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

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ABSTRACT

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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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 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 IBD tests. Genetic patterns of Mediterranean marine plant and animal populations often display a West–East differentiation

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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 (Clausen et al., 2002) and partially answered in favour of birds as effective vectors (Charalambidou and Santamaria, 2005; Rodriguez-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 subbasins 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 30 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 BRVU (herbarium of the Vrije Universiteit Brussel). A total of 1546 individual shoots was investigated for cpDNA sequence variability in five genes (ccmp-2, ccmp-3, ccmp-10, trnH-psbA, rbcL) as published in Triest and Sierens (2010). Genbank Accession numbers are listed for ccmp-2 (JN1013249–JN113255), ccmp-3 (JN113257–113259), ccmp-10 (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, Ccmp3 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., 1990) was done with primers for the ITS1 spacer (ITS1: TCCGTAGGTGAACCTGCGG and ITS2: GCTGCGTTCTTCATCGATGC) and the ITS2 spacer (ITS3: GCATCGAT-GAAGAACGCAGC and ITS4: TCCTCCGCTTATTGATATGC) using a PCR reaction of 25 μl containing $1\times$ PCR buffer, 200 μM of each dNTP, 3 mM MgCl$_2$, 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 BRVU) from each population: leaf width, flower peduncle length, podogyne length, achene length, achene width, number of inflorescences per plant and number of fruits per plant. The number of measurements for each haplotype ranged from 10 to 95 for leaf width; 26–62 for podogyne and achene sizes; 12–48 for inflorescences and peduncle sizes and 4–58 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 (Φ_{ST}), Slatkin's $\Phi_{ST}/(1-\Phi_{ST})$ were calculated with ARLEQUIN (Excoffier et al., 2005) considering the pairwise differences between haplotypes. Slatkin's $\Phi_{ST}/(1-\Phi_{ST})$ 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 subbasins of each basin and within subbasins. We considered subbasins 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/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 along coastlines of islands of Menorca (Balearic), West Sardinia (Balearic), South East Sardinia (Tyrrhenian) and Sicily (Tyrrhenian) were considered according to their subbasin. Pairwise distances