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

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CHARACTERIZATION OF MICROSATELLITE LOCI IN THE HIMALAYAN LICHEN FUNGUS *LOBARIA PINDARENSIS* (LOBARIACEAE)

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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).

**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 nBlast 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...
considered alga-specific rather than fungus-specific. For PCR amplification, forward primers were labeled with an M13 tag (5′-CGGCCAGT-3′) and reverse primers, and 1 μL Type-it Multiplex PCR Master Mix (Qiagen). All PCRs were performed on Veriti Thermal Cyclers (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 (Schenk.) 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, and L. sathulata (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.

TABLE 1. Characteristics of 18 polymorphic microsatellite loci developed for Lobaria pindarensis and screened in 109 individuals.

| Locus   | Total N | MCA (n = 36) | SNP (n = 43) | KCA (n = 30) |
|---------|---------|--------------|--------------|--------------|
|         | A | H<sub>e</sub> | A | H<sub>e</sub> | A | H<sub>e</sub> |
| 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 | 4 | 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   | 110 | 10 | 6 | 0.816 | 9 | 0.791 | 7 | 0.788 |
| Lpi17   | 106 | 6 | 4 | 0.457 | 5 | 0.692 | 6 | 0.869 |
| Average | 6.944 | 4.667 | 0.514 | 5.167 | 0.685 |

Note: A = number of alleles; H<sub>e</sub> = Nei’s unbiased gene diversity; n = number of samples per population; N = total number of samples analyzed.

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.

http://www.bioone.org/loi/apps
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 | GenBank accession no. |
|--------|--------------------------|----------|--------------|-----------------|-----------------------|-----------|---------------------|
| Lpi01  | F: TTGGCGTATATAATGCAGGCC
         R: CACAGCACTGCTGCTGCTGCTG | 57       | (CGT)_10     | FAM             | 255–264              | 3         | KF318149            |
| Lpi02  | F: GGATTCGGAGGAGGATTTGCC
         R: CATTCCACCTGCTGCTGCTG | 57       | (GAT)_10     | VIC             | 164–182              | 2         | KF318150            |
| Lpi03  | F: CCCATTATCGATCTCTGCTG
         R: AGGGATTAGTATGCTGCTG | 57       | (CTT)_9      | VIC             | 346–358              | 2         | KF318151            |
| Lpi04  | F: CAGGACTGAGCCCGAGATTTG
         R: TAGACCGATGTTTCCTCTC | 57       | (GTT)_10     | VIC             | 89–122               | 1         | KF318152            |
| Lpi05  | F: GCTGCGGCGCGATGATTTAC
         R: TGAAAGCTGTTGTTGCTGAC | 57       | (CTT)_9      | VIC             | 111–154              | 2         | KF318153            |
| Lpi06  | F: GGTATGATGATGATGATGATG
         R: CCTACTGAGTGATGATGATG | 57       | (GAT)_10     | PET             | 148–194              | 2         | KF318154            |
| Lpi07  | F: CAAGGAGATGATGATGATGAC
         R: AGGATTGATGATGATGATG | 57       | (CTT)_10     | NED             | 250–277              | 1         | KF318155            |
| Lpi08  | F: CCTCCCTGCTGAGATTTAC
         R: GGAGGCTGTTGTTGCTGAC | 57       | (ATC)_9      | FAM             | 113–131              | 1         | KF318156            |
| Lpi09  | F: GAATTCTCTCTCTCTCTCTCT
         R: CTATCGCGAATGACTGCTTC | 57       | (CT)_10      | NED             | 154–166              | 1         | KF318160            |
| Lpi10  | F: AAGGAGATGATGATGATGAC
         R: AGGATTGATGATGATGATG | 57       | (CTT)_9      | VIC             | 236–283              | 1         | KF318158            |
| Lpi11  | F: CGTAATCCTCCGAGATGATGGC
         R: GCAGCTGCGTCTGCTGCTG | 57       | (ATC)_10     | FAM             | 142–179              | 1         | KF318159            |
| Lpi12  | F: GGCTGCTCTCTCTCTCTCTCT
         R: TGTTGCTGCTGCTGCTGCTG | 57       | (CTT)_10     | NED             | 111–154              | 3         | KF318162            |
| Lpi13  | F: ACAAGGCCGAGCGACGACGAC
         R: GCTGCGGCGCGATGATTTAC
         A: AGGGATTAGTATGCTGCTG | 57       | (AGC)_9      | VIC             | 222–242              | 2         | KF318163            |
| Lpi14  | F: CGTCTACCTTCCTCTCTCTCT
         R: ATGTTGCTGCTGCTGCTGCTG | 57       | (CTT)_10     | FAM             | 227–291              | 3         | KF318162            |
| Lpi15  | F: GCTGCTGCTGCTGCTGCTGCTG
         R: CTATCGCGAATGACTGCTTC
         A: AGGGATTAGTATGCTGCTG | 57       | (CTT)_10     | FAM             | 98–119               | 2         | KF318163            |
| Lpi16  | F: GAATCTGCTGCTGCGGATGATG
         R: GGTAGCTGCTGAGGGCTGCCC
         A: AGGGATTAGTATGCTGCTG | 57       | (AGG)_12     | VIC             | 158–194              | 3         | KF318164            |
| Lpi17  | F: CTAGTCTGCTGCTGCTGCTG
         R: GCCACTCTACTACTACTACT
         A: AGGGATTAGTATGCTGCTG | 57       | (ATC)_10     | FAM             | 309–351              | 1         | KF318165            |
| Lpi18  | F: CTAGTCTGCTGCTGCTGCTG
         R: GGTAGCTGCTGAGGGCTGCCC
         A: AGGGATTAGTATGCTGCTG | 57       | (ACG)_10     | FAM             | 374–380              | 3         | KF318166            |

Note: T_a = annealing temperature.

*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 | Lpi18 |
|--------------------|---------|----------------|-------------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| L. chinensis       | CT302a  | Taiwan         | 23°28′30.4″N, 120°50′17.0″E | +     | +     | +     | i     | 0     | +     | +     | +     | +     | +     | in    | +     | +     | +     | +     | +     |
| L. gyrophorica     | TW2/03_5| Taiwan         | 24°10′37.1″N, 121°23′38.7″E | +     | +     | +     | i     | 0     | +     | +     | +     | +     | +     | +     | in    | +     | –     | +     | +     |
| L. isidiophora     | CT9/03e | Taiwan         | 24°10′13.2″N, 121°17′05.5″E | 0     | +     | +     | i     | 0     | +     | +     | +     | +     | +     | +     | in    | –     | +     | +     | +     | 0     |
| L. orientalis      | 004/15  | Russia, Sakhalin| 47°38′26″N, 142°33′24″E  | 0     | +     | +     | i     | 0     | +     | +     | +     | +     | +     | +     | +     | in    | +     | +     | –     | 0     |
| L. pulmonaria      | 289/1   | Russia, Primorsky Krai | 43°39′44″N, 134°24′32″E | +     | +     | +     | i     | 0     | –     | +     | +     | +     | +     | +     | 0     | –     | 0     | 0     | 0     | 0     |
| L. spathulata      | 001/3   | Russia, Sakhalin| 47°38′26″N, 142°33′24″E | 0     | +     | +     | i     | 0     | +     | +     | +     | +     | +     | +     | +     | –     | +     | in    | 0     | 0     |
| Lobaria sp.        | 377/2   | Russia, Primorsky Krai | 44°57′13″N, 136°30′50″E | 0     | i     | +     | +     | 0     | 0     | +     | +     | i     | in    | +     | +     | +     | 0     | +     | –     | 0     |

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