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Characterization of microsatellite markers in the cosmopolitan lichen-forming fungus *Rhizoplaca melanophthalma* (Lecanoraceae)

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Abstract

Rhizoplaca melanophthalma s.l. is a group of morphologically distinct and chemically diverse species that commonly occur in desert, steppe and montane habitats worldwide. In this study, we developed microsatellite markers to facilitate studies of genetic diversity, population structure, and gene flow in the nominal taxon of this group, *Rhizoplaca melanophthalma*. We characterized 10 microsatellite markers using a draft genome of *R. melanophthalma* s. str. assembled from Illumina reads. These loci were tested for 21 *R. melanophthalma* s. str. specimens and also with a subset of 18 specimens representing six additional species in the *R. melanophthalma* complex. The number of alleles per locus in *R. melanophthalma* s. str. ranged from 3 to 11 with an average of 6.7. Nei's unbiased gene diversity ranged from 0.35 to 0.91. Amplifications of the microsatellite loci were largely successful in the other six species, although only three markers were found to be polymorphic. The new markers will provide an additional resource for studying genetic, population- and landscape-level processes in the cosmopolitan taxon *Rhizoplaca melanophthalma* s. str.

Key words

Ascomycetes, gene flow, landscape genetics, lichen-forming fungi, microsatellites, *Rhizoplaca melanophthalma*

Introduction

Rhizoplaca melanophthalma (DC.) Leuckert & Poelt s.l. represents a group of morphologically distinct and chemically diverse species of lichen-forming fungi with broad ecological and geographical distributions. Species in this group occur all over the world in disjunct populations in continental climates, although species in this complex are notably absent from Australia. *Rhizoplaca melanophthalma* s.l. commonly grows on siliceous or calcareous rock in arid climates, but can also be found in montane coniferous forests, alpine tundra habitats, and bi-polar populations in the Arctic and Antarctica (McCune 1987). Members of this group are commonly used in air-quality biomonitoring research, making it an important species for conservation (Aslan et al. 2004; Dillman 1996). The species complex belongs to the recently re-circumscribed monophyletic genus *Rhizoplaca* in Lecanoraceae (Zhao et al. 2016).

Previous multi-locus and phylogenomic studies support the circumscription of multiple species within *R. melanophthalma* s.l. (Leavitt et al. 2011, 2013, 2016b), many of which occur in sympatry in Western North America. In Western North America the distribution area of these species extends from the northern boreal zone to Mexico along the Rocky Mountains with a center of diversity in the Great Basin region (Leavitt et al. 2011). *Rhizoplaca melanophthalma* s. str. has the broadest ecological and geographic distribution of all known species within this complex, with populations occurring in desert, montane and steppe ecosystems in Antarctica, Central Asia, Europe, and North and South America (Leavitt et al. 2013).

The *Rhizoplaca melanophthalma* group provides an interesting system for assessing genetic diversity, population structure and gene flow in symbiotic fungal species with broad ecological and geographic distributions. To facilitate additional research into population- and landscape-level processes, 10 microsatellite markers were developed for *R. melanophthalma* s.str.

Materials and methods

A total of 42 specimens representing seven different species in the *Rhizoplaca melanophthalma* species complex were included in this study. Twenty-one of these represented *R. melanophthalma* s. str., three *R. haydenii*, four *R. novomexicana*, two *R. parilis*, four *R. polymorpha*, six *R. porteri* and two *R. shushanii* (Table 1). DNA was extracted from these specimens as described previously (Leavitt et al. 2011).

A draft genome of an axenic culture of *R. melanophthalma* was obtained from a previous study (Leavitt et al. 2016a). The program MSATCOMMANDER 1.0.8 (Faircloth 2008) was used to search for di-, tri-, tetra-, penta-, and hexanucleotide microsatellite repeats in contigs >5 kb from the draft assembly. Only repeats with a minimum length of 8 bp for dinucleotide repeats and 6 bp for the rest were accepted. A total of 244 scaffolds contained microsatellite repeats (87 di-, 127 tri-, 11 tetra-, 5 penta-, and 14 hexanucleotides). For 25 of these repeats, primers were designed with

Table 1. Voucher information for *Rhizoplaca* specimens used in this study. Herbaria codes are provided for each specimen in parentheses following voucher number.

Species	DNA No.	Voucher	Locality
R. melanophthalma	8639c	Leavitt 2013-CO-CP-8639C (F)	USA, CO
R. melanophthalma	8639d	Leavitt 2013-CO-CP-8639D (F)	USA, CO
R. melanophthalma	8654a	Leavitt 2013-CO-RM-8654A (F)	USA, CO
R. melanophthalma	8654b	Leavitt 2013-CO-RM-8654B (F)	USA, CO
R. melanophthalma	8663B	Leavitt 8663 (F)	USA, UT
R. melanophthalma	8663j	Leavitt-8663 (F)	USA, UT
R. melanophthalma	8665b	Leavitt-8665 (F)	USA, NV
R. melanophthalma	8665e	Leavitt-8665 (F)	USA, NV
R. melanophthalma	8665i	Leavitt-8665 (F)	USA, NV
R. melanophthalma	8665M	Leavitt-8665 (F)	USA, NV
R. melanophthalma	8668b	Leavitt-8668 (F)	USA, NV
R. melanophthalma	8668f	Leavitt-8668 (F)	USA, NV
R. melanophthalma	8668q	Leavitt-8668 (F)	USA, NV
R. melanophthalma	8668s	Leavitt-8668 (F)	USA, NV
R. melanophthalma	8668w	Leavitt-8668 (F)	USA, NV
R. melanophthalma	6026	H9203303 (F)	Kyrgyzstan, Ala-Buka
R. melanophthalma	6029	H9203135 (F)	Kyrgyzstan, Panfilov District
R. melanophthalma	6030	H9203327 (F)	Kyrgyzstan, Chatkal
R. melanophthalma	6435	Vondrak 9409 (PRA)	Russia, Chelyabinsk
R. melanophthalma	6604	MAF-Lich 16805 (MAF)	Spain, Teruel
R. melanophthalma	6605	MAF-Lich 16778 (MAF)	Spain, Teruel
R. haydenii	8683	Leavitt 8683 (F)	USA, ID
R. haydenii	8935p	Leavitt 8935 (F)	USA, ID
R. haydenii	8935s	Leavitt 8935 (F)	USA, ID
R. novomexicana	8684a	Leavitt 8684A (F)	USA, NM
R. novomexicana	8684b	Leavitt 8684B (F)	USA, NM
R. novomexicana	8684c	Leavitt 8684C (F)	USA, NM
R. novomexicana	8684d	Leavitt 8684D (F)	USA, NM
R. parilis	8665N	Leavitt-8665 (F)	USA, NV
R. parilis	8665u	Leavitt-8665 (F)	USA, NV
R. polymorpha	8668g	Leavitt-8668 (F)	USA, NV
R. polymorpha	8668l	Leavitt-8668 (F)	USA, NV
R. polymorpha	8668p	Leavitt-8668 (F)	USA, NV
R. polymorpha	8668r	Leavitt-8668 (F)	USA, NV
R. aff. *porteri*	8665x	Leavitt-8665 (F)	USA, NV
R. aff. *porteri*	8668j	Leavitt-8668 (F)	USA, NV
R. aff. *porteri*	8668m	Leavitt-8668 (F)	USA, NV
R. porteri	8665t	Leavitt-8665 (F)	USA, NV
R. porteri	8668e	Leavitt-8668 (F)	USA, NV
R. porteri	8668h	Leavitt-8668 (F)	USA, NV
R. shushanii	8664A	Leavitt 13-TLM-001 (BRY-C)	USA, UT
R. shushanii	8664B	Leavitt 13-TLM-001 (BRY-C)	USA, UT

Table 2. Microsatellite loci developed for *Rhizoplaca melanophthalma* s. str.

Locus	Primer sequences (5'–3')	Repeat motif	Allele size range (bp)	GenBank accession no.
Rmel1	F: *GGCTGGGTGTTGTAGTGTTG R: ATACCTGGCGCTCAAGAATG	$(GTT)_{18}$	179–203	KX755412
Rmel2	F: *TGGTGGATCTGAGAGCGTAC R: ACTTCAACCTTCACAACGCC	$(CAGGCT)_{10}$	328–382	KX755413
Rmel3	F: *CAAAGGTCAGGAGAGGAGGG R: TGGACGCGTGGCAATTTATC	$(AC)_{10}$	380–412	KX755414
Rmel4	F: *ATCGAGACTTACTTCCCGCC R: AATCGTATCTCCAGACCCGC	$(GT)_{11}$	353–385	KX755415
Rmel5	F: *TTAGCCCGAGACCACATACG R: TGGAGAGATGAAGCTGGCTC	$(CT)_{12}$	311–321	KX755416
Rmel6	F: *ACACCAGATCTCACTCAGGC R: CCGGGAGTAGGTGTAGATGC	$(AC)_{10}$	184–192	KX755417
Rmel7	F: *TCCGGAACTGGCTTGATAGG R: CTGAAGTCGATGTTGGGAGC	$(CCTT)_{11}$	314–362	KX755418
Rmel8	F: *TTTGCCCGACGTGCAATATC R: CTGCAGCACTCTAACCATGC	$(AG)_{11}$	420–438	KX755419
Rmel9	F: *ATCTCCTGCATCTTCTCCGC R: AACGTCACATTCGCAGTCAC	$(AC)_{10}$	309–331	KX755420
Rmel10	F: *TCATCACACCAGACACAGGG R: ACCTTAGGCCCAGACACATG	$(AG)_{10}$	464–468	KX755421

* M13 tail: TGTAAAACGACGGCCAGT.

Primer3 (Rozen and Skaletsky 2000) as implemented in MSATCOMMANDER. An M13 tag (5'-TGTAAAACGACGGCCAGT-3') was appended to forward primers and 5' ends of the reverse primers were tailed with 5'-GTGTCTT-3' tag.

Singleplex PCR reactions were performed in 10 µl reaction volumes consisting of 5.89 µl H_2O, 1 µl 10x buffer (Roche Diagnostics, Indianapolis, USA), 0.6 µl 8 mM dNTP, 1 µl BSA, 0.15 µl Taq (Roche Diagnostics, Indianapolis, USA), 0.16 µl 6-FAM labeled M13 primer, 0.04 µl 10 µM M13 tailed forward primer, 0.16 µl 10 µM reverse primer, and 1 µl of genomic DNA. DNA amplification was performed using a touchdown PCR with initial denaturation at 95 °C for 5 min; followed by first 11 cycles of 30 s at 95 °C, 30 s at 60–50 °C, 1 min. at 72 °C, and then 35 cycles of 30 s at 95 °C, 30 s at 50 °C, 1.5 min. at 72 °C, and a final extension of 10 min. at 72 °C.

Fragment analysis was performed on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA) using GeneScan-500 LIZ (Life Technologies, Warrington, UK) as an internal size standard. Genotyping was performed utilizing the microsatellite plugin in Geneious 9.1.2 (Biomatters Limited). Polymorphism within the microsatellites was tested in GenAlEx 6.5 (Peakall and Smouse 2012) by calculating Nei's unbiased genetic diversity.

Table 3. Sample size, number of alleles (*A*) and Nei's unbiased genetic diversity (H_e) of ten microsatellite loci developed for *Rhizoplaca melanophthalma* s. str.

	Total		
Locus	***n***	***A***	H_e
Rmel1	21	8	0.886
Rmel2	20	6	0.858
Rmel3	21	9	0.866
Rmel4	21	11	0.919
Rmel5	20	5	0.679
Rmel6	21	3	0.643
Rmel7	20	6	0.763
Rmel8	20	7	0.779
Rmel9	21	9	0.881
Rmel10	20	3	0.353
Average		6.7	0.765

Results and discussion

Of the 25 microsatellites assessed, 18 amplified successfully and 10 were polymorphic in all 21 *R. melanophthalma* s. str. specimens (Table 2). The number of alleles per locus ranged from three to 11 with an average of 6.7. Nei's unbiased genetic diversity varied between 0.353 and 0.919 with the average genetic diversity being 0.765 (Table 3). The same 18 microsatellites that amplified successfully with *R. melanophthalma* s. str. also amplified in *R. haydenii*, *R. novomexicana*, *R. parilis*, *R. polymorpha*, *R. porteri*, and *R. shushanii*, but only three loci were polymorphic in all these species. For these three loci (Rmel1, Rmel, 4, and Rmel8) the number of alleles ranged from 11 to 15 with the average of 13 and Nei's unbiased genetic diversity varied between 0.892 and 0.905 with average of 0.900.

The 10 polymorphic microsatellite markers for the lichen-forming fungus *R. melanophthalma* will help elucidate population processes that have led to the observed distribution patterns in this widespread species.

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