

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

2

3 Martínez-Garrido, J.^{1,2,*}, Bermejo, R.^{3,4}, Serrão, E.A.¹, Sánchez-Lizaso, J^{2.}, González-Wangüemert, M.¹

4

5 ¹ Centro de Ciências do Mar (CCMAR). Universidade do Algarve, Gambelas, 8005-139 Faro, Portugal.

6 ² Departamento de Ciencias del Mar y Biología Aplicada. Universidad de Alicante (DCMBA, UA). P.O. Box 99, 03080

7 Alicante, Spain.

8 ³ Irish Seaweed Research Group, Ryan Institute and School of Natural Sciences, National University of Ireland,

9 Galway, Co. Galway, Ireland.

10 ⁴ Departamento de Biología. Área de Ecología. Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz.

11 11510 Puerto Real, Cádiz, Spain.

12

13 *Corresponding author: José Martínez-Garrido. (e-mail: jmgarrido99@gmail.com). Phone: 0034 625346390

14

15

16

17

18

19

20

21

22

23

24

25

26

27

28

29

30

31 **Abstract**

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

46
47 **Keywords**

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

49
50
51
52
53
54
55
56
57
58
59
60
61
62

63 Introduction

64

65 Estuaries, coastal lagoons, and saltmarshes are economically and ecologically important habitats providing a number of
66 valuable ecosystem services (Costanza et al. 1998; Pérez-Ruzafa et al. 2011). Despite their importance, these systems
67 are highly impacted by human actions, counting amongst the most threatened habitats in the world (Airoidi and Beck
68 2007). In these transition zones between land and sea ecosystems, which are characterised by extreme variations of
69 environmental conditions (Viaroli et al. 2008), species from the seagrass genus *Ruppia* (Short et al. 2007) are important
70 ecosystem engineers that create habitat by forming dense meadows (Den Hartog and Kuo 2006; Verhoeven 1979).
71 These meadows play several key ecological roles including enhancing primary productivity, improvement of water and
72 sediment quality, and providing valuable habitat and food resources for other species (Hemminga and Duarte 2000).
73 *Ruppia* spp. have been considered characteristic of pristine lagoons (Viaroli et al. 2008), and their meadows are
74 classified as habitat of community interest by the UE (Directive 92/43/ECC; Annex I, “Coastal lagoons”). However,
75 because *Ruppia* species are difficult to identify taxonomically, their conservation status has not been properly assessed
76 by the International Union for Conservation of Nature (IUCN).

77 The population structure of aquatic clonal plants is a complex product of demographic and genetic processes,
78 life history, adaptation, reproductive systems and dispersal potential, besides environmental influences (Spalding et al.
79 2003). The genus *Ruppia* (Alismatales, Ruppiaceae) consists of clonal and hermaphroditic aquatic plants that are
80 closely related to seagrass families such as Posidoniaceae and Cymodoceaceae (Les et al. 1997). It possesses sexual
81 reproduction by cross- and self-fertilization, and asexual reproduction occurs through clonal formation. Sexual
82 reproduction forms novel genotypes thereby fostering selective responses, while asexual propagation ensures
83 persistence and spread of successful genotypes. Therefore, the balance among sexual and asexual reproduction is likely
84 one of the most important factors in determining population genetic structure in this and other seagrasses (Coyer et al.
85 2004; Olsen et al. 2004; Alberto et al. 2008). In *Ruppia* spp., pollen dispersal is normally confined to within populations
86 (Verhoeven 1979). However, at larger spatial scales different mechanisms such as sea currents (Koch et al. 2010), birds
87 (Charalambidou and Santamaría 2005; Ito et al. 2010) and fishes (Agami and Waisel 1988), have been proposed as
88 dispersal vectors of plant vegetative fragments and seeds.

89 Populations of *Ruppia* spp. tend to occur in strong isolation, and isolated populations are predicted to become
90 genetically impoverished through genetic drift (Hedrick 2006, Allendorf and Luikart 2007). Genetic connectivity may
91 therefore be important to provide higher diversity that should facilitate adaptive processes under changing conditions
92 (Davis and Shaw 2001) to maintain long-term viability of populations (Segelbacher et al. 2010). Thus, estimating
93 genetic (i.e. heterozygosity) and genotypic (i.e. clonal) diversity, and the mechanisms influencing connectivity, is
94 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 (*psbA-trnH*). 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).

127 **Material and methods**

128 Taxon identification and location sampling

129

130 *Ruppia cirrhosa* specimens were collected between 2011 and 2014 at eleven locations, nine in the Iberian Peninsula and
131 two in Sicily (Italy). We identified the samples using the morphological criteria included in Flora Ibérica (Talavera and
132 García-Murillo 2010), and with molecular tools, using nuclear and chloroplast genes markers (Martínez-Garrido et al.
133 2016).

134 Five populations of *R. cirrhosa* were collected in the Atlantic side of the Iberian Peninsula [Óbidos, (central
135 Portugal), Quinta do Lago and Guadiana (southern Portugal), San Fernando and Puerto Real (Cádiz Bay, southwest
136 Spain)], while in the Mediterranean coast of the Iberian Peninsula four populations were sampled, three inside the Mar
137 Menor coastal lagoon (Molino Calcetera, Isla Ciervo, Los Narejos; southeastern Spain), and one in Palma de Mallorca
138 (Balearic Islands, Eastern Spain). In addition, to get a more complete idea of the population structure, we included two
139 locations out of Iberian Peninsula, both in the Tyrrhenian Sea: Nubia and Marausa (Sicily, Italy) (Fig. 1). At each
140 sampled location up to 40 ramets were collected randomly in an area of 60 m². Ramets were cleaned from epiphytes and
141 stored in silica gel.

142

143 Amplification and sequencing of nuclear and chloroplast genes

144

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

151

152 Microsatellite amplification and genotyping

153

154 Samples were genotyped for twelve microsatellite loci, namely, Rupcir-01, Rupcir-02, Rupcir-03, Rupcir-04, Rupcir-
155 05, Rupcir-06, Rupcir-07, Rupcir-08, Rupcir-09, Rupcir-10 (Martínez-Garrido et al. 2014) and RUMR4, RUMR10 (Yu
156 et al. 2009). Microsatellite amplification followed protocols from Martínez-Garrido et al. (2014). Rupcir-03 was not
157 included in the analysis because of high amplification failure rates in some populations. PCR products were visualized
158 by gel electrophoresis on a Molecular Imager Gel Doc XR + system (Bio-Rad) and fragment length was analysed on an

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

161

162 Data analysis

163 *Sequencing data analysis*

164

165 Sequences were edited and aligned using the CodonCode Aligner (v. 3.7.1 Codon Code Corporation) software. For ITS,
166 and *psbA-trnH* genes, we included to the sequences obtained in this work, the sequences used in Martínez-Garrido et al.
167 (2016). *Ruppia megacarpa* was used as outgroup for all genes. Phylogenetic analyses were conducted as in Martínez-
168 Garrido et al. (2016). New sequences obtained in this study were deposited in NCBI GenBank (KX860097-KX860114).

169

170 *Microsatellite data analysis*

171 *Genetic and genotypic analysis*

172

173 Raw allele sizes were scored using STRAND (vers. 2.4.59; Toonen and Hughes 2001), binned with the R package
174 MsatAllele (Alberto 2009), and manually reviewed for ambiguities. GenoDive (ver. 2.0b25; Meirmans and Van
175 Tienderen 2004) was used to identify genets [i.e. multilocus genotypes (MLG)], and the clonal assignment was
176 determined at the 100% identical (threshold 0) and with one step mutation (threshold 1), recovering both identical
177 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
178 analyses were conducted with genets to remove clonality.

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

191

192 *Population genetic structure*

193

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

207 A hierarchical analysis of molecular variance (AMOVA; Excoffier et al. 1992) was conducted using R package
208 *ade4* (Dray and Dufour 2007) to assess the genetic variation between the different clusters obtained using *adeigenet*. For
209 the AMOVA, the genetic distances between individuals were calculated as Dice dissimilarity matrix (Dray and Dufour
210 2007).

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

222

223 Results

224

225 *Phylogenetic correspondence*

226

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

232

233 *Genotypic and genetic diversity*

234

235 The total number of identified alleles from the eleven microsatellite loci used was 105, while allelic richness (A) per
236 populations ranged from 30 (i.e. $A_{(G=21)}=29$ after standardizing the number of genets to 21) (Guadiana, southwest
237 Iberian Peninsula) to 67 (i.e. $A_{(G=21)}=61$) (Nubia, Sicily) (Table 1; Electronic Supplementary Material 1). The average
238 genetic diversity within populations measured with KW and H was of 0.755 and 0.131, respectively. The highest values
239 of genetic diversity within population were found in Puerto Real (Cádiz Bay) ($KW=0.831$; $H=0.163$), Palma de
240 Mallorca ($KW=0.821$; $H=0.145$) and Nubia (Sicily) ($KW=0.816$; $H=0.159$). Private alleles (PA) were found in six
241 locations, with the highest values in the Mediterranean samples of Palma de Mallorca (7) and Nubia (5). Comparing
242 Mediterranean and Atlantic samples, both showed similar KW (0.757 and 0.753, respectively) and H values (0.136 and
243 0.125, respectively), but the allelic richness and the number of private alleles were higher in the Mediterranean (96 and
244 32, respectively) than in the Atlantic Ocean (73 and 9, respectively) (Table 1). Furthermore, when the populations of
245 Sicily are excluded from the Mediterranean group, these differences remain with 83 alleles and 23 private alleles in the
246 Mediterranean versus 73 and 13 in the Atlantic group.

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

250

251 *Genetic structure*

252

253 The sequential K -means clustering analysis implemented in *adegenet* revealed $K=6$ as the most likely number of
254 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

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

292
293 *Genotypic and genetic diversity of Ruppia cirrhosa along the Iberian Peninsula and Sicily.*
294

295 The generally high genotypic diversity found (i.e. most ramets were not clonal copies) revealed that sexual reproduction
296 is a very important factor contributing to the prevalence of *Ruppia cirrhosa* meadows (Table 1.). A similar result was
297 shown in previous studies conducted with *Ruppia cirrhosa*, *Ruppia drepanensis* and *Ruppia cf. maritima* (Martínez-
298 Garrido et al. 2014, 2016) and with the saltmarsh species, *Triglochin maritima* and *Puccinellia maritima* (Rouger and
299 Jump 2014). In a recent study conducted with the diploid *Ruppia maritima*, only 28% of the sampled ramets showed
300 different genets, but these low values were attributed to fixation of the alleles caused by inbreeding, because around
301 70% of the ramets originated from distinct events of sexual reproduction (Triest and Sierens 2015). In other seagrass
302 species living in more permanent habitats, such as *Zostera noltei* (Coyer et al. 2004; Diekmann et al. 2005), *Zostera*
303 *marina* (Coyer et al. 2004), *Cymodocea nodosa* (Alberto et al. 2008) and *Posidonia oceanica* (Arnaud-Haond et al.
304 2007), a higher variability of the proportion among sexual and clonal reproduction was detected between populations,
305 and populations with no clonal diversity were found in several cases. In contrast, the high diversity within populations
306 in highly variable habitats as those inhabited by *R. cirrhosa* is expected to favour their local adaptation and population
307 resilience.

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

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

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

343

344 *Genetic structure and connectivity patterns in the studied populations*

345

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

351 Serra et al. 2010).

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

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

374 Two main factors have been invoked to explain the connectivity patterns of *Ruppia* species: i) sea currents and
375 ii) bird dispersal. Some marine plants present a distribution pattern associated with the main sea currents (Olsen et al.
376 2004; Diekmann et al. 2005). Therefore, we might expect a similar connectivity pattern in aquatic macrophytes that
377 inhabit coastal lagoons and saltmarshes having a connection with open waters, following the general pattern of sea
378 surface currents in the Atlantic and the Mediterranean. Nevertheless, in the studied *Ruppia cirrhosa* populations, we did
379 not find a significant correlation of genetic distance with coastal distance, although significant correlations were
380 observed between genetic distances and the other geographical distances: “distance between sampling sites” and
381 “distance between populations” (i.e. straight flight distances across land or sea) (Table 3). *Ruppia cirrhosa* meadows are
382 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

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

419

420

421 **Conclusions**

422

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

436

437 **Acknowledgements**

438

439 The authors thank Dr. O. Diekmann for collecting samples in Óbidos and Dr. C. Cox for manuscript revision. We also
440 thank two anonymous reviewers for their helpful comments on the manuscript. This study was funded by the Fundação
441 para a Ciência e Tecnologia (FCT, Portugal) PTDC/MAR/119363/2010, BIODIVERSA/0004/2015,
442 UID/Multi/04326/2013, the Pew Foundation, and SENECA Foundation, Murcia Government, Spain (11881/PI/09).
443 MGW was supported by the FCT Investigator Programme-Career Development (IF/00998/2014). During the
444 development of this work R. Bermejo held a Formación de Personal Universitario (FPU) (AP2008-01209) fellowship of
445 the Spanish Ministry of Education, and an ASSEMBLE (Association of European Marine Biological Laboratories)
446 grant (Ref. 00399/2012) from the European Community.

447

448

449 **References**

- 450 Agami, M., and Y. Waisel. 1988. The role of fish in distribution and germination of seeds of the submerged
451 macrophytes *Najas marina* L. and *Ruppia maritima* L. *Oecologia* 76: 83–88.
- 452 Allendorf, F.W., and G. Luikart. 2007. Conservation and the Genetics of Populations. Oxford: Blackwell Publishing.
453 642 pp.
- 454 Airoidi, L., and M.W. Beck. 2007. Loss , Status and Trends for Coastal Marine Habitats of Europe. *Oceanography and*
455 *Marine Biology* 45: 345–405.
- 456 Alberto, F., S. Massa, P. Manent, E. Diaz-Almela, S. Arnaud-Haond, C.M. Duarte, and E.A. Serrão. 2008. Genetic
457 differentiation and secondary contact zone in the seagrass *Cymodocea nodosa* across the Mediterranean-Atlantic
458 transition region. *Journal of Biogeography* 35: 1279–1294.
- 459 Alberto F. 2009. MsatAllele-1.0: An R package to visualize the binning of microsatellite alleles. *Journal of Heredity*
460 100: 394–397.
- 461 Arnaud-Haond, S., M. Migliaccio, E. Diaz-Almela, S. Teixeira, M.S. van de Vliet, F. Alberto, G. Procaccini, C.M.
462 Duarte, and E.A. Serrão. 2007. Vicariance patterns in the Mediterranean Sea: east-west cleavage and low dispersal in
463 the endemic seagrass *Posidonia oceanica*. *Journal of Biogeography* 34: 963–976.
- 464 Bahri-Sfar, L., C. Lemaire, O. K. Ben Hassine, and F. Bonhomme. 2000. Fragmentation of sea bass populations in the
465 western and eastern Mediterranean as revealed by microsatellite polymorphism. *Proceedings of the Royal Society of*
466 *London B* 26 929-935.
- 467 Barrett, S.C.H., C.G. Echert, and B.C. Husband. 1993. Evolutionary processes in aquatic plant populations. *Aquatic*
468 *Botany* 44: 105-145
- 469 Borsa, P., A. Blanquer, and P. Berrebi. 1997. Genetic structure of the flounders *Platichthys flesus* and *P. stellatus* at
470 different geographic scales. *Marine Biology* 129: 233-246.
- 471 Charalambidou, I., and L. Santamaría. 2005. Field evidence for the potential of waterbirds as dispersers of aquatic
472 organisms. *Wetlands* 25: 252–258.
- 473 Cidade, F.W., B.B. Vigna, F.H. de Souza, J.F.M. Valls, M. Dall’Agnol, M.I. Zucchi, T.T. de Souza-Chies, and A.P.
474 Souza. 2013. Genetic variation in polyploid forage grass: assessing the molecular genetic variability in the *Paspalum*
475 genus. *BMC genetics* 14: 50.
- 476 Cirujano, S., and P. García-Murillo. 1990. Asientos para un atlas corológico de la flora occidental, 16. Mapas 434, 435,
477 436 y 437. *Fontqueria* 28: 159–165.
- 478 Cirujano, S., and P. García-Murillo. 1992. El género *Ruppia* en la Península Ibérica. *Quercus* 74: 14-21.
- 479 Clausen, P., B.A. Nolet, A.D. Fox, and M. Klaassen. 2002. Long-distance endozoochorous dispersal of submerged
480 macrophyte seeds by migratory waterbirds in northern Europe—a critical review of possibilities and limitations. *Acta*
481 *Oecologica* 23: 191–203.
- 482 Comps, B., D. Gömöry, J. Letouzey, B. Thiébaud, and R.J. Petit. 2001. Diverging Trends Between Heterozygosity and
483 Allelic Richness During Postglacial Colonization in the European Beech. *Genetics* 157: 389–397.
- 484 Costanza, R., R. D’Arge, R. de Groot, S. Farber, M. Grasso, B. Hannon, K. Limburg, S. Naeem, R.V. O’Neill, J.
485 Paruelo, R.G. Raskin, P. Sutton, and M. van den Belt. 1998. The value of the world’s ecosystem services and natural
486 capital. *Nature* 25: 3–15.
- 487 Coyer, J.A., O.E. Diekmann, E.A. Serrão, G. Procaccini, N. Milchakova, G.A. Pearson, W.T. Stam, and J.L. Olsen.
488 2004. Population genetics of dwarf eelgrass *Zostera noltii* throughout its biogeographic range. *Marine Ecology*
489 *Progress Series* 281: 51–62.

490 Cramp, A., and G. O'Sullivan. 1999. Neogene sapropels in the Mediterranean: a review. *Marine Geology* 153: 11–28.

491 Davis, M.B., and R.G. Shaw. 2001. Range Shifts and Adaptive Responses to Quaternary Climate Change. *Science* 292:
492 673–679.

493 Diekmann, O.E., J.A. Coyer, J. Ferreira, J.L. Olsen, W.T. Stam, G.A. Pearson, and E.A. Serrão. 2005. Population
494 genetics of *Zostera noltii* along the west Iberian coast: consequences of small population size, habitat discontinuity and
495 near-shore currents. *Marine Ecology Progress Series* 290: 89–96.

496 Dixon, P. 2003. VEGAN, a package of R functions for community ecology. *Journal of Vegetation Science* 14: 927–930.

497 Doyle, J.J., and J.J. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue.
498 *Phytochemical Bulletin* 19: 11–15.

499 Dray, S., and A.B. Dufour. 2007. The ade4 Package: Implementing the Duality Diagram for Ecologists. *Journal of*
500 *Statistical Software* 22.

501 Dufresne, F., M. Stift, R. Vergilino, and B.K. Mable. 2014. Recent progress and challenges in population genetics of
502 polyploid organisms: an overview of current state-of-the-art molecular and statistical tools. *Molecular ecology* 23: 40–
503 69.

504 Excoffier, L., P.E. Smouse, and J.M. Quattro. 1992. Analysis of molecular variance inferred from metric distances
505 among DNA haplotypes: Application to human mitochondrial DNA restriction data. *Genetics* 131: 479–491.

506 Feng, J., D. Jiang, H. Shang, M. Dong, G. Wang, X. He, C. Zhao, and K. Mao. 2013. Barcoding poplars (*Populus* L.)
507 from Western China. *PloS one* 8: e71710.

508 Figuerola, J., A.J. Green, and L. Santamaria. 2002. Comparative dispersal effectiveness of wigeongrass seeds by
509 waterfowl wintering in south-west Spain: quantitative and qualitative aspects. *Journal of Ecology* 90: 989–1001.

510 Gesti, J., A. Badosa, and X.D. Quintana. 2005. Reproductive potential in *Ruppia cirrhosa* (Petagna) Grande in response
511 to water permanence. *Aquatic Botany* 81: 191–198.

512 Hammerli, A., and T.B.H. Reusch. 2003. Inbreeding depression influences genet size distribution in a marine
513 angiosperm. *Molecular Ecology*: 619–629.

514 Den Hartog, C., and J. Kuo. 2006. Taxonomy and Biogeography of Seagrasses. In: *Seagrasses: Biology, Ecology and*
515 *Conservation*, ed. Springer, Dordrecht, 1–23. The Netherlands.

516 Hedrick, P.W. 2006. Genetic Polymorphism in Heterogeneous Environments: The Age of Genomics. *Annual Review of*
517 *Ecology, Evolution, and Systematics* 37: 67–93.

518 Hemminga, M.A., and C.M. Duarte. 2000. *Seagrass ecology*. Cambridge: Cambridge University Press.

519 Hughes, R.A., and J.J. Stachowicz. 2011. Seagrass genotypic diversity increases disturbance response via
520 complementarity and dominance. *Journal of Ecology* 99: 445–453.

521 Huiskes, A.H.L., B.P. Koutstaal, P.M.J. Herman, W.G. Beeftink, M.M. Markusse, and W.D.E. Munck. 1995. Seed
522 dispersal of halophytes in tidal salt marshes. *Journal of Ecology* 83: 559–567.

523 Huston, M. 1979. A general hypothesis of species diversity. *The American Naturalist* 113: 81–101.

524 Ito, Y., T. Ohi-Toma, J. Murata, and N. Tanaka. 2010. Hybridization and polyploidy of an aquatic plant, *Ruppia*
525 (*Ruppia*), inferred from plastid and nuclear DNA phylogenies. *American journal of botany* 97: 1156–1167.

526 Ito, Y., T. Ohi-Toma, J. Murata, and N. Tanaka. 2013. Comprehensive phylogenetic analyses of the *Ruppia maritima*
527 complex focusing on taxa from the Mediterranean. *Journal of plant research* 126: 753–762.

528 Jombart, T., and I. Ahmed. 2011. Adegnet 1.3-1: New tools for the analysis of genome-wide SNP data. *Bioinformatics*
529 27: 3070–3071.

530 Jombart, T., S. Devillard, and F. Balloux. 2010. Discriminant analysis of principal components: a new method for the
531 analysis of genetically structured populations. *BMC genetics* 11: 94.

532 Koch, E.W., and C.J. Dawes. 1991. Influence of salinity and temperature on the germination of *Ruppia maritima* L.
533 from the North Atlantic and Gulf of Mexico. *Aquatic Botany* 40: 387–391.

534 Koch, E.W., M.S. Ailstock, D.M. Booth, D.J. Shafer, and A.D. Magoun. 2010. The Role of currents and waves in the
535 dispersal of submersed angiosperm seeds and seedlings. *Restoration Ecology* 18: 584–595.

536 Kosman, E., and K.J. Leonard. 2007. Conceptual analysis of methods applied to assessment of diversity within and
537 distance between populations with asexual or mixed mode of reproduction. *New Phytologist* 174: 683–696.

538 Koutstaal, B.P., M.M. Markusse, and W. de Munck. 1987. Aspects of seed dispersal by tidal movements. In: Vegetation
539 between land and sea: Structure and processes, ed. Springer, Dordrecht, 226–235. The Netherlands.

540 Kress, W.J., and D.L. Erickson. 2007. A two locus Global DNA barcode for land plants: The coding *rbcL* gene
541 complements the non-coding *trnH-psbA* spacer region. *PLoS ONE* 2: e508.

542 Les, D.H., M.A. Cleland, and M. Waycott. 1997. Phylogenetic studies in Alismatidae, II: Evolution of Marine
543 Angiosperms (Seagrasses) and Hydrophily. *Systematic Botany* 22: 443–463.

544 Marchioni-Ortu, A. 1982. Numeri cromosomici per la Flora Italiana: 873–876. *Inf. Bot. Ital.* 14: 234–237.

545 Marco-Méndez, C., P. Prado, L.M. Ferrero-Vicente, C. Ibáñez, and J.L. Sánchez-Lizaso. 2014. Seasonal effects of
546 waterfowl grazing on submerged macrophytes: The role of flower. *Aquatic Botany* 120: 275–282.

547 Martínez-Garrido, J., E.A. Serrão, A.H. Engelen, C.J. Cox, P. García-Murillo, and M. González-Wangüemert. 2016.
548 Multilocus genetic analyses provide insight into speciation and hybridization in aquatic grasses, genus *Ruppia*.
549 *Biological Journal of the Linnean Society* 117: 177–191.

550 Martínez-Garrido, J., M. González-Wangüemert, and E.A. Serrão. 2014. New highly polymorphic microsatellite
551 markers for the aquatic angiosperm *Ruppia cirrhosa* reveal population diversity and differentiation. *Genome* 57: 57–59.

552 Meirmans, P.G., and P.H. Van Tienderen. 2004. GENOTYPE and GENODIVE: Two programs for the analysis of
553 genetic diversity of asexual organisms. *Molecular Ecology Notes* 4: 792–794.

554 Nikula, R., and R. Vaäinölä. 2003. Phylogeography of *Cerastoderma glaucum* (Bivalvia: Cardiidae) across Europe: a
555 major break in the Eastern Mediterranean. *Marine Biology*: 339–350.

556 Oliva, S., J. Romero, M. Pérez, P. Manent, O. Mascaró, E.A. Serrão, N. Coelho, and F. Alberto. 2014. Reproductive
557 strategies and isolation-by-demography in a marine clonal plant along an eutrophication gradient. *Molecular Ecology*
558 23: 5698–5711.

559 Olsen, J.L., W.T. Stam, J.A. Coyer, T.B.H. Reusch, M. Billingham, C. Boström, E. Calvert, H. Christie, S. Granger, R.
560 La Lumière, et al. 2004. North Atlantic phylogeography and large-scale population differentiation of the seagrass
561 *Zostera marina* L. *Molecular ecology* 13: 1923–1941.

562 Peakall, R., and P.E. Smouse. 2006. Genalex 6: genetic analysis in Excel. Population genetic software for teaching and
563 research. *Molecular Ecology Notes* 6: 288–295.

564 Pérez-Ruzafa, A., C. Marcos, I.A. Pérez-Ruzafa, and M. Pérez-Marcos. 2011. Coastal lagoons: ‘transitional
565 ecosystems’ between transitional and coastal waters. *Journal of Coastal Conservation* 15: 369–392.

566 Reusch, T.B.H. 2006. Does disturbance enhance genotypic diversity in clonal organisms? A field test in the marine
567 angiosperm *Zostera marina*. *Molecular ecology* 15: 277–86.

568 Rosenberg, N.A. 2004. DISTRUCT : a program for the graphical display of population structure. *Molecular Ecology*
569 *Notes* 4: 137–138.

570 Rouger, R., and A.S. Jump. 2014. A seascape genetic analysis reveals strong biogeographical structuring driven by
571 contrasting processes in the polyploid saltmarsh species *Puccinellia maritima* and *Triglochin maritima*. *Molecular*
572 *ecology* 23: 3158–3170.

573 Sampson, J.F., and M. Byrne. 2012. Genetic diversity and multiple origins of polyploid *Atriplex nummularia* Lindl.

574 (Chenopodiaceae). *Biological Journal of the Linnean Society* 105: 218–230.

575 Santamaría-Gallegos, N.A., J.L. Sánchez-Lizaso, and E.F. Félix-Pico. 2000. Phenology and growth cycle of annual
576 subtidal eelgrass in a subtropical locality. *Aquatic Botany* 66:329–339.

577 Segelbacher, G., S.A. Cushman, B.K. Epperson, M.J. Fortin, O. Francois, O.J. Hardy, R. Holderegger, P. Taberlet, L.P.
578 Waits, and S. Manel. 2010. Applications of landscape genetics in conservation biology: Concepts and challenges.
579 *Conservation Genetics* 11: 375–385.

580 Serra, I., A. Innocenti, G. Di Maida, S. Calvo, M. Migliaccio, E. Zambianchi, C. Pizzigalli, S. Arnaud-Haond, C.M.
581 Duarte, E.A. Serrao, and G. Procaccini. 2010 Genetic structure in the Mediterranean seagrass *Posidonia oceanica*:
582 disentangling past vicariance events from contemporary patterns of gene flow. *Molecular Ecology* 19: 557–568.

583 Short, F., T. Carruthers, W. Dennison, and M. Waycott. 2007. Global seagrass distribution and diversity: A bioregional
584 model. *Journal of Experimental Marine Biology and Ecology* 350: 3–20.

585 Spalding, M., M. Taylor, C. Ravilious, F. Short, and E. Green. 2003 Global overview: the distribution and status of
586 seagrasses. In: World Atlas of Seagrasses, ed. University of California, 5–26. Berkeley.

587 Talavera, S., and P. García-Murillo. 2010. *Ruppia* L. In: Castroviejo S., In: Aedo C., In: Laínz M., In: Muñoz
588 Garmendia F., In: Nieto Feliner G., In: Paiva J., In: Flora Ibérica, ed. Real Jardín Botánico, CSIC, 17: 88–92. Madrid.

589 Talavera, S., P. García-Murillo, and J. Herrera. 1993. Chromosome numbers and a new model for karyotype evolution
590 in *Ruppia* L. (Ruppiaceae). *Aquatic Botany* 45: 1–13.

591 Thomaz, S.M., and E.R. Cunha. 2010. The role of macrophytes in habitat structuring in aquatic ecosystems: methods of
592 measurement, causes and consequences on animal assemblages' composition and biodiversity. *Acta Limnologica*
593 *Brasiliensia* 22: 218–236.

594 Toonen, R.J., and S. Hughes. 2001. Increased throughput for fragment analysis on an ABI PRISM 377 automated
595 sequencer using a membrane comb and STRand software. *BioTechniques* 31: 1320–1324.

596 Triest, L., and T. Sierens. 2013. Is the genetic structure of Mediterranean *Ruppia* shaped by bird-mediated dispersal or
597 sea currents? *Aquatic Botany* 104: 176–184.

598 Triest, L., and T. Sierens. 2014. Seagrass Radiation after Messinian Salinity Crisis Reflected by Strong Genetic
599 Structuring and Out-of-Africa Scenario (Ruppiaceae). *PloS ONE* 9: e104264.

600 Triest, L., and T. Sierens. 2015. Strong bottlenecks, inbreeding and multiple hybridization of threatened European
601 *Ruppia maritima* populations. *Aquatic Botany* 125: 31–43.

602 Vallejo-Marin, M., and G.C. Lye. 2013. Hybridisation and genetic diversity in introduced *Mimulus* (Phrymaceae).
603 *Heredity* 110: 111–122.

604 Verhoeven, J.T.A. 1979. The ecology of *Ruppia* dominated communities in Western Europe. I. Distribution of *Ruppia*
605 representatives in relation to their autecology. *Aquatic Botany* 6: 197–268.

606 Viaroli, P., M. Bartoli, G. Giordani, M. Naldi, S. Orfanidis, and J.M. Zaldivar. 2008. Community shifts, alternative
607 stable states, biogeochemical controls and feedbacks in eutrophic coastal lagoons: a brief overview. *Aquatic*
608 *Conservation: Marine and Freshwater Ecosystems* 18: S105–S117.

609 Vromans, D.C., J.B. Adams, and T.V. Riddin. 2013. The phenology of *Ruppia cirrhosa* (Petagna) Grande and *Chara*
610 sp. in a small temporarily open/closed estuary, South Africa. *Aquatic Botany* 110: 1–5.

611 Weising, K., and R.C. Gardner. 1999. A set of conserved PCR primers for the analysis of simple sequence repeat
612 polymorphisms in chloroplast genomes of dicotyledonous angiosperms. *Genome / National Research Council Canada*
613 = *Génome / Conseil national de recherches Canada* 42: 9–19.

614 White, T.J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes
615 for phylogenetics. *PCR Protocols : a Guide to Methods and Applications*: 315–322.

Yu, S., M.Y. Cui, B. Liu, X.Y. Wang, and X.Y. Chen. 2009. Development and characterization of microsatellite loci in *Ruppia maritima* L. (Ruppiaceae). *Conservation Genetics Resources* 1: 241–243.

Zipperle, A.M, J.A. Coyer, K. Reise, W.T. Stam, and J.L. Olsen. 2010. Waterfowl grazing in autumn enhances spring seedling recruitment of intertidal *Zostera noltii*. *Aquatic Botany* 93: 202–205.

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

651

652

653

654

655

Figure 1

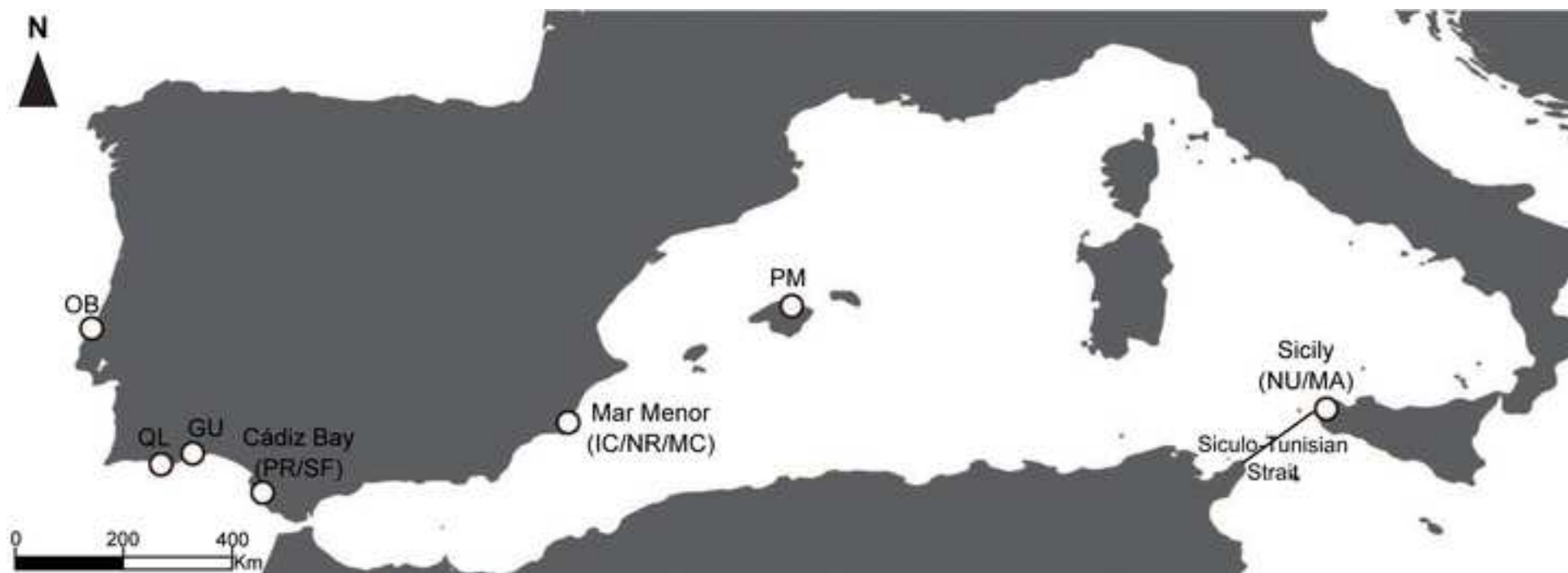


Figure 2

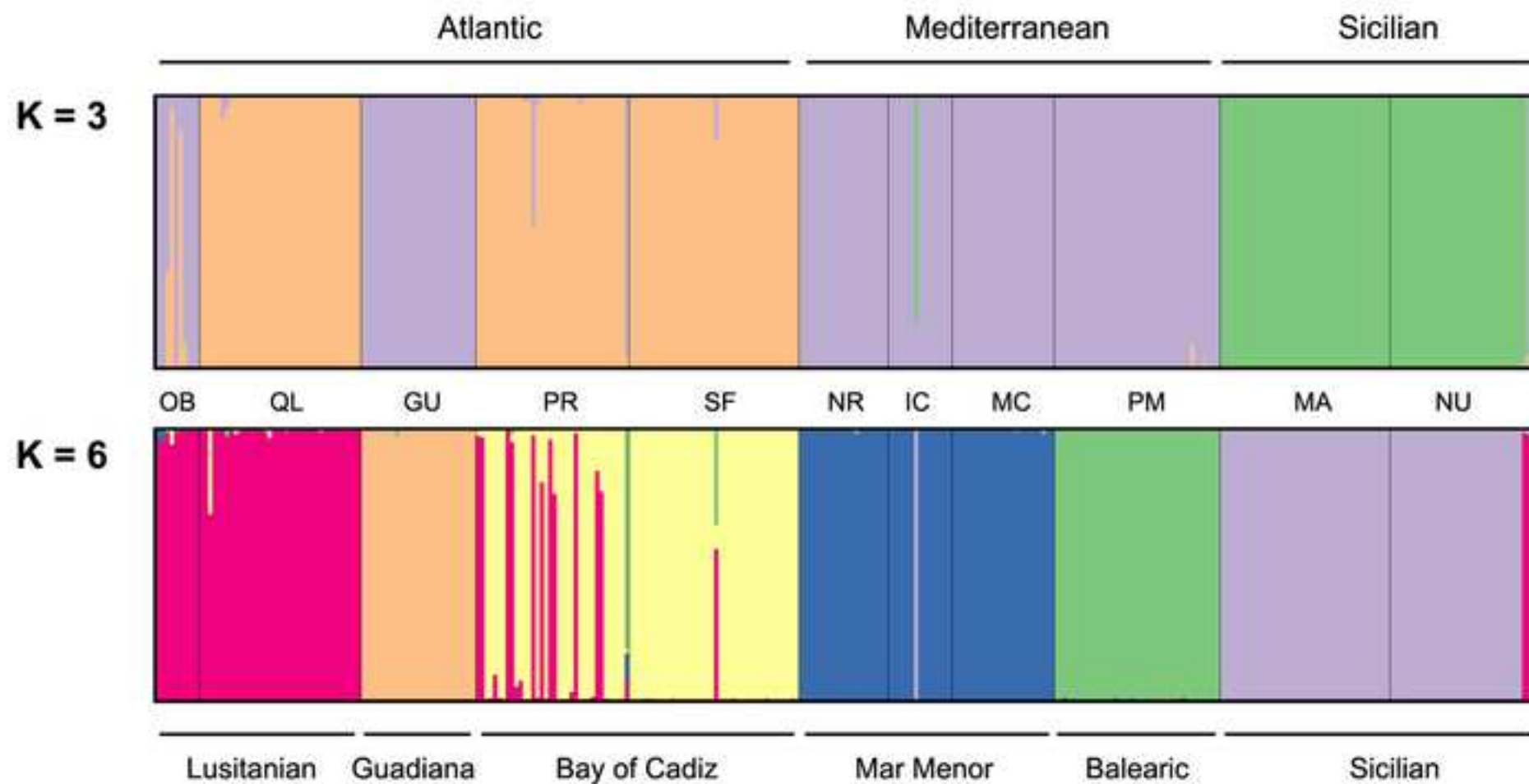


Figure 3

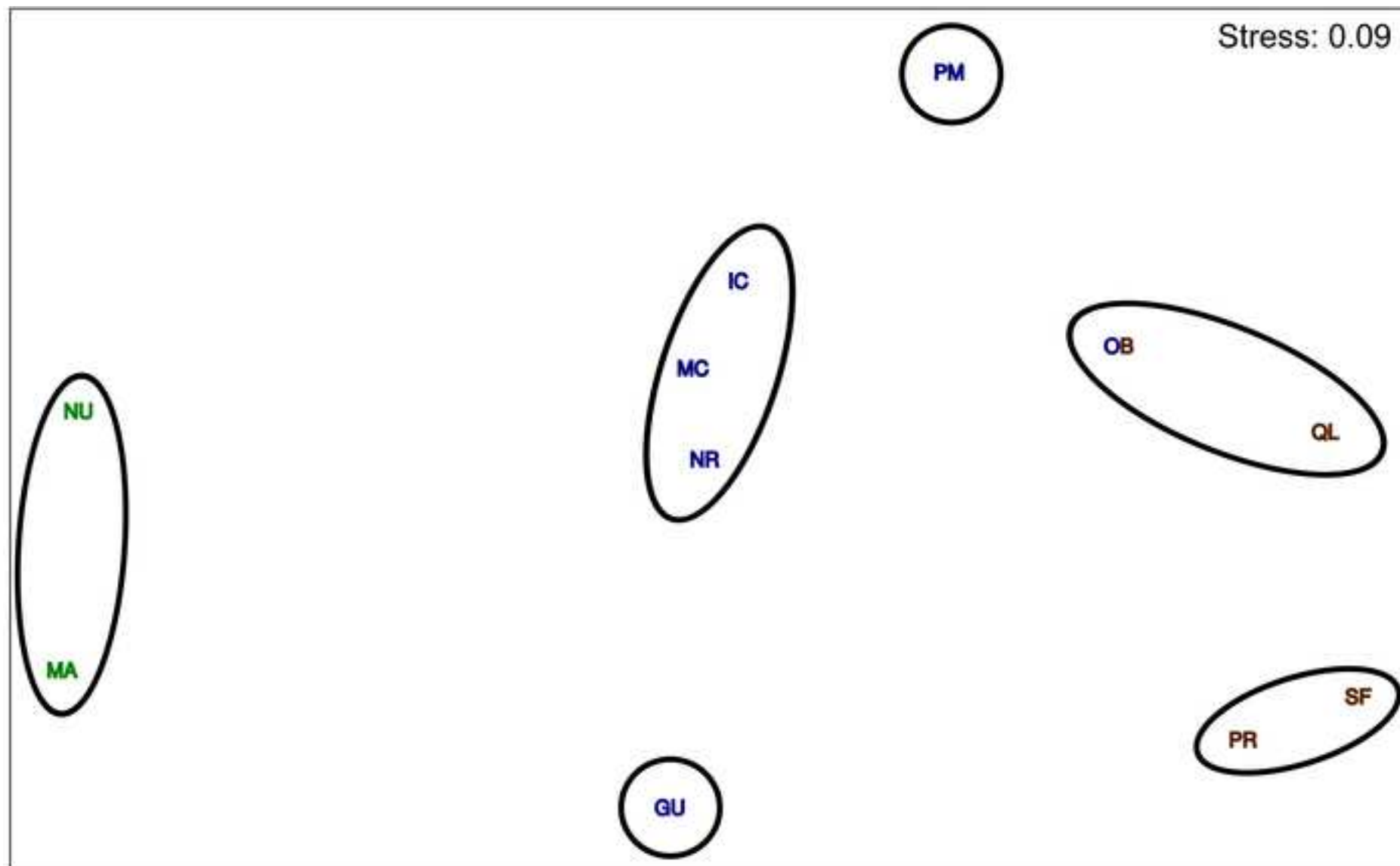


Table 1. Genotypic and genetic diversity parameters calculated for sampled populations of *Ruppia cirrhosa* and comparative parameters between the Mediterranean and Atlantic populations.

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 (\pm 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= unbiased genetic diversity calculated on the presence-absence matrix, allowing comparison with other studies and independent of the

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*.

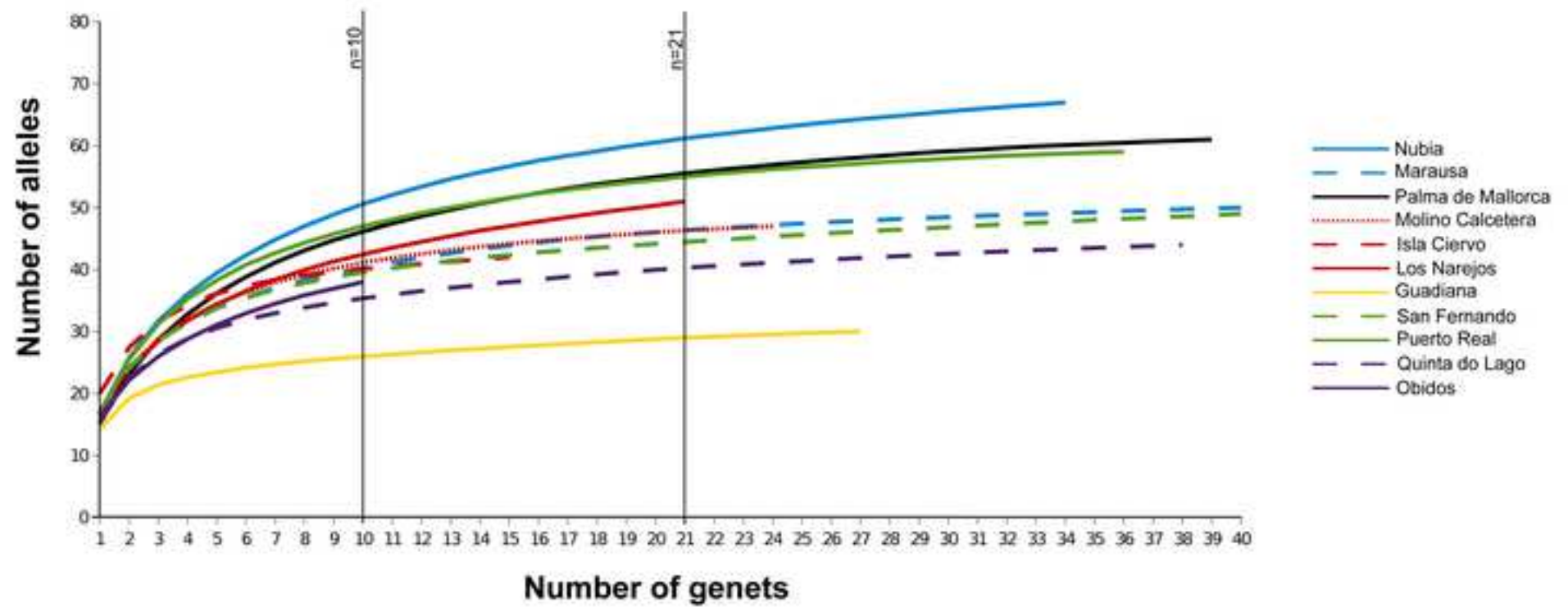
<i>K</i> -value	Source of variation	% Variation	Fixation index	<i>P</i> -value
3	Among clusters	14.43%	PhiRT= 0.144	≤0.001
	Among populations within clusters	21.37%	PhiPR= 0.249	≤0.001
	Within populations	64.20%	PhiPT= 0.358	≤0.001
6	Among clusters	20.50%	PhiRT= 0.205	≤0.001
	Among populations within 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 * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Allelic richness



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.