UNIVERSITAT DE BARCELONA

Understanding the processes that shape the genetic diversity of freshwater planarians (Platyhelminthes, Tricladida, Dugesiidae)

Estudi dels processos que modelen la diversitat genètica en les planàries d'aigua dolça (Platyhelminthes, Tricladida, Dugesiidae)

Laia Leria Florensa

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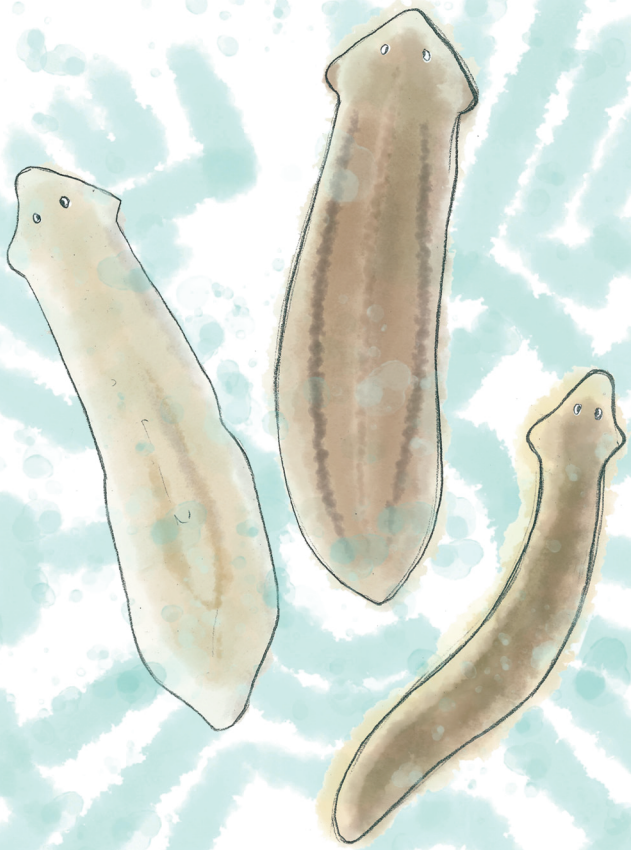

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Programa de Doctorat de Genètica

Understanding the processes that shape the genetic diversity of freshwater planarians (Platyhelminthes, Tricladida, Dugesiidae)

Estudi dels processos que modelen la diversitat genètica en les planaries d'aigua dolça (Platyhelminthes, Tricladida, Dugesiidae)

Memòria presentada per

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Els directors

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Cover illustrations: Susana Gurri

Nature is full of exceptions to our laboratory model organisms, and we need to study them to understand how they evolve and succeed

- Marta Riutort León

Acknowledgments

Everything started one summer evening, watching a football match with some friends in a bar. Àngels Tudó and me had just finished the degree in Biology and we were discussing about the subjects that we had enjoyed the most. I told her that one of my favourites had been *Phylogeography*, specially because of one bibliographic work that I had prepared about the faunal colonization of the Canary Islands. Then, she said to me that in the group where she was collaborating in the University, the Riutort's Lab, one of the topics that they worked with was about phylogeography of planarians, and that they were looking for people to do a master thesis about it. Thus, although I had never seen a planarian in my life, I went to talk with Marta Riutort (and this is how the story began). So, my first acknowledgment is for **Àngels Tudó**, the person that drove me into this scientific adventure.

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Thesis summary

The main goal of the present thesis has been to shed light into the processes that shape the genetic diversity of organisms, focussing in the following factors: the type of reproduction, the morphological diversity of genitalia, the karyological diversity, and the paleogeographic and paleoclimatic events. To do so, I have used as model organisms two different genera of freshwater planarians, *viz*. the genus *Schmidtea* and several *Dugesia* species from the Western Mediterranean region, focussing on the species *D. subtentaculata*. Freshwater planarians represent excellent models to accomplish the main objective of the present thesis because they present a wide variety of reproductive strategies, ranging from sexual to asexual by fission (which can be combined or not), they show morphological and karyological diversity, and many species inhabit regions with complex paleogeographic and paleoclimatic histories.

With this aim, I have analyzed the intraindividual genetic footprint that fissiparous reproduction leaves in individuals of the species *D. subtentaculata*, both when it is the only mode of reproduction and when it is combined with occasional sex (Chapter 1). Moreover, I have also analyzed the levels of genetic diversity, morphological diversity, and karyological diversity existing within the species *D. subtentaculata* (including a high number of populations from all its distributional range) and within the genus *Schmidtea*, under an evolutionary and a taxonomic point of view (Chapters 2 and 4). Finally, I have estimated the times of divergence between the different *Dugesia* species from the Western Mediterranean region and between the four *Schmidtea* species, to infer the putative paleogeographic and paleoclimatic processes that may have shaped its present distribution and phylogenetic relationships, which I have complemented with several analyses of species distribution modelling under different paleoclimatic scenarios (Chapters 3 and 4). Importantly, most of these analyses have been done using new nuclear molecular markers developed in the present study by performing a next-generation sequencing approach.

The results obtained in the intraindividual genetic analysis of populations of *D. subtentaculata* showing different reproductive strategies have revealed that the type of reproduction has a huge impact on the genetic characteristics of planarians. I have found that asexual fissiparous reproduction in the species *D. subtentaculata* generates outstanding levels of intraindividual genetic diversity by the putative accumulation of mutations in the planarian stem cells. Importantly, the obtained results indicate that this intraindividual genetic diversity takes place in a mosaic context within fissiparous planarians, something that has never been reported before. Thus, this genetic effect has been newly described as the *mosaic Meselson*

effect, representing a variation of the well-known Meselson effect but at the mosaic level. Moreover, I have also found evidence that events of occasional sex in fissiparous populations of *D. subtentaculata* (facultative reproduction) allow the transmission to descendants of different combinations of the alleles generated by the mosaic Meselson effect through processes of segregation and outcrossing. This situation is not only crucial for increasing the genetic diversity between the individuals of fissiparous populations but also may allow fissiparous populations to get rid of the deleterious mutations that have not been eliminated at the intraindividual level.

The integrative analysis of the molecular, morphological, and karyological characteristics within *D. subtentaculata* and between the different *Schmidtea* species has revealed that, in both groups, the degree of morphological variation in the anatomy of the copulatory apparatus is extremely low compared with the degree of molecular and karyological variation. These results indicate that the anatomy of the copulatory apparatus in planarians might be under a general state of morphological stasis, putatively due to hermaphroditism. Moreover, the high incidence of chromosomic rearrangements that has been found between the different populations of *D. subtentaculata* linked with their high genetic divergence, suggests that chromosomic rearrangements may play an important role driving speciation processes in planarians, principally in sexual populations showing a diploid karyotype. In agreement with this, the phylogenetic relationships inferred between the different *Schmidtea* species give support to a previous hypothesis of speciation between *S. nova* and *S. lugubris* due to chromosomic rearrangements. Importantly, the results found in Chapter 2 have unveiled the existence of three new species within *D. subtentaculata*, viz. *D. aurea*, *D. corbata*, and *D. vilafarrei*, which are cryptic at the morphological level but can be perfectly diagnosed by molecular and karyological data. All these results point to the need of reconsidering the taxonomic framework that is currently being used to describe planarian species, which is principally based on morphological characters.

Finally, the results obtained in Chapter 3 indicate that the paleogeographic history of the Western Mediterranean during the last 30 million years have had a huge impact driving the genetic diversification of the different *Dugesia* species from this region, either by promoting vicariant events or by connecting previously isolated areas. Moreover, the divergence time estimation performed for *Schmidtea* points out that the low species richness of this genus together with the high genetic divergence between the different species could be a result of the harsh paleogeographic history of Europe during the Cretaceous period (Chapter 4). Additionally, the species distribution modelling analyses performed in Chapters 3 and 4

indicated that, differing from many species from Europe, the last glaciations might not have represented a drastic reduction of the genetic diversity neither in the genus *Schmidtea* nor in the species *D. subtentaculata*.

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Introduction

1. Genetic diversity

1.1. What is genetic diversity?

Genetic diversity can be defined as the variation observed across the genetic characteristics of organisms. Importantly, these genetic characteristics are the set of instructions that principally determine the phenotype of individuals, thus driving their posterior interactions with the environment. For this reason, it can be said that genetic diversity is the raw material of evolution. Without genetic diversity there would be no different species on Earth, and all organisms would still look like our first ancestors.

Charles Darwin was the first person to propose that evolution depended on the existence of some kind of inheritable variation (Darwin, 1859), but it was not until Gregor Mendel's work that it was demonstrated that this variation was due to differences in the genes (Mendel, 1865). Nowadays, we know that new genetic variants appear in organisms due to spontaneous mutations in DNA during replication or due to DNA damage (Lodish et al., 2000). Importantly, in sexual organisms, if these mutations occur in the germline they can be transmitted to descendants, while if they occur in the somatic tissues they are inevitably lost when the organism dies (Weismann, 1892).

Finally, although the concept of genetic diversity is generally used at the species level, it can also be studied in a broader range of levels, going from the genetic diversity occurring within individuals to the genetic diversity occurring at the level of ecosystems.

1.2. The study of genetic diversity

1.2.1. Molecular markers

All studies on genetic diversity rely on the analysis of the variation present between sequences of DNA from certain genomic regions. These genomic regions of interest are known as molecular markers. Molecular markers can be classified into different categories depending on the genomic region where they are found (e.g., mitochondrial or nuclear) and also on the techniques that are used to obtain them (e.g., PCR-based or non PCR-based) (Alzohairy et al., 2015; Doveri, Lee, Maheswaran, & Powell, 2007). Molecular markers obtained using PCR-based methodologies, which are the most widely used, can also be classified into different categories depending on the sequencing technology that is employed, being the most relevant "Sanger sequencing" and "next-generation sequencing" (NGS) (Shendure & Ji, 2008). The

Sanger sequencing methodology is able to retrieve a limited amount of sequences simultaneously (96 or 384 in a batch), with an approximate length of 1000 base pairs (bp). On the contrary, the sequencing technology implemented in the several NGS methods is able to retrieve a much higher number of sequences (in the order of few thousands of millions), although with an approximate length of 300 bp. Importantly, while the molecular markers obtained by Sanger sequencing are targeted by the user (selected with specific primers), the molecular markers obtained with NGS generally correspond to random regions of the genome.

Among the different types of molecular markers obtained with Sanger, the ones that have been more widely used to study the genetic diversity within distinct groups of organisms are the mitochondrial markers (Ellegren & Galtier, 2016). The high mutation rate of mitochondrial genomes together with the absence of recombination, make these markers particularly useful to study patterns of genetic diversity at the intraspecific level or between closely related species (Rubinof & Holland, 2005). However, the maternal inheritance of mitochondrial genomes also prevents the detection of possible phenomena of genetic introgression if these markers are used alone (e.g., Obertegger, Cieplinski, Fontaneto, & Papakostas, 2018; Thielsch, Knell, Mohammadyari, Petrusek, & Schwenk, 2017).

The advent of the previously mentioned NGS technologies during the 2000s, opened the possibility to perform studies of genetic diversity using the information of thousands of nuclear markers. Nevertheless, the high economic cost of these technologies combined with the limited methods that were available to analyse this type of data until few years ago, promoted that many researchers opted to perform "hybrid" approaches, which consisted in searching for new specific molecular markers using the information from NGS (e.g., Lemmon & Lemmon, 2012).

One of the types of molecular markers obtained using this kind of approaches that have given better results in evolutionary studies in different organisms are the EPIC markers (e.g., Li, Riethoven, & Ma, 2010; Ströher, Li, & Pie, 2013; White, Endersby, Chan, Hoffmann, & Weeks, 2015; Yao, Li, & Dick, 2013). The search of EPIC markers (abbreviated from “Exon-Primed Intron-Crossing”) consists in looking for genes that contain intronic regions (putative variable regions) but that are flanked by conserved exonic regions, which are suitable for primer design. These molecular markers are advantageous over other genomic regions for analysing the genetic diversity of natural populations due to two principal reasons: (1) the conserved primers allow to amplify these markers in a broad taxonomic range, and (2) the fact that these markers show both conserved and variables regions allow to estimate the genetic

diversity simultaneously at different levels, which may be specially suitable when working with species complexes (Li et al., 2010).

1.2.2. Estimates of genetic diversity

Different ways exist to quantify the genetic diversity in the molecular markers. Some of the most widely used measures of genetic diversity include the following parameters: *Nucleotide diversity* (Pi) (i.e., average number of nucleotide differences per site between two sequences), *haplotype diversity* (H) (i.e., measure of the uniqueness of the different haplotypes within a population), *heterozygosity* (He) (i.e., fraction of individuals within a population that are heterozygous for a certain loci or fraction of loci within an individual that are heterozygous) and *number of alleles per locus* (A), among others (Hartl & Clark, 2007). Moreover, in the case of analysing coding molecular markers, some measures also indicate whether the genetic diversity is characterized by changes in the amino acid composition of the proteins or not, something that is very informative to infer putative processes of selection. Among the different programs that exist to calculate all these parameters of genetic diversity, one of the most widely used is DnaSP v5 (Librado & Rozas, 2009).

Another measure of genetic diversity that has been key for evolutionary studies is the *genetic distance* (Nei & Kumar, 2000). Genetic distance is defined as the degree of genetic differentiation that exists between different species or between different populations of the same species, and its calculation can be directly made by the quantification of the number of nucleotide substitutions occurring between two DNA sequences. Importantly, this quantification is generally corrected by using an evolutionary model (i.e., a model that provides information regarding the rate at which the different types of substitutions take place) (Felsenstein, 2004b), providing more accurate estimates of genetic divergence.

1.2.3. Estimates of genetic diversity under an evolutionary framework

Differing from the previously mentioned measures of genetic diversity, other methodologies exist to explore the genetic diversity of species under an evolutionary framework. Among these methodologies, two of the most widely used include haplotype networks and phylogenetic inferences.

1.2.3.a. Haplotype networks

Haplotype network reconstruction is a widely used method for visualizing the relationships that exist between haplotypes (i.e., unique DNA sequences), being particularly suitable for analyzing haplotypes that are closely related, such as those within species or within populations. Haplotype networks also display the frequency of the different haplotypes, the number of mutations separating them, and the haplotypes that are shared between individuals within populations or between populations within species (Fig. 1).

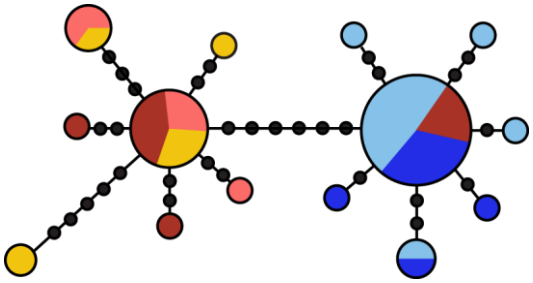

Fig. 1. Schematic representation of a haplotype network for a certain molecular marker. Each colour represents a different population within a species. Each circle corresponds to a different haplotype and the size of the circles indicate the frequency of each haplotype within the species. Black dots correspond to mutations occurring between haplotypes.

Different methodologies have been developed to reconstruct haplotype networks, including MST (minimum spanning tree) (Kruskal, 1956), MP (maximum parsimony) (Farris, 1970), TCS (statistical parsimony) (Templeton, Crandall, & Sing, 1992), MS (minimum spanning network) and MJ (median-joining network) (Bandelt, Forster, & Röhl, 1999), among others (Posada & Crandall, 2001). These methods principally differ in the type of data that they use to construct the network (distances or sequences) and whether unobserved haplotypes can be included in the reconstruction or not (Paradis, 2018). Among all these methods, the ones that have been most widely implemented in population genetic analyses are the MS and the MJ. However, it has been seen that the MJ generally provides better estimates than MS, especially when internal nodes are not sampled (Cassens, Mardulyn, & Milinkovitch, 2005).

The MJ method works with sequence data and begins by constructing several minimum spanning trees (i.e., the genetic distance between sequences is computed and the trees are built by minimizing the sum of distances between linked sequences) (Bandelt et al., 1999). Subsequently, the different possible minimum spanning trees are combined in a single reticulated minimum spanning network. In the next step, median vectors (i.e., consensus between three mutually closely related sequences) are added into the network to increase its parsimony. These median vectors may correspond either to extant unobserved haplotypes in the populations or to extinct ancestral haplotypes. Importantly, the number of median vectors added by the algorithm can be controlled by a parameter (ε).

1.2.3.b. Molecular phylogenetic inferences

Differing from haplotype networks, molecular phylogenetic inferences display the relationship between DNA sequences in a dichotomous way. In a phylogeny, DNA sequences are connected by nodes, which represent the most recent common ancestor of the two daughter lineages (Fig. 2). Moreover, this type of inference not only provides estimates of the relationship between haplotypes but also can provide estimates of the directionality of the evolutionary process (i.e., it is possible to infer which sequences diverged first and which ones diverged more recently). Importantly, to give directionality to a phylogeny is necessary to include into the analysis sequences that are closely related to the group of interest without being part of it, which are known as "outgroup" sequences.

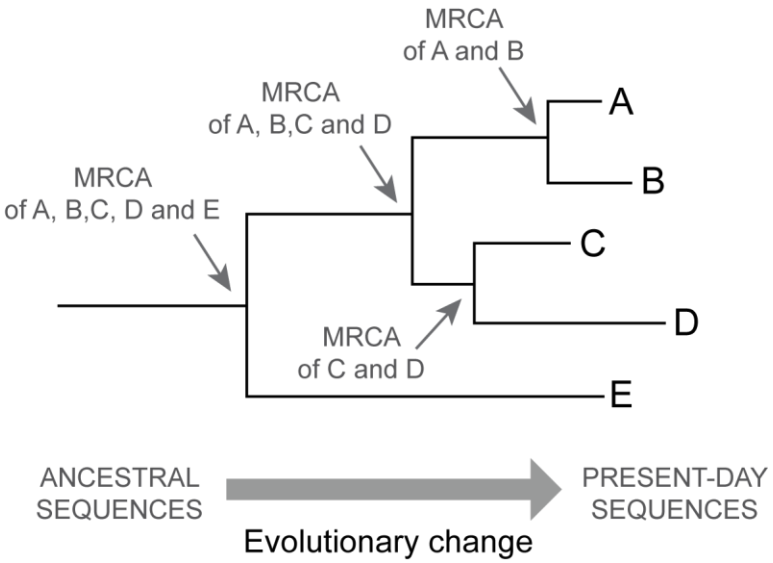

Fig. 2. Schematic representation of a phylogenetic inference for a certain molecular marker. Letters A to D correspond to haplotypes from different populations within a species. Letter E corresponds to a haplotype from the sister species of A-D (outgroup). The length of the branches is proportional to the number of nucleotide substitutions. MRCA: Most recent common ancestor.

The first methodologies that were developed to infer phylogenetic relationships using molecular data directly relied on genetic distances. The principal advantage of distance methods is that they are computationally very fast. However, they can provide inaccurate phylogenetic inferences when working with highly divergent sequences, particularly when the number of alignment gaps is high and when some positions are saturated (i.e., multiple substitutions have occurred at the same site) (Yang & Rannala, 2012). Among the different

distance-based methods for phylogenetic inference, one of the most widely used is Neighbour-Joining (Saitou & Nei, 1987).

Differing from distance-based methods, there is another group of methods that simultaneously compare all the sequences of the alignment and identify the best tree by analysing one site at a time (each site is considered a different character). The most used character-based methodologies include Maximum Parsimony, Maximum Likelihood and Bayesian Inference, which principally differ in the algorithms that are used to construct the best tree.

In Maximum Parsimony the best tree is the one that minimizes the evolutionary change (Fitch, 1971; Hartigan, 1973). This method was firstly developed to work with morphological data, under a conceptual framework directly derived from cladistics (Hennig, 1950). One of the major strengths of Maximum Parsimony relies on its simplicity, not only at computational level but also in its understanding. Nevertheless, the fact that this method does not incorporate prior information regarding the evolution of sequences, it can result in erroneous phylogenetic reconstructions, principally due to its inability to account for homoplasy (i.e., the same character state is acquired by a recurrent mutation instead of being inherited from a common ancestor) or to phenomena of long-branch attraction (i.e., sequences with higher evolutionary rates tend to be grouped together).

In Maximum likelihood, the best tree is the one that scores the higher probability of observing the character states of the data given the tree and an evolutionary model (Felsenstein, 2004a). The algorithm for the likelihood calculation on a tree that formed the basis for the modern likelihood and Bayesian methods for inferring phylogenies was developed by Felsenstein (Felsenstein, 1981). On the contrary to Maximum Parsimony, Maximum Likelihood is able to accommodate different scenarios of sequence evolution, which improves the accuracy of phylogenetic reconstructions, particularly when the sequences show complex evolutionary dynamics (Yang, 1996). However, this method is computationally more demanding. One of the most widely used programs for phylogenetic inference using Maximum Likelihood is RaxML (Stamatakis, 2006).

Bayesian phylogenetic inference is based on the Bayes' theorem. As Maximum Likelihood, it incorporates information of the model of evolution of the sequences. However, in this case, the parameters of the model are variables with statistical distributions, instead of being fixed constants. Therefore, by applying the Bayes' theorem, the posterior probability of a tree (under a certain model) is calculated as the prior probability of that tree multiplied by the

probability of the data given that tree (i.e., the likelihood) and divided by the probability of the data. This methodology uses Markov Chain Monte Carlo (MCMC) algorithms to explore the tree space (i.e., to look for the tree with the higher posterior probability among all possible trees). Similar to what happens in likelihood inferences, the incorporation of prior information of the model of evolution helps to approach biologic reality. Nevertheless, choosing inappropriate priors can lead to incorrect inferences (e.g., Rannala, Zhu, & Yang, 2012). The most popular programs used in Bayesian phylogenetic inference are MrBayes (Ronquist et al., 2012) and BEAST (Drummond, Suchard, Xie, & Rambaut, 2012).

1.2.3.c. Time-calibrated molecular phylogenetic inferences

A step beyond the inference of a molecular phylogeny is to put the estimated evolutionary relationships under a temporal framework (i.e., to time-calibrate the phylogeny). The calibration of a molecular phylogeny relies on the assumption that genes accumulate mutations at a certain rate, which is known as a molecular clock. At first, it was proposed that there was a universal molecular clock acting in all genetic regions (Zuckerkandl & Pauling, 1962). However, it was demonstrated that the rate of molecular evolution not only can vary across different genetic regions but also depends on many additional factors, such as the generation time of the organism or the selective pressure, among others (Ayala, 1999).

Three main approaches exist to calibrate a molecular phylogeny: (1) using the fossil record, (2) using paleobiogeographic events, and (3) using molecular clock estimates obtained from independent analyses (Forest, 2009). When using fossils to date certain nodes of a phylogeny it is essential that they be accurately dated and correctly placed in the phylogeny (Magallón, 2004). Importantly, the appearance of a certain taxon in the fossil record, in general, represents the period it became abundant rather than the time it originated. For this reason, calibration points with fossils are constrained as minimum times of divergence in the phylogenies, indicating that the divergence of the descendant linages can be older but not younger (Magallón, 2004).

On the contrary, when calibrating a certain node of a phylogeny using a paleobiogeographic event, such as the appearance of a geographic barrier, dispersion to a new island, or continental drift, it is assumed that the divergence of that node was caused by that event. This type of calibration is particularly useful when the taxon of interest has no representation in the fossil record, like species with soft or fragile tissues. Nevertheless, it also needs to be treated with caution, principally due to three main reasons: (a) the uncertainty of

the time when the event occurred, since most paleobiogeographic events were continuous processes that occurred during millions of years and are difficult to date with precision, (b) the real correspondence of the split of the lineages with the paleobiogeographic event, and (c) the possibility to fall into a circular reasoning (i.e., using as a calibration point the same paleogeographic event that is used to trace the phylogeographic hypothesis) (Ho et al., 2015).

Finally, is it also possible to calibrate a phylogeny by using a molecular clock inferred in an independent study. In these cases, it is necessary to use the same molecular marker and to work with closely related taxa. However, it has been seen that even closely related groups can show highly differentiated molecular clocks (e.g., rodents are reported to show a significantly accelerated molecular clock compared with other mammals) (Weinreich, 2001).

2. Factors that shape the genetic diversity

Genetic diversity can be modelled by multiple factors. Many of these factors are related to the biology of organisms itself, such as the life cycle or the type of reproduction, while other factors are extrinsic to them, such as environmental changes or interspecies interactions. However, not all these factors affect the genetic diversity in the same way. For example, some of them can have a high incidence on the genetic diversity within individuals, while others can principally drive the genetic differentiation between them. Importantly, the complex interaction of all these processes is what finally shapes the genetic diversity of species. Therefore, analysing the factors that shape the genetic background of species at different levels and under an integrative framework is fundamental not only to understand how the diversity of life on Earth has been generated, but also to infer how it is presently evolving.

In the present thesis we will focus on the study of four factors that can have an impact on the genetic diversity of species at different levels: (a) the type of reproduction, (b) morphological diversity, (c) karyological diversity, and (d) historical processes.

2.1. Type of reproduction

2.1.1. Characteristics of sexual and asexual reproduction

Reproduction is the biological process by which organisms generate new individuals. Although there are many different reproductive strategies (for example, depending on the number of

descendants or in the degree of parental care) the main types of reproduction are reduced to two: Sexual and asexual reproduction.

Sexual reproduction is characterized by the reduction of the genetic material of an individual (generally by half) via meiosis to produce the gametes, and its posterior unification with the reduced genetic material of another individual. Importantly, during gamete formation, fragments of DNA can be exchanged from one chromosome to its homologous by recombination, generating gametes that are genetically unique (Cooper, 2000). Thus, the phenomena of recombination together with outcrossing, allow sexual individuals to produce descendants that are genetically distinct to their progenitors. Sexual reproduction is the most represented type of reproduction among eukaryotic species. For instance, only 1 in 1.000 animal species are exclusively asexual (M. J. D. White, 1978). In the case of plants, strict asexuality is more widespread, although it only represents a 1% of the 250.000 angiosperm species (Asker & Jerling, 1992; Whitton, Sears, Baack, & Otto, 2008).

Asexual reproduction is characterized by the production of offspring that is genetically identical to the progenitors, due to the lack of recombination and outcrossing. Importantly, asexual reproduction can be divided into two principal classes depending if individuals need to produce gametes or not, *viz.* gametic and agametic reproduction (Hughes, 1989). Gametic reproduction in animals is known as parthenogenesis, while in plants is generally referred as apomixis. Parthenogenesis and apomixis involve the development of the female oocyte into a zygote without fertilization, although in many cases sperm is needed to trigger this process (Schlupp, 2005). Differing from gametic species, descendants of agametic organisms are produced from somatic cells or somatic structures of the progenitor. Different types of agametic reproduction can be recognized depending on the characteristics of the division process that the progenitor undergoes during reproduction. Some of the most popular examples include budding, fragmentation, and fission. Although asexual reproduction is much sparser than sexual reproduction, both gametic and agametic species are known to exist in most of the eukaryotic phyla (Meeûs, Prugnolle, & Agnew, 2007; Sköld, Obst, Sköld, & Åkesson, 2009).

2.1.2. Predicted genetic consequences of sexual and asexual reproduction

Long-term sexual and asexual reproduction leave a very different genetic footprint in species. Sexual reproduction is known to increase the genetic variability of the populations and to accelerate the evolutionary processes, as new allelic combinations can be either favored by

selection or selected against (Keightley & Otto, 2006). Asexual reproduction, on the contrary, is predicted to promote the genetic divergence between homologous alleles within individuals (i.e., the Meselson effect) due to the absence of recombination and outcrossing (Fig. 3) (Birky, 1996; Welch & Meselson, 2000). Although this effect has been investigated in several groups of parthenogenetic organisms, such as bdelloid rotifers, *Campeloma* snails, *Rhopalosiphum* aphids or *Timena* stick insects, in most of these cases the observed intraindividual allelic divergence has been finally attributed to the hybridization between sexual lineages rather than to the Meselson effect (Delmotte et al., 2003; Johnson, 2006; Schwander, 2016). Thus, to the present day, the only clear example of the Meselson effect in parthenogenetic organisms has been reported for the stick insects of the genus *Timena* (Schwander, Henry, & Crespi, 2011). Regarding agametic species, this effect has only been studied in fissiparous ribbon worms of the genus *Lineus* and in fissiparous *Trypanosoma* protozoans (Ament-Velásquez et al., 2016; Weir et al., 2016). In the case of *Lineus* worms, although high levels of genetic diversity within fissiparous populations were found, most of this genetic diversity was attributed to hybridization processes. On the contrary, clear evidences for the Meselson effect at the genome level were found in *Trypanosoma*.

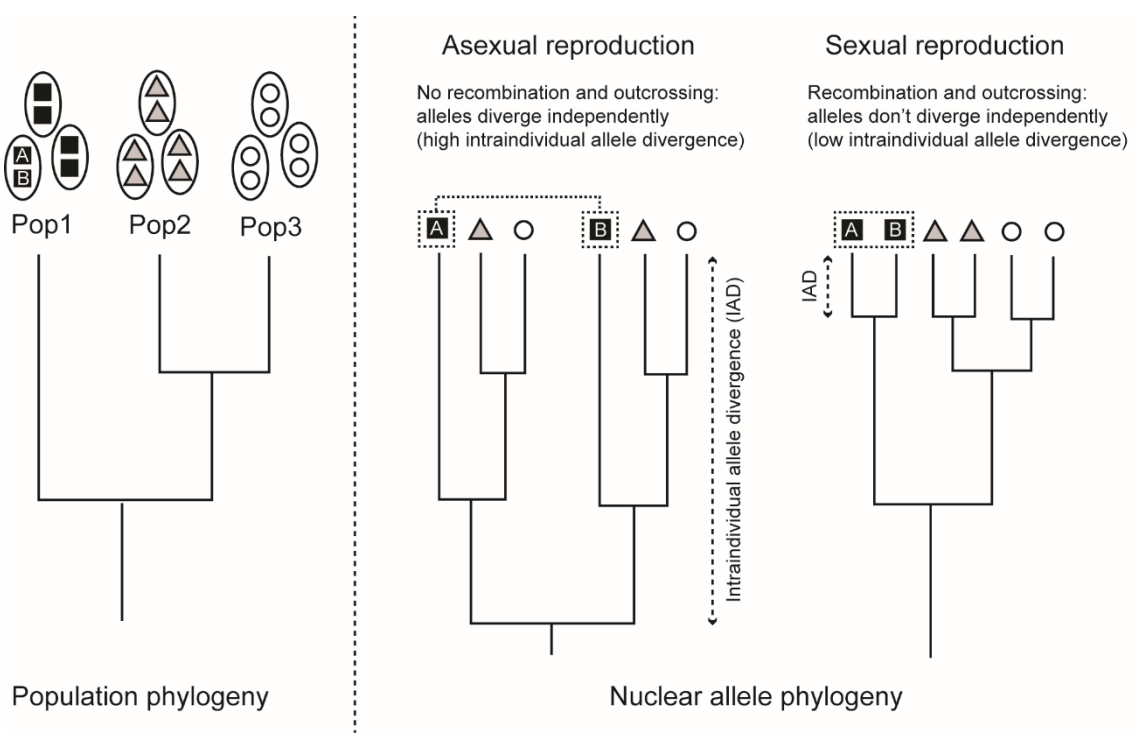

Fig. 3. Predicted nuclear allele phylogenies under asexual and sexual reproduction. Modified from Schwander *et al*. 2011.

Besides the Meselson effect, it has been proposed that long-lasting asexuality can promote an increased number of slightly deleterious mutations as a consequence of relaxed selection, since the physical linkage among loci hinders selection's ability to act upon loci independently (i.e., Muller's ratchet) (Muller, 1932). A higher accumulation of non-synonymous mutations in asexual organisms compared to their sexual relatives, has been empirically reported in some parthenogenetic groups, including stick insects and snails (Bast et al., 2018; Neiman, Hehman, Miller, Logsdon, & Taylor, 2010), as well as in some genus of apomictic plants (e.g., Hollister et al., 2015).

Finally, differing from asexual gametic species, agametic organisms are more over predicted to show high levels of genetic mosaicism in addition to the possible occurrence of the mentioned Meselson effect and Muller's ratchet (Gill, Chao, Perkins, & Wolf, 1995; Santelices, 2004), since in the absence of a zygotic bottleneck, descendants may inevitably inherit the somatic mutations of the progenitor. Mosaicism associated to agametic reproduction has long been known to occur in plants (Gill, 1986), but its existence in metazoans has only been demonstrated in colonial corals at the intracolonial level (Schweinsberg, Weiss, Striewski, Tollrian, & Lampert, 2015).

2.2. Morphological diversity

Morphological characteristics of organisms refer to their observable form and structure. These morphological characteristics can be external (such as the body shape and the body size) or internal (such as the different organ systems). Most of the morphological characteristics of organisms directly depend on their genetic characteristics (e.g., the number of segments of arthropods), while others can be altered by the environment (e.g., the body mass). Importantly, inheritable variations in the morphology of organisms can have, at the same time, a huge impact on the genetic background of species. For example, divergent selection pressure on a certain variable morphological trait can promote disruption of gene flow between some individuals within a species and consequently trigger the genetic differentiation of each lineage. Speciation processes due to divergent selection on morphological traits have been reported for a great variety of adaptive processes, such as adaptations to predation, new environments or sexual selection, among others (e.g., Ballentine, Horton, Brown, & Greenberg, 2013; Ritchie et al., 2007).

One of the morphological traits that has been proposed to play an important role driving the genetic evolution of organisms is the morphology of the genitalia (in organisms

with internal fertilization). Male genitalia have been long found to be highly variable (either at structural and functional level), even in closely related species (Hosken & Stockley, 2004). More recently, female genitalia have begun to be studied, reveling also a high degree of variation in different groups (Ah-King, Barron, & Herberstein, 2014; Anderson & Langerhans, 2015). One of the principal explanations for this high degree of diversity observed in the morphology of genitalia is called the "lock-and-key" hypothesis (Masly, 2012). This hypothesis states that different morphology of the genitalia between species prevent or reduce the success of insemination directly due to mechanical incompatibilities (e.g., Kubota, Miyazaki, Ebihara, & Takami, 2013; Wojcieszek & Simmons, 2013). Therefore, morphologic diversity in the genitalia between individuals of the same species can trigger the formation of new lineages, directly influencing the distribution pattern of interspecific genetic diversity and eventually promoting the formation of new species.

Importantly, the characteristic high degree of variation in the morphology of genitalia together with its link with reproductive isolation, has made this trait as one of the most used traits for shallow taxonomic identification in many different groups of metazoans, being especially important in the taxonomy of arthropods, platihelmints and nematodes (e.g., De Vries & Sluys, 1991; Gibbons & Khalil, 1983; Tuxen, 1956).

2.3. Karyological diversity

Variations in the chromosomic portrait of organisms can also have a huge impact on their genetic evolution. The principal processes that trigger karyological diversity include chromosomal rearrangements and polyploidization events.

Chromosomal rearrangements (CRs) refer to variations both in the structure and in the number chromosomes. They can be divided into different categories depending on the characteristics of the rearrangement, being the most important ones: (a) fusion and fission of entire chromosomes, (b) duplications, deletions and inversions of segments within the same chromosome, and (c) translocations between homologous or non-homologous chromosomes (Griffiths, Gelbart, Miller, & Lewontin, 1999). These different types of CRs have been pointed out to promote the genetic diversification of lineages due to several reasons, such as mechanical problems in chromosome pairing during meiosis in hybrid individuals (which can inhibit recombination and generate unbalanced gametes) or due to changes in the expression of the genes affected by the CRs, among others (Faria & Navarro, 2010; and references therein).

Polyploidization events refer to variations in the number of entire chromosome sets. For instance, polyploid organisms are defined as the organisms that have more than two sets of homologous chromosomes. Polyploidization events are normally generated by the formation of unreduced gametes (e.g., unreduced diploid oocytes that are fertilized with haploid sperm). Similar as for variations in the morphology of genitalia and the occurrence of chromosomic rearrangements, polyploidization events have also been related with speciation processes. In this case, speciation due to polyploidization has been proposed to occur either due to a differential fitness of the polyploids (e.g., Ramsey, 2011) or due to changes in their reproductive behavior (e.g., Herben, Suda, & Klimešová, 2017).

2.4. Historical processes: Paleogeographic and paleoclimatic events

Historical processes refer to all those past events that have had an impact into the evolutionary history of species. Two of the most important historical processes include paleogeographic and paleoclimatic events. Paleogeographic events refer to past changes in the geographic characteristics of a certain region, including the movement of landmasses as a result of plate tectonics, the formation of mountain ranges or changes in the sea-level, among others. These past geographic changes can be traced though geological studies, including the biological information found in the fossil record (Scotese, 2013). Paleoclimatic events refer to past changes in the climatic conditions of the different regions on Earth. Paleoclimatic reconstructions can be made basing on different methodologies, such as studying the composition of the air that is trapped in ice cores or by the mineralogical and biological information found in sediments (Alley, 2000; Sánchez-Rojas, Ballesteros-Barrera, & Pavón, 2011).

Paleogeographic and paleoclimatic events principally drive the genetic evolution of species by promoting changes in their geographic distribution. For example, while processes causing geographic isolation between lineages may result in a disruption of the gene flow (eventually leading to speciation), contact of previously isolated areas can promote phenomena of hybridization. The main changes in the geographic distribution of species caused by these events occur via vicariance and dispersion. Vicariance explains disjunct distribution of sister lineages by the fragmentation of the wider geographic area that their common ancestors occupied (Wiley, 1988). These processes of fragmentation can occur by different events, such as the formation of geographical barriers (e.g., mountain uplifts or changes in the sea level), the literal fragmentation of the land (e.g., due to tectonic movements) or the reduction of the area showing the optimal climatic conditions (e.g.,

isolation of lineages in different refugia during glacial periods) (Holderegger & Thiel-Egenter, 2009; Trewick, 2017). Differing from vicariant events, changes in the geographic distribution of species by dispersion are achieved by the active movement of organisms. In some cases, dispersion can be triggered by the contact of previously isolated areas, such as due to the formation of land bridges between the continent and islands during periods of low-sea level, or by the expansion of a certain lineage due to a favourable change in the environmental conditions (e.g., Barker et al., 2012).

3. Molecular data in taxonomy

Species are the fundamental units of Biodiversity and are crucial for many different fields of research, such as ecology and conservation biology, among others. At first, species were defined as groups of organisms that showed similar morphological characteristics. For instance, the term species (from the Latin *specere*) literally means "to look", referring to organisms that looked like each other. After this initial "morphological species concept", many more different species concepts were proposed, including the biological species concept, the ecological species concept or the phylogenetic species concept, among others (Mayden, 1997). However, in 1998, de Queiroz proposed a unified species concept (known as *General Lineage Species Concept*), by which species were defined as independently evolving metapopulation lineages that accumulate distinct properties (such as morphological diagnosticability or reproductive isolation) along the diversification continuum (De Queiroz, 1998). Therefore, under this conceptual framework, all previously proposed species concepts can be used as different sources of evidence to test whether lineages are evolving independently or not.

Nowadays, one of the most used sources of evidence to test evolutionary independence among lineages is molecular data, principally sequences of DNA (Pante, Schoelinck, & Puillandre, 2015). The importance that this type of data has gained in taxonomic studies is primarily due to the facility of obtaining it (compared with other sources of data) and to the amount of information that it contains. First taxonomic studies including molecular data, typically used a molecular based phylogeny or a genetic distance matrix to support the delimitation decisions based on other sources of information, which was principally morphological data. In these cases, reciprocal monophyly in the phylogeny or a certain threshold of genetic distance were invoked as evidence of molecular differentiation. Nevertheless, the seek for objectivity when using molecular data with taxonomic purposes, promoted the development of the so called "molecular methods for species delimitation".

During the past fifteen years, the number of methods for delimiting species has suffered an exponential growth.

The different molecular methods for species delimitation principally differ in the type of data that they use (e.g., genetic distances or phylogenetic trees) and in the number of loci that can incorporate (Flot, 2015). Moreover, some methods need the samples to be partitioned *a priori* into different candidate species (validation methods), while some others do not (discovery methods) (Carstens, Pelletier, Reid, & Satler, 2013). Importantly, it has been shown that the different methods for species delimitation can give different results depending on the molecular markers used and also depending on the particular scenario of speciation of each group (Knowles & Carstens, 2007; Luo, Ling, Ho, & Chao-Dong, 2018). Therefore, most authors agree that the use of different molecular markers and different methods is the best way to obtain solid conclusions when applying molecular methods for species delimitation in taxonomic studies.

4. The triclads

4.1. General characteristics of triclads

4.1.1. Anatomical features

Triclads (popularly known as planarians) represent an order of free-living Platyhelminthes, characterized at morphological level by showing the intestine ramified in three blind branches (their name coming from the Ancient Greek *tri/τρι-*, 'three'; and *klados/κλάδος*, 'branch'). Other synapomorphies of the group, besides its characteristic three-branched intestine, include a highly modified embryonic development, the cerebral position of the female gonads, the serial arrangement of the nephridiopores, and a marginal adhesive zone (Sluys, 1989).

Planarians are acoelomate organisms with bilateral symmetry in which the space between the different organ systems is filled with connective tissue, which is called parenchyma or mesenchyme. Planarians absorb oxygen directly across the whole single-layered epithelium that conforms the body wall and transport nutrients via diffusion from the gut to the rest of tissues. The three-branched blind intestine is, at the same time, ramified in many diverticula and connected in the middle part of the body to a muscular pharynx (Fig. 4A). The pharynx is a retractable tubular structure, housed in a pouch when retracted, that opens to the exterior generally by a single opening called mouth, which planarians use both to eat and to get rid of the indigestible food remains.

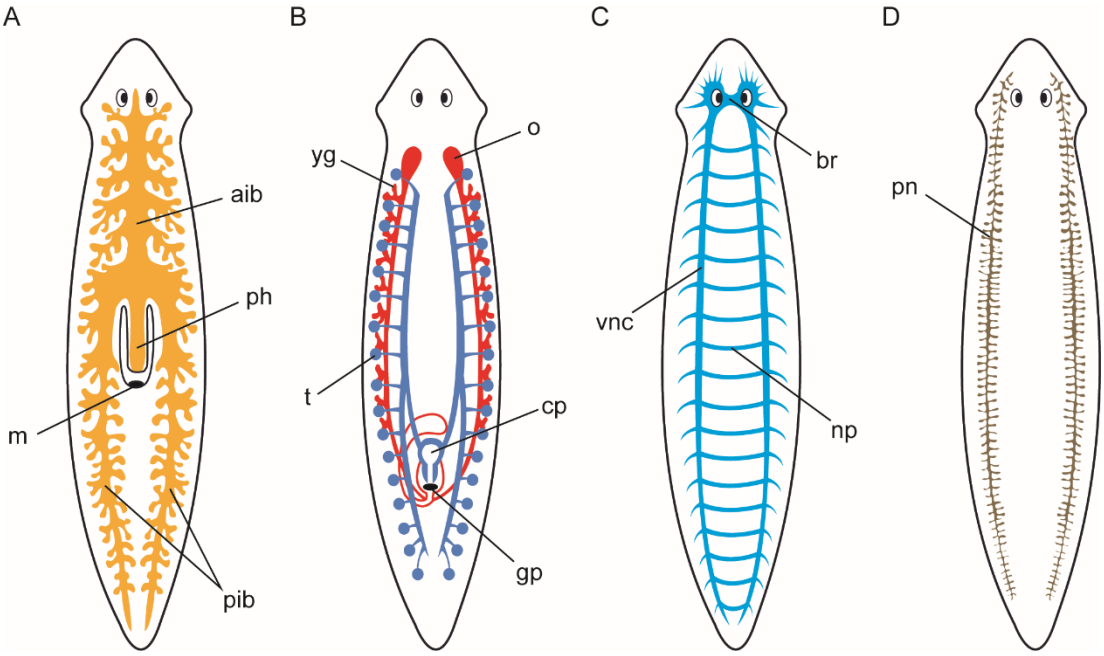

Fig. 4. Schematic representation of the different organ systems of a planarian. Digestive system (A), reproductive system (B), nervous system (C), and excretory system (D). Abbreviations: aib, anterior intestinal branch; br, brain; cp, copulatory apparatus; gp, gonopore; m, mouth; np, nerve plexus; o, ovary; ph, pharynx; pib, posterior intestinal branch; pn, protonefridia; t, testis; vnc, ventral nerve cord; yg, yolk gland. Based on Solà, 2014.

The reproductive system of planarians consists in both male and female organs, as planarians are simultaneous hermaphrodites, with only two known exceptions of dioic marine species, *viz*. *Sabussowia dioica* (Claparède, 1863) and *Cercyra teissieri* Steinmann, 1930. A pair of ovaries is usually situated in the anterior part of the animal (near the brain), while numerous follicular testicles are located in between the secondary branches of the gut. The copulatory apparatus (showing both male and female structures) is always situated in the post-pharyngeal region of the body (Fig. 4B).

The nervous system in planarians is constituted by a bilobed brain usually connected to a pair of ventral longitudinal nerve cords, which at the same time are interconnected through different nerve plexus, making the nervous system look like a ladder (Fig. 4C). Additionally, planarians show an excretory system involved in the elimination of cellular waste products. This system is comprised by a network of flame cells connected to form the protonephridia, which run dorsoventrally in two rows beneath the epidermis on each side of the body and open to the exterior via the nephridiopores (Fig. 4D).

The sensory system of planarians is constituted by the photoreceptor organs (i.e., the eyes) and the chemoreceptor organs. Planarian eyes are only able to detect the direction and intensity of the light, but they can't form clear images. On the other hand, the chemoreceptor organs in planarians are divided in three main types: the auricular grooves, the sensory fossae and the sensory pits (De Vries & Sluys, 1991). The auricular grooves are stripes of epithelium located in the laterals of the anterior region of the body (which can be folded) that do not contain gland cells but that are enriched in cilia and nerves. The sensory fossae and pits are located in the anterior margin of the body and their number and size differ among planarian groups.

The musculature that surrounds the epidermal body wall (i.e., cutaneous muscles) shows circular, longitudinal and diagonal fibres, and it is arranged in a different manner depending on the group. Parenchymatic muscles are also present, which can be longitudinal, transverse, diagonal, and dorsoventral. Finally, the entire body of planarians is covered with a mucous layer, which is segregated by distinct types of subepidermal gland cells. This mucous layer provides a non-abrasive substrate to glide over, it is related to adhesion, to sealing wounds, and to predatory functions (Pedersen, 1963; Prasniski & Leal-zanchet, 2009).

4.1.2. Stem cells and regeneration

One of the most interesting characteristics of planarians is that adult individuals are provided with an abundant population of stem cells, the neoblasts (Rink, 2013; and references therein). Stem cells are defined as cells that have both the capacity of long-term self-renewal and to produce at least one type of differentiated cells (Watt & Hogan, 2000). Stem cells can be divided into several groups depending on their potential to differentiate to distinct cell types, being the main classes: totipotent, pluripotent, and multipotent (Kalra & Tomar, 2014). Totipotent stem cells can give rise to both embryonic and extraembryonic cell types. Such cells can construct a complete, viable organism. Pluripotent stem cells are derived from totipotent cells and are able to differentiate into almost all cell types, while multipotent stem cells can generate multiple cell types of a specific tissue. The stem cell population of planarians is composed of the so called clonogenic neoblasts (c-neoblasts), which are pluripotent stem cells, and different fate-specified stem cells (specialized neoblasts) (Reddien, 2018; Wagner, Wang, & Reddien, 2011). Although planarian c-neoblasts are generally referred as pluripotent, is likely that they are actually totipotent adult stem cells. However, since most studies are carried out using asexual planarian strains, it is still presently unknown whether these c-neoblasts may be able to differentiate extraembryonic tissues (J. C. Rink, 2013).

Neoblasts reside on the parenchyma that surround the organs throughout the planarian body and represent up to 35% of the total number of cells of the planarian (Fig. 5), while the rest of planarian cells constitute differentiated cells and their progenitor stages (J. Baguñà & Romero, 1981; Plass et al., 2018). Neoblasts are the only cells of planarians that undergo mitosis, thus they are responsible for their cellular turnover and for their growth-degrowth dynamics (González-Estévez, Felix, Rodríguez-Esteban, & Aziz Aboobaker, 2012; Pellettieri & Sánchez Alvarado, 2007). Moreover, neoblasts also confer planarians extreme regeneration capabilities (planarians are able to regenerate an entire individual from a tiny piece of tissue) (Brøndsted, 1969; J. C. Rink, 2013).

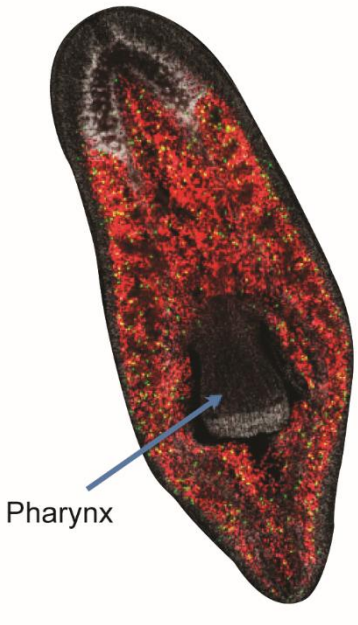

Fig. 5. Distribution of neoblasts in the planarian species *Schmidtea mediterranea*. Red dots: neoblasts; green dots: neoblasts in division. Extracted from Rossant, 2014.

This outstanding regeneration capabilities not only allow planarians to heal after accidental wounding, but also allow them to reproduce asexually by fission. Fissiparous individuals generally undergo a transverse split in two pieces, approximately at the post-pharyngeal region of the body, and after the fission process each piece regenerates the missing structures in an approximate period of two weeks (Saló, 2006). Neoblast activity after fission (as well as after accidental wounding) is characterized by a first body-wide mitotic response, followed by a neoblast migration to the wound, and a second local mitotic response corresponding to neoblast proliferation and differentiation at the wound (Wenemoser & Reddien, 2010) (Fig. 6).

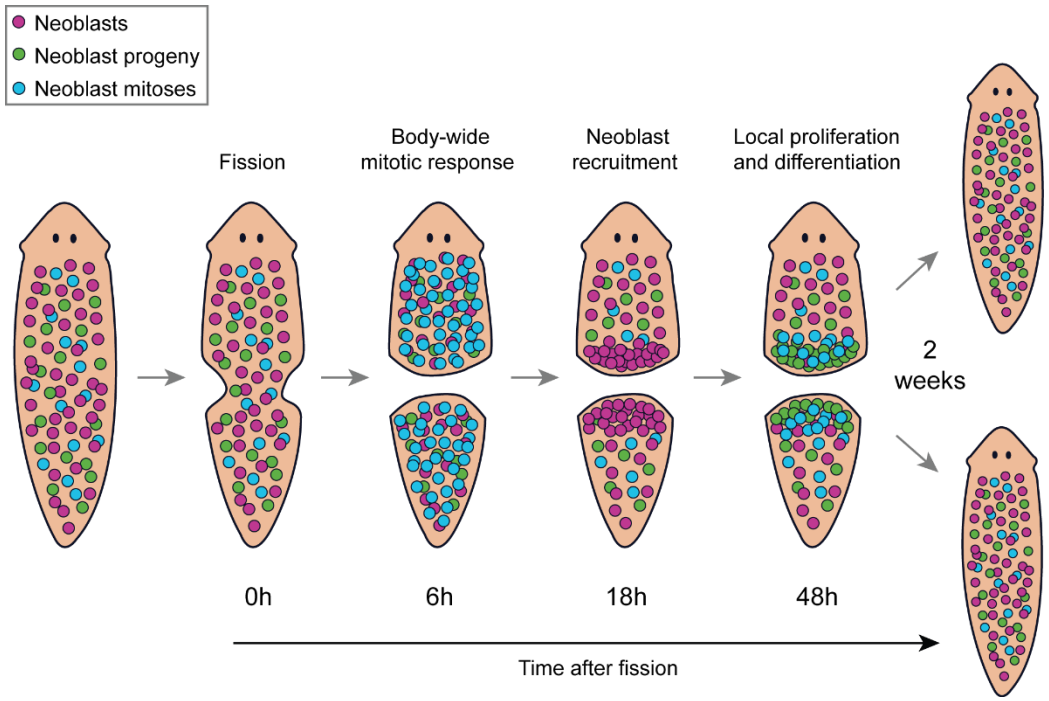

Fig. 6. Schematic representation of the planarian neoblast dynamics after a fission process. The first neoblast response after fission consists in a body-wide increase of the mitoses (6h after wounding). Subsequently, there is a neoblast recruitment to the wound (18h after wounding), which leads to a local increase of the proliferation and differentiation (48h after wounding). Finally, in an approximate period of 2 weeks, all lost structures and body proportion are completely restored. Figure based on Wenemoser & Reddien, 2010.

4.1.3. Diversity and distribution

Planarians are divided in three major groups: Sub. O. Continenticola, Sub. O. Maricola, and Sub. O. Cavernicola (Fig. 7). Continenticola includes both freshwater and terrestrial planarians. Nevertheless, these two ecological groups are not monophyletic, since the land planarians (family Geoplanidae) are the sister group of just some of the freshwater planarians, *viz*. the family Dugesiidae (Riutort, Álvarez-Presas, Lázaro, Solà, & Paps, 2012). On the other hand, Maricola is composed of marine planarians, while Cavernicola mostly includes hypogean species. Importantly, while Continenticola and Maricola show a high species richness, Cavernicola is only represented by 8 species described in 6 different genera (Abdel Halim Harrath et al., 2016; Sluys, Kawakatsu, Riutort, & Baguna, 2009; Sluys & Laumer, 2019).

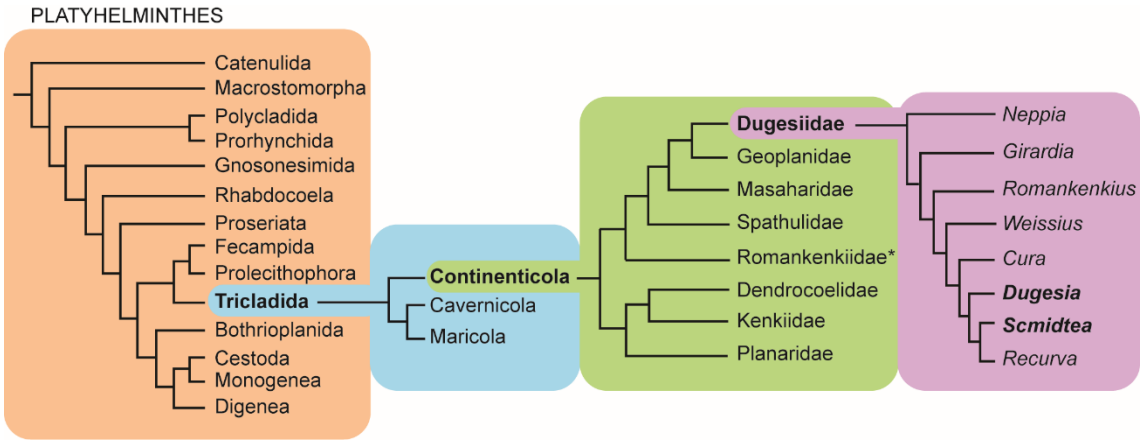

Fig. 7. Schematic phylogenetic relationships of Tricladida at different levels, focussing on the family Dugesiidae. Modified from Solà, 2014. Phylogenetic relationships based on Laumer *et al*. 2015; Harrath *et al*. 2016; Grant, 2016.

Freshwater planarians are distributed all over the world, excepting the Antarctica and some islands. They can be found in a great variety of freshwater environments, including rivers, streams, ponds, lakes, springs, caves, and even in artificial water reservoirs and canals (Vila-Farré & Rink, 2018). Like freshwater planarians, terrestrial planarians can also be found worldwide. However, in this case, there are seven regions that show a higher species diversity of terrestrial planarians compared to others, which are the Atlantic forest of Brazil, Madagascar, Sri Lanka, Java, Tasmania, Australia, and New Zealand (Sluys & Riutort, 2018). Differing from freshwater and terrestrial planarians (Sub. O. Continenticola), hypogean planarians (Sub. O. Cavernicola) show a much more limited known distribution, only having been recorded from Brazil, Mexico, Tahiti, East Malaysia, and West Africa. Finally, the present distribution of marine species is still poorly studied, with the current known distributional patterns being clearly influenced by sampling bias, which are the coasts of north-western Europe, the Mediterranean Sea, the eastern coast of North America, and the tip of South America.

4.1.4. Ecology

Planarians are predators or even top predators in some habitats, such as in cold springs (Vila-Farré & Rink, 2018). Their diet is generally based on small invertebrates, including snails, oligochaetes, nematodes, and arthropods (Armitage & Young, 1990; Cuevas-caballé, Riutort, & Álvarez-Presas, 2019; Kreuzinger-Janik, Kruscha, Majdi, & Traunspurger, 2018). Moreover, it

has been reported that they can also prey on amphibian eggs and even on other planarian species (Cuevas-caballé et al., 2019; Hull, 1947; Segev, Rodríguez, Hugemann, Hauswaldt, & Vences, 2015). Although some planarians species can show specific food preferences, including specific predatory behaviours (e.g., Boll & Leal-Zanchet, 2018), most studies have revealed that different species or even different genera can prey on the same food resource, leading to situations of ecological competition (Armitage & Young, 1990; Lock & Reynoldson, 1976).

Freshwater and marine planarians need the continuity of water bodies to survive and disperse, while terrestrial planarians need habitats with high levels of environmental humidity (planarians cannot tolerate desiccation). Planarian locomotion is based on a gliding process due to the combined action of muscles and cilia over a layer of mucus that the animals segregate over the substrate (Ball & Reynoldson, 1981). Importantly, dispersion in planarians occur due to their active movement along the substrate rather than by passive dispersal by means of external agents (such as the water current in the case of freshwater and marine species). For these reasons, planarians are considered poor dispersal organisms and thus, ideal models to carry out phylogeographic analyses.

4.2. The species *Dugesia subtentaculata*

4.2.1. Distribution

Dugesia subtentaculata is a species of freshwater planarian that belongs to the family Dugesiidae (see Fig. 7). The genus *Dugesia* is represented by approximately 80 described species, inhabiting the Paleartic, Afrotropical, Indomalayan, and Australasian biogeographic regions (Sluys, Kawakatsu, & Winsor, 1998; Solà, 2014). *D. subtentaculata* is found in the Western Mediterranean region, with approximately 17 known localities scattered in France (Montpellier), Portugal (Coimbra), Spain (Catalonia, Castilla-La Mancha and Mallorca), Morocco, and Algeria (De Vries, 1986b; Lázaro et al., 2009; Ribas, 1990; Stocchino et al., 2009) (Fig. 8).

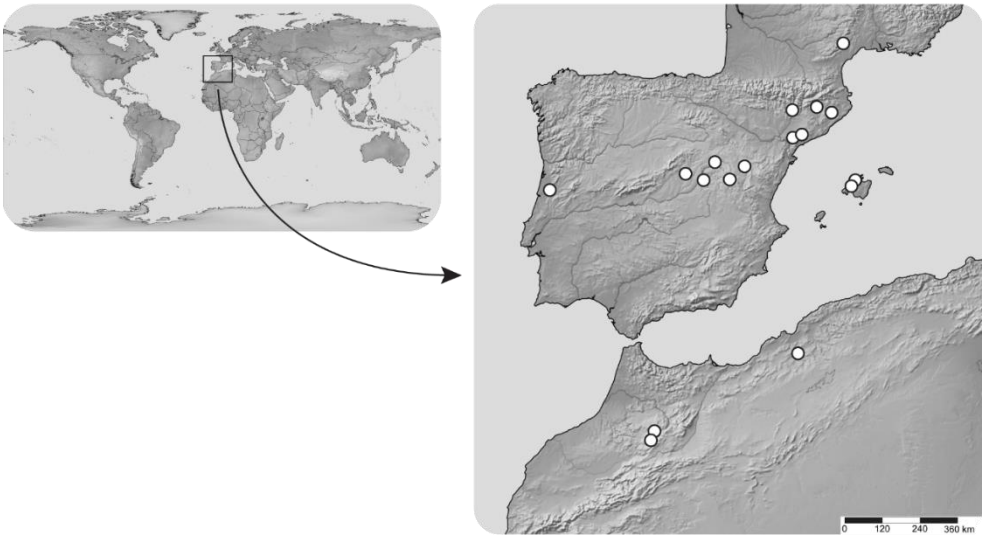

Fig 8. Distribution map of the known localities of *Dugesia subtentaculata*.

4.2.2. Morphological characteristics

As the rest of the approximately 80 described *Dugesia* species, *D. subtentaculata* is externally characterized by a triangular shaped head with two eyes in the middle region (Fig. 9). Curiously, the pigmented cup of each eye of all *Dugesia* species is situated towards the sagittal plane of the animal within the unpigmented region that surrounds it, conferring them a funny cross-eyed appearance. The coloration of all *Dugesia* species ranges from greyish to creamy-brownish, with the dorsal surface always more pigmented than the ventral surface. However, their main color can change depending on the diet (personal observation), probably due to the appreciation of the gut content through their thin epidermal layer.

Fig. 9. Photograph of a life specimen of *D. subtentaculata* from Teruel (Spain). Size of the individual 0,5 cm.

Individuals of the genus *Dugesia* can be internally distinguished from the rest of genera within Dugesiidae by two features of the copulatory apparatus: (1) the existence of a diaphragm separating the seminal vesicle and the ejaculatory duct and (2) the extension of the ectal reinforcement that surrounds the bursal canal for well over half of its length (De Vries & Sluys, 1991; Sluys, 2001). Similarly, the different *Dugesia* species can be distinguished from

each other by the anatomy of the copulatory apparatus. However, the anatomical diagnosis of any *Dugesia* species is based on the combination of diagnostic states of different characters, rather than by morphologic autapomorphies (although they can exist in some species). For example, such character states include the shape of the penis papilla (elongated, blunt or conical, among others) or the position of the openings of the oviducts and the sperm ducts into the atrium and the seminal vesicle, respectively, which can be dorsal, ventral, asymmetrical, or symmetrical (Sluys et al., 1998).

In the case of *D. subtentaculata*, its copulatory apparatus is characterized by a weakly muscular penis bulb well delimited from a short and blunt penis papilla; a central ejaculatory duct separated from a vesicle by a glandular valve-like diaphragm; abundant penial glands surrounding the seminal vesicle, the diaphragm and the ejaculatory duct; and an annular parenchymatic ring at the base of the penis papilla (this only in mature specimens) (Fig. 10) (De Vries, 1986b). Interestingly, in the description of this species, different variable morphological characters were reported between some of the populations, which were considered as intraspecific variation. These characters include the shape of the ovaries; the number of outer pharyngeal muscle layers; the distinctness, position and size of the parenchymatic ring at the base of the penis papilla, and the shape and size of penis papilla (ranging from very short and blunt to slightly longer).

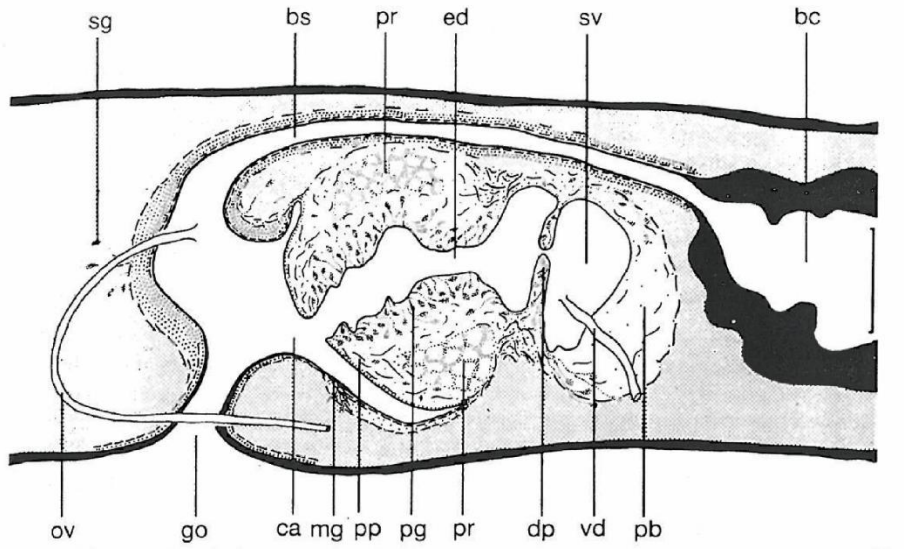

Fig. 10. Sagittal reconstruction of the copulatory apparatus of *Dugesia subtentaculata* from Montpellier (France). Modified from De Vries, 1986b. Neotype individual: ZMA V.PI.622.2. Abbreviations: bc, bursa copulatrix; bs, bursal canal; ca, common atrium; dp, diaphragm; pp, penis papilla; pr, parenchymatic ring; sg, shell glands; sv, seminal vesicle; vd, vas deferens. Scale bar indicates 200µm.

4.2.3. Karyological characteristics

The haploid chromosome complement of *Dugesia* species generally consists in a basic number of n=8 (Benazzi, 1982). Nevertheless, some *Dugesia* species show a different haploid number than 8, such as n=7 (e. g., *D. hepta* Pala, Casu and Vacca, 1981 or *D. ryukyuensis* Kawakatsu, Oki, Tamura and Sugino, 1976) or n=9 (e.g., *D. sicula* Stocchino, Corso, Manconi and Pala, 2002 or *D. maghrebiana* Stocchino, Manconi, Corso, Sluys, Casu and Pala, 2009), the latter being prevalent in most of the African and Malagasy *Dugesia* species (Stocchino, Sluys, & Manconi, 2012, 2014). Euploidies (i.e., changes that involve the gain or loss of entire chromosome sets) are frequently observed in *Dugesia* species and are generally associated to the reproductive strategy (see below). Moreover, the existence of supernumerary chromosomes (also known as B-chromosomes) as well as chromosomic translocations and pericentric inversions have also been reported to occur in some *Dugesia* species (De Vries, 1986a; Ribas, 1990; S. Tamura, Yamamoto, Takai, Oki, & Kawakatsu, 1998; Sachiko Tamura, Oki, & Kawakatsu, 1991).

Three different chromosomic portraits (named biotypes) have been described within the species *D. subtentaculata* (Ribas, 1990). Biotype A has been found only in the individuals of a population from Mallorca (Sa Calobra). It is a diploid biotype (2n=16), characterized by showing all the chromosomes with a metacentric morphology and a variable number (from 0 to 4) of small supernumerary chromosomes. Biotype B has been found in a different population from Mallorca (Soller) and, as well as in biotype A, all individuals of this biotype are diploid (2n=16). However, in this case, the specimens show three submetacentric chromosomes (pairs 3, 4 and 5) and no supernumerary chromosomes. Finally, the biotype C, is found in the rest of presently known populations of the species and, differing from the other two biotypes, it is a triploid biotype (3n=24). In this case, the morphology of the chromosomes is generally metacentric but supernumerary chromosomes can occur. Moreover, some populations of this biotype show aberrant chromosomes, which have been associated to different translocations and pericentric inversions.

4.2.4. Reproduction

Dugesia species can reproduce both sexually or asexually. Sexual individuals are hermaphrodites and reciprocal cross-fertilization is probably the rule (Stocchino & Manconi, 2013). During the copula, each individual transfers a spermatophore to the partner, which can be kept in the bursa copulatrix up to several months (Benazzi & Gremigni, 1982). The fertilization of the eggs occurs in the oviducts, during their way to the genital atrium. Once they arrive to the atrium, together with yolk cells from the vitellaria, are collected in a cocoon,

which will be deposited through the gonopore (Ball & Reynoldson, 1981). *Dugesia* cocoons are spherical and stalked, except for a species from Madagascar which deposits unstalked cocoons (Stocchino et al., 2014). After 2-4 weeks of development (depending on the species), from 1 to 10 hatchlings arise (Stocchino & Manconi, 2013). All sexual *Dugesia* species that have been studied under reproductive terms are iteroparous (i.e., can reproduce many times during its lifetime). Finally, asexual reproduction in *Dugesia* occurs mainly by fission (see section 4.1.2. for a detailed explanation on the fission process). Interestingly, fissiparous individuals don't even generate the reproductive system nor a differentiated germline (Sato et al., 2006).

Although most polyploid populations of *Dugesia* are fissiparous (e.g., Lázaro et al., 2009), triploid cocoon laying populations have also been found in natural conditions ((e.g., in *D. japonica* and *D. ryukyuensis* (Tamura, Oki, & Kawakatsu, 1995) or in *D. benazzii* (Lentati, 1966)). For many years, it was assumed that triploid cocoon laying *Dugesia* populations were reproducing asexually by pseudogamous parthenogenesis (i.e., sperm-dependant parthenogenesis), due to several cytogenetic studies carried out in tetraploid *D. benazzi* (Lentati & Puccinelli, 1959). However, recent studies on the Asian species *D. ryukyuensis* have revealed that triploid cocoon laying individuals can reproduce truly sexually through a special meiotic system (Chinone, Nodono, & Matsumoto, 2014; Kobayashi et al., 2008). Through this meiotic system, triploid planarians produce recombinant haploid sperm by the elimination of an entire chromosome set before entering to meiosis. Differing from spermatogenesis, the three chromosome sets are maintained during oogenesis at least until metaphase I. Subsequently, some of the oocytes eliminate one chromosome set and perform a normal diploid meiosis (producing haploid oocytes), while other oocytes retain the three chromosome sets, ending up with diploid and haploid oocytes.

Interestingly, for some *Dugesia* species only one type of reproduction is known under natural conditions (e.g., sexual reproduction is the only reproductive strategy known in *D. hepta*, while fissiparous reproduction is the only known reproductive strategy in *D. aethiopica*). On the contrary, other species alternate the two types of reproduction (i.e., facultative reproduction) during the year (e.g., *D. japonica* and *D. bengalensis*), while some species show different sexual and fissiparous populations in natural conditions (Stocchino & Manconi, 2013). In the case of *D. subtentaculata*, biotypes A and B (diploid populations from Mallorca) are sexual, while individuals of biotype C (triploid individuals) are fissiparous. Nevertheless, our recent samplings in the northern region of the Iberian Peninsula unravelled the existence of populations putatively belonging to *D. subtentaculata* that show a mix of sexual and fissiparous individuals. Therefore, it could be possible that this species showed the whole

variety of reproductive strategies present in *Dugesia*, *viz*. sexual, fissiparous, and facultative reproduction.

4.2.5. Genetic diversity

The analysis of DNA sequences of *Dugesia* not only have represented a key tool to identify fissiparous individuals but also have shed light into the evolutionary relationships between several *Dugesia* species (Khang, Tan, Panha, & Mohamed, 2017; Lázaro et al., 2009; Sluys et al., 2013), which could not be solved on the basis of morphological and karyological data alone (Sluys et al., 1998). Nevertheless, the genetic diversity of the genus *Dugesia* has only been analyzed using four molecular markers, *viz*. the ribosomal genes 18S, 28S, and ITS; and the mitochondrial gene Cox1. The first three genes resulted very useful to solve most of the internal phylogenetic relationships of the genus as well as to shed light into its phylogenetic relationship with other genera of planarians, but they were less informative when working at shallow level (Álvarez-Presas & Riutort, 2014; and references therein). The mitochondrial gene Cox1, on the other hand, was found to be suitable to perform analyses at the intraspecific level (e.g., Lázaro & Riutort, 2013), but its high level of variation resulted in saturation when working with divergent species (Álvarez-Presas, Baguñà, & Riutort, 2008). Therefore, new nuclear markers would be very useful to be included in molecular analyses of the genus *Dugesia*.

The available genetic data of the species *D. subtentaculata* is rather limited. Only one study has explored so far the genetic diversity of the three different biotypes (using distance measures and phylogenetic inferences of two loci, the Cox1 and the ITS) (Lázaro et al., 2009). That study showed that the sexual biotypes A and B are highly differentiated from each other, as well as from the fissiparous populations of biotype C. At the same time, that study also pointed out the close genetical relationship of *D. subtentaculata* with different species from the Western Mediterranean region, including *D. hepta* and *D. benazzi* (Sardinia) and *D. gonocephala*, *D. etrusca* and *D. liguriensies* (western Europe). Unfortunately, the phylogenetic relationships between these species remained unclear due to the low resolution of the two molecular markers used in that analysis. Some years later, a molecular biogeographic study of the whole genus *Dugesia* (using four molecular markers, *viz*. 28S, 18S, ITS and Cox1), which included two populations of *D. subtentaculata* (one from Morocco and the other from the Iberian Peninsula), uncovered a putative sister-group relationship of this species with *D. tubqalis*, an endemic species from Morocco (Solà, 2014).

In summary, *D. subtentaculata* is a genetically diverse species that exhibits different reproductive strategies, intraspecific diversity at the morphological and karyological level, and a wide disjunct distribution in the Western Mediterranean region (a region with a complex paleogeographic and paleoclimatic history). For all these reasons, we considered at the beginning of the present thesis that *Dugesia subtentaculata* could be an excellent model to study how the previously mentioned factors may shape the genetic diversity of organisms.

4.3. The genus *Schmidtea*

4.3.1. Diversity and distribution

Differing from the species rich genus *Dugesia*, the genus *Schmidtea* is constituted only by four species: *S. polychroa* (Schmidt, 1861), *S. lugubris* (Schmidt, 1861), *S. mediterranea* (Benazzi, Baguñà, Ballester, Puccinelli, & Del Papa, 1975), and *S. nova* (Benazzi, 1982). The species *S. mediterranea* is considered a model organism in regeneration research (Reddien & Alvarado, 2004; J. C. Rink, 2013), while *S. polychroa* has been used in numerous studies on embryogenesis (Cardona, Hartenstein, & Romero, 2005; Monjo & Romero, 2015) and on reproductive biology (e.g., D'Souza & Michiels, 2008; D'Souza, Storhas, Schulenburg, Beukeboom, & Michiels, 2004). On the contrary, the species *S. lugubris* and *S. nova* remain poorly studied.

Differing from the genus *Dugesia*, the genus *Schmidtea* is endemic to the Western Palearctic region. The distribution of the species *S. mediterranea* and *S. polychroa* is reasonably well known (Lázaro et al., 2011; Pongratz, Storhas, Carranza, & Michiels, 2003). *S. mediterranea* shows a scattered distribution in the western Mediterranean islands together with two localities on the coasts of Catalonia (Spain) and one in continental Tunisia. *S. polychroa* shows a broad continental distribution, expanding from the Iberian Peninsula to Hungary up to Sweden, including the islands of the United Kingdom, Sardinia, Sicily and Northern Africa (Harrath et al., 2012). In contrast, the distribution of *S. nova* and *S. lugubris* remains largely understudied, since although several localities of these species have been reported from Europe, no studies so far have compiled and analysed this information.

4.3.2. Morphological characteristics

The external morphology of the genus *Schmidtea* is similar to the genus *Dugesia*, differing from that genus in that the auricular grooves are less pronounced in *Schmidtea*, conferring them a spatulated shape of the head (Fig. 11). Individuals of the genus *Schmidtea* can be

internally distinguished from the rest of genera within Dugesiidae by two characteristics of the copulatory apparatus: (1) the existence of a double seminal vesicle and (2) the existence of a mixed muscular coat (intermingled longitudinal and circular muscles) surrounding the bursal canal (De Vries & Sluys, 1991). The four *Schmidtea* species (as happens in the genus *Dugesia*) cannot be externally distinguished. Thus, the taxonomic identification of the species within this genus also relies on diagnostic features of the copulatory apparatus, excepting for the species *Schmidtea nova*, which was described on the basis of its karyotype (a detailed description of the anatomy of the copulatory apparatus is still missing for this species).

Fig. 11. Photograph of a life specimen of *Schmidtea polychroa*. Modified from Brandl *et al*. 2016.

4.3.3. Karyological characteristics

Differing from the genus *Dugesia* (n=8), the basic haploid chromosome number of *Schmidtea* is n=4. Initially, all the species of this genus were collectively treated as *S. lugubris*. Under this scenario, seven different biotypes were identified basing on the karyotype, which were named after the first seven letters of the alphabet (Benazzi, 1957). Subsequent studies attributed the biotypes A, B, C and D to the species *S. polychroa.* The karyotype of this species is characterized by the presence of one metacentric chromosome and three acrocentric chromosomes, with the difference that individuals of biotype A are diploids (2n=8), individuals of biotypes B and C are triploids (3n=12) with differences in the gametogenesis, and individuals of biotype D are tetraploids (4n=16). Biotype E belongs to the species *S. lugubris*, and it is characterized by showing three big acrocentric chromosomes and a small submetacentric chromosome. Biotype F, the biotype that describes *S. nova*, is the only biotype within *Schmidtea* that shows a basic chromosomic number different than 4, which is n=3. It shows a

very big metacentric chromosome, an acrocentric chromosome and a small metacentric chromosome. It was proposed that this karyotype originated through a Robertsonian translocation plus a pericentric inversion from biotype E (Benazzi & Puccinelli, 1973). Finally, biotype G, corresponds to the species *S. mediterranea* and it is characterized by three metacentric chromosomes and one submetacentric chromosome. These three last biotypes (i. e., E, F and G) are generally diploid, although triploid populations of biotype G have been found (Baguñà et al., 1999).

4.3.4. Reproduction

The species *S. lugubris*, *S. nova* and the biotype A of *S. polychroa* reproduce sexually, while individuals from biotypes B, C and D of the latter species reproduce asexually by parthenogenesis. Parthenogenesis in *Schmidtea* is done by pseudogamy and it has been suggested that this type of reproduction plays an important role in the high colonization capabilities of the species (Pongratz et al., 2003). Alternatively, although most populations of *S. mediterranea* also reproduce sexually, the populations from Catalonia and the Balearic Islands reproduce asexually by fission. However, differing from *Dugesia*, fissiparity in *S. mediterranea* is not related to polyploidy but to a chromosomal rearrangement. All fissiparous populations of this species show a translocation from one chromosome of the third pair to one chromosome of the first pair, independently if the individuals are diploid or triploid, while sexual populations do not present the translocation (Baguñà et al., 1999).

4.3.5. Genetic diversity

Little was known till recently on the genetic diversity within and between the different *Schmidtea* species. The most comprehensive study of interpopulation variability within *S. mediterranea* was based on three molecular markers and showed that the different populations were genetically highly differentiated (Lázaro et al., 2011). Similarly, substantial intraspecific genetic variation was found within the species *S. polychroa*, based on the information of the Cox1 molecular marker (Pongratz et al., 2003). Finally, no study has analyzed, so far, the genetic information of different populations of the species *S. nova* and *S. lugubris*.

The situation is worse when comparing the different species of the group. Currently, only the Cox1 has been used to stablish the phylogenetic relationships between the different species of *Schmidtea*. The first Cox1 sequences of *S. mediterranea* and *S. polychroa* were obtained by Baguñà, Carranza, Paps, Ruiz-Trillo, and Riutort (2001). Two years later, the Cox1

of many populations of *S. polychroa* was sequenced, and two sequences of *S. lugubris* and *S. nova* were used as outgroup (Pongratz et al., 2003). A sequence of each of these species was included in a phylogenetic study of the triclads (Álvarez-Presas et al., 2008). In that work, the genus *Schmidtea* was recovered as monophyletic but the evolutionary relationships between the four species remained unclear. Some years later, in the previously mentioned phylogeographic study focused on the species *S. mediterranea*, the available Cox1 sequences of *S. nova* and *S. lugubris* together with new sequences of *S. polychroa* were used to infer a time-calibrated phylogeny (Additional file 3 in Lázaro et al., 2011). This phylogeny showed, in the first place, that the four *Schmidtea* species were genetically highly differentiated. Moreover, the phylogeny also pointed out that the four species are probably distributed in two monophyletic clades: one clade including *S. mediterranea* and *S. polychroa*, and the other clade including *S. lugubris* and *S. nova*. However, this topology showed very low support values, pointing out the need to perform additional studies to confirm the phylogenetic relationships within this genus.

In summary, the genus *Schmidtea*, although including two species used as model organisms in different academic areas, remains poorly studied. Particularly, neither the phylogenetic relationships between its species nor the anatomical characteristics of the species *S. nova* are presently known. Moreover, although being the sister genus of *Dugesia* (together with *Recurva*), and inhabiting the same region as several *Dugesia* species, both genera show remarkable differences principally regarding the species richness and reproductive strategies. For these reasons, we considered that including the genus *Schmidtea* in the present thesis would not only be important to shed light into the several evolutionary issues that remain unknown about it, but also that, together with *Dugesia*, would form a very comprehensive framework for analyzing the processes that shape the genetic diversity in freshwater planarians.

Objectives

General objective

The main objective of the present thesis is to shed light into the processes that shape the genetic diversity of organisms, focussing on the effect of the type of reproduction, the morphological diversity, the karyological diversity, and different historical processes. To do so, I have used as model organisms two different genera of freshwater planarians: the genus *Dugesia*, focussing on the species *Dugesia subtentaculata*, and the genus *Schmidtea*.

Specific objectives

To achieve the main goal of the present thesis, different specific objectives were underlined:

- To perform an exhaustive sampling across *D. subtentaculata* distributional range, which includes Southern France, the whole Iberian Peninsula, Mallorca (Balearic Islands) and Northern Africa.

- To find new nuclear molecular markers showing adequate levels of variability within and among *Dugesia* species using next-generation sequencing technologies.

- To analyse the intraindividual genetic footprint that fissiparous reproduction leaves in *D. subtentaculata*, both when it is the only mode of reproduction and when it is combined with sex.

- To analyse the molecular, morphological, and karyological characteristics of the different populations of *D. subtentaculata* and of the four *Schmidtea* species; and evaluate them under an evolutionary and a taxonomic point of view.

- To analyse the historical processes that may have shaped the genetic diversification of *Schmidtea* and of the different *Dugesia* species that inhabit the Western Mediterranean region, focussing on the effect of the paleogeographic and paleoclimatic events.

Publications

Supervisors report

Dr. Marta Riutort León and Dr. Miquel Vila Farré, supervisors of the doctoral thesis prepared by Laia Leria Florensa, entitled *Understanding the processes that shape the genetic diversity of freshwater planarians (Platyhelminthes, Tricladida, Dugesiidae)*, report that the thesis is made as a compendium of four publications with original data, which correspond to the chapters 1, 2, 3, and 4 of the thesis:

Article 1

Laia Leria, Miquel Vila-Farré, Eduard Solà & Marta Riutort (2019). Outstanding intraindividual genetic diversity in fissiparous planarians (Dugesia, Platyhelminthes) with facultative sex. *BMC Evolutionary Biology* 19(1): 130.

Impact factor: 3.045 **Rank:** Q2 in the category Genetics & Heredity

Article 2

Laia Leria, Miquel Vila-Farré, Marta Álvarez-Presas, Alejandro Sánchez-Gracia, Julio Rozas, Ronald Sluys & Marta Riutort (2019). Cryptic species delineation in freshwater planarians of the genus *Dugesia* (Platyhelminthes, Tricladida): Extreme intraindividual genetic diversity, morphological stasis, and karyological variability. *Molecular Phylogenetics and Evolution* published on line April 2019.

Impact factor: 3.992 **Rank:** Q1 in the category Evolutionary Biology

Article 3

Laia Leria, Miquel Vila-Farré, Eduard Solà & Marta Riutort (in preparation). New insights into the phylogeographic history of *Dugesia* (Platyhelminthes, Tricladida) freshwater planarians from the Western Mediterranean, with a special focus on *Dugesia subtentaculata*.

The manuscript corresponding to chapter 4 is currently at the last stages of preparation, it is still not decided the journal to which it will be submitted, however it will most probably be a high ranked journal within the area of Evolutionary Biology.

Article 4

Laia Leria, Ronald Sluys & Marta Riutort (2018). Diversification and biogeographic history of the Western Palearctic freshwater flatworm genus Schmidtea (Tricladida: Dugesiidae), with a redescription of *Schmidtea nova*. *Journal of Zoological Systematics and Evolutionary Research* 56(3): 335-351.

Impact factor: 2.268 **Rank:** Q1 in the category Zoology

Contributions of the candidate to the articles

The doctoral student participated in all the samplings of *Dugesia* species' populations (articles 1-3). She obtained the molecular data for all the articles, from extracting DNA to the sequencing step, and performed the phylogenetic, biogeographic, and species delimitation analyses of articles 2-4. Also performed the cloning experiments and all the analyses presented in article 1. In article 2 she was in charge of the bioinformatic search of new molecular markers. She prepared the samples for morphological analyses, obtained the sections and observed them at the microscope to reconstruct the anatomical structures for the species descriptions in article 2, as well as performed the karyotyping experiments presented in articles 2 and 4.

Finally, she wrote the initial draft of the manuscripts of all articles, and participated actively in all the process of writing them till their final version. The work presented in this thesis has not been used, implicitly or explicitly, for the preparation of another thesis.

Barcelona,

Signed: Marta Riutort León Miquel Vila-Farré

Chapter 1

Outstanding intraindividual genetic diversity in fissiparous planarians (*Dugesia*, Platyhelminthes) with facultative sex

Reference

Leria, L., Vila-Farré, M., Solà, E., & Riutort, M. (2019). **Outstanding intraindividual genetic diversity in fissiparous planarians (*Dugesia*, Platyhelminthes) with facultative sex.** *BMC Evolutionary Biology*, 19(1), 130.

Leria et al. BMC Evolutionary Biology (2019) 19:130
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BMC Evolutionary Biology

RESEARCH ARTICLE Open Access

Outstanding intraindividual genetic diversity in fissiparous planarians (*Dugesia*, Platyhelminthes) with facultative sex

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Abstract

Background: Predicted genetic consequences of asexuality include high intraindividual genetic diversity (i.e., the Meselson effect) and accumulation of deleterious mutations (i.e., Muller's Ratchet), among others. These consequences have been largely studied in parthenogenetic organisms, but studies on fissiparous species are scarce. Differing from parthenogens, fissiparous organisms inherit part of the soma of the progenitor, including somatic mutations. Thus, in the long term, fissiparous reproduction may also result in genetic mosaicism, besides the presence of the Meselson effect and Muller's Ratchet. Dugesiidae planarians show outstanding regeneration capabilities, allowing them to naturally reproduce by fission, either strictly or combined with sex (facultative). Therefore, they are an ideal model to analyze the genetic footprint of fissiparous reproduction, both when it is alternated with sex and when it is the only mode of reproduction.

Results: In the present study, we generate and analyze intraindividual cloned data of a nuclear and a mitochondrial gene of sexual, fissiparous and facultative wild populations of the species *Dugesia subtentaculata*. We find that most individuals, independently of their reproductive strategy, are mosaics. However, the intraindividual haplotype and nucleotide diversity of fissiparous and facultative individuals is significantly higher than in sexual individuals, with no signs of Muller's Ratchet. Finally, we also find that this high intraindividual genetic diversity of fissiparous and facultative individuals is composed by different combinations of ancestral and derived haplotypes of the species.

Conclusions: The intraindividual analyses of genetic diversity point out that fissiparous reproduction leaves a very special genetic footprint in individuals, characterized by mosaicism combined with the Meselson effect (named in the present study as the *mosaic Meselson effect*). Interestingly, the different intraindividual combinations of ancestral and derivate genetic diversity indicate that haplotypes generated during periods of fissiparous reproduction can be also transmitted to the progeny through sexual events, resulting in offspring showing a wide range of genetic diversity and putatively allowing purifying selection to act at both intraindividual and individual level. Further investigations, using *Dugesia* planarians as model organisms, would be of great value to delve into this new model of genetic evolution by the combination of fission and sex.

Keywords: Facultative sex, Fissiparous reproduction, Meselson effect, Mosaicism, Muller's ratchet, Multilevel selection

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Background

The fitness of an individual and its lineage largely depends on the number and viability of the offspring produced during its lifetime. In turn, the genetic background of offspring has a major role in their survival and adaptation, for example, when facing population bottlenecks or in the face of environmental changes [1, 2]. Thus, the reproductive strategy and how it shapes the genetic background of the offspring represents a key life history trait to understand how lineages survive in the wild and why some populations are maintained while others become extinct.

Sexual reproduction can generate new allelic combinations in the populations through recombination and outcrossing, which can be either favored by selection or selected against [3, 4]. This can potentially accelerate the evolutionary processes, promoting the genetic diversification of the populations [5, 6]. Asexual reproduction, on the other hand, is characterized by the production of descendants that are genetically highly similar to their progenitor, due to the absence of recombination and outcrossing. For this reason, at first, it was assumed that asexual species would show low levels of genetic diversity, both at the intraindividual level (heterozygosity) and between different individuals (from the same or from different populations). Nevertheless, unsuspected genetic variation at these two levels has been found in different asexual taxa. On the one hand, genetic variation between different individuals (within and between populations) has been attributed either to their recurrent origin from sexual lineages or to demographic expansions [7, 8]. On the other hand, genetic diversity of asexual species at the intraindividual level has been attributed to hybridization processes [9] or to the independent accumulation of mutations in the homologous alleles over generations in the absence of recombination and out-crossing (i.e., Meselson effect) [10–12]. Moreover, it has been proposed that long-lasting asexuality can promote an increased number of slightly deleterious mutations as a consequence of relaxed selection (the physical linkage among loci hinders selection's ability to act upon loci independently), which in the long term can cause detrimental effects on the populations (i.e., Muller's ratchet) [13–17]. However, most of these studies have been performed in parthenogenetic asexual organisms, while clonal reproduction by some type of fissioning is rarely considered, although this type of reproduction is known to exist in most phyla within metazoans [18].

Differing from sexual and parthenogenetic individuals, a zygotic bottleneck is absent in fissiparous organisms, and descendants inherit part of the soma of the progenitor, including somatic mutations. This adds a level of complexity since, in the long term, fissiparous individuals are predicted to show high levels of genetic mosaicism [19, 20], in addition to the possible occurrence of the Meselson effect and Muller's ratchet. Mosaicism associated with clonal reproduction has long been known to occur in plants [21], but its existence in fissiparous metazoans has only been demonstrated in colonial corals at the intracolonial level [22]. Therefore, we not only miss a confirmed example of mosaicism in noncolonial fissiparous metazoans in natural conditions but also its characterization regarding the possible occurrence of the Meselson effect and Muller's ratchet.

Planarians of the family Dugesiidae (Tricladida, Platyhelminthes) show outstanding regeneration capabilities among the metazoans [23]. Species such as *Schmidtea mediterranea* or several *Dugesia* species are indeed masters of regeneration [24, 25]. The only stem cells in the adult planarians are the neoblasts, distributed throughout most of their parenchyma (i.e., the connective tissue that fills the space between organs) and representing ~25–30% of all planarian cells [26, 27]. Neoblasts are the only cells that divide mitotically and hence are responsible for all the cell and tissue renewal during regeneration and homeostasis [28, 29]. These extraordinary regeneration capabilities of planarians, due to neoblast activity, allow some species or some populations within a species to naturally reproduce by fission. Fissiparous individuals do not develop a reproductive system. Instead, they produce new individuals by performing a binary fission and subsequently regenerating the missing body parts (Fig. 1a). Therefore, fissiparous individuals need to rebuild all the lost structures and regain the original body proportions during each reproductive event. This process implies extensive body remodeling and neoblast migration and proliferation that, together with the animal's longevity (they are theoretically immortal), opens the opportunity to amplify mutated neoblasts.

Therefore, besides being usually sexual, planarians can also reproduce asexually either by fission or by parthenogenesis, resulting in a group with an astonishing diversity of reproductive modes. Sexual individuals are simultaneous hermaphrodites (i.e., each individual possesses the entire set of male and female reproductive organs) (Fig. 1b). In general, sexual individuals are diploid and perform gametogenesis through normal meiosis from differentiated germ cells, which are in the ovaries and in the testes. Sexual individuals exhibit mutual insemination during copula and after fertilization, fertilized eggs and yolk cells are encapsulated into a cocoon, which is expelled through the gonopore and, a few weeks later, results in a variable number of juveniles hatching (Fig. 1b) [30]. Parthenogenetic individuals, on the other hand, are simultaneous hermaphrodites that need sperm to trigger the development of the zygote, without contributing its genetic content [31]. In general, asexual reproduction in planarians (either by fissiparity or by parthenogenesis) is linked to polyploidy and to chromosomal rearrangements [31]. Interestingly,

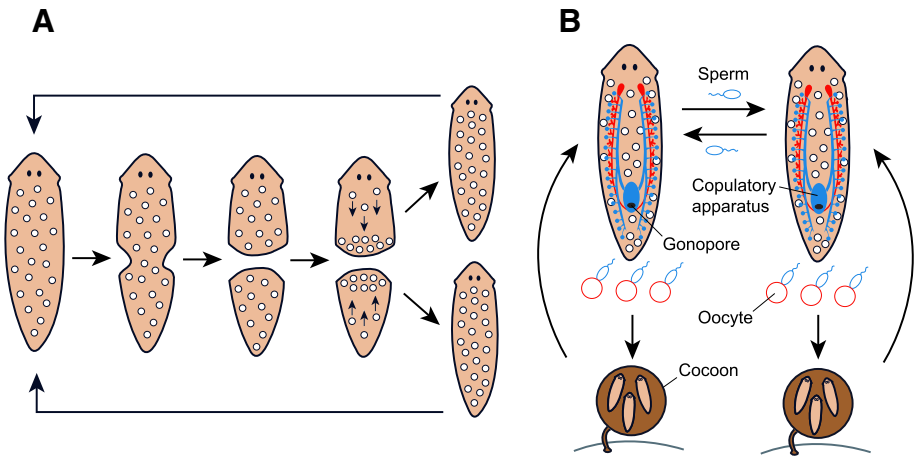

Fig. 1 Schematic representation of fissiparous (**a**) and sexual (**b**) reproduction in planarians. **a** Fissiparous individuals do not develop the reproductive system and instead they reproduce by binary fission. After the fission process, the planarian stem cells or neoblasts (white dots) are recruited to the wound and regenerate the missing part of the individual through proliferation and differentiation. **b** Sexual individuals are hermaphrodites that show the full set of male (blue) and female (red) reproductive organs. In this case, the differentiated germ cells located in the ovaries and in the testes are the responsible for gamete production, while neoblasts of sexual individuals (white dots) do not participate in the reproductive process. After the cross-fertilization by the exchange of sperm, several embryos are encapsulated into a cocoon, which will hatch after few weeks from the oviposition

these reproductive modes can operate either in different species, in different populations of the same species, or even in the same individual (facultative reproduction).

Facultative reproduction in the genus *Schmidtea* involves the alternation of parthenogenesis and sexual reproduction [32], while facultative individuals of *Dugesia* alternate fissiparity with sex ([33], and references therein). It could be thought that triploid facultative *Dugesia* individuals may in fact alternate fission with parthenogenesis, due to the disadvantages of polyploids during meiotic processes [34]. However, it has been demonstrated that triploid facultative *Dugesia* individuals can reproduce truly sexually through a special meiotic system [35]. These triploid facultative individuals are able to produce recombinant haploid sperm and recombinant diploid and haploid oocytes. Importantly, it has been shown that fissiparous planarians do not have a differentiated germline and thus, during the process of sexualization, the germline needs to be newly differentiated from neoblasts [36–38]. Therefore, in facultative *Dugesia*, somatic genetic diversity generated during periods of fissiparous reproduction could putatively be transmitted to descendants through sex.

An example of a species showing the whole variety of reproductive strategies (sexual, fissiparous and facultative) is *Dugesia subtentaculata*. At first, only strictly sexual (diploid, 2n = 16) and strictly fissiparous populations (triploid, 3n = 24) were known [39–41]. However, an extensive sampling across all its distributional range has resulted in the detection of not only more sexual and fissiparous populations, but also in many populations showing both sexual and fissiparous individuals [42]. A priori, these populations could be either a mix of strictly sexual and strictly fissiparous individuals or, could be constituted by facultative individuals (individuals that alternate between both types of reproduction). Whether they represent one or the other case can be genetically tested, in the first case we will expect to find two independent lineages in the populations, while in the second only a genetic lineage will be found. This species is therefore a potentially ideal model to analyze the genetic footprint that fissiparous reproduction leaves in organisms, and potentially also when it is combined with sex (provided that our genetic analyses demonstrate that the mixed populations bear a single genetic lineage and hence are facultative).

Here, we analyze the intraindividual genetic diversity, by cloning PCR products of two molecular markers (one mitochondrial and one nuclear), of individuals coming from a total of 10 natural populations of *D. subtentaculata* showing either sexual, fissiparous or putative-facultative reproductive strategies, to investigate the following predictions under an evolutionary framework: (1) the existence of high levels of mosaicism in purely fissiparous individuals as well as the absence of mosaicism in purely sexual individuals, (2) the existence of the Meselson effect and Muller's ratchet in fissiparous individuals and their absence in purely sexual ones, and (3) if putative-facultative populations prove to be facultative, we would expect to find in their individuals a characteristic genetic pattern different from that in exclusively sexual or fissiparous populations.

Our results demonstrate in the first place that individuals from the populations bearing sexual and fissiparous

individuals are facultative. Moreover, the data obtained provide evidence for the existence of mosaicism in freshwater planarians accompanied by the Meselson effect in fissiparous and facultative individuals, but with no Muller's ratchet. Additionally, our results point out that the combination of fissiparous reproduction with occasional sex results in an efficient way of generating high levels of genetic diversity controlled by selection that may act at two different levels (intraindividual and individual), which represents an entirely novel model of genetic evolution among metazoans.

Methods

Sampling

We studied a total of 10 natural populations of *D. subtentaculata*, 5 sexual, 2 facultative and 3 fissiparous, covering almost the maximum area of distribution of the species, which includes Southern France, the Iberian Peninsula, Mallorca (Balearic Islands) and Northern Africa (Fig. 2a; see Additional file 1: Table S1). An average of 15 individuals per population were collected and observed under the stereomicroscope under field conditions or shortly after. Sexual individuals were identified by the presence of a gonopore (external aperture of the copulatory apparatus) (indicated by an S in each individual code) and fissiparous individuals by the occurrence of a blastema (regenerating part of tissue after a process of fission) (indicated by an A in each individual code) (Fig. 2b). The individuals with neither blastema nor gonopore have no assigned identification letter. The populations were classified as sexual if most individuals presented a gonopore and none a blastema, facultative when both individuals with a gonopore and individuals with a blastema were detected, and fissiparous when individuals with a blastema were detected and none had a gonopore. Subsequently, three individuals per population (five in Hortas) were fixed in 100% ethanol for the genetic analysis, while three other individuals were kept alive for the ploidy analysis. In facultative populations, at least one sexual and one fissiparous individual were included in the analyses.

Ploidy identification

The ploidy level of the populations of Calobra, Soller and Santa Fe was extracted from the literature [40], while the ploidy level of the population Bosque was inferred by karyotyping in a companion study [42]. The ploidy level of the rest of the populations was determined by flow cytometry. Since our protocol for flow cytometry requires a high quantity of tissue (Additional file 2: Figure S1) only big sized animals could be used jointly for the ploidy identification and genetic analysis (populations of Alte, Artavia and Hortas). In the rest of populations different individuals were used. Sample preparation for flow cytometry was started by incubating a living individual for 2 min in a solution of 2% N-acetyl-L-cysteine at pH 7 to remove the mucus and thus prevent the formation of cell aggregates in the subsequent steps. Then, the animal was washed using a mixture of tap and distilled water (1:1) and subsequently placed in 1 ml of maceration solution composed of distilled water, glacial acetic acid and glycerol (13:1:1) and incubated for 15 min at room temperature. After the incubation, the cells were separated by gently pipetting using a cut tip and filtered through a nylon mesh with a pore size of 75 μm. Finally, the macerated cell suspension was stained for 5 min with 6 μl of Hoechst (stock 1 mg/ml), and the quantity of DNA was measured with a Gallios Flow Cytometer at the Unitat de Citometria dels Centres Científics i Tecnològics de la UB (CCiT, UB). To characterize the ploidy of an individual

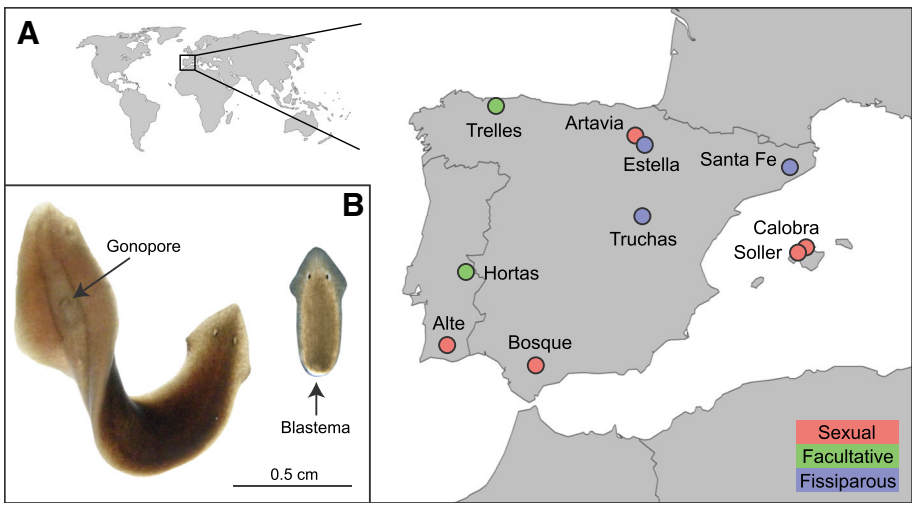

Fig. 2 Distribution map of the samples used in this study (**a**) and appearance of an individual reproducing sexually (left) and by fission (right) (**b**). For each locality, its code name and the reproductive strategy of the population are indicated

by flow cytometry, we first measured the cell suspension of that sample and counted the peaks observed (indicator of cell populations with different DNA content) and the fluorescence mode value of each peak. Then, we measured and annotated the same parameters for a cell suspension corresponding to a triploid control individual from the well-characterized population of Santa Fe del Montseny (Catalonia) [40], which we also karyotyped to verify its published ploidy. Finally, we analyzed a mix of the two suspensions thus allowing a direct comparison of both (Additional file 2: Figure S1). We used the latter values to infer the ploidy of the query individuals. This approach was conducted to avoid putative differences in the fluorescence values between samples due to slightly differences in the pH or in the duration of the staining.

DNA sequence data

Individual total genomic DNA was extracted from the 32 ethanol-fixed specimens using the commercial reagent DNAzol (Molecular Research Center Inc., Cincinnati, OH) by following the manufacturer's instructions. Two genomic regions were PCR-amplified for all the individuals: a fragment of the nuclear gene *Transmembrane p24 trafficking protein 9* (TMED9) and a fragment of the mitochondrial gene *Cytochrome oxidase subunit 1* (Cox1). The TMED9 gene was selected because it is a single copy gene in the species *Schmidtea mediterranea* containing a long intronic region (total amplified exonic region: 197 bp; total amplified intronic region: 751 bp) [43, 44]. The conditions for the PCR reactions for Cox1 were as previously published [45]. The amplification conditions for TMED9 were the following: 1) 2 min at 94 °C, 2) 45 s at 94 °C, 3) 50 s at 58 °C, 4) 40 s at 72 °C and 5) 3 min at 72 °C. Steps 2, 3 and 4 were run for 35 cycles. The primer sequences used to amplify each molecular marker are detailed in Additional file 1 Table S2.

All the PCR products were purified using a vacuum system (MultiScreen™ HTS Vacuum Manifold, Millipore Corporation, Billerica, MA, USA) and subsequently cloned using an HTP TOPO TA Cloning Kit for sequencing (Invitrogen, California, USA) following the manufacturers' protocols. At least fifteen colonies per individual were amplified using the universal T3 and T7 primers. The sequencing reactions were run either in an automated sequencer (ABI Prism 3730) by the Unitat de Genòmica of Centres Científics i Tecnològics of the Universitat de Barcelona (CCiT, UB) or by Macrogen Corporation (Amsterdam, the Netherlands) using the same universal primers. Complementary strands of DNA were edited and assembled using Geneious version 10 [46].

Sequence alignments and datasets

We aligned the sequences obtained for both genes at the nucleotide level using the online software MAFFT version 7 [47]. The alignments were cut at the same length using Geneious in order not to include missing data. The exonic and intronic regions of TMED9 were identified by comparing the gene with that of the annotated genome of *Schmidtea mediterranea* available online in SmedGD datababe [43, 48]. The reading frame of Cox1 and the coding regions of TMED9 were checked by translating the nucleotides into amino acids in Geneious. For Cox1 we used the GenBank genetic code Table 9 (mitochondrial echinoderm), while for TMED9 we used the genetic code Table 1 from GenBank (standard).

In a cloning experiment, DNA polymerases can introduce errors in the sequences during the first PCR (amplification of the target gene for each individual), during the cloning PCR (amplification of each clone), or in the sequencing reaction. Errors due to polymerase mistakes during the cloning PCR or due to sequencing errors can be generally detected as double peaks in the chromatograms. However, polymerase mistakes during the first PCR cannot be detected in the chromatograms. To evaluate the impact of polymerase errors in our data, we calculated the average number of mutations per haplotype that could be due to polymerase errors during the first PCR of the cloning process using the error rate of *Taq* DNA polymerase (2.28×10^{-5}) implemented in the PCR fidelity calculator web tool provided by Thermo Fisher [49]. For the nuclear marker TMED9, it was estimated that 75.65% of the PCR products would contain a single error due to polymerase mistakes, while for Cox1 only 59.18% of the PCR products would have a single error. To mitigate the effects of those artifact mutations, as recommended ([50], and references therein), we identified the singleton sequences of each individual that were separated by a single point mutation from other nonsingleton sequences and recoded them as the latter (Additional file 2 Figure S2). For the nuclear gene, we identified an average of 5 sequences per individual as being possible results of polymerase errors. Therefore, we recoded them. In the case of Cox1, only an average of 3 sequences per individual were identified as being a possible result of polymerase errors and were subsequently recoded.

Intraindividual genetic diversity and effect of selection

We calculated the intraindividual number of different TMED9 alleles and Cox1 haplotypes using the program DnaSP v5 [51]. We also used DnaSP v5 to calculate the intraindividual genetic diversity at both the haplotype (H_D) and nucleotide levels (π) for the two gene fragments and the intraindividual proportion of synonymous mutations (Ks), nonsynonymous mutations (Ka) and the ratio Ka/Ks (Ω). To test for significant differences in the

Table 1 Intraindividual number of cloned sequences and different haplotypes obtained for the two molecular markers

	TMED9		Cox1	
	N	h	N	h
Sexual				
Calobra1S	15	2	14	2
Calobra2S	15	6	8	2
Calobra3S	15	7	15	1
Soller1S	14	8	10	1
Soller2S	13	6	21	2
Soller3S	15	8	15	4
Bosque1S	15	6	12	2
Bosque2S	15	6	12	2
Bosque3S	15	6	14	3
Alte1	13	4	11	2
Alte2S	14	6	9	2
Alte3S	12	6	13	2
Artavia1S	11	7	7	1
Artavia2S	13	5	13	1
Artavia3S	15	6	14	2
Σ = 15	$\bar{x}$=14	$\bar{x}$=6	$\bar{x}$=13	$\bar{x}$=2
Facultative				
Hortas1A	15	10	7	1
Hortas2A	15	9	6	3
Hortas3S	12	8	5	1
Hortas4S	15	5	7	4
Hortas5S	14	8	14	3
Trelles1A	14	9	12	4
Trelles2	14	11	13	7
Trelles3S	15	12	13	5
Σ = 8	$\bar{x}$=14	$\bar{x}$=9	$\bar{x}$=10	$\bar{x}$=4
Fissiparous				
SantaFe1A	28	16	22	3
SantaFe2A	13	7	6	1
SantaFe3A	8	6	12	3
Truchas1A	14	8	15	6
Truchas2	12	12	13	7
Truchas3	14	7	13	3
Estella1A	12	9	4	1
Estella2A	13	9	3	1
Estella3	15	8	4	1
Σ = 9	$\bar{x}$=14	$\bar{x}$=9	$\bar{x}$=10	$\bar{x}$=3

N: number of cloned sequences per individual
h: number of obtained haplotypes per individual

estimates of genetic diversity and selection parameters between individuals depending on their reproductive strategy, we conducted an analysis of variance for each estimated parameter using the program Past3 [52]. We used one-way ANOVA Tests followed by Tukey's pairwise comparison for the normal variables, the nonparametric Kruskal–Wallis test followed by Dunn's post hoc pairwise comparison for the non-normal variables and Welch's F test for variables with unequal variances. All *p*-values obtained in the pairwise comparisons were corrected for multiple testing.

An analysis of molecular variation (AMOVA) was performed to see how the genetic variation was partitioned within the different reproductive strategies. AMOVA was conducted with the software ARLEQUIN 3.5.2 [53] using pairwise differences with 10,000 permutations and leaving the rest of the parameters at their defaults. We quantified how much variation was explained within the different reproductive strategies: (1) between populations, (2) between individuals within the same population, and (3) within individuals.

Phylogenetic reconstructions and haplotype networks

We took two different approaches to analyze the genetic data under an evolutionary framework: phylogenetic inferences and haplotype networks. Phylogenetic reconstructions were used to give directionality to the evolutionary processes, while haplotype networks were used to study the relationship between the alleles (this last approach is especially suitable when diversification has occurred in a short period of time and both ancestral and descendant haplotypes exist at the same time). We inferred the haplotype networks for the two genes for each individual and at the species level using the program Network version 4.6 [54]. We first imported separately the alignments of each gene into DnaSP v5 to convert them into Roehl files to be later processed in Network. The networks were constructed using the median-joining method [55], taking into account the minimum-length connections between the sequences (ε parameter equal to zero).

We inferred the phylogeny of each gene using Bayesian inference (BI) and maximum likelihood (ML) methods. Two *Dugesia* species phylogenetically close to *D. subtentaculata* [41] were used as the outgroup: *D. hepta* Pala, Cassu & Vacca, 1981 and *D. benazzii* Lepori, 1951. The degree of saturation of each alignment was assessed with the software Dambe [56] using a test of substitution saturation [57, 58], which resulted in no saturation in either of the two molecular markers, as the index of saturation in both cases was significantly lower than the index of critical saturation (TMED9: Iss = 0.192, Iss.c = 0.820, *p*-value< 0.01; Cox1: Iss = 0.129, Iss.c = 0.804, p-value< 0.01). The best substitution model for each analysis was determined using jModelTest2 [59]. The Bayesian analyses were conducted with

the program MrBayes 3.2 [60] with two runs of 5,000,000 generations with four chains and sampling at intervals of 2000 generations each. Convergence of the Markov chain Monte Carlo (MCMC) chains for the two runs was confirmed after checking that the standard deviation of split- frequencies reached a value below 0.01. To infer the best tree and posterior probabilities, the default burn-in of 25% was used after checking that the two runs had reached the stationary phase. The maximum likelihood phylogenetic inference was conducted using the program RaxML 7.0.3 [61]. Two independent analyses were performed with different strategies to obtain the support for the nodes, one using the rapid bootstrap algorithm with 2000 replicates and another one using the standard bootstrap algorithm with 1000 replicates.

Results

Ploidy level of the populations

The results of previous works showed that sexual populations of Soller, Calobra and Bosque were diploid, while the fissiparous population of SantaFe was triploid [40, 42]. The ploidy level inference for the rest of populations using flow cytometry resulted in the detection of different ploidies. The three analyzed individuals of the sexual population of Alte were diploid, while the three analyzed individuals of the sexual population of Artavia were triploid (Additional file 1: Table S3). The four analyzed individuals of the facultative population of Trelles were triploids, while in the facultative population of Hortas we found one diploid and one triploid individual (Hortas3S and Hortas4S, respectively). Finally, the five analyzed individuals from the fissiparous population of Estella were tetraploids, while the two analyzed individuals from the fissiparous population of Truchas were mixoploids (combining approximately a 35% of triploid cells and a 65% of tetraploid cells) (see Additional file 1: Table S3).

Intraindividual genetic diversity

An average of 14 and 12 intraindividual sequences were obtained for the nuclear and the mitochondrial marker, respectively (Table 1), representing a total of 810 sequences analyzed. The 453 sequences obtained for the nuclear marker were 948 bp in length, while the 357 sequences obtained for the mitochondrial gene were 649 bp in length. The analyses performed with the program DnaSP v5 revealed a total of 209 different alleles for the nuclear gene and 52 different haplotypes for the mitochondrial gene for all the individuals analyzed in the present study (GenBank accession numbers in Additional file 1: Table S4 and Table S5). The number of different intraindividual nuclear alleles varied from 2 to 8 in individuals from sexual populations, from 5 to 12 in individuals from facultative populations and from 6 to 16 in individuals from fissiparous populations (Table 1). For the mitochondrial gene, the number of different intraindividual haplotypes varied from 1 to 4 in individuals from sexual populations and from 1 to 7 in the rest of the individuals (Table 1).

The intraindividual nuclear haplotype networks showed that 14 out of the 15 analyzed individuals from sexual populations presented a star-like pattern, consisting of one or two majoritarian alleles from which other, minority and closely related ones originated (Fig. 3a). On the other hand, 15 out of the 17 analyzed individuals from fissiparous and facultative populations showed a divergent pattern, consisting of many distantly related alleles occurring at similar frequencies (Fig. 3a). The star-like pattern of sexual individuals for the nuclear gene was characterized by statistically significantly lower H_D and π values compared to the divergent pattern of fissiparous and facultative individuals (Fig. 4a; see Additional file 1: Table S6 and Table S7).

At mitochondrial level, 12 of the analyzed sexual individuals exhibited a star-like pattern similar to that for the nuclear gene, except for three of them (Soller2S, Soller3S and Bosque3S) that showed a minority haplotype highly differentiated from the majority one (Fig. 3b). On the other hand, the six analyzed individuals from the fissiparous populations of Santa Fe and Estella plus three individuals of the facultative population of Hortas (Hortas1A, Hortas2A and Hortas3S) showed a star-like intraindividual pattern (like sexual individuals), while the three individuals from the fissiparous population of Truchas and the three individuals from the facultative population of Trelles showed a divergent pattern (Fig. 3b). The statistical comparisons of haplotype and nucleotide diversity between the different reproductive strategies at mitochondrial level showed that there were only significant differences in individuals from facultative populations compared to individuals from sexual populations (Fig. 4b; see Additional file 1: Table S6 and Table S7).

Finally, the intraindividual pattern of facultative populations either at nuclear or mitochondrial level was not correlated with the type of reproduction that the individuals showed at the moment of collection, since we found individuals reproducing sexually with a divergent pattern (e.g., Hortas5S) and fissiparous individuals with a star-like pattern (e.g., Hortas2A), and the other way around (Fig. 3).

Analysis of molecular variation

The analysis of molecular variation (AMOVA) showed that the genetic variation in sexual populations was mostly explained by differences between populations (94% for the nuclear gene, 98% for the mitochondrial gene) (Fig. 5a). In facultative populations, half of the genetic variation was explained by differences within individuals (in both genes),

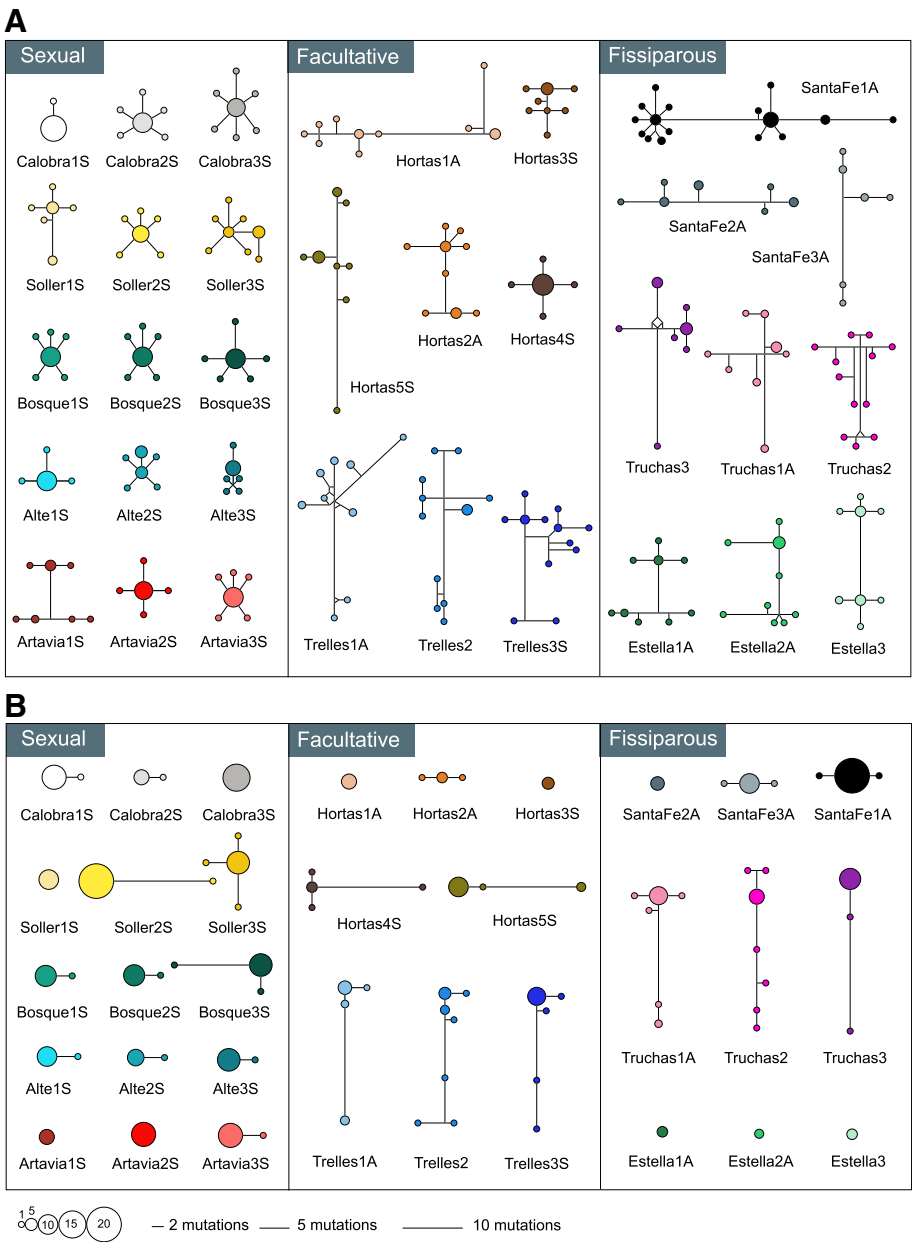

Fig. 3 Intraindividual haplotype networks of the nuclear gene (**a**) and the mitochondrial gene (**b**). Each individual is depicted with a different color, and the code of the individual is given under its network. Each circle represents a different haplotype, and the size of the circle indicates the frequency of each haplotype within the individual. Branch lengths are proportional to the number of mutations

while the rest of the genetic variation was explained by differences between populations and differences between individuals of the same population, in different proportions depending on the gene (Fig. 5b). Finally, the genetic variation in fissiparous populations was explained both by differences between populations and differences within individuals, in a different proportion depending on the gene. In the case of the nuclear gene a 77.5% of the genetic variation was explained by differences within individuals, while in the case of the mitochondrial gene a 88% of the genetic variation was explained by differences between populations (Fig. 5c).

Effect of selection

The proportion of nonsynonymous mutations (Ka) for both genes within each individual was extremely low and not significantly different between the individuals of the three reproductive strategies when comparing the mean values obtained per reproductive strategy (Fig. 6a; see Additional file 1: Table S6 and Table S7). However, the mean

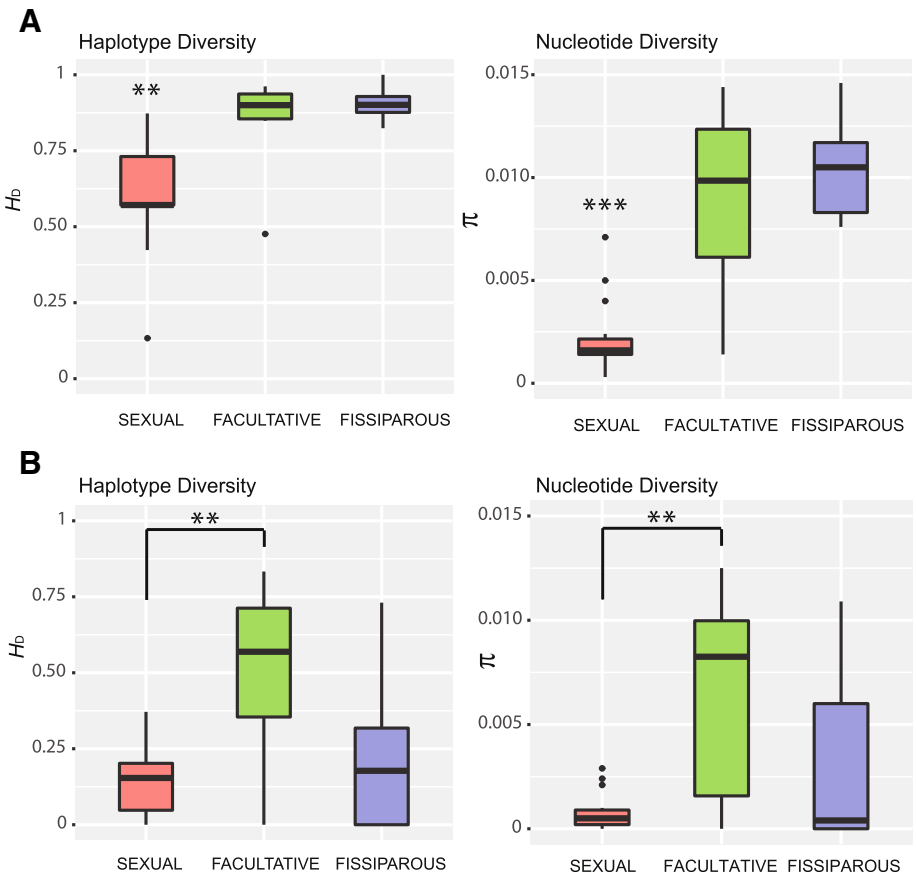

Fig. 4 Graphical representation of the intraindividual haplotype and nucleotide diversity for each reproductive strategy, for the nuclear gene (**a**) and for the mitochondrial gene (**b**). Boxes are delimited by the first and third quartiles, and the median is represented by a thick line. Whiskers delimit the minimum and maximum nonoutlier values. Outlier values are represented by black dots. Asterisks indicate significant differences in the statistical comparison between reproductive strategies (*$p = 0.01–0.05$; ** $p = 0.001–0.01$; *** $p < 0.001$). See Additional file 1: Table S6 for genetic diversity values and Additional file 1: Table S7 for statistical comparisons

intraindividual proportion of synonymous mutations (Ks) of the nuclear gene was significantly lower in individuals from sexual populations than in those from fissiparous and facultative populations (Fig. 6b; see Additional file 1: Table S6 and Table S7), an expected result given the low intraindividual π detected in this reproductive strategy. In the case of the mitochondrial gene, both individuals from sexual and fissiparous populations showed a significantly lower proportion of synonymous mutations (Ks) than individuals from facultative populations (Fig. 6b; see Additional file 1: Table S6 and Table S7). Finally, we found that there were no significant differences in the mean Ω value between the different reproductive strategies in any of the two gene fragments, which in all cases was lower than 1 (Fig. 6c; see Additional file 1: Table S6 and Table S7).

Phylogenetic inferences and species haplotype networks

The phylogenetic analyses performed with Bayesian Inference and Maximum Likelihood (for both bootstrapping algorithms) yielded the same topology for each molecular marker (the supports for ML only varied to slightly higher supports when the rapid bootstrap strategy with more replicates was used). The sexual populations of Bosque, Calobra and Soller were recovered as the first to differ, forming three monophyletic clades (named as Sexual clades I, II and III, respectively) that were highly differentiated between themselves and from the rest of populations (see Additional file 2: Figure S3 and Figure S4). Their order of appearance remained elusive, as it was different in both genes and not fully supported in any of them. On the other hand, all fissiparous and facultative populations together with the sexual populations of Alte and Artavia conformed a derived and highly supported monophyletic group (referred from now on as Mixed clade), irrespective of the gene analyzed. Moreover, the nuclear genetic diversity of the Mixed clade was distributed into four main clades (Clades A, B, C and D), while its mitochondrial genetic diversity was distributed into six main clades (Clades 1, 2, 3, 4, 5 and 6) (see Additional file 2: Figure S3 and Figure S4).

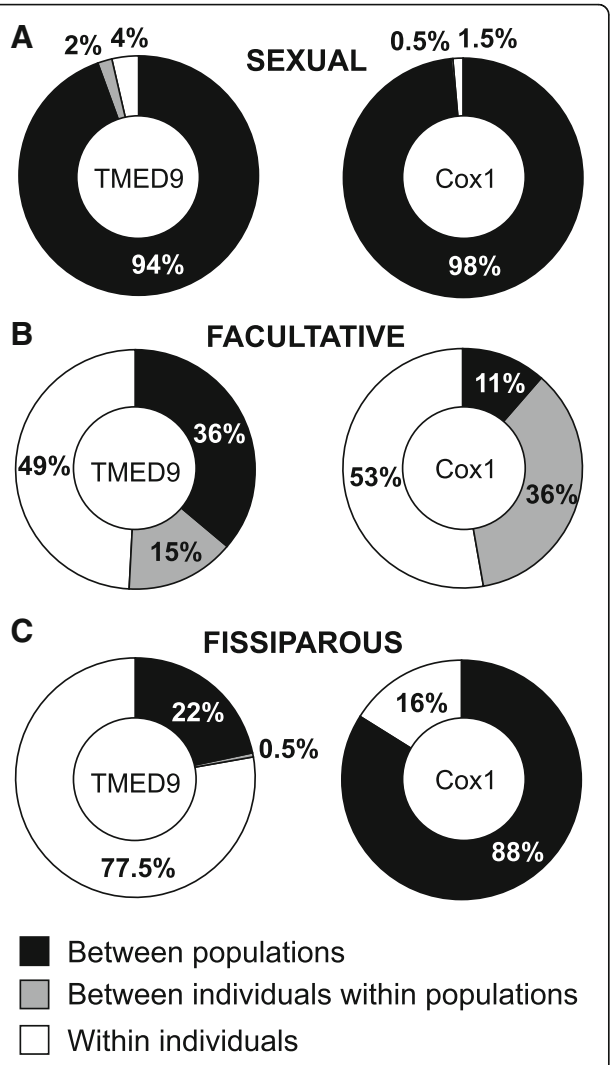

Fig. 5 Graphical representation of the analysis of molecular variance (AMOVA) for each reproductive strategy and gene. Coloured squares indicate the percentage of genetic variation that is explained within each reproductive strategy; between populations, between individuals within populations and within individuals

The species haplotype networks recovered the same main clades than the phylogenetic reconstructions (Fig. 7). They showed that while for the Sexual clades I, II and III ancestral alleles and haplotypes were lost, in the Mixed clade both derived and ancestral were present. For instance, in TMED9, alleles of clade D derived from certain alleles of C, and those of C derived from certain alleles of clade B (Fig. 7a). In the case of Cox1, haplotypes from clade 5 derived from the majoritarian haplotype of clade 6 (C-7, Fig. 7b), while clades 1, 2, 3 and 4 derived from a common ancestor with clade 6 (Fig. 7b). The two sexual populations within the Mixed clade (Alte and Artavia) only showed derived alleles and haplotypes. We found that the genetic diversity of all fissiparous and facultative individuals was distributed in both ancestral and derived clades at least in one of the two molecular markers (Fig. 7). Moreover, the derived mitochondrial genetic diversity was private of each population (the only haplotype shared between populations was the haplotype C-7 of clade 6), while both ancestral and derived nuclear genetic diversity was shared between individuals of different populations (Fig. 7). However, in the case of the nuclear marker, a maximum number of three alleles was shared between individuals of different fissiparous and facultative populations, while the number of shared alleles between the individuals of the same populations could be higher.

Importantly, the fact that both sexual and fissiparous individuals of each facultative population share the same ancestral and derived genetic diversity shows that they belong to a single genetic lineage, as expected for a population constituted by facultative individuals. This fact, together with our previous finding that some sexual individuals in these populations show a divergent haplotype pattern, demonstrate that these populations are actually facultative populations.

Discussion

High tissue turn-over and growth-degrowth dynamics as putative drivers of mosaicism in sexual planarians

Against our theoretical expectations, we found that all individuals from the sexual populations analyzed in the present study (excepting the individual Calobra1S) showed a higher number of nuclear alleles than those expected for their ploidy level, indicating that they are mosaics. Differing from most individuals from fissiparous and facultative populations, the genetic pattern of exclusively sexual individuals was characterized at both nuclear and mitochondrial level by one majority allele (generally shared with the other members of the population) and few derived low frequency alleles (private of each individual). This pattern indicates that the majoritarian allele of each individual was most probably sexually inherited, while the rest of low frequency alleles may be the result of somatic mutations.

It could be possible that some of the low-frequency alleles resulted from polymerase errors during the PCR reaction. Based on the error rate of the *Taq* polymerase (2.28×10^{-5}), we estimated that a 75% of the alleles could bear one artifactual mutation. Thus, we applied a widely accepted correction method to our dataset to minimize this effect. Nevertheless, we found that most of the cloned alleles of the same PCR product were differentiated by many more mutations than those expected by mistakes of the *Taq* polymerase. This suggests that although certain mutations are probably artificial, most of these low-frequency alleles would have originated due to other factors, such as somatic mutations.

The appearence of somatic mutations during the lifespan of individuals has been detected in several sexual organisms, including humans [62, 63], and it can be due to a

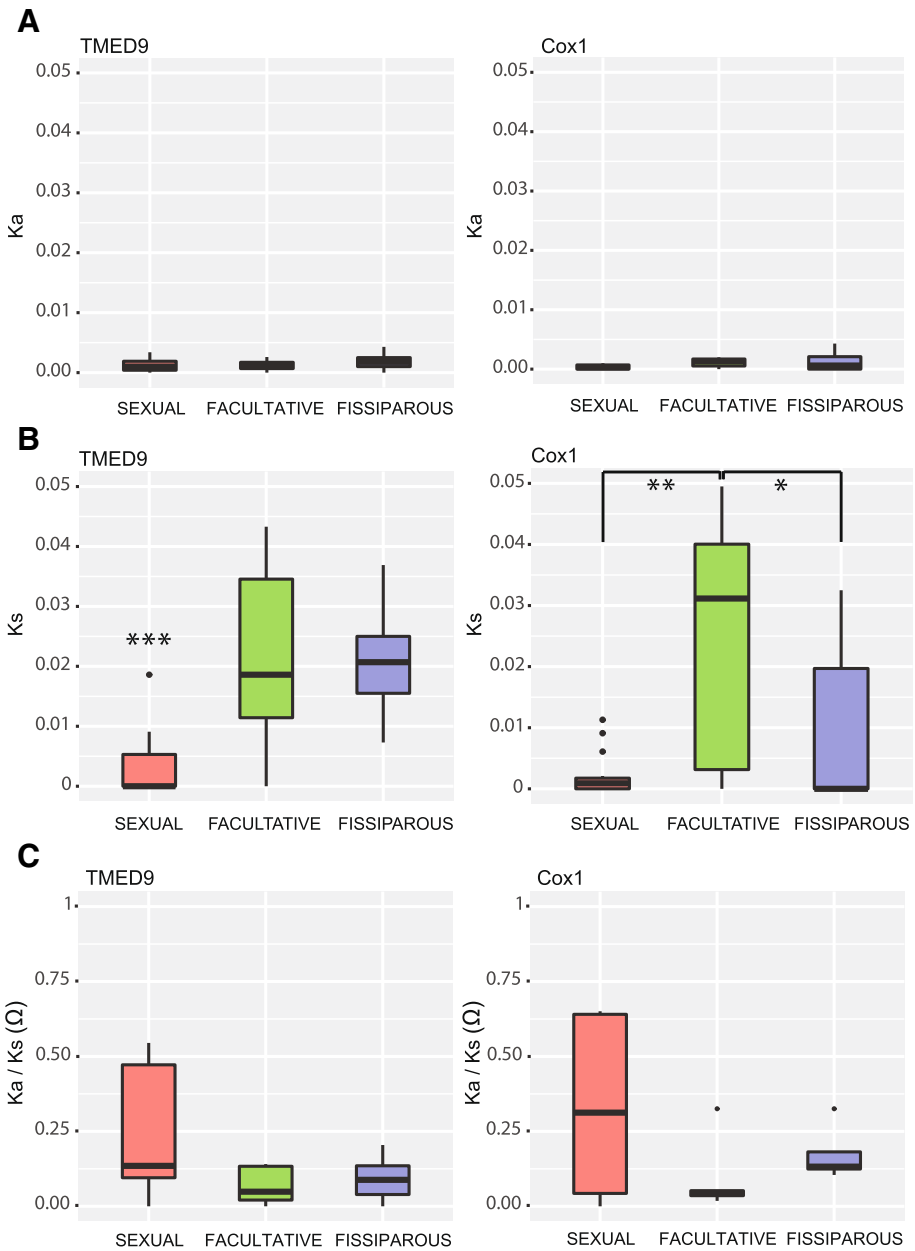

Fig. 6 Graphical representation of the intraindividual proportion of nonsynonymous mutations (**a**), synonymous mutations (**b**), and the ratio Ka/Ks (**c**). Boxes are delimited by the first and third quartiles, and the median is represented by a thick line. Whiskers delimit the minimum and maximum nonoutlier values. Outlier values are represented by black dots. Asterisks indicate significant differences in the statistical comparison between reproductive strategies (*$p = 0.01$ 0.05; ** $p = 0.001$–0.01; *** $p < 0.001$). See TableS6 for Ka and Ks values and Table S7 for statistical comparisons

wide range of causes, such as DNA damage due to environmental factors or replication errors during tissue homeostasis, among others ([64], and references therein). Planarians, moreover, have an extraordinary cell turn-over rate during their normal tissue homeostasis. Neoblast division and differentiation of the neoblast's progeny have been found to occur on an ongoing basis in nongrowing individuals ([65], and references therein). Additionally, conditions of severe starvation induced by the lack of food result in massive degrowth of the planarian body due to an increase in the cell death of differentiated cells. This situation is easily reversed by the recovery of food sources, which results in a neoblast-driven growth of the individual [25, 66]. Therefore, a potentially high tissue turnover, in addition to the growth-degrowth cycles that take place in natural conditions, may be responsible for the intraindividual genetic pattern observed in most of the sexual individuals.

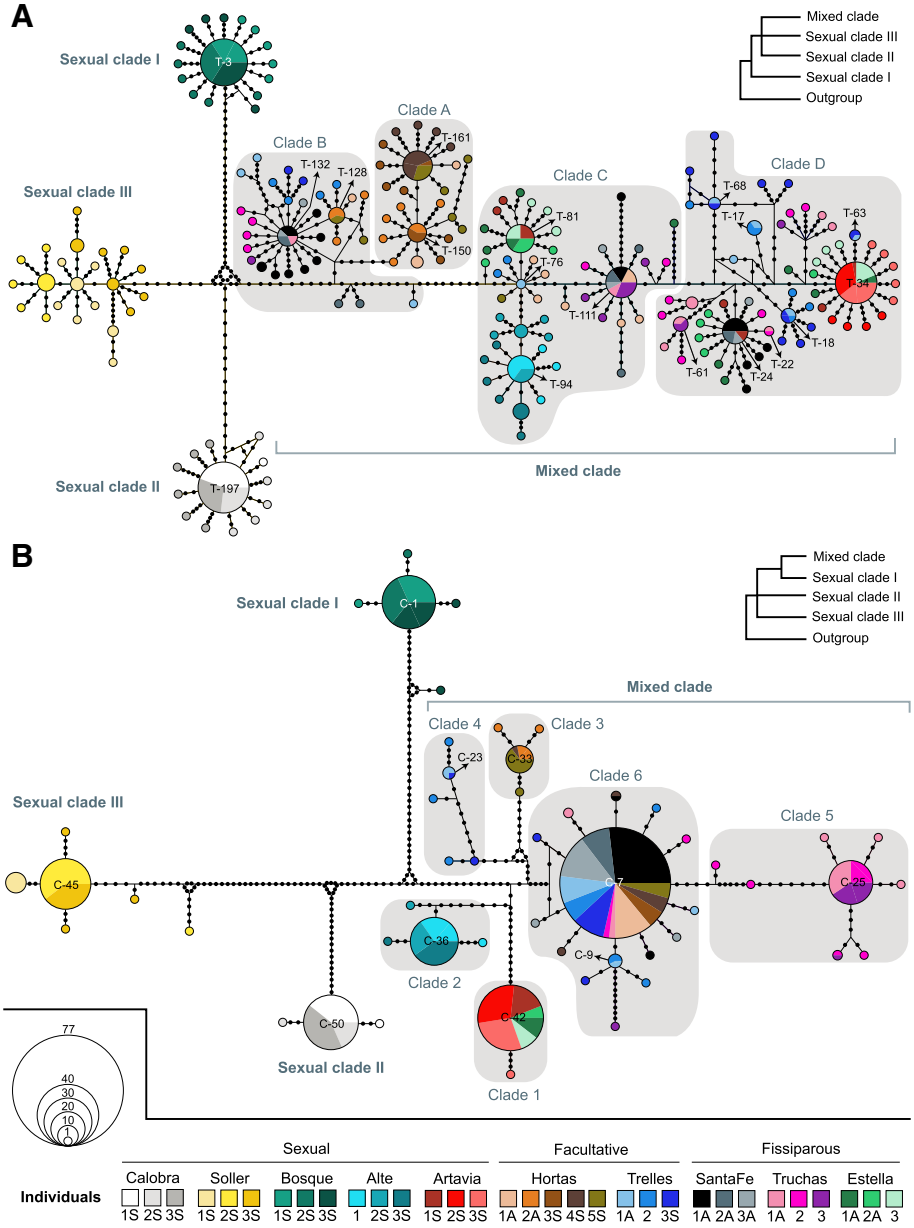

Fig. 7 Species haplotype network for the nuclear gene (**a**) and for the mitochondrial gene (**b**). Each individual is depicted with a different color. Each circle represents a different haplotype, and the size of the circle is proportional to the frequency of each haplotype in the species. Mutations are depicted as small black dots. All shared haplotypes are named with their code (see Tables S4 and S5). For each gene, a schematic representation of the phylogeny is shown in the right top corner (see Additional file 2: Figures S3 and S4), nodes with low support values are depicted as polytomies

Intraindividual genetic diversity of fissiparous planarians: evidence for a *mosaic Meselson effect*

In the case of fissiparous populations, we initially predicted the existence of mosaicism and the Meselson effect within individuals, due to the progressive accumulation of somatic mutations in the neoblasts over generations of fissiparous reproduction. The intraindividual nuclear divergent pattern (characterized by significantly higher levels of haplotype and nucleotide diversity compared to sexuals; Figs. 3 and 4) together with the high number of alleles (Table 1) observed in most individuals from fissiparous populations gives support to that hypothesis. Surprisingly, we did not expect to find equal levels of intraindividual genetic diversity in individuals from fissiparous and facultative populations, since we expected that the bottlenecks that represent the pass through a one celled zygote state plus recombination during sexual events, would reduce both the degree of mosaicism and the genetic differentiation between alleles in

the facultative populations. These findings suggest that fissiparous reproduction may be the predominant type of reproduction in the facultative populations studied. Notably, the levels of intraindividual haplotype and nucleotide diversity reported in most fissiparous and facultative individuals analyzed in the present study are so extremely high that they are comparable, at mitochondrial level, to the highest levels found between different individuals of parthenogenetic populations of different taxa (Table 4 in [8]).

A potential caveat to our hypothesis of mosaicism and the Meselson effect within fissiparous and facultative individuals is the possible existence of paralog nuclear genes and numts (i.e., mitochondrial copies in the nucleus). If part of this genetic diversity corresponded to paralogs or numts, we would expect that they would be equally present in individuals from sexual populations. Particularly, in individuals from sexual populations derived from fissiparous ones, such as Alte and Artavia. Nevertheless, they are not only absent from sexual populations in general but also from individuals from Alte and Artavia. A second caveat, as previously mentioned for sexual populations, is that some mutations of the alleles and haplotypes of fissiparous and facultative individuals could be due to mistakes in the activity of the polymerase. Nevertheless, they could only explain a minority of the intraindividual genetic diversity detected in these reproductive strategies, since most of these alleles and haplotypes are shared between different individuals or are so highly differentiated that it is extremely improbable that all mutations are a consequence of polymerase mistakes (Fig. 7). Therefore, the existence of mosaicism combined with the Meselson effect remains as the most plausible explanation to interpret the intraindividual genetic pattern found in most of the fissiparous and facultative individuals analyzed in the present study.

Importantly, given that the number of highly differentiated nuclear alleles is, for most individuals, higher than its ploidy, we can deduce that these highly differentiated alleles are distributed across different cells, in contrast to what is found when the Meselson effect occurs in parthenogenetic individuals. We propose this variation of the Meselson effect to be referred as the *mosaic Meselson effect*, which we define as the existence of a genetically heterogeneous cell population within the body of an organism, carrying highly divergent alleles in homologous genetic regions. Evidence for the occurrence of this effect in other fissiparous *Dugesia* species can be found in two studies that were focused on *D. sicula* and *D. japonica* [67, 68]. Moreover, although this is the first time that the mosaic Meselson effect is suggested, it may also occur in other fissiparous metazoans such as star-fish and corals [18], and in long-lived plants where genetic differences between branches within individuals have been studied but not quantified from a Meselson effect point of view [69–71]. At the mitochondrial level, whether the highly divergent haplotypes of the individuals are mainly found in different cells (mosaicism) or in the same cell will be discussed below.

Interestingly, the evolutionary analysis revealed that the intraindividual genetic diversity of fissiparous and facultative individuals was characterized by the existence of a mix of ancestral and derived alleles at both the nuclear and mitochondrial levels (Fig. 7). The capacity of retaining or "freezing" genetic diversity has already been proposed to occur in parthenogenetic lineages, conferring them a clear advantage compared to sexual lineages when genotypes are well adapted to the environmental conditions [72, 73], and also explaining why the genetic differentiation between parthenogenetic populations is generally lower than between sexual populations [6]. However, differing from parthenogenetic organisms, fissiparous reproduction may allow individuals to keep accumulating somatic mutations in some cells under this general "frozen" state, explaining why they can show a mixture of ancestral and derived genetic diversity. Therefore, the mosaic Meselson effect due to fissiparous reproduction may not only be characterized by the existence of highly divergent alleles in a mosaic context but also for the coexistence of ancestral and derived genetic diversity within individuals. In Fig. 8 it is depicted how the synergic effect of fissiparous reproduction and tissue homeostasis may result in this special pattern of intraindividual genetic diversity. Further investigations, based on a genomic approach, and including regional analyses of different body regions and organs, or even studying them at the single cell level, would be of great value to improve our understanding on the intraindividual genetic characteristics of these organisms.

Intraindividual selection in fissiparous planarians

Considering the evidence for the existence of mosaicism combined with the Meselson effect in fissiparous and facultative populations, we also expected to find a high incidence of Muller's ratchet in individuals from these populations. But, contrary to our expectations, our results suggest that purifying selection is occurring in all individuals analyzed in the present study (Fig. 6).

These results point out that intraindividual cell selection could be responsible for the elimination of deleterious mutations during periods of fissiparous reproduction. Processes of intraindividual cell selection are well known to occur in insects and mammals due to different mechanisms ([74], and references therein) and their consequences have been analyzed theoretically [75, 76] and empirically observed in some plants, i.e., eucalyptus trees that present one or a few branches resistant to a plague as a result of the selection of a somatic mutation [70, 77]. In our case, the Cox1 protein is known to be essential for the viability of any cell. Therefore, deleterious mutations in Cox1 would result in the elimination of the mutated

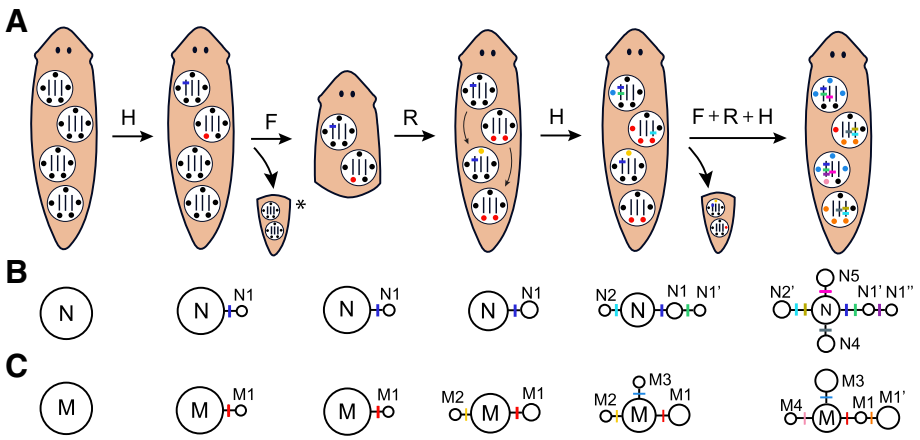

Fig. 8 Schematic representation of the mosaic Meselson effect occurring in a planarian during fissiparous reproduction. **a** Starting from a homozygous triploid individual having four neoblasts (each with three alleles and five mitochondria), mutations (depicted by different colors) start to appear randomly in the alleles and mitochondria of the neoblasts. After several cycles of homeostasis (H), fission (F) and regeneration (R), the fissiparous individual shows an increased number of derived alleles and haplotypes in different cells but still shows the ancestral. **b** and **c** depict the nuclear and mitochondrial haplotype networks at each state, N and M are the ancestral allele and haplotype, the derived alleles and haplotypes are indicated by subsequent numbering and indexing of N and M. * The same processes would happen in both pieces after a fission event

mitochondria or even the whole cell bearing it, resulting in intraindividual selection. However, in the case of the nuclear gene TMED9, it is known to code for a transmembrane protein but its exact function in planarians is not yet characterized. Hence, although our results seem to indicate that it is also under purifying selection and in consequence it may also play an important role in neoblast survival, more data on nuclear genes is needed to confirm the existence of purifying selection in the nucleus at cellular level during fissiparous periods.

A test to our hypothesis on the existence of intraindividual purifying selection in mosaic fissiparous planarians could be to analyze genes that are only expressed in certain cell types or organs in individuals who have long been reproducing by fission (such as in individuals from the population of Truchas). In this case, we would expect that all cells that do not need the expression of those genes (including neoblasts) will be able to carry mutated nonfunctional or less efficient copies, and we would detect a Muller's ratchet effect. Indirect evidence for the expected result of this test comes from a comparison of genomic and transcriptomic data from a laboratory lineage of *D. japonica* derived from a single individual that kept undergoing autonomous fission for over 20 years [68], where they detected that a 74% of the genes presented nonsynonymous polymorphisms. Nonetheless, another possibility exists to explain the lack of Muller's ratchet in the two genes analyzed in the present study: the synergic action of intraindividual selection and occasional sex (see below).

Multiple transitions between sex and fissiparity in the evolutionary history of *D. subtentaculata*

The topology of the phylogenetic trees, showing that the first lineages to diverge in *D. subtentaculata* are three sexual diploid populations while most populations within the monophyletic Mixed clade are triploid fissiparous or facultative (see Additional file 2: Figure S3 and Figure S4), suggests that the origin of the Mixed clade was due to a triploidization event from sexual diploid ancestors. This triploidization event possibly promoted a shift from sexual to fissiparous reproduction in the lineage that gave rise to the Mixed clade, since in the genus *Dugesia* polyploidy is highly associated with fissiparity [41]. Moreover, the evolutionary analyses also revealed that all individuals of the Mixed clade showed their nuclear genetic diversity distributed in the same ancestral and derived clades, indicating that after the above mentioned triploidization event, the ancestors of the Mixed clade reproduced by fission for a long time. However, the fact that most individuals of different populations nowadays share different combinations of at maximum three alleles, either ancestral or derived, can only be explained if different sexual events occurred after this period of fissiparous reproduction.

In resexualized fissiparous planarians, with the germ line appearing de novo from a population of genetically diverse neoblasts (due to the mosaic Meselson effect, Fig. 8), processes of segregation (Fig. 9a) and outcrossing (Fig. 9b) may result in descendants showing different combinations of ancestral and derived alleles but with a maximum number of shared nuclear alleles equal to their ploidy level. At the mitochondrial level, descendants could only inherit the haplotypes present in the neoblast

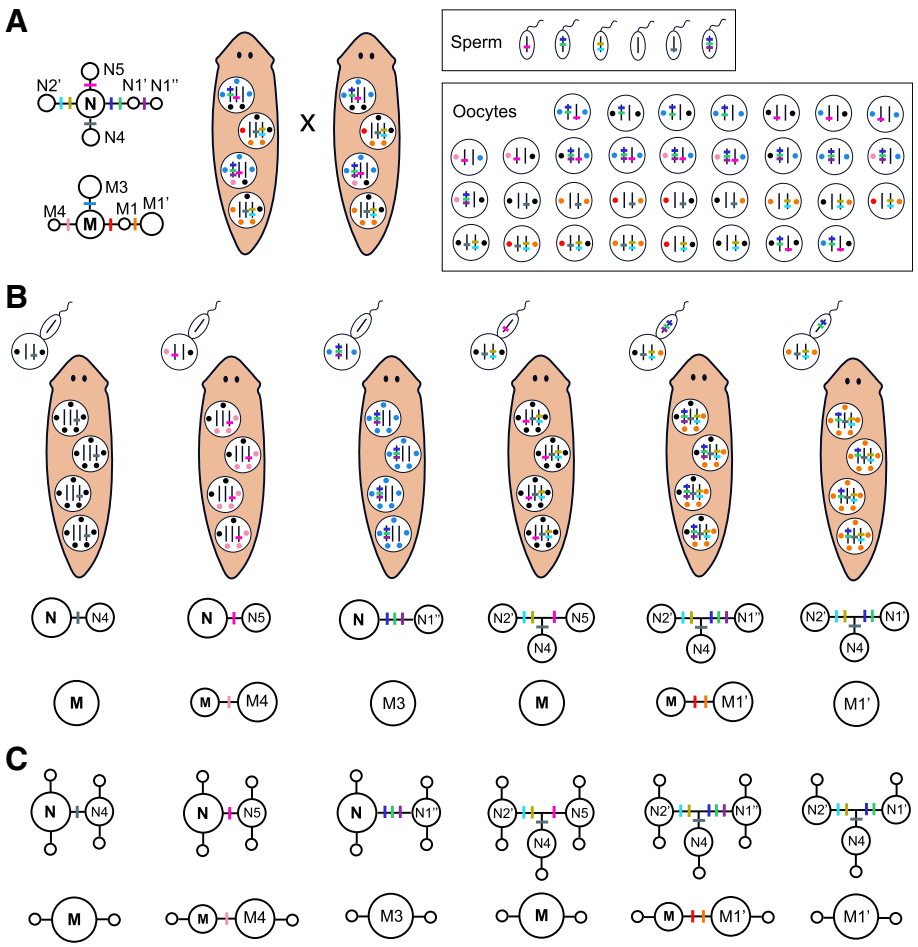

Fig. 9 Schematic representation of new genetic combinations resulting from the cross of two resexualized fissiparous planarians. **a** When fissiparous individuals showing the mosaic Meselson effect resexualize, their original (depicted in black) and new alleles and haplotypes (depicted with different colors) segregate in their gametes. While haploid sperm only carries a single nuclear allele, diploid oocytes carry two alleles and two mitochondria (the bottleneck of mitochondria during oogenesis is represented by the loss of three mitochondria per neoblast). **b** After crossing, non-mosaic descendants are born showing different combinations of ancestral (N and M) and derived (indicated by a N or M with subsequent numbering and indexing) alleles and haplotypes, such as the six different genetic combinations shown in the figure as an example. Notice that derived alleles that progenitors had in different cells (such as N1'' and N2') can be inherited together in the offspring. **c** If descendants resume fissiparous reproduction, they will result in new lineages that accumulate new mutations in the alleles and haplotypes that they inherited

precursor of the oocyte [78, 79], indicating that the progenitors of the individuals showing both ancestral and highly derived mitochondrial haplotypes were heteroplasmic at intracellular level. Nevertheless, the finding that individuals of different fissiparous and facultative populations don't share any derived mitochondrial haplotype but they share the ancestral haplotype of the group (C-7 in Fig. 7b), indicates that their ancestors were also mosaics at the mitochondrial level, as we predicted with the mosaic Meselson effect. Therefore, processes of segregation and outcrossing (the last only for the nuclear genome) during sexual events in the fissiparous ancestors of the Mixed clade allow us to explain why some fissiparous and facultative individuals show a nuclear divergent pattern but a mitochondrial star-like pattern (e.g., individuals from Santa Fe in Fig. 3), or the other way around (e.g., individual Hortas4S in Fig. 3) (Fig. 9), explaining also how the haplotype and nucleotide diversity of fissiparous individuals could be significantly different from sexual individuals only at nuclear level. If the offspring after such sexual events resume fissiparity (Fig. 9c), new mutations can start to accumulate from the different combinations of inherited alleles and haplotypes, resulting in the patterns of shared and private intraindividual genetic diversity that we have observed in the different lineages (Fig. 7).

Although our results indicate that all populations of the Mixed clade share a common evolutionary history of fissiparous reproduction with facultative sex, we found that there have been posterior transitions between reproductive strategies. On the one hand, two populations returned

to strict sex: the diploid population of Alte and the triploid population of Artavia. In both cases the return to strict sex promoted the recovery of the star-like intraindividual genetic footprint characteristic of sexual individuals. The rise of diploid sexual offspring from the outcrossing of two triploid resexualized fissiparous individuals has been directly documented under laboratory conditions in the species *D. ryukyuensis* [80]. Differing from Alte, the return to strict sexuality in the population of Artavia occurred without a change in the ploidy level. Interestingly, the existence of one individual of Artavia (Artavia1S) showing a slightly divergent pattern at the nuclear level (Fig. 3), suggests a recent return to sexuality from its fissiparous past. On the other hand, the three fissiparous populations analyzed in the present study seem to have become strict fissiparous. Individuals of these fissiparous populations have already started to generate new genetic diversity from the alleles and haplotypes that sexually inherited, but they are still genetically very similar to the other individuals of its own population (Fig. 5c). Finally, although our results indicate that fissiparous reproduction is the predominant reproductive strategy in the facultative populations we have studied, we found that present events of sex in these populations may enable the existence of an additional component of genetic diversity: differentiation between individuals (Fig. 5b), which is not found in populations where sex and fission is not regularly alternated. Different proportions of fissiparity and sex could then be selected under different ecological-climatic conditions, providing this species with a huge range of strategies to face them, as has been proposed in facultative populations of the sexual-parthenogenetic *S. polychroa* [81].

Evolutionary advantages of fissiparous reproduction with occasional sex

The evolutionary model based on the combination of fissiparous reproduction with different rates of occasional sex that we have proposed represents a paradigmatic mode of evolution for several reasons. While the somatic genetic diversity may be partially hidden from selection when fission is the only way to reproduce, the recovery of sexuality may expose this genetic diversity to selection in the offspring in different combinations (depending on the segregation and out-crossing, Fig. 9b). Therefore, besides the possible processes of intraindividual selection at the neoblast level during fissiparous reproduction (that might eliminate extremely deleterious variants), events of occasional sex not only may help to prevent the occurrence of Muller's ratchet in predominantly fissiparous linages ([82], and references therein) but also may promote a rapid adaptation to different environmental conditions. Moreover, the presence of this high intraindividual genetic diversity due to fissiparous reproduction may allow them also to overcome evolutionary problems such as those generated by population bottlenecks (a situation that, for instance, species of *Dugesia* endure each year in the Mediterranean region), since they will not result in a loss of genetic diversity. All these reasons could help explain the evolutionary success of the genus *Dugesia*, with more than 85 species distributed in Africa, Asia and Europe [83], compared to its sister genera *Schmidtea* [45] and *Recurva* [84], which principally reproduce sexually or by parthenogenesis and at present include only 4 and 2 species, respectively, distributed only in Northern Africa and Europe.

Conclusions

The intraindividual genetic data obtained in the present study provide evidence for the existence of mosaicism combined with the Meselson effect (the mosaic Meselson effect) in fissiparous metazoans, specifically in planarians, an organism of complex tissue architecture. Furthermore, our results point out that the mosaic Meselson effect enables the existence of both ancestral and highly derived genetic diversity within the same individual, a genetic characteristic never described before and very interesting under an evolutionary point of view. Concomitantly, our results provide evidence that this special genetic diversity acquired during periods of fissiparous reproduction can be transmitted to the offspring through sexual events, allowing the generation of progeny with a huge range of genetic diversity and providing a scenario of possible multilevel selection (at both intraindividual and individual level). Further investigations using *D. subtentaculata* as a model organism, which may go to the genomic level, would be of great value to understand how fissiparous organisms can orchestrate such a genetically heterogenic cell population, including putative processes of intraindividual selection. Moreover, due to the impressive plasticity of this species in shifting ploidy and reproductive strategies, it can also be a good model to analyze the mechanisms that trigger polyploidization and how they are linked to the reproductive mode and environmental conditions. Nature is full of exceptions to our laboratory model organisms, and we need to study them to understand how they evolve and succeed.

Additional files

Additional file 1: **Table S1.** List of populations used in this study, including information on the reproductive strategy, locality and collectors. **Table S2.** Sequence, source and annealing temperature of the primers used in this study. **Table S3.** Results of the ploidy inference by flow cytometry. **Table S4.** List of the different TMED9 alleles obtained in this study. The individuals showing each haplotype and its corresponding GenBank accession number are indicated. **Table S5.** List of Cox1 haplotypes, with the individuals showing each haplotype and its corresponding GenBank accession number. **Table S6.** Genetic diversity and proportion of synonymous and nonsynonymous sites at intraindividual level for the two molecular markers analyzed in the present study. **Table S7.** Results of the statistical tests used to compare

the intraindividual mean levels of genetic diversity and types of mutations between the different reproductive strategies. (PDF 407 kb)

Additional file 2: Figure S1. Workflow of the ploidy level estimation using flow cytometry. 1) Cutting the animal in two pieces, 2) cell maceration, 3) DNA staining, 4) fluorescence measurement with a Gallios flow cytometer and 5) unknown-ploidy estimation by comparison with control. **Figure S2**. Approach used in this study for the correction of the putative artifactual mutations due to polymerase mistakes. In parentheses is indicated the frequency of each haplotype within the individual before and after the correction. **Figure S3.** Bayesian inference tree of all the alleles of the nuclear gene TMED9. The colors of the terminal branches indicate to which individual each allele belongs. The color of the outer circle indicates the reproductive strategy of each individual. Numbers at the nodes indicate the support values for the Bayesian inference (posterior probability)/the maximum likelihood (bootstrap). Bootstrap values correspond to the maximum likelihood analysis conducted with rapid bootstrap. Support values lower than 0.8 (posterior probability) and 75% (bootstrap) are represented with a -. Scale bar indicates the number of substitutions per site. **Figure S4.** Bayesian inference tree of all the haplotypes of the mitochondrial gene Cox1. Haplotypes of the same individual are pictured with the same color. Numbers in white indicate the different clades within the Mixed clade. Numbers at the nodes indicate the support values for the Bayesian inference (posterior probability)/the maximum likelihood (bootstrap). Bootstrap values correspond to the maximum likelihood analysis conducted with rapid bootstrap. Support values lower than 0.8 (posterior probability) and 75% (bootstrap) are represented with a -. Scale bar indicates the number of substitutions per site. (PDF 878 kb)

Abbreviations

AMOVA: Analysis of molecular variation; BI: Bayesian inference; CCiT: Unitat de Citometria dels Centres Científics i Tecnològics; Cox1: Cytochrome oxidase subunit 1; MCMC: Markov chain Monte Carlo; ML: Maximum likelihood; TMED9: Transmembrane p24 trafficking protein 9; UB: Universitat de Barcelona

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Availability of data and materials

All the sequences obtained in this study are available in GenBank under the accession numbers: MK385658 - MK385926. The alignments used for the phylogenetic inferences are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.c5f0ps6

Authors' contributions

MR conceived the project. ES, LL, MR and MVF contributed to the samplings. LL did all the wet laboratory and data analyses and wrote the first draft of the manuscript. ES, LL, MR and MVF participated in the discussions of the results and on the writing of the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table S1. List of populations used in this study, including information on the reproductive strategy, locality and collectors.

Population code	Reproductive strategy	Locality	Coordinates	Collectors
Calobra	Sexual	Sa Calobra, Mallorca, Balearic Islands, Spain	39.82932N 2.81538E	M. Vila-Farré
Soller	Sexual	Soller, Mallorca, Balearic Islands, Spain	39.75693N 2.71193E	M. Vila-Farré
Bosque	Sexual	El Bosque, Andalucía, Spain	36.76123N 5.50581W	M. Àlvarez, M. Riutort and L. Leria
Alte	Sexual	Alte, Algarve, Portugal	37.23827N 8.17199W	A.Valls, E.Solà, M.Riutort and L.Leria
Artavia	Sexual	Artavia, Navarra, Spain	42.74488N 2.08957W	M. Vila-Farré, E.Solà and L.Leria
SantaFe	Fissiparous	Santa Fe del Montseny, Catalonia, Spain	41.77356N 2.46638E	M. Vila-Farré, E.Solà and L.Leria
Truchas	Fissiparous	Peralejos de las Truchas, Castilla La Mancha, Spain	40.59318N 1.92561W	M. Vila-Farré, E.Lázaro and L.Leria
Estella	Fissiparous	Estella, Navarra, Spain	42.6746N 2.03519W	M. Vila-Farré, E.Solà and L.Leria
Hortas	Facultative	Portalegre, Portugal	39.3232N 7.40911W	M. Àlvarez, M. Riutort and L. Leria
Trelles	Facultative	Trelles, Asturias, Spain	43.476N 6.73358W	M. Vila-Farré, E.Solà and L.Leria

Table S2. Sequence, source and annealing temperature of the primers used in this study.

Gene	Primer	Sequence 5'-3'	Source	Annealing Temp (ºC)
Cox1	BarT (F)	ATGACDGCSCATGGTTTAATAATGAT	Álvarez-Presas *et al*. 2011	43
	COIR (R)	CCWGTYARMCCHCCWAYAGTAAA	Lázaro *et al.* 2009	45
TMED9	Dunuc12_1F (F)	CTCGTATCTCTGAATCTAGCCTC	Leria *et al.* 2019	53
	Dunuc12_1R (R)	GTTCATACAACTCAT TCT TC	Leria *et al.* 2019	46

Table S3. Results of the ploidy inference by flow cytometry.

Population	Sample	Number of peaks	Number of cells	Fluorescense	Proportion	Ploidy
Alte (sexual)	Control 1	1	5706	664		
	Alte1 [a]	1	5241	414		
	Control 1 + Alte1 [a]	2	12972 and 5450	378 and 593	1'91	**2n**
	Control 1	1	11120	656		
	Alte1S [a]	1	3425	429		
	Control 1 + Alte1S [a]	2	10170 and 12652	425 and 652	1'95	**2n**
	Alte2S [a]	1	11222	421		
	Control 1 + Alte2S [a]	2	3161 and 2134	451 and 678	1'99	**2n**
Artavia (sexual)	Control 2	1	15865	684		
	Artavia1S [a]	1	7363	687		
	Control 2 + Artavia1S [a]	1	24092	773	3	**3n**
	Artavia2S [a]	1	10876	685		
	Control 2 + Artavia2S [a]	1	6353	697	3	**3n**
	Control 3	1	5027	665		
	Artavia3S [a]	1	8419	673		
	Control 3 + Artavia3S [a]	1	11821	746	3	**3n**
Hortas (facultative)	Control 4	1	11160	595		

	Hortas3S [a]	2[b]	1443 and 5388	277 and 509		
	Control 4 + Hortas3S [a]	3[b]	6151, 15910 and 6042	242, 439 and 620	1'17 and 2'12	**2n**
	Hortas4S [a]	1	5234	562		
	Control 4 + Hortas4S [a]	1	18673	638	3	**3n**
Trelles (facultative)	Control 5	1	12612	602		
	Trelles_1	1	8688	562		
	Control 5 + Trelles_1	1	20985	602	3	**3n**
	Trelles_2	1	20521	570		
	Control 5 + Trelles_2	1	20594	602	3	**3n**
	Control 6	1	10771	574		
	Trelles_3	1	17734	576		
	Control 6 + Trelles3	1	23866	612	3	**3n**
	Trelles_4	1	16609	579		
	Control 6 + Trelles_4	1	23399	603	3	**3n**
Truchas (fissiparous)[c]	Control 7	1	4790	204		
	Truchas_1	2	1371 and 2451	633 and 805		
	Control 7 + Truchas_1	2	4495 and 1700	692 and 879	3 and 3'8	**3n and 4n**
	Control 8	1	5716	617		
	Truchas_2	2	1431 and 2682	718 and 895		

	Control 8 + Truchas_2	2	4403 and 2643	651 and 880	3 and 4'05	**3n and 4n**
Estella (fissiparous)[c]	Control 9	1	5251	178		
	Estella_1	1	1805	266		
	Control 9 + Estella_1	2	1109	304 and 437	4'31	**4n**
	Control 10	1	4706	347		
	Estella_2	1	2747	479		
	Control 10 + Estella_2	2	12999	332 and 487	4'4	**4n**
	Control 11	1	20260	600		
	Estella_3	1	10093	788		
	Control 11 + Estella_3	2	8416 and 8360	589 and 819	4'17	**4n**
	C37_2	1	14388	802		
	Control 11 + Estella_4	2	7825 and 9697	606 and 858	4'24	**4n**
	Control 12	1	5453	560		
	Estella_5	1	8147	851		
	Control 12 + Estella_5	2	14072 and 12797	570 and 817	4'3	**4n**

[a] individual that was also used for the genetic analysis

[b] the peak with a lower fluorescence value corresponds to sperm

[c] decrease in the florescence values of some samples due to a modification of the cytometer parameters

Table S4. List of the different TMED9 alleles obtained in this study. The individuals showing each allele and its corresponding GenBank accession number are indicated.

Allele	Individuals	GenBank accession number
T-1	Bosque1S	MK385658
T-2	Bosque1S	MK385769
T-3	Bosque1S, Bosque2S, Bosque3S	MK385790
T-4	Bosque3S	MK385801
T-5	Bosque2S	MK385812
T-6	Bosque1S	MK385823
T-7	Bosque3S	MK385834
T-8	Bosque3S	MK385845
T-9	Bosque2S	MK385856
T-10	Bosque2S	MK385659
T-11	Bosque2S	MK385670
T-12	Bosque1S	MK385681
T-13	Bosque1S	MK385692
T-14	Bosque2S	MK385703
T-15	Bosque3S	MK385714
T-16	Bosque3S	MK385725
T-17	Trelles1A, Trelles2	MK385736
T-18	Trelles1A, Trelles2, Trelles3S	MK385747
T-19	Trelles2	MK385758
T-20	Trelles3S	MK385770
T-21	Trelles3S	MK385781
T-22	Truchas1A, Truchas2	MK385782
T-23	Truchas1A	MK385783
T-24	SantaFe1A, SantaFe2A, SantaFe3A, Artavia1S	MK385784
T-25	Trelles1A	MK385785
T-26	Estella1A	MK385786
T-27	Estella2A	MK385787
T-28	Estella2A	MK385788
T-29	Estella2A	MK385789
T-30	Estella2A	MK385791
T-31	Truchas2	MK385792
T-32	Truchas2	MK385793
T-33	Artavia1S	MK385794
T-34	Artavia1S, Artavia2S, Artavia3S, Estella1A, Estella3	MK385795
T-35	Artavia2S	MK385796
T-36	Artavia2S	MK385797
T-37	Artavia3S	MK385798
T-38	Artavia3S	MK385799
T-39	Artavia3S	MK385800
T-40	Estella2A	MK385802
T-41	Estella3	MK385803
T-42	Estella3	MK385804
T-43	Artavia2S	MK385805
T-44	Artavia2S	MK385806
T-45	Estella1A	MK385807
T-46	Artavia3S	MK385808
T-47	Estella1A	MK385809
T-48	Estella3	MK385810
T-49	Artavia3S	MK385811
T-50	SantaFe1A	MK385813
T-51	SantaFe3A	MK385814
T-52	Artavia1S	MK385815
T-53	Truchas2	MK385816
T-54	Estella1A	MK385817

T-55	SantaFe1A	MK385818
T-56	SantaFe1A	MK385819
T-57	TrellesS2	MK385820
T-58	SantaFe1A	MK385821
T-59	Trelles3S	MK385822
T-60	Truchas2	MK385824
T-61	Truchas1A, Truchas3	MK385825
T-62	Truchas1A	MK385826
T-63	Trelles1A, Trelles3S	MK385827
T-64	Truchas2	MK385828
T-65	Truchas1A	MK385829
T-66	Truchas1A	MK385830
T-67	Truchas3	MK385831
T-68	Trelles1A, Trelles3S	MK385832
T-69	Trelles3S	MK385833
T-70	Trelles3S	MK385835
T-71	Trelles3S	MK385836
T-72	Trelles3S	MK385837
T-73	TrellesS2	MK385838
T-74	Truchas2	MK385839
T-75	Trelles1A	MK385840
T-76	Hortas1A	MK385841
T-77	Trelles2	MK385842
T-78	Trelles2	MK385843
T-79	Hortas1A	MK385844
T-80	Hortas1A	MK385846
T-81	Artavia1S, Estella1A, Estella2A	MK385847
T-82	Estella1A	MK385848
T-83	Estella1A	MK385849
T-84	Estella1A	MK385850
T-85	Estella3	MK385851
T-86	Estella3	MK385852
T-87	Estella3	MK385853
T-88	Artavia1S	MK385854
T-89	Estella2A	MK385855
T-90	Artavia1S	MK385857
T-91	Hortas1A	MK385858
T-92	Hortas5S	MK385859
T-93	Alte1	MK385860
T-94	Alte1, Alte2S	MK385861
T-95	Alte1	MK385862
T-96	Alte3S	MK385863
T-97	Alte3S	MK385864
T-98	Alte3S	MK385865
T-99	Alte3S	MK385866
T-100	Alte2S	MK385660
T-101	Alte2S	MK385661
T-102	Alte3S	MK385662
T-103	Alte2S	MK385663
T-104	Alte3S	MK385664
T-105	Alte2S	MK385665
T-106	Alte2S	MK385666
T-107	Estella2A	MK385667
T-108	Alte1	MK385668
T-109	Estella2A	MK385669
T-110	Hortas1A	MK385671
T-111	Hortas1A, SantaFe1A, SantaFe2A, SantaFe3A, Truchas1A, Truchas3	MK385672

T-112	Truchas3	MK385673
T-113	Truchas3	MK385674
T-114	Truchas3	MK385675
T-115	Truchas2	MK385676
T-116	SantaFe2A	MK385677
T-117	Hortas1A	MK385678
T-118	SantaFe2A	MK385679
T-119	SantaFe3A	MK385680
T-120	Truchas2	MK385682
T-121	SantaFe1A	MK385683
T-122	Trelles1A	MK385684
T-123	Trelles1A	MK385685
T-124	Truchas2	MK385686
T-125	Trelles2	MK385687
T-126	Trelles2	MK385688
T-127	Trelles2	MK385689
T-128	Hortas2A, Hortas5S	MK385690
T-129	Trelles3S	MK385691
T-130	Hortas5S	MK385693
T-131	Truchas3	MK385694
T-132	SantaFe1A, SantaFe2A, SantaFe3A, Truchas1A	MK385695
T-133	Hortas2A	MK385696
T-134	Truchas2	MK385697
T-135	SantaFe1A	MK385698
T-136	Truchas2	MK385699
T-137	Trelles3S	MK385700
T-138	SantaFe1A	MK385701
T-139	Trelles2	MK385702
T-140	SantaFe1A	MK385704
T-141	Hortas1A	MK385705
T-142	SantaFe3A	MK385706
T-143	SantaFe2A	MK385707
T-144	SantaFe1A	MK385708
T-145	SantaFe1A	MK385709
T-146	SantaFe1A	MK385710
T-147	SantaFe1A	MK385711
T-148	SantaFe1A	MK385712
T-149	SantaFe2A	MK385713
T-150	Hortas2A, Hortas3S	MK385715
T-151	Hortas1A	MK385716
T-152	Hortas3S	MK385717
T-153	Hortas3S	MK385718
T-154	Hortas2A	MK385719
T-155	Hortas2A	MK385720
T-156	Hortas5S	MK385721
T-157	Hortas5S	MK385722
T-158	Hortas2A	MK385723
T-159	Hortas2A	MK385724
T-160	Hortas3S	MK385726
T-161	Hortas3S, Hortas4S, Hortas5S	MK385727
T-162	Hortas3S	MK385728
T-163	Hortas4S	MK385729
T-164	Hortas5S	MK385730
T-165	Hortas4S	MK385731
T-166	Hortas3S	MK385732
T-167	Hortas4S	MK385733
T-168	Hortas3S	MK385734
T-169	Hortas4S	MK385735

T-170	Hortas1A	MK385737
T-171	Hortas2A	MK385738
T-172	Hortas5S	MK385739
T-173	Trelles1A	MK385740
T-174	Hortas2A	MK385741
T-175	Soller1S	MK385742
T-176	Soller3S	MK385743
T-177	Soller3S	MK385744
T-178	Soller2S	MK385745
T-179	Soller1S	MK385746
T-180	Soller1S	MK385748
T-181	Soller2S	MK385749
T-182	Soller1S	MK385750
T-183	Soller1S	MK385751
T-184	Soller3S	MK385752
T-185	Soller2S	MK385753
T-186	Soller2S	MK385754
T-187	Soller2S	MK385755
T-188	Soller3S	MK385756
T-189	Soller3S	MK385757
T-190	Soller3S	MK385759
T-191	Soller3S	MK385760
T-192	Soller2S	MK385761
T-193	Soller1S	MK385762
T-194	Soller3S	MK385763
T-195	Soller1S	MK385764
T-196	Soller1S	MK385765
T-197	Calobra1S, Calobra2S, Calobra3S,	MK385766
T-198	Calobra3S	MK385767
T-199	Calobra3S	MK385768
T-200	Calobra3S	MK385771
T-201	Calobra1S	MK385772
T-202	Calobra3S	MK385773
T-203	Calobra3S	MK385774
T-204	Calobra3S	MK385775
T-205	Calobra2S	MK385776
T-206	Calobra2S	MK385777
T-207	Calobra2S	MK385778
T-208	Calobra2S	MK385779
T-209	Calobra2S	MK385780
Outgroup sequences		
Species	Code	GenBank accession number
Dugesia hepta	MR1960	MK385867
	MR1962	MK385868
Dugesia benazzii	MR2191	MK385869
	MR2192	MK385870

Table S5. List of Cox1 haplotypes, with the individuals showing each haplotype and its corresponding GenBank accession number.

Haplotype	Individuals	GenBank accession number
C-1	Bosque1S, Bosque2S, Bosque3S	MK385871
C-2	Bosque2S	MK385872
C-3	Bosque1S	MK385873
C-4	Bosque3S	MK385874
C-5	Bosque3S	MK385875
C-6	Trelles2	MK385876
C-7	SantaFe1A, SantaFe2A, SantaFe3A, Trelles1A, Trelles2, Trelles3S, Hortas3S, Hortas4S, Hortas1A, Hortas5S, Truchas2, Truchas1A	MK385877
C-8	Trelles2	MK385878
C-9	Trelles1A, Trelles2	MK385879
C-10	Truchas2	MK385880
C-11	Trelles1A	MK385881
C-12	Hortas4S, SantaFe1A	MK385882
C-13	SantaFe3A	MK385883
C-14	Truchas1A	MK385884
C-15	SantaFe3A	MK385885
C-16	SantaFe1A	MK385886
C-17	Trelles3S	MK385887
C-18	Trelles3S	MK385888
C-19	Hortas4S	MK385889
C-20	Trelles2	MK385890
C-21	Trelles3S	MK385891
C-22	Trelles2	MK385892
C-23	Trelles1A, Trelles3S	MK385893
C-24	Trelles2	MK385894
C-25	Truchas2, Truchas1A, Truchas3	MK385895
C-26	Truchas1A	MK385896
C-27	Truchas 1A	MK385897
C-28	Truchas1A	MK385898
C-29	Truchas2	MK385899
C-30	Truchas2, Truchas3	MK385900
C-31	Truchas2	MK385901
C-32	Hortas2A	MK385902
C-33	Hortas2A, Hortas4S, Hortas5S	MK385903
C-34	Hortas2A	MK385904
C-35	Truchas2	MK385905
C-36	Alte1, Alte2S, Alte3S	MK385906
C-37	Alte3S	MK385907
C-38	Alte1	MK385908
C-39	Alte2S	MK385909
C-40	Truchas3	MK385910
C-41	Hortas5S	MK385911
C-42	Artavia1S, Artavia2S, Artavia3S, Estella1A, Estella2A, Estella3	MK385912
C-43	Artavia3S	MK385913
C-44	Soller1S	MK385914
C-45	Soller2S, Soller3S	MK385915
C-46	Soller3S	MK385916
C-47	Soller3S	MK385917
C-48	Soller3S	MK385918
C-49	Soller2S	MK385919
C-50	Calobra1S, Calobra3S, Calobra2S	MK385920
C-51	Calobra1S	MK385921

C-52	Calobra2S	MK385922
Outgroup sequences		
Species	Code	GenBank accession number
Dugesia hepta	MR1960	MK385923
	MR1962	MK385924
Dugesia benazzii	MR2191	MK385925
	MR2192	MK385926

TableS6. Genetic diversity and proportion of synonymous and nonsynonymous sites at intraindividual level for the two molecular markers analyzed in the present study

	TMED9					Cox1				
	H_D	π	Ks	Ka	Ω	H_D	π	Ks	Ka	Ω
Sexual										
Calobra1S	0.1333	0.0003	0	0		0.1429	0.0004	0.0009	0.0003	0.3125
Calobra2S	0.5714	0.0014	0.0063	0.0025	0.4031	0.25	0.0008	0	0.0010	
Calobra3S	0.6571	0.0024	0	0.0009		0		0	0	
Soller1S	0.8571	0.0050	0.0186	0.0018	0.0978					
Soller2S	0.641	0.0019	0	0.0020		0.0952	0.0024	0.0091	0.0004	0.0430
Soller3S	0.8476	0.0040	0.0091	0.0008	0.0926	0.3714	0.0021	0.0061	0.0008	0.1342
Bosque1S	0.5714	0.0010	0	0		0.1667	0.0005	0.0021	0	0
Bosque2S	0.5714	0.0016	0	0.0008		0.1667	0.0005	0	0.0007	
Bosque3S	0.5714	0.0018	0	0.0034		0.2747	0.0029	0.0113	0.0003	0.0266
Alte1	0.4231	0.0012	0.0073	0.0010	0.1349	0.1818	0.0008	0.0011	0.0007	0.6508
Alte2S	0.7802	0.0018	0	0.0018		0.2222	0.0010	0.0014	0.0009	0.6508
Alte3S	0.6818	0.0016	0.0040	0	0	0.1538	0.0005	0.0010	0.0003	0.3175
Artavia1S	0.8727	0.0071	0.0043	0.0023	0.5410	0	0	0	0	
Artavia2S	0.5385	0.0015	0	0		0	0	0	0	
Artavia3S	0.5714	0.0014	0.0031	0.0017	0.5450	0.1429	0.0007	0.0009	0.0006	0.6406
Σ=15	$\bar{x}$=0.6193	$\bar{x}$=0.0023	$\bar{x}$=0.0035	$\bar{x}$=0.0013	$\bar{x}$=0.2592	$\bar{x}$=0.1446	$\bar{x}$=0.0008	$\bar{x}$=0.0023	$\bar{x}$=0.0004	$\bar{x}$=0.3084
Facultative										
Hortas1A	0.9143	0.0137	0.0401	0	0	0	0	0	0	
Hortas2A	0.8857	0.0074	0.0126	0.0017	0.1343	0.6	0.0021	0.0042	0.0014	0.3254
HHortas3S	0.8485	0.0023	0.0079	0.0011	0.1327	0	0	0	0	
Hortas4S	0.4762	0.0014	0	0.0017		0.7143	0.0088	0.0326	0.0017	0.0529
Hortas5S	0.8571	0.0088	0.0188	0.0009	0.0487	0.4725	0.0097	0.0400	0.0007	0.0181
Trelles1A	0.9451	0.0119	0.0327	0	0	0.7121	0.0125	0.0495	0.0017	0.0339
Trelles2	0.9341	0.0144	0.0433	0.0018	0.0416	0.8333	0.0108	0.0402	0.0020	0.0496
Trelles3S	0.9619	0.0109	0.0184	0.0026	0.1408	0.5385	0.0077	0.0297	0.0012	0.0403
Σ=8	$\bar{x}$=0.8529	$\bar{x}$=0.0089	$\bar{x}$=0.0184	$\bar{x}$=0.0026	$\bar{x}$=0.1408	$\bar{x}$=0.4838	$\bar{x}$=0.0065	$\bar{x}$=0.0297	$\bar{x}$=0.0012	$\bar{x}$=0.0403
Fissiparous										
SantaFe1A	0.918	0.0125	0.0250	0.0005	0.0183	0.1775	0.0004	0	0.0006	
SantaFe2A	0.8846	0.0117	0.0247	0.0010	0.0392	0	0	0	0	
SantaFe3A	0.9286	0.0115	0.0207	0	0	0.3182	0.0010	0	0.0007	0.3254
Truchas1A	0.9011	0.0105	0.0356	0.0018	0.0513	0.6476	0.0104	0.0311	0.0042	0.1337
Truchas2	1	0.0146	0.0369	0.0043	0.1157	0.7308	0.0109	0.0325	0.0043	0.1310
Truchas3	0.8242	0.0080	0.0207	0.0018	0.0882	0.2949	0.0060	0.0197	0.0021	0.1046
Estella1A	0.9394	0.0091	0.0155	0.0032	0.2043	0	0	0	0	
Estella2A	0.8718	0.0076	0.0073	0.0010	0.1351	0	0	0	0	
Estella3	0.8762	0.0083	0.0127	0.0025	0.2003	0	0	0	0	

Σ=9	$\bar{x}$=0.9049	$\bar{x}$=0.0104	$\bar{x}$=0.0221	$\bar{x}$=0.0018	$\bar{x}$=0.0947	$\bar{x}$=0.241	$\bar{x}$=0.0032	$\bar{x}$=0.0095	$\bar{x}$=0.0013	$\bar{x}$=0.1737

H_D: haplotype diversity; π: nucleotide diversity; Ks: number of synonymous mutations / number of synonymous sites; Ka: number of nonsynonymous mutations / number of nonsynonymous sites; Ω: Ka/Ks

Table S7. Results of the statistical tests used to compare the intraindividual mean levels of genetic diversity and types of mutations between the different reproductive strategies.

Genetic parameter	Gene	Statistical test	p-value	p-value (Sexual vs Facultative)	p-value (Sexual vs Fissiparous)	p-value (Facultative vs Fissiparous)
H_D	Tmed9	H = 16.54	0.0003***	0.0079**	0.0007***	1
	Cox1	F = 5.696	0.0082**	0.0075**	0.6245	0.0649
π	Tmed9	F = 25.31	0.0000004***	0.0002***	0.0001***	0.477
	Cox1	F =6.663	0.0042**	0.0038**	0.3129	0.1159
Ks	Tmed9	F = 13.8	0.00006***	0.001**	0.0008***	0.9957
	Cox1	F = 8.151	0.0016**	0.0015**	0.4193	0.0331*
Ka	Tmed9	F^a = 0.5634	0.5801			
	Cox1	H= 2.869	0.2233			
Ω	Tmed9	F = 2.093	0.167			
	Cox1	H = 2.633	0.274			

H_D: haplotype diversity; π: nucleotide diversity; Ks: intraindividual proportion of synonymous mutations; Ka: intraindividual proportion of nonsynonymous mutations; Ω: ratio Ka/Ks

H: statistic of the Kruskal-Wallis test; F: statistic of the One-Way Anova test; F^a = statistic of the Welch test

(*p = 0.01-0.05; ** p = 0.001-0.01; *** p <0.001)

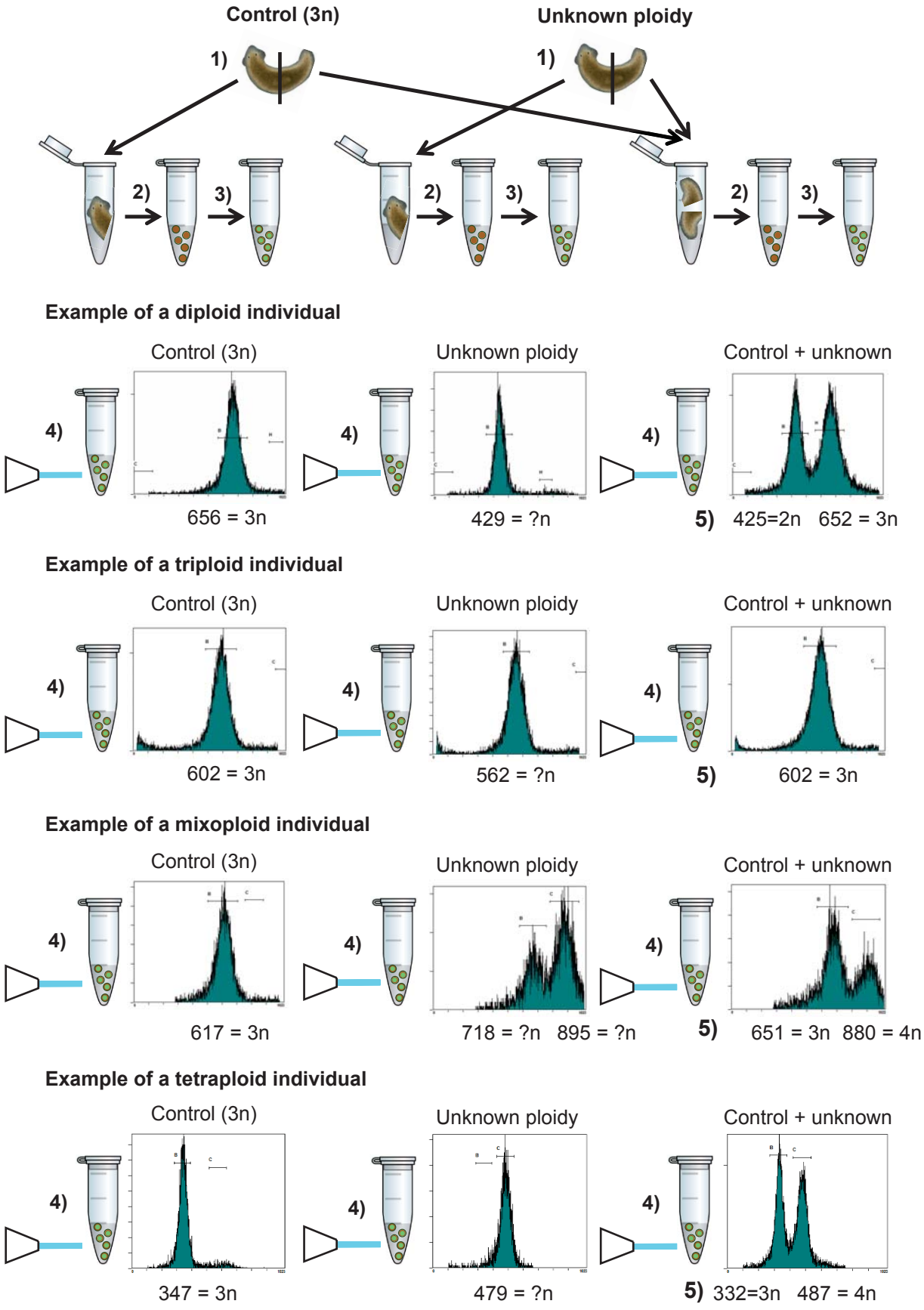
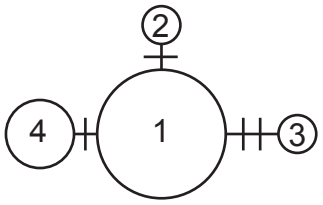


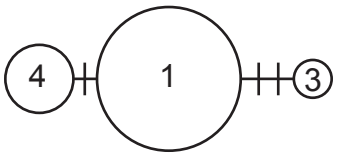
Figure S1. Workflow of the ploidy level estimation using flow cytometry. 1) Cutting the animal in two pieces, 2) cell maceration, 3) DNA staining, 4) fluorescence mesurement of each sample using a Gallios Flow Cytometer and 5) ploidy estimation of the unknown sample by comparison with the control.

Original sequence dataset for one individual



Seq 1: ATCGCATTAC (10)
Seq 2: ATCGCTTTAC (1)
Seq 3: ATGGCATAAC (1)
Seq 4: AACGCATTAC (3)

Dataset after the correction of putative artifact mutations



Seq 1: ATCGCATTAC (11)
Seq 3: ATGGCATAAC (1)
Seq 4: AACGCATTAC (3)

Figure S2. Approach used in this study for the correction of the putative artifactual mutations due to polymerase mistakes. In parentheses is indicated the frequency of each haplotype within the individual before and after the correction.

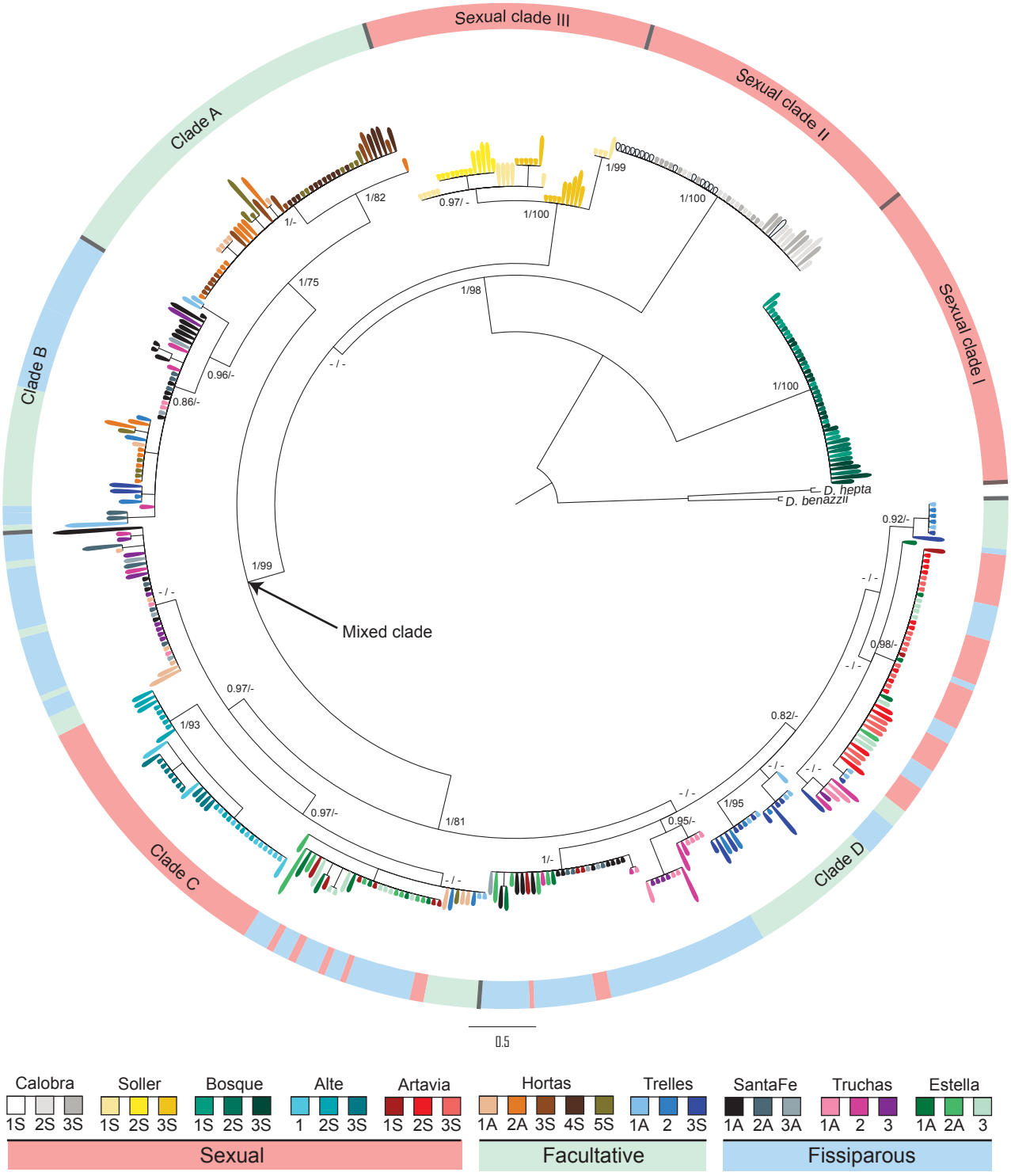


Figure S3. Bayesian inference tree of all the alleles of the nuclear gene TMED9. The colors of the terminal branches indicate to which individual each allele belongs. The color of the outter circle indicates the reproductive strategy of each individual. Numbers at the nodes indicate the support values for the Bayesian inference (posterior probability) and the maximum likelihood (bootstrap). Bootstrap values correspond to the maximum likelihood analysis conducted with rapid bootstrap. Support values lower than 0.8 (posterior probability) and 75% (bootstrap) are represented with a -. Scale bar indicates the number of substitutions per site.

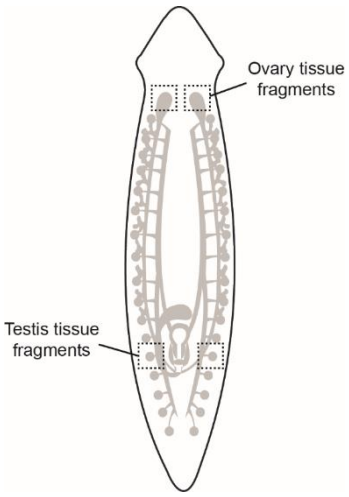
Figure S4. Bayesian inference tree of all the haplotypes of the mitochondrial gene Cox1. Haplotypes of the same individual are pictured with the same color. Numbers in white indicate the different clades within the Mixed clade. Numbers at the nodes indicate the support values for the Bayesian inference (posterior probability)/the maximum likelihood (bootstrap). Bootstrap values correspond to the maximum likelihood analysis conducted with rapid bootstrap. Support values lower than 0.8 (posterior probability) and 75% (bootstrap) are represented with a -. Scale bar indicates the number of substitutions per site.

Annex: Gametogenesis in triploid sexual individuals from facultative populations of *Dugesia subtentaculata*

In the Asian species *Dugesia ryukyuensis*, it was demonstrated that triploid ex-fissiparous individuals were able to reproduce truly sexually through a special meiotic system (Chinone, Nodono, & Matsumoto, 2014). In that work, the karyological analysis of the germ cells showed that triploid ex-fissiparous individuals not only produced recombinant haploid sperm but also recombinant haploid and diploid oocytes.

In the present thesis we aimed to test the protocol used by Chinone et al., (2014) to obtain meiotic images of triploid sexual individuals from facultative populations of the species *Dugesia subtentaculata*. To do so, we selected three individuals showing copulatory apparatus from a population with both sexual and fissiparous individuals of *D. subtentaculata* (population 4 in Chapter 2) and applied the following protocol:

1. Add 0'5 mg of Colchicine (1 mg/ml) to an Eppendorf tube containing 500 µl of distilled water.
2. Prepare 9'6 ml of a solution of KCl at 35%.
3. Add 400µl of the Colchicine solution to the 9'6 ml of the KCl solution and mix.
4. Divide the mixed solution into four Eppendorf tubes (for each individual).
5. Place two of these Eppendorf tubes at room temperature (RT) and place the other two into a water bath at 15ºC.
6. Fill a Petri dish with ice and place a glass slide, with parafilm in the middle region, over the ice.
7. Place a planarian with the ventral side upwards above the parafilm.
8. Take a surgical blade and cut the regions corresponding to the ovaries and the testis (each piece of approximately 2x2mm)



9. Place the ovary tissue fragments into one of the Eppendorf tubes at 15ºC (do the same for the testis tissue fragments).
10. Incubate for 1 hour at 15ºC in the water bath.
11. Transfer the tissue fragments to the Eppendorf tubes at RT (without breaking up the tissue) and incubate for 30 minutes.
12. Prepare 20 ml of a solution of Methanol and Acetic Acid (1:1). [This is the *Fix* solution]
13. Mix 6 ml of the *Fix* solution with 4 ml of distilled water. [This is the *Pre-fix* solution]
14. Remove the solution with Colchicine from the Eppendorf tubes and add 1'5 ml of the *Pre-fix* solution.
15. Incubate for 30 minutes at RT.
16. Remove the *Pre-fix* solution and add 1'5 ml of the *Fix* solution.
17. Incubate for 2-5 hours at RT.
18. Transfer each piece of tissue into a new Eppendorf tube containing 60 µl of the *Fix* solution.
19. Dissociate the tissue by pipetting.
20. Take the 60 µl and drop the liquid into a corner of a glass slide from approximately 10 cm high.
21. Turn the glass slide to let the liquid spread over its surface.
22. Let it dry for few seconds.
23. Take 60 µl of Acetic Acid and repeat the steps 20 and 21.
24. Cover the glass slides with foil (without touching the surface) and let dry overnight.
25. Prepare the following buffer solution: 250 ml of NaH_2PO_4 0'2M + 250 ml of $NaHPO_4$ 0'2M.
26. Adjust to PH 6'8.
27. Mix 150 ml of the buffer with 4'5 ml of Giemsa Stain at 3% into a staining glass jar.
28. Stain the glass slides for 5 minutes.
29. Clean the staining for 30 seconds using tap water.
30. Let dry.
31. Seal the karyological preparations with a cover slip using DPX.

The karyological preparations of the ovaries revealed triploid cells with both bivalent and univalent chromosomes (Fig. 1A), while in the case of the testis, we only found diploid cells with bivalent chromosomes (Fig. 1B). Although further analyses using more individuals will be needed, these preliminary results indicate that facultative triploid individuals of *D. subtentaculata* show the same meiotic system as the one found in *D. ryukyuensis*.

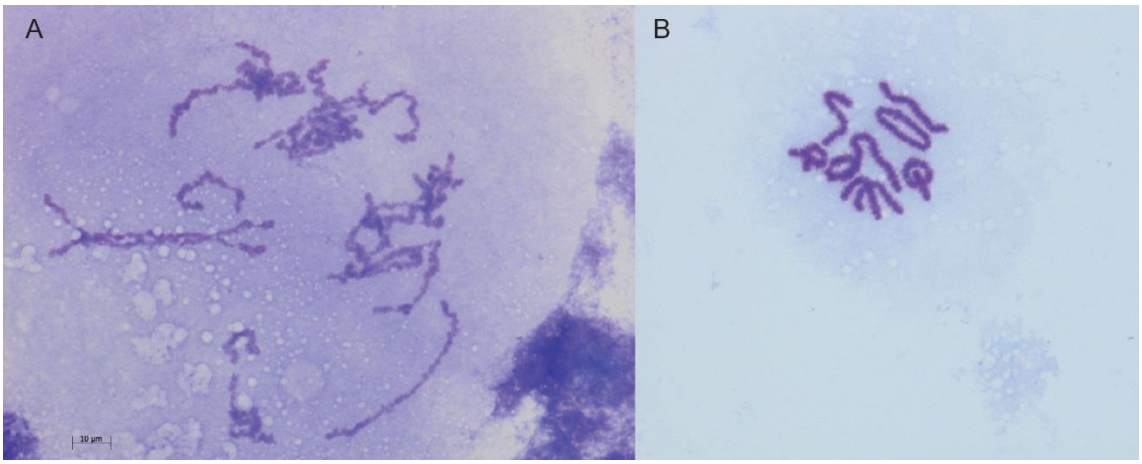


Fig. 1. Meiotic karyological images obtained of a triploid sexual individual from a facultative population of *Dugesia subtentaculata*. (A) Bivalent and univalent chromosomes from the ovary and (B) bivalent chromosomes from the testis.

References

Chinone, A., Nodono, H., & Matsumoto, M. (2014). Triploid planarian reproduces truly bisexually with euploid gametes produced through a different meiotic system between sex. *Chromosoma*, *123*(3), 265–272. https://doi.org/10.1007/s00412-013-0449-2

Chapter 2

Cryptic species delineation in freshwater planarians of the genus *Dugesia* (Platyhelminthes, Tricladida): Extreme intraindividual genetic diversity, morphological stasis, and karyological variability

Reference

Leria, L., Vila-Farré, M., Álvarez-Presas, M., Sánchez-Gracia, A., Rozas, J., Sluys, R., & Riutort, M. (2019). **Cryptic species delineation in freshwater planarians of the genus *Dugesia* (Platyhelminthes, Tricladida): Extreme intraindividual genetic diversity, morphological stasis, and karyological variability**. *Molecular Phylogenetics and Evolution*. In press.

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Cryptic species delineation in freshwater planarians of the genus *Dugesia* (Platyhelminthes, Tricladida): Extreme intraindividual genetic diversity, morphological stasis, and karyological variability

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ABSTRACT

The keystone of planarian taxonomy traditionally has been the anatomy of the copulatory apparatus. However, many planarian species comprise asexual fissiparous populations, with the fissiparous animals not developing a copulatory apparatus, thus precluding their morphological identification. Incorporation of molecular data into planarian systematics has been of great value, not only in the identification of fissiparous individuals but also as an additional source of information for determining species boundaries. Nevertheless, the discrepancy between morphological and molecular data has highlighted the need for extra sources of taxonomic information. Moreover, a recent study has pointed out that fissiparous reproduction may lead to high levels of intraindividual genetic diversity in planarians, which may mislead molecular analyses. In the present study we aim to test a new up-to-date integrative taxonomic procedure for planarians, including intraindividual genetic data and additional sources of taxonomic information, besides morphology and DNA, using *Dugesia subtentaculata* sensu lato as a model organism, a species with an intricate taxonomic history. First, we used three different methods for molecular species delimitation on single locus datasets, both with and without intraindividual information, for formulating Primary Species Hypotheses (PSHs). Subsequently, Secondary Species Hypotheses (SSHs) were formulated on the basis of three types of information: (1) a coalescent-based species delimitation method applied to multilocus data, (2) morphology of the copulatory apparatus, and (3) karyological metrics. This resulted in the delimitation of four morphologically cryptic species within the nominal species *D. subtentaculata*. Our results provide evidence that the analysis of intraindividual genetic data is essential for properly developing PSHs in planarians. Our study reveals also that karyological differentiation, rather than morphological differentiation, may play an important role in speciation processes in planarians, thus suggesting that the currently known diversity of the group could be highly underestimated.

1. Introduction

Discovering and describing species is not only important from a taxonomic point of view, but also because species are the fundamental units for other disciplines, such as ecology and conservation biology. However, defining and recognizing species is challenging, and during the history of systematic biology many different species concepts have been formulated (e.g., biological, ecological, phenetic, and phylogenetic concepts) (de Queiroz, 2007; Sluys and Hazevoet, 1999). Presently, a conceptual agreement is emerging among biologists in which species are considered as independently evolving metapopulation lineages, being known as the General Lineage Species Concept (de Queiroz, 1998) . Under this conceptual framework, other species concepts, such as, for example, the biological and phylogenetic species concepts are considered to be species *recognition criteria*, instead of *concepts*, that are used as different lines of evidence to delineate independently evolving lineages (de Queiroz, 2007; Frankham et al., 2012).

Although systematic studies may be based on only one of these lines of evidence for species delimitation, it has now become customary to

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search for congruence between several types of information, thus applying a practical method currently known under the term Integrative Taxonomy (Dayrat, 2005; Padial and Miralles, 2010; Schlick-Steiner et al., 2010). One of the most frequently used integrative taxonomic procedures consists of formulating Primary Species Hypotheses (PSHs), (i.e., hypotheses on candidate species on the basis of a single type of information, in general a single-locus molecular approach), followed by the evaluation of these PSHs with the help of other kinds of evidence, which then leads to the formulation of Secondary Species Hypotheses (SSHs) and the consequent taxonomic decisions (Pante et al., 2015a). Among the different criteria used to establish SSHs, the ones used most often concern morphology and multilocus DNA sequence data (Pante et al., 2015b). In case of congruence between these lines of evidence, confidence for the SSHs is high, which, subsequently, may be formalized through the description of new species. In case of non-congruence, additional sources of information, besides DNA and morphology, have been proven highly useful, not only for increasing confidence in the SSHs but also for inferring the putative causes underlying such incongruences between morphological and molecular evolution (Dejaco et al., 2016).

One of the most common cases of incongruence between morphological and molecular data concerns cryptic species. Cryptic species are defined as lineages that independently diversified but retained the same morphological characteristics. Basically, cryptic species have the same taxonomic status as species, albeit that the morphological information that is usually applied for recognition fails to discriminate these taxa. The notion of cryptic speciation was suggested a considerable time ago (Mayr, 1942), but it has not been until recently that the number of reported cryptic species has greatly increased, presently including examples from most animal phyla (Pérez-Ponce de León and Poulin, 2016). Different evolutionary mechanisms have been proposed to explain cryptic speciation, such as morphological stasis, convergence, or recent diversification (Struck et al., 2018). Unfortunately, a high proportion of these cryptic species remains undescribed, which precludes the integration of this important part of biodiversity into different fields of research (Fišer et al., 2018).

The increasing use of molecular data in systematic studies has promoted the development of several approaches for molecular species delimitation (Ence and Carstens, 2011; Pons et al., 2006; Puillandre et al., 2012; Yang and Rannala, 2010; Zhang et al., 2013). These methods can be classified under two principal categories, *viz*. validation and discovery methods, reflecting the condition whether the samples need to be partitioned *a priori* or not, respectively. These methods also differ in the type of input data that is used (genetic distances, phylogenetic trees, or allele sharing) and in the number of loci that can be incorporated (Flot, 2015), which may lead to some discrepancies between the delimitations obtained with the various methods (Carstens et al., 2013, Luo et al., 2018). Discovery methods can work with single-locus data and are generally used to formulate PSHs, while the validation methods that use information from multiple loci are generally used to develop SSHs (e.g., Razkin et al., 2017; Van Steenkiste et al., 2018).

Although initially only mitochondrial data had been widely used in species delimitation approaches, principally due to its high rate of sequence evolution, it has been shown that inclusion of multiple nuclear loci can help to detect putative genetic processes of introgression or incomplete lineage sorting, which, when present, may be reflected in the species hypotheses (Knowles and Carstens, 2007; Dejaco et al., 2016; Obertegger et al., 2018; Papakostas et al., 2016). Therefore, when including molecular data in a systematic study, it is important not only to apply different methods for species delimitation but also to use several mitochondrial and nuclear loci.

Species delimitation in free-living freshwater flatworms of the genus *Dugesia* Girard, 1850 (Platyhelminthes, Tricladida, Dugesiidae) traditionally has been based mostly on morphological data, concerning the anatomy of the copulatory apparatus, occasionally supplemented with karyological data. However, many *Dugesia* species may reproduce asexually by fission, with the fissiparous animals not developing a copulatory apparatus, thus precluding their morphological identification. In these cases, incorporation of molecular data has been of great value, not only in the identification of asexual individuals of known species (Lázaro et al., 2009) but also in tracing new species boundaries in *Dugesia* (Sluys et al., 2013). However, the only gene that has been used so far for species delimitation in this genus is the mitochondrial gene *Cytochrome c oxidase I* (Cox1).

Features such as geographic distribution, mode of reproduction, and karyology are sometimes reported in *Dugesia* species descriptions as extra characteristics of the species, but generally these data are not used as diagnostic characters. Different geographical distribution among lineages generally should not be used as a diagnostic trait of their evolutionary independence because of the possibility of changes in their distribution, either due to natural events (e.g., dispersion or extinction) or to human-mediated translocations (Pongratz et al., 2003; Solà, 2014). Similarly, although information on reproductive strategy may be very informative for detecting speciation processes, it is known that individuals of many *Dugesia* species are able to alternate between reproductive strategies (Stocchino and Manconi, 2013; and references therein). Thus, in this genus, information on reproductive strategy may not constitute a reliable source of evidence for tracing species boundaries. In contrast, differences in ploidy level and centromeric position of the chromosomes have been reported for different *Dugesia* species (Pala et al., 1999; Ribas, 1990). Furthermore, several *Dugesia* species have been described with a chromosome portrait that differs from the most common haploid complement of n = 8, exhibiting complements such as n = 7 or n = 9 (Ball, 1970; Gourbault, 1981; Kawakatsu et al., 1976; Pala et al., 1981; Stocchino et al., 2004). Therefore, karyological data may be a very informative additional line of evidence to be included in systematic studies of this genus.

The focal species of our study, *Dugesia subtentaculata* (Draparnaud, 1801), inhabits the Western Mediterranean region, with a total of 13 known localities scattered in Southern France, the Iberian Peninsula, Northern Africa, and the Balearic Islands (De Vries, 1986a; De Vries, 1988a; Lázaro et al., 2009). *Dugesia subtentaculata* was first described from near Montpellier (France) as being oviparous in spring and fissiparous in summer (Draparnaud, 1801). Some years later, the sexually reproducing specimens were assigned to the species *Dugesia gonocephala* (Dugès, 1830), while the fissiparous individuals remained as *D. subtentaculata*. However, in 1925, after a morphological re-examination of the two species, it was concluded that they were conspecific, and therefore the junior synonym (*D. gonocephala*) was assigned to the specimens, due to the difficulty of identifying the asexual individuals (Vandel, 1925). A good number of years later the new species *Dugesia iberica* Gourbault & Benazzi, 1979 was described from Mallorca and the Iberian Peninsula; this new species was externally and anatomically similar to *D. gonocephala* (Gourbault and Benazzi, 1979). Finally, in a taxonomic revision of these three species it was concluded that (1) *D. subtentaculata* is a different species than *D. gonocephala,* and (2) that *D. iberica* is conspecific with *D. subtentaculata*, the latter being the junior and valid species name (De Vries, 1986a). Thus, the separate taxonomic status of *D. subtentaculata* was re-established.

A few years later, in a karyological study on freshwater flatworms of the Iberian Peninsula, certain populations of *D. subtentaculata* showed differences in ploidy level, centromeric index and number of supernumerary chromosomes (Ribas, 1990). Inclusion of representatives of this species in molecular phylogenetic studies revealed very high genetic divergences between them and, in some cases, the species was not recovered as a monophyletic unit (Baguñà et al., 1999; Lázaro et al., 2009). Furthermore, a recent study on the impact of reproductive strategies on the genetic characteristics of individuals of this species showed that many individuals present very high levels of mosaicism and intraindividual genetic diversity due to their fissiparous reproduction (Leria et al., 2019).

In view of these previous studies on possible differentiation within the current nominal species *D. subtentaculata* (further below referred to as *D. subtentaculata* sensu lato (s.l.)), we considered it opportune to conduct a systematic revision of this species, not only for finally establishing its taxonomic status but also for exploring how inclusion of karyological data and up-to-date molecular methodologies might improve the usually complex species delimitation procedures in the genus *Dugesia* in general. Additionally, the existence of mosaicism and high intraindividual genetic diversity in this species makes the application of these new methodologies challenging and, therefore, *D. subtentaculata* forms a good model species to test whether these methods form a reliable tool for molecular species delimitation in planarians as well as other organisms with similar genetic characteristics. In view of our aim to perform an integrative species delimitation analysis of *D. subtentaculata* s.l., our procedure consisted of the following four sequential steps: (1) an extensive sampling across the species' distributional range, (2) a search for new, phylogenetically informative nuclear markers by means of a low-coverage genome sequencing approach, (3) formulation of PSHs on the basis of three different molecular discovery methods applied to a single mitochondrial locus and a single nuclear locus, with and without intraindividual information, and (4) formulation of SSHs by validating the PSHs with multilocus data (using a molecular validation method), morphological data, and karyological data.

2. Materials and methods

2.1. Taxon sampling

We sampled a total of 200 localities across all fluvial basins of the Iberian Peninsula and Southern France, including the species' type locality at Montpellier (France), as well as the two known populations from Mallorca (Balearic Islands) previously assigned to *D. iberica*. Moreover, *Dugesia* individuals from about 20 additional localities in the Iberian Peninsula and Northern Africa were obtained from collaborators. Thus, our dataset not only covered almost the complete distributional range of the species but even extended it considerably. *Dugesia subtentaculata* s.l. was found at 63 of these localities, which were all used in the present study (Fig. 1, Supplementary data S1). From each population some of the animals that showed a copulatory apparatus (indicated by presence of a gonopore at the ventral surface of the animal) were cut into two pieces: the anterior part of the individual (from the head to the prepharyngeal region) was fixed and stored in 100% ethanol for subsequent molecular work, while the rest of the body was fixed in Steinmann's fluid (see Winsor and Sluys, 2018) and thereafter preserved in 70% ethanol for morphological analysis of the copulatory apparatus. However, most of the animals from each of the populations that showed a copulatory apparatus were fixed entirely in Steinmann's fluid in order to have anatomical information for the whole body. Animals devoid of a copulatory apparatus were fixed in 100% ethanol. Additionally, some animals from each population were kept alive for karyological analysis. A few samples made available to us by colleagues (some populations from the Iberian Peninsula and Northern Africa) were used only for molecular analyses, as all of these animals were fixed in 100% ethanol and, therefore, were less suitable for histological studies.

2.2. Search for new molecular markers

2.2.1. Selection of individuals to be sequenced at genomic level

In order to identify new nuclear genes with adequate levels of variability within the species, we sequenced at low-coverage the genome of individuals from different populations, which in a Cox1-based phylogenetic tree showed among them different levels of genetic divergence. For constructing that tree, we extracted genomic DNA from at least two specimens from each population sampled, using the commercial reagent DNAzol (Molecular Research Center Inc., Cincinnati, OH), following the manufacturer's instructions. Subsequently, the mitochondrial gene Cox1 was PCR-amplified for all individuals, using the primers and PCR conditions described in Solà et al. (2013). The phylogeny was inferred through Bayesian Inference using the program MrBayes 3.2 (Ronquist et al., 2012), running 1,500,000 generations and sampling a tree every 150 generations. Phylogenetic inference was carried out with two independent runs and with a burn-in of 25% to infer the tree topology and the posterior probability of the nodes. Before applying the burn-in, it was checked through the standard deviation of splits value that convergence of the two runs had been achieved and that each run had arrived at the stationary region. The substitution model was previously determined with jModelTest2 (Darriba et al., 2012). Subsequently, using the Cox1 phylogeny as a reference, we chose four individuals belonging to the populations 15, 22, 46 and 48 for sequencing their genome at low-coverage. Additionally, we selected also some individuals from a population of *Dugesia sicula* Lepori, 1948 from Mallorca to be sequenced at the genome level, as this species is genetically highly differentiated from *D. subtentaculata* s.l. (Lázaro et al., 2009) and thus would facilitate detection of highly conserved regions for primer design.

2.2.2. High-quality DNA extraction and genome sequencing

We first estimated the necessary sequencing effort for reaching a 4x coverage per genome, which would be sufficient for discovering new molecular markers for our study. Therefore, by flux cytometry (protocol in Supplementary data S2) we inferred the size of the haploid genome of selected populations of *D. subtentaculata* s.l. and *D. sicula*. Thereafter, 1 μg of high-quality DNA was extracted from single individuals from each of the four populations of *D. subtentaculata* s.l., using a customized phenol–chloroform protocol; for *D. sicula* DNA extraction was performed on a pool of three individuals. Tissue was digested overnight at 37 °C in a solution containing 200 μL of Lysis Buffer Solution (Wizard®, Promega, Madison, WI, USA) and 10 μL of Proteinase K (20 mg/mL). Then 12 μL of RNase A (10 mg/mL) was added and the solution was incubated during 1 h at 37 °C. Finally, the standard phenol–chloroform extraction protocol of Sambrook et al. (1989) was followed. Quality and quantity of total DNA was examined using a Qubit 2.0 Fluorometer (Invitrogen). The whole genome of these individuals was sequenced in a lane of the Illumina Hi-Seq2000 (tagged paired-end libraries) in Macrogen Inc., South Korea (www.macrogen.com). Raw data of each genome was filtered for poor quality and low complexity reads by using the subprogram “preprocess” from the SGA pipeline (with “—dust” and “–quality-filter = 30” options). In order to generate contigs that we could later blast against *Schmidtea mediterranea*'s (Benazzi, Baguñà, Ballester, Puccinelli, & Del Papa, 1975) genome, we mapped these preprocessed genomic reads onto the reference transcriptome assembly of *Dugesia japonica* Ichikawa & Kawakatsu, 1964 (Chan et al., 2016) using the faster mapping option based on BWA (Li and Durbin, 2009) of Stampy (Lunter and Goodson, 2011).

2.2.3. Marker selection

Dugesia contigs were blasted against the genome of *Schmidtea mediterranea* SXl v4.0, available online at SmedGD (http://smedgd.stowers.org) (Robb et al., 2015), and contigs corresponding to single copy genes containing introns flanked by conserved exonic regions were selected. Primers were designed for a total of 23 markers and were PCR-tested by using one individual from each Cox1 main clade. Finally, six markers that showed a mean divergence between the different populations ranging from 2% to 6% were used for the present study. These markers, which were named Dunuc's (from *Dugesia* nuclear) followed by a number, corresponded to: (1) a MAP Kinase death domain (Dunuc2), (2) an anonymous marker (Dunuc3), (3) a disulphide isomerase (Dunuc5), (4) a translation initiation factor (Dunuc10), (5) a transport protein transmembrane domain (Dunuc12, referred to as TMED9 in Leria et al., 2019), and (6) a transcription factor (Dunuc20).

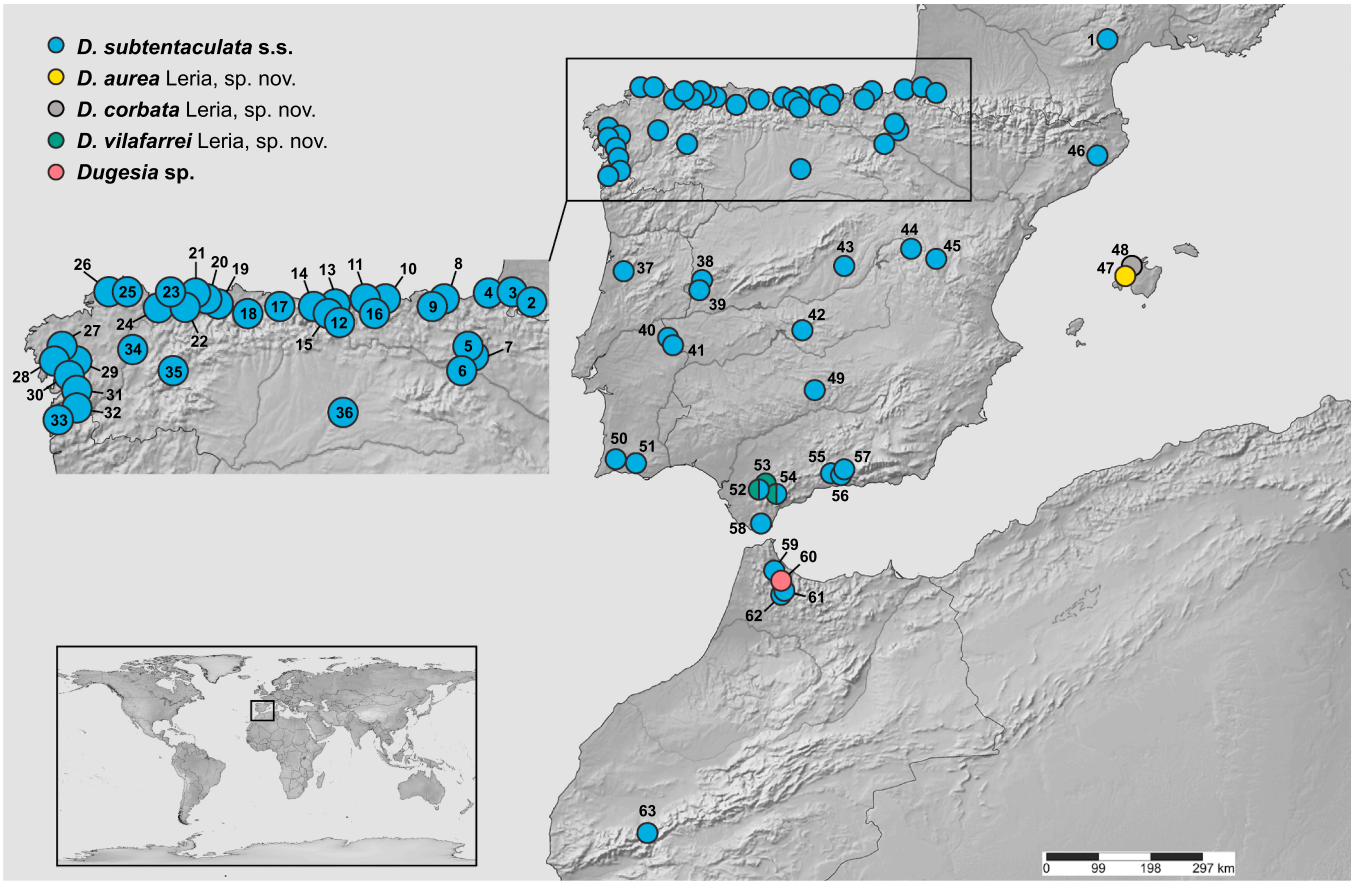

Fig. 1. Geographic populations of the *Dugesia subtententaculata* s.l. species complex examined in the present study. Numbers correspond to population codes listed in Supplementary data S1. At localities 52 and 54 *D. vilafarrei* and *D. subtentaculata* s.s., occur together, as indicated by the two separate halves of the circles filled with their respective colours.

2.3. DNA sequences and datasets

In addition to the two individuals per locality that were PCR-amplified for Cox1, nine additional gene fragments were amplified for a subset of individuals belonging to the 20 main Cox1 clades. These gene fragments included: (a) six new nuclear fragments (Dunuc2, Dunuc3, Dunuc5, Dunuc10, Dunuc12 and Dunuc20), (b) *28S ribosomal 1* (28S), (c) *Internal Transcribed Spacer 1* (ITS), (d) a mitochondrial fragment containing part of the *NADH: ubiquinone oxidoreductase core subunit 1* (Nad1), (e) *transfer RNA-Trp* (tRNA-W), and (f) part of *Cytochrome c oxidase II* (Cox2). Amplification of the two ribosomal genes was carried out by using already available primers (Álvarez-Presas et al., 2008; Baguñà et al., 1999), while the mitochondrial fragment was amplified with the help of newly designed primers by using the available mitogenomes of *D. japonica* and *D. ryukyuensis* Kawakatsu, Oki, Tamura & Sugino, 1976 as reference (Sakai and Sakaizumi, 2012). The Cox1 and Dunuc12 gene fragments were also PCR-amplified for several individuals of different *Dugesia* species closely related to *D. subtentaculata* s.l. (Lázaro et al., 2009; Solà, 2014), which were used as outgroup taxa in the various molecular species delimitation analyses, *viz. D. hepta* Pala, Casu & Vacca, 1981, *D. benazzii* Lepori, 1951, *D. etrusca* Benazzi, 1944, and *D. liguriensis* De Vries, 1988. Primer sequences and PCR conditions used in this study are detailed in Supplementary data S3.

Amplification products were purified using a vacuum system (MultiScreen™HTS Vacuum Manifold of Millipore) and were subsequently sequenced in both directions at Macrogen Europe, Inc. (Amsterdam). Complementary strands of DNA were assembled into consensus using Geneious R8 (Biomatters, http://www.geneious.com/). All sequences were deposited in GenBank (GenBank accession numbers detailed in Appendix A).

Finally, we took profit of sequences obtained in a parallel study on the intraindividual genetic variability of this species, for which we obtained intraindividual sequences by cloning the PCR products of the Cox1 and Dunuc12 genes for each individual (Leria et al., 2019). In the present study, we have included all intraindividual sequences (on average 10 per individual) of the two genes for 32 individuals (belonging to the main Cox1 clades). GenBank accession numbers of sequences used are MK385658 to MK385866 (Dunuc12) and MK385871 to MK385922 (Cox1).

Sequences of all gene fragments were separately aligned using the online software MAFFT (version 7) (Katoh and Standley, 2013) and revised with Geneious R8. The protein coding genes were translated into amino acids to check the reading frame (genetic code 9 was used for the mitochondrial genes, while genetic code 1 was used for the nuclear genes). Degree of sequence saturation of all alignments was checked with the program DAMBE (Xia and Xie, 2001) by performing a substitution saturation test (Xia et al., 2003; Xia and Lemey, 2009). The non-coding genes and the non-coding regions (introns) were analyzed at the nucleotide level, while the protein coding genes and the protein coding regions (exons) were analyzed at the three codon positions.

Aligned sequences of the 12 loci were organized into 7 different datasets to be used in the various molecular species delimitation analyses: (1) *Cox1*, (2) *Dunuc12*, (3) *Cox1-Cloned* (intraindividual Cox1 sequences), (4) *Dunuc 12-Cloned* (intraindividual Dunuc12 sequences), (5) *All* (12 loci), (6) *Nuclear* (6 Dunuc loci) and (7) *Mitochondrial* (4 mitochondrial loci) (Appendix A). In the datasets with no intraindividual information, each individual was represented by a single sequence (in some cases with polymorphic sites), while in the *Cloned*

datasets the 32 selected individuals had many sequences without polymorphic sites, corresponding to their different intraindividual haplotypes.

2.4. Integrative taxonomic procedure

Our integrative taxonomic procedure required the formulation of initial Primary Species Hypotheses (PSHs). These PSHs were based on the most often recurring partition of species obtained, after independent application of three different molecular discovery methods of species delimitation to the datasets 1, 2, 3 and 4 (single locus datasets with or without intraindividual information). The methods used in this step were: (a) Automatic Barcode Gap Discovery (ABGD; Puillandre et al., 2012), (b) multi-rate Poisson Tree Processes (mPTP; Kapli et al., 2017), and (c) General Mixed Yule-Coalescent (GMYC; Pons et al., 2006). The PSHs were subsequently validated, or not, by different lines of evidence: (a) multilocus data (datasets 5, 6 and 7) by using the coalescent-based method incorporated in the software Bayesian Phylogenetics and Phylogeography (BPP; Yang and Rannala, 2010); (b) morphological data; (c) karyological data. The results of these three sources of information were integrated in order to generate the Secondary Species Hypotheses (SSHs), eventually leading to pertinent taxonomic decisions (Fig. 2).

2.5. Molecular methods for species delimitation

2.5.1. Discovery

ABGD is a distance-based method that uses a DNA alignment to determine the threshold between intraspecific and interspecific diversity (the barcode gap). Genetic distances of each alignment were calculated with the help of the program MEGA version 5 (Tamura et al., 2011) under the Kimura-2-parameters model, while the resulting distance matrix was imported into the ABGD web-interface (available at http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html). All parameters were left as default, excepting the relative gap width, which was set to 1, since we are working on closely related candidate species. In each analysis the selected partition scheme corresponded to the maximum value of intraspecific genetic diversity (P) that delimited the outgroups as different species.

The mPTP method uses the number of substitutions along the branches of a phylogenetic tree to determine putative species, based on the assumption that intraspecific and interspecific substitutions follow distinct Poisson distributions. Moreover, this method allows different substitution rates to take place at intraspecies level. Before estimating the phylogenetic trees, we determined the best substitution model for each dataset, using jModelTest2. In both Cox1 datasets the molecular evolutionary model determined was the HKY + Gamma + Invariant sites, while for the Dunuc12 it was GTR + Gamma. The input phylogenies were obtained by using Maximum Likelihood with the program RaxML 7.0.0 (Stamatakis, 2006), with 2000 replicates to obtain bootstrap support. For the Cox1 datasets we also used the model GTR, since that model is the only one implemented in the program RaxML. To run the mPTP analyses we used the command line version (Kapli et al., 2017). All analyses were conducted with 4 independent runs of 5,000,0000 Markov chain Monte Carlo (MCMC) generations, sampling at every 10,000 generations in order to obtain the support values for each delimitation scheme. As all identical sequences were removed prior to the phylogenetic inference, no minimum branch length (minbr) was used.

GMYC identifies the transition between intra- and interspecific branching rates in an ultrametric tree by modelling speciation via a pure birth process (Yule model) and intraspecies divergence via coalescence. The ultrametric tree for each dataset was inferred by using the software BEAST v.1.7.4 (Drummond et al., 2012). For the Cox1 datasets we set as site priors: substitution model = HKY; bases frequencies = empirical; site heterogeneity model = gamma + invariant sites; number of gamma categories = 4. For the Dunuc12 datasets we set as site priors: substitution model = GTR; bases frequencies = empirical; site heterogeneity model = gamma; number of gamma categories = 4. For the Cox1, a lognormal relaxed molecular clock with a mean value of 0.017 substitutions per site per million years was used for time-calibrating the tree, a mean value that was estimated for the genus *Dugesia* (Solà et al., 2013), while for the nuclear marker the rate parameters were left as default. For both molecular markers the Yule Process was used as speciation model (Gernhard et al., 2008). For the datasets that contained polymorphic sites we set BEAST to use the information of the ambiguous codes, since by default the polymorphic sites in BEAST are treated as missing data. Equal sequences were removed prior to the analyses. Runs were conducted in CIPRES Science Gateway (Miller et al., 2010) with 50,000,000 generations and sampling every 5,000 generations. The resulting log files were examined in Tracer 1.7 (Rambaut et al., 2018) to check that the Effective Sample Size (ESS) values for the different parameters were higher than 200. TreeAnotator (available in the BEAST package) was used to construct the ultrametric trees, using a burn-in of 10%. The ultrametric trees obtained with BEAST were submitted to SPLITS package for R (SPecies LImits by Threshold Statistics; Ezard et al., 2009; available at http://r-forge.r-project.org/projects/splits/), which implements GMYC. The analyses were conducted under the single-threshold approach, while always checking that the results of the tests were significant.

2.5.2. Validation

BPP can use the information of multiple genes under the multispecies coalescent model (Rannala and Yang, 2003) to evaluate

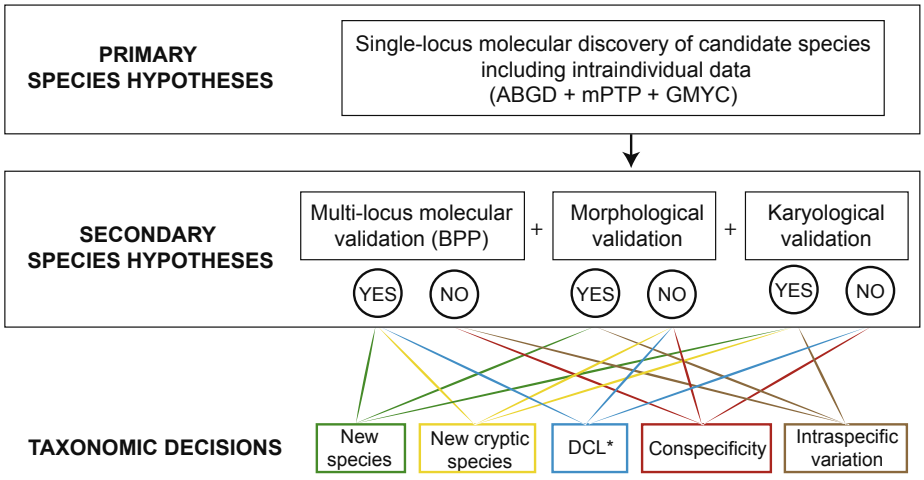

Fig. 2. Integrative taxonomic procedure used in this study. Formulation of Primary Species Hypotheses (PSHs) was based on the most recurrent partition obtained after independent application of three methods of molecular species delimitation (ABGD, mPTP, and GMYC) to four single-locus datasets (nuclear, mitochondrial, nuclear with intraindividual information, and mitochondrial with intraindividual information). Secondary Species Hypotheses (SSHs) were obtained through validation of PSHs based on multi-locus molecular data (applying BPP), morphological data, and karyological data, leading to pertinent taxonomic decisions. *: Deep Conspecific Lineage.

whether the different nodes of a given phylogeny should be collapsed or retained, depending upon whether coalescent or speciation processes are adequate explanations (model A10, species delimitation from a fixed guide tree) (Yang, 2015). The topology of the guide tree for the BPP analyses was inferred with *BEAST (Heled and Drummond, 2010) using dataset 5 (all loci) with the following priors, independently calculated for each locus: substitution model = HKY; bases frequencies = empirical; site heterogeneity model = gamma; number of gamma categories = 4; clock type: uncorrelated lognormal relaxed; Species tree = Yule Process; Population Size Model: Piecewise linear & constant root. The analysis was run in Portal CIPRES, setting 100 million generations and sampling every 10,000. Convergence was assessed in Tracer by checking the ESS values. The species tree was estimated by using *D. hepta* and *D. benazzii* as outgroups. However, the BPP analyses were conducted by using the sequences of *D. subtentaculata* s.l. alone.

As BPP needs prior information on ancestral population size (θ) as well as divergence time from the root (τ), we tested four possible different scenarios for our PSHs in three different BPP analyses, using the multilocus datasets 5, 6 and 7: (1) M1: small ancestral population size and shallow divergence (G (2 1000) for θ and G (2 1000) for τ); (2) M2: large ancestral population size and shallow divergence (G (1 10) for θ and G (2 1000) for τ); (3) M3: large ancestral population size and deep divergence (G (1 10) for θ and τ); (4) M4: small ancestral population size and deep divergence (G (2 1000) for θ and G (1 10) for τ). The combination of these priors with the three datasets gave a total of 12 different partitions to be tested with BPP.

2.6. Morphological data

Specimens that had been preserved in Steinmann's fluid were cleared in clove oil and subsequently embedded in synthetic wax. Sagittal sections were made at intervals of 8 μm and horizontal sections at intervals of 7 μm and, subsequently, were stained in Mallory-Cason/Heidenhain (cf. Winsor and Sluys, 2018) and mounted in DPX. Reconstructions of the copulatory apparatus were obtained by using a *camera lucida* attached to a compound microscope. In order to clearly visualize some anatomical structures, we also made a three-dimensional (3D) reconstruction of the copulatory complex from digitized images of serial histological sections, using the software Free-D (Andrey and Maurin, 2005). All specimens used for the morphological analysis were deposited in the collections of the Naturalis Biodiversity Center, Leiden, The Netherlands (Supplementary data S4).

Besides performing a morphological study of the different PSHs recognized within *D. subtentaculata* s.l., we also compared the morphological characteristics of the new material with the histological sections of the neotype of this species (deposited in the collections of Naturalis Biodiversity Center).

2.7. Karyological data

Individuals selected for the karyological analysis were cut into two pieces and left to regenerate for 4 days in a 1:1 mixture of tap water and distilled water at 20 °C. Then, the specimens were incubated in a solution of 0.075% colchicine for 6 h. Next, we washed the animals with a solution of 0.5% N-acetyl-L-cysteine for 1 min before fixing them in a freshly prepared mixture of methanol:glacial acetic acid (3:1) and, subsequently, incubating them for 20 min in 40%–45% glacial acetic acid. After incubation, each animal was placed onto a glass slide, and the region of the blastema and post-blastema was minced with a surgical blade and suspended in 20 μL of glacial acetic acid. The macerated cell suspension was dropped onto preheated glass slides (at 65 °C) and were left to air-dry. Thereafter, chromosome preparations were stained with 1:20 Giemsa:tap water mixture for one minute, dried, and then sealed under a cover slip by using DPX. All karyological preparations were deposited in Dpt. de Genètica, Microbiologia i Estadística; Facultat de Biologia; Universitat de Barcelona, Barcelona, Spain (Supplementary table S5).

An average of 5 metaphasic plates per individual were photographed and subsequently analyzed with the program Karyotype (Altınordu et al., 2016) in order to determine ploidy level, centromeric indices, and relative lengths of the chromosomes. Classification of the chromosomes on the basis of their centromeric index followed Levan et al. (1964). Chromosome measures of *D. subtentaculata* populations published by De Vries (1986b) and Ribas (1990) were included in the analysis.

Abbreviations used in the figures: bc, bursal canal; ca, common atrium; cb, copulatory bursa; cg, cement glands; dp, diaphragm; ed, ejaculatory duct; ec, ectal reinforcement; go, gonopore; mg, musculoglandular structure; od, oviduct; pb, penis bulb; pp, penis papilla; sg, shell glands; sv, seminal vesicle; vd, vas deferens.

3. Results

3.1. Geographic distribution of D. subtentaculata s.l.

The extensive samplings carried out for this study increased the number of known localities of *D. subtentaculata* s.l. from 13 to 67. Interestingly, *D. subtentaculata* s.l. reaches its maximum abundance in the northern sector of the Iberian Peninsula, an area where the species was not detected previous to our study. In contrast, its occurrence in southern France is restricted to the type locality (near Montpellier) and two other localities at the western coast near the Basque country (Fig. 1; Supplementary data S1). The localities where we did not find *D. subtentaculata* s.l. were either occupied by other *Dugesia* species, other planarian species, or did not have any freshwater triclad fauna at all (Supplementary data S6).

3.2. Low coverage genome assembly

We estimated a haploid genome size of approximately 2 Gb for both *D. subtentaculata* s.l. and *D. sicula*. The average of total bases sequenced per species was 7.85 Gb and 7.1 Gb, which corresponded to a coverage across the genome of 3.93X and 3.55X, for *D. subtentaculata* s.l. and *D. sicula* populations, respectively (with a very slight variation in the coverage of each individual population). After the read pre-processing, the number of reads used for the mapping step varied between 85% and 90% of raw reads, depending on the population. However, the final percentage of these pre-processed genomic reads aligned with *D. japonica* transcripts was only a 5.7%–7.3%, since the rest of the reads corresponded to freshwater protozoans. The number of *D. japonica* transcripts mapped with reads of the other species ranged from 21,087 (*D. subtentaculata* s.l. population 22) to 28,612 (*D. sicula*), although the proportion of the transcripts covered by genomic reads was highly variable. The consensus sequences across the populations of *D. subtentaculata* s.l. and *D. sicula* for these mapped regions were used as queries in BLAST searches for marker discovery (see methods).

3.3. Molecular datasets

A total of 840 new sequences of *D. subtentaculata* s.l. were obtained for the present study (Appendix A), representing approximately 7300 aligned characters. Information on each dataset, including number of sequences, alignment length, and number of variable sites, is detailed in Supplementary data S7. No stop codons were detected in the protein coding genes. Further, no gene, gene fragment, or codon position showed significant levels of sequence saturation, as in all cases the Index of Substitution Saturation was significantly lower than the Critical Index of Substitution Saturation (Supplementary data S8).

3.4. Single-locus discovery of candidate species

Phylogenetic inferences based on the Cox1 and Dunuc12 datasets

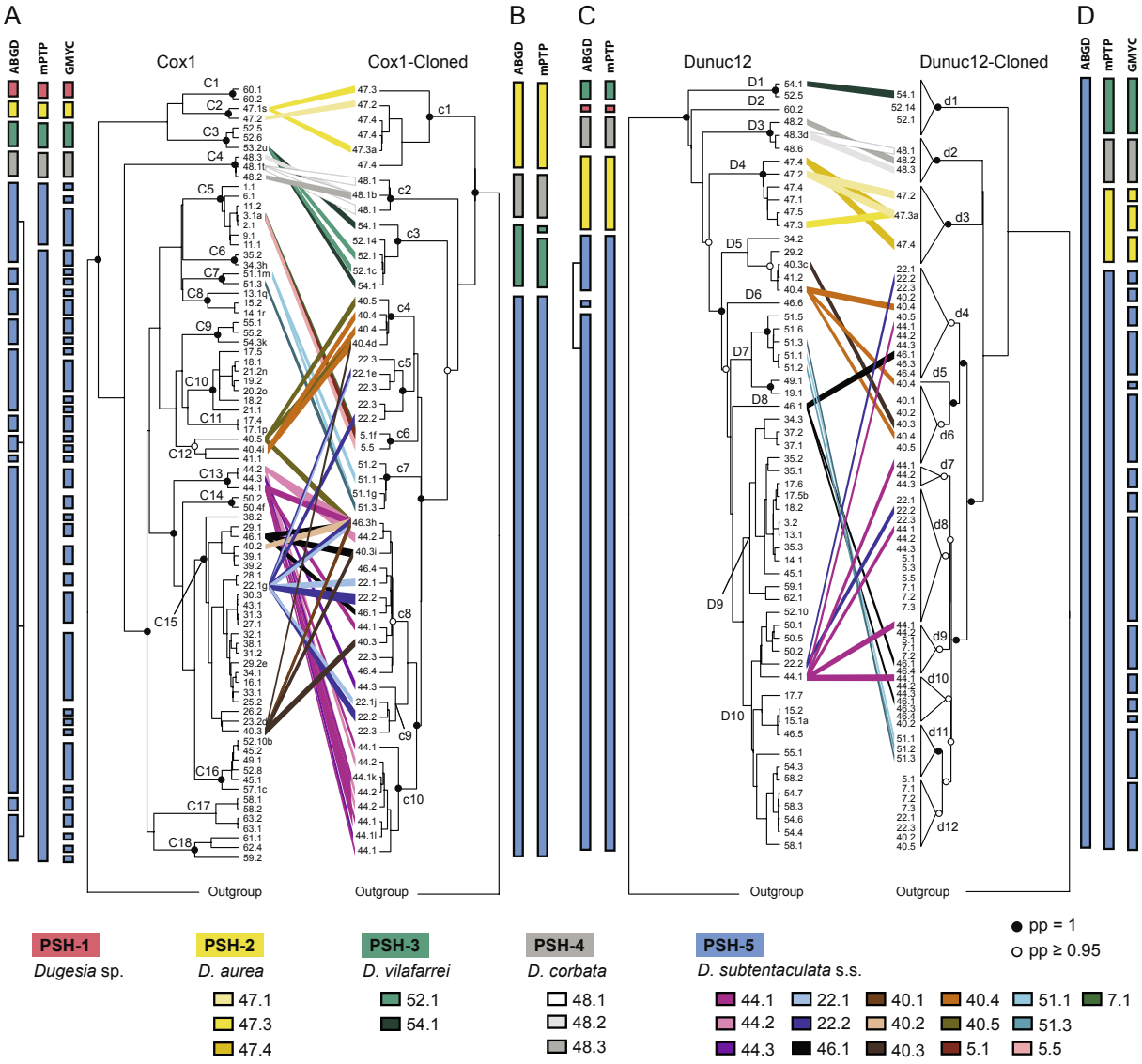

Fig. 3. Summary of the results obtained for the three molecular species delimitation methods (ABGD, mPTP and GMYC) applied to the Cox1 dataset (A), Cox1-Cloned dataset (B), Dunuc12 dataset (C), and Dunuc12-Cloned dataset (D). The partition scheme obtained for each method/dataset is indicated by coloured boxes next to the ultrametric Bayesian phylogenetic tree inferred with BEAST; boxes connected by thin black lines belong to the same candidate species. Colours of boxes correspond to the Primary Species Hypotheses resulting from the analysis. Coloured lines connect the haplotypes of some individuals (see graphical legend) obtained by direct PCR with the haplotypes of the same individuals obtained by cloning. Letters (a to v) after the codes of the individuals denote that the same haplotype was found in other individuals (Supplementary data S9). Nodes without circles indicate a pp $<$ 0.95. See Supplementary data S10 for the non-collapsed Dunuc12-Cloned tree.

without intraindividual information resulted in similar topologies (Fig. 3A and Fig. 3C, respectively). In both phylogenies the first diverging populations were the following: 47, 48, 60, and some individuals of populations 52–54. Individuals of these populations were structured in four different clades, clades C1 to C4 and D1 to D4 in Fig. 3A and Fig. 3C, respectively (further below the individuals from localities 52–54 that conform the clades C3 and D1 will be referred as 52–54*). Although each of these four clades was highly supported, evolutionary relationships between them were not fully resolved in any of the two phylogenies. The remaining populations were structured in 13 main clades in the Cox1 phylogeny (from clade C5 to clade C18 in Fig. 3A) and in 6 main clades in the Dunuc12 phylogeny (from clade D5 to clade D10 in Fig. 3C). Additional phylogenetic inferences of both genes, now using intraindividual information of individuals belonging to different clades (Fig. 3B, D), recovered each of the clades formed by the populations 47, 48, and 52–54* as monophyletic (no cloned information on population 60 being available) (clades c1 to c3 and d1 to d3 in Fig. 3B and Fig. 3D, respectively). The haplotypes of the rest of individuals were structured in 7 different clades in the Cox1-Cloned dataset (which did not have an exact match with the clades of the Cox1 dataset) and in 6 different clades in the Dunuc12-Cloned dataset (again with different correspondence with the Dunuc12 dataset clades) (Fig. 3B, D). Lack of correspondence between the clades of the cloned and non-cloned datasets was due, on the one hand, to the fact that on the basis of the cloned datasets, haplotypes of the same individual were distributed in different clades (e.g., individual 22.2 in Fig. 3B, D). On the other hand, it turned out that individuals that occurred in different clades in the non-cloned datasets, shared some haplotypes or showed very similar haplotypes in the cloned datasets (e.g., individuals 40.5-C12 and 44.2-C13 share a haplotype named 44.3 h-c8; Fig. 3A, B).

3.4.1. ABGD

The ABGD method applied to the Cox1 dataset delimited 12 different groups within *D. subtentaculata* s.l. (P 0.0027–0.0077) (Fig. 3A). But when this method was applied on the Cox1-Cloned dataset it retrieved only four groups within *D. subtentaculata* s.l. (P = 0.0077)

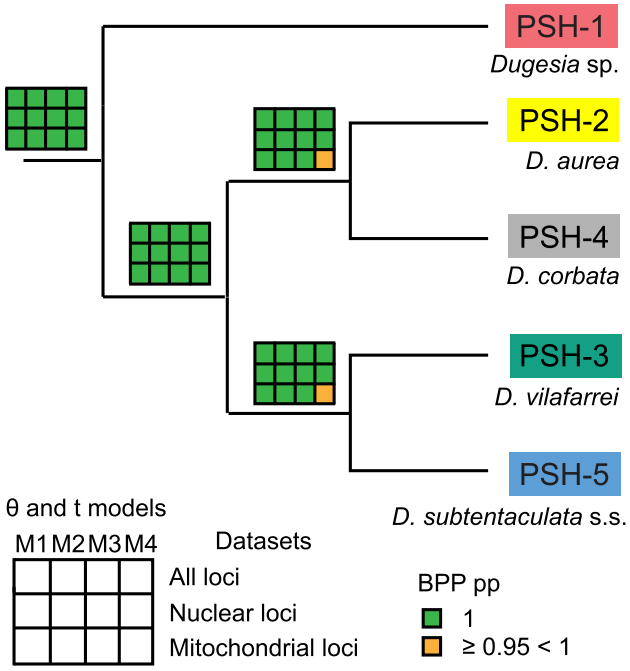

Fig. 4. Schematic representation of the results obtained with BPP on multilocus data. Colours of squares indicate the posterior probability of each node for each of the 12 BPP analyses. The topology was obtained with *BEAST for the multilocus dataset containing all loci (Supplementary data S11). Colour codes of the PSHs are the same as used in Fig. 3.

(Fig. 3B). Three of these groups were the same for both datasets, corresponding to the populations 47, 48, and 52–54*. The fourth candidate species for the Cox1-Cloned dataset comprised the rest of the populations, excepting number 60, which was not represented in the cloned dataset (this population was retrieved as an independent group in the dataset with no intraindividual information).

The ABGD method applied to the nuclear marker Dunuc12 recognized six different groups within *D. subtentaculata* s.l. (P 0.0028–0.001) (Fig. 3C). Four of these groups coincided with the Cox1 results by delimiting the following populations as candidate species: 47, 48, 52–54*, and 60. One of the other two groups was formed by a singleton from population 46, while the other group comprised the rest of the populations. ABGD on the Dunuc12-Cloned dataset retrieved all *D. subtentaculata* s.l. populations as a single species (P 0.0028–0.001) (Fig. 3D).

3.4.2. mPTP

The mPTP method delimited for the Cox1 dataset six candidate species within the group of *D. subtentaculata* s.l. populations, with an average support of 0.93 (Fig. 3A). Four of these candidate species corresponded to groups already delimited by the ABGD analysis of this dataset, *viz.* populations 47, 48, 52–54*, and 60 (all with a posterior probability (pp) of 1); the rest of the populations was divided into two groups (pp = 0.8). In the Cox1-Cloned dataset (pp = 0.93) populations 47, 48, and 52–54* were also retrieved as different candidate species (pp of 1, 1 and 0.72, respectively), while the rest of the populations formed a single group (pp = 1) (Fig. 3B). Surprisingly, in the Cox1-Cloned dataset one haplotype of individual 54.1 was positioned as representing a single candidate species (pp = 0.72).

In the case of the nuclear Dunuc12 and Dunuc12-Cloned datasets, the mPTP method delimited for both datasets the same four groups within the *D. subtentaculata* s.l. populations (with an average pp of 0.93 and 0.82, respectively), *viz.* populations 47, 48, 52–54*, and all the remaining ones (Fig. 3C, D). In addition, population 60 was also delimited as a species in the Dunuc12 dataset (this population is not present in the Dunuc12-Cloned dataset). The pp values for all groups were higher than 0.8 in both datasets, excepting the group formed by individuals of population 47 and the group comprising the rest of the populations on the Dunuc12 dataset (pp = 0.77).

3.4.3. GMYC

The GMYC analysis of the Cox1 dataset delimited populations 47, 48, 52–54*, and 60 as separate candidate species, while the rest of the populations were spread over no less than 36 different groups (Fig. 3A). However, support values for all these 36 groups were very low. In contrast, the Cox1-Cloned dataset produced no significant differences between the likelihood of the GMYC model and the null model (GMYC model = 153.5169, null model = 152.7937, likelihood ratio = 1.44 and result of LR test: 0.485 n.s.), so that no candidate species could be delineated on the basis of this dataset.

The same result was obtained for the Dunuc12 dataset (GMYC model = 258.54, null model = 257.91, likelihood ratio = 1.24 and result of LR test: 0.53 n.s.). However, the GMYC analysis performed on the Dunuc12-Cloned dataset did give significant results, in that populations 52–54* and 48 (clades d1 and d2, respectively) were retrieved as different candidate species, with high support values, while the remaining populations were arranged into 18 different groups, albeit with low support values (Fig. 3D). In the last-mentioned case, haplotypes of the same individual were distributed over two, three, four, or even five different candidate species.

3.5. Primary Species Hypotheses

Our analysis using three molecular methods for species delimitation most frequently identified the following populations as candidate species: 47, 48, 52–54*, and 60 (although population 60 was only available for the datasets without cloned information). The cloned datasets indicated that the remaining populations all constituted a single candidate species (excepting on the GMYC analysis), while in most cases the datasets without intraindividual information arranged these populations into different candidate species, because of their inability to detect that the genetic diversity occurred at the intraindividual level. Therefore, our Primary Species Hypotheses were: PSH-1 (population 60), PSH-2 (population 47), PSH-3 (populations 52–54*), PSH-4 (population 48), and PSH-5 (populations 1–63, excepting the ones constituting the other PSHs).

3.6. Validation of the Primary Species Hypotheses

3.6.1. Multilocus molecular validation

The species tree obtained with *BEAST for the multilocus dataset (Supplementary data S11) resulted in the following topology: PSH-1 was sister to a group including the other four PSHs, which comprised two sister-groups, one formed by PSH-2 and PSH-4 (two lineages from Mallorca) and the other by PSH-3 and PSH-5. The results of the BPP analyses for the three multilocus datasets using this topology (Fig. 4) suggested that the genetic differentiation among these five PSHs might be explained by speciation rather than by coalescent processes, as in all cases all nodes were recovered with a high posterior probability. Different prior values of θ (ancestral population size) and τ (divergence time) did not have a significant effect on the results of the BPP analyses in any of the datasets, excepting the mitochondrial dataset, in which the pp for the nodes PSH-2/PSH-4 and PSH-3/PSH-5 changed from 1 to 0.99 in the M4 model (small ancestral population size and deep divergence). Therefore, the multilocus molecular test validated our

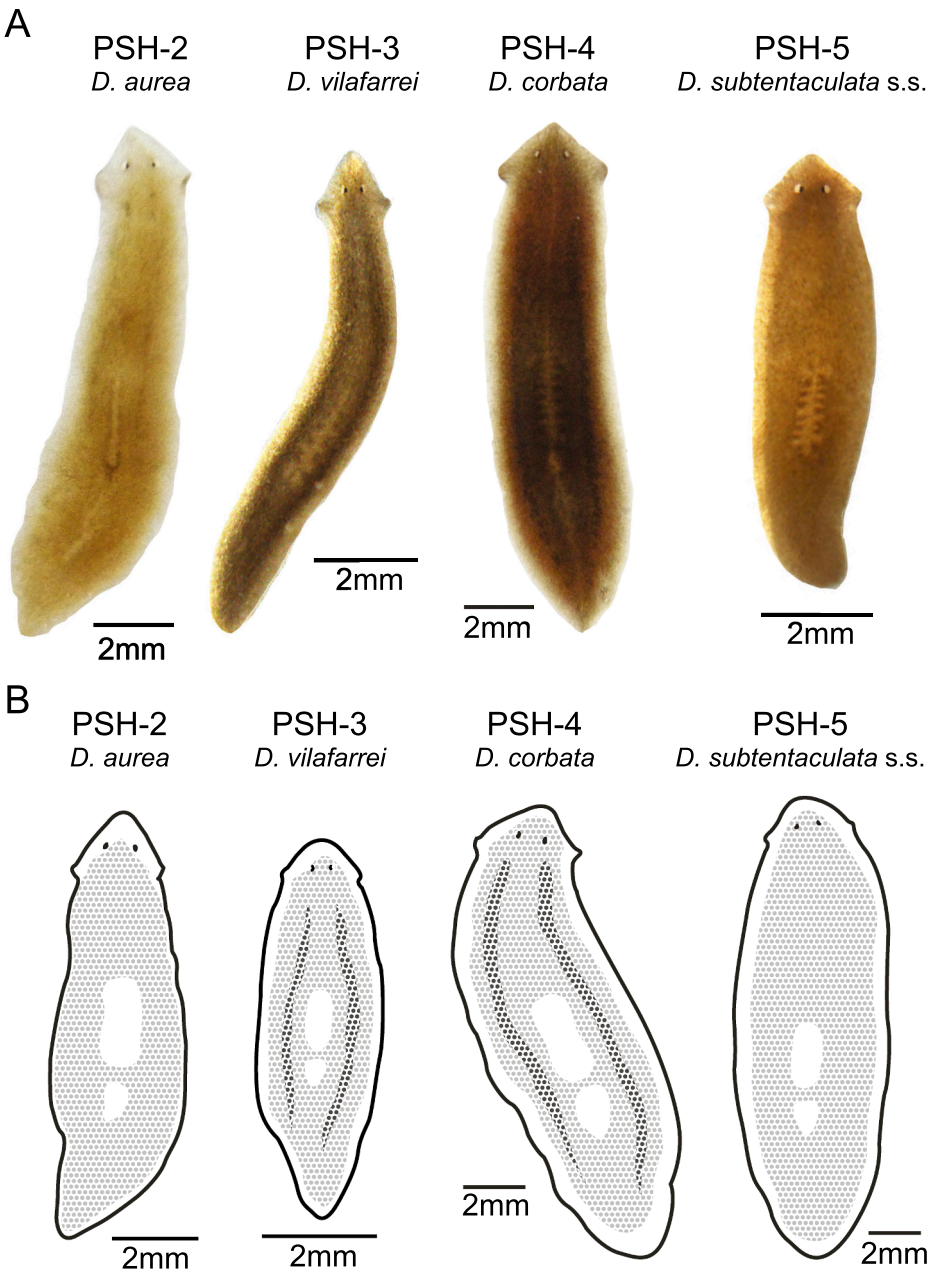

Fig 5. External morphology of the different PSHs. (A) Dorsal photographs of live specimens. (B) Drawings of specimens preserved in clove oil in dorsal view.

Primary Species Hypotheses.

3.6.2. Morphological validation

In our search for morphological diagnostic characters, we analyzed external characteristics as well as the anatomy of the copulatory apparatus of 4 individuals of PSH-2, 2 individuals of PSH-3, 3 individuals of PSH-4 and 29 individuals from 12 different populations of PSH-5 (Supplementary data S4). It was not possible to analyze individuals of PSH-1 (population 60), as we could only obtain material fixed in 100% ethanol, which is not well-suitable for histological studies.

Morphological analysis revealed that all individuals possessed most of the morphological and anatomical characteristics of *D. subtentaculata* s.l. as described by De Vries (1986a). The length of live animals ranged from 0.5 to 2 cm. All individuals had a head of a low triangular shape, with two eyes of the dugesiid type in the middle of the head (supernumerary eyes occurred in some individuals of several populations). The dorsal body surface was provided with a granular and mottled pigmentation, extending from anterior to the eyes to the posterior region, excepting the auricular grooves, which were free of pigment. Despite this broad agreement with the known external morphology of the species, we also recorded variable morphological characteristics not previously reported by De Vries (1986a). First, all analyzed individuals of PSH-2 and some populations of PSH-5 lacked dorsal pigmentation anterior to the eyes (Fig. 5). Second, two densely pigmented dorsal stripes were observed in all individuals of PSH-3 and PSH-4 (some individuals of PSH-2 also showed these stripes, although less evident). Further, all individuals of one population of PSH-5 (population 50) showed two weakly pigmented stripes on the ventral surface, only

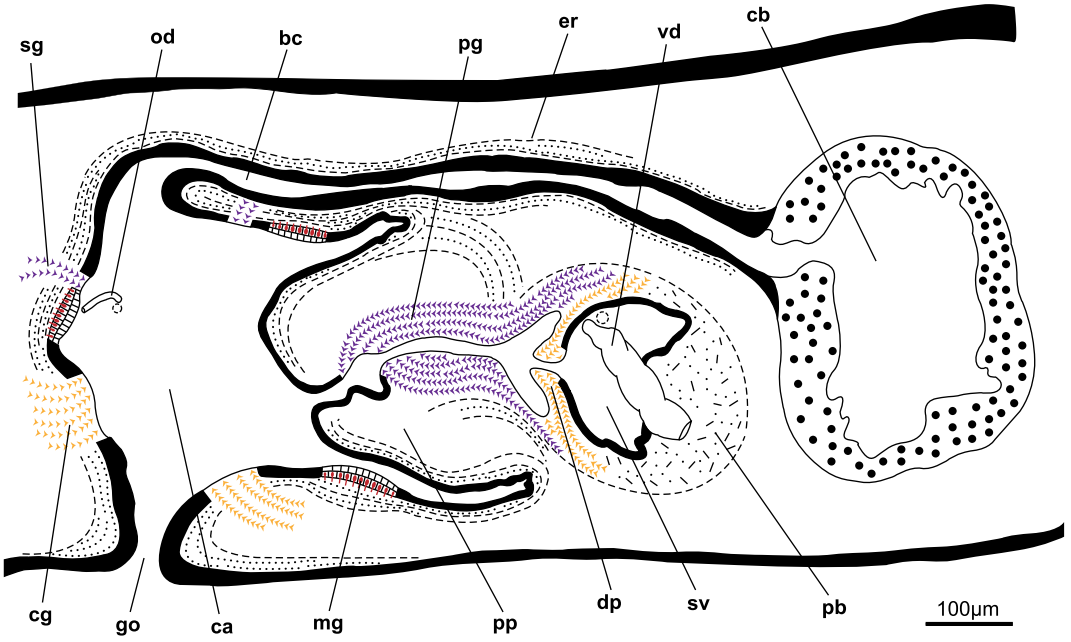

Fig. 6. Sagittal reconstruction of the copulatory apparatus of an individual of PSH-5 (*Dugesia subtentaculata* s.s., population 3, field code/number RS453); anterior to the right. Individuals of PSH-2 (*D. aurea*), PSH-3 (*D. vilafarrei*), and PSH-4 (*D. corbata*) basically show the same morphology.

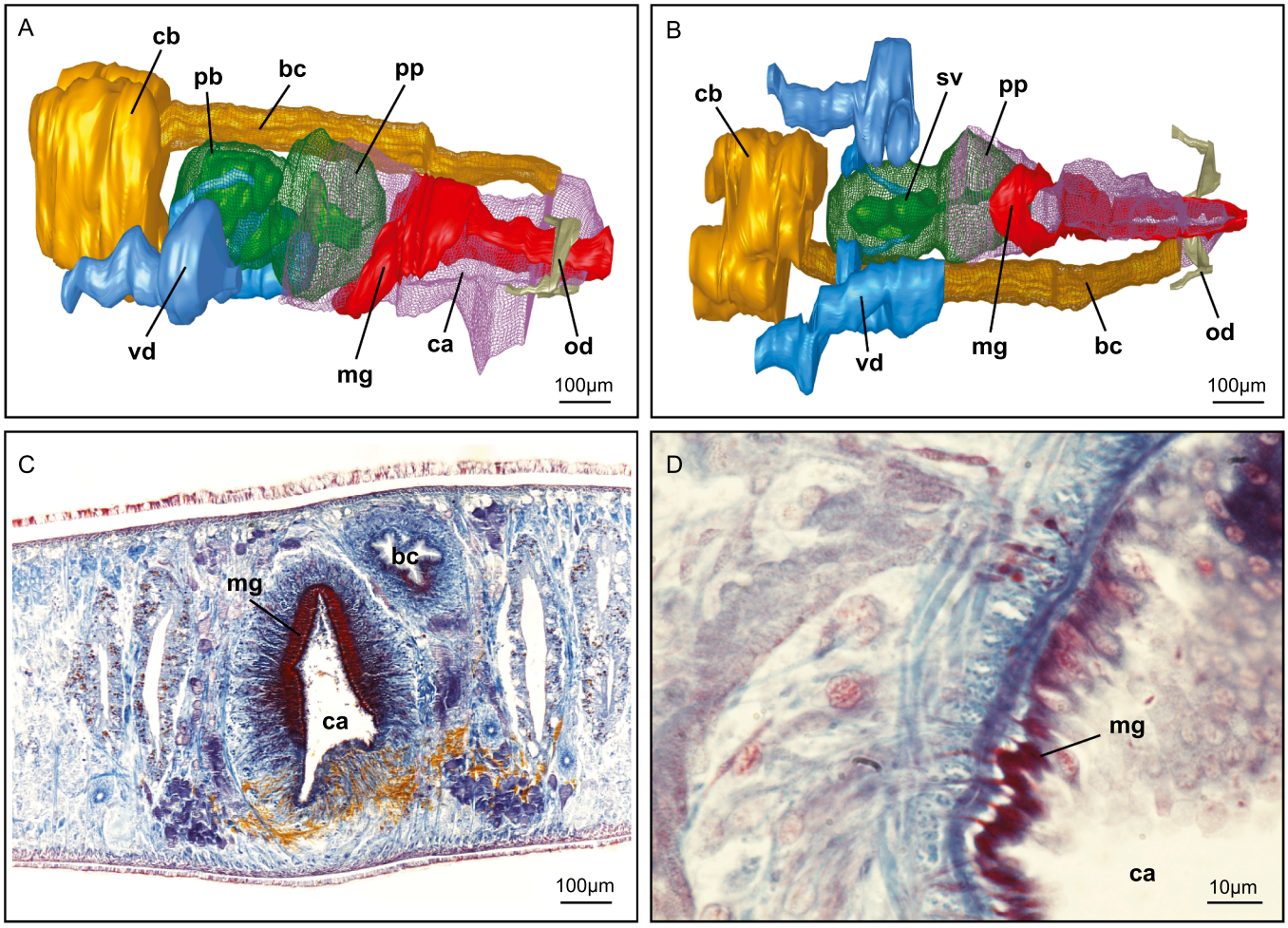

Fig. 7. 3D anatomical reconstruction (A, B) of the copulatory apparatus of an individual of PSH-5 (*Dugesia subtentaculata* s.s., population 2, field code/number RS490) and photomicrographs (C, D) of the anatomy of the musculo-glandular structure (*D. subtentaculata* s.s., population 3, field code/number RS453). (A) lateral view of the 3D reconstruction. (B) Ventral view of the 3D reconstruction. (C) Transverse section of the copulatory apparatus in the region of the common atrium. (D) Detail of the pseudo-stratified epithelium of the ampulla-shaped cells that form the musculo-glandular structure.

being visible when the individuals were observed in clove oil.

With respect to the anatomy of the copulatory apparatus, every individual from all PSHs showed the diagnostic combination of morphological characters of *D. subtentaculata* s.l. as described by De Vries (1986a) (Fig. 6): weakly muscular penis bulb, well delimited from a short and blunt penis papilla; central ejaculatory duct separated from a vesicle by a glandular valve-like diaphragm; abundant penial glands surrounding the seminal vesicle, the diaphragm and the ejaculatory duct. Besides the presence of abundant penial glands, bulb glands and shell glands, all individuals analyzed in this study possessed cement glands, discharging a yellowish secretion into the common atrium, surrounding the dorsal part of the gonoduct. Furthermore, in all specimens analyzed, a musculo-glandular structure in the atrium was present (mg in Fig. 6). Although this structure was mentioned by De Vries (1986a), she provided no detailed description of it. Examination of histological sections of the neotype of this species revealed that the glands were not well stained (all sections having an overall bluish colour), which possibly hindered the precise examination of this structure by De Vries (1986a). Our detailed examination of the histological sections of the new material, together with a 3D-reconstruction of the copulatory apparatus (Fig. 7), showed that this glandular structure extends on the major part of the atrial wall, from the ventral region under the penis papilla to the opening of the bursal canal into the atrium (Fig. 7A, B). This glandular region of the atrium showed a pseudostratified epithelium, strongly surrounded by muscles, through which an abundant erythrophilic secretion was discharged, thus making the cells look like red ampullae (Fig. 7C, D).

Despite all of these morphological resemblances, we found also some characters that differed between the various PSHs: (1) a parenchymatic ring at the base of the penis papilla was present in all individuals of PSH-4 and also in some specimens of PSH-5 (populations 17, 19, 30, and 40), (2) all individuals of PSH-2, PSH-3, PSH-4, and some individuals of PSH-5 (populations 30, 40, 41, 50, and 51) had a third layer of longitudinal muscles in the outer pharyngeal musculature, (3) all individuals of PSH-5 (excepting individuals of population 51) had elongated ovaries instead of rounded gonads, (4) all individuals of PSH-2 and population 51 of PSH-5 exhibited slightly asymmetrical openings of the vasa deferentia into the seminal vesicle; (5) in two populations of PSH-5 (populations 50 and 51) the ventral valve of the diaphragm was slightly smaller than the dorsal one. The first three of these morphological differences were already mentioned by De Vries (1986a) and were considered to be the result of intraspecific variation. The two last-mentioned differences are here reported for the first time. Finally, although PSH-5 showed some variable traits, each of these traits was shared with at least one of the other PSHs.

In conclusion, we did not find any stable diagnostic morphological feature for any of the various PSHs. Therefore, morphological data did not validate our Primary Species Hypotheses.

3.6.3. Karyological validation

Chromosomal measures of individuals from PSH-2, PSH-4, and population 46 of PSH-5 were compiled from Ribas (1990). We obtained new chromosomal measures of five individuals of PSH-3 and nine individuals of PSH-5, belonging to five different populations (Supplementary data S5). Additionally, we re-analyzed a metaphasic plate published by De Vries (1986b) concerning one individual from the type locality of *D. subtentaculata* s.l. (population 1 in the present study). As was the case also in the morphological analysis, we were not able to analyze any individual from PSH-1.

All individuals from PSH-2, PSH-3, and PSH-4 were diploid, with a chromosome complement of 2n = 16, while all populations analyzed of PSH-5 were triploid (3n = 24), excepting individuals of population 7, which were tetraploid (4n = 32), and population 51 that turned out to be diploid in a flux cytometry analysis performed in a parallel study (Leria et al., 2019). Relative length of the chromosomes was rather constant among the different PSHs (Supplementary data S12). On the other hand, our analysis revealed differences between the centromeric indices of the chromosomes among the various PSHs (Fig. 8; Supplementary data S12). All chromosomes of PSH-4 were metacentric, including four small supernumerary chromosomes. Individuals of PSH-3 showed two sub-metacentric chromosomes (pairs 2 and 3), while specimens of PSH-2 had four sub-metacentric chromosomes (pairs 3, 4, 5, and 6). In the case of PSH-5, several different aberrant chromosomes were present in each of the populations. Nevertheless, in all of them the first, sixth and eighth chromosome triplets turned out to be metacentric. The other chromosomes were either metacentric or sub-metacentric, depending on the population. Regarding the aberrant chromosomes, we identified a translocation already described by Ribas (1990), involving two chromosomes from the fourth and eighth triplet in all individuals analyzed of populations 4 and 22. Furthermore, a single large acrocentric chromosome was present in individuals from all populations, excepting population 46. This large acrocentric chromosome presumably belonged to the second triplet and its aberrant morphology possibly originated through translocation to a chromosome of the third triplet. Finally, we also found another putative translocation between one chromosome of the first triplet (donor) and one chromosome of the seventh triplet (receptor) (Fig. 8).

The karyological data described above reveal that the complements of PSH-2, PSH-3, and PSH-4 are clearly differentiated from each other and also from PSH-5. Moreover, despite the chromosomal variation detected among PSH-5 populations, all share several chromosomal characteristics that are different from PSH-2, PSH-3, and PSH-4. Therefore, karyological data validated our Primary Species Hypotheses.

3.7. Secondary Species Hypotheses

Four out of the five PSHs validated as independent lineages by BPP applied on multilocus data were also validated by karyological data, *viz.* PSH-2, PSH-3, PSH-4, and PSH-5. Unexpectedly, the status of those four taxa could not be corroborated on the basis of morphological and anatomical data. Therefore, we do here consider these four PSHs within *D. subtentaculata* s.l. to represent four different species that are cryptic at the morphological level (see Fig. 2). Three out of these four species are here described as new and thus receive a new specific epithet (see below). The name *D. subtentaculata sensu stricto* (further below referred to as *D. subtentaculata* s.s.) is retained for PSH-5, as it includes the population from the type locality of the species; the species is herein redescribed in order to account for the intraspecific variability as found in the present study. Although for PSH-1 we did not have information on either morphology or karyology, it was validated, nevertheless, by multilocus molecular data. Therefore, we considered PSH-1 to represent an unconfirmed candidate species of *Dugesia*, awaiting further morphological and karyological data that may test its taxonomic status.

3.8. Taxonomic Section

Order Tricladida Lang, 1884
Family Dugesiidae Ball, 1974
Genus *Dugesia* Girard, 1850
***Dugesia subtentaculata* s.s. (Draparnaud, 1801)**

Material examined: Individuals from populations 1–46, 49–52, 54–59, and 61–63. GenBank accession numbers are detailed in Appendix A, codes (field numbers) of the individuals analyzed histologically are detailed in Supplementary data S4, while codes of the individuals analyzed karyologically are specified in Supplementary data S5. Morphological paratypes: individuals RS453 and RS474.

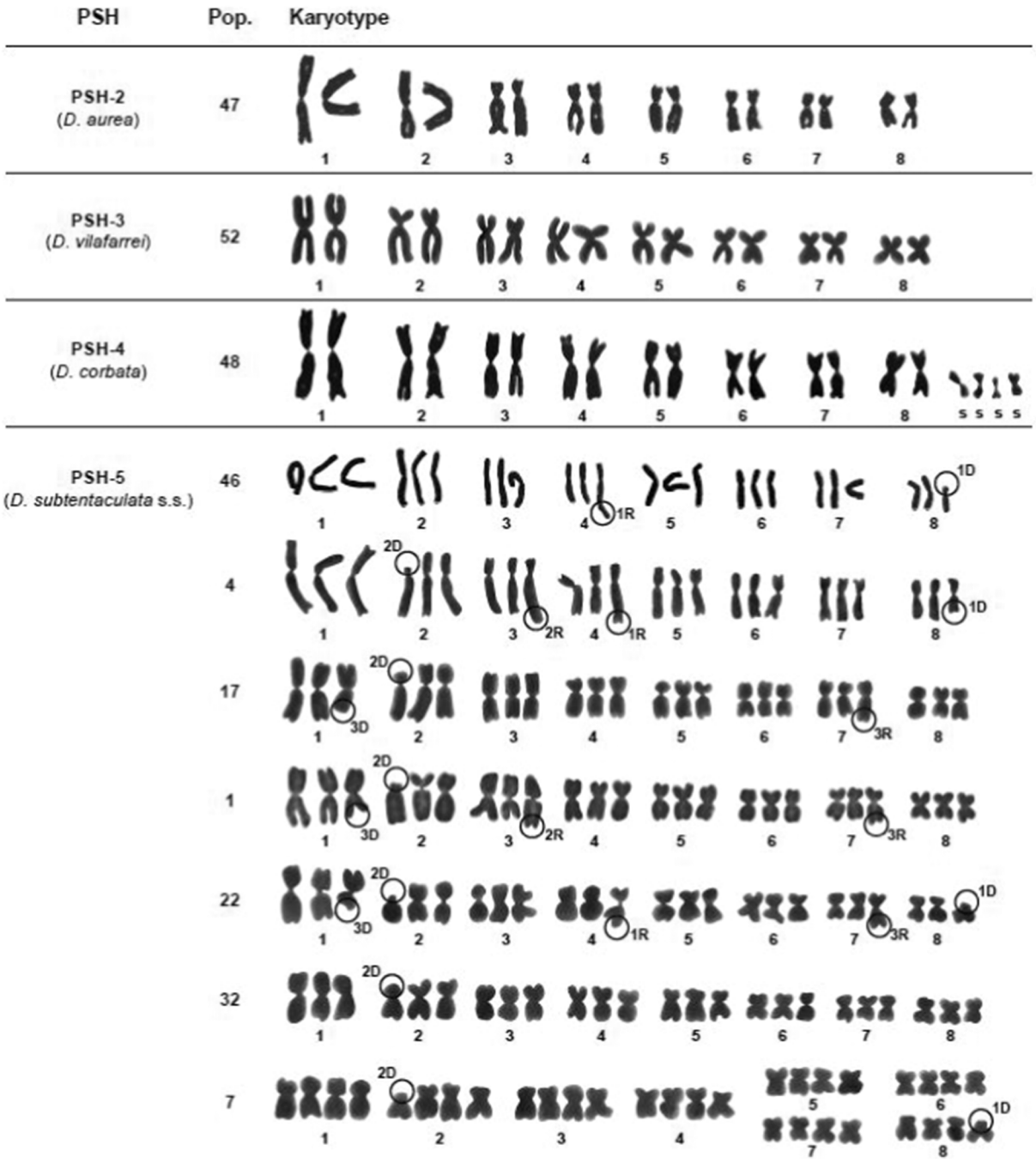

Fig. 8. Chromosome complements of the different PSHs arranged in pairs, triplets or quartets. Circles on chromosomes indicate aberrant regions. Numbers and letters near circles indicate the putative chromosomal rearrangement that gave rise to the aberrant region, as follows: 1, translocation between triplets 4 and 8; 2, translocation between triplets 2 and 3; 3, translocation between triplets 1 and 7; D, donor chromosome; R, receptor chromosome. Note that in some populations only the donor or the receptor chromosomes of these putative translocations were detected. s: supernumerary chromosomes.

Karyological paratypes: individuals P6 and C12.3. DNA vouchers and chromosome slides were deposited in Dpt. de Genètica, Microbiologia i Estadística; Facultat de Biologia; Universitat de Barcelona, Barcelona, Spain. Histological sections were deposited in the collections of Naturalis Biodiversity Center, Leiden, the Netherlands.

Diagnosis: Molecularly, *Dugesia subtentaculata* s.s. comprises individuals that are identified as a single evolutionary unit together with individuals from populations 1–46, 49–52, 54–59, and 61–63, when using the coalescence-based method BPP with the loci and settings detailed in the present study. Karyology: 2n = 16 (populations 50 and 51; Leria et al., 2019), 3n = 24, and 4n = 32 (population 7); chromosomes 1, 6, and 8 metacentric; chromosomes 2, 3, 4, 5, and 7 either metacentric or sub-metacentric, depending on the population; putative presence of aberrant chromosomes in all triplets, excepting the fifth and the sixth. In the present study corresponding to PSH-5.

Morphological re-description: Length of live animals ranges from 0.5 to 2 cm. Head of a low triangular shape, with two eyes of the dugesiid type. Dorsal body surface mottled brownish or greyish, under

natural conditions (Fig. 5); ventral surface pale or only lightly pigmented (population 50); auricular grooves devoid of pigmentation. Bi-layered or three-layered (populations 30, 40, 41, 50, and 51) outer pharynx musculature.

Numerous dorsal testes, full of sperm, extending from the level of the ovaries to the posterior end of the body. A pair of rounded or slightly elongated ovaries situated approximately at one-third to one-quarter of the distance between the brain and the root of the pharynx.

Anatomy of the copulatory apparatus characterized by: erythrophilic musculo-glandular structure covering most part of the wall of the common atrium; weakly muscular penis bulb, well-delimited from a short and blunt penis papilla; a parenchymatic ring at the base of the penis papilla present in some populations (17, 19, 30, and 40); central ejaculatory duct (in population 49 slightly ventral), separated from a vesicle by a glandular valve-like diaphragm; abundant penial glands surrounding seminal vesicle, diaphragm and ejaculatory duct; abundant cement glands surrounding the dorsal region of the gonoduct; symmetrical or slightly asymmetrical (population 51) openings of the vasa deferentia into the posterior part of seminal vesicle; symmetrical openings of the oviducts into the posterior part of the atrium, just below the opening of the bursal canal into the atrium; bursal canal with ectal reinforcement all along the canal or at least extending from the atrium to the level of the penis bulb (Fig. 6).

Distribution: Southern France, Iberian Peninsula, and Northern Africa.
Reproduction: sexual, fissiparous, or alternation between reproductive strategies (i.e., facultative reproduction).

***Dugesia aurea* Leria, sp. nov.**

Material examined: Individuals 47.1 to 47.5 preserved in ethanol 100% (GenBank accession numbers in Appendix A) and four samples fixed in Steinmann's fluid and, subsequently, preserved in 70% ethanol (codes/field numbers in Supplementary data S4). DNA holotype: individual 47.1, Soller, Mallorca, Balearic Islands, Spain, 39.75693 N 2.71193E. DNA paratypes: individuals 47.2 and 47.3. Morphological paratypes: individuals RS456 and RS458.1. DNA vouchers were deposited in Dpt. de Genètica, Microbiologia i Estadística; Facultat de Biologia; Universitat de Barcelona, Barcelona, Spain. Histological sections were deposited in the collections of Naturalis Biodiversity Center, Leiden, The Netherlands. Karyological information was extracted from Ribas (1990).

Etymology: The specific epithet refers to the etymology of the locality of Soller in Mallorca, which means "golden valley", supposedly derived from a term used by the Arabians when they arrived in this valley and saw the abundance of lemon trees. The name was also chosen to refer the gold-like coloration of the animals under natural conditions.

Diagnosis: Molecularly, *Dugesia aurea* comprises individuals that are identified as a single evolutionary unit together with individuals 47.1 to 47.5, when using the coalescence-based method BPP with the loci and settings detailed in the present study. Karyology: 2n = 16; chromosome pairs 1, 2, 7, and 8 metacentric; chromosome pairs 3, 4, 5, and 6 sub-metacentric. In the present study corresponding to PSH-2.

Morphological description: Length of live animals ranging from 0.5 to 1.5 cm. Head of a low triangular shape, provided with two eyes of the dugesiid type in the middle of the head. Dorsal surface with gold-like coloration under natural conditions, with the pigmentation being granular and mottled, extending from the eyes to the posterior region (Fig. 5). In some individuals two dorsal pigmented stripes just being visible; auricular grooves devoid of pigmentation. Three-layered outer pharynx musculature. Numerous dorsal testes, full of sperm, extending from the level of the ovaries to the posterior end of the body. A pair of rounded ovaries situated approximately at one-third to one-quarter of the distance between the brain and the root of the pharynx. Reproductive apparatus as in *Dugesia subtentaculata* s.s. (see above) (Fig. 6).

Ecology and distribution: The species is known only from one site at Soller, Mallorca. Altitude: approx. 103 m a.s.l.
Reproduction: sexual.

***Dugesia corbata* Leria, sp. nov.**

Material examined: Individuals 48.1 to 48.5 preserved in ethanol 100% (GenBank accession numbers in Appendix A) and three samples fixed in Steinmann's fluid and, subsequently, preserved in 70% ethanol (codes/field numbers in Supplementary data S4). DNA holotype: individual 48.1, Sa Calobra, Mallorca, Balearic Islands, Spain, 39.82932 N 2.81538E. DNA paratypes: individuals 48.2 and 48.3. Morphological paratypes: individuals RS461 and RS463. DNA vouchers were deposited in Dpt. de Genètica, Microbiologia i Estadística; Facultat de Biologia; Universitat de Barcelona, Barcelona, Spain. Histological sections were deposited in the collections of Naturalis Biodiversity Center, Leiden, The Netherlands. Karyological information extracted from Ribas (1990).

Etymology: The specific epithet refers to the locality where the species was found, which in Mallorca is known as "Nus de sa corbata" (in Catalan literally meaning necktie knot) because of the sharp bend in the road. The species name alludes also to the characteristic "necktie-shape" of the *Dugesia* individuals.

Diagnosis: Molecularly, *Dugesia corbata* comprises individuals that are identified as a single evolutionary unit together with individuals 48.1 to 48.5, when using the coalescence-based method BPP with the loci and settings detailed in the present study. Karyology: 2n = 16 plus 4 supernumerary chromosomes; all chromosome pairs metacentric. In the present study corresponding to PSH-4.

Morphological description: Length of live animals ranging from 0.5 to 1.5 cm. Head of a low triangular shape with two eyes of the dugesiid type in the middle. Dorsal body surface under natural conditions mottled dark reddish-brown, the granular pigmentation extending from anterior to the eyes to the posterior end of the body. With two densely pigmented broad stripes, running approximately from the region where the ovaries are located to the posterior region of the copulatory apparatus (Fig. 5). Auricular grooves devoid of pigmentation. Three-layered outer pharynx musculature. Numerous dorsal testes, full of sperm, extending from the level of the ovaries to the posterior end of the body. A pair of rounded ovaries situated approximately at one-third to one-quarter of the distance between the brain and the root of the pharynx. Reproductive apparatus as in *Dugesia subtentaculata* s.s. (see above) (Fig. 6).

Ecology and distribution: The species is known only from one site near Sa Calobra, in Mallorca. Altitude: 665 m a.s.l.
Reproduction: sexual.

***Dugesia vilafarrei* Leria, sp. nov.**

Material examined: Individuals 52.1, 52.4–52.6, 52.14, 53.1, 53.2, 54.1, and 54.2 preserved in ethanol 100% (GenBank accession numbers detailed in Appendix A) and two samples fixed in Steinmann's fluid and, subsequently, preserved in 70% ethanol (codes/field numbers in Supplementary data S4). DNA holotype: individual 52.14, El Bosque, Andalucía, Spain, 36.76123 N 5.50581 W. DNA paratypes: individuals 52.5 and 54.1. Morphological paratypes: individuals MV04-6 and MV08-1. Karyological paratypes: individuals Ind1 and Ind2. DNA

vouchers and chromosome slides were deposited in Dpt. de Genètica, Microbiologia i Estadística; Facultat de Biologia; Universitat de Barcelona, Barcelona, Spain. Histological sections were deposited in the collections of Naturalis Biodiversity Center, Leiden, The Netherlands.

Etymology: The species epithet honours collaborator and co-author of the present study Dr. Miquel Vila-Farré, who discovered two out of the three populations of this species and has extensively contributed to our knowledge of planarians in the Iberian Peninsula.

Diagnosis: Molecularly, *Dugesia vilafarrei* is constituted by individuals that are identified as a single evolutionary unit together with individuals 52.1, 52.4–52.6, 52.14, 53.1, 53.2, 54.1, and 54.2, when using the coalescence-based method BPP with the loci and settings detailed in the present study. Karyology: 2n = 16; chromosome pairs 1, 4, 5, 6, 7, and 8 metacentric; chromosome pairs 2 and 3 submetacentric. In the present study corresponding to PSH-3.

Morphological description: Length of live animals ranging from 0.5 to 1.5 cm. Head of a low triangular shape, in the middle with two eyes of the dugesiid type. Dorsal body surface under natural conditions mottled brownish and provided with two broad and densely pigmented stripes, extending from the region where the ovaries are located to the posterior region of the copulatory apparatus (Fig. 5); auricular grooves devoid of pigmentation. Three-layered outer pharynx musculature. Numerous dorsal testes, full of sperm, extending from the level of the ovaries to the posterior end of the body. A pair of rounded ovaries situated approximately at one-third to one-quarter of the distance between the brain and the root of the pharynx. Reproductive apparatus as in *Dugesia subtentaculata* s.s. (see above) (Fig. 6).

Ecology and distribution: The species is only known from three localities in the Sierra de Grazalema (Andalusia, Spain), where it coexists with *D. subtentaculata* s.s. (in localities 52 and 54). Although these localities are geographically very close to each other, populations 52 and 53 belong to the fluvial basin of the river Guadalete (Atlantic watershed), whereas population 54 lives in the fluvial basin of the river Guadiaro (Mediterranean watershed). Altitude of the localities: 270 m a.s.l. (population 52); 427 m a.s.l. (population 53); 724 m a.s.l. (population 54).

Reproduction: Sexual.

Morphological comparative discussion

De Vries (1986a) did amply show and discuss that the reproductive anatomy of *D. subtentaculata* s.l., particularly its copulatory apparatus, stands apart from all other species of *Dugesia* and thus facilitates its discrimination. Evidently, the present study revealed that cryptic diversity is hidden underneath the similar morphology of the various *D. subtentaculata* s.l. populations, resulting in the recognition of three new species.

The musculo-glandular structure present in all analyzed individuals of the four species described in the present study is unique to these species. Although De Vries (1988a) described a musculo-glandular area also in the atrial wall of *Dugesia debeauchampi* De Vries, 1988, she mentioned that only in a restricted area some glands discharge into the atrium, which may imply that in this species extension of the musculo-glandular zone is much more restricted than is the case in our four cryptic species. The parenchymatic ring of vacuolated tissue in the penis papilla of *Dugesia corbata* and some specimens of *D. subtentaculata* s.s. is present also in *Dugesia leporii* Pala, Stocchino, Corso & Casu, 2000 and *Dugesia liguriensis*. In particular, the blunt penis papilla of *D. leporii* resembles the short penis of *D. subtentaculata* s.s., *D. aurea, D. corbata,* and *D. vilafarrei.* However, *D. leporii* differs from these last-mentioned four cryptic species in that its diaphragm is pointed, and that it has a dorsal penial valve (Pala et al., 2000; Stocchino et al., 2017). *Dugesia liguriensis* has a pointed diaphragm, in contrast to the valve-like diaphragm of *D. subtentaculata* s.s., *D. aurea, D. corbata,* and *D. vilafarrei*, while it also possesses a cone-shaped penis papilla, which differs from the barrel-shaped papilla present in the last-mentioned four cryptic species (De Vries, 1988b).

4. Discussion

4.1. Molecular species delimitation in organisms with high intraindividual genetic diversity

Our study is the first in which information on mosaic intraindividual genetic diversity is used to infer molecular species boundaries (see Leria et al., 2019 for more information regarding mosaicism), while being evaluated against results obtained with datasets without this information. It is important to note that the new, exclusively sexual species (*D. aurea*, *D. corbata* and *D. vilafarrei*), have been delimited by all molecular discovery methods used, with only two exceptions (Fig. 3). Although the sample sizes for these species may seem low (at minimum 2–3 individuals per species for all methods and 5 individuals in some datasets), we consider the number of individuals analyzed sufficient for capturing the genetic variability of each species, since all of them are endemic to small geographic regions. In contrast, *D. subtentaculata* s.s., a species constituted by a large number of fissiparous and facultative populations (and only a few sexually reproducing ones), yielded different outcomes for candidate species, depending on the methods and datasets used.

From the three molecular delimitation methods applied for determining Primary Species Hypotheses, ABGD and GMYC were affected the most by inclusion or exclusion of intraindividual genetic data. For both molecular markers, when ABGD was applied to the datasets with intraindividual information, it delimited as the same candidate species those haplotypes that had been delimited as different candidate species in the non-cloned datasets. A putative explanation for this different behaviour of ABGD may be that the non-cloned datasets contained ambiguous sites, whereas in the cloned datasets all haplotypes were resolved. It has been suggested that ambiguous sites may directly influence calculation of the genetic distances among sequences, while they may bias also the topology and branch lengths of Maximum Likelihood and Bayesian phylogenetic reconstructions (Lemmon et al., 2009). Therefore, a high number of ambiguous sites in the non-cloned datasets may have resulted in an increase of genetic distances and, thus, in a consequent over-splitting of candidate species when ABGD was applied.

In two cases, GMYC failed to return significant results. For Cox1, the method failed to delimit species in the dataset with intraindividual information, while in the case of Dunuc12 it was the other way around. This lack of significant results in GMYC could be due to the fact that the trees based on these datasets showed a more regular branching pattern, which possibly hindered GMYC to detect a clear transition from speciation to coalescence. Nonetheless, in the two other cases a delimitation scheme was obtained, but then GMYC resulted in an over-splitting of *D. subtentaculata* s.s. and even of one of the sexual species (*D. aurea*). In both cases, GMYC delimited as different putative species haplotypes of the same individual. This tendency of GMYC in over-splitting candidate species has been documented for both empirical and simulated data and has been principally attributed to gene flow or incomplete lineage sorting (Luo et al., 2018; Talavera et al., 2013). Our present study shows that high intraindividual diversity due to fissiparous reproduction may cause similar over-splitting effects in the performance of GMYC.

In contrast to ABGD and GMYC, the mPTP method delivered much more stable results across the four different datasets analyzed. Although mPTP uses substitution rates and tree topology to distinguish between intraspecies and interspecies processes (which may be influenced also by the number of ambiguous sites), the ability of this method to accommodate distinct rates of molecular evolution across linages may be the reason of its consistency.

All these findings indicate that for performing molecular species delimitation in *Dugesia* species (irrespective of their reproductive

strategy, but particularly when they show fissiparous populations, such as *D. subtentaculata* s.s.), it is very important to include intraindividual genetic diversity, as the haplotypes of some individuals may be so different that some methods may identify these as belonging to separate putative species. However, when intraindividual data is not available, we recommend mPTP as the preferred method and to avoid ABGD or GMYC, as mPTP delivers more conservative results and thus minimizes over-splitting of putative candidate species. This may apply not only to other planarian genera but also to other asexual organisms with presumably high intraindividual genetic diversity due to asexual reproduction, such as starfishes, ribbon worms, or stick insects, among many others (Ament-Velásquez et al., 2016; Bast et al., 2018; Garcia-Cisneros et al., 2015).

4.2. Multilocus data in Dugesia systematics: A useful addition

Incorporation of the six new nuclear markers (Dunucs), the ribosomal gene 28S, and three small mitochondrial fragments, has greatly improved the resolution of the phylogenetic relationships among the different cryptic species previously subsumed under *D. subtentaculata* s.l., when compared to results obtained previously with single locus phylogenies (Lázaro et al. 2009). The species tree generated in the present study (Supplementary data S11) corroborated that individuals of population 60 (PSH-1) constitute the sister-group of the rest of the species included in this analysis, as already pointed out by Solà (2014). Moreover, this species tree revealed that the two new species from Mallorca (*D. aurea* and *D. corbata*) constitute a highly supported monophyletic group and that *D. subtentaculata* s.s. is the sister species of *D. vilafarrei*, albeit with a posterior probability value of only 0.82.

Furthermore, the use of new nuclear loci allowed us to apply a multilocus method to species delimitation based on coalescence (BPP), which highly increased the confidence in our delimitation scheme. BPP has already proved to be very useful in delimiting species in various groups of land planarians (Carbayo et al., 2016; Mateos et al., 2017). Therefore, the addition of the new markers used in this study together with BPP, may form a very important and adequate tool to unravel the evolutionary history of some intricate groups within the genus *Dugesia*, such as species from the Aegean region (see below) (Sluys et al., 2013).

4.3. Morphological crypsis in Dugesia

The present study revealed the existence of four morphologically cryptic species within *D. subtentaculata* s.l. that are highly differentiated at both the molecular and karyological level. To the best of our knowledge, this represents the first case of morphologically cryptic species being described for the genus *Dugesia.* Nonetheless, possibly it may not form an isolated case. For example, in a taxonomic study of *Dugesia* in the Aegean region, several lineages were molecularly highly differentiated from each other (identified as independent linages by GMYC) but did not show any morphological differentiation and, therefore, they were considered as “Deep Conspecific Lineages” (DCL) (Sluys et al., 2013). However, it may well be that inclusion of additional sources of information besides DNA and morphology, such as, for example, chromosomal characteristics, will reveal cryptic *Dugesia* species being present also in that region.

Examples of morphological crypsis in planarians are already available, such as in the land planarians of the genus *Obama* Carbayo *et al.*, 2013 (Álvarez-Presas et al., 2015), in which two pseudo-cryptic species were highly differentiated at molecular level but were indistinguishable by the commonly used morphological features. Further, putative cryptic diversity recently has been proposed also for the freshwater planarian species *Polycelis coronata* (Girard, 1891) (Rader et al., 2017) and *Crenobia alpina* (Dana, 1766) (Brändle et al., 2017) on the basis of the high genetic differentiation detected within these two species. Given the fact that planarians are frequently difficult to diagnose solely on the basis of morphological characters and that cryptic species are probably common in this group, the integrative taxonomic procedure applied in the present study (Fig. 2) could be highly useful for future taxonomic studies on planarians.

Cryptic diversity generally may be explained by three different mechanisms: (1) recent diversification, (2) morphological convergence or parallelism, or (3) morphological stasis (Fišer et al., 2018; Struck et al., 2018). In the first case, the differentiated lineages are at an early stage in the speciation continuum during which some differences in variable loci have accumulated but insufficient time has passed for the evolution of any morphological differences, as morphological characters generally are under the influence of multiple genes (Fisher, 1999). In order to test the hypothesis of recent diversification, it is necessary to put the group of interest in a temporal framework. A recent study on the historical biogeography of the genus *Dugesia* estimated the divergence between the unconfirmed candidate species PSH-1 and two populations of *D. subtentaculata* s.l. to date back to approximately 20–10 million years ago (Mya) (Solà, 2014). Although additional calibration studies would be needed, including individuals of the three new cryptic species herein described, the previously mentioned calibration analysis together with the high genetic and karyological differentiation among these cryptic species, strongly suggests that the lack of morphological differentiation among them may not be due to recent diversification.

On the other hand, the molecular monophyly of the four species suggests that morphological crypsis may not result from morphological convergence either, but it does not rule out parallelism. If parallelism would have been the underlying process, it would mean that the detailed characteristics of the copulatory apparatus in these species had evolved independently in each lineage. However, among the different features that characterize these species, there is one character that may be unique in *Dugesia*, *viz.* the musculo-glandular structure (mg in Fig. 7). The glandular component of this structure may be formed by shell glands. In most *Dugesia* species, shell glands discharge their secretion into the bursal canal around the oviducal openings or, less common, into another restricted region of the atrium, such as in *Dugesia debeauchampi* (De Vries, 1988a). But in all specimens of the four cryptic species analysed in the present study these massive, putative shell glands apparently have shifted, so that they discharge through the major part of the atrial wall, extending from dorsal to ventral surface. Therefore, the most parsimonious interpretation for the occurrence of this character state in these four cryptic species is that it evolved in their most recent common ancestor. Under this hypothesis, morphological parallelism would also be discarded as an explanation for the occurrence of morphological crypsis between these species.

Hence, only stasis remains as a plausible, alternative explanation for the phenomenon of morphological crypsis in these species. Morphological stasis in anatomical features of planarians has been proposed for South American species of the genus *Girardia* (Sluys et al., 2005). The genus *Dugesia* also exhibits a high degree of stasis in both external and anatomical features, notably the copulatory apparatus, despite the fact that the genus presumably is very old (dating back to about 240 Mya; Solà, 2014). An explanation for such a high degree of stasis might be that deviations from these morphological characteristics are under strong selective pressure (i.e., that these characters are subject to stabilizing selection), as has been proposed for other character complexes in cryptic or pseudo-cryptic species of sea urchins, unicellular algae, and lizards (Egea et al., 2016; Sáez et al., 2003; Smith et al., 2011). Importantly, the existence of morphological stasis in *Dugesia*, and very likely also in other planarian genera, suggests that the actual planarian species richness might be highly underestimated, since the anatomy of the copulatory apparatus has been and still is the principal criterion for delimiting species boundaries in these organisms.

Dugesia subtentaculata s.s. is the only species of this species complex that shows some noticeable intraspecific morphological variation. Although we cannot completely rule out that future analysis of additional material will also reveal some variability in *D. aurea*, *D. corbata,*

and *D. vilafarrei*, current absence of morphological variation within these three species might be related to their very restricted distributions. As in the case of the molecular data, such restricted geographic ranges may signal population bottlenecks that resulted in a reduction of the morphological variability in each of these three species. However, it may be the case that the intraspecific variability in *D. subtentaculata* s.s. results from various factors that are mainly linked to differences in developmental stages or processes. For example, presence or absence of a parenchymal ring in the penis papilla, or minor changes in the shape of the ovaries, might be due to different stages in the reproductive cycle of the animal. Moreover, the development of the reproductive system might be different in specimens that build it once and retain it for long periods of time (sexual populations) versus those that alternate fission and sexual reproduction. At the present moment we can only speculate on the underlying mechanisms of this morphological variability in *D. subtentaculata* s.s. and its absence in *D. aurea*, *D. corbata,* and *D. vilafarrei*. However, this variability is restricted to minor variations in some morphological characteristics (e.g., slightly asymmetrical openings of the vasa deferentia into the seminal vesicle, or small differences in the extension of the ectal reinforcement) that presumably have little impact on the functionality of the copulatory apparatus, the structure of which is highly similar among the different populations of *D. subtentaculata* s.s. and between the different species of this species complex.

4.4. Karyological variability in planarians

Our karyological analysis revealed that the four cryptic species, namely *D. aurea*, *D. corbata*, *D. vilafarrei* and *D. subtentaculata* s.s., show different chromosomal features. The species *D. aurea*, *D. corbata,* and *D. vilafarrei* are all diploid (2n = 16) but can be distinguished from each other and from *D. subtentaculata* s.s. by the centromeric indices of several chromosome pairs; the last-mentioned species can be distinguished also from the others by the ploidy level (most populations being polyploid) and by the presence of aberrant chromosomes. Similar complex karyological situations as present in *D. subtentaculata* s.s., have been observed also in many other *Dugesia* species with both sexual and fissiparous populations, such as *D. japonica*, *D. ryukyuensis*, *D. benazzii,* and *D. maghrebiana* (Benazzi-Lentati and Benazzi, 1985; Stocchino et al., 2009; Tamura et al., 1998, 1991; Vacca et al., 1993). Moreover, high levels of karyological variability among closely related species have been reported for several planarian genera of the family Dugesiidae, such as *Schmidtea* Ball, 1974*, Cura* Strand, 1942, and *Girardia* Ball, 1974 (Benazzi and Puccinelli, 1973; Benya et al., 2007; Gourbault and Benazzi, 1975).

Girardia tigrina (Girard, 1850) presents a high copy number of mariner-like transposons (Garcia-Fernàndez et al., 1995), and the genome of *Schmidtea mediterranea* harbours a novel type of giant retroelements (Grohme et al., 2018). The presence in high amounts of such elements may be related to the high incidence of chromosome rearrangements observed in this group of animals, as has been shown also for other organisms (Feschotte and Pritham, 2007). Moreover, it has been suggested that fissiparous reproduction may allow planarians to endure situations of unbalanced karyotypes, as during periods of fissiparous reproduction individuals do not undergo meiosis and, thus, would not suffer from selection against chromosomal rearrangements (Leria et al., 2018). Thus, the high motility of genome elements of planarians may explain the karyological variability detected between the four cryptic *Dugesia* species described in the present study. Moreover, in the case of *D. subtentaculata* s.s. its mainly fissiparous type of reproduction may explain the high incidence of chromosome aberrations.

The different chromosome portraits in each of these four cryptic species possibly prevent them to successfully crossbreed, as in the offspring the different centromeric positions of several homologous chromosomes would generate anomalous chromosome pairings during meiosis, thus resulting in incorrect segregation or unbalanced gametes after recombination (Faria and Navarro, 2010). It may even be the case that these chromosomal differences actually were the drivers of the speciation process, in the same way as recently proposed for the genus *Schmidtea* (Leria et al., 2018). On the other hand, the chromosomal differences simply may be due to karyotype changes that have accumulated after the speciation process. For example, speciation of the Mallorca populations, resulting in *D. aurea* and *D. corbata*, may have resulted from geographic isolation from peninsular populations. In a similar way, the two species in Mallorca may have diverged because of geographic isolation, since *D. aurea* and *D. corbata* occur in the Torrent de Soller and Torrent de Sa Calobra-Pareis, respectively, which are two separate ancient fluvial basins of the Tramuntana Range (Silva et al., 2005). Thus, geographic isolation may have been an alternative driver of speciation, instead of karyological differences. In contrast, the speciation event that gave rise to *D. vilafarrei* and *D. subtentaculata* s.s., both species co-occurring at two localities in the Iberian Peninsula, possibly was due to a triploidization event in the ancestor of *D. subtentaculata* s.s. (Leria et al., 2019). This change from diploidy to triploidy possibly triggered the shift to fissiparous reproduction, thus immediately preventing the outcrossing of the original diploids with the newly formed fissiparous triploids. Therefore, it is possible that karyological plasticity did drive indeed speciation between *D. vilafarrei* and *D. subtentaculata* s.s.

4.5. Conservation status of the species

Three out of the four cryptic species described in the present study are endemic at very restricted geographic areas. *Dugesia aurea* and *D. corbata* meet the IUCN criterion of Critically Endangered (CR), since only a single locality of each is presently known (Section V, point B.2.a), while *D. vilafarrei* meets the IUCN criterion of Endangered (EN) as it is presently known from less than five localities (Section V, point B.1.a) (IUCN, 2012). We consider that this assessment of the conservation status of these species represents their actual situation, since we performed many extensive samplings in the Balearic Islands and in the Iberian Peninsula over the past few years. As is the case with these species, many other species of the genus *Dugesia,* and also of other genera of freshwater planarians, are known only from a few localities and/or are subjected to habitat loss (see Lázaro et al., 2011; Sluys et al., 2013). Although freshwater planarians play an important role in trophic networks (they can act as top predators) (Teal, 1957; Tilly, 1968) and may be used as bioindicators of the quality of the water due to their sensitivity to pollutants (Knakievicz, 2014), no planarian species is currently included in any conservation policy, albeit some proposals have been made (e.g., Souza et al., 2016), which, unfortunately, holds true for most invertebrate species (Cardoso et al., 2011). We do here propose to include in the IUCN Red List the new species *D. aurea* and *D. corbata* under the conservation category of Critically Endangered (CR), and *D. vilafarrei* as Endangered (EN). It is our hope that then conservation policies will be formulated to protect these species and that this will form a precedent for many other conservation actions in planarians and invertebrate species in general.

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Appendix A. Genbank accession numbers of all DNA sequences used in the present study and information on the datasets in which they have been included.

Species	Ind.	Sample	Code (Field Number)	Cox1	Cox2	Nad1	tRNA W	28S	ITS	Dunuc2	Dunuc3	Dunuc5	Dunuc10	Dunuc12	Dunuc20	DATASETS*
Dugesia subtentaculata s.s.	1.1	F09_1DB	MR0672-01	MK712567				MK712521	MK713026		MK713280					1
	2.1	019_1DB	MR0747-01	MK712532												1
	2.2	019_1DC	MR0755-01	MK712561												1
	3.1	018_1DB	MR0744-01	MK712561												1
	3.2	018_1DC	MR0752-01	MK712561	MK712653	MK713175	MK713048	MK712493	MK712995	MK712707	MK713225	MK712758	MK712848	MK713099		1-2-5-6-7
	4.1	P11_1C	MR0695-01	MK712561												1
	5.1	C36_1CCi § Artavia1S	MR0647-01	MK712561												1-3-4
	5.2	C36_1D	MR0648-01	MK712561												1
	5.3	C36_2CCi § Artavia2S	MR0647-02	MK712538												3-4
	5.4	C36_2DC	MR0647-04	MK712539												
	5.5	C36_3CCi § Artavia3S	MR0647-03	MK712561												1-3-4
	6.1	C34_1DB	MR0646-01	MK712564												1
	7.1	C37_1DB§ Estella1A	MR0649-01	MK712561						MK712752	MK713258	MK712792	MK712878	MK712928		1-3-4-6
	7.2	C37.2DB§ Estella2A														3-4
	7.3	C37.3D§ Estella3														3-4
	8.1	P21_1_2F	MR0707-01	MK712561												1
	8.2	P21_2_1C	MR0710-01	MK712561												1
	9.1	P23_1C	MR0714-01	MK712566												1
	9.2	P23_1F	MR0714-02	MK712561												1
	10.1	C01_1DB	MR0571-01	MK712561	MK712659	MK713180	MK713053	MK712499	MK713002		MK713235	MK712769	MK712856	MK713118		1-2-5-6-7
	11.1	C02_1DB	MR0572-01	MK712565												1
	11.2	C02_1DC	MR0573-01	MK712562				MK712500	MK713003							1
	12.1	WP10_1	MR1242-01	MK712561												1
	13.1	C03_1DB	MR0577-01	MK712628	MK712660	MK713181	MK713054			MK712709	MK713236	MK712770	MK712857	MK713100	MK712943	1-2-5-6-7
	14.1	C05_1DB	MR0581-01	MK712630				MK712502		MK712711	MK713239	MK712773	MK712860	MK713104		1-2-6
	14.2	C05_1DC	MR0582-01	MK712630	MK712662	MK713183	MK713056	MK712503	MK713005		MK713240	MK712774	MK712861			1-5-7
	15.1	C04_1DC	MR0579-01	MK712628	MK712661	MK713182	MK713055	MK712501	MK713004	MK712722	MK713237	MK712771	MK712858	MK713118	MK712944	1-2-5-6-7
	15.2	C04_1Dt	MR0578-01	MK712629							MK713238	MK712772	MK712859	MK713119		1-2-6
	16.1	WP6_1	MR1241-01	MK712595												1
	17.1	C06_1D	MR0586-01	MK712626	MK712663	MK713184	MK713057	MK712504	MK713006		MK713241	MK712775		MK713101	MK712945	1-2-5-6-7
	17.2	C06_1DB	MR0588-01	MK712612					MK713007							1
	17.3	C06_1DC	MR0587-01	MK712626							MK713242	MK712776	MK712862		MK712946	1-6
	17.4	C06_2	MR0586-02	MK712627	MK712664	MK713185	MK713058			MK712713	MK713243	MK712777	MK712863	MK713101		1-2-5-6-7
	17.5	C06_2DC	MR0587-02	MK712616							MK713244	MK712778	MK712864	MK713101	MK712947	1-2-6
	17.6	C06_Cop1	MR0587-03	MK712534							MK713245	MK712779	MK712865	MK713102	MK712948	2-6
	17.7	C06_Cop2	MR0587-04	MK712535						MK712743	MK713246	MK712780	MK712866	MK713121	MK712949	2-6
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22.1	C12_1DB § Trelles1A	MR0600-01	MK712596												1-3-4
22.2	C12_1DC § Trelles3S	MR0601	MK712596	MK712667	MK713188	MK713061	MK712507	MK713010	MK712736	MK713250	MK712784	MK712870	MK713106	MK712951	1-3-4-2-5-6-7
22.3	C12.1D § Trelles2														3-4
23.1	C14_1D	MR0610-01	MK712574												1
23.2	C14_1DB	MR0611-01	MK712574												1
24.1	C16_1D	MR0612-01	MK712574												1
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26.1	C18_1D	MR0617-01	MK712574												1
26.2	C18_1DB	MR0618-01	MK712593												1
27.1	C22_1D	MR0619-01	MK712589												1
27.2	C22_1DB	MR0620-01	MK712574												1
28.1	C23_1D	MR0621-01	MK712580												1
28.2	C23_1DB	MR0622	MK712574				MK712508								1
29.1	C24_1DB	MR0624-01	MK712588												1
29.2	C24_1DC	MR0626	MK712578	MK712670	MK713191	MK713064		MK713013		MK713252	MK712786	MK712872	MK713132		1-2-5-6-7
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30.3	C25_2CCi	MR0926-01	MK712581												1
31.1	C26_1D	MR0628-01	MK712574												1
31.2	C26_1DB	MR0629-01	MK712587												1
31.2	PON_1D	MR0904-01	MK712583												1
32.1	C29_1D	MR0636-01	MK712579												1
32.2	C29_2CCi	MR0917-01	MK712574												1
33.1	C27_1DB	MR0631-01	MK712582												1
34.1	C32_1CCi	MR0910-01	MK712577												1
34.2	C32_2DB	MR0642-02	MK712536	MK712672	MK713193	MK713066				MK713254	MK712788	MK712874	MK713107	MK712953	2-6-7
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35.1	C33_1D	MR0645-01	MK712537						MK712719	MK713255	MK712789	MK712875	MK713103		2-6
35.2	C33_1DB	MR0644-01	MK712597	MK712673				MK713014	MK712717	MK713256	MK712790	MK712876	MK713117		1-2-5-6
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36.1	E08_1D	MR0758-01	MK712561	MK712677	MK713196	MK713069		MK713018		MK713267	MK712797	MK712884	MK712929		1-5-6-7
37.1	POR_1DB	MR0870-01	MK712545							MK713292	MK712821	MK712901	MK713108	MK712975	2-6
37.2	POR_1DC	MR0868-01	MK712546						MK712726	MK713293	MK712822	MK712902	MK713109	MK712976	2-6
38.1	E17_1D	MR0759-01	MK712592	MK712678	MK713197	MK713070	MK712513	MK713019	MK712748	MK713268	MK712798			MK712958	1-5-6-7
38.2	E17_1DB	MR0760-01	MK712594												1
39.1	E18_1D	MR0764-01	MK712590												1
39.2	E18_1DB	MR0763-01	MK712591												1

40.1	E23_1Cop § Horta3S	MR0778-01	MK712574												1-3-4
40.2	E23_1DB § Horta1A	MR0768-01	MK712584												1-3-4
40.3	E23_1DC § Horta4S	MR0775-01	MK712575	MK712679	MK713198	MK713071	MK712514	MK713020		MK713269	MK712799		MK713134	MK712959	1-2-3-4-5-6-7
40.4	E23_2DB § Horta2A	MR0768-02	MK712599	MK712680	MK713199	MK713072			MK712715	MK713270	MK712800	MK712885	MK713133	MK712960	1-2-3-4-5-6-7
40.5	E23_2DC § Horta5S	MR0776-01	MK712600	MK712681		MK713073	MK712515	MK713021		MK713271	MK712801	MK712886	MK712930	MK712961	1-3-4-5-6-7
40.7	E23_5DC	MR0769-01	MK712541												
41.1	E24_1DC	MR0779-01	MK712611	MK712682	MK713200	MK713074	MK712516	MK713022	MK712712	MK713272	MK712802	MK712887	MK713134	MK712962	1-2-5-6-7
41.2	E24_2DC	MR0772-01	MK712599						MK712716	MK713273	MK712803	MK712888	MK713135	MK712963	1-2-6
42.1	CAB_1D	MR0851-01	MK712574	MK712674	MK713194	MK713067		MK713015	MK712734	MK713259	MK712793	MK712879		MK712954	1-5-6-7
43.1	MAD_1D	MR0896-01	MK712586												1
44.1	V05_1D § Truchas2	MR0729-01	MK712608	MK712704	MK713222	MK713096	MK712531	MK713047	MK712731	MK713314	MK712843	MK712921	MK713128	MK712991	1-2-3-4-5-6-7
44.2	V05_1DB § Truchas1A	MR0730-01	MK712609	MK712705	MK713223	MK713097			MK712749	MK713315	MK712844	MK712922	MK712933	MK712992	1-3-4-6-7
44.3	V05_2D§ Truchas3	MR0729-02	MK712610	MK712706	MK713224	MK713098					MK712845				1-3-4-7
44.4	V05_3D	MR0729-03	MK712558						MK712733	MK713316	MK712846	MK712923		MK712993	6
44.5	V05_4D	MR0729-04	MK712559							MK713317	MK712847	MK712924		MK712994	6
45.1	V03_1D	MR0726-01	MK712571	MK712703	MK713221	MK713095	MK712530	MK713046		MK713313	MK712842		MK713126	MK712990	1-2-5-6-7
45.2	V03_1DB	MR0727-01	MK712572												1
46.1	StaFe1 § SantaFe1A	MR0879-01	MK712576	MK712702	MK713220	MK713094		MK713045	MK712737	MK713312	MK712841		MK713127		1-2-3-4-5-6-7
46.2	StaFe2	MR0879-02	MK712557												
46.3	StaFe6 § SantaFe2A														3-4
46.4	StaFe7 § SantaFe3A														3-4
46.5	StaFe_11	MR0879-03											MK713120		2
46.6	StaFe_15	MR0879-04											MK713145		2
46.7	Dsp01		FJ646997												
49.1	E27_1DC	MR0780-01	MK712570	MK712683	MK713201	MK713075	MK712517	MK713023	MK712724	MK713274	MK712804	MK712889	MK713139		1-2-5-6-7
50.1	POR9_1Ci	MR0803-03	MK712550						MK712729	MK713299	MK712828	MK712908	MK713122	MK712980	2-6
50.2	POR9_1D	MR0803-01	MK712601	MK712696	MK713214	MK713088			MK712730	MK713300	MK712829	MK712909	MK713123	MK712981	1-2-5-6-7
50.3	POR9_1DB	MR0803-02	MK712549						MK712727	MK713301	MK712830	MK712910		MK712982	6
50.4	POR9_1DC	MR0811-01	MK712602	MK712697	MK713215	MK713089	MK712527	MK713039							1-7
50.5	POR9_2DC	MR0812-01	MK712602	MK712698	MK713216	MK713090			MK712732	MK713302	MK712831	MK712911	MK713124	MK712983	1-2-5-6-7
51.1	POR12_1D § Alte1	MR0807-01	MK712619	MK712693	MK713211	MK713085			MK712735	MK713294	MK712823	MK712903	MK713140	MK712977	1-2-3-4-5-6-7
51.2	POR12_1DC§ Alte2S	MR0814-01	MK712619	MK712694	MK713212	MK713086	MK712526	MK713038	MK712728	MK713295	MK712824	MK712904	MK713142	MK712978	1-2-3-4-5-6-7
51.3	POR12_2DC§ Alte3S	MR0815-01	MK712620	MK712695	MK713213	MK713087				MK713296	MK712825	MK712905	MK713143		1-2-3-4-6-7
51.4	POR12_3D	MR0807-02	MK712547												
51.5	POR12_3DC	MR0816-01								MK713297	MK712826	MK712906	MK713144		2-6

	51.6	POR12_5DC	MR0808-01	MK712548						MK712725	MK713298	MK712827	MK712907	MK713141	MK712979	2-6
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	52.11	245_4	MR1169-03	MK712568												1
	52.12	30_4	MR1172-02	MK712568												1
	52.13	245_5	MR1169-04	MK712568												1
	52.7	A2_1D_M2	MR0781-02									MK712766			MK712941	
	52.8	30_2	MR1172-01	MK712569					MK712998							1
	52.9	245_2	MR1169-01	MK712568												1
	54.3	Dg1	MR1175-01	MK712623	MK712675	MK713195	MK713068	MK712510	MK713016		MK713260	MK712794	MK712880	MK713114	MK712955	1-2-5-6-7
	54.4	Dg2	MR1176-01	MK712623							MK713262	MK712796		MK713136	MK712957	1-2-6
	54.5	32_1	MR1174-01	MK712623												1
	54.6	32_3	MR1174-02	MK712623	MK712655	MK713177	MK713050	MK712496	MK712999		MK713229	MK712762	MK712851	MK713112		1-2-5-6-7
	54.7	32_5	MR1174-03	MK712623							MK713230	MK712763	MK712852	MK713113	MK712938	1-2-6
	55.1	B4P	MR1233-01	MK712624	MK712657	MK713178	MK713051	MK712497	MK713000		MK713233	MK712767		MK713131	MK712942	1-2-5-6-7
	55.2	B4T	MR1234-01	MK712625												1
	56.1	B7TT	MR1235-01	MK712573												1
	57.1	B8P	MR1236-01	MK712573	MK712658	MK713179	MK713052	MK712498	MK713001		MK713234	MK712768	MK712855			1-5-7
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	58.2	ETO2	MR1238-02	MK712622	MK712685	MK713203	MK713077	MK712520	MK713025		MK713277	MK712808	MK712891	MK713115	MK712966	1-2-5-6-7
	58.3	ETO3	MR1238-03	MK712542	MK712686	MK713204	MK713078				MK713278	MK712809	MK712892	MK713116	MK712967	2-6-7
	58.4	ETO4	MR1238-04								MK713279	MK712810	MK712893	MK712931		6
	59.1	E6_2	MR1232-02	MK712605				MK712519	MK713024	MK712738	MK713275	MK712806		MK713130		1-2-6
	59.2	R5	MR1232-01						MK713042		MK713303	MK712832				
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	62.1	E4_2	MR1231-02	MK712603				MK712518				MK712805		MK713129	MK712964	1-2
	62.4	R2	MR1231-01	MK712551					MK713041							
	63.1	MMS6_1	MR1239-01	MK712606					MK713031		MK713287	MK712817				1
	63.2	MMS6_2	MR1239-02	MK712607					MK713032		MK713288	MK712818				1
	63.3	MMS6_3	MR1239-03	MK712599					MK713033		MK713289	MK712819				1
	63.4	MMS6_4	MR1239-04								MK713290	MK712820				
	63.5	MMS6_5	MR1239-05								MK713291					
D. aurea	47.1	M2_1D	MR0182-01	MK712631	MK712687	MK713205	MK713079	MK712522	MK713027	MK712757	MK713281	MK712811	MK712894	MK713146	MK712968	1-2-5-6-7
	47.2	M2_2D § Soller1S	MR0182-02	MK712632	MK712688	MK713206	MK713080	MK712523	MK713028	MK712739	MK713282	MK712812	MK712895	MK713147	MK712969	1-2-3-4-5-6-7
	47.3	M2_3D § Soller2S	MR0182-03	MK712631	MK712689	MK713207	MK713081			MK712740	MK713283	MK712813	MK712896	MK713148	MK712970	1-2-3-4-5-6-7
	47.4	Soller1 § Soller3S	MR0182-04	MK712555						MK712742	MK713310	MK712839	MK712919	MK713150	MK712988	2-3-4-6
	47.5	Soller2	MR0182-05	MK712556						MK712741	MK713311	MK712840	MK712920	MK713149	MK712989	2-6
	47.6	Soller_1_1												MK713151		2
	47.7	Dsu01		FJ646995												1
D. corbata	48.1	M3_1D § Calobra1S	MR0526-01	MK712635	MK712690	MK713208	MK713082	MK712524	MK713029	MK712745	MK713284	MK712814	MK712897	MK713153	MK712971	1-2-3-4-5-6-7
	48.2	M3_2D § Calobra3S	MR0526-02	MK712636	MK712691	MK713209	MK713083	MK712525	MK713030	MK712746	MK713285	MK712815	MK712898	MK713152	MK712972	1-2-3-4-5-6-7
	48.3	M3_3 § Calobra2S	MR0526-03	MK712637	MK712692	MK713210	MK713084			MK712747	MK713286	MK712816	MK712899	MK713153	MK712973	1-2-3-4-5-6-7
	48.4	MLL1	MR0526-04	MK712543						MK712756			MK712900	MK712932	MK712974	6

	48.5	MLL2	MR0526-05	MK712544											
	48.6	SC_2	MR0526-06							MK713309	MK712838	MK712918	MK713154		2-6
	48.7	Dsu02		FJ646996											1
D. vilafarrei	52.1	A2_1D_M1 § Bosque 1S	MR0781-01	MK712568					MK712750	MK713232	MK712765	MK712854	MK712925	MK712940	1-3-4-6
	52.14	A2_1DC_M1 § Bosque 2S	MR0781-03	MK712533	MK712656				MK712751	MK713231	MK712764	MK712853	MK712926	MK712939	3-4-5-6-7
	52.4	29_1	MR1171-01	MK712648											1
	52.5	29_2	MR1171-02	MK712649				MK712495	MK712997	MK712710	MK713228	MK712761	MK712850	MK713155		1-2-5-6
	52.6	246_1	MR1170-01	MK712650											1
	53.1	233_1	MR1173-01	MK712648							MK713226	MK712759			MK712936	1
	53.2	233_2	MR1173-02	MK712648											1
	54.1	Dg1_1 § Bosque3S	MR1175-02	MK712648	MK712676			MK712511	MK713017	MK712753	MK713261	MK712795	MK712881	MK713156	MK712956	1-2-3-4-5-6-7
	54.2	Dg3_3	MR1177-01	MK712648											1
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	60.2	R9T	MR1230-01	MK712634	MK712700	MK713218	MK713092	MK712529	MK713044	MK712744	MK713304	MK712833	MK712913	MK713157	MK712984	1-2-5-6-7
	60.3	R9T_2	MR1230-02		MK712701	MK713219	MK713093				MK713306	MK712835	MK712915			5
	60.4	R9T_4Cop	MR1230-03	MK712553							MK713307	MK712836	MK712916		MK712986	6
	60.5	R9T_5Cop	MR1230-04	MK712554							MK713308	MK712837	MK712917		MK712987	6
	60.6	R9T6	MR1230-05	MK712552							MK713305	MK712834	MK712914		MK712985	6

Outgroups

Species	**Code**	**Cox1**	**Cox2**	**Nad1**	**tRNA W**	**28S**	**ITS**	**Dunuc2**	**Dunuc3**	**Dunuc5**	**Dunuc10**	**Dunuc12**	**Dunuc20**	**DATASETS***
D. hepta	Dhe02					MK712512								5
	D.he_B								MK713265		MK712883			5
	D.he_M							MK712754	MK713266			MK712935		5
	MR1962	MK385924					MK713035					MK713159		1-2-3-4-5
	MR1960	MK385923					MK713034					MK713158		1-2-3-4-5
	MR1274	MK712639										MK713161		1-2-3-4
	MR0071	MK712638										MK713160		1-2-3-4
D. benazzii	Dbe01					MK712509								5
	D.be_B								MK713263		MK712882			5
	D.be_M							MK712755	MK713264			MK712934		5
	MR0449	MK712640										MK713164		1-2-3-4
	MR2191	MK385925					MK713036					MK713162		1-2-3-4-5
	MR2192	MK385926					MK713037					MK713163		1-2-3-4-5
	MR0496											MK713165		2-4
	MR0492	MK712643												1-3
	MR0791	MK712641										MK713166		1-2-3-4
	MR0792	MK712642										MK713167		1-2-3-4
D. etrusca	Us.1D	MK712651										MK713169		1-2-3-4
	P45.1	MK712652										MK713168		1-2-3-4
	Det06	FJ646984										MK713170		1-2-3-4
D. liguriensis	2.43	MK712647										MK713171		1-2-3-4
	024.1D	MK712646										MK713174		1-2-3-4
	L1.1D	MK712644										MK713173		1-2-3-4
	2.41	MK712645										MK713172		1-2-3-4

*Sequences included in the datasets: (1) Cox1 Discovery; (2) Dunuc12 Discovery; (3) Cox1-Cloned Discovery; (4) Duncu12-Cloned Discovey; (5) All Validation; (6) Nuclear Validation; (7) Mitochondrial Validation
§Individual code used in Leria et al. (2019).

Appendix B. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2019.05.010.

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Supplementary data S1. List of populations of *Dugesia subtentaculata* s.l. used in the present study, with detailed information on coordinates and collectors.

Population code	Locality	Coordinates	Collectors	Date of collection
1	Lo Bosquet d'Òrb, Languedoc-Roussillon, France	43.723919N 3.147088E	M. Vila-Farré, E. Solà and L. Leria	29/09/2011
2	Louhossoa, France	43.31059N 001.35241W	M. Álvarez-Presas and L. Leria	26/03/2013
3	Saint Pée sur Nivelle, France	43.34235N 001.52650W	M. Álvarez-Presas and L. Leria	26/03/2013
4	Bidasoa, Basc Country, Spain	43.316028N 1.740778W	M. Vila-Farré, J. Martín and E. Solà	29/04/2011
5	Artavia, Navarra, Spain	42.74488N 2.089569W	M. Vila-Farré, E. Solà and L. Leria	11/10/2011
6	Agoncillo, La Rioja, Spain	42.44605N 2.311W	M. Vila-Farré, E. Solà and L. Leria	11/10/2011
7	Estella, Navarra, Spain	42.6746N 2.0352W	M. Vila-Farré, E. Solà and L. Leria	12/10/2011
8	Lariz, Basc Country, Spain	43.340444N 2.521000W	M. Vila-Farré, J. Martín and E. Solà	30/04/2011
9	Zugastieta, Basc Country, Spain	43.254472N 2.690083W	M. Vila-Farré, J. Martín and E. Solà	30/04/2011
10	Ampuero, Cantabria, Spain	43.3442N 3.41831W	M. Vila-Farré, E. Solà and L. Leria	04/10/2011
11	Liérganes, Cantabria, Spain	43.346211N 3.74445W	M. Vila-Farré, E. Solà and L. Leria	04/10/2011
12	Parque Natural Saja-Besaya, Cantabria, Spain	43.232492N 4.209677W	E. Mateos	24/05/2010
13	Casar de Periedo, Cantabria, Spain	43.3365N 4.173461W	M. Vila-Farré, E. Solà and L. Leria	04/10/2011
14	Panes, Asturias, Spain	43.325969N 4.585669W	M. Vila-Farré, E. Solà and L. Leria	05/10/2011
15	Ruente, Cantabria, Spain	43.2576N 4.266819W	M. Vila-Farré, E. Solà and L. Leria	05/10/2011
16	Parque Natural Collados del Asón, Cantabria, Spain	43.226485N 3.602011W	E. Mateos	26/05/2010

17	Villanueva de Cangas, Asturias, Spain	43.365180N 5.151719W	M. Vila-Farré, E. Solà and L. Leria	05/10/2011
18	La Nueva, Asturias, Spain	43.264611N 5.67305W	M. Vila-Farré, E. Solà and L. Leria	05/10/2011
19	Cornellana, Asturias, Spain	43.412161N 6.15166W	M. Vila-Farré, E. Solà and L. Leria	05/10/2011
20	Trevías, Asturias, Spain	43.49738N 6.431580W	M. Vila-Farré, E. Solà and L. Leria	06/10/2011
21	Luarca, Asturias, Spain	43.541891N 6.53539W	M. Vila-Farré, E. Solà and L. Leria	06/10/2011
22	Trelles, Asturias, Spain	43.476N 6.73358W	M. Vila-Farré, E. Solà and L. Leria	06/10/2011
23	Sueiro, Asturias, Spain	43.528888N 6.876688W	M. Vila-Farré, E. Solà and L. Leria	06/10/2011
24	San Tirso de Abres, Asturias, Spain	43.42315N 7.1391388W	M. Vila-Farré, E. Solà and L. Leria	06/10/2011
25	San Pedro de Viveiro, Galícia, Spain	43.621480N 7.593619W	M. Vila-Farré, E. Solà and L. Leria	06/10/2011
26	Ponte Noval, Galícia, Spain	43.63865N 7.908480W	M. Vila-Farré, E. Solà and L. Leria	07/10/2011
27	Negreira, Galícia, Spain	42.92106N 8.68771W	M. Vila-Farré, E. Solà and L. Leria	08/10/2011
28	Sinde, Galícia, Spain	42.74408N 8.59368W	M. Vila-Farré, E. Solà and L. Leria	08/10/2011
29	Pontevea, Galícia, Spain	42.764761N 8.555338W	M. Vila-Farré, E. Solà and L. Leria	08/10/2011
30	Cuntis, Galícia, Spain	42.61266N 8.566788W	M. Vila-Farré, E. Solà and L. Leria	09/10/2011
31	Ponte Caldelas, Galícia, Spain	42.39098N 8.491788W	M. Vila-Farré, E. Solà and L. Leria	09/10/2011
32	Mondariz-Balneario, Galícia, Spain	42.228111N 8.46985W	M. Vila-Farré, E. Solà and L. Leria	09/10/2011
33	Gondomar, Galícia, Spain	42.112261N 8.762869W	M. Vila-Farré, E. Solà and L. Leria	09/10/2011
34	Veiga, Galícia, Spain	42.872538N 7.527819W	M. Vila-Farré, E. Solà and L. Leria	10/10/2011
35	Trabadelo, Castilla y León, Spain	42.647211N 6.875561W	M. Vila-Farré, E. Solà and L. Leria	10/10/2011

36	Cordovilla la Real, Castilla y León, Spain	42.07849N 4.26625W	M. Álvarez-Presas, M. Riutort and L. Leria	01/06/2013
37	Pardeeiro, Portugal	40.501961N 8.344294W	A. Rodrigues	06/05/2014
38	Villasrubias, Castilla y León, Spain	40.32409N 6.64524W	M. Álvarez-Presas, M. Riutort and L. Leria	03/06/2013
39	Acebo, Extremadura, Spain	40.16863N 6.67998W	M. Álvarez-Presas, M. Riutort and L. Leria	03/06/2013
40	Hortas, Portalegre, Portugal	39.32326N 7.40911W	M. Álvarez-Presas, M. Riutort and L. Leria	04/06/2013
41	Monte de Vento, Portalegre, Portugal	39.28894N 7.38384W	M. Álvarez-Presas, M. Riutort and L. Leria	04/06/2013
42	Cabañeros, Castilla- La Mancha	39.378888N 4.500555W	L. Leria	06/2012
43	Torrejón de Ardoz, Madrid, Spain	40.418611N 3.480833W	M. Vila-Farré	2012
44	Peralejos de las Truchas, Castilla La Mancha, Spain	40.59318N 1.92561W	M. Vila-Farré, E. Lázaro and L. Leria	21/01/2012
45	Albarracín, Teruel, Spain	40.40783N 1.4474W	M. Vila-Farré, E. Lázaro and L. Leria	21/01/2012
46	Santa Fe del Montseny, Catalonia, Spain	41.77356N 2.46638E	M. Vila-Farré, E. Solà and L. Leria	11/2011
47	Soller, Mallorca, Balearic Islands, Spain	39.75693N 2.71193E	M. Vila-Farré	2012
48	Sa Calobra, Mallorca, Balearic Islands, Spain	39.82932N 2.81538E	M. Vila-Farré	2012
49	Fuencaliente, Castilla- La Mancha	38.37926N 4.30558W	M. Álvarez-Presas, M. Riutort and L. Leria	05/06/2013
50	Caldas de Monchique, Algarve, Portugal	37.283983N 8.554117W	A. Valls, E. Solà, M. Riutort and L. Leria	06/10/2013
51	Alte, Algarve, Portugal	37.23827N 8.171998W	A. Valls, E. Solà, M. Riutort and L. Leria	06/10/2013
52	El Bosque, Andalucía, Spain	36.76123N 5.50581W	M. Vila-Farré	2011
53	Benamahoma, Andalucía, Spain	36.766505N 5.466677W	M. Vila-Farré	2011
54	Benaojan, Andalucía, Spain	36.680049N 5.252672W	M. Vila-Farré	2011

55	Alhama de Granada, Andalucía, Spain	36.924730N 3.987055W	N. Bonada	11/2008
56	Játar, Andalucía, Spain	36.884008N 3.86922W	N. Bonada	11/2008
57	Jayena, Andalucía, Spain	36.914744N 3.834738W	N. Bonada	11/2008
58	Los Barrios, Andalucía	36.152777N 5.578611W	E. Mateos and E. Solà	2010
59	Beni Moussa, Morocco	35.382544N 5.337600W	N. Bonada	05/2008
60	Afaska, Morocco	35.209516N 5.187777W	N. Bonada	05/2008
61	Taria, Morocco	35.159161N 5.166674W	N. Bonada	05/2008
62	Nord Village Maggo, Morocco	35.101944N 5.184169W	N. Bonada	05/2008
63	Imlil, Toubkal, Marrakech, Morocco	31.129183N 7.975922W	H. Harrath	23/11/2009

Supplementary data S2. Genome size estimation protocol used in the present study.

1. Take eight individuals of each population (1cm length each individual).
2. Place the eight individuals in two separate Eppendorfs (four individuals in each).
3. Add 500µl of Galbraith's* buffer (at 4ºC) in each Eppendorf and incubate for 10 minutes in ice.
4. Grind up for 30 seconds using a plastic piston.
5. Filter with a mesh of 75µm.
6. Centrifuge at 800g for 10 minutes (at 4ºC).
7. Discard the supernatant and resuspend the pellet in 600µl of PBS Isoton.
8. Add 75µl of erythrocyte nuclei of chicken, trout and triploid trout (BioSure, Grass Valley, CA) only in one of the two Eppendorfs to be used as a control (tube controls).
9. Add 20µl of Propidium Iodide (1mg/ml) and incubate for 30 minutes.
10. Filter with a mesh of 75 µm.
11. Measure the fluorescence with a Gallios Flow Cytometer.
12. Identify the peak corresponding to the query sample in the "tube controls" by comparing the fluorescence value obtained in the tube without controls.
13. Compare the fluorescence value of your sample with the fluorescence values of the controls and estimate the size of the genome of your sample.

***** Galbraith's buffer composition: 45 mM magnesium chloride, 30 mM sodium citrate, 20 mM 4-morphoG linepropane sulfonate, and Triton X-100 (1 mg/ml) (ph=7) (Galbraith et al. 1983)

Supplementary data S3. Forward (F) and reverse (R) primers used for PCR amplification and sequencing with the respective PCR conditions

Gene	Primer	Sequence 5'-3'	PCR conditions	Source
Dunuc2	Dunuc2_2F (F)	ATGGAGTGGSAARTATCG	2': 94°C, (45'':94°C, 50'':57°C, 40'':72°C) x35,	This study
	Dunuc2_2R (R)	CCCAATGAACAGTGAATG	3':72°C	This study
Dunuc3	Dunuc3_1F (F)	GCTTTACATCTCTATCATTC	2': 94°C, (45'':94°C, 50'':50°C, 40'':72°C) x35,	This study
	Dunuc3_2R (R)	GATAATGATGAACCAGAAAC	3':72°C	This study
Dunuc5	Dunuc5_3Fsubte (F)	GGAATGACTAAGAATGATACTC	2': 94°C, (45'':94°C, 50'':51°C, 40'':72°C) x35,	This study
	Dunuc5_1Rsubte (R)	GCAATAACAACATCAGCG	3':72°C	This study
Dunuc10	Dunuc10_2F (F)	CTTTTATCAATCATAACTTTGTG	2': 94°C, (45'':94°C, 50'':47°C, 40'':72°C) x35,	This study
	Dunuc10_1R (R)	CCTTTCGGTTGTTCTC	3':72°C	This study
Dunuc12	Dunuc12_1Fsubte (F)	CTCGTATCTCTGAATCTAGCCTC	2': 94°C, (45'':94°C, 50'':58°C, 40'':72°C) x35,	This study
	Dunuc12_1R (R)	GTTCATACAACTCATTCTTC	3':72°C	This study
Dunuc20	Dunuc20_3F (F)	GAAACGAAAGAAGAAGAGAGCG	2': 94°C, (45'':94°C, 50'':59°C, 60'':72°C) x35,	This study
	Dunuc20_3R (R)	GATTTGCTGGTCCTAGAAG	3':72°C	This study
Cox1	COIR (R)	CCWGTYARMCCHCCWAYAGTAAA	2': 94°C, (45'':94°C, 50'':43°C, 35'':72°C) x40,	Lázaro et al. (2009)
	BarT (F)	ATGACDGCSCATGGTTTAATAATGAT	3':72°C	Álvarez-Presas et al. (2011)
	COIEF3 (F)	CCWCGTGCWAATAATTTRAG	Primer used for sequencing instead of the BarT	Solà et al. 2013
ITS-1	9F	GTAGGTGAACCTGCGGAAGG	2': 94°C, (45'':94°C, 50'':50°C, 50'':72°C) x30,	Baguñà et al. (1999)
	ITSR (R)	TGCGTTCAAATTGTCAATGATC	3':72°C	Baguñà et al. (1999)
28S †	28S1F (F)	TATCAGTAAGCGGAGGAAAAG	1': 94°C, (45'':94°C, 45'':55°C, 45'':72°C) x35, 4':72°C	Álvarez-Presas et al. (2008)
	28S4R (R)	CCAGCTATCCTGAGGG		Álvarez-Presas et al. (2008)
	28S2F (F)	CTGAGTCCGATAGCAAACAAG		Álvarez-Presas et al. (2008)
	28S6R (R)	GGAACCCCTTCTCCACTTCAGT		Álvarez-Presas et al. (2008)

Nad1-trnaW-Cox2	nd1F2 (F)	GGGGAGAGGGAGTTAG	2': 94°C, (45'':94°C, 50'':41°C, 30'':72°C) x40, 3':72°C	This study
	CoIIP (R)	CCACAAACCTCAGAAC		This study
	Cupi2 (F) ‡	ATTTGRCTYCGTTCTTC	2': 94°C, (45'':94°C, 50'':41°C, 30'':72°C) x40, 3':72°C	This study
	trnaWF (F) ‡	TTAGACTTTCAGTTTTC	2': 94°C, (45'':94°C, 50'':45°C, 30'':72°C) x40, 3':72°C	This study

† Gene amplified and sequenced in two independent fragments

‡ Primers used together with CoIIP (R)

Supplementary data S4. List of individuals included in the morphological analysis

Species	PSH	Locality	FIELD NUMBER
Dugesia aurea	2	47	RS455
	2	47	RS456
	2	47	RS458.1
	2	47	RS458.2
Dugesia vilafarrei	3	52	MV04-6
	3	52	MV08-1
Dugesia corbata	4	48	RS461
	4	48	RS462
	4	48	RS463
Dugesia subtentaculata s.s.	5	3	RS451
	5	3	RS452
	5	3	RS453
	5	3	RS454
	5	2	RS488
	5	2	RS489
	5	2	RS490
	5	2	RS491
	5	17	RS499
	5	17	RS500.1
	5	17	RS500.2
	5	17	RS500.3
	5	19	RS497.1
	5	19	RS497.2
	5	30	RS472
	5	40	RS471
	5	40	RS478
	5	40	RS479
	5	40	RS480
	5	41	RS481
	5	49	RS473
	5	4	RS492
	5	4	RS493
	5	51	RS483
	5	51	RS485
	5	50	RS474
	5	50	RS475
	5	12	RS496.1
	5	12	RS496.2

Supplementary data S5. List of individuals included in in the karyological analysis, with additional information on their reproductive strategy.

Species	PSH	Locality	Code	Reproductive strategy	Reference
Dugesia aurea	2	47	-	Sexual	Ribas 1990
Dugesia vilafarrei	3	52	Ind1	Sexual	Present study
	3	52	Ind2	Sexual	Present study
	3	52	Ind3	Sexual	Present study
	3	52	Ind4	Sexual	Present study
	3	52	Ind5	Sexual	Present study
Dugesia corbata	4	48	-	Sexual	Ribas 1990
Dugesia subtentaculata s.s.	5	46	-	Fissiparous	Ribas 1990
	5	22	C12.3	Fissiparous	Present study
	5	22	C12.7	Fissiparous	Present study
	5	32	C29.2	Fissiparous	Present study
	5	32	C29.6	Fissiparous	Present study
	5	7	C37.1	Fissiparous	Present study
	5	7	C37.7	Fissiparous	Present study
	5	4	P6	Sexual	Present study
	5	17	Piloña	Sexual	Present study
	5	1	Montpellier	Fissiparous	De Vries 1986

Supplementary data S6. List of localities sampled for the present study where *Dugesia subtentaculata* s.l. was not found. The localities were either occupied by other Tricladida species or by other organisms (only the Tricladida species are detailed).

Locality	Coordinates	Tricladida species	Collectors	
Villamartín, Andalucía, Spain	36.87869N 5.57349W	-	M. Álvarez-Presas, M. Riutort and L. Leria	17/06/2013
Torre-Alháquime, Andalucía, Spain	36.91275N 5.23014W	-	M. Álvarez-Presas, M. Riutort and L. Leria	17/06/2013
Setenil de las Bodegas, Andalucía, Spain	36.86558N 5.18445W	-	M. Álvarez-Presas, M. Riutort and L. Leria	17/06/2013
Serrato, Andalucía, Spain	36.88844N 4.97933W	*Dugesia sicula*	M. Álvarez-Presas, M. Riutort and L. Leria	17/06/2013
Igualeja, Andalucía, Spain	36.63274N 5.11842W	--	M. Álvarez-Presas, M. Riutort and L. Leria	17/06/2013
Árquez, Andalucía, Spain	36.83946N 3.99086W	*Dugesia sicula*	M. Álvarez-Presas, M. Riutort and L. Leria	18/06/2013
Salares, Andalucía, Spain	36.85230N 4.02625W	*Dugesia sicula*	M. Álvarez-Presas, M. Riutort and L. Leria	18/06/2013
Alhama de Granada, Andalucía, Spain	36.98608N 3.98378W		M. Álvarez-Presas, M. Riutort and L. Leria	18/06/2013
Fornes, Andalucía, Spain	36.94964N 3.87227W	*Dugesia sicula*	M. Álvarez-Presas, M. Riutort and L. Leria	18/06/2013
Lanjarón, Andalucía, Spain	36.92136N 3.47118W	*Dugesia sicula* and *Polycelis* sp.	M. Álvarez-Presas, M. Riutort and L. Leria	18/06/2013
El molinillo, Andalucía, Spain	37.30606N 3.42669W	*Polycelis* sp.	M. Álvarez-Presas, M. Riutort and L. Leria	18/06/2013
Molino bajo (Huéneja), Andalucía, Spain	37.16322N 2.95128W	-	M. Álvarez-Presas, M. Riutort and L. Leria	18/06/2013
Castillejar, Andalucía, Spain	37.71126N 2.64266W	-	M. Álvarez-Presas, M. Riutort and L. Leria	18/06/2013
Santuario Virgen de la Cabeza, Andalucía, Spain	37.84169N 2.58668W	-	M. Álvarez-Presas, M. Riutort and L. Leria	19/06/2013
Vegadeo, Astúrias, Spain	43.49222N 7.04444W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	06/10/2011
A Lagoa, Galícia, Spain	43.44003N 8.01670W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	08/10/2011
Teixeiro, Galícia, Spain	43.13498N 8.01448W	Polycelis sp.	M. Vila-Farré, E. Solà and L. Leria	08/10/2011

Mondariz-Balneario, Galícia, Spain	42.22811N 8.46985W	-	M. Vila-Farré, E. Solà and L. Leria	09/10/2011
A Cañiza, Galícia, Spain	42.23621N 8.28835W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	09/10/2011
San Andres de Riveira, Galícia, Spain	42.87356N 7.53841W	-	M. Vila-Farré, E. Solà and L. Leria	09/10/2011
La Escaleruela, Teruel, Spain	40.14307N 0.75536W	*Polycelis* sp.	M. Vila-Farré, E. Lázaro and L. Leria	21/01/2012
Cabra de Mora,Teruel, Spain	40.34045N 0.81256W	-	M. Vila-Farré, E. Lázaro and L. Leria	21/01/2012
Orihuela de Tremedal,Teruel, Spain	40.47208N 1.62960W	*Polycelis* sp.	M. Vila-Farré, E. Lázaro and L. Leria	21/01/2012
Peralejos de las Truchas, Guadalajara, Spain	40.59318N 1.92562W	*Polycelis* sp.	M. Vila-Farré, E. Lázaro and L. Leria	21/01/2012
Villalba de la Sierra, Cuenca, Spain	40.22659N 2.08940W	*Dugesia sicula*	M. Vila-Farré, E. Lázaro and L. Leria	22/01/2012
Reillo, Cuenca, Spain	39.90715N 1.85132W	*Girardia* sp.	M. Vila-Farré, E. Lázaro and L. Leria	22/01/2012
Yémeda, Cuenca, Spain	39.74570N 1.70798W	-	M. Vila-Farré, E. Lázaro and L. Leria	22/01/2012
Yémeda, Cuenca, Spain	39.74645N 1.71167W	-	M. Vila-Farré, E. Lázaro and L. Leria	22/01/2012
Requena, Valencia, Spain	39.47826N 1.11640W	*Schmidtea polychroa*	M. Vila-Farré, E. Lázaro and L. Leria	22/01/2012
Casas del Río, Valencia, Spain	39.29665N 1.13486W	-	M. Vila-Farré, E. Lázaro and L. Leria	22/01/2012
Casas del Río, Valencia, Spain	39.29801N 1.13866W	*Dugesia sicula*	M. Vila-Farré, E. Lázaro and L. Leria	22/01/2012
Les Borges del Camp, Tarragona, Catalonia, Spain	41.16876N 1.02195E	*Dugesia sicula*	M. Vila-Farré, E. Lázaro and L. Leria	23/01/2012
Duesaigües, Tarragona, Catalonia, Spain	41.15158N 0.92832E	-	M. Vila-Farré, E. Lázaro and L. Leria	22/01/2012
Dueñas, Castilla y León, Spain	41.89482N 4.56520W	-	M. Álvarez-Presas, M. Riutort and L. Leria	01/06/2013
Cevico de la Torre, Castilla y León, Spain	41.85218N 4.41876W	-	M. Álvarez-Presas, M. Riutort and L. Leria	01/06/2013
Cevico de la Torre, Castilla y León, Spain	41.84912N 4.41117W	-	M. Álvarez-Presas, M. Riutort and L. Leria	01/06/2013
Cevico Navero, Castilla y León, Spain	41.8684N 4.1859W	-	M. Álvarez-Presas, M. Riutort and L. Leria	01/06/2013

Antigüedad, Castilla y León, Spain	41.95565N 4.06651W	-	M. Álvarez-Presas, M. Riutort and L. Leria	01/06/2013
Cobos del Cerrato, Castilla y León, Spain	42.02774N 4.00301W	*Polycelis* sp.	M. Álvarez-Presas, M. Riutort and L. Leria	01/06/2013
Peral de Arlanza, Castilla y León, Spain	42.07813N 4.07046W	-	M. Álvarez-Presas, M. Riutort and L. Leria	01/06/2013
Benavente, Castilla y León, Spain	42.02737N 5.7085W	*Schmidtea polychroa*	M. Álvarez-Presas, M. Riutort and L. Leria	02/06/2013
Benavente, Castilla y León, Spain	42.02334N 5.71416W	-	M. Álvarez-Presas, M. Riutort and L. Leria	02/06/2013
Requejo, Castilla y León, Spain	42.03123N 6.76452W	*Polycelis* sp.	M. Álvarez-Presas, M. Riutort and L. Leria	02/06/2013
Pueblica de Campeán, Castilla y León, Spain	41.41352N 5.82893W	-	M. Álvarez-Presas, M. Riutort and L. Leria	02/06/2013
Ledesma, Castilla y León, Spain	41.09281N 6.00029W	-	M. Álvarez-Presas, M. Riutort and L. Leria	02/06/2013
Pelarrodríguez, Castilla y León, Spain	40.88507N 6.21341W	-	M. Álvarez-Presas, M. Riutort and L. Leria	02/06/2013
Castillejo de Yeltes, Castilla y León, Spain	40.75373N 6.35087W	-	M. Álvarez-Presas, M. Riutort and L. Leria	02/06/2013
Ciudad Rodrigo, Castilla y León, Spain	40.58051N 6.51324W	*Girardia* sp.	M. Álvarez-Presas, M. Riutort and L. Leria	02/06/2013
Torrejón El Rubio, Extremadura, Spain	39.80489N 6.02561W	-	M. Álvarez-Presas, M. Riutort and L. Leria	03/06/2013
Jaraicejo, Extremadura, Spain	39.66585N 5.94018W	-	M. Álvarez-Presas, M. Riutort and L. Leria	03/06/2013
Alburquerque, Extremadura, Spain	39.29749N 6.81842W	-	M. Álvarez-Presas, M. Riutort and L. Leria	04/06/2013
Portalegre, Portugal	39.34535N 7.36277W	*Polycelis* sp.	M. Álvarez-Presas, M. Riutort and L. Leria	04/06/2013
Matachel, Extremadura, Spain	38.45146N 5.96351W	-	M. Álvarez-Presas, M. Riutort and L. Leria	04/06/2013
Villanueva de Cordoba, Andalucía, Spain	38.37334N 4.81428W	-	M. Álvarez-Presas, M. Riutort and L. Leria	05/06/2013
Minas del Horcajo, Castilla-La Mancha, Spain	38.50645N 4.38462W	-	M. Álvarez-Presas, M. Riutort and L. Leria	05/06/2013
San Lorenzo de Calatrava, Castilla-La Mancha, Spain	38.45971N 3.92655W	-	M. Álvarez-Presas, M. Riutort and L. Leria	05/06/2013
Viso del Marqués, Castilla-La Mancha,	38.49410N 3.66753W	-	M. Álvarez-Presas, M. Riutort and L. Leria	05/06/2013

Spain				
Venta de Cárdenas, Castilla-La Mancha, Spain	38.41405N 3.49742W	-	M. Álvarez-Presas, M. Riutort and L. Leria	05/06/2013
Santa Elena, Andalucía, Spain	38.33549N 3.57341W	-	M. Álvarez-Presas, M. Riutort and L. Leria	05/06/2013
Úbeda, Andalucía, Spain	38.28896N 2.96383W	-	M. Álvarez-Presas, M. Riutort and L. Leria	06/06/2013
Cañada Catena, Andalucía, Spain	38.2774N 2.77168W	-	M. Álvarez-Presas, M. Riutort and L. Leria	06/06/2013
Cortijos Nuevos, Andalucía, Spain	38.28166N 2.7144W	-	M. Álvarez-Presas, M. Riutort and L. Leria	06/06/2013
Villahermosa, Castilla-La Mancha, Spain	38.86528N 2.79891W	-	M. Álvarez-Presas, M. Riutort and L. Leria	06/06/2013
Villahermosa, Castilla-La Mancha, Spain	38.87284N 2.78709W	-	M. Álvarez-Presas, M. Riutort and L. Leria	06/06/2013
Villalba de la Sierra, Catilla-La Mancha, Spain	40.27903N 2.08724W	-	M. Álvarez-Presas, M. Riutort and L. Leria	06/06/2013
L'Hospitalet-près-l'Andorre, France	42.61153N 1.81723E	*Polycelis* sp.	M.Álvarez, E.Solà and L.Leria	23/03/2013
Sinsat, France	42.79985N 1.65712E	*Polycelis* sp.	M.Álvarez, E.Solà and L.Leria	23/03/2013
Saint Pierre de Riviere, France	42.96046N 1.55859E	-	M.Álvarez, E.Solà and L.Leria	23/03/2013
Albarede, France	43.57076N 2.13479E	-	M.Álvarez, E.Solà and L.Leria	23/03/2013
Pont de Larn, France	43.51047N 2.39298E	*Girardia* sp. and *Polycelis* sp.	M.Álvarez, E.Solà and L.Leria	24/03/2013
Sauvaterre, França	43.47053N 2.54420E	-	M.Álvarez, E.Solà and L.Leria	24/03/2013
Le Martinet, France	43.48178N 2.78140E	-	M.Álvarez, E.Solà and L.Leria	24/03/2013
Seyes, France	43.50112N 1.28023E	*Schmidtea polycroa* and *Polycelis* sp.	M.Álvarez, E.Solà and L.Leria	24/03/2013
Laudine, France	43.41962N 0.81228E	-	M.Álvarez, E.Solà and L.Leria	24/03/2013
Valcabrère, France	43.03400N 0.58016E	*Polycelis* sp. and *Dugesia gonocephala*	M.Álvarez, E.Solà and L.Leria	25/03/2013

Bagen, France	43.03380N 0.68477E	*Polycelis* sp.	M.Álvarez, E.Solà and L.Leria	25/03/2013
Óleac-Dessus, France	43.16842N 0.21561E	-	M.Álvarez, E.Solà and L.Leria	25/03/2013
Montgaillard, France	43.12933N 0.11732E	*Polycelis* sp.	M.Álvarez, E.Solà and L.Leria	25/03/2013
Escoubès-Pouts, France	43.10553N 0.03144E	*Polycelis* sp.	M.Álvarez, E.Solà and L.Leria	25/03/2013
Saint Pée sur Nivelle, France	43.34235N 1.52650W	*Polycelis* sp.	M.Álvarez and L.Leria	26/03/2013
Louhossoa, France	43.31059N 1.35241W	*Polycelis* sp.	M.Álvarez and L.Leria	26/03/2013
Villanueva de Arce, Navarra, Spain	42.96038N 1.36363W	-	M.Álvarez and L.Leria	26/03/2013
Jaca, Aragón, Spain	42.57285N 0.63487W	-	M.Álvarez and L.Leria	27/03/2013
Yebra de Basa, Aragón, Spain	42.48803N 0.28330W	-	M.Álvarez and L.Leria	27/03/2013
Fiscal, Aragón, Spain	42.49902N 0.28330W	*Dugesia liguriensis*	M.Álvarez and L.Leria	27/03/2013
Belsué, Aragón, Spain	42.33543N 0.37475W	-	M.Álvarez and L.Leria	27/03/2013
Olaeta, Basque Country, Spain	43.05441N 2.62788W	*Polycelis* sp.	M. Vila-Farré, JM. Martín-Durán and E. Solà	01/05/2011
Olaeta, Basque Country, Spain	43.04775N 2.61727W	*Polycelis* sp.	M. Vila-Farré, JM. Martín-Durán and E. Solà	01/05/2011
Angostina, Basque Country, Spain	42.62727N 2.46480W	*Polycelis* sp.	M. Vila-Farré, JM. Martín-Durán and E. Solà	01/05/2011
P.N. Sierra y Cañones de Guara, Aragón, Spain	42.25191N 0.33355W	*Polycelis* sp.	M. Vila-Farré, JM. Martín-Durán and E. Solà	02/05/2011
Ugaldetxo, Basque Country, Spain	43.29697N 1.87330W	*Polycelis* sp. and *Girardia* sp.	M. Vila-Farré, JM. Martín-Durán and E. Solà	29/04/2011
San Julián de Banzo, Aragón, Spain	42.22983N 0.34491W	*Phagocata* sp.	M. Vila-Farré, JM. Martín-Durán and E. Solà	02/05/2011
La Puebla de Arganzón, Aragón, Spain	42.76758N 2.8330W	*Polycelis* sp., *Girardia* sp. and *Schmidtea polychroa*	M. Vila-Farré, JM. Martín-Durán and E. Solà	01/05/2011
Villanueva de Gállego, Aragón, Spain	41.76125N 0.83141W	*Schmidtea polychroa*	M. Vila-Farré, JM. Martín-Durán and E. Solà	27/04/2011
Barranco de San Julián de Banzo, Aragón, Spain	42.22816N 0.34773W	*Polycelis* sp.	M. Vila-Farré, JM. Martín-Durán and E. Solà	02/05/2011

Oñat, Basque Country, Spain	43.04433N 2.42688W	*Polycelis* sp.	M. Vila-Farré, JM. Martín-Durán and E. Solà	01/05/2011
Cheleiros, Portugal	38.88841N 9.32990W	*Girardia dorotocephala*	A.Valls, E.Solà, M.Riutort and L.Leria	05/11/2013
Torres Vedras, Portugal	39.09081N 9.24217W	-	A.Valls, E.Solà, M.Riutort and L.Leria	05/11/2013
Grandola, Portugal	38.10682N 8.61901W	-	A.Valls, E.Solà, M.Riutort and L.Leria	05/11/2013
Queimado, Portugal	37.50666N 8.54701W	-	A.Valls, E.Solà, M.Riutort and L.Leria	06/11/2013
Odeceixe, Portugal	37.43488N 8.76883W	-	A.Valls, E.Solà, M.Riutort and L.Leria	06/11/2013
Aljezur, Portugal	37.32390N 8.76164W	*Dugesia sicula* and *Rhyncodemus* sp.	A.Valls, E.Solà, M.Riutort and L.Leria	06/11/2013
Moinho da Rocha, Portugal	37.23558N 8.59159W	*Dugesia sicula*	A.Valls, E.Solà, M.Riutort and L.Leria	06/11/2013
Zebro de Baixo, Portugal	37.32412N 8.19678W	*Dugesia sicula*	A.Valls, E.Solà, M.Riutort and L.Leria	06/11/2013
Gomes Aires, Portugal	37.51213N 8.18328W	-	A.Valls, E.Solà, M.Riutort and L.Leria	07/11/2013
Pradas de Les, Languedoc-Roussillon, France	43.68411N 3.86050E	*Dugesia sicula, Girardia* sp. and *Dendrocoelum* sp.	M. Vila-Farré, E. Solà and L. Leria	28/09/2011
Pradas de Les, Languedoc-Roussillon, France	43.71811N 3.84261E	*Dugesia sicula*	M. Vila-Farré, E. Solà and L. Leria	28/09/2011
Pradas de Les, Languedoc-Roussillon, France	43.71595N 3.84925E	*Girardia sp.*	M. Vila-Farré, E. Solà and L. Leria	28/09/2011
Gange, Languedoc-Roussillon, France	43.93711N 3.69448E	*Dugesia sicula*	M. Vila-Farré, E. Solà and L. Leria	28/09/2011
Gornièrs, Languedoc-Roussillon, France	43.87216N 3.60485E	*Dugesia sicula*	M. Vila-Farré, E. Solà and L. Leria	28/09/2011
Sant Maurise de Navàcelas, Languedoc-Roussillon, France	43.84966N 3.53920E	-	M. Vila-Farré, E. Solà and L. Leria	28/09/2011
Lodève, Languedoc-Roussillon, France	43.72950N 3.32136E	*Dugesia sicula, Girardia* sp., *Dendrocoelum* sp. and *Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	29/09/2011

Lunaç, Languedoc-Roussillon, France	43.70821N 3.19550E	*Dugesia sicula* and *Girardia* sp.	M. Vila-Farré, E. Solà and L. Leria	29/09/2011
Avèna, Languedoc-Roussillon, France	43.75293N 3.10995E	-	M. Vila-Farré, E. Solà and L. Leria	29/09/2011
Sant Julian, Languedoc-Roussillon, France	43.57711N 2.88155E	-	M. Vila-Farré, E. Solà and L. Leria	29/09/2011
Le Pont de Reynès, Languedoc-Roussillon, France	42.49581N 2.71478E	*Dugesia sicula*	M. Vila-Farré, E. Solà and L. Leria	30/09/2011
Arenys d'Empordà, Girona, Spain	42.16310N 2.95543E	*Dugesia sicula*	M. Riutort	12/2011
Deià, Mallorca, Balearic Islands, Spain	39.74713N 2.64526E	*Dugesia sicula*	A. González	01/2012
Figueres, Girona, Spain	42.29735N 2.98716E	*Dugesia sicula*	M. Riutort	12/2011
Alòs de Balaguer, Lleida, Spain	41.91089N 0.96156E	*Dugesia sicula*	M. Vila-Farré	22/07/2011
Soller, Mallorca, Balearic Islands, Spain	39.75693N 2.71193E	*Dugesia sicula*	M. Vila-Farré	2011
Fuencaliente, Ciudad Real, Spain	38.37926N 4.3055W	*Dugesia sicula*	M. Àlvarez, M. Riutort and L. Leria	05/06/2013
Vallderoures, Aragón, Spain	40.85987N 0.15909E	*Dugesia sicula*	J.M. Blasi, A. Blasi and L. Leria	08/2013
Pena-Roja de Tastavins, Terol, Spain	40.75919N 0.02448E	*Dugesia sicula*	J.M. Blasi, A. Blasi and L. Leria	08/2013
Vilanova de Meià, Lleida, Catalonia, Spain	41.98892N 1.01342E	*Dugesia liguriensis*	M. Vila-Farré	20/05/2011
Foradada, Lleida, Catalonia, Spain	41.87762N 1.01160E	*Dugesia etrusca*	M. Vila-Farré	11/07/2011
Espadella, Lleida, Catalonia, Spain	41.90741N 0.94913E	*Dugesia etrusca*	M. Vila-Farré	22/07/2011
Les Avellanes, Llida, Catalonia, Spain	41.90035N 0.76522E	*Dugesia etrusca*	M.Vila-Farré and F. Monjo	30/07/2011
La Pobla de Segur, Lleida, Catalonia, Spain	42.24936N 0.96814E	*Dugesia etrusca*	F. Monjo	8/2011
Bagen, France	43.0338N 0.68477E	*Dugesia gonocephala*	M.Álvarez, E. Solà and L.Leria	25/03/2013
Escoubès-Pouts, France	43.1055N 0.03144E	*Dugesia gonocephala*	M.Álvarez, E. Solà and L.Leria	25/03/2013

Gondomar, Galicia, Spain	42.11217N 8.76297W	*Girardia sp.*	M. Vila-Farré, E. Solà and L. Leria	09/10/2011
Ampuero, Cantabria, Spain	43.34423N 3.41831W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	04/10/2011
Ruente, Cantabria, Spain	43.25760N 4.26681W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	05/10/2011
El Nueva, Asturias, Spain	43.26461N 5.67305W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	05/10/2011
Vegadeo, Asturias, Spain	43.48220N 7.04400W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	06/10/2011
A Igrexa, Galícia, Spain	43.62148N 7.59361W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	07/10/2011
Muras, Galícia, Spain	43.46646N 7.72588W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	07/10/2011
Vista Alegre, Galícia, Spain	43.44003N 8.01670W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	07/10/2011
Teixeiro, Galícia, Spain	43.13498N 8.01448W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	07/10/2011
Negreira, Galícia, Spain	42.92106N 8.68771W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	08/10/2011
Sinde, Galícia, Spain	42.74408N 8.59368W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	08/10/2011
Pontevea, Galícia, Spain	42.76476N 8.55533W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	08/10/2011
Cuntis, Galícia, Spain	42.61266N 8.56678W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	09/10/2011
Ponte Caldelas, Galícia, Spain	42.39098N 8.49178W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	09/10/2011
A Cañiza, Galícia, Spain	42.23621N 8.28835W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	09/10/2011
Trabadelo, Castilla y León, Spain	42.64721N 6.87556W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	10/10/2011
Agoncillo, La Rioja, Spain	42.44605N 2.31100W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	11/10/2011
Artabia, Navarra, Spain	42.74488N 2.08956W	*Polycelis* sp.	M. Vila-Farré, E. Solà and L. Leria	11/10/2011
Saja-Besaya, Cantabria, Spain	43.232492N 4.209677W	*Polycelis* sp.	E. Mateos	2012
Coyados de Asón, Cantabria, Spain	43.226485N 3.602011W	*Polycelis* sp.	E. Mateos	2012
Ballons des Vosges, France	47.70602N 6.62295E	*Polycelis* sp.	L. Leria	05/11/2012
Cirque de St Meme, France	45.40366N 5.88473E	*Polycelis* sp.	L. Leria	03/11/2012
Desfiladero de la Hermida, Cantabria,	43.26815N 4.63240E	*Polycelis* sp.	M. Álvarez, M. Riutort i L.Leria	02/06/2013

Spain				
Hortas, Portugal	39.32326N 7.40911W	*Polycelis* sp.	M. Álvarez-Presas, M. Riutort and L. Leria	04/06/2013
Cirque de St Meme, France	45.40366N 5.88473E	*Crenobia alpina*	L. Leria	03/11/2012

Supplementary data S7. Genes, number of individuals, alignment length and number of variable sites of the different molecular datasets used in this study.

Molecular discovery of candidate species †				
Dataset name	**Genes**	**Number of individuals**	**Alignment length (bp)**	**Variable sites ‡**
Cox1	Cox1	154	744	242
Cox1-Cloned	Cox1	49	649	216
Dunuc12	Dunuc12	80	996	191
Dunuc12-Cloned	Dunuc12	49	990	425
Molecular validation of candidate species				
Dataset name	**Genes**	**Number of individuals**	**Alignment length**	**Variable sites ‡**
All	Cox1	45	840	203
	Cox2	45	563	167
	Nad1	41	95	23
	tRNA-W	42	47	5
	28S	29	1502	26
	ITS	35	674	39
	Dunuc2	31	564	24
	Dunuc3	46	360	23
	Dunuc5	46	390	23
	Dunuc10	40	546	49
	Dunuc12	40	952	111
	Dunuc20	30	809	55
Total §	12	46	7342	748
Nuclear	Dunuc2	49	564	28
	Dunuc3	79	360	24
	Dunuc5	79	390	26
	Dunuc10	71	550	52
	Dunuc12	71	957	132
	Dunuc20	57	810	63
Total §	6	79	3631	325
Mitochondrial	Cox1	52	840	206
	Cox2	52	563	168
	Nad1	49	95	23
	tRNA-W	50	47	5
Total §	4	52	1545	402

† Outgroup sequences included

‡ Gaps and ambiguos sites not considered

§ The individuals that did not have sequence for some genes were considered as missing data

Supplementary data S8. Results of Xia's test for detecting saturation on the genes used in this study. The protein coding genes were analysed separately for the intronic regions and at the three coding positions of the exonic regions. Iss: index of substitution saturation; Iss.cSym: critical index of substitution saturation for a symmetrical tree topology; Iss.cAsym: critical index of substitution saturation for an asymmetrical tree topology; P: significance value in a two-tailed test. The tests were performed only on fully resolved sites, based on 32 OTUs and 60 replicates.

	Iss	Iss. cSym	P	Iss. cAsym	P
Dunuc2_Introns	0,0348	0,9555	0,0000	0,9951	0,0000
Dunuc2_Exons_1st	0,003	0,866	0,0000	0,694	0,0000
Dunuc2_Exons_2nd	-	-	-	-	-
Dunuc2_Exons_3rd	0,018	0,776	0,0000	0,535	0,0000
Dunuc3_Introns	0,0130	0,4667	0,0000	0,4781	0,0000
Dunuc3_Exons_1st	-	-	-	-	-
Dunuc3_Exons_2nd	0,0159	0,7140	0,0000	0,7707	0,0000
Dunuc3_Exons_3rd	0,0321	0,7180	0,0000	0,7845	0,0000
Dunuc5_Introns	0,0376	0,7553	0,0000	0,7295	0,0000
Dunuc5_Exons_1st	0,016	1,544	0,0000	1,853	0,0000
Dunuc5_Exons_2nd	-	-	-	-	-
Dunuc5_Exons_3rd	0,245	1,544	0,0277	1,853	0,0183
Dunuc10_Introns	0,0864	0,3520	0,0000	0,3751	0,0000
Dunuc10_Exons_1st	0,003	0,814	0,0000	0,603	0,0000
Dunuc10_Exons_2nd	-	-	-	-	-
Dunuc10_Exons_3rd	0,075	0,840	0,0000	0,648	0,0000
Dunuc12_Introns	0,022	0,689	0,0000	0,359	0,0000
Dunuc12_Exons_1st	0,002	1,189	0,0000	1,252	0,0000
Dunuc12_Exons_2nd	-	-	-	-	-
Dunuc12_Exons_3rd	0,075	1,266	0,0000	1,381	0,0000
Dunuc20_Introns	0,050	0,703	0,0000	0,378	0,0000
Dunuc20_Exons_1st	-	-	-	-	-
Dunuc20_Exons_2nd	-	-	-	-	-
Dunuc20_Exons_3rd	0,030	2,707	0,0000	3,803	0,0000
Cox1_1st	0,133	0,752	0,0000	0,493	0,0000
Cox1_2nd	0,120	0,756	0,0053	0,499	0,0377
Cox1_3rd	0,220	0,856	0,0000	0,678	0,0000
Cox2_1st	0,063	0,772	0,0000	0,527	0,0000
Cox2_2nd	0,126	0,769	0,0000	0,523	0,0000
Cox2_3rd	0,296	0,778	0,0000	0,539	0,0000
Nad1_1st	0,2489	0,6872	0,0274	1,4062	0,0001
Nad1_2nd	0,0230	0,6872	0,0000	1,4062	0,0000
Nad1_3rd	0,3480	0,6773	0,0020	1,3516	0,0000
tRNA-W	0,0588	0,9779	0,0000	1,1531	0,0000
28S	0,0543	0,7168	0,0000	0,4017	0,0000
ITS	0,096	0,707	0,0000	0,380	0,0000

Supplementary data S9. Individuals with the same haplotypes as the ones depicted in Figure 3.

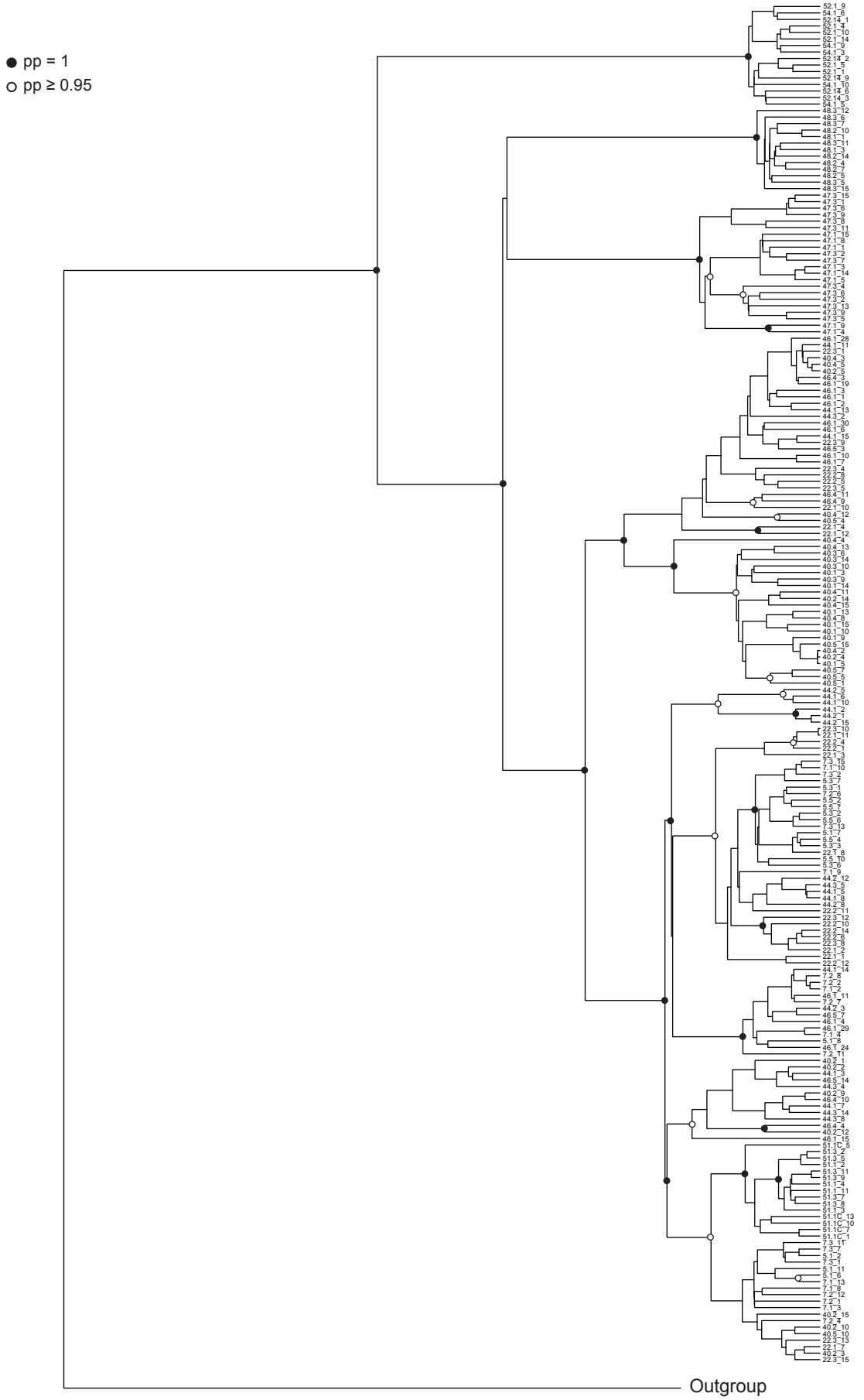
Cox1: a) 3.2, 2.2, 4.1, 5.1, 5.2, 5.5, 7.1, 8.1, 8.2, 9.2, 36.1; **b)** 52.1, 52.9, 52.11, 52.12, 52.13; **c)** 56.1; **d)** 23.1, 24.1, 24.2, 25.1, 26.1, 27.2, 28.1, 30.1, 31.1, 32.2, 40.1, 42.1; **e)** 30.2; **f)** 50.5; **g)** 22.2; **h)** 35.3; **i)** 41.2; 63.3; **k)** 54.4, 54.5, 54.6, 54.7; **m)** 51.2; **n)** 17.2, 19.1; **o)** 20.1; **p)** 17.3; **q)** 15.1; **r)** 14.2; **s)** 47.3, 47.7; **t)** 48.7; **u)** 52.4, 53.1, 54.1, 54.2.

Cox1-Cloned: a) 47.4; **b)** 48.2, 48.3; **c)** 52.14, 54.1; **d)** 40.3, 40.5; **e)** 22.2; **f)** 5.3, 5.5, 7.1; **g)** 51.1, 51.3; **h)** 22.1, 22.2, 22.3, 40.1, 40.2, 40.3, 40.5, 44.1, 46.1, 46.4; **i)** 46.1; **j)** 22.3; **k)** 44.2, 44.3; **l)** 44.3

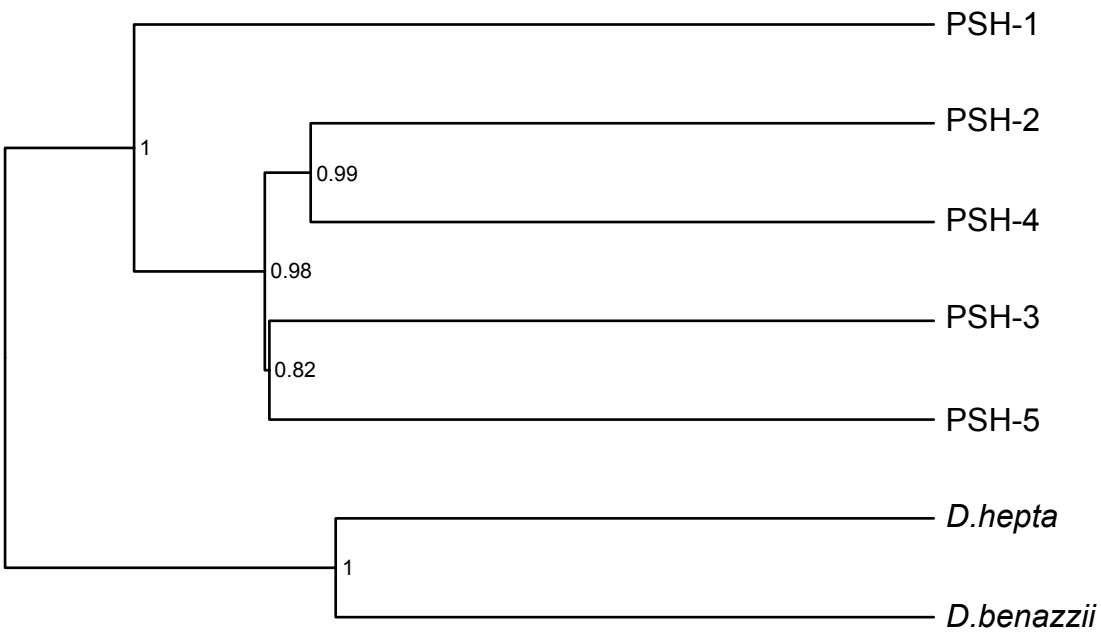
Dunuc12: a) 10.1; **b)** 17.1, 17.4; **c)** 41.1; **d)** 48.1.

Dunuc12-Cloned: a) 52.14, 54.1; **b)** 48.2, 48.3; **c)** 40.5; **d)** 44.2, 46.3, 46.4; **e)** 40.1; **f)** 40.3, 40.5; **g)** 44.3; **h)** 22.2; **i)** 5.1, 5.5, 7.1, 7.3; **j)** 22.2; **k)** 22.2, 22.3; **l)** 22.3; **m)** 45.1; **n)** 44.2, 44.3, 46.1, 46.3, 46.4; **o)** 51.2; **p)** 7.1, 7.2, 7.3

Supplementary data S10. Non-collapsed phylogenetic tree for the Dunuc12-Cloned dataset depicted in Figure 3.



Supplementary data S11. Species tree obtained with *BEAST for the multilocus dataset containing all loci (dataset 5). Values at nodes indicate the posterior probability of each node.



Supplementary data S12. Chromosome measures of the different PSHs analysed in the present study. Population codes are indicated in brackets. C: chromosome pairs, triplets or quartets ordered from longer to shorter relative lengths; CI: centromeric index; RL: relative length; T: type of chromosome depending on the centromeric position: m, metacentric; sm, submetacentric. Chromosome pairs, triplets or quartets containing aberrant chromosomes are highlighted in bold.

	PSH-2 (47)†			**PSH-3 (52)**			**PSH-4 (48)†**			**PSH-5 (1)**			**PSH-5 (4)**		
C	**CI**	**RL**	**T**	**CI**	**RL**	**T**	**CI**	**RL**	**T**	**CI**	**RL**	**T**	**CI**	**RL**	**T**
1	46.49 ± 1.22	19.04 ± 0.27	m	46.81 ± 0.65	18.69 ± 1.31	m	43.76 ± 2.07	19.37 ± 0.15	m	**47.25 ± 1.29**	**17.4 ± 2.02**	**m**	45.69 ± 1.43	18.30 ± 0.35	m
2	47.41 ± 1.05	15.94 ± 0.44	m	32.56 ± 2.98	16.04 ± 0.85	sm	45.44 ± 1.35	15.87 ± 0.45	m	**30.78 ± 10.56**	**14.4 ± 1.45§**	**sm**	**28.94 ± 4.51**	**14.56 ± 0.59**	**sm**
3	32.69 ± 3.5	13.6 ± 0.60	sm	37.63 ± 3.14	13.48 ± 0.44	sm	47.04 ± 1.09	13.04 ± 0.22	m	**46.9 ± 1.94**	**15.01 ± 1.10§**	**m**	**33.45 ± 5.18**	**13.61 ± 0.81**	**sm**
4	31.94 ± 2.78	12.78 ± 0.39	sm	41.25 ± 3.63	12.3 ± 0.60	m	43.43 ± 2.59	12.46 ± 0.27	m	42.19 ± 2.91	12.32 ± 0.27	m	**35.54 ± 6.76**	**12.75 ± 1.29**	**sm**
5	34.84 ± 0.69	10.84 ± 0.52	sm	41.95 ± 4.01	11.23 ± 0.60	m	42.76 ± 1.9	11.38 ± 0.34	m	44.77 ± 3.31	11.43 ± 0.38	m	37.17 ± 2.73	10.98 ± 0.01	sm
6	36.94 ± 2.06	9.87 ± 0.58	sm	45.63 ± 2.8	10.16 ± 0.44	m	48.06 ± 2.25	10.11 ± 0.23	m	45.71 ± 1.90	10.37 ± 0.06	m	40.93 ± 4.48	10.44 ± 0.07	m
7	40 ± 0.3	9.02 ± 0.30	m	46.08 ± 2.48	9.41 ± 0.58	m	47.22 ± 1.83	9.01 ± 0.15	m	**39.59 ± 1.28**	**10.23 ± 0.44**	**m**	32.69 ± 2.43	10.05 ± 0.16	sm
8	42.66 ± 2.6	8.85 ± 0.46	m	45.95 ± 2.38	8.7 ± 0.59	m	48.15 ± 2.38	8.64 ± 0.26	m	47.04 ± 1.44	8.85 ± 0.22	m	44.75 ± 1.60	9.32 ± 0.29	m
	PSH-5 (7)			**PSH-5 (17)**			**PSH-5 (22)**			**PSH-5 (32)**			**PSH-5 (46)†**		
C	**CI**	**RL**	**T**	**CI**	**RL**	**T**	**CI**	**RL**	**T**	**CI**	**RL**	**T**	**CI**	**RL**	**T**
1	39.96 ± 2.59	16.94 ± 0.60	m	**46.5 ± 1.06**	**16.79 ± 2.08**	**m**	**43.85 ± 1.39**	**18.11 ± 0.46**	**m**	45.99 ± 1.59	17.82 ± 0.27	m	44.56 ± 1.6	18.59 ± 0.4	m
2	**40.72 ± 5.24**	**15.28 ± 0.35**	**m**	**33.72 ± 5.45**	**16.28 ± 1.5**	**sm**	**40.47 ± 2.94**	**14.48 ± 0.55**	**m**	**39.66 ± 3.57**	**14.96 ± 0.47**	**m**	45.03 ± 2.45	15.54 ± 0.3	m
3	43.28 ± 3.01	13.99 ± 0.29	m	47.66 ± 0.37	13.52 ± 1	m	44.11 ± 2.02	13.50 ± 0.40	m	46.27 ± 4.04	13.79 ± 0.17	m	44.94 ± 2.59	13.34 ± 0.31	m
4	41.25 ± 4.04	12.48 ± 0.16	m	38.33 ± 4.26	11.75 ± 0.07	m	**40.70 ± 5.05**	**12.55 ± 0.56**	**m**	41.41 ± 5.43	12.5 ± 0.40	m	**35.23 ± 1.95**	**12.78 ± 2.1**	**sm**
5	41.21 ± 4.14	11.57 ± 0.15	m	37.03 ± 3.84	11.07 ± 0.6	sm	42.24 ± 3.31	11.66 ± 0.08	m	42.57 ± 2.33	11.57 ± 0.14	m	34.26 ± 2.18	11.02 ± 0.35	sm
6	41.24 ± 6.33	10.77 ± 0.11	m	48.7 ± 1.22	10.16 ± 0.45	m	41.61 ± 3.39	10.84 ± 0.17	m	42.19 ± 4.40	10.57 ± 0.06	m	41.3 ± 3.54	10.21 ± 0.35	m
7	42.98 ± 4.50	10.14 ± 0.13	m	**36.68 ± 4.46**	**11.15 ± 1.29**	**sm**	**42.70 ± 2.77**	**10.19 ± 0.41**	**m**	44.40 ± 2.48	9.96 ± 0.17	m	39.34 ± 1.92	9.85 ± 0.39	m
8	**42.53 ± 2.78**	**8.82 ± 0.43**	**m**	45.84 ± 0.68	9.28 ± 0.48	m	**43.67 ± 3.63**	**8.67 ± 0.38**	**m**	45.52 ± 2.74	8.84 ± 0.17	m	**43.42 ± 2.86**	**8.85 ± 2.02**	**m**

† Values extracted from Ribas 1990.

§ Triplets ordered only considering the relative length of the chromosomes that do not show the translocation (Triplet 2: 15.54 and 14.89; Triplet 3: 14.64 and 14.12). For the rest of the pairs, triplets or quartets showing aberrant chromosomes, the length of the aberrant chromosomes did not affect the order.

Annex: Reproductive strategies and ploidy level in *Dugesia subtentaculata*

The reproductive strategy of all the *D. subtentaculata* localities sampled in the present thesis was recorded. An average of 15 individuals per population were collected and observed under the stereomicroscope under field conditions or shortly after. Sexual individuals were identified by the presence of a gonopore (external aperture of the copulatory apparatus), while fissiparous individuals were identified by the occurrence of a blastema (regenerating part of tissue after a process of fission). The sampled populations were classified as sexual if most individuals presented a gonopore and none a blastema, facultative when both individuals with a gonopore and individuals with a blastema were detected, and fissiparous when individuals with a blastema were detected and none had a gonopore. The reproductive strategy of seven populations of this species was already detailed in the publication of Chapter 1 (see Fig. 2 in Chapter 1). The reproductive strategy of the rest of populations is detailed in Fig. 1.

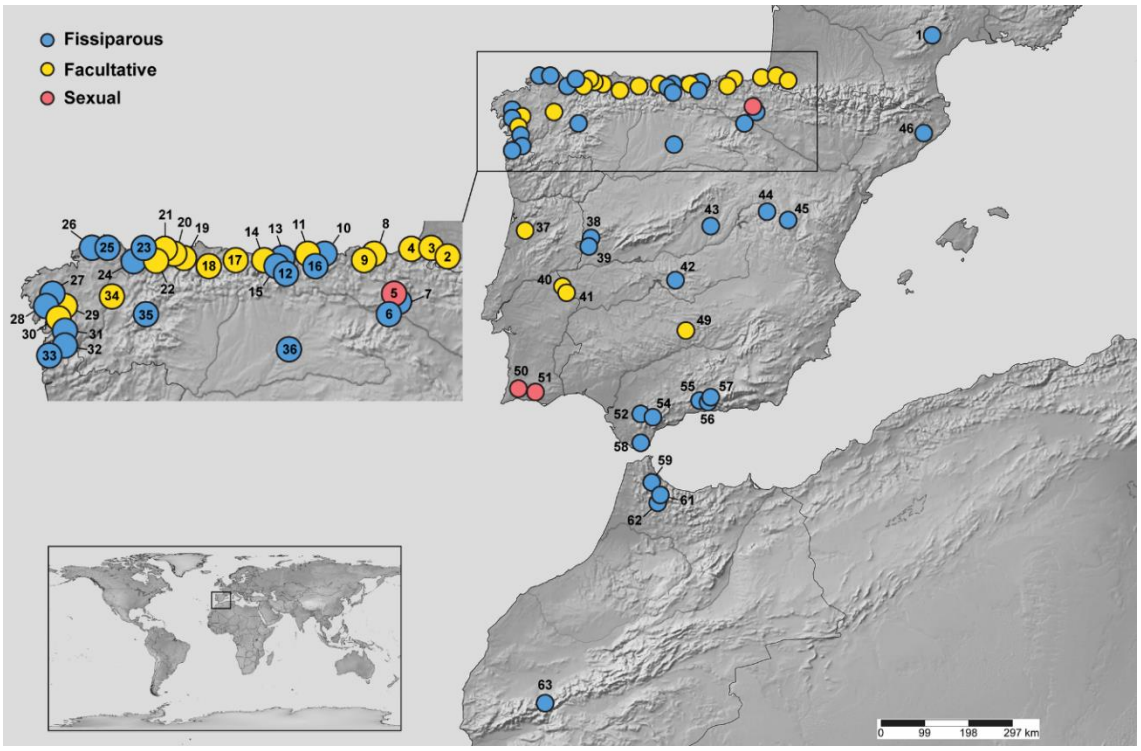


Fig. 1. Reproductive strategy of all *D. subtentaculata* populations included in the present study. The colour of the circles indicates the reproductive strategy (see legend). The numbers correspond to the code of each locality in Chapter 2 (see Supplementary data S1 for detailed information on the localities).

From the 59 sampled populations corresponding to *D. subtentaculata*, only 3 were found exclusively bearing sexual individuals, while the rest corresponded to either exclusively fissiparous populations (36 localities) or facultative populations (20 localities).

The ploidy level of eight additional populations of *D. subtentaculata* besides the ones that had already been analysed in the publications of Chapter 1 and Chapter 2 was inferred using flow cytometry (populations 3, 19, 20, 30, 34, 39, 45 and 50). Moreover, two of the populations karyologically analyzed in Chapter 2 (populations 17 and 32) were also included in this analysis. The flow cytometry protocol that we used was the same as the one detailed in Chapter 1.

The two populations that had already been karyologically analyzed in Chapter 2 (populations 17 and 32) were found to be triploid, coinciding with the results of the karyological analysis. From the eight populations that had not previously been analyzed by karyotyping, all analyzed individuals from five of them (populations 3, 19, 20, 39 and 45) were also found to be triploid in the cytometric analysis (Table 1). On the contrary, the populations 30 and 34 bared individuals with different ploidies. In both facultative populations, the analyzed individuals showing copulatory apparatus were triploid, while fissiparous individuals were either triploid, mixoploid (3n-4n), or tetraploid. Finally, all analyzed individuals of the population 50 (exclusively sexual) were diploid.

Table 1. Results of the ploidy level inferred by flow cytometry for different populations of *D. subtentaculata*. See Figure S1 in the supplementary data of Chapter 1 for detailed explanation on the inference protocol

Population	Samples	Number of peaks	Number of cells	Fluorescense	Proportion	Ploidy
3	Control 1	1	8405	705		
	18_1	1	7030	611		
	Control 1 + 18_1	1	4523	672	3	**3n**
	18_3DC	1	9511	589		
	Control 1 + 18_3DC	1	13777	625	3	**3n**
17	Control 1	1	1639	449		
	C06_1 Cop	1	5436	504		
	Control 1 + C06_1 Cop	1	1257	500	3	**3n**
	Control 2	1	2181	489		
	C06_2	1	2677	554		
	Control 2 + C06_2	1	6381	559	3	**3n**
	Control 3	1	3259	498		

	C06_3	1	6140	532		
	Control 3 + C06_3	1	5263	516	3	**3n**
19	Control 1	1	5286	579		
	C09_1 Cop	1	15325	627		
	Control 1 + C09_1 Cop	1	18465	625	3	**3n**
20	Control 1	1	19754	615		
	C11_1	1	16646	579		
	Control 1 + C11_1	1	24276	562	3	**3n**
	C11_2	1	20566	590		
	Control 1 + C11_2	1	21245	581	3	**3n**
	Control 2	1	20845	540		
	C11_3	1	13605	613		
	Control 2 + C11_3	1	21008	587	3	**3n**
	C11_4	1	10279	572		
	Control 2 + C11_4	1	20260	600	3	**3n**
30	Control 1	1	11671	590		
	C25_1 Cop*	1	9926	578		
	Control 1 + C25_1 Cop	1	31490	612	3	**3n**
	C25_2 Cop*	1	13061	600		
	Control 1 + C25_2 Cop	1	21135	582	3	**3n**
	Control 2	1	8711	544		
	C25_3	1	3018	673		
	Control 2 + C25_3	2	11474 and 3771	574 and 787	4'1	**4n**
	C25_4	2	4616 and 3769	514 and 668		
	Control 2 + C25_4	2	10671 and 2106	559 and 676	3 i 3'6	**3n and 4n**
32	Control 1	1	25199	592		
	C29_1 Cop	1	24819	667		
	Control 1 + C29_1 Cop	1	32351	612	3	**3n**
	C29_2 Cop	1	27260	705		
	Control 1 + C29_2 Cop	1	23530	671	3	**3n**
	Control 2	1	6477	523		
	C29_3	1	5422	519		
	Control 2 + C29_3	1	8189	500	3	**3n**
	C29_4	1	5223	499		
	Control 2 + C29_4	1	7926	502	3	**3n**
	Control 3	1	5212	506		
	C29_5	1	5157	520		
	Control 3 + C29_5	1	9084	546	3	**3n**
34	Control 1	1	14165	303		
	C32_1 Cop*	1	10520	325		
	Control 1+ C32_1 Cop	1	5804	267	3	**3n**
	C32_2	1	5530	330		
	Control 1 + C32_2	1	5882	283	3	**3n**
	C32_3	2	5888 and 4802	321 and 417		
	Control 1 + C32_3	2	6986 and 2058	305 and 402	3 i 3'97	**3n and 4n**
	C32_4	2	2810 and	313 and 421		

	Control 1 + C32_4	2	2711 7432 and 2294	280 and 385	3 i 4'1	**3n and 4n**
39	Control 1	1	10889	603		
	E18_5	1	10436	560		
	Control 1 + E18_5	1	10561	625	3	**3n**
	E18_6	1	3423	631		
	Control 1 + E18_6	1	12548	563	3	**3n**
	Control 2	1	5299	570		
	E18_7	1	2150	578		
	Control 2 + E18_7	1	6886	575	3	**3n**
	E18_8	1	2169	574		
	Control 2 + E18_8	1	4613	627	3	**3n**
	Control 1	1	10124	556		
	E18_1	1	10369	606		
	Control 1 + E18_1	1	13177	696	3	**3n**
45	Control 1	1	6796	440		
	V03_1	1	14390	449		
	Control 1 + V03_1	1	19205	461	3	**3n**
	V03_2	1	6713	461		
	Control 1 + V03_2	1	14528	465	3	**3n**
	Control 2	1	8195	442		
	V03_3	1	10698	471		
	Control 2 + V03_3	1	13575	476	3	**3n**
	V03_4	1	14056	435		
	Control 2 + V03_4	1	16347	485	3	**3n**
50	Control 1	1	10702	785		
	POR9_1DC	1	12629	467		
	Control 1 + POR9_1DC	2	11566 and 12634	562 and 792	2'18	**2n**
	POR9_2DC	1	6195	484		
	Control 1 + POR9_2DC	2	3923 and 3464	559 and 813	2'06	**2n**
	POR9_3DC	1	23244	432		
	Control 1 + POR9_3DC	2	2439 and 3381	531 and 813	1'95	**2n**
	Control 1	1	20477	671		
	POR9_3	1	27171	382		
	Control 1 + POR9_3	2	12544 and 4520	449 and 659	2'04	**2n**

Chapter 3

New insights into the phylogeographic history of *Dugesia* (Platyhelminthes, Tricladida) freshwater planarians from the Western Mediterranean, with a special focus on *Dugesia subtentaculata*

Reference

Leria, L., Vila-Farré, M., Solà, E., & Riutort, M. (2019). **New insights into the phylogeographic history of *Dugesia* (Platyhelminthes, Tricladida) freshwater planarians from the Western Mediterranean, with a special focus on *Dugesia subtentaculata*.** In preparation.

New insights into the phylogeographic history of *Dugesia* (Platyhelminthes, Tricladida) freshwater planarians from the Western Mediterranean, with a special focus on *Dugesia subtentaculata*

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ABSTRACT

The Mediterranean basin is a hotspot of Biodiversity, which has been principally associated to its complex paleogeographic and paleoclimatic history. For this reason, this region is considered as a perfect scenario to study how historical processes can shape the diversity of organisms. The genus of freshwater planarians *Dugesia* (Platyhelminthes, Tricladida) is one of the richest genera of freshwater planarians in terms of species number, with more than 30 described species occurring only in the Mediterranean region. However, although the processes that have driven the diversification of the species inhabiting the Eastern Mediterranean basin have been studied, the evolutionary history of the species occurring in the Western Mediterranean region remains largely unknown. In the present work, we have performed a phylogeographic study using the information of six molecular markers from most of the known *Dugesia* species of the Western Mediterranean region. Moreover, we have conducted a distribution modelling analysis for the species *Dugesia subtentaculata*, one of the species from this region showing a broader distribution. Importantly, a recent study pointed out that individuals of this species show high levels of intraindividual genetic diversity due to fissiparous reproduction, which we have taken into account to perform the phylogeographic analysis. Our results indicate that the paleogeographic history of the Western Mediterranean region during the last 30 million years has played a crucial role driving the diversification of the different *Dugesia* species from this region. Moreover, we found that the geographic expansion of *D. subtentaculata* across the Iberian Peninsula, Southern France and Northern Africa probably did not occur until the beginning of the Pleistocene (around 2.5 million years ago), when the climate shifted from tropical to more temperate conditions, resulting especially suitable for its expansion during the Last Glacial Maximum (around 22.000 years ago).

1. INTRODUCTION

The Mediterranean Basin is one of the biologically richest regions on Earth (Mittermeier, Turner, Larsen, Brooks, & Gascon, 2011), principally due to its complex geological and climatic history. The formation of the Mediterranean Basin dates back to 45 million years ago (Ma), when the African and the European tectonic plates collided (Mather, 2009). This tectonic process promoted the destruction of the Tethys Ocean via subduction and the formation of several mountain belts, such as the Alps-Betics in the west and the Dinarides, Hellenides, and Taurides belts in the east. Approximately 25 Ma, in the Western Mediterranean region, the landmass of North-Eastern Iberia and Southern France detached from the continent and started to migrate southwards (Rosenbaum, Lister, & Duboz, 2002). This landmass subsequently broke into several smaller fragments (called microplates), presently corresponding to the Kabylies, the Betic-Rif, Calabria, Corsica, Sardinia, and the Balearic Islands, which started to undergo independent migrations across the Mediterranean Sea until they reached its present position after several millions of years.

Approximately 12 Ma, while the previously mentioned microplates were still migrating across the western region of the Mediterranean Sea, the Aegean region also started to break into a western and an eastern region, a paleogeological phenomenon known as the Mid-Aegean Trench (Dermitzakis & Papanikolaou, 1981). Furthermore, the closure of the Gibraltar Arc around 5.9 Ma, promoted an almost complete desiccation of the Mediterranean (i.e., the Messinian Salinity Crisis) (Hsü, Ryan, & Cita, 1973; Krijgsman, Hilgen, Raffi, Sierro, & Wilson, 1999). During this period, non-marine sediments were extensively deposited in the sea-bed, creating land connections between areas previously separated by sea. Finally, a rapid reflooding of the Mediterranean occurred with the formation of the Strait of Gibraltar (around 5.5 Ma) and the land-connected areas became isolated again (Warny, Bart, & Suc, 2003).

The climatic history of the Mediterranean region is also rather complex. In the Early Eocene (around 50 Ma) the areas that today constitute the Mediterranean region showed a tropical climate (Wolfe, 1985). These tropical conditions extended during several Ma, but from the Late Eocene (38 Ma to 34 Ma), the temperatures started to cool down and the tropical climate in the Mediterranean region was gradually shifted by a temperate climate. However, the Mediterranean climatic conditions that we know today, characterized by dry summers and low temperatures during winter, were not established until approximately 3-2 Ma (Suc, 1984). Posteriorly, during the Pleistocene (2.58-0.12 Ma), a series of periodical glacial-interglacial cycles occurred, which strongly affected the evolutionary history of many European species

(Hewitt, 2000). One of the most studied effects of the glacial periods is the role that different Mediterranean regions (principally the Southern European Peninsulas and Northern Africa) played as refugia of the European biota, with the consequent post-glacial expansion processes (Hewitt, 2000; Husemann, Schmitt, Zachos, Ulrich, & Habel, 2014).

Freshwater planarians (Platyhelminthes, Tricladida) are free-living organisms that depend on the continuity of freshwater bodies to survive and disperse, since they cannot tolerate neither conditions of desiccation nor of high salinity (Vila-Farré & Rink, 2018). Moreover, dispersion of these animals occurs due to their active gliding movement along the substrate by the action of the cilia, rather than by swimming or by passive dispersion through the water current (Ball & Reynoldson, 1981). For these reasons, freshwater planarians (and planarians in general), are considered ideal model organisms to carry out phylogeographic analyses, as it has been already exemplified in several studies carried out on different planarian species from the Mediterranean region (e.g., Lázaro et al., 2011; Solà, Sluys, Gritzalis, & Riutort, 2013).

The genus *Dugesia* is represented by approximately 80 described species, inhabiting the Palearctic, Afrotropical, Indomalayan, and Australasian biogeographic regions (Ball, 1974; Solà, 2014). From these approximately 80 species, 15 occur in the Western Mediterranean region, *viz*. *Dugesia sicula*, *D. hepta*, *D. benazzii*, *D. liguriensis*, *D. etrusca*, *D. ilvana*, *D. gonocephala*, *D. tubqalis*, *D. magrebiana*, *D. brigantii*, *D. leporii*, *D. subtentaculata*, *D. aurea*, *D. corbata*, *D. vilafarrei* and *Dugesia* sp. from Morocco (Fig. 1; Table 1). The phylogenetic relationships between most of these species have been analyzed at the molecular level in two different studies (Lázaro et al., 2009; Solà, 2014). These studies showed that the species from this region constitute the sister group of all the *Dugesia* species from the Eastern Mediterranean region, excepting for the species *D. sicula,* which clusters with different species from Africa. These results pointed out that the former species are autochthonous from the Western Mediterranean region, while the latter is an African species that has recently colonized the Mediterranean area (Lázaro & Riutort, 2013). Unfortunately, the phylogenetic relationships between most of the Western Mediterranean species remain elusive, putatively due to the lack of information in the few molecular markers used and to incomplete taxon sampling, precluding the envision of the processes that may have shaped its present distribution.

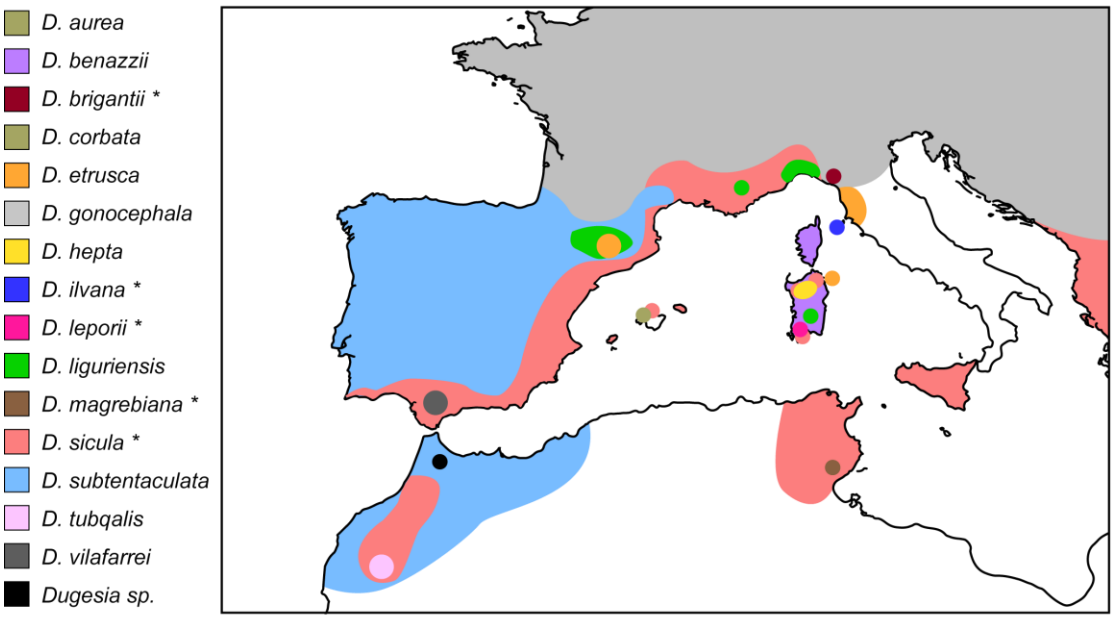

Fig. 1. Distribution map of all known *Dugesia* species from the Western Mediterranean region. Species marked with an asterisk have not been included in the present study. References for the distribution of each species are detailed in Table 1.

Table 1. References of the compiled distribution data of *Dugesia* from the Western Mediterranean region as depicted in Figure 1

Species	References
D. aurea	Leria, Vila-Farré, Álvarez-Presas, et al., 2019
D. benazzii	Benazzi-Lentati, Deri, & Puccinelli, 1987; Lázaro et al., 2009; Maria Pala, Casu, & Stocchino, 1999
D. brigantii	Vries & Benazzi, 1983
D. corbata	Leria, Vila-Farré, Álvarez-Presas, et al., 2019
D. etrusca	Lepori, 1947; Leria, Vila-Farré, Álvarez-Presas, et al., 2019; M Pala, Casu, & Vacca, 1980
D. gonocephala	De Vries, 1986; Lázaro et al., 2009
D. hepta	Pala, Casu, & Vacca, 1981; Stocchino, Corso, Manconi, Casu, & Pala, 2005
D. ilvana	Lázaro et al., 2009
D. leporii	Pala, Stocchino, Corso, & Casu, 2000
D. liguriensis	De Vries, 1988; Lázaro et al., 2009
D. magrebiana	Stocchino et al., 2009
D. sicula	Lázaro & Riutort, 2013
D. subtentaculata	Leria, Vila-Farré, Álvarez-Presas, et al., 2019
D. tubqalis	Harrath et al., 2012
D. vilafarrei	Leria, Vila-Farré, Álvarez-Presas, et al., 2019
Dugesia sp. 1 (Morocco)	Leria, Vila-Farré, Álvarez-Presas, et al., 2019

Any phylogeographic analysis relies on the establishment of the phylogenetic relationships of the studied group under a temporal framework. Phylogenetic inferences based on molecular data can be time-calibrated by three different approaches: (a) using the fossil record, (b) using paleobiogeographic events, and (c) using a standardized rate of molecular evolution (Forest, 2009). In the case of freshwater planarians (and of planarians in general) the virtual lack of a reliable fossil record (Vila-Farré & Rink, 2018), precludes to perform the phylogenetic time-calibration basing on this information. The use of standardized molecular clock estimates has been widely used to analyse the evolutionary time-scales of many different groups, for example the "standard" arthropod mitochondrial substitution rate of 1.15% substitutions per million years proposed by Brower in 1994 (e.g., Dömel, Melzer, Harder, Mahon, & Leese, 2017; Hayward & Stone, 2006; Hu, Hua, Hebert, & Hua, 2019). However, even closely related groups can show highly different rates of molecular evolution for the same genes, or different genes of the same genomic region (for example different mitochondrial genes) can also show significantly different evolutionary rates (Hebert, Remigio, Colbourne, Taylor, & Wilson, 2002; Pons, Ribera, Bertranpetit, & Balke, 2010). Therefore, the use of well-stablished paleobiogeographic events remains as one of the most suitable methodologies to time-calibrate molecular phylogenies for groups with unavailable fossil record (Landis, 2017).

Interestingly, recent extensive samplings carried out in the Iberian Peninsula and Southern France, revealed that the species *D. subtentaculata* shows a much wider distribution range than any of its sister species, including some populations in Morocco (Leria, Vila-Farré, Álvarez-Presas, et al., 2019). Concomitantly, it was seen that most populations of this species were formed by asexual fissiparous individuals. Surprisingly, the intraindividual genetic analysis of some populations of *D. subtentaculata* from distinct geographic locations revealed that the genetic diversity within this species can be found at the intraindividual level (Leria, Vila-Farré, Solà, & Riutort, 2019). To explain this observation the authors proposed that during periods of fissiparous reproduction, *Dugesia* individuals can accumulate new genetic variants (generated by somatic mutations) without losing their original alleles at the short term, ending up with a mix of ancestral and derived genetic diversity at the intraindividual level. In that work, the lack of genetic structure between the different populations of *D. subtentaculata* (putatively due to the effect of fissiparous reproduction) precluded the reconstruction of the phylogeographic history of this species. Nevertheless, the use of other methodologies that do not need genetic data, such as methods of species distribution modelling, could be of great value to shed some light on how the wide distribution of *D. subtentaculata* has been shaped over time.

In view of this information, the aim of the present study is to disentangle the phylogeographic history of the *Dugesia* species from the Western Mediterranean region, focusing on *D. subtentaculata*. To do so, we used the information of 6 molecular markers to infer a time calibrated phylogeny including most of the known *Dugesia* species from the Western Mediterranean region. Moreover, we also conducted an analysis of species distribution modelling for *D. subtentaculata*, to infer how the past environmental conditions of the Western Mediterranean region may have influenced its present distribution.

2. MATERIAL AND METHODS

2.1. Taxon sampling

We included in the present study most of the *Dugesia* species from the Western Mediterranean region that counted with available molecular data obtained in previous studies. These species included: *D. gonocephala*, *D. hepta*, *D. benazzii*, *D. liguriensis*, *D. etrusca*, *D. tubqalis*, *D. subtentaculata*, *D. aurea*, *D. corbata*, *D. vilafarrei* and *Dugesia* sp. from Morocco. Moreover, we also included molecular data from five *Dugesia* species from the Eastern Mediterranean region, *viz*. *D. improvisa, D. cretica, D. damoae, D. aenigma* and *Dugesia* sp. from Greece. We used molecular data from one individual per each species, excepting for the species *D. subtentaculata*, *D. aurea*, *D. corbata* and *D. vilafarrei*, in which two individuals per species were used. In the case of *D. subtentaculata*, one of the two individuals was from the Iberian Peninsula (V05.1D), while the other individual was from Morocco (E6.2).

2.2. DNA sequences, alignments, and datasets

DNA sequences of six different molecular markers were used per each species (Table 2). The six molecular markers used included fragments of the following genes: (a) *28S ribosomal RNA gene* (28S), (b) *Internal Transcribed Spacer 1* (ITS), (c) *18S ribosomal RNA gene* (18S), (d) *Cytochrome c oxidase I* (Cox1), (e) an anonymous marker obtained in a previous study (named as Dunuc3 in Leria, Vila-Farré, Álvarez-Presas, et al., 2019), and (f) a disulphide isomerase (named as Dunuc5 in Leria, Vila-Farré, Álvarez-Presas, et al., 2019). Importantly, for one of the individuals of *D. subtentaculata* (individual V05.1D) we downloaded its most divergent Cox1 intraindividual sequences: GenBank accession number MK385877 (ancestral sequence) and GenBank accession number MK385895 (derived Sequence).

Table 2. Information on the code, locality, and GenBank accession numbers for all DNA sequences included in the present study

Species	Code	Locality	Cox1	18S	28S	ITS	Dunuc3	Dunuc5
D. gonocephala	D01SER	100km south-west from Belgrade, Serbia	X	X	X	X	X	-
D. etrusca	Det06	Tuscany, Italy	FJ646984	X	X	FJ646898	X	-
D. liguriensis	DFR	Alpes Maritimes, France	X	X	X	X	X	X
D. benazzii	MR2192	Monte Albo, Sardinia	MK385926	X	MK712509	MK713037	MK713264	-
D. hepta	MR1960	Mascari, Sardinia	MK385923	X	MK712512	MK713034	MK713266	-
D. tubqalis	MMS5.1	Toubkal, Taddert, Morocco	X	X	X	X	X	X
Dugesia sp. (1)	R9T	Afaska, Morocco	MK712634	-	MK712529	MK713044	MK713304	MK712833
D. aurea	M2.1D	Soller, Mallorca, Balearic Islands, Spain	MK712631	X	MK712522	MK713027	MK713281	MK712811
	M2.2D		MK712632	X	MK712523	MK713028	MK713282	MK712812
D. corbata	M3.1D	Sa Calobra, Mallorca, Balearic Islands, Spain	MK712635	X	MK712524	MK713029	MK713284	MK712814
	M3.2D		MK712636	X	MK712525	MK713030	MK713285	MK712815
D. vilafarrei	29.2	El Bosque, Andalucía, Spain	MK712649	X	MK712495	MK712997	MK713228	MK712761
	Dg1.1	Benaojan, Andalucía, Spain	MK712648	X	MK712511	MK713017	MK713261	MK712795
D. subtentaculata	V05.1D	Peralejos de las Truchas, Castilla La Mancha, Spain	MK385877 MK385895	-	MK712531	MK713047	MK713314	MK712843
	E6.2	Beni Moussa, Morocco	MK712605	X	MK712519	MK713024	MK713275	MK712806
D. cretica	D02CRE1	Georgioupoli, Chania, Crete, Greece	KC006976	X	X	KC007050	X	X
D. improvisa	D01NAX2	Melanes, Naxos, Greece	KC006987	KF308696	X	KC007065	X	X
D. damoae	D01SA19	Manolates, Samos, Greece	KC006979	X	X	KC007057	X	X
Dugesia sp. (2)	D02TRI	Tripi, Laconia, Peloponnese, Greece	KC007021	X	X	KC007106	X	X
D. aenigma	D01CEF3	Agia Eirini, Cephalonia, Greece	KC006968	KF308698	X	KC007040	X	X

X: Sequences from Solà *et al*. 2019 with GenBank accession numbers pending to be obtained

DNA sequences of each gene were separately aligned using the online software MAFFT (version 7) with the default settings (Katoh & Standley, 2013) and were subsequently revised using the software Geneious R8 (Biomatters, http://www.geneious.com/) (Kearse et al., 2012). For the Cox1 we constructed two different alignments: one alignment including the Cox1 of all species plus the ancestral sequence of the individual V05.1D (named *Cox1-Ancestral*), and the other alignment including the Cox1 of all species plus the derived sequence of the individual V05.1D (named *Cox1-Derived*). For the Cox1, the genetic code 9 (Echinoderm mitochondrial) was used, while for the two nuclear markers it was used the genetic code 1 (Standard). Putative sequence saturation in the alignments was checked by performing a test of substitution saturation implemented in the program DAMBE (Xia & Lemey, 2009; Xia & Xie, 2001; Xia, Xie, Salemi, Chen, & Wang, 2003). The non-coding gene fragments (complete 28S, 18S and ITS as well as intronic regions of Dunuc3 and Dunuc5) were analysed at the nucleotide level, while for the protein coding gene fragments (complete Cox1 and exonic regions of Dunuc3 and Dunuc5) the level of saturation was analyzed independently for each codon position. Finally, the evolutionary model of each alignment was determined using the program jModelTest2 (Darriba, Taboada, Doallo, & Posada, 2012), which in all cases resulted in the GTR + G, excepting for the Dunuc3 that was determined as HKY + G.

The DNA sequence alignments of the different markers were arranged in two different datasets, which only differed in the Cox1 alignment: one dataset included the alignments of the 28S, 18S, ITS, Dunuc3, Dunuc5, and Cox1-Ancestral (Dataset-Ancestral), and the other dataset included the alignments of the 28S, 18S, ITS, Dunuc3, Dunuc5, and Cox1-Derived (Dataset-Derived).

2.3. Phylogenetic inference and divergence time estimation

Both the phylogenetic relationships and approximate times of divergence between the different *Dugesia* lineages were estimated using the software BEAST v.1.8.4 (Drummond, Suchard, Xie, & Rambaut, 2012). Prior to the analyses, the two datasets were independently imported in BEAUti v1.8.4 to set the different parameters. The parameters of the site model and the clock model were independently estimated for each alignment, while a unique tree was estimated for each dataset. For the site model the following priors were set: substitution model = GTR (excepting for the Dunuc3, which was set to HKY); bases frequencies = empirical; site heterogeneity model = gamma and number of gamma categories = 4. The clock model of each alignment was set to strict, while the tree prior of each analysis was set to "Speciation: Birth-Death Process" (Gernhard, Hartmann, & Steel, 2008). We calibrated the phylogeny using

the paleogeographic event corresponding to the Mid-Aegean Trench (fragmentation of the Aegean Peninsula into an eastern and a western region approximately 12 Ma), which was proposed to have caused the diversification of the *Dugesia* species from the Aegean region (Solà et al., 2013), as well as the diversification of many other Aegean fauna (e.g., Kornilios, Poulakakis, Mylonas, & Vardinoyannis, 2009; Papadopoulou, Anastasiou, Keskins, & Vogler, 2009). Thus, we calibrated the split between the eastern and the western *Dugesia* Greek clades (represented in the present study by *D. improvisa*-*D. damoae* and *Dugesia* sp.-*D. aenigma*, respectively) by setting the node under a normal distribution with a mean of 12 and a Stdev of 1.5. Finally, three runs for each dataset were conducted in BEAST 1.8.0. using the CIPRES Science Gateway (Miller, Pfeiffer, & Schwartz, 2010), setting 100,000,000 generations and sampling each 10,000 generations. The resulting log files were examined in Tracer 1.7 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018) to check that the Effective Sample Size (ESS) values for the different parameters were higher than 200. The log files of the three independent runs were finally combined with LogCombiner v1.8.4 and the maximum clade credibility tree was annotated with TreeAnnotator v1.8.4 (both programs available in the BEAST package).

2.4. Species distribution modelling

The potential geographic distribution of *D. subtentaculata* at different time periods was estimated using the maximum entropy method implemented in the software Maxent 3.4.0. (Phillips, Anderson, Dudík, Schapire, & Blair, 2017; Phillips & Dudík, 2008). Distribution data of this species was extracted from literature (Leria, Vila-Farré, Álvarez-Presas, et al., 2019), accounting for a total of 60 localities covering the whole distribution range of the species. Past, present and future bioclimatic layers were downloaded from the WorldClim Version 1 database (http://www.worldclim.org/version1). Past layers included the Last Inter-glacial period (around 130.000 years ago), the Last Glacial Maximum period (around 22.000 year ago) and the Mid Holocene period (~6.000 years ago). Future layers corresponded to the predicted climatic conditions at the year 2070 under two different possible scenarios regarding the concentration of Greenhouse gases: a scenario with the lowest concentration of Greenhouse gases (Representative Concentration Pathway (RCP) of 2.6) and a scenario with the highest concentration of Greenhouse gases (RCP of 8.5). All layers were downloaded at a spatial resolution of 2.5 arc-minutes (~4.5 km at the equator), excepting the layers of the Last interglacial period, that were only available at 30 arc-seconds (~1 km). Among the 19

bioclimatic variables available in WorldClim, we used the four that had been proven to be independent based on the R^2 statistic (Lázaro & Riutort, 2013): isothermality (Bio3 in WorldClim), mean temperature of the wettest quarter (Bio8 in WorldClim), mean temperature of the driest quarter (Bio9 in WorldClim), and the coefficient of variation in the precipitation seasonality (Bio15 in WorldClim). Layers in Grid format were imported to DIVA-GIS Version 7.5 (http://www.diva-gis.org/), trimmed to the region of interest and subsequently exported in ESRI ASCII format (format for Maxent). Layers in GeoTiff format were converted to BIL format using ArcMap version 10.6.1 (https://desktop.arcgis.com/es/arcmap/) previous to their importation to DIVA-GIS. Each analysis in Maxent 3.4.0. was carried out using a random test percentage of 25% and 10 replicates. The performance of the model for each analysis was evaluated by checking the area under the curve (AUC) of the receiver operating characteristic (ROC) (Fielding & Bell, 1997). Moreover, the contribution of each variable was analysed. Finally, the cloglog image output (probability of presence from 0 to 1) was used to analyse the putative distribution of the species at the different periods.

3. RESULTS

3.1. Molecular datasets

Sequences of the six molecular markers used in the present study represented a total aligned length of 5439 characters. No gene fragment or codon position showed significant levels of sequence saturation for a symmetrical tree topology, as in all tests using this topology the Index of Substitution Saturation was significantly lower than the Critical Index of Substitution Saturation (Supplementary data S1). The tests conducted under an asymmetrical tree topology showed low levels of saturation in the third codon position of the Cox1 molecular marker. However, given that the tree topology obtained in the present study was mainly symmetrical (see Fig. 2) and that this codon position was not saturated under a symmetrical topology, we decided not to dismiss this position for the subsequent molecular analyses. Finally, no stop codons were detected in the protein coding regions.

3.2. Phylogenetic relationships

The topology of the calibrated trees obtained with BEAST for both datasets (Dataset-Ancestral and Dataset-Derived) was identical and fully supported (Fig. 2). The only node that showed a different support value (posterior probability) in the two analyses was the node including *D.*

subtentaculata and *D. vilafarrei*, which changed from 0.97 (Dataset-Ancestral) to 0.92 (Dataset-Derived).

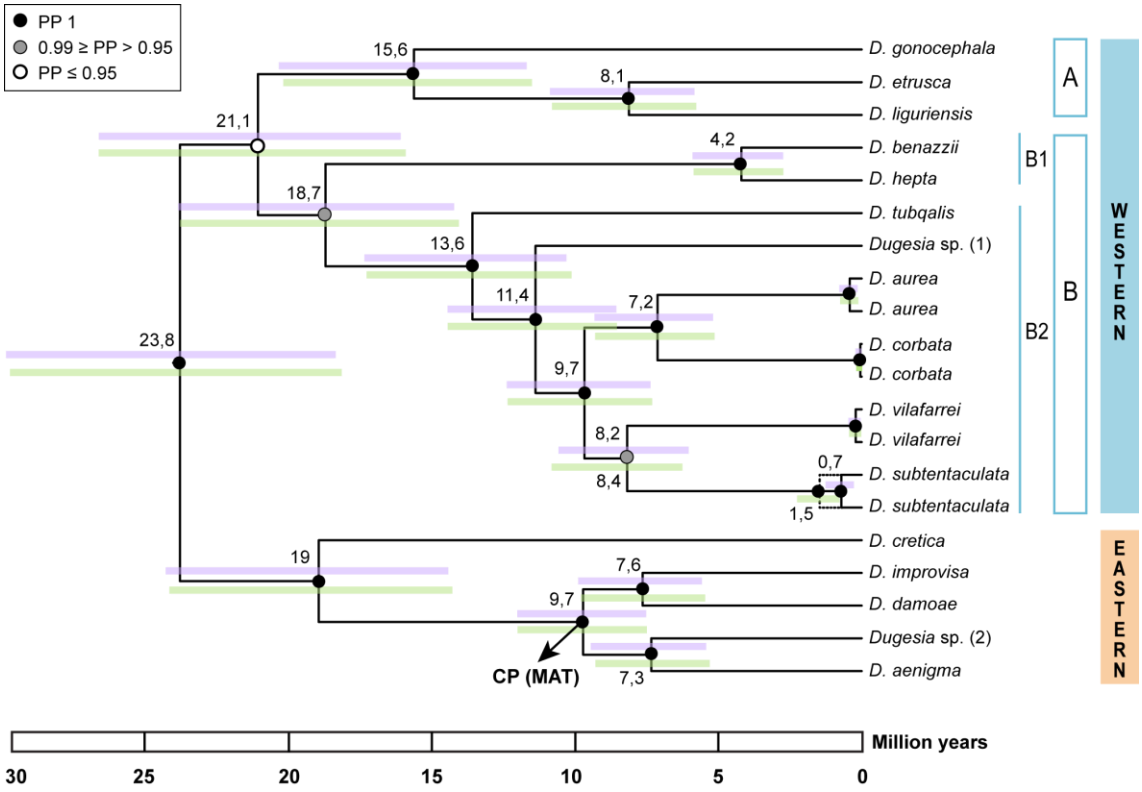

Fig. 2. Maximum credibility tree obtained with BEAST for the 6 molecular markers used in the present study. Node bars correspond to the 95% high posterior density intervals of the time estimates. Upper bars (purple) correspond to the Dataset-Ancestral and lower bars (green) correspond to Dataset-Derived. Values at nodes correspond to mean time estimates in million years ago. Different mean time estimates for the two datasets are indicated next to the corresponding bar. Coloured dots at nodes indicate the posterior probability (PP) of each node. CP (MAT): calibration point used in the present study corresponding to the Mid Aegean Trench (around 12 Ma). Scale bar represents time in million years.

As expected, all *Dugesia* species from the Aegean region (used in the present study as outgroup) formed a highly supported monophyletic group (named as Eastern clade) (Fig. 2). The rest of species also conformed a monophyletic group (named as Western clade), although with a lower support value than the Eastern clade. The first split within the Western clade divided a clade including the species *D. gonocephala*, *D. etrusca* and *D. liguriensis* (clade A) from a clade including the rest of species (clade B). Clade B was, at the same time, divided into a clade corresponding to the Sardinian species *D. hepta* and *D. benazzii* (clade B1) and another clade including the species from Africa (*D. tubqalis* and *Dugesia* sp. 1), the Balearic Islands (*D.*

corbata and *D. aurea*), and the Iberian Peninsula (*D. vilafarrei* and *D. subtentaculata*) (clade B2). The first species to diverge within clade B2 were the two species from Africa, beginning with *D. tubqalis* and following with *Dugesia* sp. 1 from Morocco. Finally, the two species from Mallorca (*D. aurea* and *D. corbata*) constituted a monophyletic clade with a sister group relationship with the species *D. vilafarrei* and *D. subtentaculata*.

3.3. Divergence time estimation

The time calibrated phylogenies inferred for both datasets (Dataset-Ancestral and Dataset-Derived) retrieved highly similar age estimates for the different nodes (Fig. 2). The only node that showed a different age in both phylogenies was the one corresponding to the split within *D. subtentaculata*, which was inferred as the double of time in the Dataset-Ancestral than in the Dataset-Derived (1.5 Ma and 0.7 Ma, respectively). For the rest of the nodes, we will refer to the age estimates of the Dataset-Ancestral (due to the high similarity between the two datasets). The mean age of the split between the Western and the Eastern clades was 23.8 Ma (95 % highest posterior density (HPD) interval of 29.87-18.36 Ma). The node that we used as a calibration point was retrieved at 9.7 Ma, fitting within the confidence interval that we set for the split. Regarding the Western clade, the age of the split between clade A and clades B was 21.1 Ma (95% HPD of 26.63-16 Ma). Subsequently, the divergence between clade B1 and clade B2 dated back to 18.7 Ma (95% HPD of 23.86-14.23 Ma). The first split within clade B2 occurred at 13.6 Ma (95% HPD of 17.35-10.31 Ma), while the divergence between *Dugesia* sp. (1) from Morocco and the group including *D. corbata*, *D. aurea*, *D. vilafarrei* and *D. subtentaculata* was estimated to take place 11.4 Ma (95% HPD of 14.46-8.56 Ma). The monophyletic clade including the species from Mallorca (*D. corbata* and *D. aurea*) started to diverge from the ancestors of *D. vilafarrei* and *D. subtentaculata* around 9.7 Ma (95% HPD of 12.39-7.37 Ma). Finally, the divergence of *D. corbata* and *D. aurea* dated back to 7.2 Ma (95% HPD of 9.33-5.19 Ma), more recent than the split between *D. vilafarrei* and *D. subtentaculata*, which was inferred to occur 8.2 Ma (95% HPD of 10.58-6.4 Ma).

3.4. Species distribution modelling

All the *D. subtentaculata* distribution models carried out with Maxent 3.4.0. yielded AUC values higher than 0.9, indicating that in all cases the model had a high predictive power of the distribution of the species (AUC values close to 0.5 indicate random chance, while AUC values

close to 1 indicate high predictive power) (Supplementary data S2). In all analyses, excepting for the Last inter-glacial period, the bioclimatic variable corresponding to the mean temperature of the driest quarter (Bio9) was the variable that contributed the most to the model, followed by the mean temperature of the wettest quarter, the precipitation seasonality, and the isothermality (Supplementary data S2). In the case of the Last inter-glacial period, the variable that contributed the most was the mean temperature of the wettest quarter, followed by the mean temperature of the driest quarter, the precipitation seasonality, and the isothermality.

The most ancient reconstruction (Last Interglacial period, ~130.000 ya) pointed out that the most suitable geographic region for *D. subtentaculata* during that period was the north-western coast of the Iberian Peninsula, with three principal hot spots around Lisbon, Galicia and Cantabria (Fig. 3A). Other locations showing a high probability of presence of *D. subtentaculata* during that period included the western region of the Atlas Mountains and different scattered regions of the coast of the whole Iberian Peninsula and Southern France. The suitable geographic region for the species during the Last Glacial Maximum (~22.000 ya) shifted to a more continental range within the Iberian Peninsula, Southern France and Northern Africa (Fig. 3B). In the Iberian Peninsula, the regions with a higher probability of presence of the species were Galicia, Cantabria and the western region of the Ebro basin. In Southern France, the putative distributions of the eastern and western coasts became connected, while in Northern Africa the region of the Riff and the north of Argelia increased in the probability of occurrence. The putative distribution of *D. subtentaculata* during the Mid Holocene period (~6.000 ya) shifted again to a more coastal range, with the hotspots occurring in the western and northern coasts of the Iberian Peninsula (Fig. 3C). This putative distribution was highly similar to the putative distribution scenario obtained with the present-day climatic conditions (Fig. 3D). In the present-day scenario, all the known localities of the species occur within the predicted geographic range showing more than a 50% of probability of presence, excepting one locality in Catalonia (0% probability) and one locality in the southern region of Castilla La Mancha (0.25% probability). Finally, the two future scenarios projected for the year 2070 (under different concentrations of Greenhouse gases) point out to a reduction of the continental area of the Iberian Peninsula suitable for this species, together with an increase of the probability of presence in the southern regions of Portugal and Andalusia (Figs. 3E and 3F).

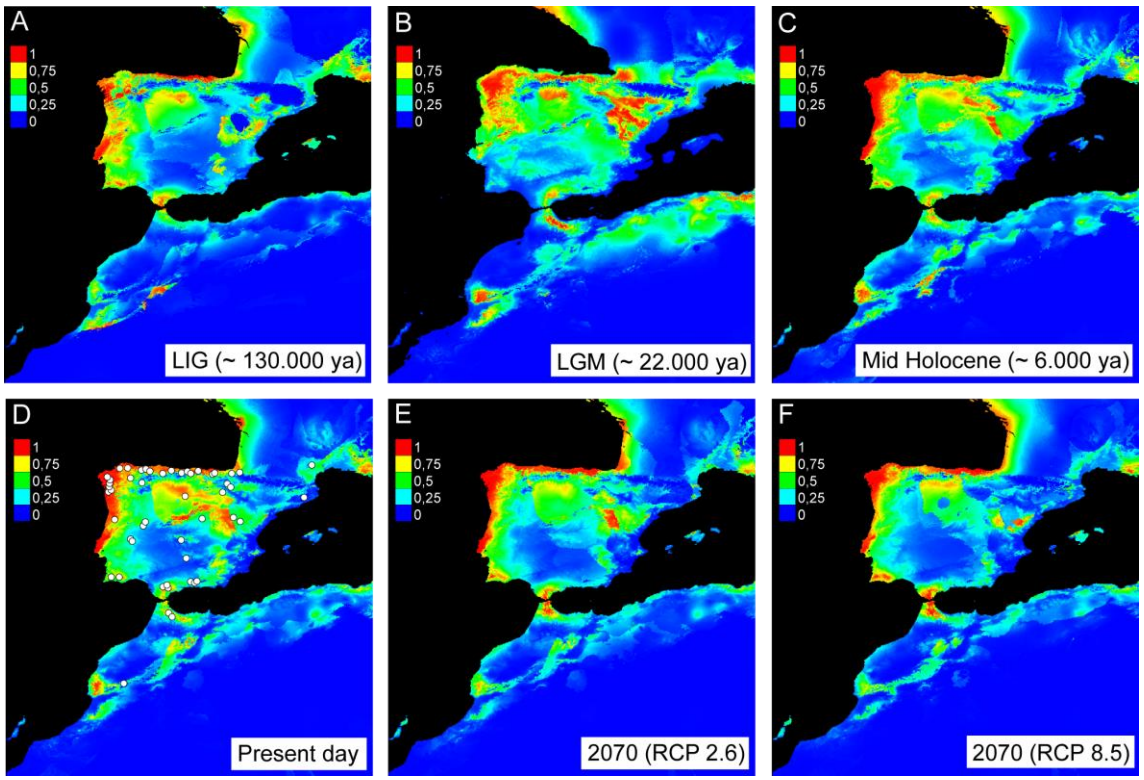

Fig. 3. Potential geographic distribution of the species *D. subtentaculata* at different time periods estimated with Maxent. A) Last Inter-Glacial, B) Last Glacial Maximum, C) Mid Holocene, D) present day, E) year 2070 with a low concentration of Greenhouse gases, and F) year 2070 with a high concentration of Greenhouse gases. Colour scale from blue to red indicates the probability of presence from 0 to 1. White dots in the present-day scenario (D) correspond to the known localities of the species.

4. DISCUSSION

4.1. Impact of high intraindividual genetic diversity in shallow divergence time estimations

The time calibrated phylogeny inferred in the present study showed that the estimated age of the most recent common ancestor between the two populations of *D. subtentaculata* included in the analysis varied in approximately in 1 million year, depending on the intraindividual Cox1 haplotype that was used of the individual from the Iberian Peninsula. For instance, the divergence between the two populations was estimated at a mean age of 1.5 Ma when the ancestral Cox1 haplotype of the individual V05.1D was used but at a mean age of 0.7 Ma when the derived Cox1 haplotype of the same individual was used (Fig. 2). Nevertheless, we found that the age estimates of the rest of the nodes within the phylogeny were not affected. Thus, even though these results are based on limited information (only varying the Cox1 sequence of one individual), they indicate that including intraindividual genetic information might be

crucial when performing divergence time estimates in planarians at the intraspecific level, particularly in species that undergo fissiparous reproduction (such as most *Dugesia* species). Therefore, additional studies including the intraindividual information of more fissiparous individuals of different *Dugesia* species (with more molecular markers) would be extremely interesting to further evaluate the effect of the high levels of intraindividual genetic diversity in molecular analyses of divergence time estimation.

4.2. Phylogenetic relationships within *Dugesia* from the Western Mediterranean region

The phylogenetic relationships obtained in the present study pointed out that the *Dugesia* species from the Western Mediterranean region are divided into two main groups: one including the species *D. gonocephala*, *D. etrusca* and *D. liguriensis*; and the other including the rest of species coming from the Western Mediterranean islands (Corsica, Sardinia and Balearic Islands), Northern Africa and the Iberian Peninsula (Fig. 2). This phylogenetic scenario was already proposed by Solà (2014), basing on the information of four molecular markers (18S, 28S, ITS, and Cox1). Nevertheless, in that study, the monophyly of the species *D. hepta* and *D. benazzii* with the rest of species from Northern Africa, Balearic Islands and the Iberian Peninsula showed no support (the support value for this group showed less than 0.95 of posterior probability in the Bayesian analysis and less than a 75% of Bootstrap support in the Maximum Likelihood analysis). On the contrary, in the phylogenetic inference obtained in the present study, the relationship between these groups of species is highly supported (with a 0.99 of posterior probability), which could be due not only to the addition of the two nuclear markers Dunuc3 and Dunuc5 but also to the inclusion of more species into the analysis (*D. corbata*, *D. aurea*, and *D. vilafarrei* were missing in the study of Solà).

Similar to what was inferred by Lázaro et al., 2009 and Solà, 2014, our phylogenetic inference showed with high support values that *D. etrusca* and *D. liguriensis* conformed a monophyletic group, which was the sister clade of the species *D. gonocephala*. Alternatively, the phylogenetic relationships obtained between the species that were previously subsumed within *D. subtentaculata* (*D. aurea*, *D. corbata*, *D. vilafarrei*, and *Dugesia* sp. 1 from Morocco) are in agreement to what was found in a previous study using *D. hepta* and *D. benazzii* as outgroups (Leria, Vila-Farré, Álvarez-Presas, et al., 2019). Therefore, although additional phylogenetic studies are needed to be performed in this group, including the species with presently no available molecular data, *viz*. *D. magrebiana*, *D. leporii*, and *D. brigantii*, the

present study has yielded further support to the phylogenetic relationships between most of the *Dugesia* species from the Western Mediterranean region.

4.3. Phylogeographic history of *Dugesia* species from the Western Mediterranean

Our time calibrated phylogeny suggests that the present distribution of the Western Mediterranean *Dugesia* species has been driven by the paleogeographic history of this region as exposed by Rosenbaum and collaborators (2002), although some inconsistencies exist. The estimated age of the Western clade together with the present distribution of all its species, point out that the ancestors of this clade were probably distributed in the landmass that today constitutes the Iberian Peninsula plus all the regions corresponding to the microplates, around 30 Ma (Fig. 4B). Subsequently, the initial detachment of the landmass constituting the microplates from the Iberian Peninsula approximately 25 Ma most likely resulted in the split between the ancestors of the species *D. gonocephala*, *D. etrusca* and *D. liguriensis* (which are presently mainly distributed in continental Europe) and the ancestors of the species that are presently distributed in the Western Mediterranean islands, Northern Africa and the Iberian Peninsula (Fig. 4C). This paleogeographic event has been pointed out to have driven the diversification of many other fauna, such as different genus of spiders (Bidegaray-Batista & Arnedo, 2011; Opatova, Bond, & Arnedo, 2016) and the freshwater flatworm species *Schmidtea mediterranea* (Lázaro et al., 2011), among others.

Our results also pointed out that the following diversification event occurred between the species from Corsica and Sardinia and the rest (species from Morocco, Balearic Islands and the Iberian Peninsula) around 19 Ma (± 5 Ma), which could correspond to the first fragmentation of the landmass constituting the microplates (Fig. 4D). Subsequently, the basal position of the two species from Morocco (*D. tubqalis* and *Dugesia* sp. 1) within the clade that includes both the Balearic and Iberian species, points out that the ancestors of all these species probably occurred in the Betic-Rif microplate. This is supported by the estimated time that this clade started to diverge (between 13.5 and 11.5 Ma), which coincide with the collision of the Betic-Rif microplate with Africa (Fig. 4E), subsequently to the Iberian Peninsula and, finally, its breaking off (isolating the African and the Iberobalearic species) (Fig. 4F). The inclusion in further studies of *D. subtentaculata* reported from the Kabylies (which actually could correspond to a new species) together with *D. magrebiana* from Tunisia (Stocchino et al., 2009), would help refining the phylogeographic scenario proposed in the present study, maybe revealing a parallel arrival to Africa of *Dugesia* through the Kabylies microplates.

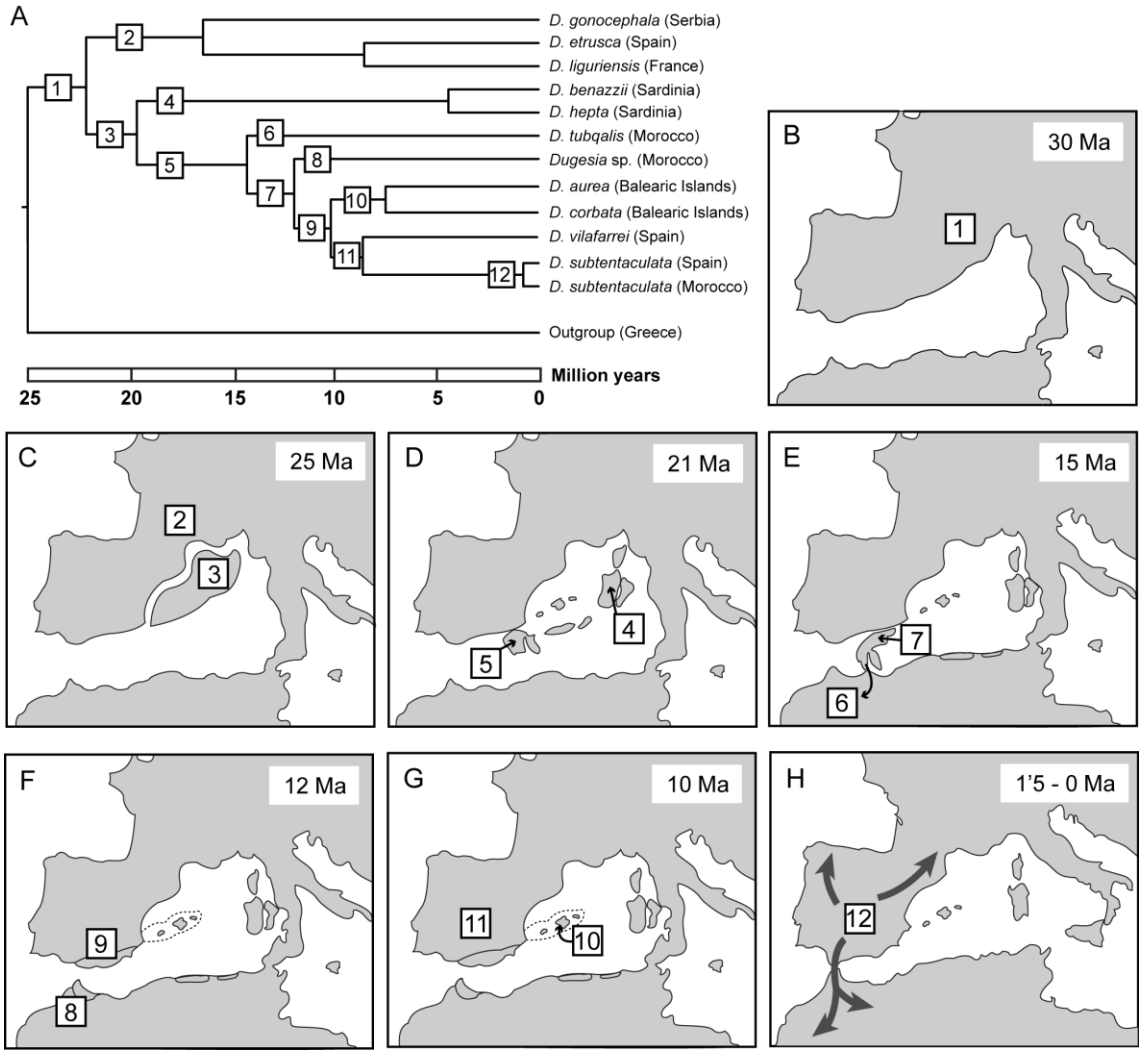

Fig. 4. Schematic representation of the phylogeographic history of *Dugesia* from the Western Mediterranean region proposed in the present study. Paleogeographic reconstructions (B-G) based on Rosenbaum et al. (2002). A) Phylogenetic relationships between *Dugesia* species under a temporal framework. Ages of nodes correspond to the mean estimates obtained in the time-calibrated phylogeny (see Fig. 2). Framed numbers from 1-12 indicate the geographic location and temporal framework of each lineage in pictures B-H. B) The ancestors of all *Dugesia* species from the Western Mediterranean region were distributed in continental Europe around 30 Ma. C) The detachment of the eastern part of the Iberian Peninsula (approximately 25 Ma) promoted the divergence between the ancestors of the species *D. gonocephala*, *D. etrusca* and *D. liguriensis* from the rest of species. D) The subsequent fragmentation of this detached landmass into several microplates (around 21 Ma) isolated one lineage in the Corsico-Sardinian microplate (ancestors of *D. hepta* and *D. benazzii*) and another lineage in the Betic-Rif microplate (ancestors of the African and the Iberobalear species). D) The collision of the Betic-Rif microplate with Africa (around 15 Ma) allowed some individuals to disperse to Africa (ancestors of *D. tubqalis*). F) The final fragmentation of the Betic-Rif microplate isolated the lineages distributed in the Riff from the Betic lineages (around 12 Ma). G) The ancestors of the Balearic species (*D. aurea* and *D. corbata*) dispersed to Mallorca, while the ancestors of *D. vilafarrei* and *D. subtentaculata* remained in the Iberian Peninsula. H) Dispersion of *D. subtentaculata* across the Iberian Peninsula and Southern France, including a return to Northern Africa.

Rosenbaum reconstructions (2002) suggest that the Balearic Islands became isolated at the early stages of the fragmentation of the plate, around 25-21 Ma. Nevertheless, alternative paleogeographic studies have suggested that they remained attached to the Iberian Peninsula by the Betic region until the early Tortonian (around 11 Ma) (Schettino & Turco, 2011) or that, at least, different periods of connection existed between the Balearic Islands and the Iberian Peninsula during this period (Roca, 1996). Those hypotheses are supported by the origin of some endemic biota of the Balearic Islands (Bover et al., 2014; Chueca, Madeira, & Gómez-Moliner, 2015). In accordance to that, our data analysis suggests that the ancestors of *D. aurea* and *D. corbata* dispersed to Mallorca from the region of the Betic-Rif microplate that became attached to the Iberian Peninsula around 10 Ma and subsequently became isolated in the island (Fig. 4G), diverging relatively soon after their arrival (around 7.2 Ma).

The divergence between the Iberian species, *D. vilafarrei* and *D. subtentaculata*, dates back to 8.2 Ma. It has been proposed that this diversification event occurred due to a change in the reproductive strategy in the ancestors of *D. subtentaculata* (Leria, Vila-Farré, Solà, et al., 2019). In particular, due to a shift from sexual to fissiparous reproduction, which immediately promoted the reproductive isolation between the lineage corresponding to the present *D. vilafarrei* (diploid sexual species) and *D. subtentaculata* (species with principally polyploid fissiparous populations).

Our time calibrated phylogeny also indicated that at least one lineage of *D. subtentaculata* returned to Africa during the early Pleistocene (between 1.5 - 0.7 Ma) (Fig. 4H). One of the major fluctuations of terrestrial and freshwater biota between Northern Africa and the Iberian Peninsula has been correlated with the desiccation of the Mediterranean Sea during the Messinian Salinity Crisis, which occurred from 5.59 to 5.33 Ma (e.g., Veith, Mayer, Samraoui, Donaire, & Bogaerts, 2004). Nevertheless, the period when this paleogeographic event occurred does not fit with the estimated period of dispersion of some populations of *D. subtentaculata* from the Iberian Peninsula to Northern Africa. Interestingly, many examples of post-Messinian connections between these two regions have also been documented in different taxa, principally associated to the active dispersion of the biota during the glacial periods (Fernández-Mazuecos & Vargas, 2011; Kaliontzopoulou, Pinho, Harris, & Carretero, 2011; Soria-Boix, Donat-Torres, & Urios, 2017), indicating that *D. subtentaculata* could have dispersed from the Iberian Peninsula to Morocco during one of the low-sea level periods after the Messinian Salinity Crisis. Additional studies analysing the intraindividual genetic data of the

African populations would be needed to trace its specific origin among the different lineages from the Iberian Peninsula.

4.4. The effect of paleoclimatic fluctuations in the distribution of *Dugesia subtentaculata*

The phylogeographic analysis performed in the present study indicated that the ancestors of *D. subtentaculata* arrived at the Iberian Peninsula through the Betic-Rif microplate at some point between 15 to 10 Ma. Surprisingly, this analysis also indicated that the radiation of the different *D. subtentaculata* populations in the Iberian Peninsula did not probably occur until approximately 1.5 Ma (2.26-0.78 Ma), coinciding with the establishment of the Mediterranean climatic conditions that we know today in this region (Suc, 1984). Therefore, it could be possible that the ancestors of *D. subtentaculata* remained ecologically trapped in certain localities from the southern region of the Iberian Peninsula until more favourable conditions developed, allowing the expansion of this species northwards.

The distribution predicted for this species during the Last Interglacial Period (130.000 ya) (which is the oldest period with available climatic data), showed that the most suitable areas for *D. subtentaculata* at that moment were the western and the northern coasts of the Iberian Peninsula (Fig. 3A). Therefore, although this species could have previously reached a more central-eastern distribution range within the Iberian Peninsula, its occurrence was probably predominant in these western-northern regions during the Last Interglacial period. Subsequently, the distribution of this species should have suffered an expansion to a more continental range during the Last Glacial Maximum (22.000 ya) (Fig. 3B). Interestingly, this was the only period in which the regions of Catalonia and Eastern-Southern France were connected to the rest of suitable distribution. Thus, it could be possible that the species reached these locations during this period, where it became isolated from the rest of suitable areas afterwards. Finally, the putative distribution of this species during the Mid-Holocene (6.000 ya) is highly similar to its present distribution, indicating that *D. subtentaculata* could be already occupying the range where it is presently found from the last 6000 years.

4.5. Present and future distribution of *Dugesia subtentaculata*

The predicted present-day potential distribution of *D. subtentaculata* fits well with the localities where the species has been observed (Fig. 3D). Unfortunately, there are some areas, including the northern coast of Portugal, the Central Mountain Range and some scattered

regions in Morocco, that show the maximum suitability for this species but that have never been sampled. Therefore, it could be possible that the distribution of *D. subtentaculata* was still broader than it is presently recorded.

Additionally, the predicted distribution model indicated that there are two populations of this species, *viz*. the population of Southern France (which is the type locality of the species) and the population of Catalonia, that are environmentally isolated from the rest of populations. As previously mentioned, these localities probably represent a relic from a period when the species occupied a broader geographic range (putatively during the Last Glacial Maximum). Thus, these localities could be considered as two present microrefugia of the species, since they are small areas outside the core distribution of the species where it persists despite that the surroundings are inhospitable (Hylander, Ehrlén, Luoto, & Meineri, 2015). Recent extensive samplings performed in the surrounding regions of these localities revealed that they are occupied by other *Dugesia* species (Leria, Vila-Farré, Álvarez-Presas, et al., 2019). Therefore, the isolation of these two localities could be presently limited not only by the environmental conditions but also by ecological competition with other *Dugesia* species.

Interestingly, the predicted suitable distribution of *D. subtentaculata* matches with the regions presently occupied by all its closely sister species, *viz*. *D. corbata*, *D. aurea*, *D. vilafarrei*, *Dugesia* sp. 1 from Morocco, and *D. tubqalis*. These results indicate that these species not only are very similar at the morphological level (for instance *D. corbata*, *D. aurea*, *D. vilafarrei* and *D. subtentaculata* are indistinguishable at the morphological level; see Leria, Vila-Farré, Álvarez-Presas, et al., 2019) but they may also have very similar ecological requirements.

Finally, the predicted potential future distribution of *D. subtentaculata* points to a reduction of the continental region of the Iberian Peninsula that is presently suitable for the species, together with an increase in the suitability of some regions in the south of the Iberian Peninsula and the north of Morocco. This scenario might be problematic for *D. subtentaculata*, since most of these areas are presently occupied by *D. sicula* (Lázaro & Riutort, 2013), a species that has been already pointed to outcompete other planarian species, such as *Schmidtea mediterranea* (Leria, Sluys, & Riutort, 2018; and references therein). Nevertheless, our predicted future distribution model for *D. subtentaculata* also pointed out that the northern and western coasts of the Iberian Peninsula would remain as highly suitable for this species, potentially ensuring its viability in the nearly future.

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Supplementary data S1. Results of Xia's test to detect putative saturation on the gene fragments used in the present study. The protein coding genes were analysed separately for the intronic regions and at the three coding positions of the exonic regions. Iss: index of substitution saturation; Iss.cSym: critical index of substitution saturation for a symmetrical tree topology; Iss.cAsym: critical index of substitution saturation for an asymmetrical tree topology; P: significance value in a two-tailed test. The tests were performed only on fully resolved sites.

	Iss	**Iss. cSym**	**P**	**Iss. cAsym**	**P**
Dunuc3_Introns	0,0421	0,6096	0,0000	0,5847	0,0000
Dunuc3_Exons_1st	0,0088	0,3781	0,0000	0,4907	0,0000
Dunuc3_Exons_2nd	0,0342	0,3781	0,0000	0,4907	0,0000
Dunuc3_Exons_3rd	0,1851	0,3738	0,0091	0,4956	0,0001
Dunuc5_Introns	0,2969	0,8862	0,0000	0,9279	0,0000
Dunuc5_Exons_1st	0,0127	0,7865	0,0000	0,8735	0,0000
Dunuc5_Exons_2nd	0,0141	0,7807	0,0000	0,8600	0,0000
Dunuc5_Exons_3rd	0,1406	0,7865	0,0000	0,8735	0,0000
Cox1-Ancestral_1st	0,0810	0,6134	0,0000	0,4087	0,0000
Cox1-Ancestral_2nd	0,0266	0,6127	0,0000	0,4084	0,0000
Cox1-Ancestral_3rd	0,4756	0,6072	0,0005	0,4063	0,0617
Cox1-Derived_1st	0,0811	0,6134	0,0000	0,4087	0,0000
Cox1-Derived_2nd	0,0266	0,6127	0,0000	0,4084	0,0000
Cox1-Derived_3rd	0,4749	0,6072	0,0004	0,4063	0,0639
28S	0,0381	0,7545	0,0000	0,5288	0,0000
18S	0,0390	0,7497	0,0000	0,5376	0,0000
ITS	0,2593	0,7235	0,0000	0,5065	0,000

Supplementary data S2. AUC value and relative contribution to the model of the different bioclimatic layers for each of the six temporal scenarios analyzed in the present study

Temporal scenario	AUC	Relative contribution to the model			
		Bio3	Bio8	Bio9	Bio15
Last Inter-Glacial (130.000 ya)	0.918 ± 0.039	3 %	55.1 %	32.6 %	9.3 %
Last Glacial Maximum (22.000 ya)	0.909 ± 0.053	1.9 %	19.5 %	60.9 %	17.8 %
Mid Holocene (6000 ya)	0.910 ± 0.037	1.4 %	32.7 %	54.4 %	11.5 %
Present day	0.912 ± 0.038	1.2 %	24.1%	62.8 %	11.9 %
2070 (RCP = 2.6)	0.913 ± 0.047	3 %	25.7 %	57.6 %	13.8 %
2070 (RCP = 8.5)	0.916 ± 0.048	4.4 %	25.2 %	55.9 %	14.5 %

Chapter 4

Diversification and biogeographic history of the Western Palearctic freshwater flatworm genus *Schmidtea* (Tricladida: Dugesiidae), with a redescription of *Schmidtea nova*

Reference

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ORIGINAL ARTICLE

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Diversification and biogeographic history of the Western Palearctic freshwater flatworm genus *Schmidtea* (Tricladida: Dugesiidae), with a redescription of *Schmidtea nova*

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Abstract

The freshwater flatworm genus *Schmidtea* is endemic in the Western Palearctic region, where it is represented by only four species, thus contrasting with the high species diversity of the closely related genus *Dugesia* within Europe. Although containing an important model species in developmental and regeneration research, viz. *Schmidtea mediterranea*, no evolutionary studies on the genus *Schmidtea* have been undertaken. For the first time, we present a well-resolved molecular phylogenetic tree of the four species of the genus, inferred on the basis of two molecular markers, and provide also the first detailed morphological account of *Schmidtea nova*. The phylogenetic tree generated corroborates an earlier speciation hypothesis based on karyological data and points to chromosomal rearrangements as the main drivers of speciation in this genus. The high genetic divergence between the four species, in combination with previous dating studies and their current geographic distribution, suggests that *Schmidtea* could have originated in Laurasia but lost most of its diversity during the Oligocene. Thus, its present distribution pattern may be the result of the expansion of three of its four relictual species over Europe, probably after the Pleistocene glaciations. Our detailed morphological study of *S. nova* revealed that it shows a number of remarkable features: interconnected testis follicles, parovaria, an ejaculatory duct exiting into the primary as well as the secondary seminal vesicle by means of a nipple, and the wall of the distal section of the ejaculatory duct being sclerotic or chitinized.

KEYWORDS
biogeography, molecular phylogeny, *Schmidtea*, speciation, taxonomy

1 | INTRODUCTION

The freshwater planarian genus *Schmidtea* Ball, 1974 is endemic to the Western Paleartic region and represented by only four species: *S. polychroa* (Schmidt, 1861), *S. lugubris* (Schmidt, 1861), *S. mediterranea* (Benazzi, Baguñà, Ballester, Puccinelli, & Del Papa, 1975), and *S. nova* (Benazzi, 1982). It should be noted that occurrence of *S. polychroa* in North America is the result of human-mediated dispersal (Ball, 1969; Benazzi & Benazzi-Lentati, 1976). The species *S. mediterranea* is a model organism in regeneration research (Reddien & Alvarado, 2004; Rink, 2013) with a fully sequenced genome (Robb, Ross, & Alvarado, 2008; Grohme et al., 2018), while *S. polychroa* has been used as a model organism in studies on embryogenesis (Cardona, Hartenstein, & Romero, 2005; Monjo & Romero, 2015)

and reproduction (D'Souza & Michiels, 2009; Pongratz, Storhas, Carranza, & Michiels, 2003). In comparison with these two model species, *S. lugubris* and *S. nova* are poorly studied.

The genus *Schmidtea* belongs to the family Dugesiidae Ball, 1974 and it is phylogenetically related to the genera *Neppia* Ball, 1974, *Girardia* Ball, 1974, *Cura* Strand, 1942, *Dugesia* Girard, 1850, and *Recurva* Sluys, 2013 (Riutort, Álvarez-Presas, Lázaro, Solà, & Paps, 2012). From these genera, only *Dugesia* and *Recurva* are found in the Palearctic region (*Girardia* can also be found in Europe, but this is due to recent human introduction of *G. tigrina* (Girard, 1850)) (Ball & Reynoldson, 1981 and references therein).

Although merely four *Schmidtea* species are presently known, their taxonomic history and resolution have been rather complex. The description of the first two species by Schmidt (1861), *Planaria lugubris* Schmidt, 1861, and *Planaria polychroa* Schmidt, 1861, immediately introduced much confusion because his description of their copulatory apparatus was incorrect and misleading. Thus, for many years, their specific distinctness was not clear, which was only resolved by Reynoldson and Bellamy (1970) and Benazzi, Puccinelli, and Del Papa (1970). Historical accounts on the nomenclature and features of these two species have been provided by Luther (1961) and Reynoldson and Bellamy (1970). During the course of their taxonomic history, the two species were eventually assigned to the genus *Dugesia*. In his taxonomic revision, Ball (1974) recognized three subgenera within *Dugesia* and moved Schmidt's two species to his subgenus *Schmidtea* Ball, 1974. The last taxonomic step was taken when De Vries and Sluys (1991) elevated the subgenus *Schmidtea* to the level of genus on the basis of two morphological synapomorphies in the copulatory apparatus: a double seminal vesicle and mixed bursal canal musculature. And, thus, Schmidt's two species finally settled taxonomically and nomenclaturally as *Schmidtea lugubris* (Schmidt, 1861) and *S. polychroa* (Schmidt, 1861).

Meanwhile, it had been shown that, on the basis of their karyotypes, seven different strains of *Schmidtea* could be recognized, which were called biotypes and were indicated by the first seven letters of the alphabet (Table 1). Biotypes A, B, C, and D form a homogeneous series with polyploid evolution, possibly starting from biotype A. On the other hand, biotypes E, F, and G are much differentiated from each other as well as from the A–D series (Benazzi & Benazzi-Lentati, 1976 and references therein).

Further morphological research revealed that the biotypes A-B-C-D correspond to the species *S. polychroa*, while the biotypes E and F were assigned to *S. lugubris* (Benazzi et al., 1970), and that biotype G represented a third, new species, viz. *Schmidtea mediterranea* (Benazzi et al., 1975). Some authors (Benazzi & Puccinelli, 1973) pointed out that the only morphological difference between biotype E and F was a permanent nipple on the penis papilla, but they were not recognized as different species until Benazzi (1982) proposed the new species name *Schmidtea nova* for specimens belonging to biotype F. However, no detailed description of the new species was provided.

Another addition to this complex taxonomic history was made when Ball (1979) reported a population from Corfu (Greece) that exhibited a karyotype similar to *S. mediterranea* but was morphologically much more similar to *S. polychroa*; unfortunately, morphological information was not presented, while no more studies were performed; material for study is presently unavailable.

As for their taxonomy, the phylogenetic relationships between the different *Schmidtea* species have been controversial. At a morphological level, *S. mediterranea* seemed to be closely related to *S. lugubris* (Benazzi et al., 1975), but their karyology suggested that *S. mediterranea* is closely related to *S. polychroa* and that *S. lugubris* is closely related to *S. nova* (Benazzi, 1982; Benazzi & Puccinelli, 1973). Molecular sequence data are still scarce for the group, a very short fragment of the *mitochondrially encoded cytochrome c oxidase I* (*Cox1*) being the only gene that has been sequenced for the four species until the present work. The first *Cox1* sequences of *S. mediterranea* and *S. polychroa* were obtained by Baguñà, Carranza, Paps, Ruiz-Trillo, and Riutort (2001). Two years later, *Cox1* of many populations of *S. polychroa* was sequenced for a phylogeographic study and two sequences of *S. lugubris* and *S. nova* were used as

TABLE 1 Biotype, karyology, and mode of reproduction of the *Schmidtea* species

Species	Biotype	Chromosome number	Karyotype[a]	Reproduction
S. polychroa	A	$2n = 8$	M-A-A-A	Sexual
	B	$3n = 12$	M-A-A-A	Parthenogenetic
	C	$3n = 12$	M-A-A-A	Parthenogenetic
	D	$4n = 16$	M-A-A-A	Parthenogenetic
S. lugubris	E	$2n = 8$	A-A-A-SM	Sexual
S. nova	F	$2n = 6$	M-A-M	Sexual
S. mediterranea	G	$2n = 8$	M-M-SM-M	Sexual
	Gasex[b]	$2n = 8$	M-M-SM-M	Fissiparous
Schmidtea sp.[c]	X	$2n = 8$	M-M-SM-M	Sexual

[a]Chromosomes ordered from greater to smaller relative lengths (M: metacentric, SM: submetacentric, A: acrocentric)
[b]Translocation from one chromosome of the first pair to one chromosome of the third pair (Baguñà et al., 1999)
[c]Putative new species from Corfu (Ball, 1979)

outgroup (Pongratz et al., 2003). Some years later, a *Cox1* sequence of each *Schmidtea* species was included in a study on the molecular phylogeny of the triclads (Álvarez-Presas, Baguñà, & Riutort, 2008). In that study, the four species constituted a monophyletic clade, but the phylogenetic relationships between them remained unclear.

Thus, despite the fact that members of the genus *Schmidtea* are common model organisms in different fields of research, many aspects of their evolutionary history and biogeography are still unknown, which even holds true for the basic anatomy of the species *S. nova*.

In this study, we aim to fill these gaps in our knowledge by (i) inferring a molecular phylogeny of the genus *Schmidtea* that forms a framework for analyzing and understanding its morphological, karyological, and geographic evolution and (ii) describing the anatomy of the species *S. nova*.

2 | MATERIALS AND METHODS

2.1 | Taxon sampling

Sequences of the mitochondrial gene *Cox1* and the *28S ribosomal RNA* gene (*28S*) of the different *Schmidtea* species were downloaded from GenBank. In the case of *S. mediterranea* and *S. polychroa*, those sequences were selected that capture the maximum genetic variability of the species, based on previous studies (Lázaro et al., 2011; Pongratz et al., 2003), while for *S. lugubris* and *S. nova*, all available *Cox1* sequences were included in the analyses. New material was also collected for *S. lugubris* and *S. nova* to obtain new DNA sequence information of both species as well as morphological information of *S. nova*. A total of three newly sampled individuals of *S. lugubris* and nine newly sampled individuals of *S. nova* were included in the analyses. Before performing the genetic and morphological analyses, individuals from the new localities, putatively belonging to *S. lugubris* and *S. nova*, were identified by karyotyping, in order to corroborate that they indeed belonged to the biotypes E and F, respectively. Unfortunately, we were not able to obtain material belonging to the possibly new *Schmidtea* species from Corfu (see Ball, 1979).

With respect to *S. lugubris*, each of three animals was cut into two pieces and treated in the following manner: (i) The anterior part (from the head to the prepharyngeal region) was placed in 1:1 tap water/distilled water mixture—which we call planarian water—to keep the animals alive for the karyological analyses, while (ii) the rest of the body was fixed in 100% ethanol for molecular work. Four of the nine *S. nova* animals were cut into the following three pieces: (i) head region (for karyological identification), (ii) head region to prepharyngeal region (for molecular work), and (iii) rest of the body (fixed in Bouin's fluid for the morphological analysis of the copulatory apparatus). However, the morphological analysis was only performed on two of these individuals. The five remaining specimens of *S. nova* were fixed in their entirety in Bouin's fluid to obtain morphological information from other parts of the body than only the copulatory apparatus. Sequences of the species *Recurva postrema* Sluys & Solà, 2013, were included as outgroup in the phylogenetic analysis, as this genus has been shown to be the sister group of *Schmidtea* (Sluys et al., 2013). Detailed information on the localities and the individuals used in each analysis is given in Table 2.

2.2 | Karyological identification of new material of *S. lugubris* and *S. nova*

Anterior fragments of animals from the new populations putatively belonging to *S. lugubris* and *S. nova* that were kept in planarian water were used for karyological identification. Chromosome preparations were obtained following a protocol established by M. Vila-Farré and J. Rink (personal communication), with some modifications: A small piece of 1–2 mm was cut off from each fragment and incubated for 24 hr in a solution containing 1% DMSO and 0.04% nocodazole (1 mg/ml) in planarian water. After 24 hr of incubation, the small pieces were washed with planarian water and fixed with 3:1 solution of methanol/glacial acetic acid, after which they were washed with a solution of 45% glacial acetic acid in water and, subsequently, incubated at room temperature for 20 min. Hereafter, the fragments were placed on a glass slide and minced to small particles using a surgical blade. The particles were recovered in 100 μl of 45% glacial acetic acid in water and dissociated by pipetting. Finally, the cell suspension was dropped onto a glass slide (preheated at 65°C in a heat block) and left to air-dry during 1 hr. Hereafter, preparations were stained for 1 min with a solution of 1:20 Giemsa/tap water. The chromosome preparations were covered with a coverslip using DPX and examined with a compound microscope. At least 10 metaphasic plates per individual were examined.

2.3 | DNA sequence data

Genomic DNA was extracted from pieces fixed previously in ethanol 100% using the commercial reagent DNAzol (Molecular Research Center Inc., Cincinnati, OH), following the manufacturer's instructions. For each individual, DNA sequences of the *Cox1* and *28S* genes were obtained by PCR amplification (the sequences of the *28S* were obtained by amplifying two overlapping fragments). The final volume of each PCR reaction was 25 μl: including 1 μl of genomic DNA (50 ng/μl), 5 μl of Promega 5x Buffer, 2 μl of $MgCl_2$ (25 mM), 1 μl of dNTPs (10Mm), 0.5 μl of each primer (25 μM), 0.15 μl of Taq polymerase (GoTaq Flexi DNA Polymerase of Promega), and 14.85 μl of double distillate autoclaved water. The amplification conditions for the *Cox1* were the following: (1) 2 min at 94°C, (2) 45 s at 94°C, (3) 50 s at 43°C, (4) 35 s at 72°C, and (5) 3 min at 72°C. The steps 2, 3, and 4 were repeated for 40 cycles. Both fragments of the *28S* gene were amplified using the same conditions: (1) 1 min at 94°C, (2) 45 s at 94°C, (3) 45 s at 55°C, (4) 45 s at 72°C, and (5) 4 min at 72°C. In this case, the steps 2, 3, and 4 were repeated for 35 cycles. Primer sequences are detailed in Table 3. Amplification products were visualized in a 2% agarose gel and subsequently purified using a vacuum system (MultiScreen™ HTS Vacuum Manifold of Millipore). The purified amplification products were sequenced in both directions by Macrogen, Inc. (The

TABLE 2 Information on specimens used for the molecular, morphological, and karyological studies

Species	Locality[a]	Collectors (date)	Code	*Cox1*[b]	*28S*[b]	Biotype[c]	Morphological analysis of *S. nova*
S. polychroa	Catalonia, Spain		S.poly0	-	DQ665993[d]	-	
	Valencia, Spain		S.p2	AF287133[d]	—	A	
	München, Germany		S.p3	AF287132[d]	—	B or C	
S. mediterranea	Montjuïc, Barcelona, Spain	M. Vila-Farré (2012)	S.med00	-	MG457267[d,e]	Gasex	
			S.med01	JF837059[d]	—	Gasex	
	Lebna, Tunisia		S.med1	JF837055	—	G	
	Mazaro, Sicily, Italy		S.med2	JF837062[d]	—	G	
S. lugubris	Nottingham, Great Britain	M. Vila-Farré (2016)	MR00681-1	MG457275[d,e]	—	E[e]	
			MR00681-2	MG457276[d,e]	MG457268[d,e]	E[e]	
			MR00681-3	-	MG457269[d,e]	E[e]	
	Balaton, Hungary		S.lu4	AF290022	—	E	
	Aiud, Romania		S.lu5	AF290021	—	E	
	Lago di Como, Italy		S.lu6	AF290020	—	E	
	Weesp, The Netherlands		S.lu7	AF290019	—	E	
S. nova	Huedin, Romania		S.nova	AF290023		F	
	Kleiwiesen, Braunschweig, Germany	M. Vences (2016)	MR00680-1	MG457277[d,e]	MG457270[d,e]	F[e]	ZMA V.Pl. 7274.1[e]
			MR00680-2	MG457278[d,e]	MG457271[d,e]	F[e]	ZMA V.Pl. 7275.1[e]
			MR00680-3	MG457279[d,e]	MG457272[d,e]	F[e]	
			MR00680-4	MG457280[d,e]	MG457273[d,e]	F[e]	
		O. Segev (2013)					ZMA V.Pl. 7273.1[e]
							ZMA V.Pl. 7273.2[e]
							ZMA V.Pl. 7273.3[e]
							ZMA V.Pl. 7273.4[e]
							ZMA V.Pl. 7273.5[e]
R. postrema[f]	Laerma, Rhodes, Greece		Rpo01	KF308763[d]	MG457274[d,e]		

[a]See Table 4 for detailed information on the localities
[b]GenBank Accession Numbers of the sequences used in this study
[c]See Table 1 for detailed information on the Biotypes
[d]Sequences used in the concatenated dataset for the phylogenetic analysis
[e]Information obtained in this study
[f]Species used as outgroup

TABLE 3 Primer sequences used in this study

Gene	Primer	Sequence 5′-3′	Source
Cox1	BarT (F)	ATGACDGCSCATGGTTTAATAATGAT	Álvarez-Presas et al. (2011)
	COIR (R)	CCWGTYARMCCHCCWAYAGTAAA	Lázaro et al. (2009)
28S	*28S*A1F (F)	TATCAGTAAGCGGAGGAAAAG	Álvarez-Presas et al. (2008)
	*28S*4R (R)	CCAGCTATCCTGAGGG	
	*28S*2F (F)	CTGAGTCCGATAGCAAACAAG	
	*28S*6R (R)	GGAACCCCTTCTCCACTTCAGT	

Netherlands) with the same primers used to amplify the fragments. Complementary strands of DNA were assembled into consensus and edited using Geneious version 10 (http://www.geneious.com; Kearse et al., 2012).

A total of 16 sequences of *Cox1* and nine sequences of *28S* were aligned using the online software MAFFT version 7 (Katoh & Standley, 2013) and revised with Geneious. The *Cox1* alignment was translated into amino acids to check the reading frame.

The degree of saturation of both alignments was checked with the program DAMBE (Xia & Xie, 2001) by performing the test of substitution saturation (Xia & Lemey, 2009; Xia, Xie, Salemi, Chen, & Wang, 2003). For *28S*, it was done at nucleotide level and for *Cox1*,

being a protein-coding gene, it was checked at the three codon positions.

Genetic distances of *Cox1* and *28S* genes between the four *Schmidtea* species and between the genera *Schmidtea* and *Recurva* were calculated under the Kimura 2-parameters substitution model using the software MEGA version 5 (Tamura et al., 2011) to compare them with the distances found between other freshwater planarian species in previous works.

2.4 | Phylogenetic analysis

To infer the phylogeny of the genus *Schmidtea*, three molecular datasets were used, viz. *Cox1*, *28S*, and the concatenated alignments. For the phylogenetic inference of the concatenated dataset, three chimerical sequences were used: (a) *Cox1* of MR00681-1 + *28S* of MR00681-3 (S.lu in the concatenated phylogeny), (b) *Cox1* of S.p2 + *28S* of S.poly0 (S.poly in the concatenated phylogeny), and (c) *Cox1* of S.med01 + *28S* of MR00899 (S.med in the concatenated phylogeny). All sequences used in this study are detailed in Table 2. The program JmodelTest 2.1.1 (Darriba, Taboada, Doallo, & Posada, 2012) was used to test which evolutionary model fitted best each molecular marker under the Akaike information criterion (AIC). The best fitting model for both molecular markers was GTR + G, and the evolutionary parameters of each marker were estimated independently for each gene in the concatenated dataset while inferring the phylogeny. Two different methods were used to carry out the phylogenetic inference analysis: maximum likelihood (ML) and Bayesian inference (BI). The ML inference was carried out using the program RaxML 7.0.0 (Stamatakis, 2006) and ran with 1,000 replicates to obtain the bootstrap support of the nodes, excepting the concatenated dataset, which was run with 2,000 replicates. The program MrBayes 3.2 (Ronquist et al., 2012) was used to perform the Bayesian analysis. For the single gene datasets, we ran 1,000,000 generations, sampling a tree every 100 generations, while for the concatenated dataset, we ran 2,000,000 generations, sampling a tree every 200 generations. All analyses were carried out with two independent runs and with a burn-in of 25% to infer the tree topology and the posterior probability of the nodes. Before applying the burn-in, it was checked through the standard deviation of splits value that convergence of the two runs had been achieved and that each run had arrived at the stationary region.

2.5 | Morphological analysis of *S. nova*

Specimens of *S. nova* that had been preserved for anatomical analysis were cleared in clove oil and, subsequently, embedded in synthetic wax. Sagittal sections were made at intervals of 8 μm and horizontal sections at intervals of 7 μm and, subsequently, were stained in Mallory-Cason/Heidenhain (cf. Winsor & Sluys, 2018) and mounted in DPX. Reconstructions of the copulatory complex were obtained using a camera lucida attached to a compound microscope. The material is deposited in Naturalis Biodiversity Center, Leiden, The Netherlands (ZMA collection code).

2.6 | Distribution data of *Schmidtea*

Distribution records of the four *Schmidtea* species were extracted from (i) publications, (ii) the Turbellarian Taxonomic Database version 1.7 http://turbellaria.umaine.edu. (Tyler, Schilling, Hooge, & Bush, 2006–2016; last consulted March 2017), or (iii) were based on our own new records. Information and references about all distribution records compiled in this study are detailed in Table 4.

Abbreviations used in the figures: am, ampulla; bc, bursal canal; cb, copulatory bursa; ed, ejaculatory duct; go, gonopore; lu, lumen; od, oviduct; ov, ovary; ph, pharynx; pp, penis papilla; pov, parovarium; psv, primary seminal vesicle; sg, shell gland; ssv, secondary seminal vesicle; te, testis; vd, vas deferens; vi, vitellarium.

3 | RESULTS

3.1 | Karyological identification of new material of *S. lugubris* and *S. nova*

The four individuals studied putatively belonging to *S. nova* showed a karyotype of $2n = 6$ with a haploid complement consisting of one large metacentric chromosome, an acrocentric chromosome, and a short metacentric chromosome (Figure 1a), which corresponds to the biotype F and thus to the species *S. nova*. The three individuals putatively belonging to *S. lugubris* showed a karyotype of $2n = 8$, with a haploid set consisting of three acrocentric chromosomes and a small submetacentric chromosome (Figure 1b), which corresponds to biotype E and thus to the species *S. lugubris*.

3.2 | DNA sequence data

All sequences are deposited in GenBank (Table 2) and alignments in TreeBASE (TB2:S21823). The alignment of the *Cox1* gene fragment had a total length of 321 bp, while the alignment of the *28S* had a final length of 1,529 bp. The alignment of the *Cox1* revealed some saturation in the 3rd codon position, but no significant differences between the Index of Substitution Saturation (Iss = 0.5717) and the Critical Index of Substitution Saturation (Iss.c = 0.5921) in a two-tailed test (p-value = 0.6580) was found. However, the saturation in this position was detected between *Schmidtea* and the outgroup (*Recurva postrema*), and, therefore, we carried out the phylogenetic inference basing on *Cox1* only with information for the four *Schmidtea* species. The *Cox1* dataset showed a total of 107 variable sites. On the other hand, no signals of saturation were detected in the alignment of the *28S* marker, which showed a total of 212 variable sites (including the outgroup). The *Cox1* sequence of *R. postrema* was used in the concatenated dataset, because with fewer sequences the degree of saturation was not critical (Iss = 0.6034; Iss.c = 0.7426; p-value = .0056).

The genetic distances of *Cox1* and *28S* between the four *Schmidtea* species and between *Schmidtea* and *Recurva* are detailed in Table 5. The mean genetic differentiation for *Cox1* within *Schmidtea* ranges from 18.7% (between *S. mediterranea* and *S. polychroa*) to 26.6% (between *S. mediterranea* and *S. lugubris*). For the

TABLE 4 Sources of the compiled distribution data of *Schmidtea* as depicted in Figure 3

	S. polychroa	*S. mediterranea*	*S. lugubris*	*S. nova*	*Schmidtea* sp.
Bibliography	- Pongratz *et al.* (2003) and references therein - Vila-Farré *et al.* (2011) - Harrath *et al.* (2012)	- Lázaro *et al.* (2011) and references therein	- Benazzi & Puccinelli (1973) and references therein - Baas (1986) - Pongratz *et al.* (2003) - GenBank Acc. AF290022 - GenBank Acc. AF290019 - Turbellarian Taxonomic Database	- Benazzi & Puccinelli (1973) and references therein - Baas (1986) - Pongratz *et al.* (2003) - Segev *et al.* (2015)	- Ball (1979)
	S. polychroa		*S. lugubris*		
New localities/ Collectors*	- Benavente, Spain (42.02737N 5.7085W) / MAP, MR and LL - Sèishes, France (43.50112N 001.28023E) / MAP, ES and LL - Liérganes, Spain (43.346211N 3.74445W) / MVF, ES and LL - Riba-roja, Spain (41.2415152N 0.4365181E) / AM - Cinca, Spain (41.8904491N 0.1650096E) / JO - Fluvià,Spain (42.161142N 2.958923E) / MR - Fluvià, Spain (42.174400N 2.823688E) / MR - Montjuïc, Spain (41.366375N 2.158747E)		- Nottingham, Great Britain (52.947711N 1.097431W)/ MVF		

*AM: A. Munté; ES: E. Solà; JO: J. Oscoz; LL: L. Leria; MAP: M. Álvarez-Presas; MR: M. Riutort and MVF: M. Vila-Farré

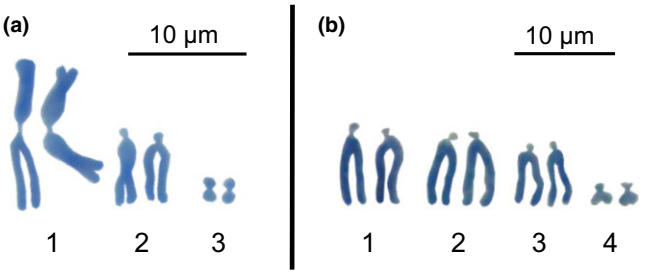


FIGURE 1 Chromosome complements, arranged in pairs, of the new populations of *Schmidtea nova* and *Schmidtea lugubris* used in this study. (a) *S. nova* from Kleiwiesen (Germany); (b) *S. lugubris* from Nottingham (United Kingdom)

28S marker, the genetic differentiation within *Schmidtea* ranges from 3.6% (between *S. mediterranea* and *S. polychroa*) to 6% (between *S. polychroa* and *S. nova*). The genetic distance between *Schmidtea* and *Recurva* in the *28S* marker is about 10%.

3.3 | Phylogenetic analysis

The phylogenetic trees obtained with the *28S* (Figure S1) and the concatenated datasets (Figure 2) resulted in the same topology for both Bayesian and maximum likelihood inference methods. In all analyses, each of the four *Schmidtea* species was monophyletic and grouped into two main clades: one containing *S. lugubris* and *S. nova* and the other comprising *S. mediterranea* and *S. polychroa*. The phylogenetic tree based on *Cox1* was rooted according to the obtained topology basing on *28S* and concatenated analyses. In the *Cox1* phylogeny (Figure S2), the sequence of *S. nova* from Huedin (Romania) clustered with the new samples of *S. nova* from Kleiwiesen (Germany). On the other hand, the six samples belonging to five localities of *S. lugubris* formed two different groups: one containing the localities of Aiud (Romania), Balaton (Hungary), and Lago di Como (Italy) and the other comprising the locality of Weesp (The Netherlands) and the new locality of Nottingham (Great Britain). However, these two groups had low support.

3.4 | *Schmidtea* distribution

The distribution of the four *Schmidtea* species is shown in Figure 3. *S. mediterranea* shows a scattered distribution in the western Mediterranean islands together with two localities on the coasts of Catalonia (Spain) and Tunisia. *S. polychroa* presents a wider distributional range in western Europe that extends from the Iberian Peninsula to Hungary and further north up to Sweden, as well as the United Kingdom; it includes also the Mediterranean islands of Sardinia and Sicily and two localities in northern Africa. The distribution of *S. lugubris* has a more eastern emphasis in that it ranges from the United Kingdom and The Netherlands to eastern Europe and Russia. However, it should be borne in mind that due to past confusion between *S. lugubris* and *S. polychroa* older records may not be fully trustworthy. An example of such confusion is the presumed occurrence of *S. lugubris* in North America, as documented by Ball (1969), which actually concerns *S. polychroa*. Finally, the few known localities of *S. nova* are scattered over a region extending from northern Italy to Central and Northern Europe. According to Benazzi and Puccinelli (1973, and references therein), biotype F, that is, *S. nova*, had been found in Sweden, Austria, and Italy. Chromosomal portraits of biotype F were also reported from four localities in The Netherlands

TABLE 5 Mean genetic distances (±*SD*) of *Cox1* and *28S* between the four *Schmidtea* species, as well as between these four taxa and the outgroup (only for *28S*)

	S. mediterranea	*S. polychroa*	*S. lugubris*	*S. nova*
Cox1				
S. mediterranea	0.037 ± 0.032			
S. polychroa	0.187 ± 0.016	0.032		
S. lugubris	0.266 ± 0.006	0.222 ± 0.013	0.020 ± 0.014	
S. nova	0.203 ± 0.01	0.218 ± 0.005	0.218 ± 0.015	0.003 ± 0.002
28S				
S. mediterranea				
S. polychroa	0.036			
S. lugubris	0.047 ± 0.0008	0.047	0	
S. nova	0.053 ± 0.0005	0.060 ± 0.0007	0.037 ± 0.0004	
R. postrema[a]	0.105	0.110	0.103 ± 0.0012	0.114 ± 0.0012

[a]Species used as outgroup

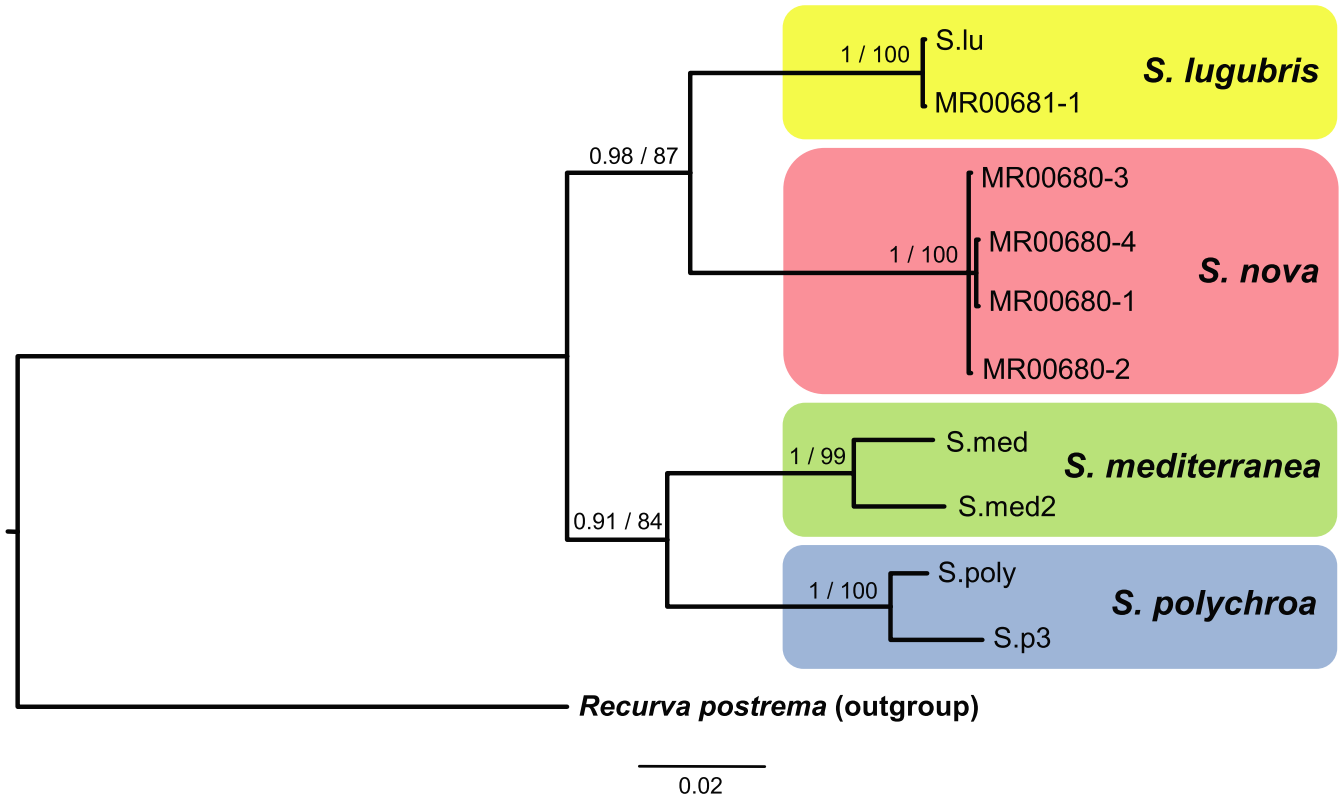


FIGURE 2 Bayesian Inference tree of the concatenated dataset (*Cox1* + *28S*). Numbers at nodes indicate posterior probability/bootstrap support values. Scale bar is proportional to the number of substitutions per site. Color of panels corresponding to color codes used in the distribution map of the species (see Figure 3)

(Baas, 1986). The animals described in this study represent the first record of *S. nova* for Germany.

3.5 | Systematic and morphological account of *S. nova*

Order **TRICLADIDA** Lang, 1884

Suborder **CONTINENTICOLA** Carranza, Littlewood, Clough, Ruiz-Trillo, Baguñà, & Riutort, 1998

Family **DUGESIIDAE** Ball, 1974

Genus *Schmidtea* Ball, 1974

Schmidtea nova (Benazzi, 1982)

3.5.1 | Material examined

Neotype, ZMA V. Pl. 7273.1, Kleiwiesen, Braunschweig, Germany, N52°32′84,4″ E10°20′20,57″, May 2013, sagittal sections on 14 slides.

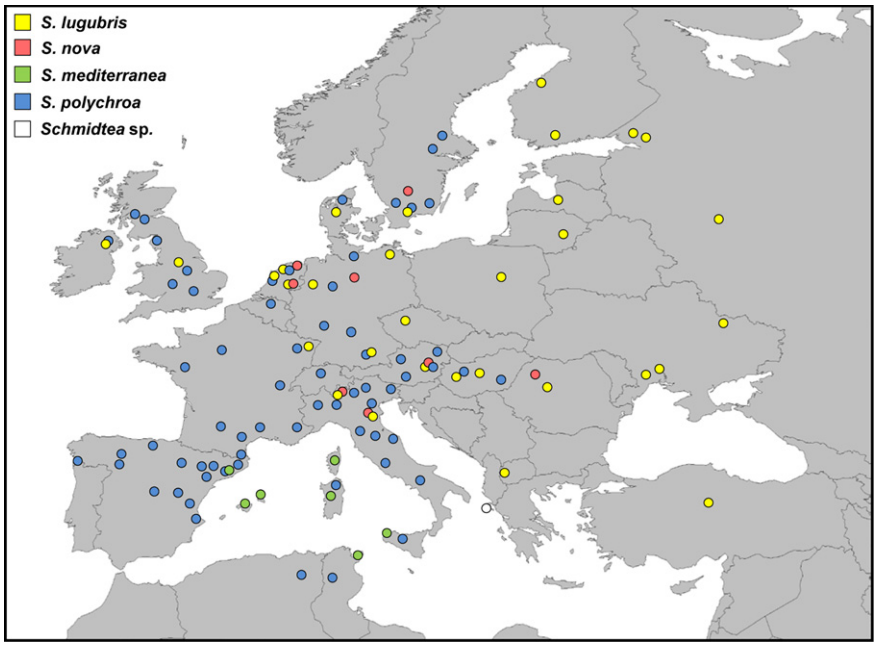


FIGURE 3 Geographic distribution of *Schmidtea* species, compiled from bibliography and from our own new samplings (see Table 4)

Other material: ZMA V.Pl. 7273.2, ibid., sagittal sections on 17 slides; ZMA V.Pl. 7273.3, ibid., sagittal sections on 17 slides; ZMA V.Pl. 7273.4, ibid., sagittal sections on 15 slides; ZMA V.Pl. 7273.5, ibid., horizontal sections on 13 slides.

ZMA V.Pl. 7274.1, ibid., sagittal sections on 20 slides.

ZMA V.Pl. 7275.1, ibid., sagittal sections on 12 slides.

3.5.2 | Diagnosis

Schmidtea species with (i) interconnected testis follicles, (ii) par-ovaria interpolated between the ovaries and the anteriormost ends of the oviducts, (iii) the ejaculatory duct exiting into the primary as well as the secondary seminal vesicle by means of a small papilla or nipple, (iv) the wall of the distal section of the ejaculatory duct being sclerotic or chitinized, (v) a bursal canal showing at about half-way its length a large, egg-shaped expansion of its surrounding coat of muscles, (vi) a chromosome complement of $2n = 6$, with a haploid set of one long metacentric chromosome, one acrocentric, and one very small metacentric chromosome, and (vii) as molecular diagnosis: including all specimens that cluster with individuals MR00680-1, MR00680-2, MR00680-3, and MR00680-4, (Table 2) from this study, with significant support in an adequate molecular delimitation model.

3.5.3 | Neotype locality

When Benazzi (1982) coined the new specific epithet *nova* for *Schmidtea* specimens belonging to biotype F, he did not deposit any type specimens, nor indicated a type locality. However, from earlier publications (Benazzi et al., 1970 and references therein), it becomes clear that the first animals for which he established the biotype F karyotype came from a population sample specifically taken at the topotypical locality of *S. lugubris*, that is, somewhere near Graz, Austria. Therefore, one may be inclined to consider the environs of Graz to be the type locality of *S. nova*. However, we consider this to be unsatisfactory and undesirable for three reasons. First, it is known that the biotypes E (in our current understanding, *S. lugubris*) and F (*S. nova*) may coexist at the same locality (Baas, 1986; Benazzi et al., 1970), which is also evident from the situation that apparently *S. nova* occurs near Graz, being already the type locality of *S. lugubris*. Second, although Benazzi et al. (1970) provided a reconstruction drawing of the copulatory apparatus of a biotype F topotypical specimen from Graz, none of their material was specifically designated as constituting the type specimen(s), while it was neither deposited in a natural history museum nor any other zoological collection from which it can be easily retrieved. Furthermore, it seems that the Benazzi collection of slides has been lost (G. Stocchino pers. comm.) and that therefore there is only a very remote possibility that this material may become available again at some time in the future. Third, although there are indications that the specimens examined by Benazzi et al. (1970) share similarities with our account of *S. nova* (see Comparative discussion below), there is thus presently no possibility to compare that material with our specimens. In view of the above, we prefer to designate as neotype locality of *S. nova* the site from where the specimens originated that we extensively analyzed in our present study, that is, Kleiwiesen, Germany (N52°32′84,4″ E10°20′20,57″). This is in accordance with Recommendation 76A.1.4 of the current International Code of Zoological Nomenclature (ICZN 1999) in that the neotype locality falls well within the known range of biotype F.

3.5.4 | Description

Head obtusely pointed, with two eyes, set in pigment-free patches, rather close to the anterior margin of the body (Figure 4). Specimen ZMA V.Pl. 7273.5 has a pair of supernumerary eyes, thus having

FIGURE 4 *Schmidtea nova*. External features of live specimen. Scale bar is not available

four eyes in total, the supernumerary eyes being somewhat smaller than the other ones. Posterior to the auricular region, the body narrows to form a slightly slenderer neck region. Live animals measure up to 1.7 cm in length and 0.4 cm in width; preserved specimens up to 11.5 mm in length and 4.5 mm in width. Dorsal surface is dark brown; ventral surface is dark greyish-brown.

Pharynx situated in the mid-region of the body, measuring between one-eighth and one-ninth of the body length in preserved specimens; it is of the normal, planariid type (Figure 5). Mouth opening situated at the posterior end of the pharyngeal cavity.

Testes situated dorsally, extending from the level of the ovaries to almost the posterior tip of the body. The well-developed follicles are located in the lateral region of the body, being situated between the tips of the intestinal diverticula. The testis follicles are interconnected, thus forming a ramified, irregularly shaped, tubular structure, running in the laterodorsal region of the body (Figure 6).

At the level of the posterior wall of the pharyngeal cavity, the vasa deferentia expand to form spermiducal vesicles, filled with sperm. Hereafter, the ducts recurve and run dorsomedially, meanwhile decreasing in diameter. In the dorsal portion of the body, the ducts head toward the anterior end of the body to open, separately, into the posterodorsal section of the anterior or primary seminal vesicle. From the ventral surface of the latter arises the proximal section of the ejaculatory duct, which gradually decreases in diameter and eventually exits at the tip of a small papilla, projecting into the lumen of the posterior or secondary seminal vesicle (Figure 7). This portion of the ejaculatory duct and the primary seminal vesicle are lined with a flat, nucleated epithelium.

The posterior section of the secondary seminal vesicle narrows to form the long, distal section of the ejaculatory duct, which traverses the penis bulb and penis papilla and ultimately opens at the tip of a small papilla that projects into the wide, most distal section of the ejaculatory duct. This widened, distal section of the ejaculatory duct exits at the tip of the penis papilla.

The secondary seminal vesicle is lined with a flat, nucleated epithelium. In contrast, the wall of the long second part of the ejaculatory duct is fully sclerotic or chitinized, up to its exit at the tip of the small papilla or nipple (Figure 8). At least in the holotype, the lumen of this long, sclerotic portion of the ejaculatory duct is filled with sperm. The entire sclerotic portion of the ejaculatory duct is surrounded by a comparatively thick layer of fine circular muscles, followed by a single layer of longitudinal muscle fibers (Figure 9).

The rather long, cylindrical penis papilla completely fills the male atrium. In some specimens, the distal half of the papilla shows a sharp, knee-shaped bend, so that the tip faces toward the ventral body surface. In such cases, the coat of circular musculature surrounding the bend is highly swollen, thus contrasting with the zone of circular muscle underneath the dorsal surface of the penis papilla (Figure 10). In any case, this zone of subepithelial circular muscles is followed by a thin layer of longitudinal muscles. The penis papilla is lined with a very flat, nucleated epithelium.

The musculature of the penis bulb is extremely well developed and consists of two parts, separated by a constriction. The first part consists of the strong muscle fibers that surround the primary seminal vesicle; these fibers are arranged in an irregular, decussate fashion.

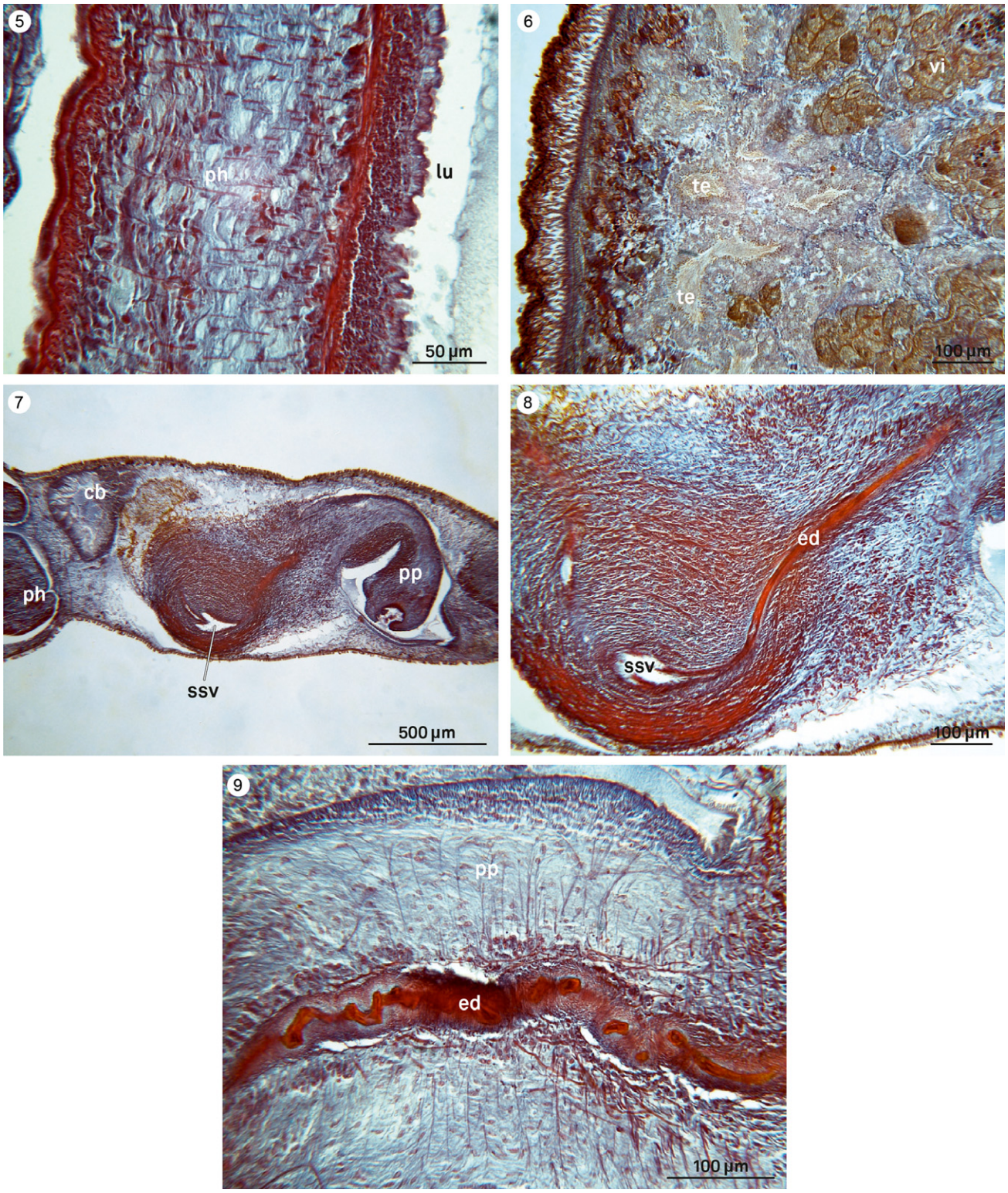
The second part of the bulbar musculature is made up of muscle fibers that surround the ejaculatory duct, being separated from the first part by a kind of constriction at the point where the proximal section of the ejaculatory duct originates from the primary seminal vesicle. From that point to almost the root of the penis papilla, or somewhat anterior to it, the ejaculatory duct is surrounded by regularly crosswise arranged, concentric layers of muscle fibers (Figures 7, 8, and 11). On its periphery and toward the point of insertion of the penis papilla, the zone with concentric layers of muscles grades into a zone with loosely arranged, mostly circular muscles, bounded by some longitudinal fibers.

The paired ovaries are situated at a short distance (generally 700 μm; in one specimen 400 μm) behind the brain, that is, generally at about one-fourth - one-fifth of the distance between the brain and the root of the pharynx (in one specimen at about one-eighth of this distance). The ovaries are rounded, with a maximum diameter of about 250 μm.

After having communicated with the bursal canal, the oviducts run forward immediately dorsally to the ventral nerve cords. In the proximity of the ovaries, the anterior section of each oviduct expands to form an ampulla, which may be filled with sperm. This ampulla communicates with an elongated, sac-shaped structure that is completely filled with small, nucleated cells. This sac, or parovarium, connects with the ventrolateral surface of the ovary (Figure 12).

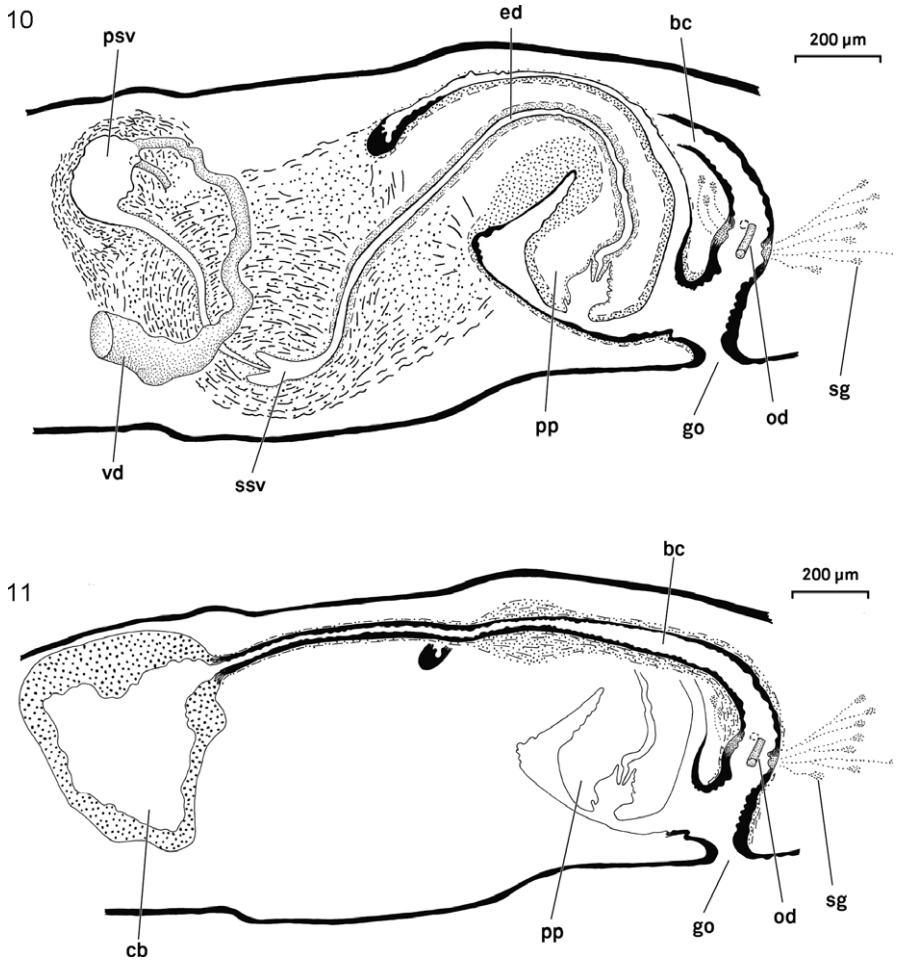
At the level of the gonopore, the narrow oviducts curve dorsomedially to open separately into the ventral part of the bursal canal. Erythrophil shell glands discharge their secretion into the section of the bursal canal immediately ventrally to the oviducal openings, this portion of the canal being slightly expanded (Figures 10 and 11).

From the point where the bursal canal arises from the atrium, it smoothly curves dorso-anteriad, to continue its course more or less parallel to the dorsal body surface, eventually opening into a large, sac-shaped copulatory bursa (Figure 11). The latter lies directly behind the pharyngeal pocket and anterolaterally to the anteriormost portion of the penis bulb (Figure 13). The bursal canal is lined with an epithelium consisting of cuboidal, nucleated cells.



FIGURES 5–9 Microphotographs of *Schmidtea nova*. Figure 5. ZMA V.Pl. 7273.5, horizontal section of the pharynx. Figure 6. ZMA V.Pl. 7273.5, horizontal section, showing the interconnected testes. Figure 7. ZMA V.Pl. 7273.1, sagittal section, showing the gross morphology of the copulatory apparatus. Figure 8. ZMA V.Pl. 7273.1, sagittal section, showing the sclerotic ejaculatory duct; anterior to the left. Figure 9. ZMA V.Pl. 7273.3, sagittal section, showing the sclerotic ejaculatory duct; anterior to the right

At its proximal section, from its origin at the atrium to about the point where it starts to run parallel to the body surface, the bursal canal has a rather wide lumen but then it gradually narrows to become a tube with a regular, narrow lumen. This proximal section of the bursal canal is surrounded by a well-developed coat of muscles, made up of intermingled circular and longitudinal fibers. At about half-way its



FIGURES 10–11 *Schmidtea nova*. Neotype, ZMA V.Pl. 7273.1, sagittal reconstruction of the copulatory apparatus; anterior to the left

length and at about the point where the bursal canal starts to become a regular tube, this coat of muscles is greatly expanded to form an egg-shaped bundle of mixed circular and longitudinal muscles (Figures 11 and 14). This egg-shaped expansion is present in all specimens examined. Anteriorly to the egg-shaped section, the thickness of the bursal canal musculature strongly decreases in diameter, merely consisting of a thin layer of intermingled circular and longitudinal fibers.

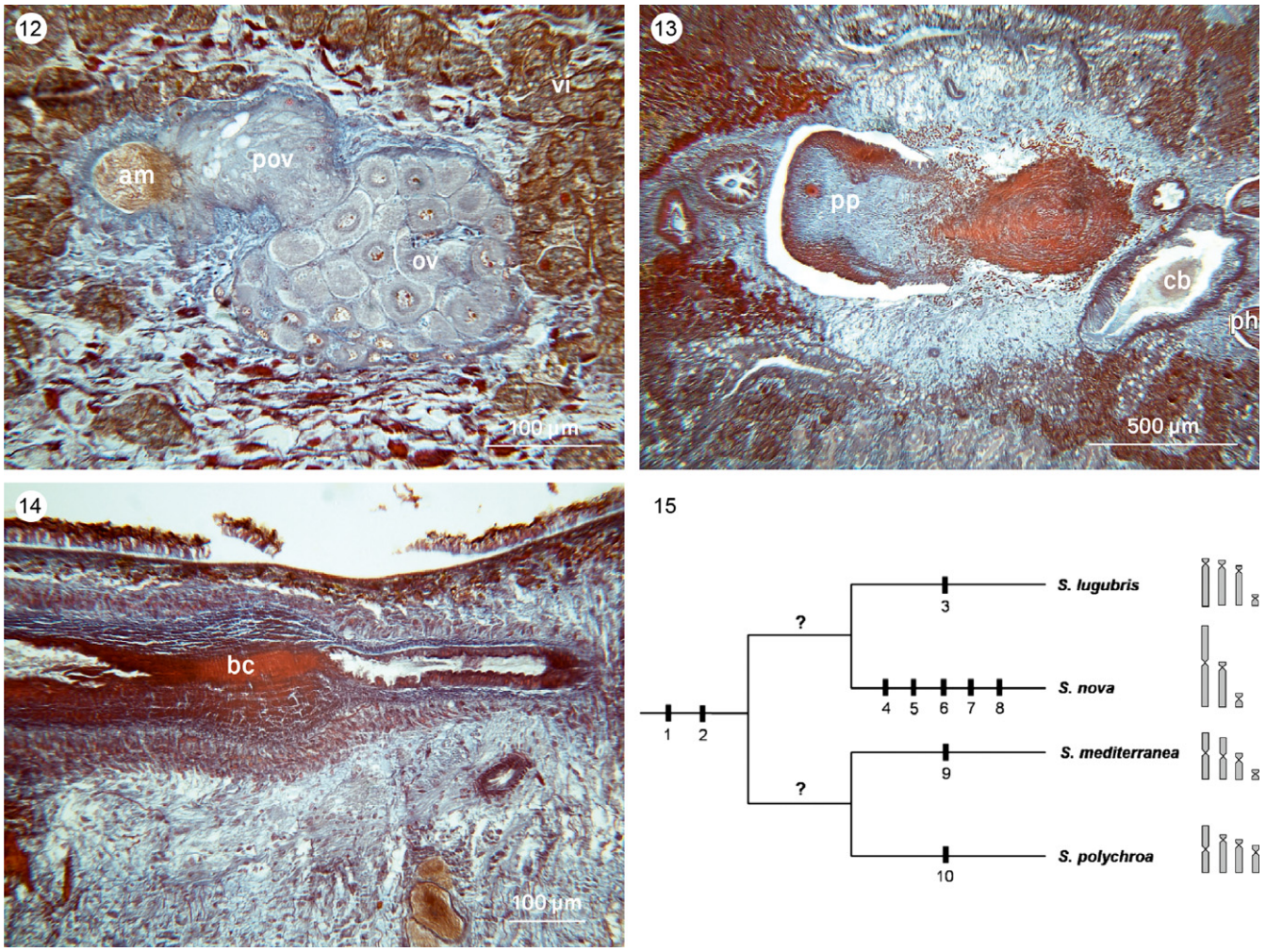
The large, sac-shaped copulatory bursa generally fills the entire dorsoventral space.

3.5.5 | Comparative discussion

In this study, we have provided for the first time an anatomical account of the species *S. nova*. Our specimens from Kleiwiesen, Germany, are here assigned to the genus *Schmidtea* on the basis of the facts that they (i) constitute a molecular monophyletic group with the other three *Schmidtea* species, (ii) exhibit the *S. nova* karyotype, and (iii) show the two morphological apomorphies of this genus, viz. the double, intrabulbar seminal vesicle system, and intermingled bursal canal musculature (cf. Sluys, 2001). In addition to differences in their karyology and molecular markers, there are also anatomical characters that preclude assignment of our specimens to any other known species of *Schmidtea* than *S. nova*. From that perspective, it is opportune to note first the few similarities shared between the rather superficial morphological description of *S. nova* by Benazzi and coworkers and the animals from Kleiwiesen.

First, the gross morphology of the copulatory apparatus of a biotype F animal from Graz (cf. Benazzi et al., 1970, Figure 3.2) is very similar to the situation in the German specimens. In particular, (i) the vasa deferentia open into the posterodorsal section of the primary seminal vesicle, (ii) there is a distinct constriction between the musculature around the primary seminal vesicle and the rest of the bulbar musculature, (iii) concentric layers of muscles surround the proximal section of the ejaculatory duct, (iv) the inside of the distal section of the ejaculatory duct stains strongly erythrophil, (v) there is no permanent nipple at the tip of the penis papilla, and (vi) the penis papilla shows a knee-shaped bend. Several of these features will be discussed below.

Benazzi et al. (1970) stress the fact that there is marked constriction in the musculature of the penis bulb. However, such a constriction occurs not only in *S. nova* but also in *S. lugubris*, *S. polychroa*, and *S. mediterranea* (cf. Luther, 1961; Figure 4; Harrath,



FIGURES 12–15 Microphotographs of *Schmidtea nova* and character tracings. Figure 12. ZMA V.Pl. 7273.5, horizontal section through ovary, parovarium, and ampulla; anterior to the right. Figure 13. ZMA V.Pl. 7273.5, horizontal section, showing the location of the copulatory bursa; anterior to the right. Figure 14. ZMA V.Pl. 7273.4, sagittal section of the bursal canal showing the egg-shaped bundle of muscles; anterior to the right. Figure 15. Presumed synapomorphic and autapomorphic characters as well as karyotypes plotted onto the topology of the molecular tree. Characters: 1: double seminal vesicle; 2: mixed bursal canal musculature; 3: permanent nipple on the tip of the penis; 4: two nipples or papillae in the ejaculatory duct; 5: part of ejaculatory duct being sclerotic; 6: expansion of bursal canal musculature; 7: interconnected testis follicles; 8: parovaria; 9: well-developed atrial folds; 10: penis papilla with a dorsal hump. Question marks represent synapomorphic characters of two lineages of *Schmidtea* that as yet remain elusive

Charni, Sluys, Zghal, & Tekaya, 2004; Figure 1; Harrath et al., 2012, Figure 6).

Benazzi et al. (1970, p. 373) remark that "The eosinophil glands inside the ejaculatory duct are usually more abundant in *lugubris* [i.e., their biotype F animals = *S. nova*], while in *polychroa* eosinophil glands are more frequent near the seminal vesicle." We interpret their description such that the observed strongly erythrophil staining of the ejaculatory duct actually coincides with our observation of the brightly red-stained, sclerotic wall of the ejaculatory duct in *S. nova*.

Benazzi and Puccinelli (1973) and Benazzi et al. (1975) pointed out that animals of biotype F, that is, specimens of *S. nova*, lack the permanent nipple on the tip of the penis papilla that is so characteristic of *S. lugubris*.

Although not all of our German specimens show the downward knee-shaped bend in the penial papilla, it is noteworthy that such a bend is expressed also in the specimen from Graz examined by Benazzi et al. (1970, Figure 3.2).

It is curious that Benazzi and coworkers did not observe or describe the conspicuous egg-shaped thickening of the bursal canal musculature, which, we presume, must have been present also in their specimens from Graz.

Apart from karyological and molecular markers (see above), the anatomy of our *S. nova* animals from Germany differs in a good number of details from that of *S. lugubris*, *S. mediterranea*, and *S. polychroa*. In particular, *S. lugubris* exhibits a permanent nipple on the tip of the penis, while the penis papilla of *S. polychroa* is provided with a dorsal hump, structures that are absent in

both *S. mediterranea* and *S. nova*. Characteristic for *S. mediterranea* is well-developed atrial folds, rich in eosinophil glands, that as such do not occur in its congeners. Within *Schmidtea*, fusion or interconnection between testis follicles is unique to *S. nova*. Such fusion or partial fusion of the testicular follicles is a rare phenomenon among triclads and has been reported for only a small number of other species (cf. Sluys & Riutort, 2018).

Along its course through the penis, on two occasions, the ejaculatory duct of *S. nova* forms a small papilla or nipple through which it opens into a more distal continuation of the duct. These well-developed papillae or nipples are unique to *S. nova*, albeit that occasionally specimens of *S. polychroa* may show the anterior nipple, but then only weakly developed (cf. Harrath et al., 2012, Figure 6).

In the above, we described a sac-shaped structure that lies interpolated between each of the ovaries and the ampulla at the most anterior end of the oviducts and for which we used the term parovarium. This term parovarium has been applied to a variety of structures attached to the ovaries of triclads, such as rudimentary ovaries and rudimentary yolk glands (cf. Jones & Sluys, 2016 and references therein). But even with such a broad definition, parovaria are rare among triclads and, to the best of our knowledge, structures similar to the parovaria of *S. nova* have not been reported previously for planarians.

3.6 | Character tracings

On the phylogenetic tree of the genus *Schmidtea*, we have plotted the presumed morphological and karyological synapomorphies and autapomorphies of the several taxa (Figure 15). At a morphological level, all four species exhibit the two synapomorphies of the genus (Figure 15, characters 1 and 2). Morphological autapomorphies for each species are also present (Figure 15, characters 3–10) but, thus far, synapomorphies supporting the sister group relationship between *S. lugubris* and *S. nova*, as well as for the monophyletic clade *S. mediterranea–S. polychroa*, remain elusive. Unfortunately, outgroup comparison is unable to provide any hint as to the number of chromosomes of the ancestor because the phylogenetically nearest genera present completely different haploid complements or are unknown (*Girardia* $n = 4$, $n = 8$, and $n = 9$; Cura $n = 6$; *Dugesia* $n = 7$, $n = 8$, and $n = 9$; Recurva n=?). However, the most parsimonious explanation for the distribution of the character states on the tree may be to presume that the ancestor of the genus had a chromosomal set of $n = 4$ and that subsequently *S. nova* changed to $n = 3$; otherwise, multiple changes to haploid sets with four chromosomes have to be postulated.

4 | GENERAL DISCUSSION

4.1 | Rapid speciation within *Schmidtea*

Our well-resolved molecular phylogeny for the genus *Schmidtea* (Figure 2) reveals two lineages, viz. *S. lugubris* and *S. nova* on the one hand, and *S. mediterranea* and *S. polychroa* on the other hand. These phylogenetic relationships give support to an earlier evolutionary hypothesis based on karyological data, stating that *S. nova* and *S. lugubris* diverged from a common ancestor with a chromosome complement of $n = 4$ (which would be the plesiomorphic state for *Schmidtea*) through a Robertsonian translocation plus a pericentric inversion that thus resulted in the three basic chromosomes, including a very short metacentric one, of *S. nova* (Benazzi & Puccinelli, 1973). On the other hand, the ancestor of *S. mediterranea* and *S. polychroa* may have evolved through different translocations and pericentric inversions (Benazzi, 1982). These results suggest that in both cases speciation in this genus was associated with chromosomal rearrangements. Speciation events due to chromosomal rearrangements tend to be rapid, as they can entail a sudden reproductive isolation between the new lineages (Faria & Navarro, 2010). This situation can be reflected as morphological and molecular radiations.

From a morphological point of view, autapomorphies for each *Schmidtea* species are present, while morphological synapomorphies for the sister group relationships between *S. lugubris* and *S. nova* and between *S. mediterranea* and *S. polychroa* are currently unknown (see above). Moreover, the branch lengths of the two lineages in the molecular phylogenies are very short, as compared with the branch lengths of each of the species (Figure 2; Figures S1 and S2). This suggests that the diversification of these two lineages occurred in a relatively short period of time, giving support to the hypothesis that speciation within *Schmidtea* may have been a consequence of chromosomal rearrangements.

Speciation events associated with such chromosome rearrangements have been suggested for other planarian genera (Benazzi, 1982) as well as for other turbellarian groups (Curini-Galletti, Puccinelli, & Martens, 1985; Galleni & Puccinelli, 1986). It demonstrates that the morphologically rather conservative triclads may exhibit a great plasticity in chromosomal composition. This plasticity may be a problem for many groups of animals as changes in chromosome structure and number will tend to result in unbalanced karyotypes (at least temporarily) that may disrupt meiotic processes, thus resulting in a failure in reproduction. However, planarians may avoid this problem thanks to their ability to reproduce asexually, similarly to what happens in plants. For instance, in *S. mediterranea*, there are populations that reproduce exclusively asexually by fission that in their chromosome portrait present a translocation between two chromosomes, with only one chromosome of each pair being affected (Baguñà et al., 1999). In contrast, all diploid populations that do not have this translocation reproduce sexually. One may interpret this situation such that in planarians changes in karyotype are not selected against because the animals can endure situations with unbalanced chromosomes due to their ability to reproduce asexually (either by fission or by parthenogenesis). Eventually, this condition may evolve to a new balanced karyotype and the recuperation of sexuality.

4.2 | Biogeography of *Schmidtea*: four relictual species that recently spread over Europe?

The inferred existence of two lineages within *Schmidtea* is concordant also with the geographic distribution of the species. The two sister species *S. mediterranea* and *S. polychroa* are found in western Europe, while *S. lugubris* and *S. nova* principally occur in northeastern Europe and the Middle East, albeit that there is overlap between these two groups in central Europe (Figure 3). This biogeographic pattern suggests that a western and an eastern lineage could have evolved after the division of the range of the ancestor of *Schmidtea*. However, to be able to formulate an hypothesis on the timing of this first split, a larger dataset, together with a calibration analysis, would be needed. Nevertheless, previous studies already provide some hints.

A study by Lázaro et al. (2011) on the phylogeography of *S. mediterranea* dated the split between this species and its sister *S. polychroa* at around 43 Mya (72.23–24.96 Mya), which would situate the origin of the genus at an even older date. Furthermore, a study on the diversification within the genus *Dugesia*, using these two *Schmidtea* species as outgroup, gave an age for this split at around 52 Mya (90–27 Mya), while the separation of *Schmidtea* from *Recurva* may go back to 150 Mya (Solà, 2014); these results imply that the first split within *Schmidtea* lies somewhere between 52 and 150 Mya. Although all of these datings are based on geographic and geological calibration points and, therefore, should be taken with some caution, they are based on different events, thus providing mutual reinforcement. Moreover, the observed high *Cox1* genetic distances between *Schmidtea* species (reaching 27% between *S. mediterranea* and *S. lugubris*) support the presumably ancient origin of the genus. When compared to the genus *Dugesia* (which is the sister genus of *Schmidtea* plus *Recurva*), the genetic distance values between the *Schmidtea* species are equivalent to the values found for the separation of the *Dugesia* lineages from Africa and Eurasia, while the distances between the European *Dugesia* species are much lower (below 15%) (Lázaro et al., 2009; Solà, Sluys, Gritzalis, & Riutort, 2013).

It is surprising that, despite its putative ancient origin, diversity in *Schmidtea* is so low, with currently only four described species. This low species diversity is shared with its sister genus *Recurva*, which only has two described species, endemic to the Greek Islands (Sluys et al., 2013). This differs greatly from the genus *Dugesia* (the only other dugesiid genus inhabiting the Palearctic Region), which has more than 80 described species distributed throughout Africa, Europe, Asia, and part of Oceania (Solà, 2014), with about 20 species in Europe (covering the same region occupied by *Schmidtea* and *Recurva*). The diversification of European *Dugesia* has been dated at 50–10 Mya (Solà, 2014; Solà et al., 2013), which is younger than the putative diversification of *Schmidtea* (see above). This suggests that the current species of *Schmidtea* and *Recurva* could be the relicts of two genera that ranged over Laurasia a long time ago but that lost their diversity due to paleogeological or paleoclimatic events, such as the risings and fallings of the sea level that affected Europe until the lower Oligocene (around 32 Mya) and resulted in the division of the continent into multiple islands (Rasser et al., 2008). On the other hand, *Dugesia* could have arrived later in Europe, just in time to colonize a recently reunified continent. Evidently, ecological or physiological differences between the two genera may contribute also to such a difference in their present diversity.

Apart from an eastern versus western biogeographic pattern in *Schmidtea*, three species are sympatric in central Europe (*S. polychroa*, *S. nova*, and *S. lugubris*), even coexisting at some localities. In a study on the biogeography of parthenogenetic and sexual populations of *S. polychroa*, it was proposed that this species may have diversified over Europe long before the glacial period and that recolonization from different refugia in southern and central Europe after the Last Glacial Maximum (approx. 21,000 years ago) had put into contact highly diverged populations (Pongratz et al., 2003). In the present study, no clear phylogenetic structure, based on the *Cox1* marker, has been found within *S. nova* and *S. lugubris* (Figure S2, Table 5), suggesting that the same relatively recent paleoclimatic events that affected S. *polychroa* could have affected also the range evolution of *S. nova* and *S. lugubris*, with the difference that their refugia may have been situated in eastern Europe. This suggests that current sympatry of these three species may be the result of secondary contact. On the other hand, a population level study of *S. mediterranea* (Lázaro et al., 2011) found no evidence for glaciations having affected the geographic evolution of this species. However, this species seems to be confined mainly to a few islands in the western Mediterranean and to the northern part of Africa already before the Pleistocene and, therefore, may not have been subjected to very low temperatures and may not have had a possibility to recolonize continental Europe. The only continental population of *S. mediterranea* currently present in the north of Catalonia in Spain may be the relict of a once wider distributional range of this species in continental Europe that was disrupted by the glaciations.

Our results, together with previous data, suggest that a more elaborate analysis of the biology, population genetics, and phylogeny of *Schmidtea*, as well as *Recurva*, may be of great interest. Such a study might be able to pinpoint which factors (historical, geological, ecological, physiological, and karyological) drive the diversification and dispersion of this group of freshwater animals, thus forming a model for a larger group of animals with similar characteristics.

4.3 | Competition within *Schmidtea*

As no ecological studies have been performed at the localities where different *Schmidtea* species coexist, it is not known yet if they occupy different niches or whether they actually compete for resources. In this context, it is worth noting that in the western lineage some changes in distribution have been observed during recent years, suggesting that *S. polychroa* may be expanding, while *S. mediterranea* is disappearing from several localities. For instance, *S. polychroa* had no known sites in Catalonia (Spain) in the 1980s (Baguñà, Saló, & Romero,

1981) and only one population near Valencia was reported in 1991 (Baguñà, 1991), whereas, recently, this species has been found in many rivers in Catalonia (Vila-Farré, Sluys, Almagro, Handberg-Thorsager, & Romero, 2011). One may be inclined to think that the species simply may have gone unnoticed. However, we have clear examples at localities that we have been sampling intensively for years, such as the Tres Pins locality in Barcelona. From here, previously, only *S. mediterranea* was known, while, currently, *S. polychroa* together with other introduced planarians (*Dugesia sicula* for instance) are more frequent (Lázaro & Riutort, 2013; Vila-Farré et al., 2011). Also human activities, as draining marshes and plant trade, may have affected the distribution of species of *Schmidtea*. For example, the draining of marshes was hypothesized to explain the disappearance of *S. mediterranea* from some localities in Girona over the last 30 years (Lázaro & Riutort, 2013; Vila-Farré et al., 2011). The Tres Pins locality in Barcelona concerns a municipal plant nursery, which makes it very likely that *S. polychroa* was introduced there, together with aquatic plants, and nowadays its population is increasing and involved in a natural competition with its local congener, *S. mediterranea*. These examples of putative competition between *S. polychroa* and *S. mediterranea* suggest that at localities where some of the *Schmidtea* species coexist they could actually compete, which may eventually result in the replacement of some species by others.

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SUPPORTING INFORMATION

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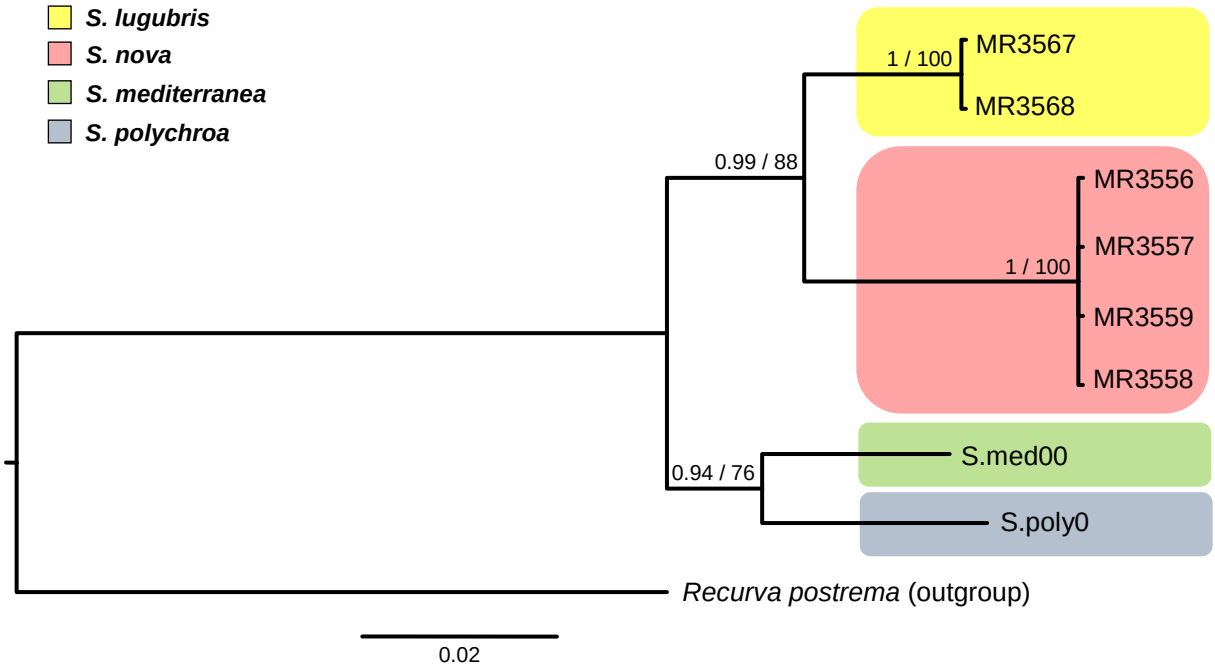

Fig. S1. Bayesian Inference tree of the 28S molecular marker. Numbers at nodes indicate posterior probability/bootstrap support values. The name of each sample indicates the code of the sample (See Table 2). Scale bar proportional to the number of substitutions per site. Colour of panels corresponding to colour codes used in the distribution map of the species (See Fig. 3).

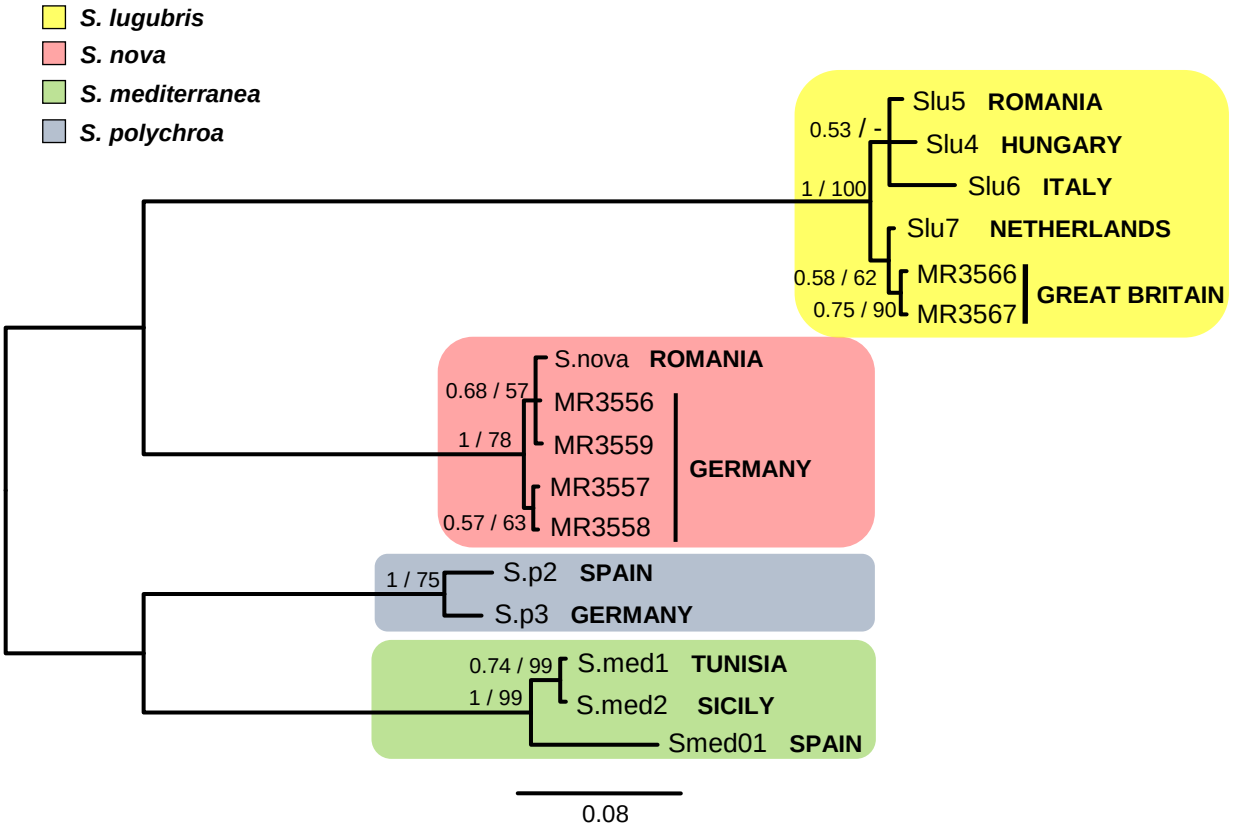

Fig. S2. Bayesian Inference tree of the Cox1 molecular marker. Numbers at nodes indicate posterior probability/bootstrap support values. The name of each sample indicates the code of the sample and its origin (See Table 2). Scale bar proportional to the number of substitutions per site. Colour of panels corresponding to colour codes used in the distribution map of the species (See Fig. 3).

Annex: Divergence time estimation and distribution modelling in the genus *Schmidtea*

In this annex, two different analyses have been performed to complement the results obtained in the publication included in the Chapter 4 of the present thesis, regarding the diversification history of the genus *Schmidtea*. One of the analysis consists in the estimation of the divergence times between the different *Schmidtea* species, by using the molecular data newly obtained in Chapter 4. Additionally, we have also used the geographic information that we compiled in Chapter 4 for the four *Schmidtea* species to predict their putative distribution at different past, present and future scenarios.

Divergence time estimation

As well as for the divergence time estimation analysis performed for the *Dugesia* species from the Western Mediterranean region (Chapter 3), we used the paleogeographic event corresponding to the Mid Aegean Trench (MAT) to calibrate the phylogeny of *Schmidtea*. Therefore, we included molecular information not only of the four *Schmidtea* species but also of most *Dugesia* species from the Western Mediterranean region, including one species of the sister genus of *Schmidtea* (*Recurva postrema*) (Table 1). The analysis was carried out using the 28S molecular marker, since the third position of the Cox1 marker was found to be saturated between *Schmidtea* and *Recurva* (Chapter 4). The existence of putative saturation in the 28S marker was checked by performing a test of substitution saturation with the software DAMBE (Xia & Lemey, 2009; Xia & Xie, 2001; Xia, Xie, Salemi, Chen, & Wang, 2003), which resulted in no saturation detected neither under an asymmetrical or a symmetrical tree topology (Index of Substitution Sataration = 0.0909; Critical Index of Substitution Saturation under a symmetrical topology = 0.7559; Critical Index of Substitution Saturation under an asymmetrical topology = 0.4917; p-value for both topologies = 0.0000).

Table 1. Information of the sample code, locality, and GenBank Accession number for all the species included in the present analysis

Species	Code	Locality	28S GenBank Accession Number
S. polychroa	S.poly	Catalonia, Spain	DQ665993
S. mediterranea	S.med	Montjuic, Barcelona, Spain	MG457267
S. nova	S.nova_9	Kleiwiesen, Germany	MG457271
	S.nova_31		MG457273
S. lugubris	S.lu_2	Nottingham, Great Britain	MG457268
	S.lu_3		MG457269
R. postrema	Rpo01	Laerma, Rhodes, Greece	MG457274
D. gonocephala	D01SER	100km south-west from Belgrade, Serbia	X
D. etrusca	Det06	Tuscany, Italy	X
D. liguriensis	DFR	Alpes Maritimes, France	X
D. benazzii	MR2192	Monte Albo, Sardinia	MK712509
D. hepta	MR1960	Mascari, Sardinia	MK712512
D. tubqalis	MMS5.1	Toubkal, Taddert, Morocco	X
Dugesia sp.	R9T	Afaska, Morocco	MK712529
D. aurea	M2.1D	Soller, Mallorca, Balearic Islands, Spain	MK712522
	M2.2D		MK712523
D. corbata	M3.1D	Sa Calobra, Mallorca, Balearic Islands, Spain	MK712524
	M3.2D		MK712525
D. vilafarrei	29.2	El Bosque, Andalucía, Spain	MK712495
	Dg1.1	Benaojan, Andalucía, Spain	MK712511
D. subtentaculata	V05.1D	Peralejos de las Truchas, Castilla La Mancha, Spain	MK712531
	E6.2	Beni Moussa, Morocco	MK712519
D. cretica	D02CRE1	Georgioupoli, Chania, Crete, Greece	X
D. improvisa	D01NAX2	Melanes, Naxos, Greece	X
D. damoae	D01SA19	Manolates, Samos, Greece	X
Dugesia sp.	D02TRI	Tripi, Laconia, Peloponnese, Greece	X

X: Sequences from Solà *et al*. 2019 with GenBank accession numbers pending to be obtained

The divergence time estimation analysis was conducted using the software BEAST v.1.8.4 (Drummond, Suchard, Xie, & Rambaut, 2012). All 28S sequences were imported in BEAUti v1.8.4 to set the prior parameters. The evolutionary model of the sequences was previously estimated using the program jModelTest2 (Darriba, Taboada, Doallo, & Posada, 2012), which resulted in GTR + Gamma. The site parameters in BEAUti were set as following: Substitution model = GTR; base frequencies = empirical; site heterogeneity model = gamma; and number of gamma categories = 4. The clock model was set to strict and the tree prior was set to "Speciation: Birth-Death Process" (Gernhard, Hartmann, & Steel, 2008). The node corresponding to the split between the lineage of *D. improvisa* – *D. damoae* and *Dugesia* sp. from western Greece was calibrated at 12 Ma (MAT), by setting the node under a normal distribution with a mean of 12 and a Stdev of 1.5. Three independent runs of 100,000,000 generations and sampling each 10,000 were conducted with Beast 1.8.0. in the CIPRES Science Gateway (Miller, Pfeiffer, & Schwartz, 2010). The resulting log files were examined in Tracer 1.7 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018) to confirm that the Effective Sample Size (ESS) values of all estimated parameters had reached a higher value than 200. Finally, the

maximum clade credibility tree of the run showing the highest ESS values (all runs showed higher ESS values than 200 and very similar posterior estimates for all the parameters) was constructed with TreeAnnotator v.1.8.4.

The phylogenetic relationships between the four *Schmidtea* species in the obtained time calibrated phylogeny matched with the ones obtained in Chapter 4, *viz*. the four species were grouped with high support into two clades: one including *S. nova* and *S. lugubris*, and the other including *S. polychroa* and *S. mediterranea* (Fig. 1). The age estimated for the divergence between *S. nova* and *S. lugubris* corresponded to 66.15 Ma (95 % highest posterior density (HPD) interval of 120.38 – 29.38 Ma), very similar to the divergence between *S. polychroa* and *S. mediterranea*, which was estimated at 67.18 Ma (95% HPD interval of 122.76 – 29.41 Ma). Additionally, the age estimated for the divergence between these two clades was 109.37 Ma (95% HPD interval of 194 – 50 Ma).

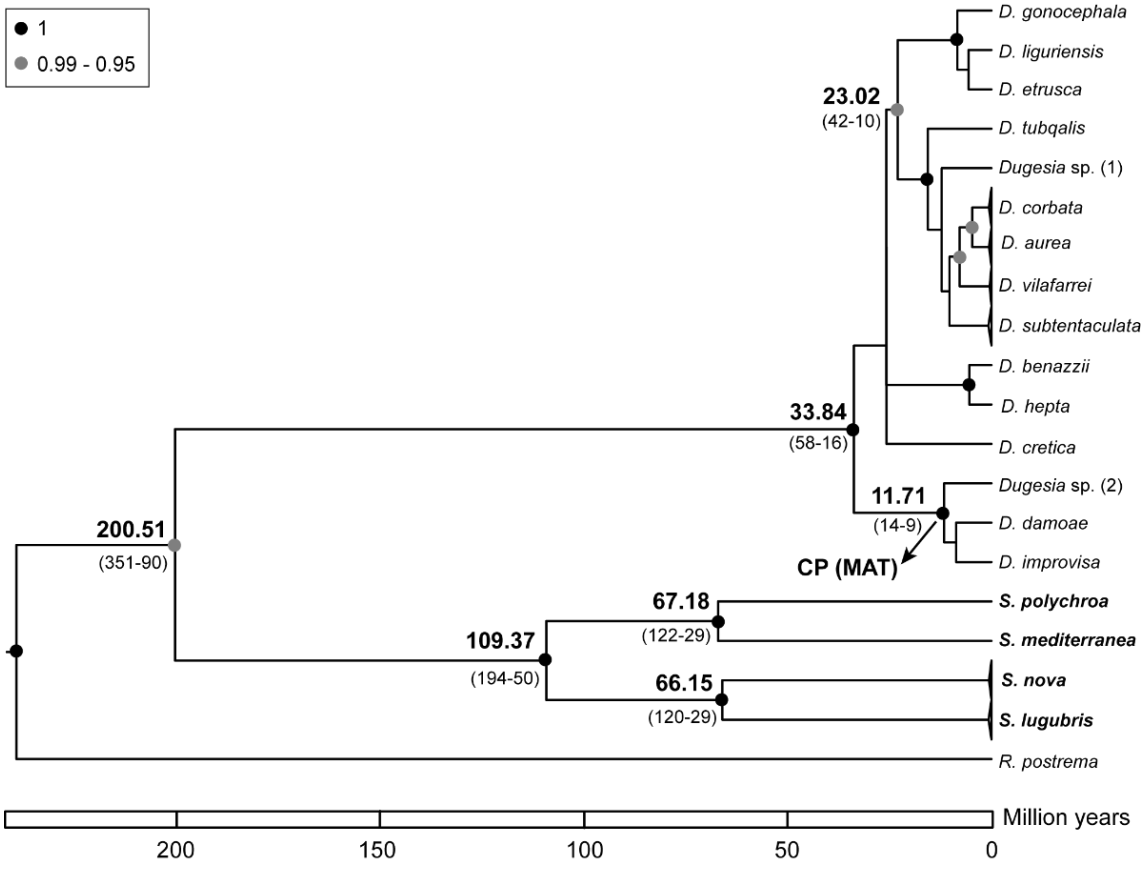

Fig. 1. 28S time-calibrated phylogenetic tree obtained with BEAST for the genus *Schmidtea*. Values at nodes correspond to the mean time estimates of divergence in million years ago. Values in brackets correspond to the 95% highest posterior density interval of the divergence time estimates. Dots at nodes indicate the posterior probability of each node (see legend). CP (MAT): biogeographic calibration point corresponding to the Mid Aegean Trench (around 12 Ma). Scale bar represents time in million years.

Species distribution modelling

The potential geographic distribution of each of the four *Schmidtea* species at different past, present and future conditions was independently estimated using the maximum entropy method implemented in the software Maxent 3.4.0. (Phillips & Dudík, 2008) as described in the Chapter 3 of the present thesis (excepting data for the Last Interglacial period, which was not included in this analysis). Present geographic information of the four species was extracted from Leria, Sluys, & Riutort (2018) (Chapter 4), corresponding to a total of 31 localities of *S. lugubris*, 8 localities of *S. nova*, 8 localities of *S. mediterranea*, and 71 localities of *S. polychroa*, which putatively cover the whole distribution range of each species.

All distribution models performed with Maxent for the four *Schmidtea* species retrieved AUC values higher than 0.88, indicating that all models showed a high predictive power (Table 2).

Table 2. AUC values obtained for each temporal scenario analyzed in the present study for the four *Schmidtea* species.

Species	Temporal scenario	AUC
S. lugubris	Last Glacial Maximum	0.894 ± 0.089
	Mid Holocene	0.959 ± 0.034
	Present day	0.965 ± 0.028
	2070 (RCP = 2.6)	0.949 ± 0.036
	2070 (RCP = 8.5)	0.941 ± 0.038
S. nova	Last Glacial Maximum	0.934 ± 0.058
	Mid Holocene	0.934 ± 0.066
	Present day	0.921 ± 0.075
	2070 (RCP = 2.6)	0.887 ± 0.094
	2070 (RCP = 8.5)	0.883 ± 0.088
S. mediterranea	Last Glacial Maximum	0.938 ± 0.017
	Mid Holocene	0.954 ± 0.010
	Present day	0.952 ± 0.012
	2070 (RCP = 2.6)	0.951 ± 0.010
	2070 (RCP = 8.5)	0.945 ± 0.013
S. polychroa	Last Glacial Maximum	0.946 ± 0.020
	Mid Holocene	0.977 ± 0.012
	Present day	0.977 ± 0.012
	2070 (RCP = 2.6)	0.973 ± 0.013
	2070 (RCP = 8.5)	0.972 ± 0.014

The image outputs of Maxent corresponding to the predicted distribution of each species under different temporal scenarios are shown in Fig. 2. One of the first noticeable differences between the predicted distribution of the different species (at any temporal scenario) was that the predicted distribution of *S. lugubris*, *S. nova* and *S. polychroa* was mostly restricted to a European range, while the predicted distribution of *S. mediterranea* showed a more southern range, including all the Mediterranean Peninsulas and the whole region of northern Africa (Fig. 2). Although the predicted distribution of *S. lugubris*, *S. nova* and *S. polychroa* were highly similar, the species *S. polychroa* showed a more slightly western/southern range than the other two species.

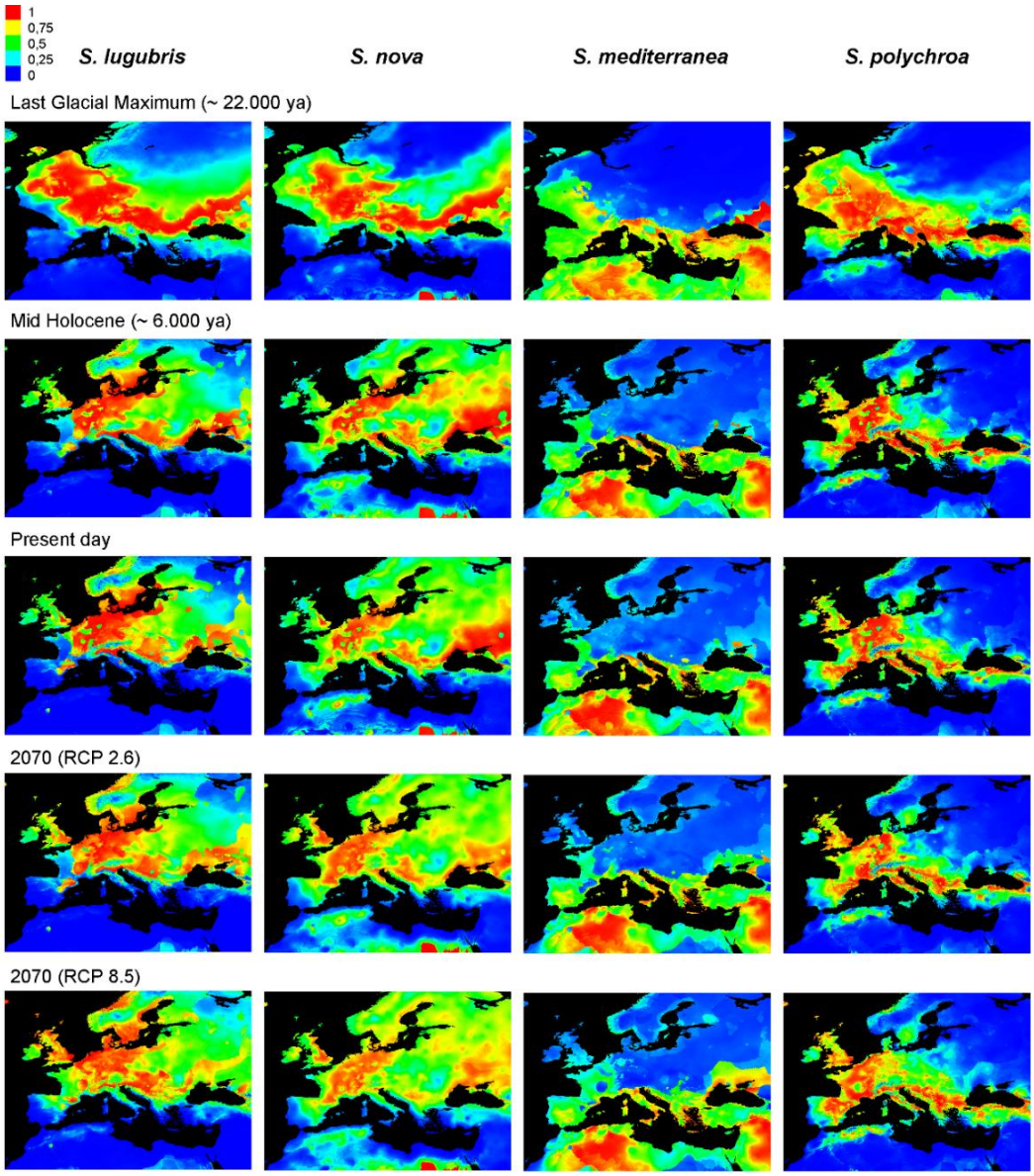

Fig. 2. Predicted geographic distribution for the four *Schmidtea* species at different temporal scenarios estimated with Maxent. Colour scale from blue to red indicates the probability of presence from 0 to 1, respectively. RCP 2.6: scenario with a low concentration of Greenhouse gases. RCP 8.5: scenario with a high concentration of Greenhouse gases.

The predicted distribution of each species did not show important differences among the various temporal scenarios that were analyzed. Nevertheless, the period corresponding to the Last Glacial Maximum (22.000 years ago) was the one in which the four species putatively showed more continuity in their distributions. Interestingly, the region corresponding to the connection between Great Britain and Europe showed a high probability of presence of the species *S. lugubris*, *S. nova* and *S. polychroa*, indicating that *S. lugubris* and *S. polychroa* may reached the Britannic Islands during that period.

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Discussion

The main goal of this thesis is to shed light into the processes that impact the genetic diversity of organisms, focussing on the effect of the following factors: the reproductive strategy, the morphological and the karyological diversity, and different historical processes. To get an original perspective on this topic I have used two genera of freshwater planarians as model organisms, *Dugesia* and *Schmidtea*.

Planarians show a variety of reproductive strategies that range from sexual to asexual, the last being either by fission or by parthenogenesis, and different populations or individuals can combine them in several ways. Importantly, while planarian regeneration ability has been intensively studied under laboratory conditions, the evolutionary consequences of having this ability have only barely been studied in the group. In fact, asexual reproduction by fission or by similar methods (e.g., budding in Hydra) has only exceptionally been analyzed from an evolutionary perspective in metazoans, even less when it occurs in combination with other reproductive strategies. Similarly, although the morphological and karyological characteristics of planarians have been widely studied, it has principally been done under a pure taxonomic point of view, while studies on these topics from an evolutionary perspective are still very scarce for the group. Finally, the low dispersion capabilities of planarians together with the fact that many planarian species are distributed in regions that show complex paleogeographic and paleoclimatic histories, make them ideal models to analyse the impact that these historical factors can have into the genetic trajectory of species.

In the following section I start by discussing three methodological topics. The first two correspond to the first two objectives of the present thesis, while the third is related to different methodological aspects I believe are needed to be considered when working with highly divergent intraindividual genetic data. Subsequently, I discuss how the results obtained in the four chapters of the present thesis can help us to understand how the genetic diversity of planarians is shaped over time, ending with some taxonomic considerations.

All along the section it has been my aim to highlight our main findings, their limitations, and future perspectives. Moreover, I have also extended the discussion of some topics derived from our findings that, although being a bit speculative, I believe can provide new interesting ideas about the evolution of this fascinating group of animals.

1. The importance of performing extensive samplings in evolutionary studies in planarians

1.1. An updated picture of the known distributional range of *Dugesia subtentaculata*

The first objective of the present thesis was to carry out an extensive sampling across all the known distributional range of the species *D. subtentaculata*, which included only 17 scattered localities in Southern France, the Iberian Peninsula, Northern Africa, and Mallorca (Balearic Islands) (Fig. 12A). With this aim, we sampled more than 200 localities from these regions, widening our search towards the north and the south of the Iberian Peninsula, and searching in the previously unexplored areas of Southern France, Mallorca and Morocco (the samples from Morocco being obtained by collaborators). We found that 59 out of these 200 localities were inhabited by *D. subtentaculata*, which multiplied by more than 3 the original number of known localities for this species (Fig. 12B). Importantly, we also found that individuals from 5 different localities corresponded to three new species, *viz*. *D. aurea*, *D. corbata*, and *D. vilafarrei*, which were previously subsumed within *D. subtentaculata* (Chapter 2).

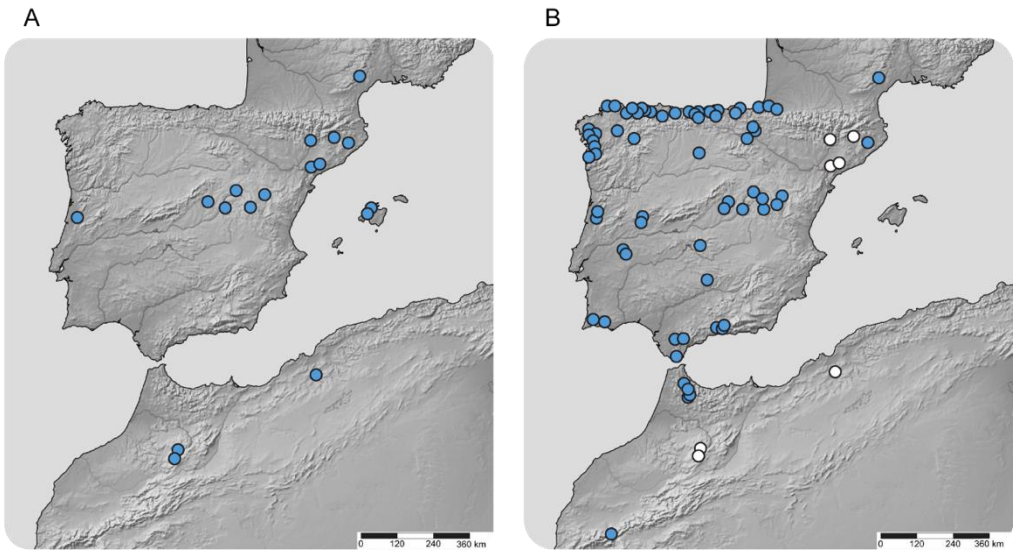

Fig. 12. Distribution map of *Dugesia subtentaculata* before (A) and after (B) the present study. Locations obtained in the present study are detailed in Supplementary data S1 of Chapter 2. White dots correspond to the locations where the existence of *D. subtentaculata* needs to be confirmed. The two previous localities known from Mallorca correspond to the new species *D. aurea* and *D. corbata*.

Our samplings revealed that the species *D. subtentaculata* is particularly abundant in the northern region of the Iberian Peninsula, a region where the species was not detected prior to this study, while the rest of the localities are scattered across its distributional range (Fig. 12B). We found *D. subtentaculata* in most of the localities that were previously reported for this species, excepting in the following 4 Catalan localities: Les Borges del Camp (Tarragona), Duesaigües (Tarragona), Pedret (Berguedà), and Font Aimat (Lleida), in which the species had been identified at the karyological level (Ribas, 1990). In the locality of Duesaigües we did not find any *Dugesia* species, while we found that the other three localities were occupied either by *D. sicula* (Les Borges del Camp), *D. etrusca* (Lleida) or *D. liguriensis* (Berguedà and Lleida). The occurrence of *D. liguriensis* in the locality of Berguedà was already reported at the molecular level by Lázaro et al., 2009, by using a sequence that had been previously misidentified as *D. etrusca* (Baguñà et al., 1999). Four different hypotheses exist to explain the absence of *D. subtentaculata* in these localities: (1) *D. subtentaculata* is cohabiting with these species in these localities but we did not sample it due to stochastic reasons, (2) these species have recently displaced *D. subtentaculata* in these localities, (3) *D. subtentaculata* has disappeared from these localities due to other factors than competition with these species (for example due to punctual episodes of water contamination or due to the occurrence of parasites), or (4) some of the karyotypes attributed to *D. subtentaculata* by Ribas (1990) actually correspond to *D. etrusca*, *D. liguriensis* or *D. sicula.*

The species *D. sicula* could rarely be confounded with *D. subtentaculata*, since the two species show different haploid chromosome numbers (being n=9 and n=8, respectively) (Ribas, 1990). Therefore, considering that *D. sicula* is a species that possess high colonization capabilities (Lázaro & Riutort, 2013), the most reasonable explanation for the absence of *D. subtentaculata* in the locality of Les Borges del Camp might be that *D. sicula* has outcompeted it. Differing from *D. sicula*, the species *D. etrusca* and *D. liguriensis* have been described with a karyotype of n=8, although detailed information on the characteristics of the chromosomes were not reported (Lázaro et al., 2009; Pala, Casu, & Vacca, 1980). Thus, although the karyotypes performed by Ribas in the localities of Lleida and Berguedà pointed out to the existence of one of the chromosomic translocations that we found in some localities of *D. subtentaculata*, it could be possible that these karyotypes corresponded either to *D. etrusca* or *D. liguriensis*. Therefore, further sampling efforts in these two localities, analysing the individuals at both molecular and karyological level, would be needed to disentangle the putative occurrence of *D. subtentaculata* in these locations.

Although our samplings covered most of the Iberian Peninsula, Southern France, and Mallorca, there are still some patches that remain unexplored, such as the regions of the Central mountain chain and the western area of Sierra Morena in Spain, or the northern region of Portugal. Similarly, it would be necessary to sample the western coast of Southern France, to confirm that the two localities of *D. subtentaculata* found in Aquitaine correspond to the upper limit of the distribution of the species in this region. Moreover, it would be of great value to perform exhaustive samplings across the northern region of Africa corresponding to Morocco and Algeria, a region where we were not able to personally sample, although we could obtain material from 5 localities from Morocco from collaborators. In this region there are several areas that show the optimal environmental conditions for *D. subtentaculata* (see Fig. 3 of Chapter 3), pointing out that it could bear not only more populations of this species but also different undescribed *Dugesia* species closely related to *D. subtentaculata*.

1.2. Consequences of increasing the sampling effort in the study of *D. subtentaculata*

The first consequence of our extensive sampling was that it allowed us to analyse at the genetic level a high number of populations and individuals of *D. subtentaculata*, becoming the molecular study that has included more intraspecific representation of *Dugesia* so far. As a result, we unveiled that the maximum genetic diversity of this species can be nearly found within some individuals (Fig. 3 in Chapter 2), something that would remain unknown if we only included few populations of this species. Moreover, in the case of the species *D. aurea*, *D. corbata*, and *D. vilafarrei*, the extensive samplings allowed us to unveil not only that these species show very low levels of genetic diversity but also that they are endemic from very small geographic regions. Thus, being sure that these findings correspond to the real situation of the different species, we were able to assign them into the categories of Critically Endangered (*D. aurea* and *D. corbata*) and Endangered (*D. vilafarrei*), with the hope that they will be included in different conservation policies.

At the morphological level, analysing individuals from a high number of different populations allowed us to recognize some character states that a priori seemed to be exclusive of certain species but that finally were not. An illustrative example is the case of the opening position of the vasa defferentia into the seminal vesicle. We found that most analyzed individuals of the different species showed a symmetrical opening, excepting all individuals of the species *D. aurea* and one population of the species *D. subtentaculata*, in which the opening was asymmetrical. If this single population of *D. subtentaculata* had not been included

into the analysis, we would have elevated this character state as a diagnostic trait for the species *D. aurea*, generating a case of misleading taxonomical information. In general, taxonomic descriptions in *Dugesia* (as well as in most planarian groups) are carried out using a low number of different populations, including many cases of species being described based on only one or two single populations, or even based in single individuals (Harrath et al., 2019; Sluys et al., 2013; Stocchino et al., 2009). Therefore, although the morphological characteristics reported for some of the *Dugesia* species that have been described only from one or two populations could be a reliable representation of the morphological diversity within the species (principally in the species that are endemic from small geographic regions), the results found in the present study indicate that putative intraspecific morphological variation could be present in the group.

At the karyological level, we found a striking degree of variation between the different analyzed populations of *D. subtentaculata*. Such phenomena of high karyologic diversity had been previously reported in populations of *D. benazzii, D. japonica* and *D. ryukyuensi*s (Oki, Tamura, Yamayoshi, & Kawakatsu, 1981; Maria Pala, Casu, & Stocchino, 1999; Tamura et al., 1991; Vacca, Casu, & Pala, 1993). However, similarly to what happens in morphological studies, the karyotype of most *Dugesia* species has been analyzed only from one or from a low number of different populations, suggesting that overlooked intraspecific karyological variation could also exist in this group.

All these results indicate that increasing the number of analyzed populations in evolutionary studies in planarians might be necessary to obtain a reliable picture of both the intraspecific and interspecific diversity of the group, at the molecular, morphological, and karyological levels.

2. Pros and cons of obtaining new nuclear markers for *Dugesia* using a genomic approach

The second objective of the present thesis was to obtain several nuclear markers that worked both at the intraspecific and interspecific level in *Dugesia*, by using a genomic approach. With this aim, we sequenced the whole genome of three individuals belonging to three different populations of *D. subtentaculata,* one individual of *D. corbata*, and several individuals (analysed in a pool) of one population of *D. sicula* and, subsequently, searched for EPIC markers (protocol detailed in Chapter 2).

The first obstacle that we encountered was to obtain enough quantity of high-quality DNA to perform the genome sequencing from single individuals. We found that the standard extraction methods, such as using DNAzol or different extraction kits, did not retrieve the minimum quantity nor quality of DNA needed, so we had to optimize a protocol using phenol-chloroform. However, the main problem that we had to face was related to the search of the EPIC genomic regions. Since there is no available genome of *Dugesia*, we had to assemble our genomic reads with the transcriptome of *D. japonica* and search for the EPIC markers using the annotated genome of *Schmidtea mediterranea*. Considering that *Dugesia* and *Schmidtea* are closely related (see Fig. 7), we expected that the length of the intronic regions would be similar between the two genera. Nevertheless, we found that approximately 3/4 of all selected markers showing an appropriate length in *Schmidtea*, in *Dugesia* were either too long to be amplified in a standard PCR reaction or too short to bear enough information (including some markers that were either too conserved or too variable). Although we did expect to capture intronic regions with different levels of variability (including those too variable and too conserved), we did not expect that the length of the same intronic regions between *Schmidtea* and *Dugesia* would be so different. This is indicating that although *Schmidtea* and *Dugesia* are closely related genera, their genomic characteristics are quite different, which is in agreement with our finding that they might diverged approximately 200 Ma (Chapter 4 – Annex). Hence, the divergent genomic characteristics between *Dugesia* and *Schmidtea* hindered us from obtaining a high number of EPIC markers.

Nevertheless, despite the mentioned drawbacks, we obtained six new EPIC markers (named in the present study as Dunuc2, Dunuc3, Dunuc5, Dunuc10, Dunuc12, and Dunuc20), which resulted very useful in the genetic diversity analyses performed at different levels in the present thesis. The high variability of the gene Dunuc12 (named in Chapter 1 as TMED9), allowed us to analyse the intraindividual nuclear genetic diversity of the sexual species *D.*

aurea, *D. corbat*a, and *D. vilafarrei* together with several populations of *D. subtentaculata* showing different reproductive strategies (Chapter 1). Moreover, these six EPIC markers, allowed us to apply a multilocus method for species delimitation that resulted fundamental to newly describe *D. aurea*, *D. corbata*, and *D. vilafarrei* (Chapter 2). Finally, two of the most conserved new EPIC markers (Dunuc3 and Dunuc5) were key to unveil the phylogenetic relationships between these species and the rest of *Dugesia* species from the Western Mediterranean region, which had not been possible to be solved basing on the information of the molecular markers used previously to this study (Chapter 3).

Moreover, besides being used for the analyses of the present thesis, some of these EPIC molecular markers have also been used in three additional studies on different *Dugesia* species. The gene Dunuc12, has been used to infer the phylogenetic relationships within two different species complexes: the group comprised by *D. etrusca*, *D. liguriensis*, and *D. ilvana* (Pérez-García, 2017), and the species group including *D. hepta* and *D. benazzii* (Dols, Leria, Aguilar, Stocchino, & Riutort, 2019). In both works, as in the case of *D. subtentaculata*, the inclusion of this gene highly improved the resolution of the evolutionary history within the respective groups of species. Finally, the genes Dunuc3 and Dunuc5 have also provided valuable phylogenetic information in a recent molecularly based biogeographic study of the whole genus *Dugesia* (Solà, Leria, Stocchino, & Riutort, 2019).

Therefore, although the number of EPIC markers developed in the present study is quite low, they have proven to be very useful in several evolutionary studies focused on different *Dugesia* species and at different taxonomic levels. Although I believe that future evolutionary studies on *Dugesia* need to be done at the genomic level, for example using a reduced genomic sequencing approach, such as RAD-seq or transcriptomic data, I think that counting on the information of discrete nuclear markers is always useful for many different reasons, such as for rapidly identifying already described species or for testing which individuals are best to be included in a genomic analysis to optimize the sequencing effort. Moreover, many of the results obtained during the search of nuclear markers in the present work, including the optimization of high-quality DNA extraction, estimation of the size of the genome (both protocols detailed in Chapter 2), and identification of the divergent characteristics of the *Dugesia* genome compared to *Schmidtea*, can be of great value for future genomic analyses in this genus.

3. Methodological considerations when performing molecular analyses in organisms with putative high levels of intraindividual genetic diversity

3.1. Molecular phylogenetic inferences

One of the most important problems that we detected when working with individuals of *D. subtentaculata* showing high levels of intraindividual genetic diversity is that it can lead to misleading molecular phylogenetic inferences (Fig. 3 in Chapter 2). Let's set as an example that a phylogeny of different populations within a certain species is being reconstructed using several nuclear molecular markers amplified by standard PCR. If it is not known a priori that most of the genetic diversity of the different populations can be found at the intraindividual level (as we found in *D. subtentaculata*) two different types of sequences can be obtained after the PCR amplification: (1) sequences showing many polymorphic sites, and (2) sequences without polymorphic sites.

In the case of obtaining sequences with many polymorphic sites, the high number of these sites is indicating that different alleles of the same individual have been amplified during the PCR reaction. If it is assumed that these different alleles correspond to a standard case of heterozygosis and any of the available phasing methods is applied (Flot, 2007; Flot, Tillier, Samadi, & Tillier, 2006; Stephens, Smith, & Donnelly, 2001), several completely artificial alleles will probably be obtained. A misleading situation like this was directly observed in a molecular analysis of the species *D. sicula*, in which sequences with polymorphic sites were tried to be resolved by phasing (Lázaro, 2012). In that case, it was found that the haplotypes reconstructed by phasing showed a higher number of mutations than the real haplotypes obtained by cloning, resulting in a more complex and artificial haplotype network.

Sequences without polymorphic sites can be obtained if one of the intraindividual alleles has been more efficiently amplified than the others during the PCR reaction. This is a more problematic situation, since the intraindividual genetic diversity of the individuals will remain unnoticed. Therefore, if a random amplified allele of each individual is used to infer a molecular phylogeny, the obtained phylogenetic relationships will be random as well (Fig. 13), as we found in the phylogenetic inferences of *D. subtentaculata* using the sequences without cloning (Fig. 3 in Chapter 2). In the case that different genes are amplified, unnoticed intraindividual genetic diversity may be easier to detect, since the phylogenetic relationships between the different individuals would probably differ for each marker. However, one may think of other possible explanations for the discrepancy between gene trees, rather than

considering the existence of intraindividual genetic diversity. Therefore, I strongly recommend to always clone the PCR products before doing molecular phylogenetic analyses when working with organisms susceptible to bear high levels of intraindividual genetic diversity, such as asexual fissiparous species.

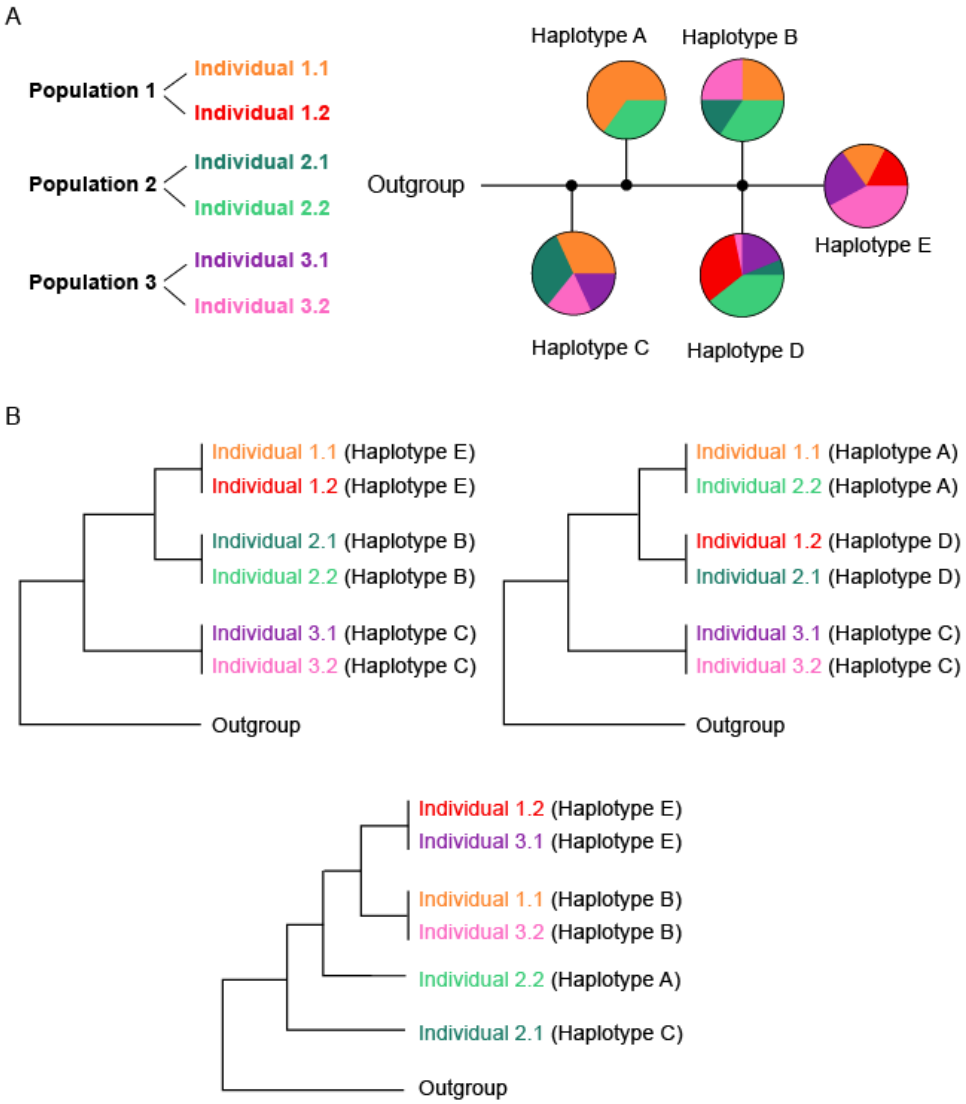

Fig. 13. Schematic representation of the consequences that can have using standard PCR amplification to infer the phylogenetic relationships between individuals showing high levels of intraindividual genetic diversity. A) Haplotype network showing the true relatedness of the different alleles of each individual and, B) Example of three different phylogenies that can result from the random amplification of certain alleles of each individual.

3.2. Molecular species delimitation

Another problematic issue that we detected when analysing the genetic diversity of *D. subtentaculata* is related to molecular species delimitation. Surprisingly, we found that some alleles within individuals were so highly differentiated that some molecular methods for species delimitation identified them as different candidate species (Fig. 3 in Chapter 2). Therefore, if it is not known a priori that the organisms included in a molecular delimitation analysis show high levels of intraindividual genetic diversity, it could be possible to over-estimate the number of different molecular candidate species. Similar as for the phylogenetic inferences, the best practice when working with organisms that can putatively present high levels of intraindividual genetic diversity is to clone the PCR products before applying the different methods for species delimitation, to exactly know the amount of genetic diversity that can be found within individuals.

Importantly, if cloning is not possible, I recommend using the methods that are more conservative, such as m-PTP (Kapli et al., 2017), and to avoid the methods that are more prone to over-splitting, such as GMYC (Pons et al., 2006). Finally, another possible and very interesting solution could be to develop new molecular methods for species delimitation that considered the type of reproduction of the individuals to trace species boundaries. For example, such as the method ABGD needs the user to specify a prior value of intraspecies genetic diversity, a minimum threshold of genetic diversity could be selected by the user for fissiparous individuals to be considered as different species.

3.3. Molecular divergence time estimation

In Chapter 3, we investigated how the occurrence of high levels of intraindividual genetic diversity affected the results obtained during an analysis of molecular divergence time estimation. Interestingly, we found that the use of either the most ancestral Cox1 haplotype of a fissiparous individual of *D. subtentaculata* or the use of its most derived Cox1 haplotype, changed the estimate age of divergence between this individual and its sister lineage in approximately 1 million year (Fig. 2 in Chapter 3). Nevertheless, this analysis also showed that the divergence time estimates between the rest of species were not affected by the intraindividual haplotype of *D. subtentaculata* that was used. These results indicate that considering or not the intraindividual genetic diversity in organisms with high levels of it may

not be important when estimating divergence times at the interspecific level but that may be crucial when doing such estimates at the intraspecific level.

3.4. Future perspectives in the analysis of intraindividual genetic diversity in planarians

To study the intraindividual genetic diversity of *D. subtentaculata* we decided to use a molecular cloning approach. In our hands, this approach has proven to be useful to unveil the existence of high levels of genetic diversity within individuals. However, obtaining a reliable representation of the intraindividual genetic diversity of a planarian (or of any other organism) by using a cloning approach is methodologically impossible, since it would require to manually analyse thousands of colonies per individual.

A very appealing possibility, though challenging, would be to use genomic or transcriptomic data from single cells (Wang & Navin, 2015). By analysing a representative number of different cells within an individual, it would not only be possible to detect artifactual mutations more easily than by cloning (since the number of sequences per individual would be higher), but it would also allow to differentiate between the genetic diversity that is found within cells (i.e., heterozygosis) from the genetic diversity that is found between cells (i.e., mosaicism), something that cannot be directly observed by cloning. This methodology is presently giving very good results in the analysis of somatic mosaicism in humans, which is specially contributing in making advances on cancer research (Dou, Gold, Luquette, & Park, 2018; Filbin et al., 2018). Interestingly, it has been recently applied to the planarian species *Schmidtea mediterranea*, providing highly valuable information on the different cell types of these organisms and on their differentiation trajectory from neoblasts (Fincher, Wurtzel, Hoog, Kravarik, & Reddien, 2018; Plass et al., 2018; Swapna, Molinaro, Lindsay-Mosher, Pearson, & Parkinson, 2018). Therefore, molecular analyses based on single cell data could represent the next step in the study of the intraindividual genetic diversity of planarians.

4. Genetic consequences of fissiparous reproduction in planarians

4.1. At the intraindividual level

The analyses performed in Chapter 1 of the present thesis indicated that fissiparous reproduction in *D. subtentaculata* leads to extreme levels of intraindividual genetic diversity in a mosaic context (at both nuclear and mitochondrial level) putatively due to the progressive accumulation of mutations in the neoblasts of the individuals during long periods of fissiparous reproduction (Fig. 8 in Chapter 1). We proposed that this situation corresponded to a variation of the well-known Meselson effect but at the mosaic level, the *mosaic Meselson effect*, which we defined as the the existence of a genetically heterogeneous cell population within the body of an organism, carrying highly divergent alleles in homologous genetic regions. Surprisingly, the genetic differentiation between some of the intraindividual Cox1 haplotypes that we found in *D. subtentaculata* reached a 3.2 % of divergence, a value that is equivalent to the divergence reported for this gene between some *Dugesia* species, such as between *D. hepta-D. benazzii* or between *D. improvisa-D. ariadnae* (Lázaro et al., 2009; Solà, Sluys, Gritzalis, & Riutort, 2013).

One of the first questions that arises from the occurrence of the mosaic Meselson effect is how fissiparous individuals can orchestrate such high levels of intraindividual genetic diversity. Interestingly, the low levels of intraindividual non-synonymous mutations that we detected in the two genes analyzed indicated that processes of intraindividual selection at the neoblast level could help fissiparous planarians to prevent protein heterogeneity within individuals. These processes can easily explain the elimination of the mutations that directly affect the fitness of the neoblasts (which may be the case of the Cox1 and the TMED9 genes), but what about the mutations that affect the fitness of the differentiated cells but not the fitness of the neoblasts?

One possibility would be that fissiparous planarians are indeed not able to get rid of the deleterious mutations that affect the differentiated tissues. Indirect evidence supporting this hypothesis comes from a recent study carried out in fissiparous individuals of the species *D. japonica* (Nishimura et al., 2015). In that study, genomic and transcriptomic data from a laboratory lineage derived from a single individual that had kept undergoing autonomous fission for over 20 years was analyzed, resulting in the detection of a 74% of the genes having accumulated non-synonymous polymorphisms. However, an important point that needs to be considered for interpreting these results is that these fissiparous planarians were maintained in laboratory conditions (fed twice a week with chicken liver and kept in autoclaved water at a constant temperature), a situation that strongly differs from natural conditions. Therefore, it

could be possible that mutations that can be accumulated under laboratory conditions are actually deleterious in the wild, something that may represent a caveat for these results to clearly evidence that fissiparous planarians cannot get rid of the deleterious mutations that affect the differentiated tissues.

Another very different possibility than the inevitable accumulation in the neoblasts of the deleterious mutations that affect the differentiated tissues, would be that the fitness of the differentiated tissues played a feedback role on the dynamics of their progenitor neoblasts. It has been shown that changes in the expression of certain genes in the differentiated tissues (or in the intermediary cellular stages) can have an impact on neoblast maintenance and proliferation dynamics (Lei et al., 2016; Tu et al., 2015). For example, Lei and collaborators observed that the extracellular signal done by an epidermal growth factor (EGF-3), strongly determined both the self-renewal and differentiation dynamics in neoblasts. Therefore, it could be possible that neoblasts bearing deleterious mutations for a certain differentiated tissue would be replaced by other neoblasts by a process of feedback selection from the tissue.

Everyday new advances are made in the study of the dynamics of neoblasts during normal tissue homeostasis and regeneration, which, in my opinion, will be key to understand how planarians can deal with the high levels of intraindividual genetic diversity that are generated due to fissiparous reproduction.

4.2. At the species level

The mosaic Meselson effect occurring during fissiparous reproduction can also have strong implications at the whole species level. First, the fact that the complete genetic diversity of a species can be found within fissiparous individuals, may allow planarians to overcome evolutionary problems such those generated by population bottlenecks, since these events will not result in a loss of the genetic diversity of the species. For instance, population bottlenecks may be recurrent in the Mediterranean region, where many small rivers and creeks inhabited by *Dugesia*, completely dry out during the summer period. Second, our results indicated that fissiparous individuals showing the mosaic Meselson effect are characterized by presenting a mix of ancestral and derived genetic diversity. This situation may result from the absence of genetic bottlenecks during fissiparous reproduction (descendants from fission do not pass through a one-cell state). Thus, although mutations keep appearing in certain cells (generating

the derived variants), most cells at the short term may still show the ancestral genetic diversity of the individuals. Therefore, fissiparous individuals can also be considered as a "reservoir" of the ancestral genetic diversity of the species, a phenomenon that may be very advantageous when the genotypes are well adapted to the environmental conditions (Von Saltzwedel, Maraun, Scheu, & Schaefer, 2014).

Although fissiparous reproduction is especially common in *Dugesia*, it can also be found in other planarian genera of the family Dugesiidae such as *Schmidtea* (Baguñà et al., 1999) and *Girardia* (Knakievicz, Moura-Vieira, Erdtmann, & Bunselmeyer-Ferreira, 2006). Similarly, several freshwater planarian species of the family Planariidae also show fissiparous reproduction in natural conditions, such as *Crenobia alpina*, *Polycelis felina*, and *Phagocatta vitta* (Ball & Reynoldson, 1981). Importantly, fissiparous reproduction has been described also in many other groups of metazoans, with fissiparous representatives in approximately half of the animal phyla, including several groups that, like planarians, use somatic stem cells to regenerate (Sköld et al., 2009). Thus, it would be extremely interesting to see whether similar genetic characteristics associated to fissiparous reproduction as the ones described in the present study for the freshwater planarians of the genus *Dugesia* occur not only in other planarian genera but also in other metazoans.

5. Genetic consequences of fissiparous reproduction with occasional sex in planarians

5.1. Giving shape to the mosaic Meselson effect

Our results in Chapter 1 indicate that the genetic diversity generated by the mosaic Meselson effect in fissiparous *Dugesia* specimens can be transmitted to the offspring through sexual events, a phenomenon with major implications for the genetic diversity of these populations. We found that through facultative reproduction a new component of genetic diversity was generated: genetic diversity between individuals (Fig. 5 in Chapter 1). This way, three types of descendants may be obtained after a re-sexualization event in a fissiparous population: descendants only bearing the ancestral alleles of the population, descendants only bearing the derived alleles of the population, and descendants bearing all possible different combinations of ancestral and derived alleles (Fig 9 in Chapter 1). Importantly, genetic diversity between individuals was not observed neither in strict sexual populations (in which the genetic diversity

was mostly found between different populations) nor in strict fissiparous populations (in which most of the genetic diversity was found within individuals) (Fig. 5 in Chapter 1).

Although we did expect that most of the genetic diversity in fissiparous populations could be found at the intraindividual level, we did not expect that all sexual populations analyzed in the present study would be so homogeneous at the genetic level, since sexual reproduction is precisely characterized by generating genetic diversity within populations (Otto, 2009). One possibility could be that the low genetic diversity of the five sexual populations analyzed in the present study (three of them corresponding to the species *D. aurea*, *D. corbata*, and *D. vilafarrei*) respond to different stochastic circumstances, such as recent population bottlenecks having occurred in all these populations. Nevertheless, a more plausible hypothesis might be that the low genetic diversity detected in these sexual populations is a consequence of their endemicity from very small geographic regions (they probably show low effective population sizes), a situation that is found in most sexual *Dugesia* species from the Mediterranean region (Lázaro et al., 2009; Solà et al., 2013). Therefore, it could be possible that the combination of fissiparous reproduction with occasional sex evolved in planarians as a mechanism for generating genetic diversity between individuals within populations, a component of genetic diversity that is crucial for adaptation and that is not generated when the two reproductive strategies are done alone. Further studies, comparing the genetic diversity between the sexual species of *Dugesia* that are distributed in small regions and those sexual species showing broader distributions (such as *D. gonocephala*) would be needed to confirm this hypothesis.

Besides increasing the genetic diversity of fissiparous populations by the combination of the alleles generated due to the mosaic Meselson effect, events of occasional sex may also play an important role regarding the processes of selection, since the genetic diversity that was putatively present only in some cells of the progenitors shifts to be the whole genetic background of the descendants. Therefore, genetic diversity previously exposed only to selection at the cellular level, may change to be exposed to selection at the individual level, generating a scenario of multilevel selection. This process could be key for fissiparous populations not only to fix the advantageous genetic variants but also to eliminate the deleterious variants that could have not been eliminated solely by intraindividual selection.

5.2. Are facultative planarians breaking the soma-germline barrier?

Taking all this information into account, a very interesting question arises: Can occasional sex in fissiparous planarians be considered as a break of the traditionally assumed barrier between the soma and the germline?

In a study carried out in facultative individuals of the species *D. japonica*, it was seen that a subpopulation of neoblasts was responsible of the differentiation of the germ cells (Sato et al., 2006). Interestingly, these precursors of the germline (the germline neoblasts) were distributed in the region were the testes and the ovaries had to be differentiated in the future and, moreover, it seemed that they did not contribute to the formation of new tissues during the regeneration process. Under this scenario, it may seem logic to assume that occasional sex in fissiparous planarians does not represent a break of the soma-germline barrier, since the only mutations that will be able to be transmitted to the offspring through sexual events will be the ones occurring in the germline neoblasts. However, it is still unknown how the population of these germline neoblasts is restored after a fission process. On the one hand, it could be possible that this restoration occurred due to the mitotic activity of the remaining germline neoblasts, which would support the prevalence of the soma-germline barrier. On the other hand, it could occur that after a fission process part of the somatic neoblasts differentiated into germline neoblasts. Under this scenario, certain genetic variants occurring at the somatic level could theoretically be transmitted to the germline during sexual events, even those mutations already selected at the intraindividual level, representing a break of the soma-germline barrier.

In the case of fissiparous planarians, maintaining a subpopulation of neoblasts with a lower mitotic activity to differentiate the germline might contribute to mitigate the Muller's ratchet in the populations. Nevertheless, the high genetic differentiation that we detected between some of the alleles that were putatively sexually inherited in *D. subtentaculata* point out that (1) even the germline neoblasts inevitably accumulate high levels of genetic diversity during fissiparous reproduction or (2) that somatic neoblasts can indeed contribute to the subpopulation of germline neoblasts and pass their variants to descendants through sexual events. Further analyses, comparing the genetic characteristics between the somatic tissues and the different subpopulations of neoblasts would be necessary to shed light into this complex but extremely interesting situation.

6. Morphological stasis in planarians

6.1. Speciation in the absence of morphological diversity in the copulatory apparatus

One of the hypotheses that we had at the beginning of this thesis was that variations in the anatomy of the copulatory apparatus of planarians could trigger the genetic differentiation between lineages due to reproductive incompatibilities. This idea was based on the "Lock-and-Key" hypothesis for the evolution of genitalia, which has been found to occur in many groups of organisms, with a particular high incidence in arthropods (Brennan & Prum, 2015; and references therein).

In the case of *Dugesia*, we encountered a situation in which different species are highly differentiated at both genetical and karyological level (*D. subtentaculata*, *D. corbata*, *D. aurea*, and *D. vilafarrei*) but show an almost identical anatomy of the copulatory apparatus. A similar situation was found in the genus *Schmidtea*. In this case, although some diagnostic characters do exist between the four *Schmidtea* species (Fig. 15 in Chapter 4), the degree of morphological variation is minor in comparison with the high level of differentiation that exists between them at both molecular and karyological level. Importantly, the minor anatomical variable characters in the copulatory apparatus between the four *Schmidtea* species may not involve reproductive incompatibilities. All these findings indicate that the anatomy of the copulatory in planarians is not one of the main factors triggering the speciation processes within this group.

6.2. Is the stasis in the morphology of the copulatory apparatus in planarians a consequence of hermaphroditism?

The results obtained in the divergence time estimation analyses performed in *Dugesia* and *Schmidtea*, indicated that morphological similarity between the different species within each group may not be a consequence of their recent diversification. In fact, we found that the most closely related *Dugesia* species analyzed in the present study (*D. aurea* and *D. corbata*) diverged around 7 Ma (Fig. 2 in Chapter 3), while the most closely related *Schmidtea* species analyzed in the present study (*S. nova* and *S. lugubris*) did so around 65 Ma (Fig. 1 in Chapter 4 - Annex). These results yielded further support to a previous hypothesis, based on *Dugesia* and *Girardia*, suggesting that the anatomy of the copulatory apparatus in planarians is under a general state of morphological stasis (Sluys, Kawakatsu, & De León, 2005; Solà, 2014).

Morphological stasis has been principally related to the effect of stabilizing selection (i.e., selection that eliminates the divergent traits) (Egea et al., 2016; A. G. Sáez et al., 2003; Smith, Harmon, Shoo, & Melville, 2011). Thus, it could be possible that important variations in the anatomy of the copulatory apparatus in planarians are under a strong selective pressure, while minor variations (such as the ones detected in *Dugesia* and *Schmidtea*) are able to indistintly appear in the different lineages. However, why the morphology of genitalia could be so strongly selected in planarians?

One possible explanation might be related to hermaphroditism. Theoretically, the maximum fitness of simultaneous hermaphrodites (as planarians) is achieved when reciprocal fertilization occurs, since the genetic material of each parental individual is transmited to the offspring of both partners. Therefore, variations on a certain male or female morphological trait could increase one of the two sex-specific fitness components but would reduce the general fitness of the individual, resulting in an overall selection against variation (Morgan, 1994). For example, an increment in the size of the penis papilla in one individual could increase its fitness of acting as a male during the copula, but would decrease its fitness of acting as a female (wich would also decrease the fitness of its partner of acting as a male). Interestingly, studies on the morphology of the copulatory apparatus and of the sperm in flatworms of the order Macrostomida, revealed that drastic changes in the morphological characteristics of both organs occur coupled with changes in the reproductive behaviour, specifically when reciprocal fertilization in hermaphroditic animals is shifted to hypodermic insemination (Schärer, Littlewood, Waeschenbach, Yoshida, & Vizoso, 2011). These results support the hypothesis that morphological stasis of genitalia might be selected in hermaphroditic planarians that show reciprocal cross ferilization, which accounts for the vast majority of planarians (Sluys & Riutort, 2018).

7. The role of karyological variability driving speciation processes in planarians

7.1. Shifts in the ploidy level

The karyological analysis of multiple populations of *D. subtentaculata* revealed that shifts in the ploidy level are highly frequent in this species, including both polyploidization and diploidization events (Chapter 1 and Chapter 2). Interestingly, we found that diploid populations were sexual (excepting one triploid sexual population), while polyploid populations reproduced either by fission or combining both reproductive strategies (Chapter 1; Chapter 2 - Annex). Similar situations of sexual populations being diploid and fissiparous populations being triploid have been observed in many other *Dugesia* species, such as *D. liguriensis*, *D. etrusca*, and *D. sicula* (Harrath et al., 2012; Lázaro et al., 2009).

One possible explanation for the relationship between polyploidy and fissiparous reproduction in *Dugesia*, could be that polyploidy confers a higher rate of tissue regeneration than diploidy. This phenomenon has been directly observed when comparing the rate of antennae tissue regeneration between diploid and polyploid freshwater snails of the genus *Potamopyrgus* (Krois, Cherukuri, Puttagunta, & Neiman, 2013). In that study, the observed higher regeneration rate of polyploids compared to diploids, was suggested to be a consequence of the higher levels of RNA and protein produced by polyploid individuals. Thus, if this was the case for *Dugesia*, it could be possible that polyploid fissiparous individuals outcompeted diploid fissiparous during regeneration after fission, explaining why most polyploid *Dugesia* populations are fissiparous in natural conditions, while most diploid populations are sexual.

Importantly, if shifts in the ploidy level trigger shifts between sexual and fissiparous reproduction in *Dugesia*, they may be also linked to speciation events. For instance, we proposed that the speciation between *D. vilafarrei* and *D. subtentaculata* occurred due to a triploidization event in the ancestors of *D. subtentaculata*, which possibly triggered a shift from sexual to fissiparous reproduction, isolating the diploid sexual individuals (lineage of *D. vilafarrei*) from the triploid fissiparous (lineage of *D. subtentaculata*). Similarly, the diploidization event within *D. subtentaculata* that putatively originated the sexual lineage of Alte (Chapter 1), probably resulted in the reproductive isolation between these individuals and the rest of polyploid fissiparous individuals, indicating that members of this population could eventually evolve into a different species. However, differing from *Dugesia*, in other planarian

genera, such as in *Schmidtea*, polyploidization events promote shifts from sexual to parthenogenetic reproduction, instead of shifts from sexual to fissiparous reproduction. In these cases, polyploidization events may not represent such strict situations of reproductive isolation, in fact, it has been seen that sexual and parthenogenetic individuals of the species *S. polychroa* are able to interbreed and generate completely viable offspring (D'Souza, T. G. & Michiels, 2009). Therefore, shifts in the ploidy level may be an important driver of speciation in planarian groups that show both sexual and fissiparous reproduction but not in groups that alternate sex with parthenogenesis.

7.2. Chromosomic rearrangements

In the case of the genus *Schmidtea*, the molecular phylogenetic relationships that we found between the four species supported a previous speciation hypothesis for this genus based on different chromosomic rearrangements (Chapter 4). On the one hand, *S. nova* and *S. lugubris* could have diverged from a common ancestor with a chromosome complement of n = 4 through a Robertsonian translocation plus a pericentric inversion, resulting in the three basic chromosomes of *S. nova* (Benazzi & Puccinelli, 1973). On the other hand, although it is not so clear as in the previous case, the ancestors of *S. mediterranea* and *S. polychroa* could also have diverged through different translocations and pericentric inversions (Benazzi, 1982). Similarly, we also found that the karyotypes of *D. aurea*, *D. corbata*, *D. vilafarrei*, and *D. subtentaculata* could be perfectly differentiated one from another by different chromosomic rearrangements. Hence, although the chromosomic rearrangements that characterize these species may not directly triggered their speciation (which in some cases could indeed be the case, such as for *S. nova* and *S. lugubris*), they probably constitute present barriers to gene flow.

Two principal reasons exist to explain why chromosomic rearrangements can promote reproductive isolation between lineages. First, they can be detrimental for the viability of the hybrids (for example, changing the expression of crucial loci by altering the regulatory regions or by altering the structure of the genes) and, second, they can generate sterility of the hybrids (principally by the generation of unbalanced gametes due to incorrect chromosome pairing during meiosis) (see Faria & Navarro, 2010; and references therein). These situations may have a huge impact in the evolutionary trajectory of diploid species, such as for the different *Schmidtea* species and for the species *D. aurea*, *D. corbata*, and *D. vilafarrei*. However, for planarian species with higher ploidies, such as *D. subtentaculata*, the occurrence of chromosomic rearrangements may not promote reproductive isolation between linages, since

polyploid individuals inheriting different chromosomic rearrangements would probably have at least a pair of equal chromosome sets, which would ensure the formation of a certain number of viable gametes and would also maintain adequate levels of expression for the genes affected by the rearrangement. This hypothesis is supported by the fact that we detected a striking number of polyploid individuals of *D. subtentaculata* bearing aberrant chromosomes, while the karyotypes observed in the diploid species were always balanced (Chapter 2). All these results indicate that chromosomic rearrangements may play an important role driving speciation processes in diploid planarians but that they may not be a principal driver of speciation in polyploid planarian species.

A promising model to study the effect of chromosomic rearrangements in the fitness of diploid and polyploid planarians is the case of two sympatric and closely related species from Sardinia, *viz*. *Dugesia hepta* and *D. benazzii*. *D. hepta* shows a haploid chromosomic number of n=7, which is unique in the Western Palearctic region, while *D. benazzi* shows the most common haploid chromosomic number in *Dugesia*, n=8 (Pala, Casu, & Vacca, 1981). *D. hepta* is a diploid species, while *D. benazzii* shows either diploid, triploid, and tetraploid populations. Moreover, the comparison between the karyotypes of *D. hepta* and *D. benazzii* pointed out that these two karyotypes may be the result of different processes of chromosomic rearrengements, rather than a loss of a single chromosome in *D. hepta*. Interestingly, a recent molecular study including individuals of both species has shown that they are able to hybridize in natural conditions, putatively generating hybrids bearing two chromosome sets from *D. hepta* and one chromosome set from *D. benazzii* (Dols et al., 2019). Thus, further analyses comparing the viability and reproductive capabilities of these hybrids compared to the parental species (among other genetic analyses), could be very interesting to undestand how chromosomic rearrengements drive planarian evolution.

7.3. Putative causes of karyological variability in planarians

A very intriguing question that arises after observing the above-mentioned high incidence of both shifts of ploidy level and chromosomic rearrangements in planarians, is what are the causes that generate this extreme karyological variability.

One of the principal mechanisms that have been reported to trigger polyploidization events is the formation of unreduced gametes during meiosis. In the plant species *Arabidopsis thaliana*, it has been seen that mutations in certain genes can alter the different meiotic phases, resulting in most of the gametes not being haploid (Moghe & Shiu, 2014). Thus, one

possibility could be that mutations in the genes that regulate the meiotic process in diploid sexual planarians resulted in the obtention of polyploid offspring. On the other hand, in the case of triploid sexual planarians, such as most of the facultative populations of *D. subtentaculata* analyzed in the present study (Chapter 2- Annex), obtaining both diploid and triploid offspring is easier to envision. In fact, the preliminary results obtained in the annex of Chapter 1 indicated that triploid sexual individuals of this species use the same meiotic system as the one reported for *D. ryukyuensis*, which is characterized by the recurrent production of haploid sperm and both haploid and diploid oocytes (Chinone et al., 2014). Therefore, this meiotic system may constrain sexual triploid individuals to systematically produce diploid and triploid descendants, with eventual cases of tetraploidization by the formation of unreduced triploid oocytes.

Another very interesting point which I believe is worth to be mentioned regarding the putative causes for the shifts in the ploidy level in planarians, is the recent finding that the genome of the species *Schmidtea mediterranea* lacks some of the genes that are responsible for the spindle assembly checkpoint (SAC) (Grohme et al., 2018), which is the cellular mechanism that ensures the correct segregation of the chromosomes during cellular division (Zhou, Yao, & Joshi, 2002). Thus, although the lack of these genes does not prevent planarians to show a functional SAC, it could be possible that the SAC function in planarians was somewhat more "relaxed" than in other organisms, explaining the high incidence of polyploidization events and cases of aneuploidy in this group.

Regarding the striking incidence of chromosome rearrangements in planarians, one of the principal causes could be related to their high tissue turnover and to the process of regeneration after fission. These two processes involve a tremendous rate of cell division via mitosis (Pellettieri & Sánchez Alvarado, 2007; Wenemoser & Reddien, 2010). Therefore, although planarians possess a broad genetic toolkit for DNA repair (Barghouth, Thiruvalluvan, Legro, & Oviedo, 2019), it could be possible that errors during the repair of double strand DNA breaks during cell division generated chromosomic rearrangements, as has been reported to occur in mammal cells (Iarovaia et al., 2014; Qiu, Zhang, Roschke, Varga, & Aplan, 2017).

Finally, an additional possibility to explain the high incidence of chromosomic rearrangements in planarians might be related with transposable elements, as it has been proposed to occur in other organisms (Feschotte & Pritham, 2007). In the case of planarians, it has been seen that *Girardia tigrina* presents a high copy number of mariner-like transposons (Garcia-Fernàndez et al., 1995). Moreover, the recent sequencing of the genome of *Schmidtea*

mediterranea has also revealed the existence of a novel type of giant retroelements in this planarian species (Grohme et al., 2018).

Further analyses, using any planarian species showing high levels of karyological diversity as model organism, such as *D. subtentaculata,* would be of great value to unveil the factors that trigger the extreme levels of karyological variability that occur within this group, including the gain and loss of entire chromosome sets, the gain and loss of single chromosomes, and the high incidence of chromosomic rearrangements.

8. Paleogeographic and paleoclimatic processes as determinants of the genetic diversification in planarians

8.1. Is the genus *Schmidtea* a European planarian relic from the Cretaceous?

The results obtained in the present thesis in the analysis of divergence time estimation in *Schmidtea* pointed out that that the first split within this genus occurred approximately 110 Ma (194-50 Ma), while the divergence between *S. mediterranea-S. polychroa* dated back to 67 Ma (122-29 Ma), very similar to the estimated divergence between *S. nova-S. lugubris* (Chapter 4 - Annex). These times of divergence are slightly older than the ones obtained in a previous work focused on the phylogeographic history of *S. mediterranea* (Lázaro et al., 2011). In that work, which used the information of the Cox1 and the Cyb markers, the first split within *Schmidtea* was dated to 85 Ma (interval of confidence not shown), while the divergence between *S. mediterranea-S. polychroa* was estimated around 43 Ma (72.23-24.96 Ma) and the divergence between *S. nova-S. lugubris* around 35 Ma (interval of confidence not shown). The calibration of that phylogeny was performed by constraining the node separating the genus *Girardia* from the genera *Schmidtea* and *Dugesia* at a maximum age of 100 Ma, considering that the biogeographic event that promoted the diversification of these groups corresponded to the breakup of Gondwana into South America and Africa, as proposed by Ball (1974). However, it has been recently pointed out that the diversification between *Girardia* and the rest of Dugesiidae genera might be much older than the breakup of Gondwana (Solà et al., 2019), explaining why the ages of divergence estimated by Lázaro are younger than the ones obtained in the present study. Moreover, in this recent work, the estimated age of diversification between the *Dugesia* species from Eastern and Western Greece coincided with

the biogeographic event known as the Mid-Aegean Trench (MAT) when using other calibration points for the root, indicating that this biogeographic event (the one that we used to calibrate our *Schmidtea* phylogeny) may be a good calibration point. Altogether, although additional divergence time estimates for the genus *Schmidtea* would be needed to confirm the results obtained in the present thesis (by increasing the number of molecular markers and the sampling effort within *Schmidtea*), the current most plausible temporal scenario for this genus indicates that its origin and posterior diversification might date back to the Cretaceous period (145-66 Ma).

During the Mid-Late Cretaceous period (100-66 Ma), the lands that today constitute Europe, corresponded to an island archipelago (Csiki-Sava, Buffetaut, Ősi, Pereda-Suberbiola, & Brusatte, 2015; and references therein) (Fig. 14). This paleogeographic situation was putatively caused by a dramatic rise of the sea-level resulting from the increase of the seafloor speeding rate that occurred after the breakup of Pangea (Seton, Gaina, Müller, & Heine, 2009). Importantly, this island archipelago was extremely dynamic, principally due to the intense tectonic activity of the region. Thus, it could be possible that a vicariant event during this period isolated the ancestors of the four *Schmidtea* species into two different islands, originating the lineages corresponding to *S. mediterranea-S. polychroa* and *S. lugubris-S. nova*. Although it is not possible to know the exact islands where these lineages became isolated, the present distribution of the different species indicates that the lineage of *S. mediterranea-S. polychroa* was probably isolated in a more western region, while the lineage of *S. lugubris-S. nova* was probably isolated in a more eastern region. Concomitantly, the poor species richness of *Schmidtea* suggests that other putative lineages of this genus may possibly had gone extinct during this period, probably due to the marine inundation of the lands where they inhabited. Similar processes of speciation and extinction during this period have been reported for several endemic terrestrial and freshwater vertebrate fauna from Europe, principally basing on information from the fossil record (Csiki-Sava et al., 2015). Although most of this fauna is now long extinct, there are some cases of lineages that survived. The best-known examples are the family of frogs Alytidae (which includes the popular Midwife toad) and the family of salamanders and newts Salamandridae. Therefore, the four species of the genus *Schmidtea* could correspond to the descendants of the only two lineages of this genus that survived the harsh conditions that struck Europe during the Cretaceous, suggesting that this genus of freshwater planarians may represent a true European faunal relic.

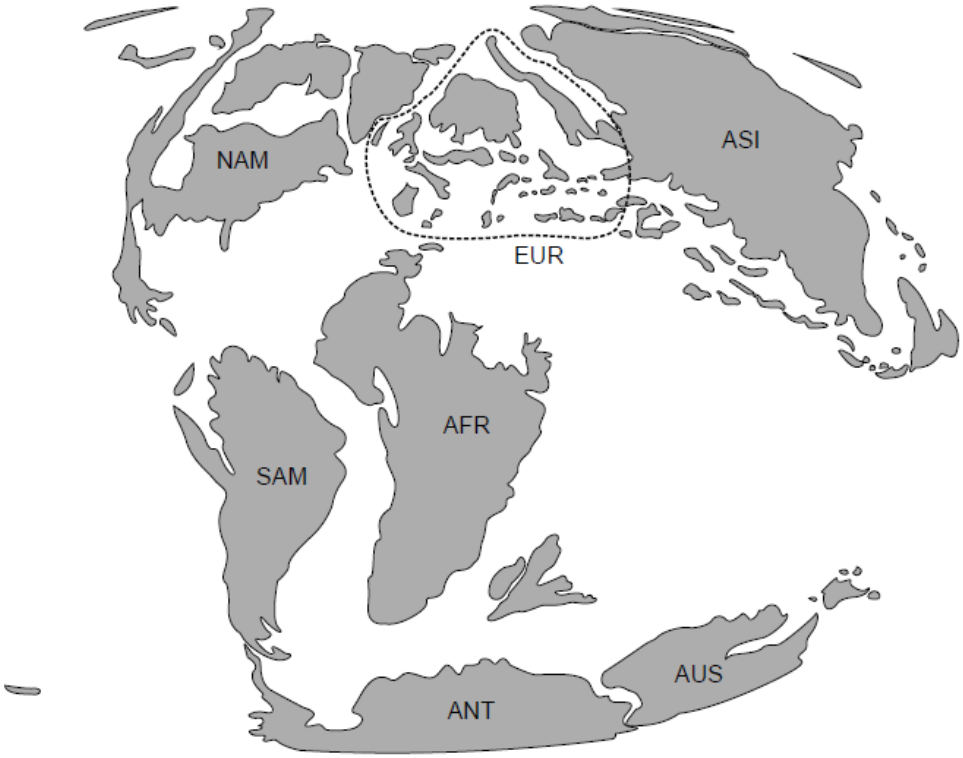

Fig. 14. Global paleogeographic reconstruction of the Middle Cretaceous period (105 Ma). The lands that today constitute Europe are highlighted with a dashed line. EUR: Europe; NAM: North America; SAM: South America, AFR: Africa; ANT: Antartida; AUS: Australia; ASI: Asia. Modified from Blakey, n.d.

8.2. The role of the Western Mediterranean microplate paleodynamics in planarian diversification

The results found in the Chapter 3 of the present thesis indicate that the paleogeographic history of the Western Mediterranean region during the Oligocene – Miocene (33.9 - 5.3 Ma) played an important role driving the diversification of the *Dugesia* species that presently inhabit in this region. The first diversification event that we detected corresponded to the split between the group of species including *D. gonocephala*, *D. liguriensis* and *D. etrusca* and the rest of species from the Western Mediterranean region, coinciding with the detachment of the landmass that today constitutes all the Western Mediterranean Islands (together with the Kabylies and Calabria), as proposed by (Rosenbaum, Lister, & Duboz, 2002). Moreover, our results indicated that the following fragmentation of the plate into different microplates and

their migration across the Mediterranean, not only probably isolated the ancestors of the species *D. hepta* and *D. benazzi* in the islands of Corsica and Sardinia but also allowed, on the one hand, the arrival of the ancestors of *D. tubqalis* and *Dugesia* sp. 1 to Northern Africa and, on the other hand, the return of the ancestors of *D. aurea*, *D. corbata*, *D. vilafarrei*, and *D. subtentaculata* to the Iberian Peninsula after a long journey across the Western Mediterranean.

This paleogeographic event has also been proposed to have driven the diversification of the freshwater planarian species *S. mediterranea* (Lázaro et al., 2011). However, differing from *Dugesia*, the fragmentation and migration of the microplates was proposed to drove this species from the eastern coast of the Iberian Peninsula to Sardinia, and from Sardinia to Sicily and Tunisia via Calabria. Therefore, while some of the *Dugesia* species were probably being transported to Africa through the Betic-Rif microplate, *S. mediterranea* was probably being transported to Italy through Calabria, and from there to the north of Africa through Sicily. A similar phylogeographic pattern as the one proposed for *S. mediterranea* was proposed to occur in the genus of terrestrial spiders *Parachtes* (Bidegaray-Batista & Arnedo, 2011), while the genus of terrestrial spiders *Ummidia* showed a similar phylogeographic pattern to the one proposed in the present thesis for the genus *Dugesia* (Opatova, Bond, & Arnedo, 2016).

Interestingly, this very same paleogeographic event led to alternative diversification scenarios in other organisms. For example, in the genus of earthworms *Postandrilus* the first breakup of the microplates from the Iberian Peninsula seem that only promoted the diversification between the Iberian and the Balearic lineages, but that it did not trigger the dispersion of this group to other Mediterranean regions (Pérez-Losada, Breinholt, Porto, Aira, & Domínguez, 2011). On the contrary, the phylogenetic relationships and distribution pattern of different species of the land snail genus *Tudorella*, indicates that the breakup and posterior migration of these microplates not only promoted the diversification of the lineages from the Balearic Islands and the Betic-Riff region but also allowed the colonization of Tunisia, Sicily, Malta, Sardinia, and Provence from the Kabylies, after their arrival to Africa (Pfenninger et al., 2010). These examples illustrate how the same paleogeographic event can result in different diversification histories depending on the organisms. In the case of the microplate paleodynamics of the Western Mediterranean, differences on the distribution of the groups within the microplates, on their dispersal capabilities, and on the suitability of the new environmental conditions for each group, possibly played an important role in shaping these multiple diversification scenarios.

8.3. Islands within islands: The historic factors that might have driven the diversification of the Balearic planarian species *D. aurea* and *D. corbata*

Similar to the situation observed in other organisms (see above), it could have been possible that the first breakup of the Western Mediterranean microplates triggered the diversification between the ancestors of the two *Dugesia* species from Mallorca (*D. aurea* and *D. corbata*) from the rest of *Dugesia* from this region, by promoting their geographic isolation in this island. However, our results indicated that the arrival of these two species to Mallorca was posterior (around 10 Ma), probably by dispersion from the Betic-Rif microplate through a land bridge connection, as has been proposed to have occurred in other endemic fauna from the Balearic Islands during this period (Bover et al., 2014; Chueca, Madeira, & Gómez-Moliner, 2015).

Interestingly, the deep genetic divergence found between *D. aurea* and *D. corbata* (around 7 Ma), indicates that these two species speciated soon after their arrival to the island. It has been proposed that during the Tortonian (11-7 Ma) the Balearic Islands were subjected to serial changes of the sea level, which reduced the emerged land to a group of small islands (corresponding to the present uplands) during the periods of high sea level (Pomar, 1988). The only known present localities of the species *D. aurea* and *D. corbata* are found in the Tramuntana range, which is the highest region of Mallorca (*D. aurea* is found in Torrent de Soller and *D. corbata* in Torrent de Sa Calobra – Pareis). Therefore, it could be possible that these two species became isolated in their present locations during some of the high sea level periods that took place during the Tortonian.

However, why are these two species still isolated in these two locations of the Tramuntana range? A possible explanation might be due to the environmental conditions. Interestingly, the results of the species distribution modelling analysis performed for *D. subtentaculata* revealed that the central region of the Tramuntana range (where *D. aurea* and *D. corbata* are found) is the only optimal region for this species in the Balearic Islands (see Fig. 3 in Chapter 3). Thus, if *D. aurea* and *D. corbata* have similar environmental requirements than *D. subtentaculata*, it could be possible that these two species are ecologically restricted to the Tramuntana range within Mallorca. A phenomenon of endemicity in the Tramuntana range has been found to occur in many groups of organisms, including both plant and animal species, such as the famous leaf beetle *Timarcha balearica* or the Mallorcan midwife toad *Alytes muletensis* (Pons & Palmer, 1996; Sáez, Fraga, & López-Alvarado, 2011). This phenomenon of endemicity has been associated to the special environmental characteristics of the

Tramuntana range together with its location within an island. Hence, the results obtained in the present thesis suggest that the genetic characteristics of the two *Dugesia* species endemic from Mallorca are the result of their first isolation in the island, followed by a second isolation in the Tramuntana range, representing a case of diversification resulting from a phenomenon of double insularity.

8.4. The role that European Quaternary climatic conditions played in planarian diversification

The dating analysis performed for *Dugesia*, revealed that the ancestors of the species *D. subtentaculata* probably arrived at the Iberian Peninsula around 10 My ago, when the Betic-Rif microplate collided with the southern region of the Iberian Peninsula (Chapter 3). However, we also found that the radiation of the different *D. subtentaculata* populations in the Iberian Peninsula did not probably occurred until approximately 1.5 Ma (2.26-0.78 Ma), coinciding with the gradual change of the climate conditions from tropical to the Mediterranean conditions that we know today in this region (Suc, 1984). Thus, it could be possible that the ancestors of *D. subtentaculata* remained ecologically restricted to certain localities from the southern region of the Iberian Peninsula for some millions of years, until more favourable conditions developed, allowing the expansion of this species northwards.

Interestingly, although the species *D. vilafarrei* has putatively remained the same amount of time in the Iberian Peninsula as *D. subtentaculata*, it can only be found in three geographically close localities in the southern region of the Iberian Peninsula. Thus, assuming that both species may have similar ecological requirements, why *D. subtentaculata* was able to expand across the whole Iberian Peninsula, while *D. vilafarrei* remained restricted in the south? One of the most plausible hypotheses may be that *D. subtentaculata* shows higher colonization capabilities than *D. vilafarrei*, possibly due to its fissiparous type of reproduction. Hence, it could be possible that when climatic conditions become favourable at the early Quaternary, fissiparous populations of *D. subtentaculata* rapidly colonized the Iberian Peninsula, while *D. vilafarrei* could not outcompete them. A similar situation can be found in the species *D. hepta* (sexual) and *D. benazzii* (sexual and fissiparous). In this case, although both species inhabit in Sardinia, *D. hepta* is only found in four rivers of the same fluvial basin, while *D. benazzi* is widespread across the island (Dols et al., 2019). All these results indicate that the combined effect of the change in the climatic conditions that took place at the early Quaternary (around 2 Ma) together with putative processes of competition between species

related to the reproductive strategy drove the diversification of *Dugesia* in the Iberian Peninsula during that period.

The results of the species distribution modeling for the four *Schmidtea* species and *D. subtentaculata*, indicated that the optimal distribution range of these species have remained rather constant during the last 130.000 years. Interestingly, we also found that the period corresponding to the Last Glacial Maximum (22.000 ya) was the period when the putative distribution of all species showed more continuity, indicating that the last glaciations might not reduce the distribution area of these planarian species. For instance, it could be even possible that *S. polychroa* and *S. lugubris* expanded to Great Britain during that period, while *D. subtentaculata* expanded to the eastern limits of its present distribution, reaching the east coast of France and Catalonia. In the case of *Schmidtea*, this hypothesis is supported by the high mitochondrial divergence that was found between some populations of *S. polychroa* in a previous study, in which it was suggested that the quaternary ice ages did not drastically reduce the distribution and genetic diversity of this species (Pongratz et al., 2003).

In the case of *D. subtentaculata*, a species principally inhabiting the Iberian Peninsula, a range expansion during the Last Glacial Maximum may be easy to envision, since multiple glacial refugia during that period have been identified in this region (Gómez & Lunt, 2007). However, the case of the different *Schmidtea* species is in controversy to what has been proposed for many European species, in which the last glaciations have been pointed out to promote drastic reductions both in their distribution and genetic diversity (Hewitt, 2000). Interestingly, different cases of cold-adapted species have also been reported to have survived the last glaciations in different regions of Northern Europe (e.g., Hänfling, Hellemans, Volckaert, & Carvalho, 2002; Quinzin, Normand, Dellicour, Svenning, & Mardulyn, 2017), suggesting that this could also be the case of *Schmidtea*. Therefore, although additional genetic analyses including more samples of the different *Schmidtea* species together with analysis of other European planarian genera would be needed, the species distribution analysis performed in the present thesis points out that the climatic oscillations that took place during the Quaternary might not reduce the genetic diversity of the European freshwater planarian species.

9. Do we need to adopt a new taxonomic framework for planarians?

The anatomy of the copulatory apparatus is the main source of information used to trace species boundaries in planarians. Thus, although many taxonomic studies nowadays include other sources of information, such as molecular data, new planarian species are never described if they are not accompanied by diagnostic morphological characters of the copulatory apparatus.

In the present thesis, we have proposed that the anatomy of the copulatory apparatus of planarians might be under a general state of morphological stasis, putatively due to hermaphroditism. This situation is especially problematic for the taxonomy of the group, since there could be many morphologically cryptic species. Evidences of putative morphological crypsis in the copulatory apparatus have already been found in other *Dugesia* species. For example, in a recent taxonomic work in the Aegean region, it has been found that several populations morphologically diagnosed as *D. sagitta*, cluster into two paraphyletic lineages highly differentiated at the molecular level (Sluys et al., 2013). Similarly, in that work, a high degree of molecular divergence has been found between different populations of the species *D. cretica,* which do not show any differential characteristics at the morphological level. Importantly, the putative existence of morphologically cryptic species has been reported in other planarian genera besides *Dugesia*, such as in land planarians of the genus *Obama* or in freshwater planarians of the genera *Polycelys* and *Crenobia* (Álvarez-Presas, Amaral, Carbayo, Leal-Zanchet, & Riutort, 2015; Brändle, Sauer, Opgenoorth, & Brandl, 2017; Rader, Unmack, & Moore, 2017). All these results suggest that the present diversity of planarian species could be highly underestimated as a consequence of using as the main source of taxonomic information a character that is under stasis.

Additionally, the putative high selective pressure acting upon the copulatory apparatus, implies that the only characteristics that may be able to vary are those that probably do not generate reproductive incompatibilities. These characters include minor morphological variations, such as the position of the ejaculatory duct into the penis papilla or the position of the openings of the vasa defferentia into the seminal vesicle, among others. In the present thesis, we have seen that some of these characters, which are the ones generally used to diagnose planarian species, can be found to vary at the intraspecific level when a high number of populations are analyzed, suggesting that many planarian species could actually be described basing on a misleading combination of characters due to low sampling efforts.

For all these reasons I consider that the morphology of the copulatory apparatus should not be used as the main source of information to diagnose planarian species. With this, I do not mean that morphological data should be no longer included in taxonomical studies of planarians, on the contrary, I think that more populations should have to be analyzed in each study in order to detect putative intraspecific morphological diversity. For instance, as what we applied in Chapter 2 of the present thesis, I believe that the combination of molecular, morphological, and karyological information could form a very complete taxonomic framework for planarians, which may be valuable not only to be used in future descriptions of new species but also to re-evaluate the taxonomic status of many species that have been described solely on the basis of morphological information.

Finally, I would like to end by encouraging planarian taxonomists to not refrain from describing species that are cryptic at the morphological level, since, in my opinion, they reflect an evolutionary reality that might account for an important portion of the current planarian diversity.

Conclusions

1. The species *D. subtentaculata* shows a broader distributional range than previously thought, being particularly abundant in the northern region of the Iberian Peninsula, an area where the species was previously unknown.

2. Investing in a high sampling effort when performing evolutionary studies in planarians is necessary to obtain a reliable picture of the genetic, morphologic, and karyologic diversity existing within species.

3. The high divergence between *Schmidtea* and *Dugesia* makes the genome of *Schmidtea mediterranea* not the most appropriate reference to search for variable nuclear markers in *Dugesia*.

4. The methodological approach used in the present study to search for nuclear markers bearing information at both the intraspecific and interspecific level in *Dugesia* has yielded good results, despite of not having a good genome of reference.

5. Fissiparous reproduction in *Dugesia* generates high levels of nuclear and mitochondrial intraindividual genetic diversity in a mosaic context, putatively due to the progressive accumulation of mutations in the different alleles of the neoblasts. This genetic phenomenon represents a variation of the Meselson effect, which we have named as the *mosaic Meselson effect*.

6. Events of occasional sex in fissiparous populations of *Dugesia* increase the genetic diversity between individuals due to the segregation and outcrossing of the alleles generated by the *mosaic Meselson effect*.

7. Processes of intraindividual selection at the neoblast level during periods of fissiparous reproduction together with selection at the individual level during events of occasional sex might allow *Dugesia* individuals to get rid of deleterious mutations.

8. Unnoticed high levels of intraindividual genetic diversity can lead to erroneous estimates of molecular phylogenetic relationships, number of molecular candidate species, and molecular ages of divergence.

9. Speciation processes in planarians can occur in the absence of variation in the morphology of the copulatory apparatus.

10. Shifts in the ploidy level may trigger speciation processes in *Dugesia* by promoting changes between sexual and fissiparous reproduction.

11. Chromosomic rearrangements may play an important role in the speciation processes of diploid planarians, but they may not be a principal driver of speciation in polyploid planarian species.

12. Paleogeographic events have played a crucial role driving the genetic diversification within *Dugesia* and *Schmidtea*, either by promoting vicariant processes or by connecting previously isolated areas.

13. Climatic changes during the last 130.000 years may have not drastically reduced neither the distribution nor the genetic diversity of the different planarian species inhabiting the Palearctic region.

14. The integrative analysis of molecular, morphological, and karyological information represents a suitable framework to perform taxonomic studies in planarians, which in the present work has unveiled the existence of three new *Dugesia* species.

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Annex:
Other publications

Dugesia hepta and *Dugesia benazzii* (Tricladida, Platyhelminthes): two sympatric species with occasional sex?

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Abstract

Dugesia hepta Pala, Casu & Vacca, 1981 and *Dugesia benazzii* Lepori, 1951 are two autochthonal morphologically described freshwater planarian species from the islands of Corsica and Sardinia. *D. hepta* is endemic of Sardinia and found in four northern hydrographic basins in cohabitation with *D. benazzii*, which has a wider Tyrrehnian distribution. Although these species have been broadly studied -especially *D. benazzii*- as regards to their variety of reproductive patterns as well as for their karyological diversity, little is known of them from a molecular phylogenetic perspective. For the first time, we present a molecular phylogenetic tree of the two species and their populations based on two molecular markers. Our results not only confirm that both species are molecularly distinct but show that *D. benazzii*'s Corsican and Sardinian populations could belong to separate species. Furthermore, we present molecular evidences that support what would be the first record of natural hybridization of the genus *Dugesia.*

Keywords: *Dugesia*, hybridization, hybrid speciation, molecular phylogeny, haplotype network

1. Introduction

Tricladida –most commonly known as planarians- have been the subject to an in-depth research in a wide-range spectrum of scientific fields, such as regeneration, pattern formation, genomics and transcriptomics (Abril et al., 2010; Newmark & Alvarado, 2002; Reddien & Alvarado, 2004; Robb, Gotting, Ross, & Alvarado, 2015; Robb, Ross, & Alvarado, 2007) as well as on diversity and phylogeographical analyses (Álvarez-Presas & Riutort, 2014; Leria, Sluys, & Riutort, 2018; Leria, Vila-Farré, et al., 2019; Sluys et al., 2013; Solà, Sluys, Gritzalis, & Riutort, 2013). However, historically, the Tricladida (Lang, 1884) have been a challenging group for taxonomists and systematists due to the unsuspected complexity for classifying their specimens which relies, ironically, in their morphological simplicity. Within any genus most planarian species share a common external morphology, hence the diagnostic characters are mainly found in their most complex organ, the copulatory apparatus. Nonetheless, the existence of fully fissiparous species (Stocchino & Manconi, 2013) as well as the fact that freshwater planarians are known for being able to resorb their own reproductive organs during starvation periods (Berninger, 1911; Newmark & Alvarado, 2002; Schultz, 1904) can sometimes render the morphological approach useless.

A good example of this situation is the longstanding freshwater flatworm genus *Dugesia.* This genus inhabits the Afrotropical, Palearctic, Oriental, and Australasian biogeographic regions and comprises an approximate number of 85 described species (Stocchino, Sluys, Riutort, Solà, & Manconi, 2017). In this genus three main types of life cycle related to their reproductive strategy can be found: sexual, asexual (fissiparous) and facultative, that alternate between the formers (Stocchino & Manconi, 2013). Asexual reproduction in this genus occurs by transverse fission of the architomic type - differentiation does not precede the fissioning (as in paratomy). Due to the existence of the aforesaid fissiparous reproducing populations, that do not develop the copulatory organs, it has not been unusual to group together several different species into one unique species or species group. For instance, the *Dugesia gonocephala* s.l. (Dugès, 1830) was a hodgepodge where all the asexual and non-conclusive forms of European *Dugesia* were confined (Benazzi, 1955; Benazzi & Banchetti, 1972; Benazzi & Deri,

1980; De Vries, 1984, 1986; Sluys & De Jong, 1984). The incorporation of molecular data not only helped to identify fissiparous individuals but also to delimit new species and to clarify the phylogenetic relationships within the genus (Álvarez-Presas & Riutort, 2014; Lázaro et al., 2009; Leria, Vila-Farré, et al., 2019; Leria, Villa-farré, Solà, & Riutort, 2019; Riutort, Álvarez-Presas, Lázaro, Solà, & Paps, 2012; Sluys et al., 2013).

Two species once belonging to the *D. gonocephala* s.l. complex, *Dugesia benazzii* Lepori, 1951 and *Dugesia hepta* Pala, Casu & Vacca, 1981 are the main focus of the present study. The former inhabits the islands of Corsica, Sardinia and Capraia (De Vries, 1985) whereas the latter is an endemism of Sardinia restricted to four fluvial basins in the northern region, where cohabits in sympatry with *D. benazzii*. *D. hepta* differs from other Sardinian *Dugesia* species in its haploid chromosomal number (n = 7; 2n = 14), which is unique in the Western Palearctic region (Stocchino, Corso, Manconi, Casu, & Pala, 2005), while in contrast *D. benazzii* presents the most common haploid chromosomal number among European and Asiatic *Dugesia* species, n = 8. Moreover, *D. benazzii* is known for comprising diploid (2n = 16), triploid (2n = 3x = 24), tetraploid (2n = 4x = 32) and aneuploid (with a mean chromosomal number of 32) populations (Lepori, 1951; Pala, Casu, & Lepori, 1982). Concurrently, *D. benazzii* characteristically presents sexual and fissiparous natural populations whereas for *D. hepta* there are no reports on asexual and/or polyploid individuals (Stocchino & Manconi, 2013). At first, both species were considered to be identical and indistinguishable except for their karyotypes (Pala et al., 1981), yet in a later study (Stocchino et al., 2005) differential morphological features arose in the copulatory apparatus and in the external morphology. Molecularly, *D. benazzii* and *D. hepta* have had a meager presence in the current molecular phylogenetic era, being represented in all cases only by a few individuals (Lázaro et al., 2009; Solà et al., 2013). From those studies we learnt that they are sister groups and closely related to the *Dugesia* species from the Western European Region.

These two sister morphologically nearly identical, and biogeographically sympatric species pose an interesting case of study. Since no thorough molecular study with a broad taxon sampling centered on these species have been carried out before and adding to the fact that *D. hepta* is restricted to only four fluvial basins where cohabites with *D. benazzii* individuals, one may wonder whether *D. hepta* could have had multiple origins or if it is really a monophyletic species. The first case poses a scenario where *D. hepta* would be the result of a recurrent chromosomal disorder from *D. benazzii* specimens and, thus, should not be considered as a species *per se*. In the second, we could face a possible case of biogeographical sympatric speciation due to a chromosomal rearrangement, with a concomitant parallel dispersion over the same fluvial basins. Speciation due to chromosomic rearrangements has been proposed to take place in other planarian genera (Benazzi, 1982; Leria et al., 2018) as well as in several turbellarian groups (Curini-Galletti, Puccinelli, & Martens, 1985; Galleni & Puccinelli, 1986). On the other hand, regardless of the true taxonomical status of *D. hepta*, given its morphological similarity to *D. benazzii* and their spatial distribution, one could wonder whether they are able to intercross and if they do it naturally. In fact, there are reports on an aneuploid (2n = ?x = 32) population of *D. benazzii* -referred as the 'biotype G' (Benazzi, 1949)- located in Rio Bunnari that was considered to be a stabilized natural hybrid population (Pala et al., 1982) based on karyological data, yet further research rejected that hypothesis (Benazzi-Lentati & Benazzi, 1985).

Hence, *D. hepta* and *D. benazzii* pose a case in which first, sympatric speciation may have occurred as the consequence of a chromosomal reorganization, and in the second place, posterior hybridization may have occurred. In the case of plants, approximately 25% of flowering species are considered to be involved in natural hybridization and introgression (Mallet, 2007), and the prevalence of this processes has been demonstrated to facilitate speciation and adaptive radiation (Mallet, 2007; Pennisi, 2016). In animals, hybridization is less frequent, affecting only a 10% of species (Arnold, Sapir, & Martin, 2008; Mallet, Besansky, & Hahn, 2016). Moreover, the existence of this reticulate evolutionary process leads to difficulties in phylogenetic inferences and species delimitation studies, challenging for instance de concept of species grounded on the evolutionary independence of lineages. Hence, studying the patterns and processes of reticulate evolution is not only important to understand evolutionary processes generating new species but also may help to resolve the relationship among closely related taxa affected by them.

In the present study, we aim to answer the following questions using molecular tools: (i) is *D. hepta* a monophyletic distinct species from *D. benazzii* or the result of a recurrent chromosomal disorder from the last species? (ii) Do these two species (or taxonomic entities) hybridize? To accomplish our goals, we have performed a broad sampling of both species from Sardinia; we make use of karyological data to identify some individuals and molecular data to infer the phylogeny of the Sardinian populations of *D. hepta* and *D. benazzii* as well as to reconstruct a haplotype network to assess the existence of hybridism. The results obtained show that *D. hepta* and *D. benazzii* are indeed two different species. However, we also find a complex scenario that can be compatible either with hybridization between the two species or with a case of recent speciation with incomplete lineage

sorting, or even both phenomena. In any case, the molecular result points to a reticulate relationship between species that will require analyses at the genomic level to fully understand the evolution of these group of species.

2. Material and methods

2.1. Sampling

Dugesia benazzii and *Dugesia hepta* specimens were sampled from 32 localities distributed on the islands of Corsica and Sardinia (Figure 1, Table S1) between 1997 and 2010. For each locality, some specimens were fixed and preserved in absolute ethanol for molecular analysis. Others were kept alive and taken to the laboratory to obtain karyotypes.

2.2. Assignment of individuals to species

Diagnostic differences between the two species rely on their karyotypes and some small differences in the copulatory apparatus. We found the external morphological differences proposed by Stocchino et al. (2005) to be misleading (Aguilar, 2011; unpublished master thesis) but in contrast we envisaged that *D. hepta* and *D. benazzii* differed in 6 SNPs in their *Internal Transcribed Spacer-1* sequences (*ITS-1*) of the ribosomal cluster (Table 1). A situation that may allow the assignment of individuals from sympatric populations without a tedious work of karyotyping and/or obtaining histological sections from all the individuals. In the present work in the first place, we checked whether this correlation was univocally true by karyotyping several individuals from different populations to assign them to a species based on their chromosomal number. Once the karyological assignation was established, *ITS-1* sequences were obtained. Our newly obtained data corroborated Aguilar's (2011) hypothesis. Thereafter, *ITS-1* sequences were obtained to assign individuals to species.

2.3. Karyotyping

Karyotypes were obtained for 31 animals from four populations where *D. hepta* and *D. benazzii* were known to coexist (Table S1) in order to assign them to species and corroborate the *ITS-1* criterion. Chromosome metaphasic plates were obtained by the squashing method. Regenerative blastemas of caudal fragments were treated with a solution of colchicine (0.3%) for 4 hours. The blastemas were then transferred on slides and treated with a solution of acetic acid (5%) for 5 minutes. Subsequently they were stained with acetic orcein for 2 hours and squashed using a small coverslip. (cf. Stocchino et al., 2014 and references therein).

The criterion to assign the individuals to species based on karyological data was the following: specimens were assigned to *D. hepta* when the karyotype was 2n = 14 and any other case was considered a *D. benazzii* individual. This criterion was used because *D. hepta* is only described from diploid sexual populations, while *D. benazzii* is known to present diploid, triploid and also different types of aneuploids (Lepori, 1951; Pala et al., 1982).

2.4. DNA extraction, quantification and sequence amplification

Total genomic DNA extraction was performed for 161 individuals using DNAzol Reagent (Molecular Research Center Inc., Cincinnati, OH) and Wizard Genomics DNA Purification Kit (Promega Corporation) following the manufacturer's instructions. DNA quantification was performed for each sample using a spectrophotometer NanoDrop™ 1000 (Thermo Fisher Scientific Inc.) using 2 µL per sample.

Specific primers were used to amplify a fragment of the mitochondrial gene *Cytochrome c oxidase I* (*Cox1*), the *ITS-1* and the *transmembrane p24 trafficking protein 9* (*TMED9*; referred in the present study as *Dunuc12*). Sequences and annealing temperatures of each pair of primers are given in Table 2. Final PCR reaction volume for all markers was 25 µL, consisting of: (1) 5µL of Promega 5x Green GoTaq Flexi Buffer, (2) 2 µL of $MgCl_2$ (25 mM), (3) 1 µL of dNTP (0.5 mM), (4) 0.5 µL of each primer (either 10 or 25 µM), (5) 0.15 µL of *Taq* polymerase (5 u/µL) (GoTaq Flexi DNA Polymerase of Promega), (6) 1 µL of genomic DNA sample (50 ng/ µL). Autoclaved miliQ water was added to obtain the final PCR volume. It was necessary in many cases to vary the annealing temperatures or the amount of $MgCl_2$ and/or DNA in order to achieve sequence amplification. The resulting PCR products were visualized in a 1% agarose gel in order to verify the correct amplification of the different molecular markers.

Viable-checked PCR products were purified before sequencing using a vacuum system (MultiScreen™$_{HTS}$ Vacuum Manifold of Millipore) or a digestion with exonuclease I (0.2 u/µL; Tebu-Bio) and shrimp alkaline phosphatase (0.2 u/µL; SAP, Sigma-Aldrich) (1:2 ExoSAP per PCR product at 37°C for 15 min plus an additional phase at 80°C for 15 min). Sequencing reactions were performed using Big Dye (3.1, Applied Biosystems) and ran in an automated sequencer ABI Prism 3730 (Unitat de Genòmica dels Serveis Científico-Tècnics de la Universitat de Barcelona) or at Macrogen Inc. (Amsterdam). The same primers used to amplify were used for sequencing both strands. Chromatograms were visually checked for quality with Geneious R8 (Biomatters, http://www.geneious.com/ last visited June 2019) and then contig and consensus sequences were obtained.

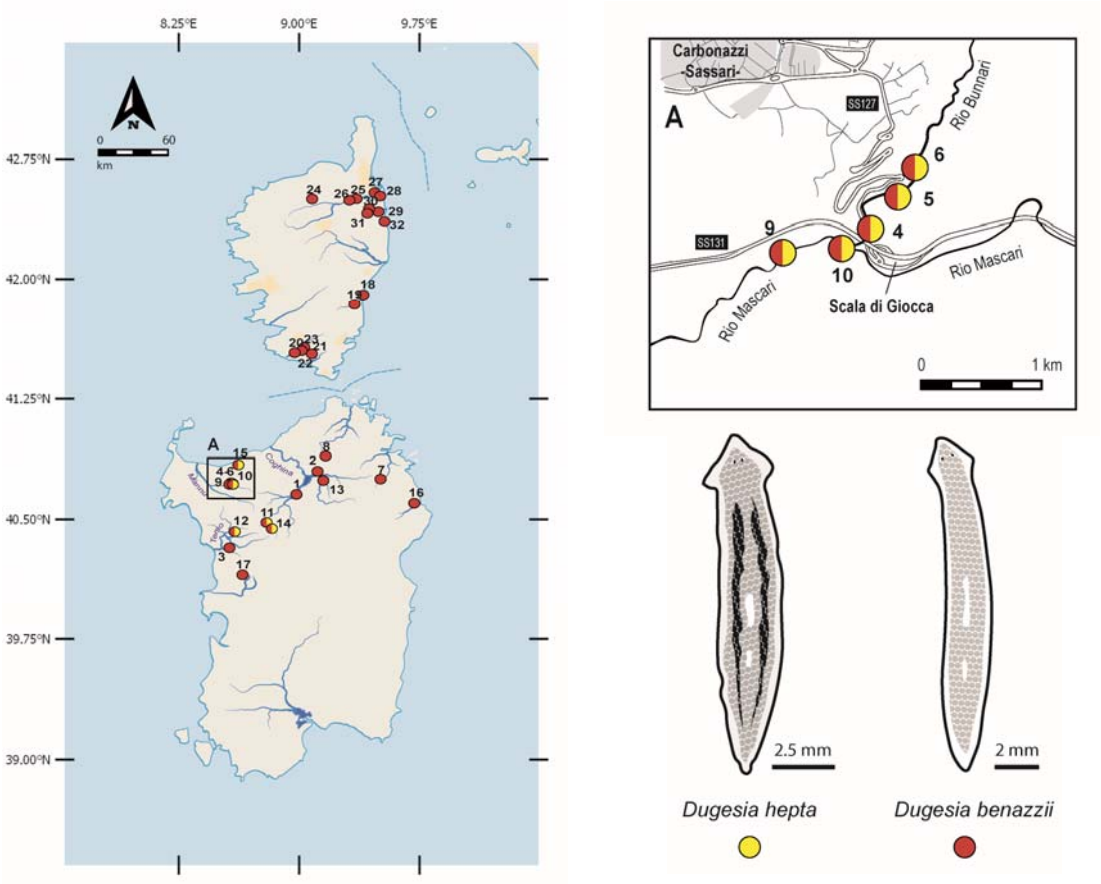

Figure 1. Sampling localities used for this study and geographical distribution of *D. hepta* and *D. benazzii*. Localities are numbered according to Supporting information Table S1. Red-colored circles indicate the presence of *D. benazzii* species whereas yellow-colored circles indicate *D. hepta*'s presence. (A) zoom in of Scala di Giocca. The map was created with Q-GIS v.3.2.2 (https://qgis.org/es/site/ last visited June 2019) and edited with Illustrator CC v.22.0.1 (https://www.adobe.com/products/illustrator.html last visited June 2019).

Table 1. Polymorphic diagnostic site positions of *D. benazzii* and *D. hepta* in the *ITS-1* alignment and Gen Bank accession numbers for the type haplotypes.

Species\Alignment position	12	24	49	223	372	458	*Acc. number*
D. benazzii	C	-	G	C	A	C	Pending
D. hepta	T	T	A	T	G	T	Pending

Since some individuals presented double-band patterns in their *Dunuc12* chromatograms, we decided to clone their PCR products to inspect their origin; we also cloned some animals that presented a mito-nuclear discordance or other peculiar molecular features (see results), and animals not presenting such features as a control. In total 17 individuals (4 controls and the rest presenting double bands, mito-nuclear discordances or other peculiarities) were cloned using a TOPO® TA Cloning Kit (Thermo Fisher Scientific Inc.) following the manufacturer's instructions. Approximately fifteen to thirty colonies from each individual were sequenced using the T3 and T7 primers (included in the kit).

2.5. Phylogenetic analysis

Alignments of the sequences were performed with the online software MAFFT v.7 (Katoh & Standley, 2013) and posteriorly revised in BioEdit v.7.2.5 (Hall, 1999). Prior to analyses, *Cox1* and *Dunuc12* sequences were translated into amino acids to verify that there were no stop codons within coding regions. Three alignments were obtained: (1) *ITS-1*, used to perform the species assignment; (2) *Cox1* (dataset I) and (3) *Dunuc12* (dataset II), which were used to perform the subsequent phylogenetic analyses. *Cox1* and *Dunuc12* sequences of four specimens belonging to three other *Dugesia* species were used as outgroup for the phylogenetic inferences (Supporting

Table 2. Sets of primers used in this study.

Gene	Primers	Sequence (5'-3')	Ann. Temp. †	Source
Dunuc12	Jon_12F	GATTACGAAAGCTATTTATAATT	52	Present study
	Snow_12R	CATGCACAAGATTACAAAG	52	Present study
	Elo_12F	AAGCTATTTATAATTCAGCG	54	Present study
	Hell_12R	AAAGAAATTGCTGCTAAAG	54	Present study
	Dunuc12_1F	CTCGTATCTCTGAATCTAGCCTC	55	Leria, Vila-Farré, et al., 2019
	Dunuc12_1R	GTTCATACAACTCATTCTTC	55	Leria, Vila-Farré, et al., 2019
Cox1	SamCF	GCTAATAATTTGAGTTTTTG	51	Present study
	TarlyCR	CATTTTAAAACAACATTACC	51	Present study
	COIF	CCNGGDTTTGGDATDRTWTCWCA	49	Lázaro et al., 2009
	COIR	CCWGTYARMCCHCCWAYAGTAAA	49	Lázaro et al., 2009
ITS-1	ITS-9F	GTAGGTGAACCTGCGGAAGG	45	Baguñà et al., 1999
	ITSR	TGCGTTCAAATTGTCAATGATC	45	Baguñà et al., 1999

†: *Annealing temperature*

information Table S1).

Levels of sequence saturation were assessed by means of the Xia et al. (2003) test implemented in the software DAMBE (X. Xia & Xie, 2001). The best substitution model was selected with jModelTest (Posada, 2008) based on the Akaike information criterion (AIC). In both cases the best fitting model resulted in a HKY+G. Phylogenetic analyses were performed using two inference methods: Maximum Likelihood (ML) and Bayesian inference (BI). ML analyses were performed with RaxML 7.0.3 (Stamatakis, 2006), applying a GTR+G substitution model -owing to the absence of the HKY model in the aforementioned RaxML version. 5,000 replicates were calculated to obtain bootstrap supports (bs) conducting a rapid bootstrap analysis and the ML search was performed starting from a random tree. Furthermore, we applied an optimization of both branches and model parameters on bootstrapped trees. BI analyses were conducted using MrBayes v.3.2 (Ronquist et al., 2012) and applying a HKY+G substitution model. Prior to run the analyses, nexus files were generated with MEGA6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). We ran one cold and three heated chains for two parallel runs. Both topological and model parametrization convergence were surveyed by checking that the standard deviation of the split frequencies reached a value below 0,01. 10,000,000 generations were performed for each gene, saving a tree every 5,000 generations. We applied the default burn-in –set at 25%- in order to avoid the inclusion of trees obtained before likelihood values had stabilized to infer the topology and the posterior probabilities (pp). Obtained trees were visualized with FigTree v.1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/ last visited June 2019).

2.6. Haplotype networks

In order to construct the haplotype networks, we generated two additional alignments only comprising specimens from Sardinia: 1) consisting of cloned *Dunuc12* sequences from 17 individuals (dataset III), and 2) comprising haplotypes from the *Cox1* sequences (dataset IV). In the former we included Sardinian individuals that presented chromatograms either with double-bands or patterns indicating possible heterozygosity for indels which had aroused our interest (MR0092-05, MR0092-11, MR0025-02, MR0091-01, MR0092-04 and MR0030-06). We also included individuals belonging to the COI mixed clade (see results) that did not present polymorphic bands (MR0088-02 and MR0092-03) as well as two individuals whose karyotype was known yet formed part of the mixed clade (MR0353-01 and MR0352-01). Furthermore, individuals MR0022-04, MR0022-05 and MR0172-01 were included due to possible mito-nuclear discordance. In addition, MR0022-04 and MR0172-01 had double-band patterns too. Finally, four individuals previously identified as *D. hepta* (MR0354-01 and MR0355-01) and *D. benazzii* (MR0368-01 and MR0370-01) based on karyological data were also analyzed as controls.

As for the *Cox1* haplotype network (dataset IV), all individuals with polymorphic positions in dataset I were excluded from the alignment. Sequences' ends were trimmed using BioEdit v.7.2.5 to avoid overestimating the number of underlying haplotypes due to the terminal missing data. DnaSP v.5 (Librado & Rozas, 2009) was used to determine and assign the

haplotypes for each individual analyzed and Network v.5.0.0.0 (Fluxus Technology Ltd.) was used to construct the haplotype networks. We used a default *epsilon* value set at zero.

2.7. Genetic diversity

Levels of nucleotide and haplotype diversity were calculated using DnaSP v.5. for each *Cox1* haplogroup. We also calculated a *Cox1* distance matrix using Kimura 2P evolutionary model for all the individuals included in the study.

3. Results

3.1. ITS-1 as diagnostic marker

The karyological analysis of the 31 individuals from localities 4, 5, 7, 9 and 14 revealed that nine of them matched to *D. benazzii* and 22 of them to *D. hepta*. *ITS-1* sequences could only be recovered for 23 of the aforesaid individuals. In 19 cases, the six species-specific SNPs proposed by Aguilar (2011) revealed the individuals to belong to *D. hepta* and four cases to *D. benazzii*, coinciding with the expected for the karyological species. These results confirm the *ITS-1*'s validity as a marker for molecular diagnosis of the two species. Henceforth we refer to it as the ITS-1 criterion in the present manuscript.

We attained an overall number of 146 *ITS-1* sequences; none presented double peaks in the chromatograms. The resulting alignment had a length of 496 bp. The six SNPs were highlighted as key elements for the species diagnose. An overall number of 91 individuals were identified as *D. benazzii,* 55 as *D. hepta* and 15 remained unidentified since our attempts to amplify and sequence their respective *ITS-1* were unsuccessful (*Dugesia* sp. in the Supporting information Table S1).

3.2. Dataset characteristics

We set four alignments to be analyzed either to estimate phylogenies (dataset I and II) or to construct the haplotype networks (dataset III and IV). Dataset I was comprised of 163 *Cox1* sequences (706 bp, 44 from Corsica, 115 from Sardinia and 4 outgroup sequences) and dataset II was comprised of 102 *Dunuc12* sequences (644 bp total, 83 exonic bp, 2 from Corsica, 96 from Sardinia and 4 outgroup sequences). Dataset III was constructed with non-polymorphic *Cox1* sequences (685 bp, 53 sequences representing the 43.1% of the original Sardinian alignment) -from which 19 haplotypes were identified (Supporting information Table S2). Lastly, dataset IV consisted of cloned *Dunuc12* sequences (532 bp, 57 exonic bp, 259 sequences) from 17 individuals, from which a total of 176 haplotypes were recovered (Supporting information Table S3) with a mean of 10.0 ± 4.83 different haplotypes per individual. Some of these haplotypes may have resulted from polymerase errors during the PCR step before the cloning procedure; however, our results exceed whatever expectations of errors caused by the malfunction of the polymerase. On the other hand, a study by Leria et al. (2019b) has demonstrated in a closely related group of species (*D. subtentaculata*, *D. aurea*, *D. corbata* and *D. vilafarrei*) the presence of mosaicism as a consequence of somatic mutation accumulation due to homeostatic processes, that results in multiple closely related haplotypes in sexual animals (star-like patterns).

The tests revealed no significant saturation signals from any of the alignments.

3.3. Phylogenetic analyses

The phylogenetic inferences carried out by means of both Bayesian Inference (BI) and Maximum likelihood (ML) yielded no topological incongruities as regards to each data set analyzed.

Based on the mitochondrial marker (dataset I), the BI tree showed four major clades (Figure 2): (1) *D. benazzii* specimens belonging to Corsica (*D. benazzii* A), (2) Sardinian *D. benazzii* specimens (*D. benazzii* B), (3) *D. hepta* individuals belonging to Sardinia (*D. hepta*) and (4) an unexpected, apparently mixed clade comprised of 13 individuals identified via *ITS-1* as *D. benazzii*, 5 individuals identified as *D. hepta* and 8 individuals whose *ITS-1* sequences we were unable to retrieve successfully –either due to amplification or sequencing failure- and, therefore, remained as *Dugesia sp*. Most of the clades were well-supported as indicated by posterior probability (pp; ≥0,95) and *bootstrap* (bs; ≥75) values -with the exception of the *D. hepta* clade, yet the resolution within clades was scarce. *D. benazzii* did not result in a monophyletic group but a paraphyletic one, being the Corsican clade (A) the sister group to a clade constituted by *D. benazzii* from Sardinia (B), *D. hepta* and the mixed clade. However, the phylogenetic relationship among these last three clades was unclear due to low support values.

The topology yielded by the nuclear marker (dataset II; Figure 3) differed from the one obtained with *Cox1*. First of all, the populations of *D. benazzii* from Corsica and Sardinia clustered as a monophyletic clade. Likewise, the main clades -namely one for *D. benazzii* and one for *D. hepta*- were unambiguously supported as indicated by pp and bs values, albeit the resolution within the groups was yet again poor. Secondly, there were no traces of the aforementioned mixed clade. Instead, the individuals belonging to the mitochondrial mixed clade were integrated within either the *D. hepta* or the *D. benazzii* group, in concordance with the *ITS-1* criterion. However, two individuals (MR0022-04 and MR0022-05) showed signs of mito-nuclear discordance given that their

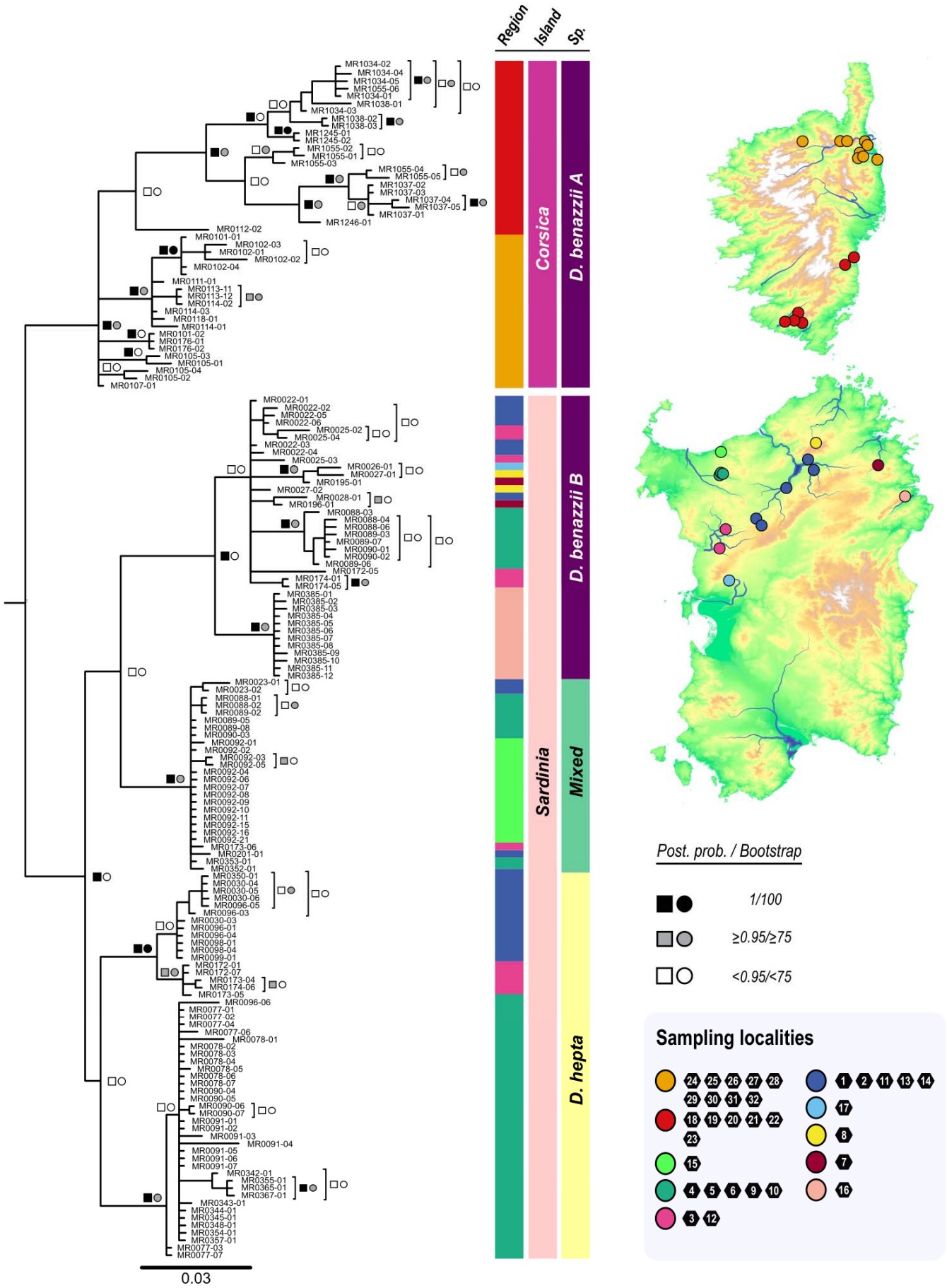
Region
Island
Sp.
Corsica
Sardinia
D. benazzii A
D. benazzii B
Mixed
D. hepta
0.03
Post. prob. / Bootstrap
1/100
≥0.95/≥75
<0.95/<75
Sampling localities

Figure 2. Bayesian Inference tree of dataset I (*Cox1*). Node values are displayed qualitatively using squares for posterior probability (pp) and circles for bootstrap support (bs) values. Used colors indicate fully supported (black), significantly supported (gray) and non-supported (white) nodes. Locality numbers from Table S1 are highlighted in black hexagons. Sampling localities are displayed in the map as follows: in Sardinia - grouped according to hydrographical distribution and in Corsica – grouped into northern and southern geographical regions as showed in the tree.

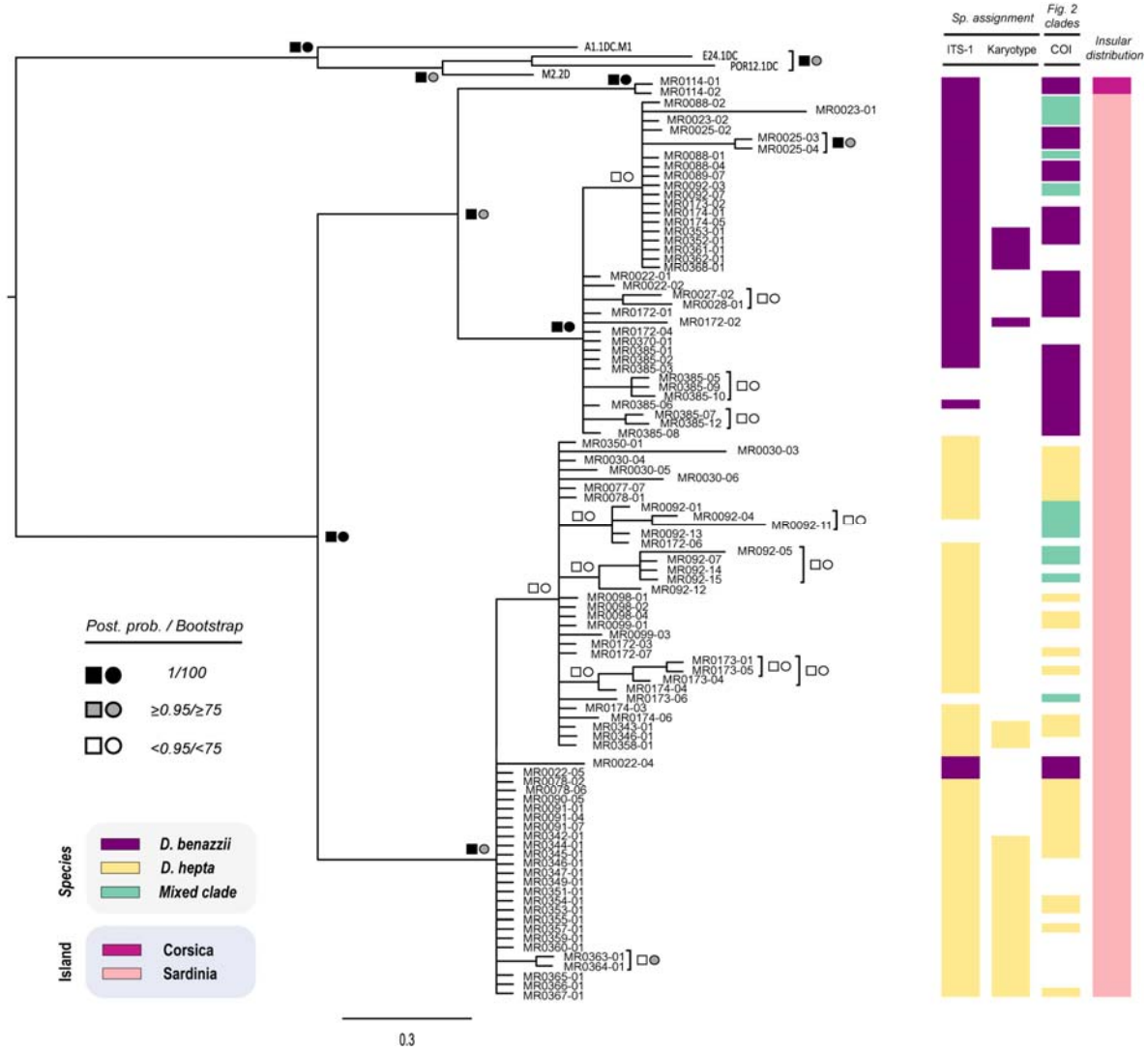

Figure 3. Bayesian inference tree of dataset II (*Dunuc12*). Node support values are displayed as in Figure 2. Bar diagrams indicate the species assignation of samples based on the *ITS-1* criterion and karyology. It is also showed the phylogenetic position in dataset I's tree and the insular distribution (fuchsia – Corsica and light pink – Sardinia).

contrary to the *ITS-1* identification- whilst the mitochondrial sequences placed them unequivocally within the *D. benazzii* B clade.

3.4. Haplotype networks

As regards to the *Dunuc12* (dataset IV) cloned haplotype network (Figure 4A), we found two distinct haplotype clusters separated by at least 24 substitutions and a 6-nucleotide indel. The individuals karyologically identified as *D. hepta* (MR0354-01 and MR0355-01) had all their haplotypes assigned to one cluster, while those karyologically identified as *D. benazzii* (MR0368-01 and MR0370-01 – from *D. benazzii* B clade) had their haplotypes in the other cluster, thus indicating that each cluster could be matched to a different species: namely a *D. benazzii* cluster and a *D. hepta* cluster. Although some of the haplotypes sequenced may have been artificially generated by errors of the polymerase, the high differentiation between the two species' clusters ensures that their differentiation is real, so that we can assign haplotypes from individuals as belonging to one species or the other depending on the cluster they belong to. Out of all the individuals assigned to the species *D. hepta* based solely on the *ITS-1* criterion, MR0030-06 and MR0091-01 had all their haplotypes unequivocally assigned to the *D. hepta* cluster, in accordance with

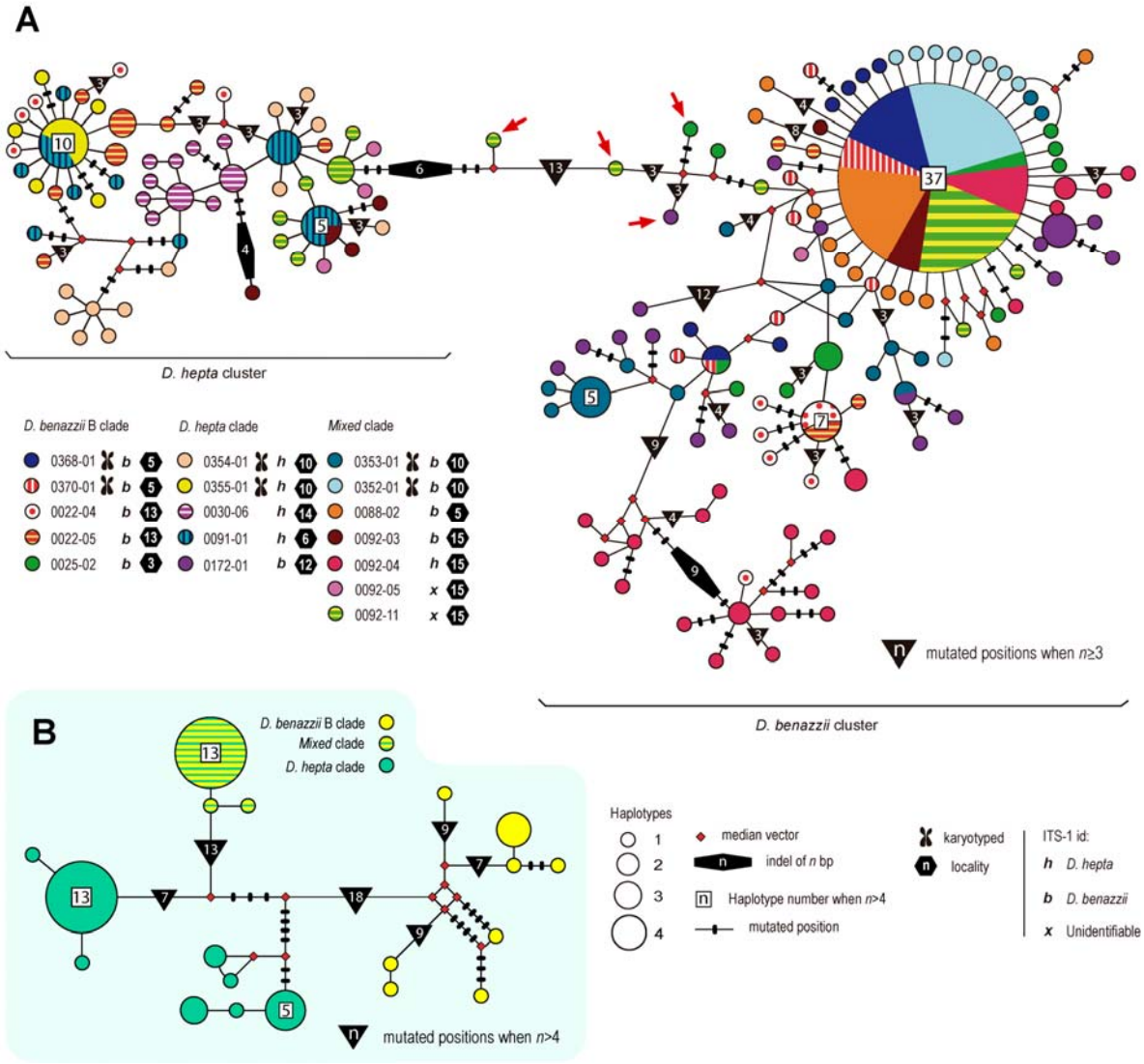

Figure 4. Haplotype networks for datasets III (A – *Dunuc12*) and IV (B – *Cox1*). Haplotypes are depicted as individual circles which are proportional to their abundancy (number of sequences), highlighted in a white square. Mutations are either depicted with black bars or black triangles when the number of mutations between linked haplotypes is equal to or exceeds a certain threshold number. Insertions and deletions are represented with an elongated hexagon indicating numerically the length of the *indel.* (A) For each cloned individual, information regarding *ITS-1* species identification, sampling locality and availability of karyotype is given. Recombinant haplotypes are highlighted with red arrows.

their mitochondrial data. In contrast, MR0092-04 had all its haplotypes associated to the *D. benazzii* cluster despite being part of the mitochondrial mixed clade. On the other hand, the individuals assigned to *D. benazzii* based on the *ITS-1* criterion showed more disparate results. Out of the specimens from mitochondrial *D. benazzii* B clade (Figure 4A), MR0022-04 and MR0022-05 presented haplotypes in both clusters whilst MR0025-02 had all its haplotypes in the *D. benazzii* cluster. Individual MR0172-01 from mitochondrial *D. hepta* clade presented only *benazzii* haplotypes, coinciding with its *ITS-1* assignment. As regards to the individuals belonging to the mitochondrial mixed clade, individual MR0088-02 had all its haplotypes associated to the *benazzii* cluster while MR0092-03 showed haplotypes in both clusters. At the same time, karyotyped individuals MR0353-01 and MR0352-01 showed only *benazzii* haplotypes. Lastly, individuals MR0092-05 and MR0092-11 that were not possible to identify based on *ITS-1* had haplotypes assigned to both clusters. It should be pointed out that intercluster recombinant haplotype variants were found in three individuals –MR0025-02, MR0092-11 and MR0172-01-. In summary, (Figure 5) the clonal analyses of the nuclear gene of different "anomalous" individuals have resulted in the finding of four groups of individuals. Some that are pure *D. hepta* or *D. benazzii*, and probably presented double bands in their sequences due to their heterozygosity (Figure 5, group NH). A second group that presents mitochondrial haplotypes either from *hepta* or

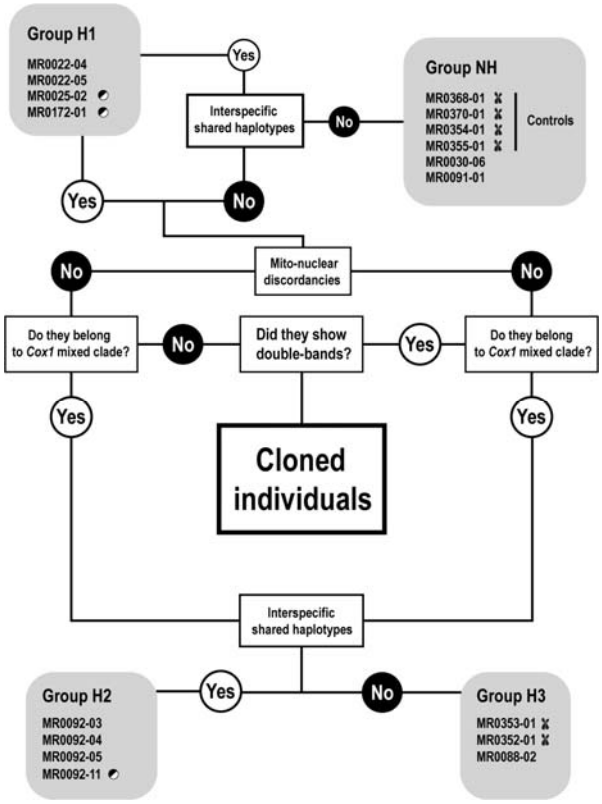

Figure 5. Summarization of dataset III groups and their characteristics. Individuals whose karyotype is stablished are highlighted with bivalent chromosome symbol. Black and white split-up circles indicate individuals with recombinant sequences.

benazzii but in which nucleus we can find haplotypes from both species or some recombinant (group H1). A third group presenting the mixed clade mitochondrial haplotype and also presenting in the nucleus haplotypes from both species (group H2), and finally a group of mixed mitochondrial haplotype but only *benazzii* nuclear information (group H3).

As per the *Cox1* haplotype network (Figure 4B), the same three Sardinian clades observed in the tree (Figure 2) were recovered. Curiously enough, in this case the mixed clade derives from within the *hepta* cluster, separated by 13 substitutions, instead of being closer to the *benazzii* cluster as shown in the phylogenetic tree (a relationship that receives a low support, Figure 2). The *benazzii* clade is separated from the *hepta* clade by 18 substitutions. Hence, the three groups are well separated but in fact the internal differentiation within the *hepta* and the *benazzii* clades is also quite high.

3.5. Genetic diversity

The *Cox1* distances estimated with Kimura 2P are shown in Table 3. The mean distance between *D. benazzii* from Sardinia and Corsica are 5.8 ± 0.8%. Within Corsica (*D. benazzii* A clade) the mean genetic distance value is 2.9 ± 1.4%, and within Sardinia (*D. benazzii* B clade) 1.6 ± 0.9%. For *D. hepta* the mean distance value is 1.3 ± 1.2%. Mean genetic distances value for the mixed clade is 0.1 ± 0.1%.

We also calculated nucleotide (π) and haplotype diversity (H_D) within each of the three *Cox1* haplogroups (Table 4). The values of H_D were high for the *hepta* and *benazzii* haplogroups in contrast to a low value for the mixed group. For π again *hepta* and *benazzii* presented higher values than the mixed clade, however its values were also moderately low.

4. Discussion

4.1. D. benazzii species status

The clues provided by the phylogenetic *Cox1* tree obtained in this study (Figure 2) point out that *D. benazzii* could constitute more than one species. We base this suggestion on the paraphyletic arrangement of the *D. benazzii* clades in the tree and especially on the high genetic differentiation among them. The genetic differentiation between the populations from Corsica and Sardinia for COI has a mean value of 5.8 ± 0.8%, far superior to the ones found between populations within each of the islands (Table 3). Moreover, the values found between islands concur with some of the interspecific genetic distance values for *Dugesia* species from the Western and Eastern Mediterranean (Lázaro et al., 2009; Solà et al., 2013) that vary between 2.8% for closely related species in the Aegean region, and 11% for some species on the Western region. On the other hand, the phylogenetic tree obtained from the nuclear marker (*Dunuc12*, Figure 3) showed a monophyletic *D. benazzii* clade, yet the two individuals from Corsica appeared again to be highly differentiated from the Sardinian populations. Nonetheless, the fact that only two sequences for the aforementioned nuclear marker of Corsican individuals were used in this study give us little information regarding genetic diversity of the populations of the allegedly Corsican *D. benazzii*. On the other hand, populations of *D. benazzii* from both islands are identical regarding the *ITS-1*, which will support the monophyly of *D. benazzii*, but not its division in more than one species unless this is a very recent event and ribosomal clusters are still being kept similar by concerted evolution. As for the morphology, the original description of the species given by Lepori (1951) did not establish any remarkable differences between Corsican and Sardinian *D. benazzii* populations as regards the copulatory apparatus but it did point out some minor dissimilitude that could lead to consider Corsican and Sardinian populations as distinct geographical subspecies. Hence, there is incongruence between the mitochondrial history and the nuclear and morphological accounts. In some cases, a potentially high degree of genetic variation may only be reflected by recondite morphological traits (according to

Table 3. Distance matrix of mean K2P distances (%) with standard deviations for the *Cox1* marker. Actual number of sequences is given inside the parentheses.

	D. benazzii A	*D. benazzii B*	*Mixed clade*	*D. hepta*
***D. benazzii A* (44)**	2.9 ± 1.4			
***D. benazzii B* (39)**	5.8 ± 0.8	1.6 ± 0.9		
***Mixed clade* (26)**	5.2 ± 0.8	4.2 ± 0.4	0.1 ± 0.1	
***D. hepta* (52)**	4.9 ± 0.7	4.0 ± 0.5	3.2 ± 0.1	1.3 ± 1.2

Kucera & Darling 2002) that are not evident at first sight. Sibling species often have minor morphological differences that are only noticed once species are recognized for other reasons –such as karyological data or molecular evidences-. The species that fit this profile are known as pseudo-cryptic (Knowlton, 1993), and this may be the case for Corsican and Sardinian *D. benazzii* populations. On the other hand, speciation is a continuum. Theoretically, the further we stray from the starting point the clearer and more evident should be the differences between descendant lineages but in the first stages of speciation there can be divisiveness among sources of evidence -i.e. genetic data *versus* morphological data, nuclear *versus* mitochondrial DNA- because changes do not accumulate uniformly and at a fixed rate. This interval of speciation is known as the 'gray zone' (De Queiroz, 2007) and could explain why we find differences regarding the *Cox1* sequences between Corsican and Sardinian *D. benazzii* populations but not in the *ITS-1* or in their morphology.

These results point to the need for a revision of the taxonomic status of *D. benazzii*, based on more data going from an increase of the number of nuclear markers and the use of molecular methods for species delimitation to a morphological and karyological revision of the individuals. A similar situation has been resolved in a close relative, *D. subtentaculata*, by the concurrent use of all these lines of evidence in an integrative way, resulting in the description of three new species that are morphologically cryptic with *D. subtentaculata* (Leria, Vila-Farré, et al., 2019).

4.2. Species status and origin of D. hepta

Dugesia hepta is a monophyletic species beyond questioning, as ascertained by both phylogenetic trees. We sustained a reasonable doubt regarding its taxonomical status owing to (1) the atypical chromosomal number (n = 7), and (2) their geographical distribution -restricted to four fluvial basins and in cohabitation with the morphologically near identical *D. benazzii* individuals. Our results rule out the possibility that *D. hepta* could be an aberrant chromosomal form of *D. benazzii* in which case we might expect to find *D. hepta* as a polyphyletic ensemble appearing in the phylogenetic trees independently from different *D. benazzii* clades. Our phylogenetic trees do not support that hypothetical scenario but rather show that *D. hepta* and *D. benazzii* are two different species that shared a common ancestor.

It is not clear from our results whether *D. hepta* is sister to only the Sardinian *D. benazzii* or to an older lineage that gave rise to the *D. benazzii* group from Sardinia (group B) and Corsica (group A). Nuclear data seem to indicate that *D. hepta* could be in fact the sister group of *D. benazzii* A and B (Figure 3), yet such relationship is questioned when the mitochondrial data is considered. Alas, discordances in nodal support depending on the inference method used to build the tree (Figure 2) let open the possibility that *D. hepta* could be the sister group of *D. benazzii* A and B based on the present *Cox1* data. It is worth noticing that although both species present similar values of diversity for their *Cox1* sequences (Tables 3 and 4) they differ in how this genetic variation is geographically distributed. The Sardinian populations of *D. benazzii* from the sampled localities showed no remarkable signs of geospatial structure or isolation, with the exception of the samples from Monte Albo (Table S1, Figure 1 locality 16) that are appreciably genetically isolated from the rest of *D. benazzii* B populations. In contrast, the populations of *D. hepta* appeared to be more structured with no apparent admixture of individuals from different fluvial basins (Figure 2). A plausible explanation for the differences in mitochondrial structuration degree could reside within the reproductive strategy of each species. *D. hepta* is exclusively sexual while *D. benazzii* is strategically more flexible having both sexual and fissiparous populations which could be advantageous towards rapidly colonizing new fluvial basins, as it has been shown for other *Dugesia* species (Lázaro & Riutort, 2013; Leria, Villa-farré, et al., 2019). This also could explain why *D. benazzii* has a broader geographic distribution. A speculative scenario will be that, *D. hepta*'s ancestral populations may had undergone through a constrain in numbers due to direct competition with other species -possibly *D. benazzii* itself who could have colonized Sardinia from Corsica and displaced *D. hepta*-. However,

Table 4. Estimations of nucleotide (π) and haplotype diversity (H_D) for the haplotype groups in dataset IV (*Cox1*).

Group	Sequences	Haplotype num.	H_D	π
Hepta	n = 27	8	0.738 ± 0.005	0.0131
Benazzii	n = 11	8	0.891 ± 0.008	0.0178
Mixed	n = 15	3	0.257 ± 0.020	0.0006

similar results could be expected if recent bottlenecks caused by abiotic phenomena -such as the desiccation of the brooks and springs where they can usually be found- affect *D. hepta* population's survival more than those of *D. benazzii* due to its exclusive sexual way of reproduction. There was no apparent correlation between the genetic lineages and their geographic distribution for the nuclear gene, for any of the species. This can be a result of the gene analyzed being highly conserved and hence lacking information for recent events of dispersion.

Whether *D. hepta* is sister to *D. benazzii* from Sardinia or to the lineage that gave rise to *D. benazzii* group from Sardinia and Corsica, the speciation event may have been related to a chromosomic rearrangement. Bearing in mind that *D. hepta*'s chromosomal number (n = 7) is uncommon within the whole Western Palearctic Region, and that most species of the Asia-European clade of *Dugesia* genus commonly share the n = 8 chromosomal number, we suggest that the ancestor that gave rise to the lineages leading to the current *D. hepta* and *D. benazzii* species might have shared the same chromosomal number, n = 8. Therefore, *D. hepta* could pose a case of speciation due to a chromosomal rearrangement. In most animal and plant groups there are differences regarding the chromosomal number among closely related species (King, 1993). Nonetheless, not all of the changes that may operate on the chromosomes are implicated in speciation phenomena (King, 1987), but only those that have potential to diminish the biological efficiency of the hybrids, which are known as negative heterotic (Forsdyke, 2004; King, 1987, 1992; Rieseberg, 2001), or that even imped their viability. Both cases, at shorter or longer term, give rise to reproductive isolation among populations and therefore, are likely to cause speciation (White, 1978). Chromosomal rearrangement speciation cases have gained presence over time (Coates & Shaw, 1984; Kawakami, Butlin, & Cooper, 2011; Talavera, Lukhtanov, Rieppel, Pierce, & Vila, 2013). There are other cases in freshwater planarians where a chromosomal rearrangement is suspicious of being the speciation cause, within the dugesid genus *Schmidtea*. *S. nova* and *S. lugubris* are two sibling species with haploid chromosomal numbers of n = 3 and n = 4 respectively. Within the genus, n = 4 is considered to be the plesiomorphic karyological state. *S. nova* would have originated from a common ancestor through a Robertsonian translocation plus a pericentric inversion resulting in its three basic chromosomes that would have rapidly isolated reproductively the descendent lineages (Benazzi & Puceinelli, 1973; Leria et al., 2018). In *D. hepta* we also have a reduction of the chromosomal number as well as changes in the chromosomal structure -being the most remarkable a large submetacentric chromosome 1 -within a predominantly metacentric set- that could be the by-product of a non-reciprocal translocation that led to the loss or the assimilation of the eighth chromosome. We can conclude that these two species exhibit a great karyological plasticity regarding ploidy and chromosomal composition as it has been previously proposed for other planarian groups (Leria, Vila-Farré, et al., 2019; Leria, Villa-farré, et al., 2019; Ribas, 1990), in comparison to their conservative morphology, and this plasticity may in some cases be related to speciation events. However, does really the chromosomal difference between *D. hepta* and *D. benazzii* impede their intercrossing?

4.3. Dangerous liaisons: A complex relationship between D. hepta and D. benazzii

Pala and coworkers (1982) had proposed that *D. hepta* and *D. benazzii* might be able to intercross to explain the presence of individuals bearing a variable number of chromosomes (being the most frequent number 32 but never eutetraploid) in Scala di Giocca (Rio Bunnari); although it was posteriorly refuted by Benazzi-Lentati and Benazzi (1985) based on karyometric analyses. To try to elucidate whether hybrids exist, we have planned our analyses to detect a classical basic case of hybridization, the detection of nuclear haplotypes from both parent species in the putative hybrids, together with the mitochondrial haplotypes from only one of them. Our results, however, show a much more complex and interesting situation that should be deeply looked into in further researches. We have found evidence that prove the existence of at least three types of "anomalous" individuals (H1 to H3, Figure 5) that most probably could be the result of hybridizations. Even though it would be tempting to jump into hasty conclusions, we cannot unerringly relate Vacca's et al. individuals to our hybrids. The situation is certainly much more complex than we could expect.

H1 individuals could be the result of a recent hybridization. Those individuals have *Cox1* sequences either belonging to the Sardinian *D.*

benazzii group –as well as their *ITS-1*- or to *D. hepta* group yet the *Dunuc12* nuclear marker presents haplotypes from both species or presumptive recombinant alleles. These individuals could be the result of a recent hybridization in which *D. benazzii* or *D. hepta* will have acted as a mother so that the hybrids have one or the other mitochondrial DNA. In the nucleus we will in this case expect to find haplotypes from both parents, which is the case for individuals MR0022-04 and M0022-05, while in the other two individuals we only find *benazzii* haplotypes but some presumptive recombinants. This latter case could be a consequence of the *hepta Dunuc12* haplotypes not having been PCR amplified as efficiently as *benazzii Dunuc12* haplotypes (so a methodological artifact) or else, that hybrid individuals have been able to backcross with *D. benazzii* parental species resulting in the loss of the hepta nuclear haplotypes.

For the *ITS-1* sequences, (showing *benazzii* origin in the four H1 individuals) either a similar situation is found (lack of amplification or backcross to parental species) or else the concerted evolution processes that regularly homogenize the multiple copies of the ribosomal clusters (Dover, 1982; Hillis & Dixon, 1991) may have resulted in the original *D. benazzii* cluster having overruled the *D. hepta* cluster. Subsequently, the validity of the *ITS-1* criterion as a highly reliable method to identify the species that we originally proposed is questioned. It will work for the parental species, but it will certainly fail to determine hybrids unless it is cloned. Since a hybrid will have the genomes of both parental species, theoretically one could be amplifying the *ITS-1* of any of the two parental genomes.

The H2 group individuals also present nuclear *Dunuc12* haplotypes from both species (or a recombinant), hence likely being of hybrid origin. However, what makes these hybrids special is that they bear the mixed clade *Cox1* haplotypes that appear as a monophyletic clade in the *Cox1* tree, completely independent from the *D. hepta* and *D. benazzii* clades. Therefore, we have individuals bearing in their nucleus haplotypes coming from both species, while their mitochondrial genome seems to have differentiated from both parentals, showing a closer relationship to the *hepta* haplogroup from which most probably derived (Figure 4B). Moreover, to make the picture more complex, within this mixed clade we also find the group H3 presenting the mixed clade *Cox1* haplotypes but only *benazzii* nuclear sequences.

Many of the members in the mixed clade belong to the same river where Pala et al. (1982) found and described the alleged stabilized 32-aneuploid hybrids (Bunnari). Yet the 32-aneuploid can be also found in Rio Silis (locality 15) where a karyological study carried out on the *D. gonocephala* s.l. planarian populations by Vacca et al. (1988) discovered another anomalous karyotype of 22 chromosomes with low frequency (described in 10 individuals out of the 95 studied). They were unable to neither reconstruct the ideogram nor stablish a solid ploidy due to the differences in size and shape among chromosomes. Furthermore, all individuals had copulatory apparatus and were able to lay cocoons but these were sterile. This 22 chromosomal number could result from the sum of 14+8 chromosomes, which would be possible if a diploid *D. hepta* gamete (most likely an oocyte) and a haploid *D. benazzii* gamete (most probably a sperm) joined, which could point to these animals to be our hybrids. The fact that in the *Cox1* network (Figure 4B) the mixed clade *Cox1* haplotypes derive from the *D. hepta* haplotypes would give further support to this latter possibility. Thus, we find two karyotypes in Rio Sillis that could be a match to our H2-H3 hybrids. Alas, we only have karyological information from two individuals of the mixed clade and they were found not to bear a diploid set of chromosomes (neither 14 nor 16 chromosomes), but the exact number was not registered.

All this evidence could point to a hybrid lineage that originated through the cross of a *D. hepta* oocyte with a *D. benazzii* sperm, and that now seems to be stabilized with its individuals reproducing by their own, so that no mitochondrial genomes from any of both parental species is newly introduced in this lineage. A possibility would be that the hybrid populations could carry on reproducing by fission, a reproductive strategy frequently used in *Dugesia* when they become triploids (Stocchino & Manconi, 2013) and, on time, evolve their own mitochondrial lineage by accumulating changes, as we observe in this case. That they use this type of reproduction will be supported by the low nucleotide and haplotype diversity found within this group, especially as compared to the found for the *hepta* a *benazzii* haplogroups (Table 4), expected for clonal individuals. This hypothesis would explain why the hybrids bear nuclear haplotypes of the two parental haplogroups but would not explain the existence of a recombinant haplotype (individual MR0092-11), neither the individuals of the H3 group. Howbeit, if the hybrids are able to intercross these latter cases would be explained, but this hypothesis has the problem of how the two different karyotype compositions can combine to produce viable gametes in the hybrids. A possibility would be that these animals use a similar strategy to that observed in triploid ex-fissiparous lineages of *D. ryukyuensis* (Chinone, Nodono, & Matsumoto, 2014). In a lineage from this species before spermatogenesis begins the spermatogonia eliminate a whole set of chromosomes, hence in a triploid hybrid two sets of the same species could remain in a certain proportion of cases and pass a regular meiosis. In the female oogenesis the three sets of chromosomes are retained until the metaphase I occurs. During the meiosis two

chromosome sets pair and the third remains alone. Thus, there is a certain probability that either the two sets of chromosomes from the same species pair or that those homologous chromosomes from the two parental species pair. This process would provide some haploid genetically equilibrated (bearing one copy of each gene) oocytes and also some diploid oocytes, either bearing two sets of chromosomes coming both from one species or even recombinants between the two species' chromosomes. This situation would clearly render these animals mostly sterile (explaining for instance the observations of Vacca et al. 1988) since the probability of getting two gametes with an equilibrated set of chromosomes each to mate and give offspring would be low. Nonetheless, even if this happened with a low frequency it would be enough to explain the presence of a recombinant haplotype among so many sequenced, and specially that some individuals may have only nuclear haplotypes from one of the parent species. Thus, in this hypothesis the hybrids may mostly reproduce by fission but could be able to mate and produce some fertile offspring from time to time. In any case, both hypotheses point to a probable case of speciation by hybridization, since the hybrids would have stabilized and have stablished populations reproducing on their own.

There is also a third possibility: that these mixed clade hybrids can cross with the parental species. Almost all the individuals in the mixed clade belong to localities where both species coexist (localities 5, 6, 10, 12, 14 and 15; Supporting information Table S1; Figure 1), thus giving the hybrids that have produced some genetically viable gamete the opportunity to backcross. When the hybrids act as females, the mitochondrial lineage is retained to evolve independently while the nuclear genome is continuously being introgressed by parental species chromosomes. We wonder if a similar reproductive strategy may explain the *D. hepta*, *D. benazzii* and putative hybrids conundrum, and if environmental and/or competition factors may explain the existence of the hybrids and the cohabiting of the three lineages.

However, similar results can be expected under incomplete lineage sorting (ILS) when a radiation takes place as it has been demonstrated in other cases (Suh, Smeds, & Ellegren, 2015). What we consider to be the *hepta* and *benazzii* exclusive haplotypes for *Dunuc12* and *ITS-1* would have been population alleles in a polymorphic ancestor. Stochastically, the *benazzii* variant could have gone lost in the *D. hepta* lineage and preserved as a polymorphic state in *D. benazzii* populations. Thus, the mitochondrial mixed clade would be a distinct *D. benazzii* clade -C- that would have diverged from the other Sardinian *D. benazzii*. We could expect to find in said clade homozygous *Dunuc12* individuals for the *D. benazzii* variant -even for the *D. hepta* variant, thought we found none- as well as heterozygous individuals that would be our 'hybrids'. This could also justify why there are individuals whose karyotype is *benazzii*-like and homozygous for the *benazzii* haplotypes within the mixed clade. However, this hypothesis will not explain the individuals bearing anomalous karyotypes and showing infertility found in previous studies. It will also have the difficulty to explain why three differentiated lineages cohabit in the same localities, whilst two cohabiting with their hybrids makes more sense from an ecological point of view.

5. Conclusions

We present for the first time molecular evidences of the species status for *D. hepta*, as a sister group and not derived from, *D. benazzii*. In addition, we have found that *D. benazzii* individuals from Corsica may in fact be a different species. At the same time, we have uncovered an unexpected and complex situation in those rivers from Sardinia where the two species, *D. benazzii* and *D. hepta*, cohabit. *D. benazzii* was thought to be a complex species presenting different ploidies and even aneuploids, while *D. hepta* exclusively diploid and sexual. Our results show that some aneuploids may in fact be the result of crossings between both species, which represents the first demonstration of planarian hybridism in natural conditions on the base of molecular data, and what is more relevant that they may even have become a new species. But the complexity of the mitochondrial and nuclear haplotype combinations found makes present information not enough to solve the riddle on how these hybrids may have originated and how they reproduce (if they do) and point to the need of a thorough study. An extensive sampling in the rivers where they cohabit, followed by a study at the genomic level of karyotyped individuals, so that the reproductive behavior, karyotype and genomic information is known from each individual will most probably render an interesting view on how this complex situation has been generated and is evolving.

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