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Development and characterization of microsatellite loci for *Ocotea* species (Lauraceae) threatened with extinction

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ABSTRACT. The Atlantic rainforest species *Ocotea catharinensis*, *Ocotea odorifera*, and *Ocotea porosa* have been extensively harvested

in the past for timber and oil extraction and are currently listed as threatened due to overexploitation. To investigate the genetic diversity and population structure of these species, we developed 8 polymorphic microsatellite markers for *O. odorifera* from an enriched microsatellite library by using 2 dinucleotide repeats. The microsatellite markers were tested for cross-amplification in *O. catharinensis* and *O. porosa*. The average number of alleles per locus was 10.2, considering all loci over 2 populations of *O. odorifera*. Observed and expected heterozygosities for *O. odorifera* ranged from 0.39 to 0.93 and 0.41 to 0.92 across populations, respectively. Cross-amplification of all loci was successfully observed in *O. catharinensis* and *O. porosa* except 1 locus that was found to lack polymorphism in *O. porosa*. Combined probabilities of identity in the studied *Ocotea* species were very low ranging from 1.0×10^{-24} to 7.7×10^{-24}. The probability of exclusion over all loci estimated for *O. odorifera* indicated a 99.9% chance of correctly excluding a random nonparent individual. The microsatellite markers described in this study have high information content and will be useful for further investigations on genetic diversity within these species and for subsequent conservation purposes.

Key words: *Ocotea catharinensis*; *Ocotea odorifera*; *Ocotea porosa*; Cross-amplification; Single sequence repeat; Atlantic rainforest

INTRODUCTION

The genus *Ocotea* comprises approximately 350 species that are distributed throughout the tropical and subtropical climates. Most species occur in the Americas from Mexico to Argentina, with one species found in the Canary Islands, 7 in Africa, and around 50 in Madagascar (Rohwer, 2000). Due to the value of their timber and essential oils, natural populations of *Ocotea catharinensis* Mez., *Ocotea odorifera* (Vellozo) Rohwer, and *Ocotea porosa* (Nees & Mart.) Barroso have been heavily exploited from the Atlantic rainforest of Brazil and are consequently experiencing large reductions in population size and area (Araújo, 1948; Reitz et al., 1978; Klein, 1980; Carvalho, 1994). These species are currently classified as being vulnerable to extinction by the IUCN Red List (Varty, 1998; Varty and Guadagnin, 1998a,b) and listed as threatened by the Brazilian list (MMA, 2008). Moreover, large portions of their former habitat have also been modified or destroyed due to urban growth, cattle ranching, and monocultures. Since these factors might have reduced the levels of within-population genetic diversity, potentially increasing the risk of extinction, an investigation to determine how genetic diversity is partitioned within and among populations of these 3 species of *Ocotea* is important for developing strategies for both *in situ* and *ex situ* conservation. Here, we report the development and characterization of 8 polymorphic microsatellite markers for *Ocotea odorifera* and the transferability of these markers to *O. catharinensis* and *O. porosa* with regard to their application in population genetic assessments and the subsequent formation of efficient conservation strategies for each of the species.

MATERIAL AND METHODS

Genomic DNA was extracted from silica gel-dried leaves of 1 individual of *O. odorifera* sampled from a natural population located in Rio de Janeiro, RJ (22°15'5, 44°34'W). A modified CTAB method (Doyle and Doyle, 1987) was used for DNA extraction for initial microsatellite library development. The enriched microsatellite library was developed using the methodology proposed by Billotte et al. (1999) by using the *Rsa*I restriction enzyme (Invitrogen) and 2 dinucleotide repeat sequences. DNA was digested with *Rsa*I, and fragments were linked to *Rsa*I adapters. Microsatellite enrichment was performed using biotin-labeled microsatellite oligonucleotide probes $(CT)_8$ and $(GT)_8$ with subsequent capture by streptavidin MagneSphere paramagnetic particles (Promega, Fitchburg, WI, USA). Captured DNA was amplified by polymerase chain reaction (PCR) by using primer sequences complementary to the adapters and linked into the pGEM-T vector (Promega). *Escherichia coli* XL-1 Blue cells (Stratagene, La Jolla, CA, USA) were used for cloning. Forty-eight clones were selected and sequenced on ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA), of which 20 contained microsatellite motifs suitable for primer design. The program WebSat (Martins et al., 2009) was used to determine the single sequence repeat (SSR) before primer design by using the Primer 3 version 0.4.0 program (Rozen and Skaletsky, 2000). PCR analyses were optimized using DNA from 8 individuals of each of the 3 species obtained from different sampled populations. PCR was performed in a total volume of 12.5 µL containing approximately 20 ng template DNA, 1 U MyTaq DNA Polymerase (Bioline), 5X MyTaq Reaction Buffer (5 mM dNTPs, 15 mM $MgCl_2$, stabilizers, and enhancers; Bioline), and 0.2 µM each primer. Cycling conditions were as follows: an initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, specific annealing temperature (Table 1) for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. Amplification products were checked using agarose gel (2%) electrophoresis stained with ethidium bromide and compared to a 100-bp size standard (Axygen). Polymorphism analyses were conducted using an AB3500 Genetic Analyzer (Life Technologies Inc., Grand Island, NY, USA) for 2 populations (N = 60/species) of each of the 3 species.

Table 1. Characteristics of eight microsatellite markers developed for *Ocotea odorifera*.

SSR Locus	Repeat motif	Primer sequence (5'-3')	Ta (°C)	Allele size (bp)	GenBank accession No.
Ood 05	$(GA)_7$	F:GACACAGTAATGCTGGGGAAA R:ACCCTCAACCTCATCATTGC	57	246-252	KC261494
Ood 07	$(GA)_{25}$	F:TAATGGGTCCCCTGTTTTGA R:CCCCTTTCTTTCCCTCTCAC	56	206-258	KC261495
Ood 09	$(AG)_{14}$	F:ATATGCTACTCTTTGGAAGC R:CTAGTAAAATTGTCCAACGA	56	160-186	KC261496
Ood 14	$(TC)_{16}$	F:CCTTAAACTTCACCCTCTCC R:CCAAGTTCAAAAGAGGAAAA	56	204-236	KC261497
Ood 15	$(CT)_{17}$	F:AACAGAGTGGACTCGAAGAA R:TATGGAAGTGCCTCTTTCTC	56	146-176	KC261498
Ood 16	$(AG)_{17}$	F:TCCATTCGGAGAGAAAAATA R:CTCTAGTGACGGAATGGAAG	56	182-226	KC261499
Ood 17	$(CT)_7CC(CT)_9$	F:AGTAGCTTCACCAACCAAGA R:TGGCTTGTTTTACTCCCTTA	60	212-252	KC261500
Ood 20	$(TC)_{17}$	F:TTAGTCTCACCTTCCATTCC R:TGGACACGAGGTTAGTTTCT	56	191-221	KC261501

F = forward; R = reverse; Ta = optimal annealing temperature.

The presence of null alleles, scoring errors, and large allele dropouts was checked for

all loci using Microchecker (Van Oosterhout et al., 2004). GenAlEx 6.5 (Peakall and Smouse, 2006) was subsequently used to estimate the mean number of alleles per locus (*A*) and observed (H_O) and expected heterozygosity (H_E) for each locus in each population. Genotypic linkage disequilibrium (LD) and deviations from Hardy-Weinberg equilibrium (HWE) were determined using FSTAT version 2.9.3 (Goudet, 2001). Probability of identity (PI) and probability of exclusion (Q) were also calculated using GenAlEx 6.5.

RESULTS AND DISCUSSION

Eight polymorphic loci were obtained for *Ocotea odorifera*, and their characteristics are shown in Table 1. Cross-amplification of all SSR loci was successfully observed in *O. catharinensis* and *O. porosa*, although there was no polymorphism in the locus *Ood16* for the latter species. For *O. odorifera*, the mean number of alleles detected over all loci was 10.2. H_E and H_O ranged from 0.41 to 0.92 and from 0.39 to 0.93 across the populations, respectively. The loci *Ood15*, *Ood16*, and *Ood17* in population 2 of *O. odorifera* showed significant departure from HWE after Bonferroni correction. In *O. catharinensis*, the mean number of alleles observed was 8.56, and H_E and H_O ranged from 0.28 to 0.91 and from 0.09 to 0.83 in each of the populations, respectively. In *O. porosa*, the mean number of alleles found across loci was 8.37; H_E ranged from 0.64 to 0.88 and H_O from 0.42 to 1.00 across the populations (Table 2). Significant departures from HWE were found in *O. catharinensis* for loci *Ood15*, *Ood20* (Pop. 1), and *Ood17* (Pop. 2). In *O. porosa*, the loci *Ood 05* (Pop. 1) and *Ood07* and *Ood17* (Pop. 2) deviated significantly from HWE (Table 2). There was no LD detected between pairs of loci for all *Ocotea* species analyzed.

Table 2. Variability of eight microsatellite loci in *Ocotea odorifera*, *O. catharinensis* and *O. porosa* within two populations of each species.

SSR Locus	*O. odorifera*				*O. catharinensis*				*O. porosa*			
	Population	N_A	H_E	H_O	Population	N_A	H_E	H_O	Population	N_A	H_E	H_O
Ood 05	Nova Iguaçu/RJ	4	0.58	0.43	Ituporanga/SC	4	0.64	0.48	Mafra/SC	7	0.75	0.42*
Ood 07	(pop.1)	17	0.91	0.79	(pop.1)	12	0.86	0.71	(pop.1)	12	0.88	0.64
Ood 09		11	0.89	0.93		10	0.83	0.81		8	0.77	0.66
Ood 14		10	0.86	0.79		8	0.77	0.63		13	0.84	0.73
Ood 15		10	0.83	0.70		8	0.52	0.29*		6	0.71	0.88
Ood 16		12	0.85	0.71		7	0.47	0.52		1	-	-
Ood 17		11	0.88	0.78		5	0.75	0.53		9	0.75	1.00
Ood 20		13	0.91	0.73		9	0.80	0.09*		13	0.83	0.75
	Means ± SD	11.00	0.84	0.73	Means ± SD	7.87	0.70	0.51	Means ± SD	8.62	0.69	0.63
		(1.28)	(0.04)	(0.05)		(0.91)	(0.05)	(0.08)		(1.45)	(0.10)	(0.11)
Ood 05	Guaratuba/PR	3	0.41	0.50	Santa Teresa/ES	3	0.28	0.31	Ponta Grossa/PR	9	0.75	0.56
Ood 07	(pop.2)	12	0.90	0.64	(pop. 2)	10	0.85	0.79	(pop. 2)	9	0.84	0.48*
Ood 09		11	0.86	0.84		12	0.82	0.79		13	0.86	0.68
Ood 14		10	0.86	0.90		9	0.77	0.71		14	0.82	0.79
Ood 15		7	0.80	0.39*		15	0.91	0.79		3	0.64	0.88
Ood 16		13	0.92	0.56*		8	0.83	0.83		1	-	-
Ood 17		8	0.81	0.47*		8	0.79	0.52*		5	0.74	0.46*
Ood 20		11	0.87	0.80		9	0.75	0.68		11	0.85	0.84
	Means ± SD	9.37	0.80	0.64		9.25	0.75	0.68		8.12	0.69	0.58
		(1.15)	(0.06)	(0.07)		(1.22)	(0.07)	(0.06)		(1.66)	(0.10)	(0.10)

N_A = number of alleles; H_E = expected heterozygosity; H_O = observed heterozygosity. *Significant departure from HWE after Bonferroni's correction (Rice 1989).

Null alleles were detected for *O. odorifera* in loci *Ood07* and *Ood20* (Pop. 1) and

Ood07, *Ood15*, *Ood16*, and *Ood17* (Pop. 2); for *O. catharinensis,* in loci Ood 15 and Ood 20 (Pop. 1) and Ood 17 (Pop. 2); and for *O. porosa*, in loci Ood 05 and Ood 07 (Pop. 1) and Ood 05, Ood 07, Ood 09, and Ood 17 (Pop. 2). Cumulative probability of identity in *Ocotea odorifera* for all loci in each population was very low (1.0×10^{-24} to 7.7×10^{-24}), and the probability of exclusion indicated a 99.9% chance of correctly excluding a random nonparent individual. The highly polymorphic microsatellite markers presented in this study will be appropriate for the analysis of genetic diversity, population structure, and parentage analyses and constitute a useful tool for the conservation of these *Ocotea* species in the future.

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