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Chloroplast sequences reveal a diversity gradient in the Mediterranean *Ruppia cirrhosa* species complex

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ABSTRACT

Two subcosmopolitan species *Ruppia maritima* and *Ruppia cirrhosa* are recognized throughout Europe, whereas *Ruppia drepanensis* is endemic to SW Europe. We aimed at characterizing the geographic structure of the chloroplast DNA haplotype diversity of 56 *Ruppia* populations across the European part of the Mediterranean. On the basis of five cpDNA markers (total length of 2300 bp) 16 haplotypes were obtained for 1546 individuals. Three populations of *R. maritima* showed a single haplotype and differed in five insertions/deletions and 16 substitutions from a highly variable *R. cirrhosa* species complex, which included *R. drepanensis*. The haplotype diversity of this species complex was much higher in the western Mediterranean basin than in the eastern basin. Analysis of molecular variance (AMOVA) showed significant differentiation of *R. cirrhosa* between the two basins and also within the western Mediterranean thereby suggesting the latter as an important centre of *Ruppia* diversity. An isolation-by-distance (IBD) pattern was stronger between the West-East basin populations than within basins. A PCO analysis of the western basin populations indicated a diversity gradient with those of Sardinia as polymorph intermediates. The low diversity within the eastern basin suggests that the observed gradient could be hypothesized as a historical dispersal of only a limited number of haplotypes from west to east.

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, northward to the White Sea and Iceland (Green and Short, 2003). Ruppiaceae are considered to be phylogenetically associated closely to seagrass families such as Posidoniaceae and Cymodoceaceae (Les et al., 1997; Waycott et al., 2006). Generally, four species are recognized with *Ruppia cirrhosa* (Petagna) Grande and *Ruppia maritima* L. as widespread, nearly cosmopolitan taxa and *Ruppia megacarpa* Mason and *Ruppia tuberosa* Davis & Tomlinson limited to Australia (Brock, 1982). Globally, seagrass diversity is the highest in tropical Asia and western Australia and among the lowest in Europe and the Mediterranean. Several studies confirmed additional diversity of *Ruppia* in the Mediterranean, notably *Ruppia drepanensis* Tineo (or the variety *R. cirrhosa* (Petagna) Grande var. *drepanensis* (Tineo) Symoens) in the SW Mediterranean. 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), autoecological and isozyme polymorphism studies (Triest and Symoens, 1991) attempted to document on Mediterranean *Ruppia* diversity. *Ruppia* populations of the eastern Mediterranean basin are shown to have annual or perennial growth cycles (Malea et al., 2004). Rhizomes can colonize rapidly whereas seeds persist in dry and hypersaline conditions (Verhoeven, 1979). Although much work on the ecology, biomass, productivity and ecophysiology of *Ruppia* was achieved, the ecotypic and genotypic variation at population level remains partly understood (Triest and Symoens, 1991; Green and Short, 2003; Den Hartog and Kuo, 2006).

There are several reports on restricted gene flow of both plant and animal populations among the historical Mediterranean refugia since the last glaciation. Genetic patterns often display a west-east cleavage with the Siculo-Tunisian Strait, which was narrower between Sicily and Tunisia during the last glaciations. Arnaud-Haond et al. (2007) suggested a barrier to gene flow between the east and west Mediterranean for the seagrass *Posidonia oceanica* (L.) Delile. In *Zannichellia* taxa (Triest et al., 2007) and *Potamogeton pectinatus* L. (*Stuckenia pectinata* (L.) Börner) populations (Mader et al., 1998) cpDNA revealed few but unique haplotypes in the SW Mediterranean, indicating a potential for differentiation in this part of the European continent, most likely as an African flora element. In water plants, chloroplast sequences (cpDNA) are mostly used to infer phylogenetic relationships (e.g. Zhang et al.,

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Table 1
Collection localities of 1546 *Ruppia* individuals in 38 Mediterranean wetlands (Abbreviations: SP: Spain; F: France; I: Italy; SLO: Slovenia; GR: Greece; the wetland number and ABC as neighboring waterbodies; *N*: number of plants; * refer to *R. maritima*, all others belong to a *R. cirrhosa* complex).

10 regions	35 wetlands (56 populations)	*N*	Localities	Longitude	Latitude
1. SW-Inland Spain					
La Mancha	SP1	30	Laguna de Manjavacas	39°24.69	−2°51.68
Donana NP	SP2ABC	92	Valverde	37°04.28	−6°16.33
2. Alboran subbasin					
Gulf Almeria	SP3	30	Almerimar, Lago Victoria	36°42.57	−2°49.61
	SP4AB	58	Roquetas de Mar	36°42.78	−2°38.76
Gulf Alicante	SP5ABC	90	Santa Pola, Torre de Tamarit	38°11.07	−0°36.81
3. Balearic subbasin					
Gulf Valencia	SP6	30	Albufera of Valencia NP, Lago de El Saler	39°20.77	−0°18.94
	SP7	30	Marjal dels Moros, Puçol	39°36.98	−0°15.50
	SP8*	15	Prat de Cabanes, Torreblanca NP	40°11.11	0°12.58
Ebro delta	SP9	25	Illa de Buda	40°38.24	0°44.78
Costa Brava	SP10AB	60	L'Estartit, els Griells	42°01.89	3°11.57
	SP11	30	Empuriabrava, Aiguamolls del Ampurdà	42°15.56	3°08.77
Menorca	SP12AB	60	Es Grau, saltmarsh and Albufera	39°56.47	4°15.58
	SP13	29	Addaia, Salinas Mongofre	39°59.09	4°12.25
	SP14*	30	Son Bou	39°54.03	4°04.04
Lion Gulf	F15	23	Between Carnon and La Grande Motte	43°33.15	3°59.96
Rhône delta	F16	30	Camargue, Trou de l'oie	43°21.57	4°48.48
	F17	29	Camargue, Le Capouillet	43°21.57	4°48.48
	F18*	31	Camargue, La Pallisade	43°22.75	4°48.28
4. Sardinia					
W-Sardinia	I19	28	Oristano, Santa Giusta	39°52.07	8°36.29
	I20	27	Oristano, Stagno Istai	39°58.09	8°27.39
SE-Sardinia	I21	30	Cagliari, western lagoons in salinas	39°10.53	9°01.25
	I22	30	Chia, Monte Cogoni	38°53.43	8°52.37
	I23	30	Su Giudeu	38°53.22	8°52.03
	I24	30	Porto Corallo lagoon	39°26.06	9°37.03
5. Sicily					
W-Sicily	I25ABC	30	Trapani Saline	37°51.58	12°29.07
6. Tyrrhenian subbasin					
Northern coast	I26AB	60	Castiglione della Pescaia, Badiola	42°46.43	10°56.15
	I27	30	Orbetello lagoon	42°25.43	11°14.50
Southern coast	I28AB	60	Circeo, Borgo Grappa and Foglianu	41°23.05	12°55.32
8. Adriatic subbasin					
Gulf Trieste	I29	30	Grado, Valle Cavanata	45°42.50	13°28.36
	I30	26	Valle di Comacchia	44°34.46	12°14.31
	SLO31AB	46	Portoroz, Secovlje Salina	45°29.28	13°36.26
	SLO32	30	Piran, Strunjan Salina	45°31.40	13°36.22
9. Ionian subbasin					
W-Greece	GR33ABCD	120	Arta, Logarou, Koronissiu & Tsoukalio	39°00.55	20°55.32
	GR34AB	60	Messolonghi lagoon	38°19.57	21°25.52
Peleponesos	GR35AB	60	Ilia, Lake Kotychi	38°09.28	21°23.15
	GR36AB	60	Achaia, Lake Prokopos	38°00.47	21°17.55
	GR37	1	Aigio, Aliki lagoon	38°15.54	22°06.31
10. Aegean subbasin					
Evros delta	GR38ABC	36	Evros, Monolimni	40°46.33	26°03.33

2008). Within species phylogeography at population level requires much sequencing effort. Maternal genetic markers are relevant at different geographical levels to infer dispersal by seeds or clonal propagation (e.g. Koga et al., 2008).

We investigated the genetic diversity of *Ruppia* populations across the European part of the Mediterranean. The objective of this study was to characterize the haplotypic chloroplast diversity for each species, to estimate the diversity in the two Mediterranean basins and to interpret apparent geographical structuring.

2. Material and methods

2.1. Study sites and plant materials

Ruppia plants were collected in 2006, 2007, 2008 and 2009 in 56 waterbodies from 38 wetland areas in the European part of the Mediterranean (Table 1 and Fig. 1). In each site we collected up to 30 individual shoots along a 30 m transect, except for the smallest stands. Leaves were dried on silica gel and a reference herbarium for each population was deposited at BRVU. A total of 1546 individual shoots could be investigated. The 53 *R. cirrhosa* populations were further analysed at population level or grouped at the level of two basins. Only for an exploration of phylogenetic structure with a permutation test we pooled the populations in 10 regions corresponding to relevant Mediterranean biogeographical subdivisions, taking into account the major currents (http://www.ifremer.fr/lobtln/OTHER/Terminology.html and http://www.mediterranean-yachting.com) and definitions of the Mediterranean Sea. These are the coastlines of the Alboran, Balearic, Tyrrhenian, Adriatic, Ionian and Aegean subbasins. The islands of Menorca, Sardinia, Sicily and the inland localities of SW and central Spain each were considered as separate regions (Table 1 and Fig. 1). The blank map was derived from http://histgeo.ac-aix-marseille.fr.

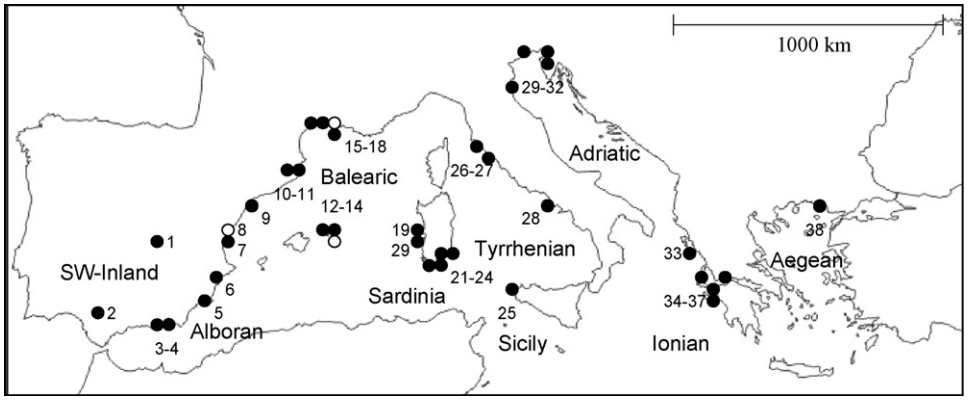

Fig. 1. Locations of 38 Mediterranean wetlands, and 56 populations sampled for *Ruppia* cpDNA haplotype sequencing.

2.2. DNA extraction, amplification and sequencing

Genomic DNA extractions were performed on dry material stored in silica gel (15–20 mg) using the E.Z.N.A. SP Plant DNA Mini Kit (Omega bio-tek) and checked for polymorphism in nine chloroplast intron markers. Three cpSSR primer pairs (Ccmp 2, Ccmp 3 and Ccmp 10) derived from the complete sequence of tobacco (*Nicotiana tabacum*) chloroplast genome (Weising and Gardner, 1999), a non-coding region (trnH-psbA) and a coding (rbcL) region (Kress and Erickson, 2007) were used. The PCR amplification was carried out in 25 μL of reaction mixture containing 0.1 μL of genomic DNA, 2.5 μL 10× PCR buffer, 0.2 mM of each dNTP, 1.6 mM $MgCl_2$, 200 nM of the forward and reverse primer, 80 $\mu g\,mL^{-1}$ bovine serum albumin (BSA) and 1 U Taq DNA polymerase. The PCR reactions were performed in a thermal cycler (MJ research PTC-200 and Bio-Rad MyCycler) and started with 2 min at 94 °C, followed by 30 cycles of 45 s at 92 °C, 1 min at 50 °C for the reactions with ccmp-primers and 1 min at 55 °C for the reactions with trnH-psbA and rbcL, 2 min at 72 °C, and a final extension at 72 °C for 5 min. The amplification products were separated on 5% non-denaturing polyacrylamide gels (acryl-bisacrylamide 19:1, 7 mM urea) with ethidium-bromide detection (Gel Scan 2000, Corbett research) and visualized with One-Dscan software (Scanalytics). Amplicon length was estimated using the GeneRuler 50 bp DNA ladder (Fermentas). Amplicon sequencing (in both forward and reverse direction) was performed by Macrogen Inc. (Seoul, South Korea).

2.3. Data treatment

Chloroplast DNA sequences were aligned with CLUSTAL W (Thompson et al., 1994) and further adjusted manually at sites with mononucleotide repeats and insertions/deletions (indels). The 2300 bp long haplotypes were defined on basis of transitions, transversions, indels and mononucleotide repeats. Indels were considered as a single event and recoded as proposed by Müller (2006). A minimum spanning network using NETWORK 4.5.1.0 (Fluxus Engineering) and a correspondence analysis (CA) using relative frequencies served as basis for the haplotype definition. A neighbor joining tree of all 16 haplotypes excluding the mononucleotide repeats was performed using p-distances and bootstrap tests (1000 permutations) in MEGA 4 (Tamura et al., 2007) giving all changes equal weight regardless the indel length. All further population analyses were done on 15 haplotypes of the *R. cirrhosa* complex (thus excluding three wetlands with *R. maritima* haplotype D1). A PCO (Principal Coordinate Analysis) was performed on the haplotype frequencies of all western Mediterranean populations of *R. cirrhosa*. CA and PCO were carried out with the software NTSYS-PC (Rohlf, 1993). A permutation test of the haplotype frequencies taking their mean number of differences (Dm) into account was performed for a data set either including or excluding mononucleotide repeats to verify the significance of a phylogenetic structure through comparison of G_{ST} with N_{ST} (option PERMUT) or R_{ST} (option cpSSR) with PERMUT (Pons and Petit, 1996). The tests (1000 permutations) were done at three levels, namely 53 populations, 34 wetland areas and 10 biogeographical regions as defined above.

Non-parametric analysis of molecular variance (AMOVA) for two Mediterranean basins was calculated with ARLEQUIN (Excoffier et al., 2005). The 53 *R. cirrhosa* populations were pooled as two groups (34 of the western and 19 of the eastern Mediterranean basin). Both the conventional *F*-statistics (each haplotype treated as equally distant) and the method with pairwise differences between haplotypes were calculated. Variance was apportioned to three components (among groups, among regions within each group, within regions) for calculating fixation indices at different levels: among groups (Φ_{CT}); among populations among groups (Φ_{ST}); among populations within groups (Φ_{SC}) and their significance level (>1000 permutations). All analyses were performed with and without considering mononucleotide repeats.

Genetic differentiation between pairs of regions (Φ_{ST}), Slatkin's $\Phi_{ST}/(1-\Phi_{ST})$ and gene flow estimation (Nm) with N as the female effective population size and m the female migration rate were calculated with ARLEQUIN considering pairwise differences between haplotypes. Again, these analyses were done with and without mononucleotide regions. Slatkin's $\Phi_{ST}/(1-\Phi_{ST})$ was used for testing isolation-by-distance (IBD) between pairs of regions with geographical distances obtained as the average distance between populations. The product–moment correlation and regression equation were obtained with STATISTICA software.

3. Results

3.1. Haplotype definition

A total of 16 haplotypes could be detected in 1546 samples. A network analysis (Fig. 2) and correspondence analysis (the first axis explained 55% of the variation whereas the second axis explained an additional 20%; figure not shown) revealed five groups of chloroplast haplotypes (named A, B, C, D, E) that distinguished the following taxa: *R. maritima* (haplotype D1) and a diverse complex (haplotypes A1, A2, B1, B2, B3, B4, B5, C1, C2, C3, C4, E1, E2, E3) corresponding morphologically to various forms of *R. cirrhosa* ranging from many-coiled peduncles to single-coiled peduncles, nearly resembling those of *R. maritima*. This polymorphic group is further referred here as the *R. cirrhosa* s.l. complex and includes the putative *R. drepanensis* (or *R. cirrhosa* var. *drepanensis*) samples.

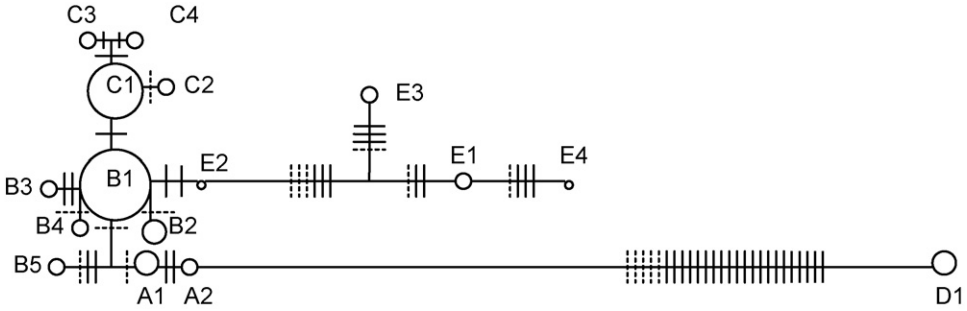

Fig. 2. Network of 16 *Ruppia* haplotypes. Filled squares each represent different insertions/deletions; full lines are substitutions and dotted lines refer to mononucleotide repeats.

Table 2
Ruppia cirrhosa cpDNA haplotype frequencies, gene diversity (h) and nucleotide diversity (π) in 53 Mediterranean populations (N, number of populations).

Regions	N	A1	A2	B1	B2	B3	B4	B5	C1	C2	C3	C4	E1	E2	E3	E4	h	π
1. West basin	34	6.1	2.2	24.6	7.1	2.5	0.5	2.8	37.4	2.2	1.4	2.0	3.6	–	0.4	0.1	0.240	0.000335
2. East basin	19	–	–	95.5	4.8	–	–	–	0.2	–	–	–	–	0.2	–	–	0.104	0.000034
3. Mediterranean	53	4.3	1.6	46.1	6.1	1.7	0.3	1.9	26.1	1.6	1.0	1.4	2.5	0.1	0.3	0.1	0.185	0.000214

Haplotype D1 (*R. maritima*) was highly divergent from all other haplotypes with up to 16 substitutions and five insertions/deletions over the 2300 bp long cpDNA sequence. 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. Within the *R. cirrhosa* complex, haplotypes B1 and C1 were most common, representing respectively 46% and 26% of all samples (Table 2). All other variants had lower frequencies ranging from 0.1% to 6%. Most were observed in a single population or wetland. Of the 53 waterbodies with *R. cirrhosa*, 32 had only a single haplotype whereas 12, 5 and 4 populations contained two, three or four haplotypes, respectively. Gene diversity (h) varied from 0 to 0.6 and nucleotide diversity (π) from 0 to 0.0021. A neighbor joining tree separated *R. maritima* (D1) as a clearly distinct taxon (Fig. 3). Within the *R. cirrhosa* complex, bootstrap values of 66% were obtained for the cluster of haplotypes E1, E3 and E4. All others had less than 50% bootstrap support.

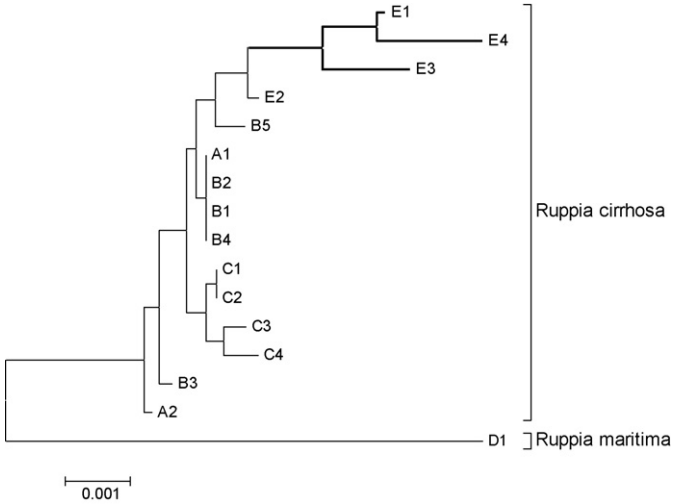

Fig. 3. Neighbor joining tree of 16 cpDNA haplotypes of Mediterranean *Ruppia* without considering mononucleotide repeats.

3.2. Geographical structuring

A common haplotype B1 was present in all regions but was observed at higher frequencies in the eastern Mediterranean basin (Table 2). The related haplotype B2 (differing in a single T-repeat) also occurred in these regions with a high abundance of B1 from Sardinia towards the eastern Mediterranean regions. Haplotype C1 was more abundant in the western Mediterranean and was not observed along the Ionian and Aegean subbasin. Overall, the gene diversity (h) values were above 0.5 in regions of the west basin, below 0.5 in the eastern basin but nearly zero in the Ionian subbasin along several wetlands of western Greece and the Peleponesos. The nucleotide diversity (π) was highest in the western Mediterranean, more precisely for the wetlands along the Balearic subbasin, Alboran subbasin and Sardinia.

An AMOVA quantified the partitioning of the cpDNA variation between two basins of the Mediterranean Sea (Table 3) and showed a significant genetic differentiation ($\Phi_{CT} = 0.1222$) between both basins when considering conventional F-statistics, treating all haplotypes as equally different ($p < 0.000$). The $\Phi_{ST} = 0.7512$ (differentiation among populations among basin) was even more pronounced ($p < 0.000$). When taking into account the pairwise dif-

Table 3
Analysis of molecular variance (AMOVA) of *Ruppia cirrhosa* cpDNA haplotypes from 53 Mediterranean populations divided over two basins (d.f., degree of freedom). All fixation indices are $p < 0.0000$.

Source of variation	d.f.	Sum of squares	% of variation	Fixation indices
Pairwise differences (*with mononucleotide repeats*)				
Among 2 groups (East and West basin)	1	96.780	12	Φ_{CT}: 0.1222
Among regions within each basin	51	906.481	63	Φ_{ST}: 0.7512
Within regions	1417	354.607	25	Φ_{SC}: 0.7166
Pairwise differences (*no mononucleotide repeats*)				
Among 2 groups (East and West basin)	1	78.814	16	Φ_{CT}: 0.1602
Among regions within each basin	51	554.729	58	Φ_{ST}: 0.7450
Within regions	1417	238.894	26	Φ_{SC}: 0.6964

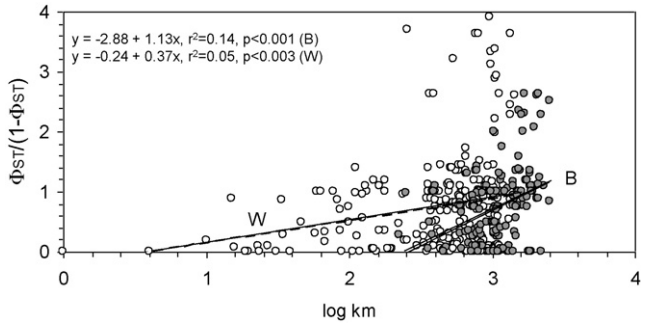

Fig. 4. Pairwise cpDNA haplotype differentiation of 53 *Ruppia cirrhosa* populations plotted against their geographical distance. Dark symbols refer to comparisons between eastern and western basin (B); open symbols refer to within basin comparisons (W).

ferences among haplotypes, then $\Phi_{CT} = 0.1602$ and $\Phi_{ST} = 0.7450$ remained highly significant ($p < 0.000$). The Φ_{SC} (among population within basin differentiation) also was high for both approaches (0.7166 and 0.6964, respectively containing 63% and 59% of the variation; $p < 0.000$). Despite the large number of monomorphic populations, the overall within population variation reached 24% (26% when considering pairwise differences).

3.3. Geographical gradient and isolation-by-distance

At the level of populations, the pairwise Φ_{ST} values differed significantly in 66% of all comparisons. However, most of this occurred within the western basin (33%) and not in the eastern basin (1%). Another 32% represented significantly differing pairwise Φ_{ST} values between both basins. Gene flow estimates mostly had *Nm* lower than 1 for the western basin (*Nm* < 1 indicates reduced levels of seed flow) but showed much higher values for populations of the eastern basin.

On a larger scale a significant isolation-by-distance pattern occurred across the European part of the Mediterranean Sea (Fig. 4). Estimates of pairwise differentiation plotted against the geographical distances (log kilometres) of population pairs between the western and eastern basin resulted in a regression $y = -2.88 + 1.13x$ with $r = 0.37$, $r^2 = 0.14$ ($p < 0.000$). The isolation-by-distance for pairs of populations within basins showed much scatter and a lower slope with $y = -0.24 + 0.37x$ with $r = 0.23$, $r^2 = 0.05$ ($p = 0.003$). IBD for populations was significant within the western basin ($p < 0.000$) but not for the eastern basin.

A PCO of the haplotype frequencies of 34 populations of the *R. cirrhosa* complex of the western Mediterranean basin revealed a gradient of haplotype diversity and abundance (Fig. 5). The first axis explained 46% of the variation and separated according to abundances of B and C haplotypes. The second and third axes separated the polymorphic populations of the western basin and explained an additional 20% and 12%, respectively. Most diverse and intermediate populations were from inland Spain (Donana NP) and those bordering the Alboran subbasin, Balearic subbasin and Sardinia. Populations of western Sardinia grouped separately from southeastern Sardinia along different axes of the PCO.

A test of phylogenetic structure of the *R. cirrhosa* complex across the Mediterranean was not significant at the level of 55 waterbodies (Dm = 2.70 and $G_{ST} < N_{ST}$, not significant) or the 34 wetlands (Dm = 3.35 and $G_{ST} < N_{ST}$, not significant). When pooling the populations in 10 biogeographical regions and taking into account the differences in microsatellite length repeats, support for a phylogenetic structure was obtained with cpSSR (Dm = 3.59; $G_{ST} = 0.287$; $R_{ST} = 0.379$; $p = 0.03$).

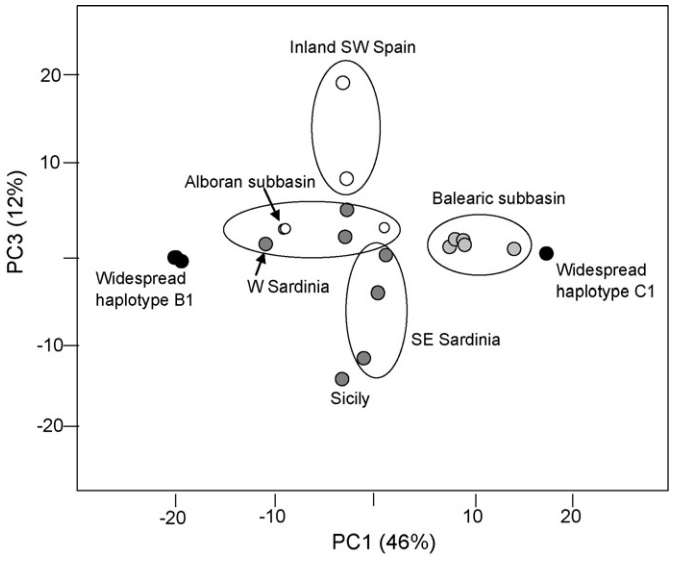

Fig. 5. Principal coordinate analysis (PCO) of cpDNA haplotype diversity in 34 populations of the western Mediterranean basin. Regions of higher diversity levels are encircled.

4. Discussion

4.1. Haplotypes and taxonomic considerations

Chloroplast sequences revealed haplotype diversity within *Ruppia* and our results indicate that *R. maritima* and a *R. cirrhosa* complex are evolutionary rather distant as they differ in five indels and 16 substitutions for the five considered cpDNA genes. Our results with cpDNA confirmed the identity of three *R. maritima* populations among 53 *R. cirrhosa* populations. Not many cpDNA data at population level have been published for submerged aquatic plants but generally, polymorphism seems to be low (Mader et al., 1998; Talbot et al., 2004; Triest et al., 2007; Koga et al., 2008; Provan et al., 2008). We detected 15 different haplotypes within *R. cirrhosa* which seems to indicate a fairly large cpDNA diversity when compared to e.g. *Zostera*, *P. pectinatus*, mangrove trees (Triest, 2008) or the freshwater *Batrachium* (Koga et al., 2008). Very low cpDNA diversity was reported for *Zostera marina* and *Zostera noltii* with a few indels and substitutions when considering *Z. marina* from very distant coasts in e.g. Ireland, Italy and U.S. (Provan et al., 2008). Likewise, few *matK* haplotypes were observed between *Z. marina*, *Zostera asiatica* and *Zostera japonica* (Talbot et al., 2004) or with cpDNA in *P. pectinatus* across Europe (Mader et al., 1998). In the latter species, a single widespread haplotype was observed and only one unique haplotype in Donana (SW Spain) and another in subarctic regions, hence very distant localities and subjected to more extreme climatic conditions. These haplotype numbers are very low, but maybe a more profound search for polymorphic regions of the chloroplast genome could reveal higher levels of polymorphism in e.g. *P. pectinatus*. The freshwater *Batrachium* showed eight cpDNA haplotypes across Japan (Koga et al., 2008) which is comparable to our observations on *Ruppia*. In the light of these few comparisons with other aquatic plants, the 15 haplotypes observed in Mediterranean *R. cirrhosa* should be regarded as a reasonably high number.

4.2. Diversity gradient across the Mediterranean

We hypothesized that *Ruppia* could harbour additional cpDNA genetic diversity in the western Mediterranean on basis of such earlier but limited findings with *Zannichellia* (Triest et al., 2007) and *P. pectinatus* (Mader et al., 1998). The present study indeed confirmed a high cpDNA diversity of *Ruppia* in the SW Mediterranean in partic-

ular and in the western Mediterranean basin in general. It suggests a hotspot of *Ruppia* diversity in the latter. Finer-scales hotspots at regional level seem more realistic for plants of the Mediterranean region with some of the most important hotspots situated on the islands of the western Mediterranean (Médail and Quézel, 1999). The higher *Ruppia* haplotype diversity caused an overall west-east gradient and IBD pattern across the European part of the Mediterranean basin. Significant differentiation between western and eastern basins and also within the western Mediterranean was obtained using various approaches such as AMOVA, permutation tests, ordination and IBD testing through correlation with pairwise differentiation estimates. The absence of an overall significant phylogeographic structure at population level can be explained by the presence of similar haplotypes in populations of both the eastern and western basin. More precisely, haplotype B1 is common throughout the study area and the very related B2 differing only in a single T-repeat, occurred in the eastern basin but also in the adjacent part of the western basin (i.e. Tyrrhenian subbasin, Sicily, SW Sardinia). An additional explanation could be the low frequency of unique haplotypes and their occurrence in few populations.

Clear-cut differences on a large geographical scale are documented and could be observed for cpDNA haplotypes of several water plant and mangroves populations. A phylogeographical analysis of *Batrachium* across Japan showed a significant geographical barrier for eight haplotypes, a genetic structure which was enforced mainly by the clonal growth of *Ranunculus* (subgenus *Batrachium*) species resulting in similar haplotypes within each population (Koga et al., 2008). Other case-studies on cpDNA of freshwater plants but with high fruit set e.g. *Sagittaria potamogetifolia* Merr. showed that one common haplotype combined with a few unique alleles resulted in low genetic differentiation across an endemic area in China (Tan et al., 2008). Water plants with mixed reproduction systems such as *P. pectinatus* and *Ruppia* species can be expected to show intermediate patterns of large-scale geographical structuring of their cpDNA diversity if their populations are not separated by strong barriers. When historical barriers such as land bridges or narrowed connections between regional seas were strong, one might expect unique haplotypes on either side of such a barrier, such as for mangrove species on both sides of the Malay Peninsula (Liao et al., 2007; Minobe et al., 2010). Possible dispersal routes then can be inferred from the similarity between haplotypes as shown for a mangrove tree species (*Ceriops tagal* (Pers.) C.B. Robinson) across the Sunda shelf, east of the Malay peninsula barrier (Liao et al., 2007).

Such strong barriers did probably not exist between the western and eastern basin of the Mediterranean Sea, because the Siculo-Tunisian Strait still connected both basins during the last glacial maximum. Nevertheless, this barrier seemed to be sufficient to cause significant genetic differentiation of several pelagic and benthic marine animals, but also of the seed dispersed seagrass *Posidonia* (Arnaud-Haond et al., 2007). Because the nuclear microsatellites deliver other information on the genetic structure of populations, these cannot be compared directly to the situation we observed with cpDNA for *R. cirrhosa*. The west-east cleavage of *Posidonia* was best explained by vicariance and recent contact, whereas the cpDNA in *Ruppia* showed a less strong barrier at the border between both basins. No evidence of vicariance is available. The diversity gradient in *Ruppia* is however clear-cut between Sardinia and Sicily, which is not exactly located at the Siculo-Tunisian strait which is considered as a potential historical barrier between the west and east basin. There are large geographical distances between wetlands sharing similar *Ruppia* haplotypes in the east basin such as those between the northern Adriatic subbasin and the Ionian or Aegean subbasin. The resulting high levels of gene flow suggest a historical contact and long distance dispersal between the wetlands of the eastern basin. A historical eastward dispersal of a limited number of haplotypes can be hypothesized. Whether a long distance dispersal was realized through waterfowl or through sea currents needs to be documented further. Within a region such as south-west Spain, *R. maritima* seeds in the diet of a waterfowl community were shown to partially survive gut passage and germinate (Figuerola et al., 2002). Feces contain intact seeds and the high number of concerted waterbird movements among wetlands is hypothesized to potentially result in an effective dispersal of many aquatic organisms (Charalambidou and Santamaria, 2005). Intact seeds of a series of mainly emergent wetland species were shown to pass mallard guts. Smaller seeds passed faster and were retrieved most after feeding (Soons et al., 2008). However, *P. pectinatus* was the only submerged species they investigated and had low retrieval percentage. Clausen et al. (2002) listed a number of prerequisites to be all fulfilled for a successful dispersal of propagules and establishment of submerged waterplant populations of e.g. *Zostera*, *Ruppia* and *Potamogeton*. In my opinion, long distance dispersal through waterfowl should also be tested against the possibility of long distance dispersal through water currents in the case of coastal *Ruppia* populations.

We can expect still more unique cpDNA haplotypes in populations across the Mediterranean or elsewhere in the subcosmopolitan distribution area of *Ruppia*. Although the present study revealed a basic pattern of cpDNA haplotype distribution, it might be refined by adding more wetland locations, especially in the African and Minor Asian part of the Mediterranean basin. Nuclear microsatellite studies will allow further hypothesis testing of phylogeographical and dispersal patterns of the *R. cirrhosa* complex.

As a conclusion, this study of *Ruppia* populations of the Mediterranean clearly showed that there is a different distribution of chloroplast DNA variability at the level of species. *R. maritima* was detected by a single haplotype from only three wetlands suggesting that this taxon might be more rare in the Mediterranean than previously accepted. Fifteen haplotypes were found in a *R. cirrhosa* species complex. A higher diversity and more unique haplotypes were observed in the western Mediterranean basin. It is hypothesized that the western Mediterranean basin represents a centre of *Ruppia* diversity and that the observed west-east gradient can be explained as a historical dispersal of a limited number of haplotypes from western to eastern Mediterranean basins.

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