Characterization of Microsatellite Loci in the Himalayan Lichen Fungus Lobaria pindarensis (Lobariaceae)

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Source: Applications in Plant Sciences, 2(5)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1300101

Applications in Plant Sciences 2014 2(5): 1300101

Primer Note

Characterization of microsatellite loci in the Himalayan lichen fungus *Lobaria pindarensis* (Lobariaceae)[1]

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- *Premise of the study:* Microsatellite loci were developed for the rare, Himalayan, endemic haploid lichen fungus, *Lobaria pindarensis*, to study its population subdivision and the species' response to forest disturbance and fragmentation.
- *Methods and Results:* We developed 18 polymorphic microsatellite markers using 454 pyrosequencing data and assessed them in 109 individuals. The number of alleles per locus ranged from three to 11 with an average of 6.9. Nei's unbiased gene diversity, averaged over loci, ranged from 0.514 to 0.685 in the three populations studied. The cross-amplification success with related species (*L. chinensis*, *L. gyrophorica*, *L. isidiophora*, *L. orientalis*, *L. pulmonaria*, *L. spathulata*, and *Lobaria* sp.) was generally high and decreased with decreasing relationship to *L. pindarensis*.
- *Conclusions:* The new markers will allow the study of genetic diversity and differentiation within *L. pindarensis* across its distribution. Moreover, they will enable us to study the effects of forest management on the genetic population structure of this tree-colonizing lichen and to carry out population genetic studies of related species in East Asia.

Key words: Ascomycetes; Himalayas; lichen-forming fungi; *Lobaria pindarensis*; microsatellites; population subdivision.

Lobaria pindarensis Räsänen (Lobariaceae, Peltigerales) is a foliose lichen species known from mountain forests and open woodlands in the Himalayas of Bhutan, India, and Nepal. The lichen thallus is haploid and it mainly disperses with vegetative propagules, but sexual reproduction with ascospores can also occur (Scheidegger et al., 2010). The lichen disperses locally, thus sharing ecological traits with *L. pulmonaria* (L.) Hoffm. (Scheidegger and Werth, 2009; Scheidegger et al., 2012). Although microsatellite markers are available for *L. pulmonaria* (Dal Grande et al., 2010; Werth et al., 2013), only three markers (LPu32425, LPu40211, and LPu34888) published by Werth et al. (2013) reveal small, multiple bands when amplified with *L. pindarensis.* All other published markers do not amplify with *L. pindarensis*. Here, we develop microsatellite markers to study the impact of land use and habitat fragmentation on gene flow of this dispersal-limited lichen (Scheidegger et al., 2010).

[1]Manuscript received 23 December 2013; revision accepted 6 February 2014.

The authors thank the Genetic Diversity Centre, ETH Zurich, for technical assistance; the Swiss National Science Foundation (grant JRP IZ70Z0_131338/1 to C.S.); the European Commission (Marie Curie Actions "Lichenomics" to S.W.); and the Department of National Parks and Wildlife Conservation, Ministry of Forest and Soil Conservation, Government of Nepal, and the National Trust for Nature Conservation, Nepal, for providing collecting permits in the study areas.

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doi:10.3732/apps.1300101

METHODS AND RESULTS

Ten specimens of *L. pindarensis*, collected in two valleys in Nepal (Table 1; Manaslu Conservation Area [MCA] and Sagarmatha National Park [SNP]), were used for total DNA extraction with the QIAGEN Plant Mini Kit (QIAGEN, Hilden, Germany). Subsequently, whole genome 454 pyrosequencing of pooled DNA samples was performed using a Roche GS FLX sequencer to generate a sufficient number of microsatellite loci. Library preparation and sequencing were performed by Microsynth (Balgach, Switzerland). Shotgun libraries were prepared using the GS FLX Titanium Rapid Library Preparation Kit (Roche Diagnostics, Basel, Switzerland), while Microsynth provided barcode adapters. Out of a 1/4th run, we obtained 233,260 reads of an average length of 314 bases for a total of 73,191,881 bases. The unassembled sequences were screened for all possible sequence motifs of di-, tri-, tetra-, and pentanucleotide microsatellites using Primer3, as implemented in MSATCOMMANDER version 1.0.2 alpha (Rozen and Skaletsky, 2000; Faircloth, 2008). Microsatellites with motifs repeated at least eight times (for dinucleotides) or six (for all others) were chosen. For each locus, primer pairs were developed with MSATCOMMANDER using the default parameters.

Using all reads, MSATCOMMANDER found 1021 primer pairs that fulfilled the default primer parameters. Subsequently, 656 pairs were discarded either because they contained unfavorable secondary structure, primer dimer formation, or mononucleotide repeats in the flanking region, or because they were duplicates, which were detected after alignment using CLC DNA Workbench 5 (CLC bio, Aarhus, Denmark). The remaining 365 sequences were verified one by one using ntBLAST with the megablast option (http://www.ncbi.nlm.nih.gov/blast) to exclude those that were highly similar to algae, plants, or microorganisms that are often present in environmental samples. To test for cross-amplification with the photobiont of *L. pindarensis*, *Dictyochloropsis reticulata* (Tschermak-Woess) Tschermak-Woess, PCRs of the remaining 116 primer pairs (including 44 di-, 65 tri-, and 7 tetranucleotides) were run using DNA from an axenic culture of *D. reticulata* (Dal Grande et al., 2010, 2012; Widmer et al., 2010, 2012). The 56 loci that produced positive PCR reactions were excluded from further analyses because they were

Table 1. Characteristics of 18 polymorphic microsatellite loci developed for *Lobaria pindarensis*[a] and screened in 109 individuals.

	Total		MCA (n = 36)		SNP (n = 43)		KCA (n = 30)	
Locus	N	A	A	H_e	A	H_e	A	H_e
Lpi01	106	4	3	0.643	4	0.615	3	0.587
Lpi02	109	5	4	0.652	4	0.568	4	0.524
Lpi03	109	5	3	0.160	4	0.295	4	0.582
Lpi04	108	8	6	0.635	7	0.762	6	0.800
Lpi05	109	7	4	0.162	5	0.666	6	0.715
Lpi06	109	7	4	0.463	5	0.636	6	0.747
Lpi07	105	9	7	0.567	6	0.681	5	0.690
Lpi08	108	5	3	0.565	5	0.741	5	0.594
Lpi09	109	10	8	0.700	5	0.260	5	0.556
Lpi10	109	11	5	0.754	8	0.856	8	0.779
Lpi11	108	8	5	0.459	3	0.671	6	0.820
Lpi12	109	4	3	0.256	3	0.456	4	0.724
Lpi13	109	7	7	0.752	5	0.617	7	0.726
Lpi14	109	10	6	0.308	7	0.780	7	0.786
Lpi15	109	6	4	0.760	5	0.767	5	0.501
Lpi16	108	10	6	0.816	9	0.791	7	0.788
Lpi17	96	6	4	0.437	5	0.692	6	0.869
Lpi18	81	3	2	0.170	3	0.462	2	0.533
Average		6.944	4.667	0.514	5.167	0.629	5.333	0.685

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

[a] Populations used in the study: MCA = Manaslu Conservation Area, Gorkha District (28°27.641′N, 85°2.803′E); SNP = Sagarmatha National Park, Solukhumbu District (27°48.871′N, 86°43.016′E); KCA = Kanchenjunga Conservation Area, Taplejung District (27°41.546′N, 87°45.607′E). Voucher specimens (collector numbers SD1164_IZ70ZO_131338/1_MCA, SD135_IZ70ZO_131338/1_SNP, and SD268_IZ70ZO_131338/1_KCA) were collected in 2011 and 2012 and were deposited at Tribhuvan University, Kirtipur, Nepal (TUCH), and in the frozen herbarium at the Swiss Federal Research Institute WSL, Birmensdorf, Switzerland.

considered alga-specific rather than fungus-specific. For PCR amplification, forward primers were labeled with an M13 tag (5′-TGTAAAACGACGGCCAGT-3′) (Schuelke, 2000). PCR reactions were performed in a total volume of 10 μL containing 1 μL of ~1–5 ng genomic DNA, 0.5 μL of 5 μM forward and reverse primers, and 1× Type-it Multiplex PCR Master Mix (QIAGEN). All PCRs were performed on Veriti Thermal Cyclers (Life Technologies, Carlsbad, California, USA). The PCR reactions were assessed in a temperature gradient increasing by one-degree increments from 56–61°C, performed with the Type-it Microsatellite PCR Kit (QIAGEN) according to the manufacturer's protocol, and under the following conditions: denaturation for 5 min at 95°C; followed by 33 cycles of 30 s at 95°C, 90 s at 56–61°C, and 30 s at 72°C; then for the M13-tag binding an additional eight cycles of 30 s at 95°C, 90 s at 53°C, and 30 s at 72°C; with a final extension of 30 min at 60°C.

The 60 remaining loci were assessed for amplification for the fungal component of *L. pindarensis* under the same conditions as above and using the total DNA of *L. pindarensis*. Out of these 60 loci, 48 produced specific single products, all at an annealing temperature of 57°C. Polymorphism of the 48 microsatellite loci was initially tested on a subset of seven individuals from three valleys, including three individuals from MCA, three from SNP, and two from Kanchenjunga Conservation Area (KCA), resulting in the detection of 25 polymorphic loci. These 25 loci were then tested in one population of 48 specimens from MCA, resulting in 18 loci with satisfactory amplification.

To characterize these 18 polymorphic *L. pindarensis* loci, PCRs of 109 individuals from three valleys were conducted (Table 1). The PCR protocol used fluorescent forward primers and the reaction was adjusted to the following conditions: 5 min at 95°C; followed by 25 cycles of 30 s at 95°C, 90 s at 57°C, and 30 s at 72°C; with a final extension of 60 min at 60°C. All PCR products obtained were multiplexed (Table 2) and run on a 3130xl DNA Analyzer with a GeneScan 500 LIZ Size Standard (G5 dye set) for fragment analysis (both by Life Technologies). Alleles were sized using GeneMapper version 3.7 (Life Technologies), and the variability of each microsatellite locus was measured by counting the number of alleles and calculating gene diversity using Arlequin version 3.11 (Excoffier et al., 2005). Trinucleotide microsatellites (n = 15) were the most common loci detected among the 18 microsatellite motifs (Table 2). The microsatellite loci produced 3–11 alleles per locus with an average of 6.9, and mean gene diversities over three populations varied from 0.514 to 0.685 (Table 1).

Cross-species amplification of seven closely related species of *Lobaria* (Schreb.) Hoffm. was tested on one specimen of each species (Appendix 1), applying the same PCR conditions established for *L. pindarensis*. All fragments were sequenced according to Cornejo and Scheidegger (2010) except Lpi01 and Lpi05, which were verified on an agarose gel but not sequenced. The transferability was high. Only one locus (Lpi05) did not amplify in *L. chinensis* Yoshim. However, several loci contained insertions within the flanking regions, and in others the microsatellite was disrupted (imperfect or interrupted microsatellite). In some loci, the microsatellite sequences were reduced or disappeared completely, as in Lpi10 in *L. isidiophora* Yoshim. and Lpi16 in *L. gyrophorica* Yoshim., *L. pulmonaria*, *L. spathulata* (Inum.) Yoshim., and *Lobaria* sp. (Fig. 1). In general, the cross-amplification success of Lpi markers decreased with decreasing relationship to *L. pindarensis*, being lowest in *L. pulmonaria* and *Lobaria* sp. (with four and six loci not amplifying, respectively).

CONCLUSIONS

Fungi, algae, and/or cyanobacteria live in close contact within the lichen thallus and hence the manual separation of symbionts for later molecular analyses is technically unfeasible. Therefore, symbiont-specific genetic markers have to be used in population genetic studies of lichens (Widmer et al., 2010). The newly developed, highly variable fungus-specific markers reported here will allow detailed studies on regional genetic differentiation, effects of forest disturbance on genetic diversity, and the contributions of clonal and sexual reproduction in this lichen species. Moreover, the flanking regions of the microsatellites will be used for sequence analyses in future phylogenetic studies of related taxa of the genus *Lobaria*.

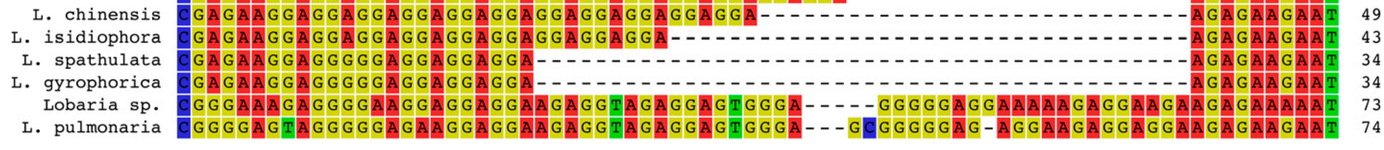

Fig. 1. Alignment of the Lpi16 sequence containing a trinucleotide microsatellite region. The flanking region was excluded from the graphics. This locus was initially developed for *Lobaria pindarensis*. The first four species contain a microsatellite with n > 9 repeats. The following two species have n = 3 repeats and are not considered microsatellites. Finally, in *L. pulmonaria* and *Lobaria* sp. this locus did not evolve a microsatellite sequence.

Table 2. Overview of the microsatellite loci developed for the lichen fungus *Lobaria pindarensis*.

Locus	Primer sequences (5′–3′)	T_a (°C)	Repeat motif	Fluorescent dye	Allele size range (bp)	Multiplex[a]	GenBank accession no.
Lpi01	F: TTTGCGGTATAATCGACGCG	57	$(CGT)_{10}$	FAM	255–264	3	KF318149
	R: CACACGACGTCACTGTCTTG	57					
Lpi02	F: GGGATTGCAGAGGGATTTGC	57	$(GAT)_{10}$	VIC	164–182	2	KF318150
	R: CATTTCCACTCCGTCAACCC	57					
Lpi03	F: CCCATTATGCCATGTCCTGC	57	$(CTT)_9$	FAM	346–358	2	KF318151
	R: AGGAGGATAGTGATGGTCGC	57					
Lpi04	F: CAGAACTAGGCCGCATTGTG	57	$(GTT)_{10}$	VIC	89–122	1	KF318152
	R: TGAGCACGTTGTTTCACTCG	57					
Lpi05	F: GACTGGCCGGCAATTAGTAG	57	$(CTT)_9$	VIC	111–154	2	KF318153
	R: TGAAAGGGTCTTGTTGCGAAC	57					
Lpi06	F: GCGTATTGGAGATGGCGATG	57	$(GAT)_9$	PET	148–194	2	KF318154
	R: GGCCTGAACATGGAATGCAG	57					
Lpi07	F: CAAGCCACCCACTCATTTCG	57	$(CTT)_{10}$	NED	250–277	1	KF318155
	R: GCTACACGTTTGGGTCTTCC	57					
Lpi08	F: CTTTCCTCGCTGCAGAACTG	57	$(ATC)_9$	FAM	113–131	1	KF318156
	R: GGAAGGCAAAGGAAGATGGTG	57					
Lpi09	F: AAATTTCCTCCGGCTGTTGG	57	$(ATC)_{18}$	PET	254–316	3	KF318157
	R: TCACTCGACGAATTTCCACC	57					
Lpi10	F: AAGAGAGGTATGGGCGGAAC	57	$(AAG)_9$	VIC	236–283	1	KF318158
	R: ACAGATTCGGAGTGGGAAGG	57					
Lpi11	F: CGTAATCTTCTGGCCTGCTG	57	$(CT)_9$	PET	142–179	1	KF318159
	R: CCAGCTCCGGTATGATGTTG	57					
Lpi12	F: GGGTGCTTTCGTTCCATTCC	57	$(CT)_{11}$	NED	154–166	1	KF318160
	R: TGGTTTCATGGTGGAGAGGG	57					
Lpi13	F: ACAAAGGCCAGACAACAACC	57	$(AGC)_9$	NED	222–242	2	KF318161
	R: GCTGTGACTGTTGCTGTGAC	57					
Lpi14	F: CTTCCCAGGCAGTATCCCTC	57	$(CTT)_{11}$	NED	227–291	3	KF318162
	R: ATCGTGCTCTGTTCTACCGG	57					
Lpi15	F: GTTTGATAGAGCAGGCGACG	57	$(CTT)_9$	FAM	98–119	2	KF318163
	R: CTATCGCGAATGACTGGCTTC	57					
Lpi16	F: GAATCTTCTGCCCGCACTTC	57	$(AGG)_{12}$	VIC	158–194	3	KF318164
	R: GGTAGCACTTTGAAGGCGTC	57					
Lpi17	F: AATAGTCTCAGCCACTCCCG	57	$(AT)_{11}$	FAM	309–351	1	KF318165
	R: CGTCAAGCAGTTCGAATGGG	57					
Lpi18	F: CCATGTTAAGGCACGGGAAC	57	$(ACG)_{11}$	FAM	374–380	3	KF318166
	R: CCTACTGAGCCGGTGTACTC	57					

Note: T_a = annealing temperature.
[a] Multiplex indicates loci that were mixed in the same capillary electrophoresis run.

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Appendix 1. Cross-amplification of *Lobaria pindarensis* loci with related species of the genus *Lobaria*. Specimens are stored in the personal herbarium of Christoph Scheidegger at WSL. All samples are kept frozen at –20°C.

Species	Voucher	Locality	Geographic coordinates	Lpi01	Lpi02	Lpi03	Lpi04	Lpi05	Lpi06	Lpi07	Lpi08	Lpi09	Lpi10	Lpi11	Lpi12	Lpi13	Lpi14	Lpi15	Lpi16	Lpi17	Lpi18
L. chinensis Yoshim.	CT3/02a	Taiwan	23°28′30.4″N, 120°50′17.0″E	+	+	+	i	0	+	+	+	in	+	+	+	+	+	in	+	+	+
L. gyrophorica Yoshim.	TW2/03_5	Taiwan	24°10′37.1″N, 121°23′18.7″E	+	+	+	i	0	+	+	+	+	+	+	+	+	in	+	–	+	+
L. isidiophora Yoshim.	CT9/03e	Taiwan	24°10′13.2″N, 121°17′05.5″E	0	+	+	i	0	+	+	+	in	–	+	+	+	+	in	+	+	0
L. orientalis (Asahina) Yoshim.	004/15	Russia, Sakhalin	47°38′26″N, 142°33′24″E	0	+	+	i	0	+	+	+	+	+	+	+	+	in	+	+	–	0
L. pulmonaria (L.) Hoffm.	289/1	Russia, Primorsky Krai	43°39′44″N, 134°24′32″E	+	+	+	i	0	–	+	+	in	–	+	+	+	+	0	–	0	0
L. spathulata (Inum.) Yoshim.	001/3	Russia, Sakhalin	47°38′26″N, 142°33′24″E	0	+	+	i	0	+	+	+	+	+	+	+	+	+	+	–	+	in
Lobaria sp.	377/2	Russia, Primorsky Krai	44°57′13″N, 136°30′50″E	0	i	+	+	0	0	+	+	i	in	+	+	+	0	+	–	0	0

Note: + = microsatellite present; – = no microsatellite present; 0 = no PCR product obtained; i = insertion within the flanking region; in = microsatellite interrupted.