CHARACTERIZATION OF MICROSATellite LOCI IN
lichen-forming fungi of Bryoria section Implexae
(PARMELIaceae)\textsuperscript{1}

OLGA NADYEINA\textsuperscript{2,3,6}, CAROLINA CORNEJO\textsuperscript{2}, CARLOS G. BOLUDA\textsuperscript{2,4},
LEENA MYLLYS\textsuperscript{5}, VÍCTOR J. RICO\textsuperscript{3}, ANA CRESPO\textsuperscript{4}, and CHRISTOPH SCHEIDEGGER\textsuperscript{2}

\textsuperscript{2}Swiss Federal Research Institute WSL, Zürcherstrasse 111, CH-8903 Birmensdorf, Switzerland; \textsuperscript{3}M. H. Kholodny Institute of Botany, Tereschenkivska 2, Kyiv (Kiev) 01601, Ukraine; \textsuperscript{4}Departamento de Biología Vegetal II, Facultad de Farmacia, Universidad Complutense de Madrid, Plaza de Ramón y Cajal s/n, Madrid 28040, Spain; and \textsuperscript{5}Botanical Museum, Finnish Museum of Natural History, FI-00014 University of Helsinki, Helsinki, Finland

\textbf{Premise of the study:} The locally rare, haploid, lichen-forming fungi Bryoria capillaris, B. fuscescens, and B. implexa are associated with boreal forests and belong to Bryoria sect. Implexae. Recent phylogenetic studies consider them to be conspecific. Microsatellite loci were developed to study population structure in Bryoria sect. Implexae and its response to ecosystem disturbances.

\textbf{Methods and Results:} We developed 18 polymorphic microsatellite markers using 454 pyrosequencing data assessed in 82 individuals. The number of alleles per locus ranged from two to 13 with an average of 4.6. Nei’s unbiased gene diversity, averaged over loci, ranged from 0.38 to 0.52. The markers amplified with all three species, except for markers Bt05, Bt15, and Bt18.

\textbf{Conclusions:} The new markers will allow the study of population subdivision, levels of gene introgression, and levels of clonal spread of Bryoria sect. Implexae. They will also facilitate an understanding of the effects of forest disturbance on genetic diversity of these lichen species.

\textbf{Key words:} Ascomycetes; Bryoria implexa; lichen-forming fungi; microsatellites; Trebouxia spp.

The members of Bryoria sect. Implexae are pendent, copiously branched lichens with circumboreal distribution (Brodo and Hawksworth, 1977; Myllys et al., 2011a). They are an important component of the boreal forests (Glavich et al., 2005), and their frequency depends on forest fragmentation (Hilmo and Holien, 2002). These lichen-forming fungi are haploid and disperse with vegetative propagules; sexual reproduction with ascospores is uncommon (Brodo and Hawksworth, 1977). Bryoria sect. Implexae includes seven morphologically and chemically recognized species in Europe (Myllys et al., 2011a), which have different frequency across longitudinal and altitudinal gradients (Hawksworth, 1972; Myllys et al., 2011a). Molecular data confirm the monophyly of the section, although the relationships among the currently recognized species remain poorly understood because phylogenetic analyses suggest that several species are conspecific (Myllys et al., 2011b). Highly variable microsatellite markers of the fungal partner of lichen symbioses (Widmer et al., 2010; Devkota et al., 2014) will be used to study the genetic diversity and differentiation in Bryoria sect. Implexae, to determine the gene flow across and within the currently recognized species, and to assess the impact of land use and habitat fragmentation on population structure of these locally rare and threatened, boreal forest–associated lichens.

\textbf{METHODS AND RESULTS}

Eighty-two specimens representing the three morphologically and chemically characterized species, Bryoria capillaris (Ach.) Brodo & D. Hawksw., B. fuscescens (Gyeln.) Brodo & D. Hawksw., and B. implexa (Hoffm.) Brodo & D. Hawksw., were collected in three regions (Spain, Switzerland, and Finland; Appendix 1). All specimens are deposited in the Lichens Herbarium of the Universidad Complutense de Madrid (MAF-Lich), and duplicates are stored at the Swiss Federal Research Institute WSL at $-20\,^\circ\text{C}$. A subset of 30 specimens was used for total DNA extraction with the MoBio PowerPlant Pro DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California, USA). The pooled DNA was used to create a shotgun multiplex identifier library using the GS FLX Titanium Rapid Library Preparation Kit (Roche Diagnostics, Basel, Switzerland), and Microsynth AG (Balgach, Switzerland) provided the barcode adapters. The library was sequenced on 1/4th of a plate on a Roche 454 Genome Sequencer FLX at Microsynth. We obtained 533,962 reads of an average length of 812 bp (National Center for Biotechnology Information [NCBI] Sequence Read Archive [SRA] accession no. SRR1283191; http://www.ncbi.nlm.nih.gov/sra). The unassembled sequences were screened for di-, tri-, tetra-, and pentanucleotide microsatellites using MSATCOMMANDER 1.0.2 alpha (Rozen and Skaltsky, 1999; Faircloth, 2008), ensuring a minimum repeat length of 8 bp for dinucleotides and 6 bp for all others.

\textsuperscript{1}Manuscript received 25 April 2014; revision accepted 23 May 2014.

\textsuperscript{6}Author for correspondence: olga.nadyeina@wsl.ch
doi:10.3732/apps.1400037
MSATCOMMANDER recovered 6329 primer pairs that fulfilled the default primer parameters among all reads. Of those, 5952 pairs were discarded from further studies because they contained unfavorable secondary structure, primer-dimer formation, monorepeats in the flanking region, or because they were duplicates, which we detected after alignment using CLC Main Workbench 6 (CLC bio, Aarhus, Denmark). Putative sequences of algae, plants, animals, or microorganisms, which are often present in epiphytic samples, were identified and removed using the nBLAST search on http://www.ncbi.nlm.gov.

This inspection resulted in 58 primer pairs used for further analysis, i.e., to test for amplification with the symbiotic partner of these lichen-forming fungi. We used DNA from five axenic cultures of *T. jamesii* (Hildreth & Ahmadjian) *Tréb.* and *T. simplex* (Lindgren et al., 2014): *T. simplex* Friedl & Gärtner (SAG48.88), *T. jamesii* (Hildreth & Ahmadjian) Gärtner (SAG2103), and *T. simplex* Tschermak-Woess (SAG101.80). Forward primers were labeled with an M13 tag (5′-TGTAAAAACGGTGACGCAC-3′) for PCR amplification (Schuelke, 2000). All PCR runs were performed on Veriti Thermal Cyclers (Life Technologies, Carlsbad, California, USA). The PCR reactions were evaluated in a temperature gradient with one-degree steps from 56–61°C. The PCRs performed with the JumpStart REDTaq ReadyMix (Sigma-Aldrich, St. Louis, Missouri, USA) according to the manufacturer’s protocol, with the following conditions: denaturation for 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 45 s at 56–61°C, and 45 s at 72°C; then for the M13-tag binding addition of eight cycles of 30 s at 94°C, 45 s at 53°C, and 45 s at 72°C, with a final extension of 30 min at 72°C. In total, 14 primer pairs produced positive PCR reactions with at least one of the five *Trebouxia* species, and were excluded from further analyses because they were considered alga-specific.

The amplification of the fungal component of *Bryoria sect. Implexae* was tested with the 44 remaining loci under the same conditions as mentioned above. There were 14 loci that produced specific single products at an annealing temperature of 56°C, 12 at 57°C, six at 58°C, six at 60°C, and six at 61°C. Polymorphism of the 44 microsatellite loci was initially tested on a subset of 12 individuals (four individuals from each of three countries: Spain, Switzerland, and Finland), resulting in 18 polymorphic loci with satisfactory amplification. All PCR products obtained were multiplexed (Table 1). PCR reactions were performed in a total volume of 10 μL containing 1 μL of ~5 ng genomic DNA, 1 μL each of forward and reverse primers of varying concentration (Table 1), and 5 μL of Type-it Multiplex PCR Master Mix (QIAGEN, Hilden, Germany). The PCR protocol used fluorescent forward primers and the reaction was adjusted to: 5 min at 95°C; followed by 30 cycles of 30 s at 95°C, 90 s at 56, 58, or 60°C (Table 1), and 30 s at 72°C; with a final extension of 60 min at 60°C. PCR products were run on a 3130xl DNA Analyzer with GeneScan 500 LIZ as the size standard for fragment analysis (both by Life Technologies).

The 18 polymorphic microsatellite markers were tested for locus variability and marker consistency on three populations (Table 2). Alleles were sized using GeneMapper 5.0 (Life Technologies). The linkage disequilibrium (LD) between microsatellite loci and their variability were measured by counting the number of alleles and calculating Nei’s unbiased gene diversity using Arlequin 3.11 (Excoffier et al., 2005). Dinucleotide microsatellites (n = 13) were the most common microsatellite motifs among the 18 loci (Table 1). The microsatellite loci revealed significant LD based on 999 permutations (P < 0.001). They show two to 13 alleles per locus with a mean of 4.6, and average gene diversities varied from 0.38 to 0.52 over three populations (Table 2).

| Locus | Primer sequences (5′–3′) | Repeat motif | Multiplex<sup>a</sup> | T<sub>a</sub> (°C) | Fluorescent dye | Primer conc. (μM) | Allele size range (bp) | GenBank accession no. |
|-------|-------------------------|--------------|----------------------|-----------------|---------------|-----------------|-----------------------|-----------------------|
| Bi01  | F: GGACGACGCATACACACCCTC <br> R: GATTCGGGTAGTGTGTTCTC | (AACAGC)<sub>h</sub> | 1 56 | FAM | 0.32 | 94–129 | KJ739845 |
| Bi02  | F: GCTGATGTTGCTGCAATGCTC <br> R: GATGGGCACGTTTCTCTTC | (AG)<sub>12</sub> | 1 56 | FAM | 0.80 | 369–372 | KJ739846 |
| Bi03  | F: GTGACCTGTCGTCATGCTC <br> R: AGTGGCAGGTGCTTCTTC | (AG)<sub>12</sub> | 1 56 | FAM | 0.80 | 279–281 | KJ739847 |
| Bi04  | F: CATCCTGCTGTCATGCTC <br> R: CTTCAGGATGACCGCCATTCTC | (TO)<sub>7</sub> | 1 56 | PET | 0.80 | 320–325 | KJ739848 |
| Bi05  | F: CAAAGGCTGCAGTCGAGCTC <br> R: CAACGCTGCAGTCGAGCTC | (AAGG)<sub>7</sub> | 1 56 | NED | 0.50 | 127–143 | KJ739849 |
| Bi06  | F: GGGGGTGGAGTGTGTTCTC <br> R: CTGACCTCTCCTCCATATCTC | (GT)<sub>7</sub> | 1 56 | PET | 0.32 | 114–168 | KJ739850 |
| Bi07  | F: GAATCGCTGCTGTCGAGCTC <br> R: GAAATCCGCCCAACAACCA | (CCCTT)<sub>7</sub> | 2 58 | PET | 0.80 | 123–144 | KJ739851 |
| Bi08  | F: CATGCGGATGATAAGGAGCC <br> R: CGCAGCTTATACCGCTTCTT | (TC)<sub>7</sub> | 2 58 | NED | 0.32 | 367–372 | KJ739852 |
| Bi09  | F: CGTCTGCTGTCGAGGATGTA <br> R: GCATCCACCATCTCATGACT | (AT)<sub>5</sub> | 2 58 | PET | 1.10 | 341–343 | KJ739853 |
| Bi10  | F: CTGCTGCTGCTGTCGAGCTC <br> R: GTAGATGCTGAGGTGTGCTC | (TC)<sub>7</sub> | 2 58 | FAM | 0.90 | 434–437 | KJ739854 |
| Bi11  | F: GCACAAATCCACCATCCTTC <br> R: CATGCGGATGATAAGGAGCC | (AC)<sub>12</sub> | 2 58 | FAM | 0.50 | 314–318 | KJ739855 |
| Bi12  | F: CGAGATGCTGTTTGGGAGCG <br> R: CTACGCTCTGTAACACCAAG | (TTG)<sub>12</sub> | 2 58 | FAM | 0.32 | 100–124 | KJ739856 |
| Bi13  | F: CTGCTGCTGCTGTCGAGCTC <br> R: CTTTACACCATCCTCCAC | (TTC)<sub>13</sub> | 3 60 | FAM | 0.90 | 93–134 | KJ739857 |
| Bi14  | F: CTAGACGAGAACGTGACC <br> R: CATGCGGATGATAAGGAGCC | (TC)<sub>T</sub> | 3 60 | FAM | 0.60 | 316–365 | KJ739858 |
| Bi15  | F: GTGAGGAGCACTTACATTCA <br> R: GCAGTCAACATCGCTGACG | (TC)<sub>B</sub> | 3 60 | PET | 3.00 | 379–381 | KJ739859 |
| Bi16  | F: CAGAGCTTTAGCACTACAGCT <br> R: CGTCTGCTGTCGAGGATGTA | (AG)<sub>3</sub> | 3 60 | FAM | 1.50 | 405–457 | KJ739860 |
| Bi17  | F: CGAGCTTACGAGGCTACGCG <br> R: GCAGTCAACATCGCTGACG | (TC)<sub>T</sub> | 3 60 | VIC | 0.60 | 387–396 | KJ739861 |
| Bi18  | F: CGAGCTTACGAGGCTACGCG <br> R: GCAGTCAACATCGCTGACG | (TC)<sub>B</sub> | 3 60 | PET | 0.80 | 346–352 | KJ739862 |

Note: T<sub>a</sub> = annealing temperature.<br><sup>a</sup>Multiplex indicates loci that were mixed in the same capillary electrophoresis run.
Cross-species amplifications within three congeneric species were analyzed with the chi-square test. *B. capillaris* was shown to not amplify consistently, while *B. fuscescens* and *B. implexa* amplified more regularly (Appendix 2). Most markers amplified with all three species. However, the microsatellite marker B115 only amplified with *B. fuscescens*, B60 with *B. fuscescens* and *B. implexa*, and B118 with *B. capillaris* and *B. fuscescens*.

## CONCLUSIONS

The fungus-specific markers developed here will facilitate studies on genetic diversity and differentiation in *Bryoria* sect. *Implexae* throughout its geographic distribution, and on effects of forest management on genetic diversity of populations in this species group. Furthermore, putative phylogenetic signal within the flanking regions of the microsatellite sequences might help to delimit closely related species and to assess the taxonomic value of the morphological and chemical characters of these regionally rare and threatened lichens.

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**Table 2.** Results of microsatellite screening in 82 individuals of lichen-forming fungi of *Bryoria* sect. *Implexae* between species of *Bryoria* sect. *Implexae*, and between compared regions.

| Locus | Total | *B. capillaris* (n = 36) | *B. fuscescens* (n = 37) | *B. implexa* (n = 9) | Spain (n = 31) | Switzerland (n = 35) | Finland (n = 16) |
|-------|-------|--------------------------|--------------------------|----------------------|----------------|---------------------|-----------------|
|       |       | A | $H_e$ | A | $H_e$ | A | $H_e$ | A | $H_e$ | A | $H_e$ | A | $H_e$ |
| B001  | 82    | 7 | 0.82 | 6 | 0.71 | 6 | 0.79 | 4 | 0.58 | 5 | 0.73 | 6 | 0.71 | 4 | 0.44 |
| B002  | 67    | 4 | 0.74 | 3 | 0.64 | 4 | 0.68 | 2 | 0.43 | 3 | 0.68 | 4 | 0.69 | 3 | 0.59 |
| B003  | 82    | 2 | 0.24 | 2 | 0.32 | 2 | 0.15 | 2 | 0.22 | 2 | 0.12 | 2 | 0.36 | 2 | 0.13 |
| B004  | 82    | 3 | 0.36 | 3 | 0.54 | 2 | 0.39 | 2 | 0.54 | 2 | 0.33 | 2 | 0.36 | 2 | 0.13 |
| B005  | 79    | 4 | 0.61 | 3 | 0.57 | 3 | 0.47 | 2 | 0.22 | 3 | 0.52 | 4 | 0.66 | 2 | 0.13 |
| B006  | 82    | 10 | 0.83 | 10 | 0.88 | 5 | 0.64 | 3 | 0.64 | 3 | 0.53 | 8 | 0.85 | 4 | 0.64 |
| B007  | 82    | 3 | 0.49 | 3 | 0.37 | 2 | 0.11 | 1 | 0.00 | 2 | 0.28 | 3 | 0.46 | 2 | 0.13 |
| B008  | 82    | 4 | 0.54 | 3 | 0.52 | 3 | 0.49 | 3 | 0.56 | 2 | 0.49 | 3 | 0.54 | 3 | 0.57 |
| B009  | 60    | 2 | 0.50 | 2 | 0.25 | 2 | 0.28 | 1 | 0.00 | 2 | 0.40 | 2 | 0.31 | 2 | 0.33 |
| B10   | 82    | 2 | 0.44 | 2 | 0.44 | 2 | 0.05 | 1 | 0.00 | 2 | 0.23 | 2 | 0.49 | 2 | 0.13 |
| B11   | 82    | 3 | 0.36 | 3 | 0.45 | 2 | 0.28 | 2 | 0.39 | 2 | 0.12 | 3 | 0.54 | 2 | 0.33 |
| B12   | 82    | 7 | 0.67 | 5 | 0.39 | 6 | 0.49 | 4 | 0.81 | 3 | 0.34 | 6 | 0.48 | 5 | 0.82 |
| B13   | 82    | 13 | 0.84 | 9 | 0.80 | 8 | 0.68 | 6 | 0.92 | 6 | 0.67 | 9 | 0.83 | 7 | 0.88 |
| B14   | 82    | 3 | 0.47 | 3 | 0.40 | 2 | 0.05 | 1 | 0.00 | 2 | 0.23 | 3 | 0.48 | 2 | 0.13 |
| B15   | 52    | 2 | 0.04 | 1 | 0.00 | 2 | 0.05 | 1 | 0.00 | 1 | 0.00 | 2 | 0.15 | 1 | 0.00 |
| B16   | 82    | 6 | 0.76 | 6 | 0.57 | 5 | 0.61 | 3 | 0.72 | 3 | 0.61 | 6 | 0.67 | 4 | 0.69 |
| B18   | 81    | 4 | 0.56 | 4 | 0.35 | 3 | 0.62 | 3 | 0.68 | 3 | 0.59 | 4 | 0.27 | 3 | 0.68 |
| B19   | 82    | 3 | 0.65 | 3 | 0.11 | 3 | 0.53 | 3 | 0.72 | 3 | 0.60 | 3 | 0.43 | 3 | 0.69 |
| Mean  | 4.58  | 0.53 | 6 | 0.71 | 6 | 0.79 | 4 | 0.58 | 2.63 | 0.38 | 4.11 | 0.52 | 2.84 | 0.40 |

Note: $A =$ number of alleles; $H_e =$ Nei’s unbiased gene diversity; $n =$ total number of samples analyzed.
### APPENDIX 1. Voucher information for species of *Bryoria* sect. *Implexae* used in this study.

| Species     | Voucher specimen accession no. | Collection locality and date | Geographic coordinates | No. of individuals |
|-------------|--------------------------------|-----------------------------|------------------------|--------------------|
| *B. capillaris* | 18964–18967                     | Spain, Prov. Segovia, 1854 m a.s.l., *Pinus sylvestris* forest, 6 Nov. 2012 | 40°47'35.0"N, 03°59'12.6"W | 4                  |
| *B. capillaris* | 18968–18993                     | Switzerland, Canton of Berne, 1511 m a.s.l., *Picea abies* forest, 25 Nov. 2012 | 46°35'28.3"N, 07°20'26.9"E | 26                 |
| *B. capillaris* | 18997–18999                     | Finland, Prov. Etelä-Häme, Liesjärvi, 110 m a.s.l., *Picea abies* forest, 17 Nov. 2012 | 60°40'17.0"N, 23°51'10.4"E | 3                  |
| *B. capillaris* | 18994–18996                     | Finland, Prov. Etelä-Häme, 110 m a.s.l., *Picea abies* forest, 17 Nov. 2012 | 60°42'04.3"N, 23°54'41.9"E | 3                  |
| *B. fuscescens* | 19001–19014                     | Spain, Prov. Madrid, 1490 m a.s.l., *Pinus sylvestris* forest, 6 Nov. 2012 | 40°46'05.4"N, 03°59'35.9"W | 14                 |
| *B. fuscescens* | 19015–19027                     | Spain, Prov. Segovia, 1854 m a.s.l., *Pinus sylvestris* forest, 6 Nov. 2012 | 40°47'35.0"N, 03°59'12.6"W | 13                 |
| *B. fuscescens* | 19028–19034, 19036              | Switzerland, Canton of Berne, 1511 m a.s.l., *Picea abies* forest, 25 Nov. 2012 | 46°35'28.3"N, 07°20'26.9"E | 8                  |
| *B. fuscescens* | 19000, 19035                    | Finland, Prov. Etelä-Häme, Liesjärvi, 110 m a.s.l., *Picea abies* forest, 17 Nov. 2012 | 60°40'17.0"N, 23°51'10.4"E | 2                  |
| *B. implexa*   | 19037                           | Switzerland, Canton of Berne, 1511 m a.s.l., *Picea abies* forest, 25 Nov. 2012 | 46°35'28.3"N, 07°20'26.9"E | 1                  |
| *B. implexa*   | 19038–19042                     | Finland, Prov. Etelä-Häme, 110 m a.s.l., *Picea abies* forest, 17 Nov. 2012 | 60°42'04.3"N, 23°54'41.9"E | 3                  |
| *B. implexa*   | 19043–19045                     | Finland, Prov. Etelä-Häme, Liesjärvi, 110 m a.s.l., *Picea abies* forest, 17 Nov. 2012 | 60°40'17.0"N, 23°51'10.4"E | 5                  |

*Vouchers deposited at Lichens Herbarium of the Universidad Complutense de Madrid (MAF-Lich).*

### APPENDIX 2. Percentage of successful amplification between species of *Bryoria* sect. *Implexae*, and between compared regions.

| Group      | n  | Bi01 | Bi02 | Bi03 | Bi04 | Bi05 | Bi06 | Bi07 | Bi08 | Bi09 | Bi10 | Bi11 | Bi12 | Bi13 | Bi14 | Bi15 | Bi16 | Bi18 | Bi19 |
|------------|----|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| *B. capillaris* | 36 | 0.008 | 100  | 94   | 100  | 100  | 100  | 100  | 97   | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 22   | 100  | 100  |
| *B. fuscescens* | 37 | 0.81  | 100  | 68   | 100  | 100  | 100  | 100  | 100  | 51   | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 100  |
| *B. implexa*   | 9  | 0.99  | 100  | 89   | 100  | 100  | 100  | 100  | 100  | 67   | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 78   | 100  | 89   |
| Spain        | 31 | 0.97  | 100  | 65   | 100  | 100  | 100  | 100  | 100  | 52   | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 90   | 100  |
| Switzerland  | 35 | 0.86  | 100  | 97   | 100  | 100  | 100  | 100  | 100  | 94   | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 43   | 100  | 97   |
| Finland      | 16 | 0.39  | 100  | 81   | 100  | 100  | 100  | 100  | 100  | 69   | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 56   | 100  | 100  |
| Total        | 82 | 0.81  | 100  | 81–84| 100  | 100  | 100  | 100  | 100  | 72   | 100  | 100  | 100  | 100  | 100  | 100  | 100  | 63–67| 100  | 96–99|

*Note: n = total number of samples analyzed; p = probability (according to chi-square test) that each group will equally amplify with all markers.*