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PRIMER NOTE

CHARACTERIZATION OF MICROSATELLITE LOCI IN THE LICHEN FUNGUS *LOBARIA PULMONARIA* (LOBARIACEAE)[1]

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- *Premise of the study:* Microsatellite loci were developed for the threatened haploid lichen fungus *Lobaria pulmonaria* to increase the resolution to identify clonal individuals, and to study its population subdivision.
- *Methods and Results:* We developed 14 microsatellite markers from 454 DNA sequencing data of *L. pulmonaria* and tested for cross-amplification with *L. immixta* and *L. macaronesica*. The number of alleles per locus ranged from two to 23. Nei's unbiased gene diversity, averaged over loci, ranged from 0.434 to 0.517 in the three studied populations.
- *Conclusions:* The new markers will increase the genetic resolution in studies that aim at disentangling clones in *L. pulmonaria* and may be useful for closely related species within *Lobaria* sect. *Lobaria*.

Key words: Ascomycetes; Ascomycota; lichen fungi; *Lobaria pulmonaria*; microsatellites; population subdivision.

Lobaria pulmonaria (L.) Hoffm. (Lobariaceae, Peltigerales) is a widely distributed lichen in the northern hemisphere and afro-temperate forests in South Africa. In central Europe, the species has faced a severe decline in the past decades, and the species is therefore of conservation concern. In the past decade, *L. pulmonaria* has become a model species for the population biology and conservation biology of lichens (Scheidegger and Werth, 2009). Eight microsatellite markers have thus far been published for the lichen fungus *L. pulmonaria*, a species that mainly reproduces clonally (Dal Grande et al., 2012; Werth and Scheidegger, 2012). Here, we develop 14 additional microsatellite markers to increase the genetic resolution for detailed studies of population subdivision and the reproductive system of this species, and we test for cross-amplification with two Macaronesian endemics closely related to *L. pulmonaria*—*L. immixta* Vain. and *L. macaronesica* C. Cornejo & Scheid.

METHODS AND RESULTS

We collected thallus fragments from 190 individuals from São Miguel Island, Azores (SM1: 37.85132°N, 25.78249°W), and from El Hierro, Canary Islands (SH2: 27.74317°N, 17.98651999°W; SH3: 27.73292°N, 18.01134°W) (see Appendix 1 for voucher information). Samples were air-dried after collection and stored at −20°C until DNA extraction using the DNeasy Plant Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The fungus *L. pulmonaria* was grown in axenic culture from ascospores (voucher: GB1-10; 36.69658°N, 5.02322°W), and total genomic DNA isolated from the axenic culture was used for 454 pyrosequencing following standard protocols on a GS FLX instrument (Roche, Schlieren, Switzerland) performed at Microsynth (Balgach, Switzerland). The data amounted to 233 260 reads of an average length of 313.8 bases, in total 73 171 881 bases.

MSATCOMMANDER version 0.8.2 (Faircloth, 2008) was used to find di-, tri-, tetra-, penta-, and hexanucleotide repeats and design primers with Primer3 (Rozen and Skaletsky, 2000) using default values (annealing temperature [T_a] = 60°C, GC content 35–75%, primer length 19 bp) based on the 454 database. A total of 478 contigs contained microsatellite repeats (43 di-, 359 tri-, 37 tetra-, 11 penta-, and 28 hexanucleotides). For 304 of these contigs, flanking regions allowed primer design. Subsequently, 16 loci were tested, each with more than 11 repeats. Fourteen of these loci amplified successfully, and polymorphism was assessed based on 99 thalli of *L. pulmonaria*, 59 of *L. macaronesica*, and 32 of *L. immixta*. Multiplex PCR reactions were carried out in 5 µL reaction volumes using the M13 method (Schuelke, 2000), adding 1 µL of primer mix containing all loci to be labeled with the same fluorescent dye (primer without M13-tail: 0.15 µM, M13-tailed primer: 0.01 µM, dye-labeled M13 primer: n × 0.15 µM, n = number of loci in multiplex), 2.5 µL Jump-Start *Taq* ReadyMix (Sigma-Aldrich, Buchs, Switzerland), 0.5 µL of genomic DNA, and 1 µL of ddH_2O. For primer sequences, see Table 1. PCR amplifications used an initial denaturation at 94°C for 5 min; followed by 30 cycles of 30 s at 94°C, 45 s at the annealing temperature of 60°C, 45 s at 72°C; followed by eight cycles of 30 s at 94°C, 45 s at 53°C, 45 s at 72°C to incorporate the dye-labeled M13 primer (5′-TGTAAAACGACGGCCAGT-3′); and a final extension at 72°C for 60 min.

Fragment analysis was performed after pooling PCR products labeled with four dyes, using GeneScan-500 LIZ (Life Technologies, Rotkreuz, Switzerland) as an internal size standard on a 3130xl Genetic Analyzer (Life Technologies). Genotyping was performed with GeneMapper version 3.7 (Life Technologies). Polymorphism was determined using our own code (available upon request) in R (R Development Core Team, 2011).

Of the 14 loci assessed, 13 amplified and were polymorphic in *L. pulmonaria*, eight in *L. macaronesica*, and seven in *L. immixta.* None of the loci amplified within a culture of *Dictyochloropsis reticulata*, the green-algal photobiont of *L. pulmonaria*. The number of alleles per locus ranged from two to 23, and the maximum gene diversity was 0.846 (Table 2). Eight of the new markers and five of the previously published markers had 10 or more alleles in the studied individuals. Hence, the resolution for future studies will be increased. Moreover, with seven and eight loci working for *L. macaronesica* and *L. immixta*, the new markers appear to be useful for population genetic studies of closely related species in *Lobaria* sect. *Lobaria*.

CONCLUSIONS

By increasing the marker resolution, the newly developed polymorphic microsatellite loci will allow us to perform detailed

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TABLE 1. Overview of the microsatellite loci designed for the lichen fungus *Lobaria pulmonaria*.

Locus	Primer name	Primer sequences (5′–3′)[a]	T_a (°C)	Repeat motif	Dye	Size (bp)	Multiplex	GenBank accession no.
LPu04843	M13-LPu04843F	F: TGTAAAACGACGGCCAGTACCTACGAAGCCAAGGTCG	60	$(AAG)_{16}$	PET	304–389	1	JX126841
	LPu04843R	R: CATCGTCAGGCTTGAGCAC	60					
LPu08412	M13-LPu08412F	F: TGTAAAACGACGGCCAGTACACAAGGGCTCCGGTAAG	60	$(CT)_{14}$	PET	220–236	1	JX126842
	LPu08412R	R: ACCAGTCGGAAACCAGGAC	60					
LPu17457	M13-LPu17457F	F: TGTAAAACGACGGCCAGTTGAGGTCAGGAAGCACCG	60	$(CTGCTT)_{12}$	VIC	182–294	1	JX126845
	LPu17457R	R: GGAGACACTCGTAGCCACC	60					
LPu26427	M13-LPu26427F	F: TGTAAAACGACGGCCAGTGCAACGGGAGTATATCGGC	59	$(CTT)_{14}$	VIC	344–389	1	JX126846
	LPu26427R	R: CGCGAAGCGTATGATAAGAGG	60					
LPu32425	LPu32425F	F: ATACCGAATACCGCGTCCC	60	$(AAG)_{18}$	NED	109–336	1	JX126847
	M13-LPu32425R	R: TGTAAAACGACGGCCAGTCCTGCTGCCTTGTACGAC	60					
LPu39713	M13-LPu39713F	F: TGTAAAACGACGGCCAGTGCTTACCAGAAGGTGGGTC	60	$(GCT)_{19}$	FAM	226–428	1	JX126852
	LPu39713R	R: TCCTTCTAATCCGAACCCTG	57					
LPu40211	M13-LPu40211F	F: TGTAAAACGACGGCCAGTCGGATGATGGCAAACGG	60	$(CTT)_{13}$	FAM	240–273	1	JX126854
	LPu40211R	R: AACGGACCCTGGGATTTCG	60					
LPu34888	LPu34888F	F: TCGGACGATGTGGGAATGG	60	$(ATC)_{14}$	FAM	315–360	2	JX126849
	M13-LPu34888R	R: TGTAAAACGACGGCCAGTGGCTGAGTCACTTGGTTGC	60					
LPu38061	LPu38061F	F: ATTCTGGAAACCCGGACTG	58	$(AG)_{14}$	FAM	261–272	2	JX126851
	M13-LPu38061R	R: TGTAAAACGACGGCCAGTATGCACAGCAGGTCAAACG	60					
LPu37451	LPu37451F	F: CGCGCCTTTGAACACCTG	60	$(AGT)_{13}$	NED	367–445	2	JX126850
	M13-LPu37451R	R: TGTAAAACGACGGCCAGTCTCCGCTGGCAAATAACCG	60					
LPu39912	LPu39912F	F: CATCGTCAGGCTTGAGCAC	60	$(CTT)_{16}$	NED	303–388	2	JX126853
	M13-LPu39912R	R: TGTAAAACGACGGCCAGTACCTACGAAGCCAAGGTCG	60					
LPu13707	LPu13707F	F: GGTGGAAACAGAAGGTGCG	60	$(ACT)_{15}$	PET	165–516	2	JX126843
	M13-LPu13707R	R: TGTAAAACGACGGCCAGTCGCCAACAGCTGGATTGAG	60					
LPu30668	LPu30668F	F: GGGTTAGGGCATGGATTGC	59	$(AG)_{13}$	NED	198–200	2	JX126848
	M13-LPu30668R	R: TGTAAAACGACGGCCAGTAGCCCAACTGACCTGGATG	60					
LPu14122	LPu14122F	F: CACCGCTGGAATAGGTACG	58	$(ATCAGT)_{11}$	PET	322–364	2	JX126844
	M13-LPu14122R	R: TGTAAAACGACGGCCAGTAATTACGGCCGGGATCAGG	60					

Note: T_a = annealing temperature.
[a]The M13-sequence is underlined.

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TABLE 2. Polymorphism in 14 new and eight previously published microsatellite loci developed for the tree lungwort *Lobaria pulmonaria*, and cross-amplified for its close relatives *L. immixta* and *L. macaronesica*.[a]

	Total		*L. pulmonaria* SH2 (N = 54)		*L. pulmonaria* SH3 (N = 37)		*L. pulmonaria* SM1 (N = 8)		*L. macaronesica* SM1 (N = 56)		*L. immixta* SM1 (N = 32)	
Locus[b]	N	A	A	H_e	A	H_e	A	H_e	A	H_e	A	H_e
LPu04843	190[Υ]	19	9	0.831	10	0.785	2	0.250	5	0.702	1	0.000
LPu08412	190	5	4	0.539	4	0.685	3	0.607	—	—	—	—
LPu17457	190	10	4	0.523	3	0.419	4	0.750	—	—	2	0.272
LPu26427	96	5	1	0.000	1	0.000	2	0.250	2	0.198	2	0.175
LPu32425	190	10	2	0.201	2	0.105	4	0.821	3	0.482	2	0.516
LPu39713	190	7	2	0.331	2	0.450	1	0.000	3	0.395	2	0.121
LPu40211	96	10	6	0.652	3	0.520	3	0.607	2	0.071	3	0.567
LPu34888	190	10	3	0.461	2	0.054	5	0.893	2	0.226	6	0.756
LPu38061	190	4	3	0.542	2	0.054	3	0.667	1	0.000	—	—
LPu37451	190	14	10	0.846	6	0.668	4	0.821	—	—	—	—
LPu39912	190	19	9	0.835	10	0.785	2	0.250	5	0.705	1	0.000
LPu13707	190	23	6	0.440	4	0.683	3	0.464	11	0.615	2	0.389
LPu30668*	190	2	—	—	—	—	1	0.000	2	0.333	2	0.533
LPu14122*	190	6	—	—	—	—	4	0.750	1	0.000	2	0.272
Average		10.9	4.2	0.517	3.5	0.434	2.9	0.509	2.6	0.339	1.8	0.327
LPu03	190	3	2	0.075	1	0.000	1	0.000	1	0.000	—	—
LPu09	190	10	4	0.519	5	0.630	3	0.750	2	0.335	4	0.587
LPu15	190	16	4	0.623	5	0.714	3	0.679	10	0.867	2	0.121
LPu23	190	11	3	0.479	3	0.160	1	0.000	1	0.000	6	0.552
LPu24	190	5	2	0.042	1	0.000	1	0.000	1	0.000	1	0.000
LPu25	190	25	14	0.863	7	0.784	6	0.929	1	0.000	4	0.504
LPu28	190	40	13	0.898	9	0.786	3	0.679	18	0.858	2	0.175
MS4	190	3	2	0.082	2	0.056	1	0.000	—	—	—	—
Average		14.1	5.5	0.448	4.1	0.391	2.4	0.380	4.9	0.294	3.2	0.323

Note: A = number of alleles; H_e = Nei's unbiased gene diversity; N = total number of samples analyzed.

[a] Populations used in the study: SM1 = São Miguel, Azores, 37.85132°N, 25.78249°W; SH2 = El Hierro, Canary Islands, 27.74317°N, 17.98651999°W; and SH3 = El Hierro, Canary Islands, 27.73292°N, 18.01134°W.

[b] Loci printed in italics have been published previously.

* Marker was not analyzed for populations SH2 and SH3 in *L. pulmonaria*.

[Υ] Overall number of samples includes three additional thalli of *L. macaronesica* from site SH2.

studies of the reproductive system of *L. pulmonaria* (and its close relatives), and hence aid our understanding of the population biology of a fascinating lichen symbiosis.

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APPENDIX 1. Herbarium vouchers of *Lobaria* species used in this study residing in the personal herbarium of Christoph Scheidegger at WSL. All samples analyzed are stored frozen at −20°C.

Site	Species	Voucher	Record
SM1	*L. immixta*	SM1-01a	14703
SM1	*L. macaronesica*	SM1-01c	14705
SM1	*L. pulmonaria*	SM1-01e	14707
SH2	*L. pulmonaria*	SH2-01a	10746
SH3	*L. pulmonaria*	SH3-02b	11170
GB1	*L. pulmonaria*	GB1-10	10164

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