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Microsatellite loci development in mycoheterotrophic *Corallorhiza maculata* (Orchidaceae) with amplification in *C. mertensiana*[1]

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- *Premise of the study:* Microsatellite primers were developed for the first time in the species *Corallorhiza maculata*, a nonphotosynthetic orchid that is becoming a model for studying mycorrhizal specificity.
- *Methods and Results:* Eight polymorphic microsatellite markers were developed using an enrichment and cloning protocol. The number of alleles for each locus ranged from two to seven. The loci were tested in three populations, and the resulting high F_{ST} values suggested that these loci have utility in illuminating population structure. Cross-amplification was also achieved in the sister species *C. mertensiana.*
- *Conclusions:* These microsatellite markers will be useful in further study of the population genetics of this species, including that of previously described mycorrhizal races.

Key words: *Corallorhiza maculata*; cross-species amplification; microsatellite; orchids.

Corallorhiza maculata (Raf.) Raf. is a nonphotosynthetic terrestrial orchid that is becoming a model for studies of mycorrhizal specificity. It typically grows in late succession forests of pine, fir, or oak and is distributed throughout Mexico and the entire United States with the exception of the Great Plains region and the Southeast (Freudenstein, 1997). Because the plant is achlorophyllous, all of its carbon is acquired via a symbiotic relationship with a fungus in which the orchid appears to be acting parasitically. Like many parasite–host relationships, the group of fungi that the orchid exploits is narrow (Taylor and Bruns, 1999). Using three single nucleotide polymorphisms, Taylor et al. (2004) determined that there are at least six distinct genotypes or races of *C. maculata*, each of which associates with a different clade of ectomycorrhizal fungi in the family Russulaceae. Despite this fine-scale specialization, however, different genotypes can be found growing intermixed at a single site with each genotype utilizing a different fungus. Because prior genotyping was coarse, there is a need for higher-resolution genetic markers. Microsatellite markers should enable improved understandings of the patterns of gene flow in relation to mycorrhizal specificity within this species. Because *C. maculata* is a member of a species complex consisting of *C. mertensiana* Bong., *C. bulbosa* A. Rich. & Galeotti, and *C. williamsii* Correll, the microsatellite markers may also be useful for several other species. Here we report the development and application of eight microsatellite loci. The loci were developed for *C. maculata* and tested on three populations (Coastal California, Interior Northern California, and the Rocky Mountains in Colorado). The loci were also tested on a population of *C. mertensiana*, which is the only other widespread species within the *C. maculata* complex.

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METHODS AND RESULTS

Floral and rhizome tissues were used as sources for genomic DNA of five orchid species (including *C. maculata*) by extraction using the Plant DNeasy Mini kit (QIAGEN, Valencia, California, USA). DNA of the five species were gel quantified and pooled in equimolar amounts. Here we consider only loci developed for *C. maculata*.

Genetic Identification Services (Chatsworth, California, USA) were provided with 100 μg of pooled genomic DNA, and enrichment and cloning were carried out as described previously (Jones et al., 2002). Concurrent partial digestion with *Rsa*I, *Hae*III, *Bsr*BI, *Pvu*II, *Stu*I, *Sca*I, and *Eco*RV was carried out and followed by adapter ligation. Biotinylated $CA_{(8)}$, $GA_{(8)}$, $ATG_{(8)}$, and $TAGA_{(8)}$ oligonucleotides were used for enrichment and capture of 300–750 bp fragments on magnetic beads (CPG, Lincoln Park, New Jersey, USA). Digestion with *Hind*III removed the adapters and was followed by ligation in pUC19 and transformation of DH5α cells. One library for each motif (4) was sent to SeqWright (Houston, Texas, USA) for colony picking and insert sequencing.

In total, 768 colonies were sequenced. We designed primers for promising sequences using PRIMER3 (Rozen and Skaletsky, 2000) with an optimum primer length of 23 and T_m of 62°C. The origins of prospective loci were determined via amplification from seven DNAs (three of which were individuals of *C. maculata*) representing the five orchid species (Lombardo et al., 2008). Amplification was attempted using an initial denaturation at 96°C for 2 min followed by 35 cycles of 92°C for 20 s, 58° for 30 s, and 72°C for 30 s with a final elongation at 72°C for 10 min, with a total reaction volume of 10 μL containing 0.25 μL JumpStart REDTaq polymerase (Sigma-Aldrich, St. Louis, Missouri, USA), 200 μM dNTPs, 2.15 mM MgCl, 10 mM Tris-HCl, 50 mM KCl, and 0.001% gelatin (w/v).

Primer pairs that successfully amplified *C. maculata* DNA were further screened, including bidirectional sequencing to verify the presence of a microsatellite.

Twenty-one individuals of *C. maculata* from the Colorado Rocky Mountains (San Juan NF: 37°31′N, 107°53′W, elevation 2017 m), 20 individuals of *C. maculata* from coastal California (37°52.16′N, 122°31.66′W, elevation 45 m), and six individuals of *C. maculata* from interior California (Mendocino NF:

TABLE 1. Characterization of the eight microsatellite loci for *Corallorhiza maculata*. Shown for each locus are forward and reverse primer sequences, repeat motif, allele size range, annealing temperature (T_a), and GenBank accession numbers.

Locus name	Primer sequence (5′–3′)	Repeat motif	Allele size (bp)	T_a (°C)	GenBank Accession No.
SW1–8	F: ATCATGTGGTTGGAAGTGCCTAT	$(GT)_5GA(GT)_7$	263–303	58	HQ904068
	R: AATAGAGGCTGAGGCTGAGTTGA				
SW1–22	F: TCAAATGGGATAACTTGACAACCT	$(CA)_{12}$	222–234	58	HQ904066
	R: TCAATGAATCCTATTTTCTTGGATTG				
SW1–35	F: TGACTATTACTCATCGCCCTCCT	$(CA)_{19}GA(CA)_4$	190–211	58	HQ904067
	R: ATGCATCACGTGCAAAAAGTAGA				
SW1–109	F: TCTGTGGTCGTCTTCTTCTGTTG	$(GAT)_{15}$	159–178	58	HQ904064
	R: CCTAATGGGACATCATCACCAC				
SW1–122	F: ATATCATGTCACCTGCCTCACAG	$(GAT)_{16}(GGT)_5$	201–221	58	HQ904069
	R: TGAGAGAGAACCACAATCAACCAG				
SW1–140	F: CGGAAGATCTTGTTGCTTCTGAT	$(GAT)_{10}$	171–174	58	HQ904065
	R: CTCCCCTGACATCTTATGGATGA				
SW2–73+	F: CAACCAAATTCACAACCCACTTT	$(GT)_{14}$	277–289	58	HQ904070
	R: TGAAATGGGATAACTTGACAACCT				
SW2–162	F: GAGGGGTGTAGCGTCTTGTATTT	$(GT)_{11}$	221–238	68	HQ904071
	R: AACAGACAAACCTTCGGAACAAG				

39°59.75′N, 122°59.32′W, elevation 1420 m) were used to test for polymorphism in successfully amplified and sequenced loci. Ten individuals of *C. mertensiana* Bong. collected from throughout Vancouver Island, Canada (no GPS coordinates are available because the samples were collected ca. 15 yr ago), were used to test the transferability of these markers. Voucher specimens for the *C. maculata* populations (V170405: Coastal CA; V170406: Interior CA; and V170408: CO Rocky Mountains) were deposited in the herbarium at the University of Alaska Fairbanks. The PCR products were amplified using a standard reverse primer and a FAM-labeled forward primer except for primer SW1-162, where the forward primer was standard and the reverse primer was FAM labeled. PCR amplifications were performed with a 25 μL final volume, and amplification conditions were the same as those of initial screening except that an optimized annealing temperature was used for each primer pair (Table 1). Allele sizes were approximated using GeneMapper software version 3.7 (Applied Biosystems, Foster City, California, USA) after visualization on an ABI3130XL with Applied Biosystems GeneScan –500 ROX size standard.

The following summary statistics found in Table 2 were estimated using GENEPOP version 4.0.10 (Raymond and Rousset, 1995): expected (H_e) and observed (H_o) heterozygosity and deviation from Hardy–Weinberg equilibrium.

Overall there were a moderate number of alleles per locus in both *C. maculata* (2–6) and *C. mertensiana* (1–7). Observed heterozygosity is lower than expected heterozygosity in all populations of *C. maculata* while the population of *C. mertensiana* has much more similar values of observed and expected heterozygosity. These findings are consistent with the putative high levels of selfing occurring in *C. maculata* and the lower levels of selfing in *C. mertensiana*. Departure from Hardy–Weinberg equilibrium ($P < 0.05$) was found in all populations of *C. maculata.* Testing for disequilibrium ($P < 0.05$ using Fisher's exact test in GENEPOP [Raymond and Rousset, 1995]) globally across all *C. maculata* populations showed that 16 of 28 pairwise locus comparisons exhibited significant disequilibrium whereas only one out of 28 pairwise comparisons showed significant disequilibrium in the *C. mertensiana* population. Spatially restricted subsets of *C. maculata* were also tested for disequilibrium and suggested substantially fewer linked loci, suggesting that limited gene flow among geographic areas or lineages, rather than physical clustering in the genome, may explain much of the apparent linkage.

CONCLUSIONS

The significant deviations from Hardy–Weinberg equilibrium in all populations of *C. maculata* can be expected because of the highly selfing nature of the orchid (Freudenstein, 1997). Selfing and distinct lineages may also underlie the significant linkage disequilibrium in *C. maculata*, which was also found in the single nucleotide polymorphism study of Taylor et al. (2004). These new polymorphic microsatellite loci clearly delineate genetic variation and will aid in investigating the extent of gene flow taking place between populations and races of *C. maculata*. The cross-amplification of *C. mertensiana* will serve to increase our understanding of population dynamics in this species as well.

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TABLE 2. Results of initial primer screening on samples from three populations of *Corallorhiza maculata* and one population of *C. mertensiana*. Shown for each locus are the number of alleles (*A*), observed and expected heterozygosity (H_o and H_e), and the *P* values for Hardy–Weinberg equilibrium (HWE) for each population.

	C. maculata												*C. mertensiana*			
	Colorado Rocky Mountains				Coastal California				Interior California							
Locus	*A*	H_o	H_e	HWE	*A*	H_o	H_e	HWE	*A*	H_o	H_e	HWE	*A*	H_o	H_e	HWE
SW1–8	5	0	0.632	0	5	0.500	0.609	0.0001	3	0	0.667	0.0029	6	0.800	0.821	0.935
SW1–22	4	0.048	0.700	0	6	0.200	0.805	0	3	0	0.667	0.0040	2	0	0.189	0.050
SW1–35	6	0.333	0.444	0.0735	3	0.400	0.363	0.0230	3	0.167	0.426	0.1109	3	0.800	0.563	0.110
SW1–109	6	0.048	0.733	0	6	0.300	0.759	0	3	0	0.667	0.0041	5	0.800	0.711	0.749
SW1–122	6	0.143	0.674	0	4	0.300	0.712	0	2	0	0.545	0.224	5	0.800	0.711	0.762
SW1–140	2	0	0.177	0.0023	2	0	0.324	0	2	0	0.485	0.0302	1	—	—	—
SW2–73	4	0.048	0.702	0	8	0.200	0.838	0	3	0	0.667	0.0041	3	0.200	0.195	1.000
SW2–162	3	0	0.168	0.0040	5	0.050	0.520	0	1	—	—	—	3	0.625	0.500	0.731

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