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ISOLATION OF MICROSATELLITE MARKERS FOR THE COMMON MEDITERRANEAN SHRUB *MYRTUS COMMUNIS* (MYRTACEAE)[1]

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- *Premise of the study*: The development of microsatellite markers was conducted in the Mediterranean common shrub *Myrtus communis* (myrtle) to assess levels of genetic diversity and patterns of gene flow across fragmented landscapes in southern Spain.
- *Methods and Results*: Fourteen primer pairs were isolated showing clear and consistent patterns of amplification, three of which were apparently monomorphic. Levels of polymorphism in the other 11 markers were checked in 48 individuals from two populations. The number of alleles per locus ranged from 3 to 11 and the total number of alleles was 83.
- *Conclusions*: These highly polymorphic markers will allow us to improve our understanding of the genetic consequences of chronic fragmentation in Mediterranean landscapes.

Key words: gene flow; habitat fragmentation; Mediterranean landscapes; microsatellites; *Myrtus communis*.

There is evidence that habitat loss and fragmentation can reduce within-population levels of genetic diversity of both rare and widespread species (Honnay and Jacquemyn, 2007). However, the number of studies dealing with rare or endangered species is large compared to those focused on common species. This is particularly relevant since common species are usually keystone species in the ecosystems due to the high number and complex interactions they display (Gaston, 2010). Studies in common species require the availability of appropriate tools, including new sets of polymorphic codominant markers that could be used to assess genetic diversity and to perform parentage analyses to understand patterns of pollen and/or seed flow across fragmented landscapes.

Myrtus communis L. (myrtle) is a common and widespread shrub, and the sole representative of the Myrtaceae in the Mediterranean Basin. In previous studies using isozymes and AFLPs, we have demonstrated that the species shows a density-dependent mixed-mating system (González-Varo et al., 2009), and that it has a great ability to disperse the seeds across the chronic and extremely fragmented Guadalquivir River valley (southern Spain) (Albaladejo et al., 2009). However, questions regarding finer details in the patterns of genetic diversity, mating system, and gene flow (e.g., the fitting of effective dispersal kernels) are lacking since available biochemical and molecular markers (isozymes, AFLPs) were not suitable for such studies. In addition, tests aiming at transfer microsatellite markers from other Myrtaceae species (see, for example, Zucchi et al., 2002) failed in myrtle (R. G. Albaladejo et al., unpublished results). Consequently, we have developed a new set of microsatellite markers.

METHODS AND RESULTS

For the isolation of the microsatellite markers, we directly sequenced a large number of clones from a non-enriched random genomic library. Briefly, we extracted ca. 2 μg of total genomic DNA from a single myrtle individual with the Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany) following the manufacturer's protocol. Genomic DNA was sheared with a Nebulizer Kit (Invitrogen Corporation, Carlsbad, CA) applying high-pressure nitrogen to obtain DNA fragments ranging between 500 and 1000bp. Sheared DNA was repaired with the End-It DNA End-Repair Kit (Epicentre Biotechnologies, Madison, WI), and then the fragments were routinely cloned using the Zero Background Cloning Kit (Invitrogen Corporation, Carlsbad, CA). Four hundred twenty-five randomly chosen clones were sequenced in the forward direction with the M13 universal primer and using the Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and run on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) automatic sequencer.

Sequences were checked for the occurrence of di-, tri, and tetranucleotide repeats with the on-line software Sputnik (available at http://www.cbib.u-bordeaux2.fr/pise/sputnik.html). Microsatellite motifs were detected in 56 (13%) of the sequences, among which half were discarded because microsatellite motifs were too short, nucleotide repeats were too close to the vector for primer design, or because the clones showed high sequence homology. We sequenced the reverse direction of the selected clones and designed 28 primer

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pairs using Primer3 software (Rozen and Skaletsky, 2000). In a first test for variability of the markers, we carefully examined 16 individuals from four populations. Of the 28 primer pairs, 14 originated clear and consistent patterns of amplification, among which three were monomorphic (and therefore excluded from subsequent analyses). The remaining 14 primer pairs were discarded because they failed to amplify, produced multibanding patterns, or showed too pronounced stuttering.

To further characterize the 11 selected polymorphic microsatellite markers, we genotyped 48 myrtle individuals, consisting of 24 individuals from each of two populations, El Chaparral (CHP) and Dehesa Boyal (DBL), located in the Guadalquivir River valley about 2 km apart (geographical coordinates: CHP 37°14.5′N, 6°17.0’W and DBL 37°13.3′N, 6°18.0’W). Genomic DNA was isolated from dried young leaves as described above, and amplification reactions were carried out following the nested PCR method by Schuelke (2000) in a final volume of 10 μL containing ca. 30 ng of template DNA, 1 × PCR buffer, 0.2 mM of each dNTP, 1 U of Go*Taq* polymerase (Promega, Madison, WI), 1.5 mM of $MgCl_2$, 0.2 μM of the reverse and the M13 universal primer (the latter labeled with FAM, NED, VIC, or PET to the 5′ end), and 0.07 μM of the modified forward primer with the M13 primer sequence (18 bp) added at its 5′ end. Three PCR profiles were employed for different primers (see Table 1), leading to the successful amplification of the microsatellite loci: (A) denaturation at 94°C for 4 min followed by 10 touchdown cycles at 94°C (30 s), 60°C (30 s; −1°C/cycle), 72°C (1 min), 30 cycles at 94°C (30 s), 50°C (30 s), 72°C (1 min), and a final extension step at 72°C 7 min; (B) 94°C for 4 min followed by 10 touchdown cycles at 94°C (30 s), 60°C (30 s; –0.5°C/cycle), 72°C (1 min), 30 cycles at 94°C (30 s), 55°C (30 s), 72°C (1 min), and a final extension step at 72°C for 7 min; and (C) 94°C for 4 min followed by 30 cycles at 94°C (30 s), 59°C (30 s), 72°C (1 min), and a final step at 72°C for 7 min. Primer sequences, repeat motif, GenBank accession number, and PCR amplification profile are shown in Table 1. Amplification reactions were carried out in an Eppendorf Mastercycler ep realplex (Hamburg, Germany), and fluorescently labeled amplified products were run in an ABI 3730 DNA Analyzer automatic sequencer (Applied Biosystems, Foster City, CA). Microsatellite scoring was automatically done with GeneMapper v.3.7 (Applied Biosystems, Foster City, CA) and manually inspected for corrections. Expected (H_e) and observed (H_o) heterozygosity, and fixation index (F) were calculated with Genalex, v.6 (Peakall and Smouse, 2006). The frequency of null alleles following Dempster et al. (1977), and linkage disequilibrium between pairs of loci were estimated using Genepop v.4 (Rousset, 2008).

All polymorphic markers showed a dinucleotide repeated motif but two of them, *Myrcom*6 and *Myrcom*9, amplified compound motifs, showing an additional mononucleotide region close to the dinucleotide repeat (Table 1). Nevertheless, the amplification profiles in these two markers showed low stuttering, which allowed confident scoring of the peaks. The number of alleles per locus ranged from 3 to 11, with a total of 83 alleles scored in 48 individuals. None of the loci pairs showed significant linkage disequilibrium after Bonferroni correction. Some markers (especially *Myrcom*1, *Myrcom*2, and *Myrcom*4) showed relatively high fixation index values in the two analyzed populations, which in part might be due to the presence of null alleles (Table 2). However, the small sample size analyzed and/or the existence of local genetic structure within populations (L. Fernández-Carrillo, unpublished results) could also account for the significant excess of homozygotes detected at these loci.

CONCLUSIONS

The newly developed polymorphic markers showed high levels of polymorphism, suggesting a great potential in genetic diversity and parentage studies. Specifically, these markers will be used to gain a better understanding of the patterns of gene flow, at ecological time scales, among myrtle populations across extremely fragmented Mediterranean landscapes. This information

Table 1. Characteristics of the 11 polymorphic and three monomorphic microsatellites markers developed for *Myrtus communis*.

Locus name	Primers sequence 5′-3′	Repeated motif	PCR profile[b]	Allele size range (bp)	Total no. of alleles[c]	GenBank accession no.
*Myrcom*1	[a]F: CGTGATGCACACTGAACTGA R: AACCCCTTTTGCCAACATTT	$(AC)_6$	B	227–231	3	GU584108
*Myrcom*2	F: ATAGCTCTTACCCGCCATTG R: GTGCATGGTCCTCGATAGGT	$(TC)_{18}$	B	187–213	11	GU584109
*Myrcom*3	F: GGCAGCTACCAGTCATACCC R: TTTGCAGCATTTCAAAGTGG	$(CT)_{13}$	B	180–186	4	GU584110
*Myrcom*4	F: CAACCACATCCACCCATAGA R: CCACAGTCAAGAGGGAGAGC	$(TC)_{20}$	C	197–219	9	GU584111
*Myrcom*5	F: TGAGAGATCAGCAACCAAAAAG R: CATGAATGGCAACGATGAAA	$(CT)_8$	A	274–288	5	GU584112
*Myrcom*6	F: AAATGAAAAAGCTAAAAGTTAAACA R: AACAGGAAGAGCAAGCCAAG	$(A)_{12}(CA)_{10}$	B	171–187	8	GU584113
*Myrcom*7	F: AGACATGCTCAAACTTGTATGC R: AATGTATCCCAACATGTCAGA	$(GA)_{19}$	B	169–191	9	GU584114
*Myrcom*8	F: TGCTCGGTCATTAATTGGTGT R: TCAAAACCGTCTCCATGAAA	$(TA)_6$	B	232–270	9	GU584115
*Myrcom*9	F: GAAAGTTGCACTGTTTATTTCCAA R: TCTTCCTTCCAATCCTCATCA	$(A)_{15}GG(GA)_9$	B	181–186	6	GU584116
*Myrcom*10	F: TTAAGTGCCTTTGGCATTTGT R: AGAGGACCTCGCGATAGACA	$(CT)_{17}$	B	216–248	9	GU584117
*Myrcom*11	F: GCAAATAAAAAGCGAGTTAAATGA R: CCACACTTTTAAGAATTTGTGGTC	$(TA)_9$	B	232–250	10	GU584118
*Myrcom*12	F: CCCTCCATTTTTCCCTTCTC R: AGCCGAAGCTCCAAGAAAC	$(GA)_9$	A	168	1	GU951529
*Myrcom*13	F: TCCACTTTGCTCACACAAGC R: CATCTCCTCACCCTGCTACC	$(AAG)_4(AAC)_5$	A	207	1	GU951530
*Myrcom*14	F: TCATCATCTAAACCGCACCA R: TTACTGTTCATGCCCCACAC	$(TC)_3TG(TC)_3TG(TC)_2TG$ $(TC)_2TG(TC)_2TG(TC)_6$	A	290	1	GU951531

[a] F; forward primer, R; reverse primer.

[b] PCR profiles A and B contain 10 touchdown cycles with annealing temperatures of 60–50°C (−1°C/cycle; profile A), and 60–55°C (−0.5°C/cycle; profile B), and C is a standard profile of 30 cycles with annealing temperature of 59°C(see text for details).

[c] Number of alleles for loci *Myrcom*1–11 refer to the whole sample of 48 individuals (see Table 2), while at loci *Myrcom*12–14 to the subsample of 16 individuals (see text for details).

Table 2. Average genetic diversity for two *Myrtus communis* populations (sample sizes in parentheses) using the 11 newly developed polymorphic microsatellite markers.

	No. of alleles	H_e	H_o	F	Null allele frequency
Population DBL (N = 24)[a]					
*Myrcom*1	3	0.258	0.125	0.515	0.246
*Myrcom*2	7	0.722	0.500	0.308	0.224
*Myrcom*3	3	0.455	0.500	−0.099	0.000
*Myrcom*4	6	0.574	0.235	0.590	0.226
*Myrcom*5	5	0.581	0.652	−0.122	0.000
*Myrcom*6	6	0.643	0.478	0.256	0.193
*Myrcom*7	8	0.792	0.708	0.105	0.025
*Myrcom*8	4	0.502	0.261	0.480	0.236
*Myrcom*9	5	0.723	0.609	0.158	0.168
*Myrcom*10	6	0.740	0.174	0.765	0.359
*Myrcom*11	8	0.771	0.870	−0.127	0.000
Population CHP (N = 24)					
*Myrcom*1	2	0.219	0.050	0.771	0.316
*Myrcom*2	8	0.798	0.211	0.736	0.330
*Myrcom*3	4	0.596	0.583	0.022	0.110
*Myrcom*4	8	0.782	0.190	0.757	0.362
*Myrcom*5	5	0.570	0.652	−0.144	0.055
*Myrcom*6	7	0.667	0.739	−0.108	0.000
*Myrcom*7	8	0.780	0.826	−0.059	0.000
*Myrcom*8	6	0.355	0.273	0.233	0.082
*Myrcom*9	5	0.702	0.583	0.169	0.087
*Myrcom*10	7	0.699	0.500	0.284	0.083
*Myrcom*11	9	0.802	0.875	−0.091	0.000

H_e = expected heterozygosity, H_o = observed heterozygosity, F = fixation index.

[a] Vouchers of populations: Dehesa Boyal (DBL), II-2010, *González-Varo JP & Albaladejo RG* (SEV 249870); El Chaparral (CHP), II-2010, *González-Varo JP & Albaladejo RG* (SEV 249869).

will be invaluable since available data on how common species are affected by habitat fragmentation are scarce.

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