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Isolation and characterization of new polymorphic microsatellite loci in the mixotrophic orchid *Limodorum abortivum* L. Swartz (Orchidaceae)

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Abstract

Here, we report the isolation and characterization of 11 polymorphic microsatellites in *Limodorum abortivum*. Allele variability has been characterized in three populations from Southern Italy and France. The number of alleles ranged from one to six per locus with an average of 3.8 alleles per locus. Observed and expected heterozygosity values ranged from 0.000 to 1.000 and from 0.492 to 0.806, respectively, with striking differences among populations. These microsatellites should be valuable tools for studying fine-scale genetic structure of scattered *Limodorum abortivum* populations, patterns of relationship with closely related taxa and the evolutionary ecology of its mycorrhizal interactions.

Keywords: cross-species amplification, genomic library, *Limodorum abortivum*, microsatellite, orchids

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Limodorum abortivum (tribe Neottiae, Orchidaceae) has a patchy distribution and typically grows in small populations in Mediterranean pine and oak forests. Plants are characterized by much reduced leaves and an overall violet colour; its vegetative parts contain low levels of photosynthetic pigments. A recent study showed that plants have a reduced photosynthetic activity and are specifically associated with different *Russula* sp. clades, a group of fungi mycorrhizal on surrounding trees (Girlanda *et al*. 2006). This suggested a partial mycoheterotrophy: through mycorrhizal fungal connection, mycoheterotrophic plants gain access to a large pool of organic carbon and are therefore sometimes considered as *cheating* parasites with respect to the interconnecting fungus and the surrounding photoautotrophs (Taylor *et al*. 2002). This unusual dual (photosynthetic and mycoheterotrophic) carbon-gaining strategy of *L. abortivum*, termed *mixotrophy* (Selosse *et al*. 2004), is often mirrored by frequent shifts to an autogamous mating system. The use of microsatellite markers may thus help to understand the mating system of *Limodorum* spp., and how the resulting patterns of gene flow are related to the evolution of mycorrhizal specificity.

Here, we report the development of microsatellite loci and their application to three *L. abortivum* populations: two populations from southern Italy (Vesuvio National Park-Italy1 and Cilento National Park-Italy2) with 20 and 15 individuals, respectively, and one from southern France (Restinclières near Montpellier-France) with 22 individuals.

Genomic DNAs were isolated from floral or rhizome tissue of five orchid species (including *L. abortivum*) using the Plant DNeasy Mini kit (QIAGEN). DNAs of the five species were gel quantified and pooled in equimolar amounts. Here, we consider only loci developed for *L. abortivum*.

A total of 100 μg of pooled genomic DNA was provided to Genetic Identification Services, where enrichment and

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cloning were carried out as described previously (Jones *et al*. 2002). In brief, simultaneous partial digestion with *Rsa*I, *Hae*III, *Bsr*BI, *Pvu*II, *Stu*I, *Sca*I and *Eco*RV (New England BioLabs) was carried out followed by adapter ligation. Biotinylated $CA_{(8)}$, $GA_{(8)}$, $ATG_{(8)}$ and $TAGA_{(8)}$ oligos were used for enrichment and capture of 300–750-bp fragments on magnetic beads (CPG, Inc.). Adapters were removed by digestion with *Hin*dIII following by ligation into pUC19 and transformation of DH5α cells. Four libraries, one for each motif, were sent to SeqWright Inc. for colony picking and insert sequencing.

In total, 768 colonies were sequenced. Primers were designed for promising sequences using PRIMER 3 program (Rozen & Skaletsky 2000) with optimum primer length of 23 bp and T_m of 62 °C. To determine the origins of prospective loci, amplification from seven DNAs representing the five orchid species was attempted using an initial denaturation at 96 °C for 2 min followed by 35 cycles of 92 °C for 20 s, 58 °C for 30 s and 72 °C for 30 s and final elongation at 72 °C for 10 min, with a total reaction of 10 µL containing 0.125 µL Sigma JumpStart RED*Taq* polymerase, 200 µM dNTPs 2.15 mM MgCl, 10 mM Tris-HCl, 50 mM KCl, 0.001% gelatin (w/v).

Thirty primer pairs were initially selected and further screened. Eight individuals from the three different *L. abortivum* populations were used for a preliminary test of polymorphism following optimization of polymerase chain reaction (PCR) conditions for each locus. Total plant DNA was extracted from 100 mg of frozen (or silica gel dried) leaf samples as above. PCR amplifications were performed in 15-µL final volume containing approximately 15–20 ng template DNA, 1× *Taq* buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 1.5 mM $MgCl_2$), 200 µM of each dNTP, 2 pmol of each primer and 0.5 U *Taq* polymerase (Pharmacia Amersham Biotech).

The amplifications were carried out with a MyCycler gradient (Bio-Rad) under the following conditions: 3 min at 94 °C, *n* cycles composed of 30 s denaturing at 94 °C, 1 min at the annealing temperature, 2-min extension at 72 °C, plus an extra 10-min final extension at 72 °C. The annealing temperature was set within a gradient ranging ±4 °C from the estimated primer T_m. The optimal annealing temperature and amplification cycle numbers are given in Table 1 for each primer.

All loci displaying more than one band or without polymorphism among individuals were excluded from further analysis. We have developed 11 polymorphic dinucleotide and trinucleotide microsatellite motifs of repeat length five or greater, with a clear and reproducible amplification signal and no multiple banding patterns (Table 1). Amplification products were cycle sequenced from both directions with a BigDye version 3.1 terminator kit and visualization on an ABI PRISM 3130 Genetic Analyser (Applied Biosystems), and perfectly matched sequences from the cloned genomic fragments used for primer design.

For population analysis, the PCR products were amplified with 6-FAM or HEX end–labelled primers (Table 1). Allelic sizes were then estimated with GENEMAPPER version 3.7, using an ABI PRISM 3130 Genetic Analyser (Applied Biosystems) and custom ROX (500) or LIZ (500) labelled size standards.

The following parameters were calculated in ARLEQUIN 3.01 (Excoffier *et al*. 2005): number of alleles per locus, observed and expected heterozygosities, Hardy–Weinberg equilibrium and linkage disequilibrium.

Overall, the number of alleles per locus ranged from one to six (Table 1). The mean number of alleles per locus was higher in the Italy1 population (3.0) than in the Italy2 population (2.0) and in the France population (1.6).

Observed and expected heterozygosities ranged from 0.000 to 1.000 and from 0.492 to 0.797, respectively, in the Italy1 population, from 0.000 to 1.000 and from 0.517 to 0.806 in the Italy2 population, and from 0.000 to 1.000 and from 0.511 to 0.799 in the French population.

Several loci showed significant deviation from Hardy–Weinberg equilibrium (see Table 1). In particular, in the Italy1 population (where all loci were polymorphic), significant deviations from Hardy–Weinberg equilibrium ($P < 0.05$) were detected for all loci except SW1-78 and SW1-76.

All examined individuals were successfully genotyped. In the three populations, the software MICRO-CHECKER version 2.2.3 (van Oosterhout *et al*. 2004) suggests the presence of null alleles for some loci. However, among those, only locus SW2-362, where a single allele was fixed within populations and polymorphic between populations, consistently showed evidence for a null allele in all three examined populations (see Table 1).

Potential linkage ($P < 0.05$) was recorded among loci when analysing the single populations but only SW1-120 and SW2-185 were consistently linked in all three populations.

The low or zero level of observed heterozygosity and the marked differences in within-population genetic diversity among regions (particularly the low genetic diversity in the French population) is likely to be a consequence of the high rate of vegetative multiplication and autogamous pollination in addition to other historic and demographic factors (bottlenecks, founder effects, isolation by distance).

We also tested for cross-species amplification of the 11 *L. abortivum* primers in the closely related *Limodorum brulloi* and *Limodorum trabutianum* and in *Epipactis helleborine* using the same PCR conditions as described above. All microsatellite loci successfully cross-amplified these species. These newly developed microsatellite loci are therefore potentially useful markers for investigating both the mating system and habitat connectivity in the genus *Limodorum* and the nature of its specific mycorrhizal interactions in deeper detail.

Table 1 Characteristics of 11 polymorphic loci in *Limodorum abortivum*: T_a, annealing temperature; N_a, number of allele; H_O, observed heterozygosity; H_E, expected heterozygosity; TOT$_a$, total number of alleles per locus; †dye linked with forward primer in each case. A 'Yes' in the MC column indicates a warning of null alleles by the software MICRO-CHECKER; *Locus with significant departure from Hardy–Weinberg equilibrium

Locus	Primer sequence 5′–3′	Repeat motif	Dye†	T_a (°C)	PCR cycles	Size range (bp)	Italy1				Italy2				France					GenBank Acc. no.
							N_a	H_O	H_E	MC	N_a	H_O	H_E	MC	N_a	H_O	H_E	MC	TOT$_a$	
SW1-144	F: TGCCATAAGATTTTCAGCAACAA R: TCATATGCAACAAATGACCATAAAA	$(GAT)_{12}$	FAM	60	35	233–266	4	0.050*	0.716	Yes	2	0.466	0.577	—	1	—	—	—	5	EU302477
SW1-60	F: AAAGGGACCACTGGGTTAGGTTA R: CTCATCCCTCCTTATGCACTGTT	$(GT)_{14}$	HEX	60	35	200–226	3	0.100*	0.674	Yes	1	—	—	—	1	—	—	—	3	EU302478
SW1-78	F: ATTGATGATGAAGTGGATGTGGA R: TCTGGAAAACTTCGATCCCATAA	$(GT)_{17}$	HEX	63	35	297–301	2	0.200	0.633	—	3	0.000*	0.806	Yes	1	—	—	—	3	EU302479
SW2-215	F: GGCTGACCTAAGAACCCAAGAAT R: AACCCTAAATGGGAGGAAGCATA	$(GT)_9$CTATGTAT $(GT)_6$	FAM	61	35	137–153	2	0.150*	0.675	Yes	2	1.000*	0.517	—	1	—	—	—	3	EU302480
SW2-152	F: ATATTTGACCGTTGGGGTGGT R: TTGTGTGGCTTGATCTCACTTGT	$(GT)_{14}$	FAM	61	35	249–263	6	0.150*	0.797	Yes	1	—	—	—	1	—	—	—	6	EU302481
SW1-120	F: AATCCGTCCAAACTCAAACATTG R: GTTGAAGATGCCGTACGGAAAAG	$(CTA)_7$	FAM	60	30	210–225	3	1.000*	0.558	—	2	1.000*	0.517	—	2	1.000*	0.511	—	3	EU302482
SW2-185	F: CGCACTCATGAAAAATCCAAGTA R: ACATCCCTTCAGGTTCCATACAC	$(GT)_{21}$	FAM	65	30	278–312	5	1.000*	0.661	—	4	0.733*	0.737	—	6	1.000*	0.799	—	10	EU302483
SW1-71	F: GAGAGAATGGATGCGTGAGTCTT R: AGGTGCGGTTCTTTTCTCTCTCTT	$(CA)_{14}$	FAM	65	35	169–185	2	0.800*	0.492	—	2	1.000*	0.517	—	1	—	—	—	2	EU302484
SW1-76	F: AGGATGCAGACTGTTGTCTCTCA R: TCACTGGCTGATGGAGTTCACTA	$(GT)_{29}$	HEX	65	35	188–204	2	0.100	0.537	Yes	1	—	—	—	1	—	—	—	3	EU302485
SW2-362	F: TGCATTCAAAATCCCAAGTGATA R: GATCACCATTCAAAAGCGAAGAG	$(GT)_{25}$	FAM	65	35	278–280	2	0.000*	0.684	Yes	2	0.000*	0.634	Yes	2	0.000*	0.761	Yes	2	EU302486
SW1-171	F: AAGTCCAGAATGTTCGATAAACAAA R: CAAGGTCGTGGGATTATAAGTGG	$(CTA)_6$	HEX	63	30	269–281	2	1.000*	0.512	—	2	1.000*	0.517	—	1	—	—	—	2	EU302487

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References

Excoffier L, Laval G, Schneider S (2005) ARLEQUIN (version 3.0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online*, **1**, 47–50.

Girlanda M, Selosse MA, Cafasso D *et al*. (2006) Inefficient photosynthesis in the Mediterranean orchid *Limodorum abortivum* is mirrored by specific association to ectomycorrhizal Russulaceae. *Molecular Ecology*, **15**, 491–504.

Jones KC, Levine KF, Banks JD (2002) Characterization of 11 polymorphic tetranucleotide microsatellites for forensic applications in California elk (*Cervus elaphus canadensis*). *Molecular Ecology Notes*, **2**, 425–427.

van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 435–438.

Rozen S, Skaletsky H (2000) PRIMER 3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, New Jersey.

Selosse MA, Faccio A, Scappaticci G, Bonfante P (2004) Chlorophyllous and achlorophyllous specimens of *Epipactis microphylla* (Neottieae, Orchidaceae) are associated with ectomycorrhizal septomycetes, including truffles. *Microbial Ecology*, **47**, 416–426.

Taylor DL, Bruns TD, Leake JR, Read DJ (2002) Mycorrhizal specificity and function in myco-heterotrophic plants. In: *Mycorrhizal Ecology* (eds Sanders I, van der Heijden MGA), *Ecological Studies*, **157**, 375–413. Springer, Berlin, Germany.