Regional genetic structure in the aquatic macrophyte *Ruppia cirrhosa* suggests dispersal by waterbirds

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Abstract

The evolutionary history of the genus *Ruppia* has been shaped by hybridization, polyploidisation and vicariance, that have resulted in a problematic taxonomy. Recent studies provided insight into species circumscription, organelle take-over by hybridization, and revealed the importance of verifying species identification to avoid distorting effects of mixing different species, when estimating population connectivity. In the present study, we use microsatellite markers to determine population diversity and connectivity patterns in *Ruppia cirrhosa* including two spatial scales: 1) from the Atlantic Iberian coastline in Portugal to the Siculo-Tunisian strait in Sicily, and 2) within the Iberian peninsula comprising the Atlantic-Mediterranean transition. The higher diversity in the Mediterranean Sea suggests that populations have had longer persistence there, suggesting a possible origin and/or refugial area for the species. The high genotypic diversities highlight the importance of sexual reproduction for survival and maintenance of populations. Results revealed a regional population structure matching a continent-island model, with strong genetic isolation and low gene flow between populations. This population structure could be maintained by waterbirds, acting as occasional dispersal vectors. This information elucidates ecological strategies of brackish plants species in coastal lagoons, suggesting mechanisms used by this species to colonize new isolated habitats and dominate brackish aquatic macrophyte systems, yet maintaining strong genetic structure suggestive of very low dispersal.

Keywords

Ruppia, connectivity patterns, genotypic diversity, waterfowl, coastal lagoon

Introduction

Estuaries, coastal lagoons, and saltmarshes are economically and ecologically important habitats providing a number of valuable ecosystem services (Costanza et al. 1998; Pérez-Ruzafa et al. 2011). Despite their importance, these systems are highly impacted by human actions, counting amongst the most threatened habitats in the world (Airoldi and Beck 2007). In these transition zones between land and sea ecosystems, which are characterised by extreme variations of environmental conditions (Viaroli et al. 2008), species from the seagrass genus *Ruppia* (Short et al. 2007) are important ecosystem engineers that create habitat by forming dense meadows (Den Hartog and Kuo 2006; Verhoeven 1979). These meadows play several key ecological roles including enhancing primary productivity, improvement of water and sediment quality, and providing valuable habitat and food resources for other species (Hemminga and Duarte 2000). *Ruppia* spp. have been considered characteristic of pristine lagoons (Viaroli et al. 2008), and their meadows are classified as habitat of community interest by the UE (Directive 92/43/ECC; Annex I, "Coastal lagoons"). However, because *Ruppia* species are difficult to identify taxonomically, their conservation status has not been properly assessed by the International Union for Conservation of Nature (IUCN).

The population structure of aquatic clonal plants is a complex product of demographic and genetic processes, life history, adaptation, reproductive systems and dispersal potential, besides environmental influences (Spalding et al. 2003). The genus *Ruppia* (Alismatales, Ruppiaceae) consists of clonal and hermaphroditic aquatic plants that are closely related to seagrass families such as Posidoniaceae and Cymodoceaceae (Les et al. 1997). It possesses sexual reproduction by cross- and self-fertilization, and asexual reproduction occurs through clonal formation. Sexual reproduction forms novel genotypes thereby fostering selective responses, while asexual propagation ensures persistence and spread of successful genotypes. Therefore, the balance among sexual and asexual reproduction is likely one of the most important factors in determining population genetic structure in this and other seagrasses (Coyer et al. 2004; Olsen et al. 2004; Alberto et al. 2008). In *Ruppia* spp., pollen dispersal is normally confined to within populations (Verhoeven 1979). However, at larger spatial scales different mechanisms such as sea currents (Koch et al. 2010), birds (Charalambidou and Santamaría 2005; Ito et al. 2010) and fishes (Agami and Waisel 1988), have been proposed as dispersal vectors of plant vegetative fragments and seeds.

Populations of *Ruppia* spp. tend to occur in strong isolation, and isolated populations are predicted to become genetically impoverished thought genetic drift (Hedrick 2006, Allendorf and Luikart 2007). Genetic connectivity may therefore be important to provide higher diversity that should facilitate adaptive processes under changing conditions (Davis and Shaw 2001) to maintain long-term viability of populations (Segelbacher et al. 2010). Thus, estimating genetic (i.e. heterozygosity) and genotypic (i.e. clonal) diversity, and the mechanisms influencing connectivity, is important in conservation planning for these habitats. This is especially important for species such as *Ruppia*, which

increase the heterogeneity and complexity of the habitat, promoting the establishment of additional species and enhancing ecosystem resilience and function (Hughes and Stachowicz 2011; Thomaz et al. 2010).

Traditionally, three *Ruppia* species have been recognized in the Iberian Peninsula and Mediterranean region: *R. drepanensis* Tineo, *R. maritima* L., and *R. cirrhosa* (Petagna) Grande (Cirujano and García-Murillo 1990, 1992; Talavera et al. 1993). However, recent phylogenetic analyses revealed the existence of hybrids and a complex evolutionary history where hybridisation and polyploidisation processes have been implicated (Ito et al. 2013; Triest and Sierens 2014; Martínez-Garrido et al. 2016). *Ruppia drepanensis,* endemic of SW Mediterranean and adjacent Atlantic coastlines, is in the same phylogenetic clade than *R. cirrhosa* for both nuclear and chloroplast genes, and are considered sister species. The diploid *R. maritima* is in a more distantly related clade supported by both nuclear (internal transcriber spacer) and chloroplast genes (*psb*A-*trn*H). However, two new entities, namely *R. cf. maritima* and "R. hybrid", showed incongruent results between the nuclear and chloroplast trees, suggestive of hybridisation and introgression effects (Martínez-Garrido et al. 2016).

Commonly known as the "ditchgrass", *Ruppia cirrhosa* is unique among the Iberian *Ruppia* species by being restricted to habitats influenced by fully marine waters, usually connected to open seawater. It is also the most robust species of *Ruppia* in the Iberian Peninsula, with the leaf having three nerves and being up to ~1.2 cm wide (Talavera and García-Murillo 2010). Floral peduncles have a variable length depending of the depth of the water body the plant inhabits and the life cycle is annual or perennial depending on water seasonality (Gesti et al. 2005). Karyotyping studies conducted in Italy and the Iberian Peninsula have shown that *R. cirrhosa* is tetraploid (2n=4x=40) with a base chromosome number of X=10 (Marchioni-Ortu 1982; Talavera et al. 1993). Studies conducted with microsatellite markers identified high genetic diversity and strong structuring in the Iberian populations (Martínez-Garrido et al. 2014; Martínez-Garrido et al. 2016). Hence, the environmental and ecological conditions in which *R. cirrhosa* grows, widely distributed in coastal habitats from the Iberian Peninsula, and its high genetic diversity and population structure, enable us to use *R. cirrhosa* as a model species to analyse evolutionary and genetic connectivity among coastal lagoons.

In this study we used highly variable molecular markers, which allow us distinguish genets (i.e. genetic individual) from ramets (i.e. modular units of the same genetic individual), to perform detailed analyses to assess genotypic diversity and gene flow of *R. cirrhosa*, identified as described morphologically in Flora Iberica (Talavera and García-Murillo 2010) and genetically by Martínez-Garrido et al. (2016). Our mains objectives are i) to study the genotypic (i.e. clonal) and genetic diversity of *R. cirrhosa* populations at different spatial scales: along the southern Iberian Peninsula and between the regions of Iberia and Sicily; and ii) to asses the genetic structure and the putative factors that have been invoked to explain the population connectivity patterns (i.e. dispersion through the sea or across the land).

Material and methods

Taxon identification and location sampling

Ruppia cirrhosa specimens were collected between 2011 and 2014 at eleven locations, nine in the Iberian Peninsula and two in Sicily (Italy). We identified the samples using the morphological criteria included in Flora Ibérica (Talavera and García-Murillo 2010), and with molecular tools, using nuclear and chloroplast genes markers (Martínez-Garrido et al. 2016).

Five populations of *R. cirrhosa* were collected in the Atlantic side of the Iberian Peninsula [Óbidos, (central Portugal), Quinta do Lago and Guadiana (southern Portugal), San Fernando and Puerto Real (Cádiz Bay, southwest Spain)], while in the Mediterranean coast of the Iberian Peninsula four populations were sampled, three inside the Mar Menor coastal lagoon (Molino Calcetera, Isla Ciervo, Los Narejos; southeastern Spain), and one in Palma de Mallorca (Balearic Islands, Eastern Spain). In addition, to get a more complete idea of the population structure, we included two locations out of Iberian Peninsula, both in the Tyrrhenian Sea: Nubia and Marausa (Sicily, Italy) (Fig. 1). At each sampled location up to 40 ramets were collected randomly in an area of 60 m^2. Ramets were cleaned from epiphytes and stored in silica gel.

Amplification and sequencing of nuclear and chloroplast genes

DNA was extracted using the CTAB protocol (Doyle and Doyle 1987). Two molecular markers were amplified using different PCR protocols in an Applied Biosystems 2720 Thermal Cycler. The complete ITS region of the nuclear ribosomal DNA (ITS1, 5.8S rRNA and ITS2) (White et al 1990) and the non-coding *trn*H-*psb*A chloroplast inter-genic region (Kress and Erickson 2007). The amplifications were performed under the conditions described in Martínez-Garrido et al. (2016), and the amplified products were sequenced using an ABI PRISM 3130XL automated genetic analyser (Applied Biosystems).

Microsatellite amplification and genotyping

Samples were genotyped for twelve microsatellite loci, namely, Rupcir-01, Rupcir-02, Rupcir-03, Rupcir-04, Rupcir-05, Rupcir-06, Rupcir-07, Rupcir-08, Rupcir-09, Rupcir-10 (Martínez-Garrido et al. 2014) and RUMR4, RUMR10 (Yu et al. 2009). Microsatellite amplification followed protocols from Martínez-Garrido et al. (2014). Rupcir-03 was not included in the analysis because of high amplification failure rates in some populations. PCR products were visualized by gel electrophoresis on a Molecular Imager Gel Doc XR + system (Bio-Rad) and fragment length was analysed on an

ABI PRISM 3130XL automated genetic analyser (Applied Biosystems) using the GeneScan ROX 350 as a size standard.

Data analysis

Sequencing data analysis

Sequences were edited and aligned using the CodonCode Aligner (v. 3.7.1 Codon Code Corporation) software. For ITS, and *psb*A-*trn*H genes, we included to the sequences obtained in this work, the sequences used in Martínez-Garrido et al. (2016). *Ruppia megacarpa* was used as outgroup for all genes. Phylogenetic analyses were conducted as in Martínez-Garrido et al. (2016). New sequences obtained in this study were deposited in NCBI GenBank (KX860097-KX860114).

Microsatellite data analysis

Genetic and genotypic analysis

Raw allele sizes were scored using STRAND (vers. 2.4.59; Toonen and Hughes 2001), binned with the R package MsatAllele (Alberto 2009), and manually reviewed for ambiguities. GenoDive (ver. 2.0b25; Meirmans and Van Tienderen 2004) was used to identify genets [i.e. multilocus genotypes (MLG)], and the clonal assignment was determined at the 100% identical (threshold 0) and with one step mutation (threshold 1), recovering both identical number of MLGs (324). The proportion of different genets in each sample (genotypic richness), was estimated as (G-1)/(N-1), with G representing the number of genets and N representing the number of sampled specimens. Further analyses were conducted with genets to remove clonality.

Polyploids can potentially include multiple copies of the same allele that would not be detected by our analyses and hence cause uncertainty about the real frequency of each allele. To avoid this problem we transformed the MLG data into a binary (presence-absence) matrix (Sampson and Byrne 2012; Vallejo-Marin and Lye 2013; Martínez-Garrido et al. 2016), and then we calculated the total, private numbers of alleles and genetic diversity (*H*) for each population using GenAlex (vers. 6.5; Peakall and Smouse 2006) software. We used this genetic diversity (*H*) index because it allows to compare our results with previous studies while being also independent of the ploidy level. In addition, genetic diversity of each population was also calculated using the Kosman index of diversity within populations (*KW*) following equation 5 from Kosman and Leonard (2007) and employing the R script from Rouger et al. (2014). For that, Dice similarity index between individuals was calculated using the R package "ade4" (Dray and Dufour 2007) from the presence/absence matrix as commonly used for other polyploids to estimate genetic distances (Cidade et al. 2013; Vallejo-Marin and Lye 2013).

Population genetic structure

To study the patterns of population genetic structure, a discriminant analysis of principal components (DAPC's; Jombart et al. 2010) was conducted using R package *adegenet* (vers. 2.0-1; Jombart and Ahmed 2011) allowing us to identify clusters conformed by genetically similar genets (Vallejo-Marin and Lye 2013; Dufresne et al. 2014). The most likely number of clusters in the data was calculated using the *K*-means clustering algorithm *find.clusters* (K=1 to K= 22, all principal components (PC) retained, 10^6 iterations) and diffNgroup option (measured using Bayesian Information Criterion (BIC)) (Jombart et al. 2010). A-score to determine the number of PC retained at each *K*-values was calculated as recommended by Jombart et al. (2010). The posterior probability of assignment of each genet at different *K*-values was represented using the *distruct* software (Rosenberg 2004). To supplement DAPC results, we performed a non-metric multi-dimensional scaling (nMDS) ordination of populations using the R package *vegan* (Dixon 2003). The distance matrix (Kosman distance between populations (KB)) was determined based on the matrix of distances between individuals (Kosman and Leonard 2007). For this, the population size was standardized to 21 and 10 with 1000 bootstrap replicates, but only results standardized to 21 are shown for the nMDS and the Mantel test (see below) since the results were similar for both approaches.

A hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992) was conducted using R package *ade4* (Dray and Dufour 2007) to assess the genetic variation between the different clusters obtained using *adegenet*. For the AMOVA, the genetic distances between individuals were calculated as Dice dissimilarity matrix (Dray and Dufour 2007).

To test the pattern of spatial autocorrelation among the populations studied and to hypothesize about the model of species connectivity, a Mantel test was performed using the package *ade4* in R between the matrix of population dissimilarity (calculated based on KB) and three different types of geographical distances. The geographical distances were classified as: i) “coastal distance”: measured using coastal paths with the shorter distance between sampling sites; ii) “distance between sampling sites”: measured using the shorter straight geographical distances between sampling sites and iii) “distance between populations”: calculated using the largest of the distances between known populations of *R. cirrhosa* located between the two sampling sites. This reflects the largest dispersal distance necessary for migration between the sampling sites. To calculate “distance between population”, we considered the *R. cirrhosa* populations recorded in ANTHOS database (http://www.anthos.es). Consequently, if a *Ruppia* population has been recorded between two of our sampled populations, the distance between our sampled populations is the maximum of the two straight distances between each of the two sampled populations and the intermediate population.

Results

Phylogenetic correspondence

The sequenced nuclear ribosomal ITS region had 653 bp and the *psb*A-*trn*H from 265 to 310 bp in length. All the samples showed the same haplotype for both, the ITS and *psb*A-*trn*H genes. The unique ITS haplotype detected was ITS-b, and in the chloroplast *psb*A-*trn*H, the haplotype found corresponded to the haplogroup B-C. All samples clustered in the clade of *R. cirrhosa* according to the phylogeny of Martínez-Garrido et al. (2016), genetically confirming the species identification (results not shown).

Genotypic and genetic diversity

The total number of identified alleles from the eleven microsatellite loci used was 105, while allelic richness (A) per populations ranged from 30 (i.e. $A_{(G=21)}$=29 after standardizing the number of genets to 21) (Guadiana, southwest Iberian Peninsula) to 67 (i.e. $A_{(G=21)}$=61) (Nubia, Sicily) (Table 1; Electronic Supplementary Material 1). The average genetic diversity within populations measured with *KW* and *H* was of 0.755 and 0.131, respectively. The highest values of genetic diversity within population were found in Puerto Real (Cádiz Bay) (*KW*=0.831; *H*=0.163), Palma de Mallorca (*KW*=0.821; *H*=0145) and Nubia (Sicily) (*KW*=0.816; *H*=0159). Private alleles (PA) were found in six locations, with the highest values in the Mediterranean samples of Palma de Mallorca (7) and Nubia (5). Comparing Mediterranean and Atlantic samples, both showed similar *KW* (0.757 and 0.753, respectively) and *H* values (0.136 and 0.125, respectively), but the allelic richness and the number of private alleles where higher in the Mediterranean (96 and 32, respectively) than in the Atlantic Ocean (73 and 9, respectively) (Table 1). Furthermore, when the populations of Sicily are excluded from the Mediterranean group, these differences remain with 83 alleles and 23 private alleles in the Mediterranean versus 73 and 13 in the Atlantic group.

All populations showed high variability in clonality, showing the maximal genotypic richness (*R*) in Marausa, Palma de Mallorca, San Fernando, Quinta do Lago (ca. 98%) and minimal in the populations inhabiting the coastal lagoon of Mar Menor (Molino Calcetera, Isla Ciervo, Los Narejos) and Óbidos (ca. 50 %) (Table 1).

Genetic structure

The sequential *K*-means clustering analysis implemented in *adegenet* revealed K=6 as the most likely number of clusters (Fig. 2). This pattern was further confirmed with the nMDS (Fig. 3). In addition, a putative three clusters (K=3)

pattern was also considered to test the existence of the Atlantic-Mediterranean break in *Ruppia cirrhosa* from the Iberian Peninsula (Fig. 2a). In general terms, populations were grouped according to their geographical distribution. In this way, when using K=3, the cluster correspond to: (1) the Sicilian populations (NU, MA; $A_{(G=21)}$ =53.78; PA=9), (2) the Mediterranean populations (PM, MC, IC, NR; $A_{(G=21)}$=50.95; PA=9) and (3) the Atlantic populations (SF, PR, QL; $A_{(G=21)}$=46.57; PA=4) (Fig. 2; Electronic Supplementary Material 2). However, two populations were not clustered according to their geographical location: Guadiana (southwest Iberia) which was associated to the Mediterranean cluster, and Óbidos (central Portugal) showing an admixture model between the Atlantic and Mediterranean cluster, but mainly assigned to the Mediterranean populations. In the case of the most likely number of clusters obtained (K=6) (Fig. 2b), the Sicilian populations (Nubia and Marausa) (cluster 1; $A_{(G=21)}$=53.78; PA=9) continued clearly segregated from the Iberian populations (cluster 2, 3, 4, 5, 6). The Iberian populations conformed five different clusters that correspond to: Balearic (Palma de Mallorca; cluster 2; A=55.52; PA=7); Mar Menor (Molino Calcetera, Isla Ciervo and Los Narejos; cluster 3; $A_{(G=21)}$=48.66; PA=0); Guadiana (cluster 4; $A_{(G=21)}$=29; PA=2); Bay of Cádiz (Puerto Real and San Fernando; cluster 5; $A_{(G=21)}$=49.72; PA=3); and Lusitanian (Quinta do Lago and Óbidos; cluster 6; $A_{(G=21)}$=40.29; PA=1) (Fig. 2b; Electronic Supplementary Material 2). However, Óbidos, also had some of the samples included in cluster 3 (Mar Menor), but these results should be taken carefully because of the low number of genets.

The nMDS supported the results obtained with the DAPC confirming the regional clustering (Fig. 3). AMOVA results using the regions K=3 and K=6 confirmed the genetic structure, showing in both cases significant differentiation, explaining approximately 14% and 21% of the variance among clusters, respectively (Table 2). Moreover, significant differentiation between populations within regions (around 21% and 14% of variance, respectively; $P<0.001$) and within populations (around 64% and 66% of variance, respectively; $P<0.001$) was also detected (Table 2).

The Mantel test performed using all the populations showed significant correlation between Kosman genetic distances (KB) and three distinct geographical distances: coastal, between sampling sites and between populations. However, these results are conditioned because of the great genetic distances showed by the Sicilian populations that are also far geographically. Therefore, to avoid this effect, a new Mantel test was conducted with the Sicilian populations removed from the analysis. It is remarkable that, the correlation with the "distance between population" was the model that better explained the results, with a higher Mantel's r (0.608) and stronger significance ($P<0.01$) than the "distance between sampling sites" ($r=0.425$; $P<0.05$). No significant correlation ($P>0.05$) was detected using the "coastal distance" (Table 3).

Discussion

This study revealed high genetic and genotypic diversity of *Ruppia cirrhosa* along the coast of the Iberian Peninsula

and Sicily and high differentiation between populations. The patterns of population genetic structure observed supported the hypothesis that dispersion might be mediated by vectors that can travel across the land (e.g., aquatic birds) rather than along the coast. These results provide a better understanding of the reproduction strategies of the species, and improve our knowledge of the mechanisms used by this species to colonize and persist in new habitats and to maintain genotypic and genetic diversity and structure.

Genotypic and genetic diversity of Ruppia cirrhosa along the Iberian Peninsula and Sicily.

The generally high genotypic diversity found (i.e. most ramets were not clonal copies) revealed that sexual reproduction is a very important factor contributing to the prevalence of *Ruppia cirrhosa* meadows (Table 1.). A similar result was shown in previous studies conducted with *Ruppia cirrhosa*, *Ruppia drepanensis* and *Ruppia cf. maritima* (Martínez-Garrido et al. 2014, 2016) and with the saltmarsh species, *Triglochin maritima* and *Puccinellia maritima* (Rouger and Jump 2014). In a recent study conducted with the diploid *Ruppia maritima*, only 28% of the sampled ramets showed different genets, but these low values were attributed to fixation of the alleles caused by inbreeding, because around 70% of the ramets originated from distinct events of sexual reproduction (Triest and Sierens 2015). In other seagrass species living in more permanent habitats, such as *Zostera noltei* (Coyer et al. 2004; Diekmann et al. 2005), *Zostera marina* (Coyer et al. 2004), *Cymodocea nodosa* (Alberto et al. 2008) and *Posidonia oceanica* (Arnaud-Haond et al. 2007), a higher variability of the proportion among sexual and clonal reproduction was detected between populations, and populations with no clonal diversity were found in several cases. In contrast, the high diversity within populations in highly variable habitats as those inhabited by *R. cirrhosa* is expected to favour their local adaptation and population resilience.

Genotypic diversity in clonal organisms should be determined by the differential success rate between seed (i.e. sexual reproduction) and vegetative propagules or expansion (i.e. asexual reproduction). This balance varies with the environmental conditions (e.g. in aquatic plants: substrate stability, intraspecific competition for space, resistance to extreme conditions, hydrodynamic conditions and sediment nutrients). It is remarkable that our data shows less sexual reproduction in the populations that inhabit more stable hydrological habitats (coastal lagoon of Mar Menor and Óbidos) than those populations that occur in saltmarshes and locations that might suffer drought periods. We raise the hypothesis that the low genotypic diversity found in these populations could be linked with the demographic stability of the meadows (Huston 1979). *Ruppia cirrhosa* is perennial, although by chance, due to drought conditions, it can display an annual life cycle (Gesti et al. 2005), and the formation of clonal meadows is only possible in the most stable habitats. Stable habitats increase the probability that the rhizomes of a clone will survive over the years, allowing them to spread and colonise over a long period. In contrast, in the less stable habitats where periodically unfavourable (e.g., dry)

conditions do not allow persistence, then recruitment is only possible from the seed bank and is therefore associated to persistence of high genotypic diversity. A similar adaptive response has been observed between annual and perennial *Zostera marina* meadows, with seed banks allowing persistence in the Gulf of California over the summer when the plants could not survive because of high seawater temperature (Santamaría-Gallegos et al. 2000). In perennial habitats, sexual reproduction by populations of *Ruppia cirrhosa* also produces a seed bank but, their recruitment success could be affected by clonal density (intra-specific competitive dominance and priority colonization effects). In contrast, in the populations that suffer drought periods, disturbance causes physical discontinuities in the meadow that by reducing intra-specific competition might facilitate sexual recruitment (e.g., Zipperle et al. 2010) and the ability of seed banks to germinate and grow rapidly into new meadows in these extreme conditions. Consequently, seed banks play an important role in hydrologically disturbed habitats as demonstrated by empirical studies: seed banks were decisive for the persistence of *R. cirrhosa* after dry periods in a temporary estuary of South Africa (Vromans et al. 2013). These findings are in agreement with the lower number of genets in meadows with low disturbance detected in other seagrass studies (Hammerli and Reusch 2003; Reusch 2006), although the opposite would result where seagrass disturbance does not prevent survival but prevents reproduction (e.g., Oliva et al. 2014).

Genetic diversity of *R. cirrhosa* was not related with geographical distribution (i.e. Mediterranean Sea vs Atlantic Ocean). The differences in diversity detected between populations could be the result of processes associated to the particular characteristics of each site. Nevertheless, all the populations presented high heterozygosity, equal or higher than other aquatic plants such as *T. maritima* and *P. maritima* from UK (Rouger and Jump 2014). These values are similar to other *Ruppia cirrhosa* populations and higher than *R. cf. maritima* (Martínez-Garrido et al. 2016) and the diploid *R. maritima* (Triest and Sierens 2015). Differences in the reproduction strategies could explain these results, with *R. cirrhosa* shedding pollen at the water-surface promoting cross-fertilization whereas in *R. cf. maritima* and *R. maritima* fecundation might develop mainly in the interior of the flowers causing self-fertilization, as discussed in Martínez-Garrido et al. (2016). In addition, apomixis (i.e. seed production without fertilisation) may be present and/or acting at different intensities in some of these species, however this has yet to be studied.

Genetic structure and connectivity patterns in the studied populations

The genetic structure of the populations shows that Sicilian populations are clearly differentiated from the Iberian Peninsula. In this sense, the Siculo-Tunisian Strait, from Mazara del Vallo (Sicily, southern Italy) to Cape Bon (Tunisia), may be an important genetic boundary between the eastern and western Mediterranean basins for *Ruppia*, as previously discussed by Triest and Sierens (2014) based on chloroplast genes, and in accordance with findings for a variety of other species (Arnaud-Haond et al. 2007; Borsa et al. 1997; Bahri-sfar et al. 2000; Nikula and Vaäinölä 2003;

Serra et al. 2010).

In the Iberian Peninsula, the populations showed a general pattern among Atlantic and Mediterranean populations, but two populations showed unexpected assignments, Óbidos and Guadiana. The sample from Óbidos had few genets, a result which could be influencing the assignment of the population, which showed admixture among Mediterranean and Atlantic clusters (K=3) (Fig. 2) and was also associated with Quinta do Lago (K=6). However, the Guadiana population showed high genetic distance from clusters that are geographically close, the Lusitanian and Bay of Cádiz (Fig. 3). This suggests a distinct spatial and/or temporal colonization history. It can be hypothesized that it might have an ancient Mediterranean origin, which is in agreement with the observed results. Other possible hypotheses are that it might have suffered a strong bottleneck, causing a large loss of allelic richness and low genetic diversity (but this does not explain its unique alleles), and/or be the result of strong selective pressures (e.g., to the fine sediment and low salinity of the site). Adaptive responses to the habitat features (i.e. nutrients availability, sediment type, temperature, salinity) have been suggested to affect population genetic diversity in other *Ruppia* species, namely for salinity (*Ruppia maritima* L.; Koch and Dawes 1991) and for salinity and sediment type (*Ruppia occidentalis*; Barrett et al. 1993). In this sense, this population could be described as a separate ecotype of *R. cirrhosa*. It presented two private alleles (only found once in the full set of ramets) that were found only in a previous study in *Ruppia cf. maritima* (Martínez-Garrido et al. 2016).

According to the most likely number of clusters detected (K=6), *R. cirrhosa* populations showed a regional structure more based on the specific lagoon they inhabit, despite large differences at broader scale, between Atlantic and Mediterranean populations (K=3). Although the number of populations used in the present study did not allow us to make an exhaustive biogeographical analysis, the Mediterranean populations showed higher allelic richness and number of private alleles than the Atlantic populations, supporting previous studies suggesting a Mediterranean origin of *R. cirrhosa* (Triest et al. 2014). In addition, island populations (i.e. Palma Mallorca, Nubia and Marausa), showed values of allelic richness and private alleles equal or higher than the continental populations.

Two main factors have been invoked to explain the connectivity patterns of *Ruppia* species: i) sea currents and ii) bird dispersal. Some marine plants present a distribution pattern associated with the main sea currents (Olsen et al. 2004; Diekmann et al. 2005). Therefore, we might expect a similar connectivity pattern in aquatic macrophytes that inhabit coastal lagoons and saltmarshes having a connection with open waters, following the general pattern of sea surface currents in the Atlantic and the Mediterranean. Nevertheless, in the studied *Ruppia cirrhosa* populations, we did not find a significant correlation of genetic distance with coastal distance, although significant correlations were observed between genetic distances and the other geographical distances: “distance between sampling sites” and “distance between populations” (i.e. straight flight distances across land or sea) (Table 3). *Ruppia cirrhosa* meadows are restricted to very isolated ponds with a narrow connection to open waters. Previous studies have suggested that the

exchange of genetic material between isolated saltmarshes was possible due to the action of tidal currents on seeds (Koutstaal et al. 1987). However, according to our results, the influence of tidal and sea currents in the dispersion of seeds and vegetative fragments of *R. cirrhosa* seems to be less important than in other species, at least in the Iberian Peninsula. This could be explained by a combination of biological, geomorphological and hydrodynamic factors, such as: i) the negative seed buoyancy that promote seed sinking close to parent plant, ii) the muddy bottom predominant in the ponds which could trap seeds, iii) the low tidal influence in the studied sites not allowing a great distance dispersal, iv) the fact that most of the propagules are exported out of the saltmarsh rather than imported within (Huiskes et al. 1995); and v) diverse waterbirds feed on *Ruppia* spp.

Waterbirds are likely the vector that could facilitate gene flow between isolated neighbouring populations across the land, transporting seeds in the gut. Figuerola et al. (2002), working with *R. maritima* L in wetlands of southwest Iberian Peninsula, demonstrated the presence of seeds in the diet of several ducks and coots and the capacity of the seeds to germinate after being defecated by the birds. In addition, our results show that population patch distribution plays an important role in the connectivity of *R. cirrhosa* across the land. Consequently, the model that best fits our data over large dispersal distances in the Iberian Peninsula, is one that includes "distance between populations" ($r=0.608$; $P<0.01$), although "distance between sampling sites" ($r=0.425$; $P<0.05$) also showed significant correlation. This suggests that, as in the theory of island biogeography (Macarthur and Wilson 1969), the distance between islands will be negatively correlated with the arrival of settlers, and it will determine the intensity in the exchange of migrants (alleles in this case) between the different islands. *Ruppia* populations, whose distribution is restricted to very particular environmental conditions, should be considered as islands (i.e. habitat suitable for *R. cirrhosa*), surrounded by unsuitable habitat. Stepping stone populations are thus expected to act as a bridge favouring the connectivity; implying that connectivity will be lower when stepping stone populations are not present.

Behaviour and physiological traits of waterbird species should determine the threshold distance for seed-dispersion such as speed and route flight, seed retention and degradation time in the gut, which could have a positive influence in the probability of *Ruppia cirrhosa* seed germination and/or to be able to reach suitable habitats when the distance among sites is shorter. These results are in agreement with studies that suggest an important role of waterbirds as seed dispersers of *Ruppia* spp. and other aquatic plants at local spatial scales, and a more discussed potential role at larger spatial scale (Clausen et al. 2002; Charalambidou and Santamaría 2005). Furthermore, a discontinuous genetic pattern found for two *Ruppia* species from Asia and Oceania, suggested that the disjunct distribution was bird-mediated (Ito et al. 2010). Similarly, in a recently study performed with microsatellites, bird-mediated dispersal was also suggested to promote the isolated structure found with the diploid *R. maritima* (Triest and Sierens 2015). However, in the case of *R. cirrhosa*, populations have lower seed production and it is more coastal than *R. maritima*, therefore sea currents have been proposed until now as the main dispersal factor (Triest and Sierens 2013). However, the low

mutation rate of the chloroplast genes used, the broad spatial scale and the fact that several entities of *Ruppia* were examined together, could be masking the effects of bird-mediated gene flow on those results (Triest and Sierens 2013). Also, it is important to stress that despite lower fruit production than *R. maritima*, *R. cirrhosa* exhibits a considerable amount of flowers and seeds, which are a very attractive food resource for some birds (Marco-Méndez et al. 2014).

Conclusions

In the present study, we highlight the importance of sexual reproduction in *Ruppia cirrhosa* populations, which seems to be more important in populations inhabiting temporary habitats in hydrologically disturbed sites. These results point out the key role of seed banks on the survival of plant populations living in extreme environments. The high genetic diversity indicates that despite the distance between populations, high variability is maintained within, which favours adaptation to changing environments. Although we could not detect a clear pattern of genetic diversity among Atlantic and Mediterranean populations, based on the allelic richness and number of private alleles, we hypothesize a Mediterranean origin of *R. cirrhosa* and/or a climatic refugial zone there. Finally, in the case of *R. cirrhosa* in southern Iberia, our results based on correlation and population structure, suggest that waterbird seed-dispersion is more intense at distances among neighbouring habitats (and probably inside the same lagoon) and has a smaller, albeit significant, influence at larger spatial scales. In contrast, the influence of tidal and sea currents on the connectivity patterns might be more restricted to the populations that inhabit the same water body. Nevertheless, further detailed studies tracking the birds species that ingest *Ruppia* spp are required to determine precisely the extent that waterbird-dispersal events have on influencing the species' genetic structure.

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Figures

Figure 1. Locations of *Ruppia cirrhosa* populations sampled in the Iberian Peninsula and Italy.

Figure 2. Results of the discriminant analysis of principal components (DAPC's) showing the membership probability of assignment for each population at different K values. **a)** K=3, **b)** K=6. Population names are indicated between both figures and cluster correspondence of populations are showed above (K3) and below (K=6) each figure. NU, Nubia; MA, Marausa; PM, Palma de Mallorca; MC, Molino Calcetera; IC, Isla Ciervo; NR, Los Narejos; GU, Guadiana; SF, San Fernando; PR, Puerto Real; QL, Quinta do Lago; OB, Óbidos.

Figure 3. Non-metric Multi-Dimensional Scaling (nMDS) ordination of *Ruppia cirrhosa* conducted with Kosman genetic distances between populations. Population names are coloured based on the cluster correspondence at K=·3 and populations names are circled based on the obtained K=6 in the DAPC.

Tables

Table 1. Genotypic and genetic diversity parameters calculated for sampled populations of *Ruppia cirrhosa* and comparative parameters between the Mediterranean and Atlantic populations.

Table 2. Analysis of molecular variance (AMOVA) for *Ruppia cirrhosa* populations, showing the partitioning of genetic variation among and within the successive values of *K*.

Table 3. Mantel test considering Kosman genetic distances (KB) and the three geographical distances calculated between the populations of *Ruppia cirrhosa*.

Electronic Supplementary Material

ESM 1. Standardized allelic richness in the studied populations of *Ruppia cirrhosa*.

ESM 2. Allelic richness, privative alleles and genetic diversity parameters calculated for sampled populations of *Ruppia*

650 *cirrhosa*, segregated based on discriminant analysis of principal component at K=3 (excluding GU & OB) and K=6.

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Figure 1

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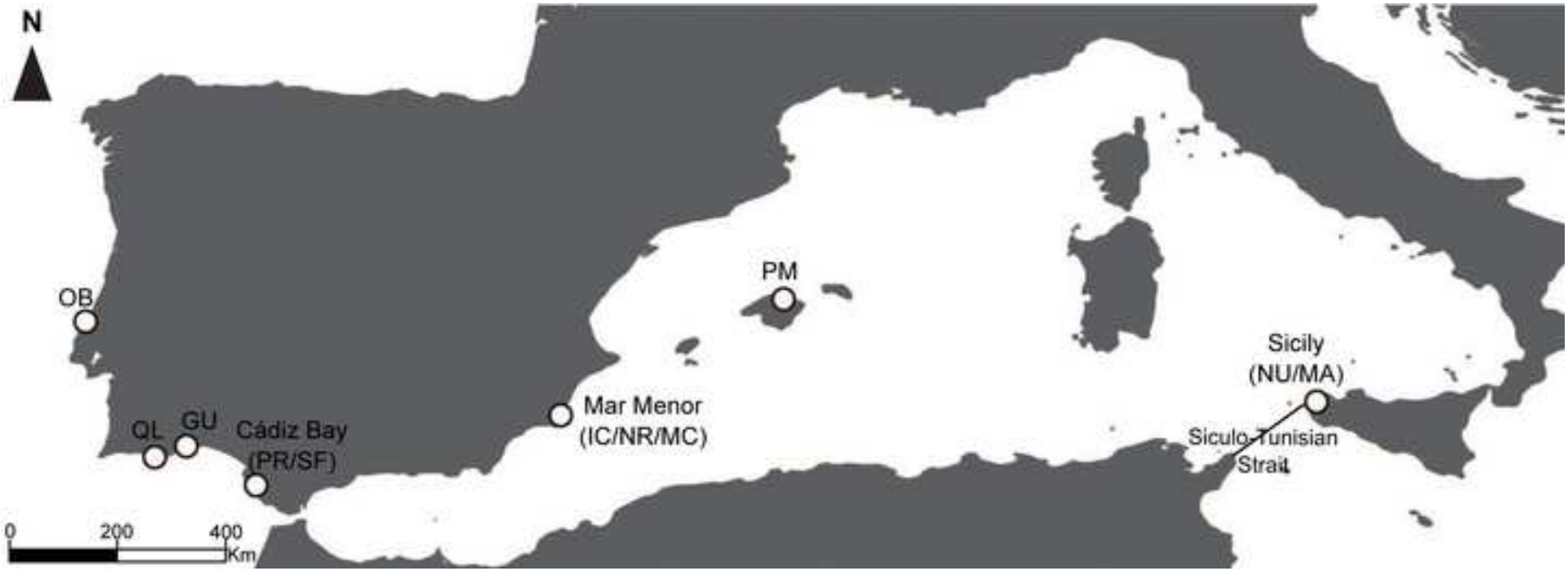

Figure 2

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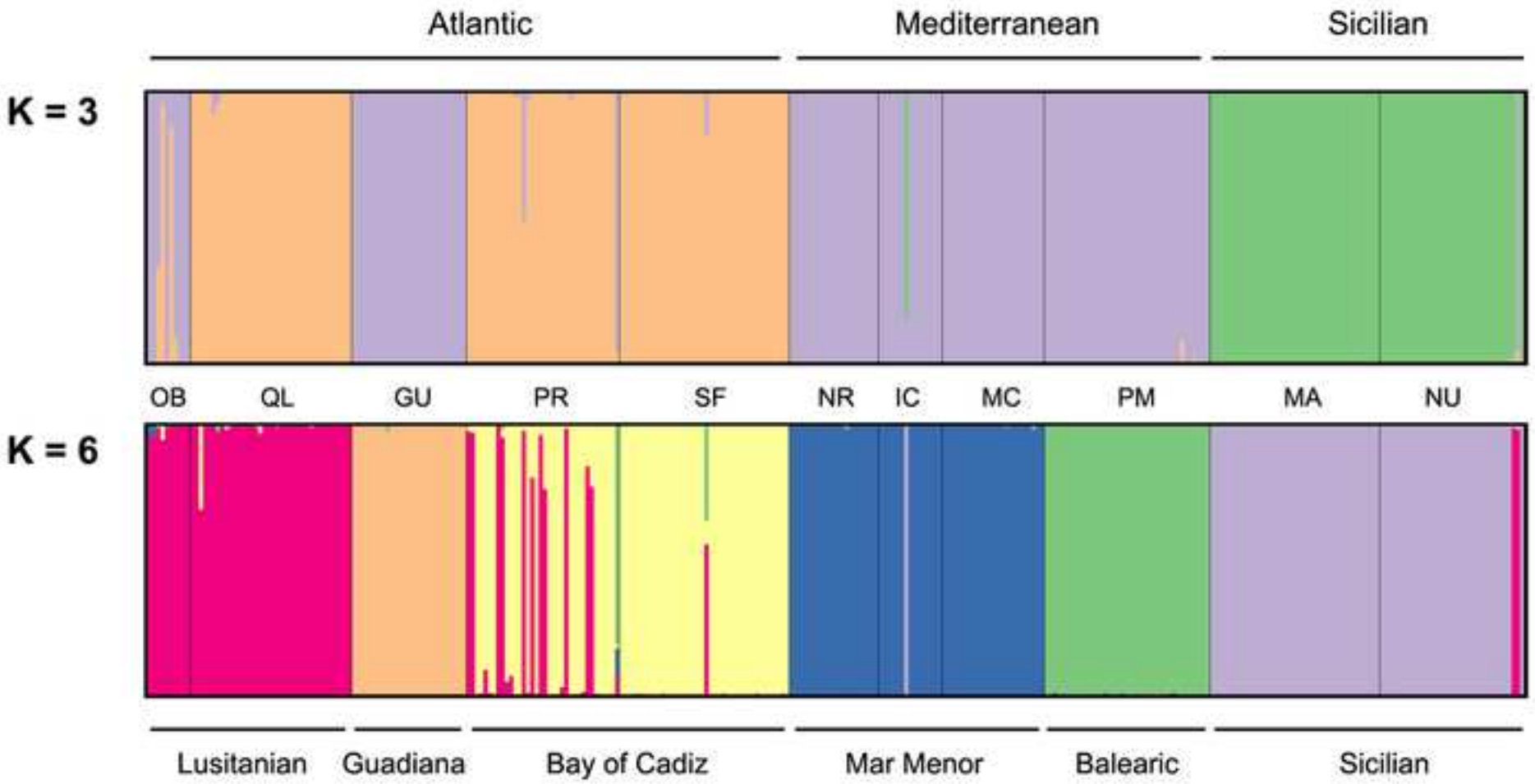

Figure 3

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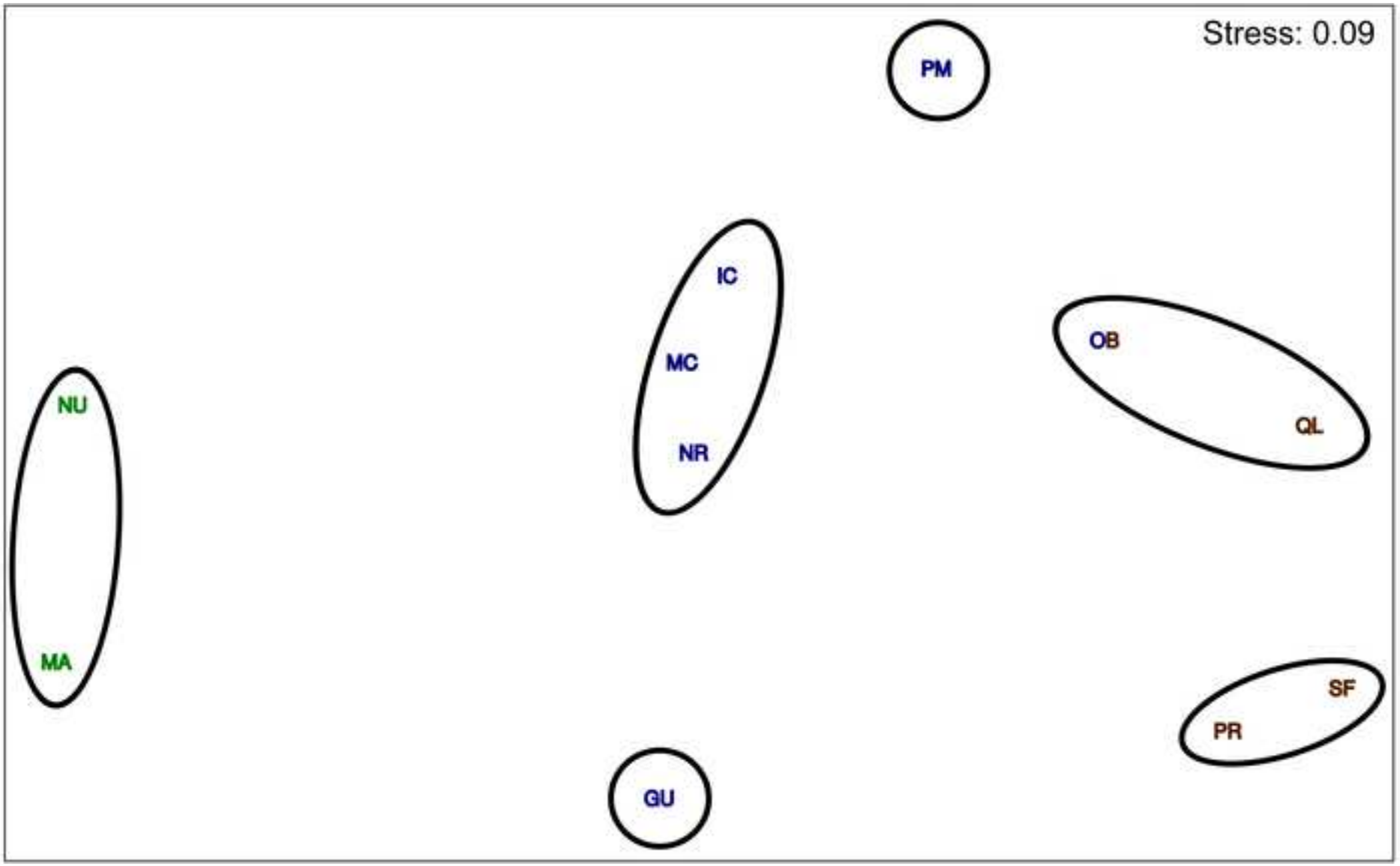


Table 1

Table 1. Genotypic and genetic diversity parameters calculated for sampled populations of *Ruppia cirrhosa* and comparative parameters between the Mediterranean and Atlantic populations.

Population	N	G	R	An	$A_{(G=10)}$	$A_{(G=21}$	PA	*KW*	*H*
Nubia (NU)	40	34	0.846	67	50.60±2.76	61.20±1.92	5	0.816	0.159
Marausa (MA)	40	40	1.000	50	40.38±2.36	46.37±1.62	1	0.751	0.130
Palma de Mallorca (PM)	40	39	0.974	61	46.14±2.97	55.52±2.02	7	0.821	0.145
Molino Calcetera (MC)	40	24	0.590	47	41.12±1.97	46.32±0.74	0	0.750	0.133
Isla Cievo (IC)	40	15	0.359	42	40.12±1.34		0	0.692	0.124
Los Narejos (NR)	40	21	0.513	51	42.46±2.32	51.00±0.00	0	0.710	0.123
Mediterranean populations	240	173	0.720	96	43.45±2.28	52.08±1.26	32	0.757	0.136
Guadiana (GU)	40	27	0.667	30	25.96±1.40	29.00±1.02	2	0.662	0.089
San Fernando (SF)	40	40	1.000	49	39.57±2.04	44.46±1.67	2	0.789	0.138
Puerto Real (PR)	40	36	0.897	59	47.02±2.76	54.97±1.78	0	0.831	0.163
Quinta do Lago (QL)	40	38	0.949	44	35.37±1.65	40.29±1.40	0	0.792	0.128
Obidos (OB)	17	10	0.563	38	38.00±0.00		1	0.694	0.106
Atlantic populations	177	151	0.852	73	37.18±1.57	42.18±1.46	9	0.753	0.125
TOTAL	417	324	0.755	105	80.64	94.26	18	0.755	

N, number of ramets sampled; G, number of genets found; R, genotypic richness R=(G-1)/(N-1)*;* An, allelic richness in each population and allelic richness (±SE) estimated after standardizing G to 10 (G=10) and G to 21 (G=21) (except where G<21); PA, private alleles; *KW*= average population genetic diversity measured using the Kosman index of diversity within populations and standardized to 21. *H*= unbiassed genetic diversity calculated on the presence-absence matrix, allowing comparation with other studies and independent of the

Table 2

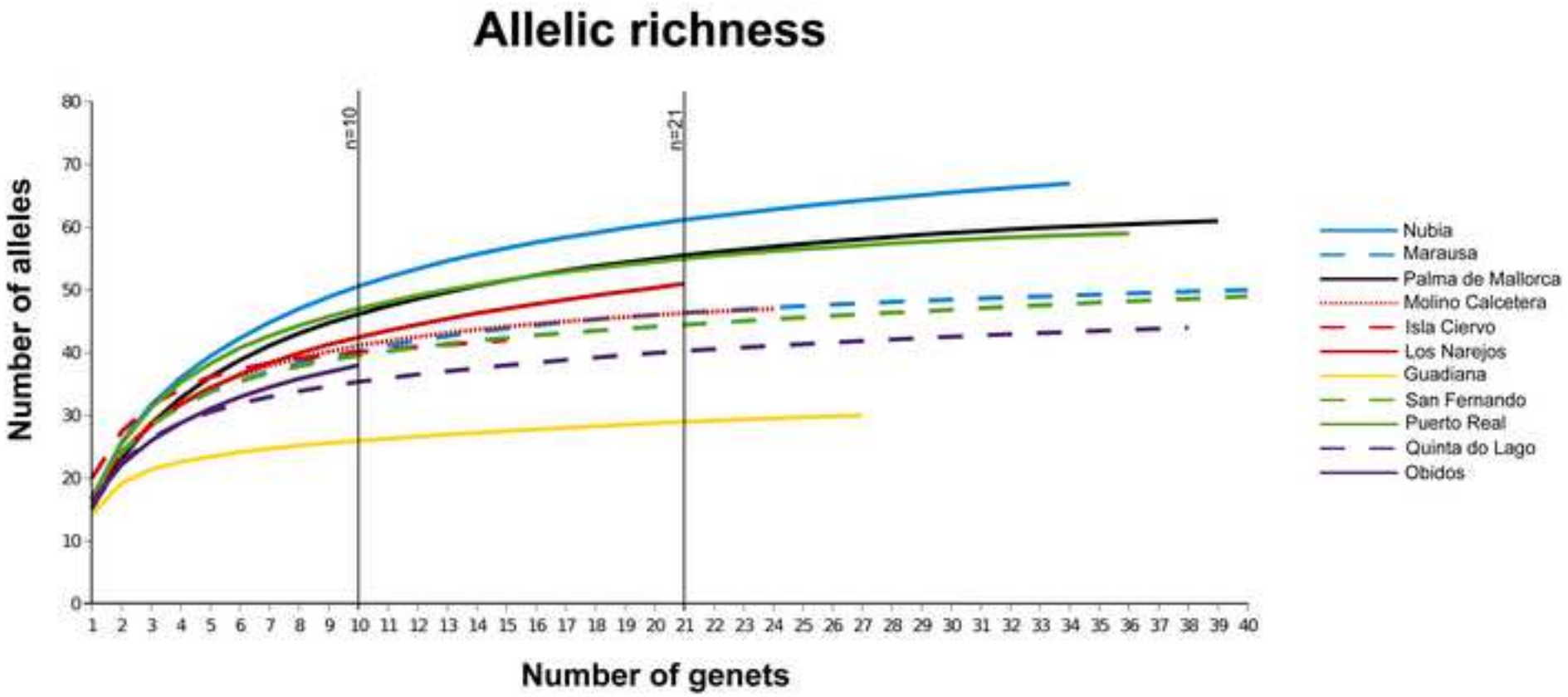
Table 2. Analysis of molecular variance (AMOVA) for *Ruppia cirrhosa* populations, showing the partitioning of genetic variation among and within the successive values of *K*.

K-value	Source of variation	% Variation	Fixation index	*P*-value
3	Among clusters	14.43%	PhiRT= 0.144	≤0.001
	Among populations within clusters	21.37%	PhiPR= 0.249	≤0.001
	Within populations	64.20%	PhiPT= 0.358	≤0.001
6	Among clusters	20.50%	PhiRT= 0.205	≤0.001
	Among populations within clusters	13.88%	PhiPR= 0.174	≤0.001
	Within populations	65.62%	PhiPT= 0.343	≤0.001

Table 3. Mantel test considering Kosman genetic distances (KB) and the three geographical distances calculated between the populations of *Ruppia cirrhosa*.

	Coastal distance		Sampling site distance		Populations distance	
Populations	r	*P*-value	r	*P*-value	r	*P*-value
Eleven (included all studied populations)	0.654	0.001***	0.688	0.001***	0.755	0.001***
Nine (excluded Sicilian populations)	0.341	0.059	0.425	0.022*	0.608	0.003**

Statistically significant at *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$



ESM 2. Allelic richness, privative alleles and genetic diversity parameters calculated for sampled populations of *Ruppia cirrhosa,* segregated based on discriminant analysis of principal component at K=3 (excluding GU & OB) and K=6.

Number of clusters	G	An	$An_{(G=10)}$	$An_{(G=21)}$	PA
K=3 (excluding GU & OB)					
Cluster 1 (NU/MA)	74	77	45.49	53.78	9
Cluster 2 (PM/MC/IC/NR)	99	83	42.46	50.95	9
Cluster 3 (SF/PR/QL)	114	68	40.65	46.57	4
K=6					
Cluster 1 (NU/MA)	74	77	45.49	53.78	9
Cluster 2 (MC/IC/NR)	60	60	41.23	48.66	0
Cluster 3 (PM)	39	61	46.14	55.52	7
Cluster 4 (GU)	27	30	25.96	29	2
Cluster 5 (SF/PR)	76	63	43.3	49.72	3
Cluster 6 (QL/OB)	48	53	36.69	40.29	1

G, number of genets found; An, allelic richness in each cluster and allelic richness estimated after standardizing G to 10 (G=10) and G to 21 (G=21; OB and IC not counted because G<21). PA, private alleles.