Characterization of Microsatellite Loci in Lichen-Forming Fungi of Bryoria Section Implexae (Parmeliaceae)

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Primer Note

Characterization of microsatellite loci in lichen-forming fungi of *Bryoria* section *Implexae* (Parmeliaceae)[1]

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- *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.
- *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 Bi05, Bi15, and Bi18.
- *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.

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.

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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°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 Skaletsky, 1999; Faircloth, 2008), ensuring a minimum repeat length of 8 bp for dinucleotides and 6 bp for all others.

MSATCOMMANDER recovered 6329 primer pairs that fulfilled the default primer parameters among all reads. Of those, 5932 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 ntBLAST 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 *Trebouxia* spp., which are hypothesized to be the photobionts of *Bryoria* sect. *Implexae* (Lindgren et al., 2014): *T. angustilobata* Beck (SAG2204), *T. asymmetrica* Friedl & Gärtner (SAG48.88), *T. arboricola* Puymaly (SAG219-1a), *T. jamesii* (Hildreth & Ahmadjian) Gärtner (SAG2103), and *T. simplex* Tschermak-Woess (SAG101.80). Forward primers were labeled with an M13 tag (5′-TGTAAAACGACGGCCAGT-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, 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 additional 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).

Table 1. Overview of the microsatellite loci developed for the group of lichen-forming fungi *Bryoria* sect. *Implexae.*

Locus	Primer sequences (5′–3′)	Repeat motif	Multiplex[a]	T_a (°C)	Fluorescent dye	Primer conc. (μM)	Allele size range (bp)	GenBank accession no.
Bi01	F: GGACGACGACATACCACTC R: GAGTTCGGGTTTAGGTCGTC	$(AACAGC)_6$	1	56	FAM	0.32	94–129	KJ739845
Bi02	F: GCGTGAATGTGTCCGAATCG R: GAATGGGCGCTCACTGTCTT	$(AG)_{12}$	1	56	FAM	0.80	369–372	KJ739846
Bi03	F: GTGAACTCGCTCGTATCGTC R: CCTAGGGATGACACGCAGAA	$(AG)_{12}$	1	56	FAM	0.80	279–281	KJ739847
Bi04	F: CAGTGCGGCAAACAGTTAGT R: GCACAAATCCACCCACTCCT	$(TG)_{10}$	1	56	PET	0.80	320–325	KJ739848
Bi05	F: CAAGGAGGTCGACTGTGAGT R: CAACCGATCCCACGCTCTC	$(AAGG)_6$	1	56	NED	0.50	127–143	KJ739849
Bi06	F: GGGAGGGTGGAAGTTGGTTT R: CGACCACTTCCACTTCCATATC	$(GTT)_9$	1	56	PET	0.32	114–168	KJ739850
Bi07	F: GAAATCGGCTTGTTGTCCTCC R: GAACTACCGCCCACAAACAA	$(CCTTT)_6$	2	58	PET	0.80	123–144	KJ739851
Bi08	F: CATGCGGAGTTAAAGGAGGC R: CGCACCTATTTACGGCCTTT	$(TC)_8$	2	58	NED	0.32	367–372	KJ739852
Bi09	F: CGTTCGTTCGTAGGTAGGTA R: GCCTACCCACCATCTGAACT	$(AT)_8$	2	58	PET	1.10	341–343	KJ739853
Bi10	F: CTCGCGTTTCCCTGTTTCTT R: GTATGAGGTCGGAGTGTGCT	$(TC)_8$	2	58	FAM	0.90	434–437	KJ739854
Bi11	F: GCACAAATCCACCCACTCCT R: CAGTGCGGCAAACAGTTAGT	$(AC)_{12}$	2	58	FAM	0.50	314–318	KJ739855
Bi12	F: GCAGAAAGTGAGTTAGCCGG R: CTCAGCCTCAACCACAACGA	$(TTG)_{12}$	2	58	FAM	0.32	100–124	KJ739856
Bi13	F: TCTTTCCTCTCCTGTCCACC R: CCTTACAGACCGGAGAAGCC	$(TTC)_{11}$	3	60	FAM	0.90	93–134	KJ739857
Bi14	F: CTAACCACGACAAGCTGACC R: GTACCGACGCAACTTACCTA	$(TC)_7$	3	60	FAM	0.60	316–365	KJ739858
Bi15	F: GTAGCAGGACATACGGAGGT R: CGTCCTAGCATCTCGGTTCT	$(TC)_9$	3	60	PET	3.00	379–381	KJ739859
Bi16	F: CCAGGTCCTTCACTACAGCT R: CGGTACAAGTCCAGTTGCAG	$(AG)_8$	3	60	FAM	1.50	405–437	KJ739860
Bi18	F: GCAGCTATCAGGAGTCACGT R: GCAGCTATCAGGAGTCACGT	$(TC)_7$	3	60	VIC	0.60	387–396	KJ739861
Bi19	F: CCACCTCGAAGAGTACTGCT R: CTGAGCTATGTCCTCGCACA	$(TC)_{10}$	3	60	PET	0.80	346–352	KJ739862

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

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)	
	n	*A*	H_e	*A*	H_e	*A*	H_e	*A*	H_e	*A*	H_e	*A*	H_e	*A*	H_e
Bi01	82	7	0.82	6	0.71	6	0.79	4	0.58	5	0.73	6	0.71	4	0.44
Bi02	67	4	0.74	3	0.64	4	0.68	2	0.43	3	0.68	4	0.69	3	0.59
Bi03	82	2	0.24	2	0.32	2	0.15	2	0.22	2	0.12	2	0.36	2	0.13
Bi04	82	3	0.36	3	0.45	2	0.28	2	0.39	2	0.12	3	0.54	2	0.33
Bi05	79	4	0.61	3	0.57	3	0.47	2	0.22	3	0.52	4	0.66	2	0.13
Bi06	82	10	0.83	10	0.88	5	0.64	3	0.64	3	0.53	8	0.85	4	0.64
Bi07	82	3	0.49	3	0.37	2	0.11	1	0.00	2	0.28	3	0.46	2	0.13
Bi08	82	4	0.54	3	0.52	3	0.49	3	0.56	2	0.49	3	0.54	3	0.57
Bi09	60	2	0.50	2	0.25	2	0.28	1	0.00	2	0.40	2	0.31	2	0.33
Bi10	82	2	0.44	2	0.44	2	0.05	1	0.00	2	0.23	2	0.49	2	0.13
Bi11	82	3	0.36	3	0.45	2	0.28	2	0.39	2	0.12	3	0.54	2	0.33
Bi12	82	7	0.67	5	0.39	6	0.49	4	0.81	3	0.34	6	0.48	5	0.82
Bi13	82	13	0.84	9	0.80	8	0.68	6	0.92	6	0.67	9	0.83	7	0.88
Bi14	82	3	0.47	3	0.40	2	0.05	1	0.00	2	0.23	3	0.48	2	0.13
Bi15	52	2	0.04	1	0.00	2	0.05	1	0.00	1	0.00	2	0.13	1	0.00
Bi16	82	6	0.76	6	0.57	5	0.61	3	0.72	3	0.61	6	0.67	4	0.69
Bi18	81	4	0.56	4	0.35	3	0.62	3	0.68	3	0.59	4	0.27	3	0.68
Bi19	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.

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 Bi15 only amplified with *B. fuscescens*, Bi05 with *B. fuscescens* and *B. implexa*, and Bi18 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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Appendix 1. Voucher information for species of *Bryoria* sect. *Implexae* used in this study.

Species	Voucher specimen accession no.[a]	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

[a]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*	*p*	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	92	100	100	100	97	100	100	100	100	100	22	100	100	100
B. fuscescens	37	0.81	100	68	100	100	100	100	100	100	51	100	100	100	100	100	100	100	100	100
B. implexa	9	0.99	100	89	100	100	100	100	100	100	67	100	100	100	100	100	78	100	89	100
Spain	31	0.97	100	65	100	100	100	100	100	100	52	100	100	100	100	100	90	100	100	100
Switzerland	35	0.86	100	97	100	100	91	100	100	100	94	100	100	100	100	100	43	100	97	100
Finland	16	0.39	100	81	100	100	100	100	100	100	69	100	100	100	100	100	56	100	100	100
Total	82		100	81–84	100	100	97	100	100	100	72	100	100	100	100	100	63–67	100	96–99	100

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