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Effects of habitat transition on the evolutionary patterns of the microgastropod genus *Pseudamnicola* (Mollusca, Hydrobiidae)

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Molecular phylogenies of extant species are considered effective tools to infer mechanisms of speciation. Here, we benefit from this utility to investigate the evolutionary history of an organismal group linked to different aquatic ecosystems, the microgastropod genus *Pseudamnicola* (family Hydrobiidae). Previous studies have found around 45 species of the nominal subgenus *P. (Pseudamnicola)*, most of them in coastal stream localities of several Mediterranean islands and mainland territories; whereas only 12 species of the other subgenus, *P. (Corrosella)*, have been collected from springs and headwaters of mountainous regions of the Iberian Peninsula and South of France. Since springs often act as isolated habitats affecting dispersion and constraining gene flow, we supposed that the temporal history and mode of diversification of species from both subgenera should differ and therefore be reflected in their phylogenetic patterns. To assess this hypothesis, we performed a molecular phylogeny based on mitochondrial and nuclear DNA sequences and later conducted an independent analysis to examine the potential effect of certain geographical and ecological variables in the genetic divergences of the subgenera. Additionally, we estimated the ancestral area of diversification of both groups. Published anatomical revisions and our molecular analyses suggest that the genus *Pseudamnicola* should be divided into three genera: the two previous subgenera plus a new one described here. As postulated, the evolution of the spring organisms was strongly related to habitat fragmentation and isolation, whereas dispersal followed by divergence seem to have been the most common speciation processes for euryhaline species inhabiting coastal streams and low river stages in which waters remain connected. On the contrary, rather than habitat fragmentation or dispersion, environmental conditions have played a larger role during the deep divergent split leading to the three genera.

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Introduction

The current species diversity is a result of the interaction of several evolutionary and ecological processes which generate and define each species. In the last decades evolutionary biologists have investigated such driving forces through the reconstruction of species-level phylogenies, since they represent hypothesis about the speciation events that originated the extant organisms (Barraclough & Nee 2001; Stadler & Bokma 2013). Traditionally, lineages divergence in allopatry is considered the most frequent scenario for speciation (Endler 1977; Coyne & Orr 2004), modulated by population dispersion and the presence of geographical barriers that maintain isolated the split populations (Gavrilets & Losos 2009; Glaubrecht 2011). However, a barrier does not need to be a geographical entity and may be also considered as a sudden shift of environmental conditions where the suitable habitat for a species ends (Fitzpatrick *et al.* 2009; Pyron & Burbrink 2010). For instance, changes in the chemical parameters of the aquatic environment can favor allopatric separation between populations, especially for those groups that exhibit scarce dispersal abilities and opportunities, resulting in restrictive distributions (Ponder & Colgan 2002; Perez *et al.* 2005). Freshwater gastropods, which are typically habitat specialist, gather those properties of limited dispersal capabilities and narrow-ranged distributions, and tend therefore to be strongly affected by variation in their habitat. For this reason they represent an ideal model to investigate speciation processes associated with isolating mechanisms (e.g., Mavárez *et al.* 2002; Albrecht *et al.* 2007; Rintelen *et al.* 2012; Schreiber *et al.* 2012; Delicado *et al.* 2013). In particular, one potential candidate taxon that may provide valuable information about evolutionary processes in different environments is the microgastropod family Hydrobiidae Stimpson, 1965.

Hydrobiids are known to be presumably the most species-rich family of freshwater gastropods, characterized besides by a long evolutionary history, wide distribution and ecological and morphological diversity. Recently Wilke *et al.* (2013) published the most complete phylogenetic hypothesis on the superfamily Rissooidea (newly considered as Truncatelloidea: Criscione & Ponder 2013) delineating the family Hydrobiidae *s. str.*, as well as its distribution range mainly to the western Palearctic and eastern Nearctic. Accordingly, this family comprises around 70 genus-level and 550 species-level taxa. Moreover, the diversity of habitat types which they inhabit is also remarkable. Approximately 35 hydrobiid species are brackish and the rest occurs in freshwater ecosystems such as springs (the majority of the species according to Strong *et al.* 2008),

ponds, lakes, rivers, etc. One inference of the hydrobiid phylogeny published by Wilke *et al.* (2013) is that species from the same subfamily seem to share similar ecological requirements. A notable exception seems to be the genus *Pseudamnicola* Paulucci, 1878, whose evolutionary history appears to have been influenced by a transition between two different environments. Consequently, this group may be considered one of the key elements to understand the origin and causes of the great hydrobiid diversity.

Pseudamnicola was first proposed by Paulucci (1878) to differentiate between European and American *Amnicola* Gould & Haldeman, 1840 species, characterizing both groups mainly by their conchological features. Nearly a century later, Boeters (1970) studied the genus anatomically and based on differences in female genitalia, defined a new genus, *Corrosella,* designating *Corrosella falkneri* as its type species. However, given the lack of other diagnostic characters for identifying each genus, Boeters (1984) concluded that *Corrosella* should be a subgenus within *Pseudamnicola*. This exemplification demonstrates the necessity of further studies in order to produce more exhaustive morphological and anatomical descriptions as well as well-supported, consistent phylogenies. Hydrobiids *s. str.*, and especially *Pseudamnicola*, have weakly sculptured shells that exhibit scarce number of diagnostic characters (Arconada & Ramos 2003; Bichain *et al.* 2007; Strong *et al.* 2008), and therefore making it difficult to establish clear species boundaries. By incorporating molecular techniques and morphological and anatomical descriptions, Delicado *et al.* (2012, 2013) and Delicado & Ramos (2012) identified seven new species of *P. (Corrosella),* thus increasing the known species richness of this subgenus from five to 12. These studies not only revealed cryptic species diversity within the genus, but also differences in habitat requirements and distribution range between the two subgenera. In fact, Delicado *et al.* (2013) found that these 12 species of *P. (Corrosella)* are mainly restricted to headwaters of mountainous regions of the Iberian Peninsula and South of France, whereas around 45 nominal species of *P. (Pseudamnicola)* have been recorded in streams, lakes and low river courses of several Mediterranean islands and mainland territories (Pallary 1926; Schütt & Bilgin 1970; Boeters 1976; Ghamizi *et al.* 1997; Schütt & Sesen 1993; Glöer *et al.* 2010; Bank 2011). These current biodiversity patterns suggest different dispersal strategies between the subgenera: *P. (Corrosella)* may scarcely disperse via habitat connection and suitability of habitats, which results in a pattern of isolation by distance (Wright 1943), whereas the wider distribution range of *P. (Pseudamnicola)* may be a result of long-distance dispersions possibly via passive mechanisms (Delicado *et al.* 2013, 2014).

Previous works revealed that diversification patterns of *Pseudamnicola* species belonging to the same subgenus are related to geographical isolation rather than ecological divergence (Delicado *et al.* 2013, 2014). Beyond what has been shown by these studies, we hypothesize that the temporal history and mode of diversification of the exclusively springsnail *P. (Corrosella)* species should differ of *P. (Pseudamnicola)* species, which are more euryhaline in habit. Springs and headwaters of streams, the habitat type of *P. (Corrosella)* species, typically present more stable conditions, being however more vulnerable to severe environmental changes (as flooding or desiccation, pollution, etc.), which makes them isolated habitats and limiting factor for dispersion (Wilke *et al.* 2010). Moreover, due to their spatial location in small areas at high altitudes, most of these springsnail species occur in a very confined number of localities or even are single-site endemisms, increasing their risk for extinction (Strong *et al.* 2008). Conversely, only a few species of *P. (Pseudamnicola)* occur in mountainous springs and most of them dwell streams and lakes, in which the environmental conditions are more variable and waters remain connected. In an attempt to compare genetic patterns among populations occurring in upland and lowland streams, Hughes (2007) recovered information of several freshwater animal groups, including mollusks, and concluded that genetic differences of lowland populations are generally lower than those inhabiting in headwaters locations. Despite this study did not compare upstreams and downstreams populations belonging to the same taxonomic group, its results suggest that habitat type is a very influential factor on dispersal capabilities and, therefore, on the genetic structure of the populations.

Thus, based on this background, in the present study we gathered for the first time mitochondrial and nuclear DNA sequences data of species from both subgenera to: i) build a molecular phylogeny and thus compare the time and mode of diversification of both, the strict springsnails and lowland stream, subgenera, ii) conduct an independent analysis to assess the potential effect of habitat transition on the divergence of the lineages, iii), estimate their ancestral biogeographic areas of diversification, and iv) finally clarify the systematic status of the genus *Pseudamnicola*, and its subgenera. On the whole, through this multidisciplinary study, we aim at providing a holistic overview of the evolutionary framework of an organismal group linked to different inland aquatic ecosystems.

Material and methods

Samples and sequences

To assess *Pseudamnicola* evolutionary relationships, we examined a total of 202 individuals from 91 localities (Fig. 1) belonging to the genus and two outgroup species, *Peringia ulvae* (Pennant, 1777) and *Mercuria emiliana* (Paladilhe, 1869). The sequences of these outgroups were acquired from GenBank under accession numbers JX081779–80, JX081888–89 and JX081990–91 (Delicado *et al.* 2013). Ingroup localities included: 19 for *P. (Pseudamnicola)* species from the Ibero-Balearic region (previously sequenced in Delicado *et al.* 2014), 51 for *P. (Corrosella)* species (generated by Delicado and Ramos 2012; Delicado et al. 2012, 2013) and 21 for additional *P. (Pseudamnicola)* populations from other areas of the Mediterranean basin (Fig. 1, **Table S1**).

"[Insert Figure 1 about here]"

DNA isolation, amplification and sequencing

DNA of a total of 40 specimens from those 21 supplementary Mediterranean populations were isolated in this work following the CTAB protocol of Wilke *et al.* (2006). Two mitochondrial genes, cytochrome c oxidase subunit I (COI) and the large subunit rDNA (16S rRNA), as well as the nuclear large subunit rRNA (28S) were amplified with the primers: LCO1490 and HCO2198 (Folmer *et al.* 1994) for COI fragment, 16Sar-L and 16Sbr-H (Palumbi *et al.*, 1991) for 16S, and F63.2 and LSU3 primers for 28S (Park & Foighil 2000) modified by Benke *et al.* (2009). The PCR cycling conditions were as described in Delicado *et al.* (2013), including the annealing temperatures: 48, 50, and 51 °C for COI, 16S, and 28S, respectively. Products were sequenced in an ABI 3730 XL sequencer (Life Technologies) using a Big Dye Terminator Kit (Life Technologies).

Phylogenetic study design

Once the new sequences for each individual were obtained, these were edited in Bioedit v. 7.0.5.3 (Hall 1999) and compiled together with the other 164, gleaned from GenBank, in three individual data matrices corresponding to each gene fragment. Sequences of the

three partitions could be unambiguously aligned by using Se-Al version 2.0a11 (Rambaut 2002).

First, data matrices for each gene were analyzed separately and subsequently combined to reconstruct the phylogenetic trees. A partition homogeneity test (ILD) (Mickevich & Farris 1981; Farris *et al.* 1994) implemented in PAUP* 4.0 b10 (Swofford 2002), was used on the different partitions (i.e., each gene) to check congruence among data for the different genes. Prior to the phylogenetic analyses, we employed jModelTest v. 0.1.1 (Posada 2008) under Akaike's information criterion (AIC; Akaike 1974) to identify the best molecular evolutionary model of nucleotide substitution that fits for each dataset. For the COI partition the model selected was HKY+I (Invariable sites) +G (rate variation among sites) model (Hasegawa *et al.* 1985) and for 16S and 28S fragments GTR+I+G (Generalized time-reversible model; Tavaré 1986). Phylogenetic inference was obtained by conducting Maximum Likelihood (ML), Maximum Parsimony (MP) and Bayesian Inference (BI) methods.

MP analyses were performed in PAUP* through a heuristic search with a tree bisection and reconnection TBR swapping algorithm, including ten random stepwise additions. ML analyses were conducted in PHYML v. 2.4.4 (Guindon & Gascuel 2003) using the evolutionary models selected by jModelTest. Clade support for the MP and ML phylogenies was assessed by nonparametric bootstrapping (Felsenstein 1985) using 1000 pseudoreplicates in each case. BI was run using the software MrBayes version 3.1.2 (Huelsenbeck 2000; Huelsenbeck & Ronquist 2001), performing two independent and parallel runs of four Metropolis-coupled chains with 5 million generations each, and sampling one tree per 1,000 replicates. After assessing convergence between runs by checking that the standard deviation of split frequencies fell below 0.01 in MrBayes 3.1.2, the initial 10% of the trees were discarded as burn-in. The robustness of Bayesian trees was assessed by posterior probabilities (BPPs).

Additionally to our approach of species delineation by combining mitochondrial and nuclear markers, we here tested the assignment of the sequences to the species identified by our multi-locus inferences as new, and quantified the lineage diversity in an objective and reproducible way by employing Automatic Barcode Gap Discovery (ABGD: Puillandre *et al.* 2012). The ABGD analysis was performed at the web interface http://wwwabi.snv.jussieu.fr/public/abgd/ using the aligned fasta file of COI sequences, and the default settings, i.e. the uncorrected genetic distances, a relative gap width of X=1.5, and intraspecific divergence (*P*) values between 0.001 and 0.100,

Temporal history and mode of speciation

An ultrametric species tree of the genus *Pseudamnicola* was inferred by coalescence approach in the program *BEAST (Heled & Drummond 2010). This extension of the package BEAST works to combine datasets from multiple gene loci and multiple individuals per species, crumpled conforming to a grouping file, to generate a species tree. In the absence of outgroups, a total of 202 individuals was grouped in the previously described *Pseudamnicola* species and those potentially identified as new in the phylogenetic analyses. All the priors as well as the grouping file were compiled in an input file generated by the interface BEAUti v. 1.7.1 (Drummond *et al.* 2012). In order to ascertain whether the substitutions rates were constant in all the branches, we performed a relative rate test (Takezaki *et al.* 1995) included in the program PHYLTEST 2.0 (Kumar 1996). As no uniformity in the rates was detected, we used an uncorrelated lognormal relaxed molecular clock model (Drummond *et al.* 2006) to estimate divergence time between species. Besides for the calibration of the analysis we utilized a substitution rate for COI of 0.81% ± 0.24% per million years (percentage of substitutions per lineage per Myr) as calculated in Delicado *et al.* (2013). This mean rate integrates others published for hydrobiids (Wilke 2003; Falniowski *et al.* 2008; Hershler & Liu 2008; Wilke *et al.* 2009) based on geological events. The substitution rates of 16S and 28S were estimated from this COI substitution value.

As prior of the topology of the tree, we used birth-death model (Gernhard 2008), suitable for species-level phylogenies with almost complete sampling. Nucleotide substitution models obtained from jModelTest were applied to the corresponding gene partition of the data set. We ran the analysis with Markov Chain Monte Carlo lengths of 50 million generations, sampling every 2,000 generations (initial 10% discarded as burn-in). The effective sample size of each parameter required to reach stationarity of the posterior distribution (above 200) was examined in Tracer v. 1.5. (Rambaut & Drummond 2009). The maximum clade credibility tree of all sampled trees was compiled in TreeAnnotator v. 1.7.1. The topology of this tree as well as BPPs, node ages and 95% high posterior density (HPD) intervals were finally visualized in FigTree v. 1.3.1 (Rambaut 2010).

As independent analyses, we performed several Mantel test (Mantel 1967) in order to assess the possible influence of some environmental factors, such as water conductivity, altitude and geographic distance, on the genetic divergences between *Pseudamnicola*

individuals. As this method resulted in no significant correlations between the environmental variables and genetic divergences of individuals within the same subgenus (Delicado *et al.* 2013, 2014), here we aimed at combining the information of each variable, yielded in those publications, for *P. (Corrosella)* and *P. (Pseudamnicola)* specimens simultaneously with the same purpose. A total of 52 and 88 localities of *Pseudamnicola* were included for conductivity and geographical variables (distance and altitude), respectively. The significance of the correlation between environmental and genetic variables was tested based on 9,999 permutations using the vegan package version 2.0-4 (Oksanen *et al.* 2013) for the R statistical environment version 2.15 (R Development Core Team 2011).

Historical biogeography

Ancestral range estimation for *Pseudamnicola* species was performed in the R package BioGeoBEARS version 0.2.1 (Matzke 2013). This method infers, in a likelihood framework, the potential ancestral areas by modeling events of range evolution along a phylogeny. As input files, we included the species tree obtained in the program *BEAST and a matrix with the distribution areas of the tips (species) of this tree. In order to designate discrete range states for each species, we utilized the worldwide freshwater ecoregions presented in Abell *et al.* (2008) and depicted in Fig. 3. We set the maximum areas per lineage and node at two, because typically *Pseudamnicola* species occur in just one ecoregion. Afterward, we conducted the analysis under the models: dispersal–extinction–cladogenesis model (DEC; Ree 2005) and dispersal–vicariance analysis (DIVA; Ronquist 1997) and the BayArea model (Landis *et al.* 2013), both in a likelihood version (referred as DIVALIKE and BayAreaLIKE; Matzke 2013). These models include two free parameters (*d* = dispersion or range extension and *e* = extinction or range contraction); however, BioGeoBEARS also includes the founder event *j* parameter for each model, resulting in three additional models: DEC +J, DIVALIKE +J and BayArea +J. Here we compared the model fit of these six models through AIC. The most likely ancestral range was estimated for each node according to the most likely model and subsequently plotted on the maximum clade credibility tree of Fig. 3.

Results

Phylogenetic reconstruction and species boundaries

283

284 *Mitochondrial data set.* After combining COI and 16S fragments, a total of 1170 characters
285 was obtained, of which 658 were for COI and 512 for 16S. The COI data set contained 257
286 variable sites, 236 of which were parsimony informative. The 16S data set contained 169
287 variable sites, 118 of which were parsimony informative. In all reconstructions with this
288 data set, the two subgenera of *Pseudamnicola* were revealed as two well-supported
289 monophyletic groups, with the exception of the species *P. (P.) gasulli* that constituted an
290 independent clade (Fig. 2). However, the relationships between these groups were not
291 obvious from the study of these mitochondrial genes, and this was reflected in the position
292 of *P. (P.) gasulli* in the different tree topologies. In the COI topology, *P. (P.) gasulli* was
293 clustered within the subgenus *P. (Corrosella)* with high support values in all analyses; in
294 contrast, in the 16S reconstruction, this species was more closely related to the other *P.*
295 *(Pseudamnicola)* species, though this was poorly supported in the ML analysis. Overall, the
296 relationships among *Pseudamnicola* species seemed better resolved in the COI analysis
297 (Fig. 2). The percentage of sequence divergence was higher for COI than for 16S, with
298 maximum genetic divergence of 16.6% for COI (between *P. (P.) granjaensis* and *P. (C.)*
299 *marisolae*) and 10.6% for 16S (between *P. (P.) sp5* and *P. (C.) sp1*).

300 *Nuclear data set.* The 28S alignment consisted of 204 sequences each with 1057
301 characters. Of these, 815 were invariant, 54 parsimony uninformative and 188 parsimony
302 informative. Interspecific genetic variation ranged from 0% (as between *P. (P.) beckmanni*
303 and *P. (P.) granjaensis*) to 6.9% (between *P. (P.) gasulli* and *P. (P.) artanensis*). The ML and
304 BI topologies recovered *Pseudamnicola* as a monophyletic group, clustering *P. (P.) gasulli*
305 with the other *P. (Pseudamnicola)* species, with a ML bootstrap value and BPPs near 80%
306 and 0.8, respectively. All the species were grouped as a polytomy within their
307 corresponding clades (Fig. 2).

308 *Combined data set*. All the multi-locus inferences recovered a total of 26 species of
309 *Pseudamnicola s. l.*, 20 out of them previously identified through integrative methods and
310 six discovered as new taxa. ABGD analysis confirmed the assignment of the sequences in
311 these new six species; however, some of the previously known species were split in two
312 groups, increasing the number of species suggested by ABGD analysis to 36, eight
313 additional species for the subgenus *Corrosella* and two for *Pseudamnicola*. Moreover, there
314 were obvious disparities between the nuclear and mitochondrial gene trees related to the
315 phylogenetic position of *P. (P.) gasulli*. Because of these disparities, the ILD test showed no
316 congruence between the mitochondrial and nuclear data. Nevertheless, this does not mean

than the resulting phylogeny was incongruent. As shown in Fig. 2, the phylogeny of the genus was well resolved at basal nodes, however the evolutionary relationships among the three lineages constituting *Pseudamnicola* were ambiguous.

The biogeographic pattern reflected by the MP, ML and BI topologies was more explicit in the subgenus *P. (Corrosella)* than in *P. (Pseudamnicola)*. In contrast to *P. (Corrosella)*, *P. (Pseudamnicola)* species did not show an apparent biogeographical pattern, for instance: i) the species *P. (P.) beckmanni* (from Majorca) that was genetically closer to the species occurring in Tunisia, Sicily and mainland Italy than to *P. (P.) meloussensis* (from Minorca), which is found in the same archipelago of islands; ii) the same species can be found in different regions, such as *P. (P.)* sp. 5, in mainland Italy and Sardinia; iii) some species of *P. (Pseudamnicola)* seem to live sympatrically in the same locality, such as *P. (P.)* sp. 2 and *P. (P.)* sp. 4, which both inhabit the same locality (Borkane ditch) of Tunisia.

"[Insert Figure 2 about here]"

Divergence time estimation

The non-constancy of the substitution rate has been demonstrated since the relative rate test showed significant differences in evolutionary rates between tree branches along the phylogeny (branches that showed significant differences in evolutionary rates are highlighted in Fig. 2). Thus, the model of constant-rate of diversification may be rejected and the relaxed molecular clock approach applied. After performing the analysis, the substitution rates inferred for each partition (substitution/Myr) were 0.3% for 16S and 0.17% for 28S. Given that the posterior values of the parameters ucld.stdev and coefficients of variation in all the markers were greater than zero, evolution has not been clock-like for these genes. Furthermore, 28S had higher deviation from a strict clock model as its coefficient of variation was greater than 1.

The topology of the chronogram with corresponding confidence intervals analyzed in *BEAST is shown in Fig. 3. Using an estimated rate of 0.81 ± 0.24% substitution/Myr for the COI fragment, and rate estimations for 16S and 28S, the most basal split leading to the three major clades within *Pseudamnicola* was calculated to have occurred ca. 22 Ma (HPD: 28-17 Ma), during the Upper Miocene. Contrary to the rest of the phylogenetic inferences, the reconstruction by coalescence showed high posterior probabilities at the level of these

three lineages, associating the species *P. (P.) gasulli* to the *P. (Pseudamnicola)* lineage and dating their subsequent split ca. 17 Ma (23-13 Ma). However, this result requires further investigation since currently only one species composes the clade of *P. (P.) gasulli*. Thus, this fact may be why the relative rate test showed significant differences on evolutionary rates when comparing the *P. (P.) gasulli* lineage with each of the other two lineages, but showed no significant differences when comparing *P. (Corrosella)* and *P. (Pseudamnicola)*. Nonetheless, the topology of each of the lineages displayed dissimilarities in tempo and mode of species diversification.

The age of the most recent common ancestor of *P. (Corrosella*) species was estimated to be older (ca. 13 Ma) than the age of the *P. (Pseudamnicola)* species ancestor, excluding *P. (P.) gasulli* (ca. 6 Ma). Consequently, the first cladogenetic events that occurred during *P. (Corrosella)* evolution were likely older than those for *P. (Pseudamnicola)*. Moreover, these two lineages may have experienced a radiation event near the base of each lineage, since the relative rate test showed rapid diversification at basal levels of both clades. In any case, the radiation event that occurred during the origin of *P. (Pseudamnicola)* involved more species and a relatively shorter period of time (ca. 8-3 Ma), characterizing the major cladogenetic event for this group. The most recent splits between sibling species of *P. (Pseudamnicola)* were likely to have occurred from ca. 5 Ma to 0.08 Ma. All of these splits appeared not well supported. Rate constancy was rejected by the relative rate test in most of the comparisons between branches within the *P. (Pseudamnicola)* lineage, which may affect the split frequencies (probably accelerated) and thus justify the low BPP values at the affected nodes.

"[Insert Figure 3 about here]"

Ancestral area estimation

According to a log-likelihood-ratio test, all the models tested in BioGeoBEARS which assume founder event speciation (+J) displayed significantly higher likelihood values than those without (all $p < 0.05$). According to model selection criteria AIC, no significant differences were found among the three +J models. However, the DEC +J model obtained the highest model fit (AIC = 81.01), whereas in DIVALIKE (AIC = 81.42) and BayAreaLIKE (AIC = 81.39) model support was slightly lower. Estimated parameters in DEC +J resulted d=0.0014, e=0 and j=0.0477. The most likely ancestral area inference according to this

model is given in Fig. 3. This analysis suggests the Iberian Peninsula as the geographic area in which the cladogenetic event that originated the three main *Pseudamnicola* lineages occurred. Speciation by vicariance seems to be the most common evolutionary process within *P. (Corrosella)* lineage. On the contrary and acknowledging the lack of a complete taxon sampling for *P. (Pseudamnicola)* species, dispersal along Mediterranean islands and peninsulas and founder event may have been the dominant processes in the evolutionary history of this group.

Exploring causes of diversification

Mantel tests performed separately for each subgenus (Delicado *et al.* 2013, 2014) revealed no correlation between the genetic distance matrix and physical variables, such as conductivity and altitude, but a pattern of isolation by distance was found. However, when both subgenera were included in the analysis, Mantel tests showed significant correlation with the three examined variables, namely conductivity, altitude and geographic distance (**Table 1**). Despite this, conductivity and altitude only had minor influences, compared to geographic distance, on the divergence of the subgenera for the COI and 16S genes, while conductivity had more influence for the 28S gene, followed by geographic distance.

"[Insert **Table 1** about here]"

Discussion

Effects of habitat transition on the evolutionary history of Pseudamnicola s. l.

Phylogenetic patterns and distribution ranges. The application of molecular tools in the systematic analysis of *Pseudamnicola* has revealed the existence of three main lineages within the genus, corresponding to the two subgenera previously described plus the species *P. (P.) gasulli*. From this molecular study, we conclude that the observed morphological differences existing between the two subgenera (discussed in Delicado *et al.* 2012) have a phylogenetic signal and moreover, that the anatomical differences recorded for *P. (P.) gasulli* in Boeters (1988) and Delicado *et al.* (2014) reflect a different origin of this species with respect to other *P. (Pseudamnicola)* species. The combined

mitochondrial and nuclear phylogeny reasonably supports each of the clades; however, the relationships among them still remain unclear.

Although well supported as monophyletic groups, *P. (Pseudamnicola)* and *P. (Corrosella)* display different phylogenetic patterns (see Fig. 2). In *P. (Pseudamnicola)*, splitting events appear more recent and with less-supported branches, whereas in *P. (Corrosella)*, the branches are longer and more robust, which is a possible sign of a more older and gradual speciation process within this group. One reasonable explanation for the different topologies may be because *P. (Corrosella)* species present more restricted distribution ranges and inhabit springs and headwaters of streams, which often act as isolated habitats (Wilke *et al.* 2010). Thus, these isolated locations may constrain gene flow between populations (Brändle *et al.* 2005) and increase the degree of endemicity. In contrast, *P. (Pseudamnicola)* species and *P. (P.) gasulli* are euryhaline species and occur in coastal streams, lakes and low river stages where the ecological conditions are less restrictive and the waters remain connected. Moreover, such locations are more exposed to the presence of birds and fishes than springs (Haase 2008), which may favor jump dispersal via vectors. In any case, these two habitat prototypes are likely associated with two different dispersal abilities, directly influencing their phylogenetic topologies.

Despite barcoding-gap method confirmed the assignment of the six new species obtained by our multi-locus phylogenetic analysis, the total number of species obtained by these two approaches differs. This testifies the need of combining, at least in hydrobiids, the information yielded by COI sequences with multi loci analyses, morphological descriptions, biogeography or ecological data (as recommended in Puillandre *et al.* (2012) or Collins & Cruickshank (2013)). Nevertheless, here we benefit from the information of the COI fragment in order to objectively compare genetic divergences between *Pseudamnicola* lineages and between this group and other microgastropods. Thereby, the average pairwise divergence in the COI partition between species (described through integrative taxonomy) is 1.5 times greater in *P. (Corrosella)* than in *P. (Pseudamnicola)*. Sequence differences (measured as uncorrected pairwise distances) between spring snails species of *P. (Corrosella)* ranged between 5.3% to 12% (with an average of 9%), which is similar to ranges described for other springsnail genera, such as *Bythinella* Moquin-Tandon, 1856 (1.5-13.4% in Bichain *et al.* 2007), and *Floridobia* Thompson & Hershler, 2002, *Marstonia* Baker, 1926 and *Pyrgulopsis* Call & Pilsbry, 1883 (0.5-6.1%, 1.0-8.5% and 2.8-11.2%, respectively in Hershler *et al.* 2003). Alternatively, genetic divergences for *P. (Pseudamnicola)* species are an average of 6.7% (ranging between 0.5% to 10%), which falls between the estimated 9% for *P. (Corrosella)* and 4.5% for the brackish genus

Hydrobia (Wilke *et al.* 2000). To a limited extent, this gradient of genetic divergences may be due to the type of environment (freshwater *vs.* brackish) occupied by these three groups.

In general, *P. (Corrosella)* species occur in isolated habitats, present a clear biogeographical pattern of northern and southern phylogenetic clustering (shown in Delicado *et al.* 2013) and genetically distinct species. On the other hand, the geographical patterns of *P. (Pseudamnicola)* are not as explicit. A possible reason could be because the number of samples examined is more limited, and thus, the entire distribution range of this subgenus has not been covered. It would be interesting to first, extend the study in the sampled regions, and second, to genetically examine the species described from Morocco and Algeria (Ghamizi *et al.* 1997; Glöer *et al.* 2010) in order to investigate whether the current distribution of the group is a result of stochastic or tectonic processes. In any case, as no clear biogeographic pattern is present among the studied populations, an alternative explanation for their distribution pattern may be long distance colonization followed by isolation. As the principal aim of this work is to study the relationships between species and their biogeographical distribution, no population genetic level analyses have been performed, thus we cannot hypothesize which dispersal mode the populations of this subgenus have followed. Further research at the population level and over a larger geographical area is required.

Nevertheless, phylogenetic analysis of the group does reveal the existence of three well-supported lineages within the genus. Previous anatomical studies (Delicado *et al.* 2012, 2014) reflect substantial differences between the subgenera and highlighted *P. (P.) gasulli* as a different entity bearing evidences of divergence. Taking all these results into account, we suggest that the three lineages may correspond to three different genera, raising *Corrosella* to the category of genus once again (as in Boeters 1970) and removing *P. (P.) gasulli* from *P. (Pseudamnicola)*, thus making itself a new genus here designated as *Didacus* n. gen. In addition to the anatomical and morphological characteristics that sufficiently distinguish them as different genera, the genetic divergences that exist between them (uncorrected distances ranged between 11.1% to 14.3% for COI and between 6.7% to 8.4% for 16S) are similar to those reported between other genera. For instance, between genera belonging to the sister subfamily Hydrobiinae (based on phylogenies in Wilke *et al.* 2001, 2013; Szarowska 2006), e.g. *Adriohydrobia*, *Hydrobia*, *Peringia* and *Ventrosia*, molecular distances range between 10.4% to 14.8% for COI and 2.3% to 5.8% for 16S (uncorrected pairwise distances, Wilke 2003). Therefore, the subfamily Pseudamnicolinae would be composed of three genera, one strict freshwater and two euryhaline freshwater.

Thus, within the current phylogeny for hydrobiids (Wilke *et al.* 2013), these two subfamilies are very interesting from an evolutionary perspective because they may represent a transition between two different environments, event that has not occurred often during the evolution of this family.

Temporal history and biogeographic origin. All of the phylogenetic inferences performed in this work have suggested the division of *Pseudamnicola s. l.* into three lineages, which may correspond to three different genera. Observing their patterns of diversification, each of these genera is likely to have experienced different evolutionary processes in space and time. Based on the available taxonomic sampling performed for each lineage and the coalescence analysis (Fig. 3) using an evolutionary rate previously cited for hydrobiids, we estimate that the split leading to these three lineages likely occurred during the upper-middle Miocene (28-17 Ma). Although not all of the described *Pseudamnicola s. l.* species have been included and not all of the areas of its distribution range have been sampled in this work, our results of the ancestral area estimation suggest that the Iberian Peninsula has played an important role in the diversification of the group as it is, to date, the only region in which all three proposed genera inhabit. Thus, given the more restrictive distribution pattern of *Corrosella* and the distribution pattern of *Didacus* gen. n. (composed of *Didacus gasulli*) with respect to that of *Pseudamnicola*, their evolution may be a result of a peripatric (allopatric) speciation that occurred in the Iberian Peninsula (as shown in Fig. 3). During the Oligocene and Miocene (between 35 Ma and 5.33 Ma), the Iberian Peninsula suffered a compressive period with the loss as well as adhesion of several continental fragments (Hevia 2004) in which most of the Iberian mountain ranges originated, thereby affecting the region's hydrological system. The creation of these physical barriers may have caused an isolation process followed by vicariance by which the geographical range of the last common ancestor of the three genera was fragmented in a relatively short period of time, thus leading to the separate lineages. However, it seems that some populations not only remained isolated but also evolved and adapted to new habitat conditions in a mountainous environment (as in the case of the genus *Corrosella*). It is noteworthy that our results revealed significant differences in habitat features (altitude and conductivity) between genera (**Table 1**), which may be a consequence of an adaptive process. Therefore, this may be a case in which there is some implicit degree of natural selection in allopatric speciation, as postulated by Wright (1931).

With respect to *Corrosella* species, the inclusion of additional *Pseudamnicola* sequences to the *BEAST analyses did not substantially change the divergence times previously

520 estimated for the three major cladogenetic events leading to diversification within the
521 *Corrosella* clade (see Delicado *et al.* 2013). In the analysis here, these events are estimated
522 to have occurred slightly earlier (ca. 12 Ma, 6 Ma and 3 Ma) to the ca. 10 Ma, 5 Ma and 2
523 Ma estimated in Delicado *et al.* (2013). In any case, these ages indicate that their
524 phylogenetic relationships and distribution could be the result of vicariant and climatic
525 events that occurred in the Iberian Peninsula during the Miocene, or of a process of
526 isolation by distance (Wright 1943) (also supported by Mantel test), or a combination of
527 both. The Miocene was a crucial period for the Iberian Peninsula because of several
528 geological processes related to Alpine orogeny that critically affected the diversification
529 and dispersal of many species of plants and animals (Miguel *et al.* 2007; Joger *et al.* 2007;
530 Pardo *et al.* 2008). Thus, the uprising of the current Iberian mountain ranges during the
531 Alpine orogeny may have led to fragmented habitats of *Corrosella* and as a consequence, to
532 allopatric speciation within the group, which increased its species richness and changed
533 distribution patterns. For instance, the second and last uprising of the Pyrenees in the
534 North (Barbadillo *et al.*1997; Hevia 2004) may have influenced the split between *C. astieri*
535 and *C. navasiana* - *C. hauffei*. In addition, the creation of the Betic Cordillera and the active
536 plate tectonics in the southern and eastern regions of the Alboran Sea (approximately 10
537 Ma, according to Rosenbaum *et al.* 2002) may have been one of the reasons of the
538 radiation involving southern *Corrosella* species. Finally, the creation of the Iberian
539 hydrological network during the Quaternary (Vargas *et al.* 1998) may have led to vicariant
540 events that influenced the most recent splitting of *Corrosella* species.

541 Furthermore, species of *Pseudamnicola s. str.* have been found on the Mediterranean basin
542 mainland, peninsulas and islands, yet only the species *P. subproducta* inhabits the Iberian
543 Peninsula. Therefore and though with low probability values (see pie charts at nodes of
544 Fig. S1), our results point toward the hypothesis that the diversification of *Pseudamnicola*
545 *s. str.* did not occur in the Iberian Peninsula, but probably toward central Mediterranean
546 areas (Fig. 3). The inclusion of more populations from different Mediterranean localities in
547 future analyses will help to clarify the most likely evolutionary scenario of the
548 *Pseudamnicola s. str.*. In any case, the coalescence analysis performed with the available
549 data shows rapid cladogenetic events whose period does not mismatch any of the
550 hypotheses discussed above, as such events seem to have been posterior to the geological
551 origin of the western Mediterranean region and anterior to Plio-Pleistocenic glaciations.
552 Thus, the tree topologies and estimated divergences suggest the geological events
553 occurred during the Messinian salinity crisis (MSC) (between 5.96-5.33 Ma, according to
554 Krijgsman *et al.* 1999) as the main drivers for the diversification of *Pseudamnicola s. str.*

Based on both the divergence time estimate and the current distribution patterns of *Pseudamnicola s. str.*, we deduce that such a cladogenetic event is more likely to have occurred by a process of colonization of several Mediterranean regions during the MSC followed by isolation, rather than by a vicariant event produced by fragmentation of a larger habitat. This is probably testified by the *j* parameter in our ancestral area estimation. The poor dispersal capability of freshwater snails (they require permanent water courses, although they may survive desiccation for several days) (Jensen *et al.* 1996; Haase *et al.* 2010; Havel *et al.* 2014), and their inability to cross marine water masses make the hypothesis of colonization less plausible. However, the estimated periods of diversification of *Pseudamnicola* species are subsequent to when the Mediterranean peninsulas and islands were formed and therefore, it is likely that the populations arrived after that event. Two alternative scenarios may also explain the colonization process: i) via land bridges connecting several microplates, for instance between Sardinia, Tunisia, Sicily and Italy (Rosenbaum *et al.* 2002; Goes *et al.* 2004) or between the Balearic Islands and the Iberian Peninsula (Carranza *et al.* 2004; Fochetti *et al.* 2009; Lázaro *et al.* 2011), both of which were established during the Messinian (6-5 Ma) and the Plio-Pleistocenic glaciations or ii) through passive extra-aquatic dispersion via water birds. This dispersal mechanism has been reported as the most feasible explanation for the distribution pattern of other hydrobioids, especially for brackish genera (Wilke & Davis 2000; Liu *et al.* 2003; Haase *et al.* 2010; Kappes & Haase 2012). Indeed, this hypothesis has recently been confirmed by Wada *et al.* (2012), who demonstrated that gastropods can pass through and survive the digestive system of birds and fishes and can even subsequently produce offspring, making predation by birds a possible method of dispersal. Kappes and Haase (2012) proposed several active and passive dispersal mechanisms for freshwater mollusks. However, in this case, no geographic criterion exists and multiple tectonic plates are involved, therefore long-distance dispersal via birds may be more likely hypothesis to explain this fact.

Systematics

Genus *Pseudamnicola* Paulucci, 1878

Type species. Bithynia lucensis Issel, 1866 (Kennard & Woodward, 1926), subsequent designation

Diagnosis. Shell ovate-conic, slightly longer than wide and an aperture wider than long; female genitalia with pyriform bursa copulatrix, pigmented renal oviduct and one elongate seminal receptacle; prostate gland between two and three times longer than wide; penis broadly triangular with the base expanded and many folds along its entire surface; penis with a dark patch of pigment, whose extension varies among species, from its middle region to the tip; pigmented nervous system generally elongate.

Genus *Corrosella* Boeters, 1970

Pseudamnicola (Corrosella) (Boeters, 1970)

Type species. Corrosella falkneri Boeters, 1970

New diagnosis. Shell ovate-conic, generally eroded, and longer than wide; female genitalia with pyriform bursa copulatrix, pigmented renal oviduct and one short seminal receptacle, either elongated or pyriform; prostate gland around three times longer than wide; penis gradually tapering with the base expanded and some folds in middle section; dark patch of pigment in penis, with different sizes, from the middle region to the tip; pigmented nervous system from moderately concentrated to elongate.

Genus *Didacus* gen. n.

Type species. Pseudamnicola (Pseudamnicola) gasulli (Boeters, 1984)

Etymology. Dedicated to Diego Moreno Lampreave, main collector of the material related to this new genus and specialist in the conservation of freshwater and marine mollusks in southern Spain.

Diagnosis. Shell ovate-conic, aperture occupying one-third of shell length; female genitalia with pyriform bursa copulatrix, bursal duct approximately one and a half times longer than bursal length, pigmented renal oviduct and absence seminal receptacle; prostate gland twice as long as wide; strap-like penis dark pigmented; nervous system brown pigmented and elongate.

Remarks. Despite sharing the morphological and anatomical traits that define the species of the subfamily Pseudamnicolinae, such as shell ovate-conic, pigmented renal oviduct, simple penis or pigmented nervous system, *Didacus* gen. n. is distinguishable from the genera *Pseudamnicola* and *Corrosella* by several features: i) shell aperture generally represents one-third of shell length in *Didacus* gen. n. and approximately the half in *Pseudamnicola* and *Corrosella,* ii) absence of seminal receptacle in the female genitalia, iii) strap-like penis and often coiled, whereas penis of the other two genera is straight and decreases in width from the base to the tip, iv) prostate gland dimensions smaller than in the other two genera.

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Figure 1. Distribution maps of samples analyzed in this work. Map **A** shows the location of the Mediterranean basin localities here sequenced (see codes in **Table S1**). Map **B** gathers the Ibero-Balearic localities of *P.* (*Corrosella*) published in Delicado *et al.* (2013) and those of *P.* (*Pseudamnicola*) studied in Delicado *et al.* (2014), plus the additional locality of Alfabia, Majorca, Spain (Alf) here included. Locality codes are given in white for *P. (Corrosella)* and in black for *P. (Pseudamnicola)*.

Figure 2. Phylogenetic relationships of *Pseudamnicola* species based on Bayesian inference of the combined data set (top) and the mitochondrial fragments (COI and 16S) and the nuclear 28S (bottom). In the combined inference bootstrap supports of the branches resulted > 90% and BPPs > 0.9, except for those branches highlighted through: black circles, in which MP and ML bootstraps range between 50% and 90% and BPPs between 0.5 and 0.9; and black squares, in which bootstraps also range between 50% and 90%, but posterior probabilities are > 0.9. In the individual trees of the bottom asterisks

924 represent bootstrap values and BBPs of branches > 90% and >0.9, respectively. Arrows
925 point to branches with no rate constancy (result from Relative Rate Test).

926 **Figure 3.** Ultrametric tree obtained with *BEAST based on the combined analysis of COI
927 (using a rate previously calibrated for hydrobiids) and the ribosomal fragments 16S and
928 28S (using rates estimated in this analysis). At nodes where *BEAST posterior
929 probabilities (BPP) are not given, the node is supported by BPP ≥ 0.90; black dots indicate
930 BPP < 0.90. Bars at nodes represent confidence intervals of divergence times. Letters from
931 A to F at nodes correspond to the most probable ancestral-area states (pie charts with the
932 distribution of the probability of the ancestral-area states are depicted in Fig. S1), and at
933 tips correspond to the current distribution of the species (extracted from Abell *et al.*
934 2008). Ma: million years ago.

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