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NOTE

New highly polymorphic microsatellite markers for the aquatic angiosperm *Ruppia cirrhosa* reveal population diversity and differentiation

J. Martínez-Garrido, M. González-Wangüemert, and E.A. Serrão

Abstract: *Ruppia cirrhosa* is a clonal monoecious plant phylogenetically associated to seagrass families such as Posidoniaceae and Cymodoceaceae. It inhabits shallow waters that are important for productivity and as a biodiversity reservoir. In this study, we developed 10 polymorphic microsatellite loci for *R. cirrhosa*. Additionally, we obtained cross-amplification for two microsatellites previously described for *Ruppia maritima*. These 12 markers were tested in four *R. cirrhosa* populations from the southwest of Europe. The number of alleles per locus was high for most of the markers, ranging from 4 to 13. Two populations (Sicily and Cádiz) showed heterozygote deficit ($p < 0.001$). The four populations (Sicily, Murcia, Cádiz, and Tavira) were significantly differentiated ($F_{ST} \neq 0$; $p < 0.001$), corroborating the usefulness of these microsatellites on *R. cirrhosa* population genetics.

Key words: Ruppia cirrhosa, clonal plant, population genetics, microsatellites.

Résumé : *Ruppia cirrhosa* est une plante monoécique clonale phylogénétiquement associée aux familles des herbiers telles que les Posidoniacea et les Cymodoceaceae. Elle se trouve dans les eaux peu profondes qui sont importantes pour la productivité et comme réservoir de biodiversité. Dans cette étude, nous avons développé 10 loci microsatellites polymorphes pour *R. cirrhosa*. De plus, nous avons obtenu des amplifications croisées pour deux microsatellites précédemment décrits chez *Ruppia maritima*. Ces 12 marqueurs ont été testés dans quatre populations de *R. cirrhosa* provenant du sud-ouest de l'Europe. Le nombre d'allèles par locus était élevé pour la plupart des marqueurs, allant de 4 à 13. Deux populations (Sicile et Cádiz) ont montré un déficit en hétérozygotes ($p < 0{,}001$). Les quatre populations (Sicile, Murcia, Cádiz and Tavira) étaient significativement différentes ($F_{ST} \neq 0$; $p < 0{,}001$), confirmant l'utilité de ces microsatellites sur la génétique des populations de *R. cirrhosa*.

Mots-clés : Ruppia cirrhosa, plante clonale, génétique des populations, microsatellites.

Introduction

Genetic, species, and ecosystem diversity are recognized as major components of biodiversity. Seagrass communities are important at these three levels by playing an essential role in marine ecosystems (Duarte 2000). However, around 25% of seagrass species are classified by IUCN as threatened or near threatened as a result of numerous human impacts (Short et al. 2011). The genus *Ruppia* (Alismatales: Ruppiaceae), consists of monoecious species phylogenetically associated to seagrass families such as Posidoniaceae and Cymodoceaceae (Les et al. 1997). *Ruppia cirrhosa* (Petagna) Grande, the ditchgrass, is widespread in Europe, Africa, Asia, and North America. Its wide tolerance to salinity, irradiation, and temperature allows *R. cirrhosa* to colonize a wide variety of shallow waters, such as coastal lagoons and estuaries. It is a clonal plant with a mixed reproductive mode for space occupation, comprising both sexual reproduction and clonal growth. The colonization processes and productivity of *R. cirrhosa* have been increasingly studied (e.g., Menéndez 2002; Mannino and Sarà 2006). However, few studies have used molecular markers to assess the genetic variability of this aquatic plant; these showed high diversity at small spatial scales using chloroplast genes (Triest and Sierens 2009, 2010, 2013). However, in clonal plants, highly variable molecular markers are essential to discriminate "genets" (i.e., multilocus genotypes, distinct clones arising from distinct seeds) from "ramets" (modular units of the same genetic individual). Microsatellite loci are thus an essential tool for such population genetics studies of clonal plants. High resolution microsatellite markers have not been developed for *R. cirrhosa*, but they have been the focus of one study on *Ruppia maritima* (Yu et al. 2009). In this study, we develop 10 new polymorphic microsatellite loci for *R. cirrhosa*. Additionally, we obtain cross-amplification for two microsatellites previously described for *R. maritima* (Yu et al. 2009).

Materials and methods

Leaf genomic DNA was extracted from 15 ramets of *R. cirrhosa* collected in Murcia (SE Spain) using the cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle and Doyle 1987). A microsatellite enriched genomic library was made by Ecogenics GmbH (Zurich, Switzerland) from size-selected fragments of genomic DNA and enriched for SSR content by using magnetic streptavidin beads and biotin-labeled CT and GT repeat oligonucleotides. The SSR enriched library was pyrosequenced on a Roche 454 platform using the GS FLX titanium reagents. The total 32 314 reads had an average length of 141 base pairs. Of these, 1685 reads contained a microsatellite insert with a tetra- or a trinucleotide of at least six

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J. Martínez-Garrido. Centro de Ciências do Mar (CCMAR), CIMAR-Laboratório Associado, Universidade do Algarve, Gambelas, 8005-139 Faro, Portugal; Departamento de Ciencias del Mar y Biología Aplicada, Universidad de Alicante (DCMBA,UA), P.O. Box 99, 03080 Alicante, Spain.
M. González-Wangüemert and E.A. Serrão. Centro de Ciências do Mar (CCMAR), CIMAR-Laboratório Associado, Universidade do Algarve, Gambelas, 8005-139 Faro, Portugal.
Corresponding author: José Martínez-Garrido (e-mail: jmgarrido99@gmail.com).

Table 1. Primer sequences, repeat motifs, and characteristics of 10 microsatellite loci developed from *Ruppia cirrhosa* and two loci obtained by cross-amplification from *Ruppia maritima*.

Locus name (dye)	Primer sequences 5′→3′	Repeat motif	T_a (°C)	t_a (s)	$MgCl_2$ (mmol/L)	A	Size range (bp)	Genbank no.
Rupcir-01 (FAM)	F: AACCATGGGGTGTTCACAAG R: CTCTCTTTCTTCGCTCTTCTCAC	$(AG)_{15}$	56	45	1.5	8	61–76	KF227908
Rupcir-02 (FAM)	F: TGTTGCACGAGAAGTGCC R: TGCCGCATCTGATAATTGCC	$(CA)_{18}$	62	30	1.3	10	60–82	KF227909
Rupcir-03 (FAM)	F: GCATGTCGTACATTCTCTCGC R: CACTATGTTGCTTGGTGGGC	$(CA)_{11}$	56	45	2.0	10	81–99	KF227910
Rupcir-04 (HEX)	F: AGGAAGTCATCTCACAGCCC R: AGGCCTCATCTCTGATCTCTC	$(GAA)_8$	56	45	1.5	5	110–122	KF227911
Rupcir-05 (NED)	F: TGAAGTCCATCTGAAAACGCC R: AACTCTCTCAAATTACCTCTCCTTC	$(AG)_{16}$	58	45	1.0	9	63–79	KF227912
Rupcir-06 (FAM)	F: CGGATTTCCCCATGTGTGAG R: AGCAGATAAGAGCAGGCAGG	$(AC)_{13}(GCAC)_7$	56	45	1.5	13	161–207	KF227913
Rupcir-07 (FAM)	F: CACCACCACAGTCCGAACAC R: TGGTGGGATTGGGACAAGAC	$(CAC)_7$	64	20	1.0	6	69–83	KF227914
Rupcir-08 (HEX)	F: AGGTAGAGAGGTGTTGTGGC R: AGGATCACATGGGTTGACCG	$(GT)_{12}$	56	45	1.5	8	148–164	KF227915
Rupcir-09 (NED)	F: CGTTCTCCTGCCCCAATTTC R: ACAGATACATCAAGCGACGAC	$(TTC)_{14}$	56	45	1.5	11	117–153	KF227916
Rupcir-10 (FAM)	F: CTCGTTGACTTCCTCCTCTC R: GAGGGCAGAGAGCACTTCG	$(TC)_{12}$	60	45	1.5	12	112–148	KF227917
RUMR4 (HEX)	F: ACTAAGTACTCCTCGAATCA R: ATCAATGGTGTTTGTATGGT	$(CT)_2CC(CT)_8$	51	30	3.0	4	123–162	GQ246488
RUMR11 (HEX)	F: GTCGGTTTGTCTATTTCTCCC R: TTCTTCCTCCCTCCTTCCTAT	$(CT)_{10}$	60	30	2.5	6	166–178	GQ246491

Note: Forward primers were labeled with fluorophores (FAM, HEX, and NED). T_a (°C), annealing temperature in degrees celsius; t_a (s), annealing time in seconds; A, number of alleles.

repeat units or a dinucleotide of at least 10 repeat units. Suitable primer design was possible in 175 reads and 48 were tested for polymorphism. Of these, 38 reads were excluded because of low amplification rate and (or) allelic patterns that were difficult to interpret.

To optimize, characterize, and determine the applicability of the microsatellites, we tested them on two Mediterranean (Sicily and Murcia) and two Atlantic (Cádiz and Tavira) populations. Thirty-one ramets from each location were sampled within 80 m^2. Amplifications used the following conditions: 5 min at 95° C; 36 cycles of 95° C for 30 s, 56–64 °C (see Table 1) for 20–45 s (see Table 1), 72° C for 45 s; and a final extension step at 72° C for 20 min. PCR was conducted in 15 μL reactions containing 1.5 μL of 10× PCR buffer, 1–2 mmol/L $MgCl_2$ (see Table 1), 200 μmol/L dNTP mix, 0.16 μmol/L of each primer, 0.5 U of *Taq* DNA polymerase (Bioline), and 3 μL of diluted DNA containing approximately 15 ng of template DNA.

Two microsatellite loci from *R. maritima* (Yu et al. 2009) could also be cross-amplified in *R. cirrhosa*. The PCR reaction contained 2.0 μL of PCR buffer, 2.5–3.0 mmol/L $MgCl_2$ (see Table 1), 0.1 μmol/L of each primer, 200 μmol/L dNTP mix, 1.0 U *Taq* DNA polymerasa (Bioline), and 4 μL of diluted DNA containing approximately 15 ng of template DNA in a total volume of 20 μL per reaction. PCR reaction protocol was 4 min at 95° C; 32 cycles of 30 s at 94° C, 30 s at 51–60° C (see Table 1), 30 s at 72° C; and a final extension of 72° C for 10 min.

PCR products from microsatellite amplification were visualized by gel (2.5% agarose) electrophoresis on a Molecular Imager Gel Doc XR + system (Bio-Rad) and analysed on an ABI PRISM 3130 automated genetic analyzer (Applied Biosystems) at Centro de Ciências do Mar (CCMAR) using the GeneScan 350ROX standard. Allele sizes were scored with STRAND (http://www.vgl.ucdavis.edu/informatics/strand.php), binned using the R package MsatAllele (Alberto 2009), and manually reviewed for ambiguities. Genets (i.e., multilocus genotypes, or distinct clones arising from distinct seeds) were discriminated from ramets using GenClone (Arnaud-Haond and Belkhir 2007). Repeated genotypes were removed for further analyses, that were conducted on genets only. The probability of "null alleles", "stuttering", and "large allele dropout" was evaluated using Micro-Checker software (van Oosterhout et al. 2003). GENETIX (Belkhir et al. 1996–2004) was used to estimate the observed (H_O) and expected heterozygosity (H_E), linkage disequilibrium, and F_{ST} estimator. F_{IS} estimator of departure from Hardy-Weinberg expectation (Weir and Cockerham 1984) was calculated using Genepop (Rousset 2008).

Results and discussion

The four populations of *R. cirrhosa* contained 94 genets out of 124 ramets and were successfully genotyped for 12 microsatellite loci. All populations of this clonal species were, therefore, relying significantly on sexual reproduction for propagation, creating new genets from seeds. Linkage disequilibrium was not detected. The number of alleles per locus considering all populations together was high for most of the markers (Table 1). At the population level, all populations were genetically diverse, with H_E values (i.e., gene diversity) ranging from 0.535 (Tavira) to 0.615 (Sicily) (Table 2). The F_{IS} estimator indicated a significant excess of homozygotes in the populations from Sicily and Cádiz. None of the microsatellite loci showed significant probability ($p < 0.05$) of "large allele dropout" or "stuttering". Four loci (Rupcir-02, Rupcir-03, Rupcir-10, and RUMR4) in Sicily and one in Cádiz (Rupcir-02) and Tavira (Rupcir-9) showed significant values ($p < 0.05$) for "null alleles", a pattern that might be affected by the homozygote excess in Sicily. All four *R. cirrhosa* populations were genetically differentiated (significant F_{ST}, $p < 0.001$, after Bonferroni correction) with differentiation levels ranging from $F_{ST} = 0.214$ (Sicily–Murcia) to $F_{ST} = 0.259$ (Cádiz–Tavira) (Table 3).

Concluding, in this study, we have developed 10 microsatellite markers from the *R. cirrhosa* genome and two more by cross-amplification with those from *R. maritima*. We have successfully used those 12 polymorphic markers in four *R. cirrhosa* populations and confirmed their applicability to population genetic studies.

Table 2. Parameters of genetic diversity from the four *Ruppia cirrhosa* populations.

Locus	Population			
	Sicily (25)	Murcia (13)	Cádiz (30)	Tavira (26)
Rupcir-01				
Allele number	6	4	6	3
H_O	0.640	0.692	0.700	0.577
H_E	0.658	0.648	0.773	0.496
F_{IS}	0.048	−0.029	0.111	−0.143
Rupcir-02				
Allele number	5	5	5	5
H_O	0.240	0.692	0.267	0.692
H_E	0.509	0.787	0.581	0.582
F_{IS}	0.543**	0.159	0.553**	−0.169
Rupcir-03				
Allele number	6	4	5	5
H_O	0.400	0.615	0.300	0.539
H_E	0.753	0.465	0.395	0.641
F_{IS}	0.484**	−0.289	0.256	0.179
Rupcir-04				
Allele number	3	3	3	3
H_O	0.600	0.615	0.633	0.539
H_E	0.622	0.500	0.545	0.462
F_{IS}	0.055	−0.193	−0.146	−0.146
Rupcir-05				
Allele number	8	5	9	3
H_O	0.680	0.923	0.800	0.846
H_E	0.710	0.784	0.753	0.541
F_{IS}	0.062	−0.138	−0.045	−0.549
Rupcir-06				
Allele number	7	3	8	3
H_O	0.840	0.539	0.600	0.346
H_E	0.774	0.429	0.644	0.538
F_{IS}	−0.064	−0.217	0.085	0.373*
Rupcir-07				
Allele number	5	4	4	2
H_O	0.920	0.923	0.600	0.346
H_E	0.623	0.612	0.656	0.493
F_{IS}	−0.460	−0.477	0.102	0.316
Rupcir-08				
Allele number	5	5	5	3
H_O	0.600	0.923	0.633	0.500
H_E	0.669	0.713	0.643	0.476
F_{IS}	0.124	−0.258	0.033	−0.030
Rupcir-09				
Allele number	8	3	6	2
H_O	0.840	0.769	0.833	0.154
H_E	0.729	0.544	0.744	0.500
F_{IS}	−0.133	−0.379	−0.104	0.702**
Rupcir-10				
Allele number	7	5	10	3
H_O	0.200	0.692	0.667	0.500
H_E	0.682	0.666	0.790	0.519
F_{IS}	0.717**	0.000	0.173	0.055
RUMR4				
Allele number	3	2	2	4
H_O	0.240	0.231	0.300	0.462
H_E	0.409	0.500	0.486	0.596
F_{IS}	0.430	0.566	0.397*	0.244
RUMR11				
Allele number	3	2	2	3
H_O	0.280	0.539	0.300	0.769
H_E	0.246	0.394	0.339	0.578
F_{IS}	−0.120	−0.333	0.133	−0.314

Table 2 (*concluded*).

Locus	Population			
	Sicily (25)	Murcia (13)	Cádiz (30)	Tavira (26)
Total allele number	66	45	65	39
Mean allele number	5.500	3.750	5.417	3.250
H_O	0.540	0.679	0.553	0.522
H_E	0.615	0.587	0.612	0.535
F_{IS}	0.142**	−0.119	0.114*	0.044

Note: The number of genets in the population is in parentheses. H_O, observed heterozygosity; H_E expected heterozygosity; F_{IS}, inbreeding coefficient. *, $p < 0.05$; **, $p < 0.001$.

Table 3. Pairwise estimates of F_{ST} between four *Ruppia cirrhosa* populations.

F_{ST}	Sicily	Murcia	Cádiz
Murcia	0.214**	—	—
Cádiz	0.254**	0.219**	—
Tavira	0.249**	0.248**	0.259**

Note: Underlining denotes significant values after sequential Bonferroni correction. *, $p < 0.05$; **, $p < 0.001$.

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