Reprint of “Is the genetic structure of Mediterranean Ruppia shaped by bird-mediated dispersal or sea currents?”

Ludwig Triest*, Tim Sierens


1. Introduction

The genus Ruppia has a cosmopolitan, but discontinuous distribution and is found on all continents, including many isolated islands from tropical to subarctic regions (Green and Short, 2003). Ruppia maritima L. is the most widely distributed species of this truly global seagrass genus (Short et al., 2007). In the Mediterranean region three taxa are recognised, namely R. maritima, Ruppia cirrhosa (Petagna) Grande and Ruppia drepanensis Tineo, the latter as an inland ecotype of the SW Mediterranean (also as variety R. cirrhosa (Petagna) Grande var. drepanensis (Tineo) Symoens). Morphological studies (Aedo and Fernandez-Casado, 1988; Cirujano and Garcia-Murillo, 1990), cytotaxonomical investigations (Cirujano, 1986; Talavera et al., 1993; Van Vierssen et al., 1981), isozyme polymorphisms (Triest and Symoens, 1991) and chloroplast DNA sequence analyses (Triest and Sierens, 2010) confirmed the Mediterranean Ruppia diversity.

Ruppia occurs in a wide variety of coastal lagoon and continental brackish to saltwater habitats. The morphological plasticity, adaptation to temporal or permanent habitats and especially traditions in national flora publications or regional wetland inventories still create much confusion in naming Ruppia species. As a consequence no reliable distribution range maps could be provided as was done for all other seagrass species (Short et al., 2007). The ecotypic and genotypic variation at population level partly remains not understood (Den Hartog and Kuo, 2006) although this type of information is essential for seagrass conservation genetics (Waycott et al., 2006). Therefore, a direct comparison of the putative diagnostic features between distinct cpDNA haplotypes using the same populations will give more clarity on the morphological variability, distinctiveness and distribution of Ruppia taxa.

Chloroplast sequences generally revealed very low variability in most aquatic plant and seagrass populations (Mader et al., 1998; Talbot et al., 2004; Triest et al., 2007; Koga et al., 2008; Provan et al., 2008; Tan et al., 2008) unlike the polymorphic Ruppia (Triest and Sierens, 2009, 2010; Ito et al., 2010). In a previous study of 53 water bodies across the European part of the Mediterranean, 15 haplotypes were revealed and showed a much higher nucleotide diversity of cpDNA in the Western than in the Eastern basin. This hotspot of diversity caused an overall gradient and isolation-by-distance (IBD) at basin level and was stronger between both basins.

A B S T R A C T

In the European part of the Mediterranean at least 15 cpDNA haplotypes of Ruppia can be distinguished and characterized the West basin as a diversity hotspot. Ruppia cirrhosa shows a West–East differentiation and clear isolation-by-distance between each basin. We investigated whether the maternal cpDNA differentiation between and within subbasins of the Mediterranean could shed light on distribution and dispersal phenomena of a morphological variable species complex. Complementary nuclear ITS markers showed three variants and allowed to detect hybrids with Ruppia maritima. Haplotypes differed significantly in leaf and fruit features for Ruppia drepanensis. Haplotypes A, D and E had numerous seeds whereas haplotypes B and C were mostly vegetative. The scattered distribution of rare haplotypes argued for occasional dispersal at long distances. However, birds as vectors of maternal cpDNA markers did not homogenize the genetic structure but it showed the presence of scattered isolated haplotypes reflecting a thin tail of long distance dispersal events. We observed a strong maternal isolation-by-distance between subbasins of the West basin and within the Balearic subbasin. It was found paradoxal that the most continuous widespread haplotype B also had lowest number of fruits. Sea currents are discussed as a potential dispersal vector at broad geographic scale for the most marine haplotype B variants of R. cirrhosa, hereby resembling other seagrasses.

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than within them (Triest and Sierens, 2010). A significant IBD was observed within the West basin but not in the East basin. The latter had too low variability and thus could not be considered in further IBT tests. Genetic patterns of Mediterranean marine plant and animal populations often display a West–East differentiation because the connection between both basins was narrower between Sicily and Tunisia during the periods of Pleistocene and Quaternary glacial maxima and because of the sea currents circulation patterns. A barrier to gene flow between the East and West Mediterranean was suggested for Posidonia oceanica (L) Delile (Arnaud-Haond et al., 2007; Serra et al., 2010) and explained as vicariance. The Ruppia cpDNA haplotypes of Eastern basin populations represent a very small subset of those from the Western basin, thereby suggesting a historical eastward dispersal of a single R. cirrhosa haplotype over long distances (Triest and Sierens, 2010).

There is ample evidence that birds are the main vector for dispersal of R. maritima seeds at short distances (Figuerola and Green, 2002; Figuerola et al., 2002; Charalambidou and Santamaria, 2005). Long-distance dispersal (LDD) is far more difficult to estimate as it is a rare event in a thin-tailed dispersal kernel (Nathan et al., 2008). The role of birds in LDD was argued (Figuerola and Green, 2002; Figuerola et al., 2002), critically reviewed (Claußen et al., 2002) and partially answered in favour of birds as effective vectors (Charalambidou and Santamaria, 2005; Rodríguez-Perez and Green, 2006; Brochet et al., 2010). Nevertheless it is not clear to what extent such dispersal events have influenced the species genetic structuring at broader geographic scales. R. maritima is a taxon with a single cpDNA haplotype whereas R. cirrhosa is more polymorph with unique allele variants (Triest and Sierens, 2010). Maternal genetic markers can be relevant at different geographic levels to infer dispersal patterns. However if chloroplast capture occurred after introgressive hybridization, then the information obtained solely from cpDNA might blur the interpretation of distribution ranges. Therefore nuclear markers are needed to reveal introgression events.

We investigated the distribution and morphological traits of Ruppia chloroplast haplotypes in the European part of the Mediterranean. The objective was to estimate whether maternal cpDNA differentiation between and within subspecies of the Mediterranean followed an isolation-by-distance model using flight distances or sea current distances. The distribution pattern of each cpDNA variant, their nuclear DNA identity, morphology, fitness traits and habitat type will be compared to discuss on the potential role of historical dispersal through either birds or sea currents. Additionally we will comment on ‘how marine’ the different Ruppia haplotypes are in the Mediterranean.

2. Materials and methods

2.1. Study sites and plant materials

Ruppia plants were collected in 2006, 2007, 2008 and 2009 in 56 water bodies from 38 wetland areas in the European part of the Mediterranean (Table 1, Fig. 1). In each site we collected up to 15 individual shoots (ramets) along a 30 m transect, thereby largely avoiding identical genets because visible clumps of a ramet were smaller than 1 m diameter. Leaves were dried on silica gel and a reference herbarium for each population was deposited at BRUV (herbarium of the Vrije Universiteit Brussel). A total of 1546 individual shoots was investigated for cpDNA sequence variability in five genes (ccmp2, ccmp3, ccmp10, trnH-psbA, rbcL) as published in Triest and Sierens (2010). Genbank Accession numbers are listed for ccmp2–jc (JN1013249–JN113255), ccmp3–jc (JN113257–J113259), ccmp10 (JN113260–JN113263), trnH-psbA (JN113266–JN113271) and rbcL (JN113275–JN113278). Here we add new information from two nuclear spacers (ITS1 and ITS2) of which Genbank Accession numbers are listed for ITS1 (JN113280–JN113282) and ITS2 (JN113283–JN113285), whereas AJ012292 and FJ495523 were previously listed for Ruppia.

2.2. DNA extraction, amplification and sequencing

Genomic DNA extractions and amplification for three cpSSR primer pairs (Ccmp 2, Ccmp 3 and Ccmp 10), a non-coding region (trnH-psbA) and a partially coding (rbcL) region were as in Triest and Sierens (2010).

PCR and direct amplicon sequencing of nuclear ITS1 and ITS2 spacers (White et al., 1999) was done with primers for the ITS1 spacer (ITS1: TCCGACTGCCAGCCGGG and ITS2: GCTGCTTCCTTCATCGGC) and the ITS2 spacer (ITS2: GCATCGATGAAAGCCGACG and ITS4: TCTCCTGCTATTGATATGC) using a PCR reaction of 25 μl containing 1× PCR buffer, 200 μM of each dNTP, 3 mM MgCl2, 200 nM of each primer, 0.5 μl of BSA (10 μg/μl) and 1 unit of Taq polymerase. Reaction: 95°C for 4 min followed by 35 cycles of 95°C for 1 min, 54°C for 1 min, 72°C for 2 min and a final extension step of 5 min.

2.3. Morphology and habitat

For each haplotype group (A, B, C, D, E) we measured following features on reference herbarium material (deposited at BRUV) from each population: leaf width, flower peduncle length, peduncle length, achene length, achene width, number of inflorescences per plant and number of fruits per plant. The number of measures for each haplotype ranged from 10 to 95 for leaf width; 26–62 for pedogyne and achene sizes; 12–48 for inflorescences and peduncle sizes and 4–8 for individual shoots. The habitat type was scored on a scale from 1 to 5 reflecting an increased marine influence: (1) inland waters, (2) coastal brackish, (3) coastal temporary saltmarsh, (4) coastal permanent lagoon, (5) coastal lagoon with Zostera and seaweeds.

2.4. Data treatment

DNA sequences were aligned with CLUSTAL W (Thompson et al., 1994). The 2300 bp long haplotypes were defined on basis of transitions, transversions, indels and mononucleotide repeats (Corrigendum: haplotype B5 as mentioned in Triest and Sierens, 2010 has to be replaced by haplotype E3). One haplotype (D) referred to R. maritima whereas 14 haplotypes (groups A, B, C, E) referred to an unresolved complex including R. drepanensis (A) and R. cirrhosa (B, C, E). A minimum spanning network using NETWORK 4.5.1.0 (Fluxus Engineering) served as basis for the haplotype definition.

Morphological measurements were tested for significant differences with one-way ANOVA (Kruskal–Wallis) and pairwise Mann–Whitney U test. Genetic differentiation between pairs of regions (ΦST) were calculated with ARLEQUIN (Excoffier et al., 2005) considering the pairwise differences between haplotypes. Slatkin’s ΦST/(1 – ΦST) was used for testing isolation-by-distance between pairs of regions with geographical distances obtained as the average distance between populations. Straight flight distances and distances following major sea currents (both log transformed) were used. The tests were done at the within basin level (East or West), between subspecies of each basin and within subspecies. We considered subspecies corresponding to relevant Mediterranean biogeographical subdivisions, taking into account the major current systems (http://www.ifremer.fr/lobtln/OTHER/Terminology.html and http://www.mediterranean-yachting.com/winds.htm) and currentology of the Mediterranean Sea (Blondel et al., 2010). These are the coastlines of the Alboran, Balearic (including Lyon Gulf), Tyrrhenian, Adriatic, Ionian and Aegean subbasins. The populations
Table 1
Chloroplast haplotypes and nuclear DNA identification of *Ruppia* in 38 Mediterranean wetlands (Abbreviations indicate SP: Spain; F: France; I: Italy, SLO: Slovenia and GR: Greece, the wetland number and ABC as neighbouring waterbodies; N = number of plants; | are *R. drepanensis*, *| are *R. maritima*, # are hybrids with cpDNA capture whereas all others belong to a *R. cirrhosa* complex).

<table>
<thead>
<tr>
<th>Basins and subbasins</th>
<th>38 Wetlands (56 sites)</th>
<th>N</th>
<th>Localities</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Chloroplast haplotype (Triest and Sierens, 2010)</th>
<th>Nuclear DNA marker (This study)</th>
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3. Results

3.1. Haplotype and ITS definition

Five groups of chloroplast haplotypes (named A, B, C, D, E) distinguished R. maritima (haplotype D1) from R. drepanensis (A1, A2), the latter not fully resolved from a diverse R. cirrhosa complex (haplotypes B1, B2, B3, B4, C1, C2, C3, C4, E1, E2, E3, E4). The haplotype groups A, B and C each differed only in a few substitutions, a microsatellite repeat or an indel whereas E was more divergent (Triest and Sierens, 2010). A total of 15 haplotypes from 53 water bodies formed the basis for further analysis of their morphology and distribution (Fig. 1). Three ITS1 amplicon (305–306 bp) and three ITS2 amplicon (443 bp) sequences were fully linked. A total of 38 substitutions and four deletions characterized those three linked ITS variants. ITS1 amplicon was somewhat more variable (19/306 variable sites) than ITS2 (19/443 variable sites) giving an overall identity of 97.1% and 98.6%, respectively.

These variants differed mainly in substitutions and were named ITS-A, ITS-B and ITS-C. The nuclear ITS-A variant was observed in the most divergent haplotypes D and group E whereas the nuclear variant ITS-B was shared by the most common haplotypes B and C (Table 1). In a few populations the nuclear ITS-A variant was observed in individuals with haplotype B1, thereby indicating hybridization. This suggests chloroplast capture of haplotype B1 (from egg cell of haplotype B) through hybridization with pollen of a taxon characterized by nuclear ITS-A sequences and was detected in three populations from Spain (Table 1, Fig. 1). These populations were from Valverde (Donana NP), Marjal dels Moros (Puçol) and Addaia (Menorca). The nuclear ITS-C variant coincided with haplotype group A. The sequence homology of nuclear ITS1 and ITS2 was lowest for comparisons between taxa with haplotypes D (or E) and other haplotypes (94–96%). The ITS sequence homology was largest for the comparison between taxa with haplotypes A and B along coastlines of islands of Menorca (Balearic), West Sardinia (Balearic), South East Sardinia (Tyrrenhenian) and Sicily (Tyrrenhenian) were considered according to their subbasin. Pairwise distances between each population were estimated from the coordinates using straight paths (Euclidean direct flight distances) and using paths constructed along with major sea currents (shortest possible routes). Distances were obtained from drawing paths in Google Earth. All statistical tests were obtained with STATISTICA software.
(or C), reaching 98.5–98.7%. Only one hybrid population (Valverde, Donana NP) showed intra-individual ITS variability.

3.2. Morphology

For each of the five haplotype groups (A, B, C, D, E) we compared leaf and fruiting inflorescence features (Table 2). Leaf width ranged from 0.1 to 1.1 mm and differed significantly between haplotypes A, D/E and B/C (ANOVA F_{1,269} = 92; p < 0.0000). Leaves were nearly capillary and about 0.1 mm wide for haplotype A, with 25–75 percentile values between 0.2–0.3 mm for D/E and 0.5–0.9 mm for B/C.

The inflorescences were clearly different between haplotype groups. In haplotype D the peduncle was less than 25 mm whereas in the other haplotypes it reached 30–120 mm in our samples. Haplotype D had straight or one-curved peduncles whereas B, C and E were spirally (sometimes only one curl), often reddish. Many-coiled and dense, often whitish, peduncles were found in haplotype A. The podogeine length was very variable, but smaller for haplotype B (F_{4,199} = 7.85; p = 0.0001).

The achenes were shorter for haplotype A (1.8–2.0 mm as 25–75 percentile values), D (1.9–2.1 mm) and E (2.0–2.2 mm) than for B/C (2.1–2.6 mm) with most groups significantly different, except D from E and B from C (F_{4,199} = 10.56; p < 0.0000). Achenes were narrowest for haplotype A (1.0–1.1 mm) than for all other groups (1.2–1.5 mm). The number of ripe fruits per inflorescence reached the largest numbers (6–8 on average) for haplotype A, whereas all other haplotypes had less fruits (on average 4–6), though with many outliers (F_{2,153} = 3.87; p = 0.005). The reproductive fitness (fruits/plant) clearly differentiated the many-seeded haplotype D (on average about 100) from haplotype A/E (about 30) and B/C (on average less than 5 and often zero) (F_{4,112} = 152; p < 0.0000).

Despite a large variability, all haplotypes were significantly different in reproductive fitness (Mann–U, all with p < 0.001) except between haplotypes A and E.

The habitat type scored on a scale from 1 to 5 (from no to a strong sea influence) was averaged for each haplotype (Table 2). This indicated that the habitat of haplotype B was more marine than haplotypes A (Mann–U; p = 0.003) and D (Mann–U; p = 0.003). Haplotype B (when in combination with ITS-B and not as a hybrid with ITS-A) thus should be considered as the most marine Ruppia genotype of the Mediterranean.

3.3. Distribution patterns, distances and IBD at subbasin level

The geographic distribution of similar haplotypes that refers to maternal dispersal events showed contrasting patterns among the variants in the Mediterranean (Fig. 1). The most continuous and widespread haplotype B1 occurred on all three peninsula and islands studied. The second common haplotype C1 was mainly confined to the Western Mediterranean (only one individual detected in samples from the Adriatic subbasin). All other Ruppia haplotypes showed a scattered distribution (A1, B2, C2, D1, E1, E3) or were – up to now – detected from a single area (A2, B3, B4, C3, C4, E1, E4, E5).

At the level of two Mediterranean basins a significant IBD was observed within the West basin but not in the East basin (Table 3). The latter had too low variability, indicating historical dispersal of only haplotype B1 (and B2 if no homoplas). The East basin therefore could not be considered in further IBD tests. In the West basin a strong IBD was found for pairwise distances between three subbasins (Alboran, Balearec and Tyrrhenian) when considering distances following major sea currents (Table 3, Fig. 2). This indicates restricted historical dispersal between those subbasins and was mainly explained by the scattered haplotypes E and local unique variants at short distances. Pairwise distances between subbasins measured according to sea currents gave significant IBD unlike straight flight distances (Fig. 2). At subbasin level an overall IBD was detected as well as within the Balearic subbasin only, indicating a restricted historical dispersal (Table 3, Fig. 3).

The slope of an IBD model was higher between basins (West versus East) than between subbasins (e.g., Alboran, Balearec, Tyrrhenian) and lowest for within subbasin comparisons. The IBD model also was stronger for distances that follow sea currents than for direct flight distances. We obtained slope values of 1.19 (flight) to 1.23 (flow) between basins, 0.47 (flight – but a non significant relationship) to 0.74 (flow) between subbasins (Western Mediterranean) and 0.04 (Eastern Mediterranean – but a non significant relationship). Lowest slope values were obtained for comparisons within subbasins of the Western Mediterranean (0.29 for flight and 0.24 for sea current). Overall the R^2 values were very low at large geographic level because identical haplotypes occurred at large distances. At a smaller geographic level, within the Balearic subbasin, the R^2 explained up to 32% of the IBD variation (Table 3).

The maximum geographic range between populations of similar cpDNA variants was used as a proxy for accumulated historical seed or shoot dispersal events. Haplotype B1 occurred at large ranges across subbasins within the East basin, from the Adriatic over the Ionian towards to Northern Aegean Sea encompassing about 2000 km (when measured along coastlines and sea currents) or 1180 km (straight flight distances across Balkan peninsula). In the West basin haplotype B1 ranged over more than 1900 km (sea currents) or 1500 km (straight flight) between Southern Iberian and Italian peninsula. The maximum observed geographic range between populations of similar haplotypes was high for A1 (>500 km inland), B2 (1450 km, but could be homoplas...
of a mononucleotide T-repeat), C2 (790 km, but could be homoplasy of a mononucleotide T-repeat), D1 (1150 km) and E3 (380 km between Italy and Sardinia), except for B3 (10 km), C3 (70 km), E1 (15 km).

4. Discussion

We revealed haplotype groups using both exons (rbcl) and introns (ccmp, trnH-psbH) of the chloroplast genome and combined these with nuclear spacer (IT1S1 and 2) information and morphological measurements. Haplotype group A with nuclear ITS-C corresponded to a morphologically distinct *R. drepanensis* characterized by capillary leaves and long, many-coiled peduncles. The unique and most distinct haplotype D combined with ITS-A corresponded to a many-seeded *R. maritima* with short peduncles. Haplotype groups B and C that shared ITS-B were morphologically distinct in having wider leaves, longer achenes and spirally peduncles. This polymorphic group was assigned to a *R. cirrhosa* complex. Haplotype group E was more related to B and C than to any other haplotype group and was considered as part of the haplotypic *R. cirrhosa* complex as a result of chloroplast capture after hybridization. The latter group contained the nuclear ITS-A and approached morphological features of *R. maritima*, except for their lower number of fruits per plant. The latter complex has caused much of the taxonomic uncertainty in Mediterranean *Ruppia*.

Several chloroplast exons and a nuclear coding gene of *Ruppia* taxa at a global scale revealed hybridization and polyploidy as important phylogenetic events (Ito et al., 2010). The Asian and Oceanic taxa *R. megacarpa* S. Mason, *R. tuberosa* Davis and Tomlinson and *R. polycarpa* S. Mason could be clearly resolved. However, Ito et al. (2010) could not clearly distinguish *R. maritima* from *R. cirrhosa* (*R. drepanensis* was not studied) and proposed a world-wide *R. maritima* complex without considering the evidence of a distinct *R. maritima* taxon and a polymorphic *R. cirrhosa* complex in Europe (Triest and Sierens, 2009, 2010). Analysis of methodological aspects reveal that Ito et al. (2010) used a small part of the rbcl (542 bp) instead of the full sequence (>1300 bp). They designed primers for a shorter rbcl on basis of a *R. maritima* sequence and hence caused a bias in resulting relationships and further interpretation as a *R. maritima* complex. Likewise, shortened sequences of *Matk* were used. Obviously, the parsimony information for *R. cirrhosa* was underestimated in their study and unfortunately more confusion was added to the already confusing taxonomy of *Ruppia*.

### 4.1. Dispersal by birds

Birds are considered as the most likely vector of LDD through seeds. Waterfowl carry seeds on their feet, feathers and in the gut. Seeds might germinate when birds deposit them in suitable habitats (Fishman and Orth, 1996). The role of birds in LDD was argued on basis of feeding experiments, simulation of flight distances versus retention time of seeds in the gut and germination capacity of *R. maritima* seeds (Figueroa and Green, 2002; Figueroa et al., 2002). The role of birds in dispersal is not at all questioned, but it is more difficult to obtain experimental evidence for their role in LDD or in homogenizing a genetic structure. Several putative steps going from fruiting period until germination and establishment in a distant habitat were critically reviewed (Clausen et al., 2002) but partially answered in favour of birds as effective vectors even if the season of seed production is decoupled from bird migration periods (Charalambidou and Santamaria, 2005; Rodriguez-Perez and Green, 2006; Brochet et al., 2010). To be effectively dispersed though birds, the various *Ruppia* haplotypes should be highly fertile. We observed that haplotype D (*R. maritima*, including the introgressed hybrids with nuclear ITS-A and capture of haplotype B) was many-seeded with on average 3 times more fruits per plant than haplotypes A and E and even 10–30 times more than haplotypes B and C, respectively. The probability of bird-mediated seed dispersal of B/C populations thus will be reduced if these plants flower and fruit less freely. Therefore we hypothesize that the many-seeded haplotypes A, D, and E have a higher probability for dispersal by birds than B and C. This did not result in a lowered genetic structuring throughout the Mediterranean distribution range because haplotype groups A, D and E show a scattered distribution and were mainly observed in the Mediterranean populations.

### Table 2

Measurements of morphological characters for five cpDNA haplotype groups (comprising three nuclear ITS-variants) in 56 Mediterranean *Ruppia* populations (mean, minimum and maximum values are given). Significant differences (Mann–Whitney-U, *p* < 0.05) are indicated as a,b,c,d.

<table>
<thead>
<tr>
<th>Diagnostic feature</th>
<th>Sample size of haplotype group (A/B/C/D/E)</th>
<th>Haplotype A</th>
<th>Haplotype B</th>
<th>Haplotype C</th>
<th>Haplotype D</th>
<th>Haplotype E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf width (mm)</td>
<td>10/55/63/31/10</td>
<td>0.4–1.0</td>
<td>0.71</td>
<td>0.29</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Podogyne length (mm)</td>
<td>26/40/62/48/28</td>
<td>1.0–1.5</td>
<td>1.0–1.5</td>
<td>1.0–1.5</td>
<td>1.0–1.5</td>
<td></td>
</tr>
<tr>
<td>Achene width (mm)</td>
<td>26/40/62/48/28</td>
<td>0.4–1.0</td>
<td>0.71</td>
<td>0.29</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Number of fruits/inflorescence</td>
<td>17/39/48/42/12</td>
<td>0.2–1.0</td>
<td>0.71</td>
<td>0.29</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Number of fruits/seed</td>
<td>4/58/44/12/9</td>
<td>0.2–1.0</td>
<td>0.71</td>
<td>0.29</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Peduncle</td>
<td>17/39/48/42/12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sea influence (scale 1–5)</td>
<td>(3/41/20/6/6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 3

Overview of significant isolation by distance models (IBD) at different geographic levels (basin and subbasin) using genetic differentiation *Φ*_{ST}(*1 – *Φ*_{ST}) against straight flight (left) and sea current (right) distances (both log km). N = number of population pairs; *r* = Pearson correlation, *p* = significance level, *R*^2^ = coefficient of determination.

<table>
<thead>
<tr>
<th>Geographic level</th>
<th>N</th>
<th>IBD (Flight distances)</th>
<th><em>r</em></th>
<th><em>p</em></th>
<th><em>R</em>^2^</th>
<th>IBD (Sea current distances)</th>
<th><em>r</em></th>
<th><em>p</em></th>
<th><em>R</em>^2^</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between basins</td>
<td>210</td>
<td><em>y</em> = −2.88 + 1.19<em>x</em></td>
<td>0.37</td>
<td>0.0001</td>
<td>0.14</td>
<td><em>y</em> = −3.40 + 1.23<em>x</em></td>
<td>0.25</td>
<td>0.0002</td>
<td>0.06</td>
</tr>
<tr>
<td>Within basins</td>
<td>255</td>
<td><em>y</em> = −0.24 + 0.37<em>x</em></td>
<td>0.23</td>
<td>0.0003</td>
<td>0.06</td>
<td><em>y</em> = −0.49 + 0.44<em>x</em></td>
<td>0.29</td>
<td>0.0001</td>
<td>0.08</td>
</tr>
<tr>
<td>Within west basin</td>
<td>210</td>
<td><em>y</em> = −0.46 + 0.50<em>x</em></td>
<td>0.27</td>
<td>0.0001</td>
<td>0.07</td>
<td><em>y</em> = −0.67 + 0.54<em>x</em></td>
<td>0.32</td>
<td>0.0001</td>
<td>0.10</td>
</tr>
<tr>
<td>Between west subbasins</td>
<td>144</td>
<td><em>y</em> = −1.24 + 0.74<em>x</em></td>
<td>0.26</td>
<td>0.0020</td>
<td>0.07</td>
<td><em>y</em> = −0.02 + 0.24<em>x</em></td>
<td>0.29</td>
<td>0.0169</td>
<td>0.09</td>
</tr>
<tr>
<td>Within West subbasins</td>
<td>66</td>
<td><em>y</em> = −0.10 + 0.29<em>x</em></td>
<td>0.34</td>
<td>0.0053</td>
<td>0.12</td>
<td><em>y</em> = −0.55 + 0.48<em>x</em></td>
<td>0.57</td>
<td>0.0003</td>
<td>0.32</td>
</tr>
</tbody>
</table>
Western part whereas haplotypes B1 and C1 were most common throughout the European part of the Mediterranean.

Dispersal vectors with low displacement over long distances and short seed passage time are expected to contribute hardly to LDD, even when the seed load is very high (Nathan et al., 2008). We found no evidence for an extensive bird-mediated dispersal lowering the IBD within the West basin because there was a significant genetic structure between the subbasins, the latter when considering sea currents, but not when considering flight distances among the subbasins. This can be explained partly from the identical haplotypes at longer distances but also from the occasionally large differentiation at short distances. At short distances (about <20 km) waterfowl was shown to have an effect on R. maritima pond populations and their seed bank densities throughout the year in SW Spain and not only at times of major concentrations of migratory fowl in autumn (Rodriguez-Perez and Green, 2006). Winter observations on teal in the Camargue showed that dispersal of propagules indeed can be decoupled from the time of seed production of aquatic plants (Brochet et al., 2010). Over larger distances, flamingos were proposed as an important factor in wetland migrations across the Mediterranean (Rodriguez-Perez and Green, 2006).

Greater Flamingos are adapted to shallow wetlands and individuals can travel long distances (Amat et al., 2005). Flamingos were reported to connect Western Mediterranean wetlands with East basin wetlands in Turkey (Balkiz et al., 2007). From our observations during the field work on Ruppia and from bird lists of protected areas, Ruppia is often found in the same areas from which flamingo populations were recorded.

In the Mediterranean, Ruppia fruits are produced at different times of the year according to the taxon and the habitat type being ephemeral or permanent (Malet et al., 2004). In large lagoons, R. cirrhosa is flowering and fruiting from June to July, whereas in ephemeral sites this can be a month earlier (Gesti et al., 2005). R. maritima set fruits in May–June, whereas R. drepananthes even might fruit earlier, depending on the ephemeral habitat (personal observations). Haplotypes A (R. drepananthes), D (R. maritima) and E (part of R. cirrhosa complex) produce a large number of seeds and such a high seed rain most likely results in a rich seed bank throughout the year or even years. These haplotypes are mostly known from isolated, shallow, sometimes ephemeral habitats depending on freshwater for germination. R. maritima seeds germinated best at low chlorinities in strong contrast to much lower germination rates of R. cirrhosa (Van Vierssen et al., 1984). R. drepananthes and R. maritima both occur in inland saline lakes in Spain whereas R. maritima also established far inland in few isolated saline habitats of Western and central Europe. Therefore, it is evident that haplotypes A (R. drepananthes), D (R. maritima) and most likely also haplotype E (part of R. cirrhosa complex) are dispersed by birds. Together with the scattered distribution pattern of these haplotypes, it makes sense to state that LDD by birds are rare events and therefore not at all lowering the genetic differentiation between population pairs at various distances.

There are only few reports on the putative homogenizing effect of bird-mediated dispersal over large geographic distances of submerged waterplants. These studies were conducted with dominant markers and are not entirely convincing that populations connected through bird migration routes indeed had an overall lowered differentiation. Using RAPD markers, Mader et al. (1998) showed that the slope of geographic against genetic distance was lower for Potamogeton pectinatus L. populations that were frequently visited by swans in comparison to those that were not visited by swans. However, in our opinion this interpretation should be revised because the lower slope for bird-visited population differentiation was caused by higher pair-wise genetic differentiation at small geographic distance and not, as should be in case of homogenization over longer distances, due to lowered genetic differentiation when compared to non-visited populations. In a related RAPD study, King et al. (2002) compared two coastlines in the Baltic Sea for genetic differentiation in P. pectinatus and concluded that the SW Baltic coastline populations known for high waterfowl concentration, showed a reduced isolation. On basis of cumulative differentiation values it was suggested that waterfowl can increase the neighbourhoods up to about 150–200 km. Their interpretation is based on a priori grouping of populations considered to have the greatest bird traffic coming and going to arctic Russia, and those that are less visited. A non significant trend towards lower differentiation was explained by bird-traffic regions. In our opinion, migrating birds are a potential explanation, however, a physical barrier such as the highly fragmented archipelago region could be an alternative explanation for the stronger IBD of the Northern most populations.

Ito et al. (2010) detected similar sequences in exons of one nuclear and four chloroplast genes for R. megarapica from North Eastern Asia and Southern Oceania. This disjunct distribution was explained as a bird-mediated long distance dispersal. Given the abovementioned constraints of using partial, shortened sequences of conservative genes, these materials need further analysis using full rbcL sequences and less conservative genes before inferring long distance dispersal events.

Albeit, it will be very difficult to detect traces of bird-mediated dispersal events in large and continuous populations with genetic methods. Distribution patterns of unique alleles might be more informative than overall gene diversity and differentiation measures. It is a paradox that for Ruppia the least fertile haplotypes B and C showed the largest and most continuous distribution whereas in comparison the most fertile haplotypes A, D and E showed a scattered distribution. The most marine chloroplast genotype can, after cpDNA capture through hybridization with R. maritima, be hitchhiked to other, even inland habitats. The three cases of introgression we found showed that pollen of R. maritima fertilized the R. cirrhosa ovules. This also fits with the observation that R. maritima is more fertile and thus has a higher probability to cross-pollinate with other taxa.

4.2. Dispersal through sea currents

R. cirrhosa can occur in saltmarshes, salinas, estuaries and coastal lakes, nowadays mostly protected from direct sea influence by dunes and dikes. R. cirrhosa populations can co-occur with other seagrasses such as Zostera. We observed R. cirrhosa together with Zostera in several lagoon areas of Italy (Oristano, Circeo, Orbetello, Grado) and Greece (Amvrakikos gulf and Messolonghi lagoons). Their establishment might have originated from drifting propagules transported by sea currents. Vegetative propagation of seagrasses can only allow the plants to colonize adjacent non vegetated area or get mixed but not far away. Seed production on flowering and drifting shoots might increase the dispersal distance. The seeds of seagrasses generally are too heavy to travel a long distance by water currents alone (Orth et al., 1994) although fruits of P. oceanica float and are potentially able to disperse for several weeks under the influence of surface currents (Serra et al., 2010). Dispersal distances of detached seeds of Zostera marina L. appear to be very limited (Orth et al., 1994; Ruckelshaus, 1996). Orth et al. (1994) frequently observed floating reproductive shoots at the sea surface. They suggested that long-distance dispersal and colonization of distant habitats may be achieved via these floating plants. Fully ripened Ruppia fruits detach and sink readily to the bottom. However, larger shoot fragments with rhizomes and flowering/fruiting parts regularly detach and survive when floating (Personal observation in Oristano, Stagno Ista, Porto Corallo lagoon and Circeo in June 2008). The most probable means of seagrass colonizing nearby shores is through transportation of seeds.
on floating flowering or fruiting shoots. These shoots may have become detached through stochastic disturbances such as stormy weather or the feeding activities of waterfowl burrowing in the sediment. An important question is whether Ruppia shoots can root again after a period of floating.

Rafing of seeds on floating reproductive shoots is an important dispersal strategy for eelgrass, allowing for colonization of new areas (Källström et al., 2008). Eelgrass disperses by floating on ocean currents sometimes for great distances and with the help of birds, but with low seed survival (Phillips and Mehez, 1988). Buoyancy for at least 26 days was observed for Z. marina. Wind-driven surface-transport velocity of detached post-flowering inflorescences gave a dispersal potential of up to 150 km in one season along the Western Swedish coast (Källström et al., 2008). This estimate of about 150 km corresponds somehow with estimates (200 km) of dispersal potential from genetic studies (Reusch et al., 1999; King et al., 2002). P. oceanica showed moderate gene flow between populations of the Tyrrhenian subbasin (Procaccini et al., 2001). Floatation of buoyant diaspores (Enhalus, Thalassia, Posidonia) and detached shoots with reproductive material can have dispersal distances of 100 m to 10 km (Harwell and Orth, 2002; Lacap et al., 2002). Z. marina reproductive fragments were estimated to have a floatation potential according to the speed of water flow of about 23 km/h but strong winds even may enhance (Harwell and Orth, 2002). Other estimates for Thalassia testudinum K.D. Koegi were up to 15 km (Kaldy and Dunton, 1999) or even up to 350 km using floating potential and 360 km using microsatellite based IBD (Van Dijk et al., 2008), 63.5 km for Enhalus acoroides (L.F.) Ryol, up to 73.5 km for Thalassia hemprichii (Ehrenb. ex Solms) Asch. (Lacap et al., 2002) and over 300–400 km during stormy weather (Kendall et al., 2004). Rafting provides a typical dispersal range within tens of kilometres (Thiel and Gutow, 2005). Genetic structure of local seagrass populations suggests that gene flow is limited and depends largely on vegetative dispersal (Procaccini and Mazzella, 1998; Reusch et al., 1999). Seed currents also were reported as dispersal vector for coastal land plants such as Beta vulgaris L. subsp. maritima (L.) Arcang. (Fievet et al., 2006) and Cakile maritima Scop. (Gandour et al., 2008). Thus even coastal terrestrial plant parts can be dispersed through sea currents.

Ocean currents are relatively slow but their vector load in principle is without limit over ecological timescales and especially so over longer timescales (Nathan et al., 2008). The Mediterranean Sea is a unique system and cannot be considered as a miniature ocean because of its microtidal system and heterogeneity in salinity, currents and temperatures (Blondel et al., 2010). If sea currents represent an effective vector for accumulated dispersal of particular taxa, then their populations should be located in the sea or at least in lagoons with an actual or historical connection to the sea. Such genotypic variants are also expected to co-occur in habitats with seagrasses or seaweeds and eventually possess adaptive traits for dispersal by water. We observed that in the habitats with most marine influences, only haplotype B or C occurred whereas in the more sheltered, shallow lakes, ponds and ditches all haplotypes could be observed. The paradox about why the least fertile haplotypes being the most widely distributed across the Mediterranean should be investigated with both genetic methods using microsatellites and ecological studies on the floating potential of R. cirrhosa rhizomes and shoots. A additional question raised here is whether lagoon R. cirrhosa are adapted to vegetative survival and dispersal on long term by water currents, hereby resembling other seagrasses or if the marine habitat merely is a secondary habitat and too harsh for allocating in fruits.

On the basis of our observations, we assume that haplotype B1 is the most continuous and widespread distributed maternal genotype of the Mediterranean Ruppia. The variants B3 and B4 were local variants whereas B2 occurred in several subbasins. The latter however contained a single mononucleotide repeat that could be the result of independent events. We summarize that IBD gave better fit when using sea current distances than direct flights. This IBD relationship was low within basins because genetic differentiation can occur at short distances and identical haplotypes might exist at large distances. Birds are considered as important vectors of dispersal at short distances and occasional LDD events of the most fertile Ruppia populations of haplotypes A (R. drepnensis), D (R. maritima) and E (part of R. cirrhosa complex). Our results highlight a paradox, namely a large Mediterranean distribution of the least fertile haplotypes and therefore we propose historical sea currents as a potential vector for accumulated dispersal of the most marine variants of R. cirrhosa. This might explain an overall continuous distribution and a lower diversity in the East Basin. The alternative explanation that waterfowl would be the only responsible vector for LDD from West to East basin is less likely. Nevertheless, good candidates of additional vectors along coastlines are flamingos that connect wetlands over large distances. Such hypothesis should be tested in more detail by studying R. cirrhosa populations in flamingo dominated habitats.

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