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PRIMER NOTE

MICROSATELLITE PRIMERS IN THE LICHEN SYMBIOTIC ALGA *TREBOUXIA DECOLORANS* (TREBOUXIOPHYCEAE)[1]

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- *Premise of the study:* Polymorphic microsatellite markers were developed for the symbiotic green alga *Trebouxia decolorans* to study fine-scale population structure and clonal diversity.
- *Methods and Results:* Using Illumina pyrosequencing, 20 microsatellite primer sets were developed for *T. decolorans*. The primer sets were tested on 43 individuals sampled from four subpopulations in Germany. The primers amplified di-, tri-, and tetranucleotide repeats with three to 15 alleles per locus, and the unbiased haploid diversity per locus ranged from 0.636 to 0.821.
- *Conclusions:* The identified microsatellite markers will be useful to study the genetic diversity, dispersal, and reproductive mode of this common lichen photobiont.

Key words: *Anaptychia ciliaris*; photobiont; population genetics; selectivity; symbiosis; *Xanthoria parietina*.

Green algae of the genus *Trebouxia* constitute a significant portion of terrestrial algal diversity as they are the most common photobionts in lichens (Friedl and Büdel, 2008). Population biology of lichen photobionts is currently poorly understood, especially due to the lack of appropriate molecular markers. To date, highly variable markers such as microsatellites have only been developed for the photobionts of two lichen species, namely *Lobaria pulmonaria* (L.) Hoffm. (Dal Grande et al., 2010) and *Parmotrema tinctorum* (Delise ex Nyl.) Hale (Mansournia et al., 2012).

Trebouxia decolorans Ahmadjian is a common haploid lichen photobiont that has been reported from several continents and was found in association with both widespread (e.g., *Xanthoria parietina* (L.) Th. Fr.) and locally endangered (e.g., *Anaptychia ciliaris* (L.) Körb.) fungal species (Helms et al., 2001). Our goal was to develop microsatellite loci to be used in high-resolution population studies in *T. decolorans*. This is a key step in understanding reproductive mode and fine-scale spatial genetic structure and diversity in trebouxioid algae.

METHODS AND RESULTS

We established an algal culture of *T. decolorans* (strain AB05019B2, Botanische Staatssammlung München) from the lichen *X. parietina* collected in Maising, Germany (47°58′19″N, 11°16′34″E, 635 m a.s.l.), using a micromanipulator (Beck and Koop, 2001). Approximately 10 μg of total DNA from the algal culture (ITS sequence GenBank JF831923) was used to construct a library (sheared DNA fragments were of 500 bp length) for 100 bp × 100 bp paired-end sequencing using an Illumina GAIIx and standard Illumina protocols (Illumina, San Diego, California, USA). The Illumina sequencing of this sample was done in the laboratory of D. Bhattacharya (Rutgers University, New Brunswick, New Jersey, USA) and is described in more detail in Beck et al. (unpublished). All 244958 contigs, totaling 64.2 Mbp of genome data with an average coverage of 3.8×, were screened in fasta files using MSATCOMMANDER 1.0.8 (Faircloth, 2008) accepting dinucleotide repeats of ≥10, trinucleotide repeats of ≥8, and tetranucleotide repeats of ≥8. One hundred out of 244958 contigs screened contained repeats consisting of 58 di-, 20 tri-, and 22 tetranucleotide repeats. Primers were developed using Primer3 (Rozen and Skaletsky, 2000). Forward primers were appended with an M13 tag (5′-TGTAAAACGACGGCCAGT-3′). Nine sequences were discarded because the flanking regions of the repeat sequences were too short in length and therefore not suitable for primer design. Primers could be designed for 91 contigs containing repeats, including 54 di-, 19 tri-, and 18 tetranucleotide repeats. Primers were checked for amplification with the original *T. decolorans* culture, and with DNA isolated from the same algal taxon of the locally endangered lichen *A. ciliaris* collected in Pähl, Germany, on *Tilia* sp. (47°55′N, 11°11′E, 662 m a.s.l., M-0102896; ITS sequence GenBank JX444960). PCR was performed in a 10 μL reaction volume containing ~1–5 ng genomic DNA, 1× Type-it Multiplex Master Mix (QIAGEN, Hilden, Germany), 0.15 μM reverse primer, 0.01 μM M13-tailed forward primer, and 0.15 μM of 6FAM–M13-labeled primer (Schuelke, 2000). PCR was carried out with an initial 5-min denaturation at 94°C followed by 30 cycles of 94°C for 30 s, 57°C for 45 s, and 72°C for 45 s, eight cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 45 s, and a final extension of 72°C for 30 min. Primer pairs that either failed to amplify in either one or both photobiont strains, or produced multiple, spurious bands during PCR were discarded. Primers that worked on both photobionts and provided clear electropherograms were selected, which left 24 loci worth further testing, comprising 13 di-, four tri-, and seven tetranucleotide repeats.

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TABLE 1. Characteristics of 20 microsatellite primers developed in *Trebouxia decolorans.*

Locus	Primer sequences (5′–3′)	Repeat motif	Dye	Size range (bp)[a]	T_a (°C)	GenBank accession no.
Tde01	F: *GGTGTCTCCATCAAGCACCT R: TGACTGGCCGAAACATGTAA	$(AAG)_{13}A(AAG)$	FAM	93–114	57	JX437011
Tde02	F: *ACCCTCGGGACTCCATCTAA R: AGCAGGAAAGGACCTGACAA	$(AC)_{11}$	VIC	410–425	57	JX437012
Tde03	F: *CACACGCACTGTCTCATCAA R: GCAAGCATCTGAGCAAACAA	$(ATA)_{14}$	PET	341–385	57	JX437013
Tde04	F: *GGAAGGTTTCAAGGGTTTCC R: AGTGACGCGCTGCTATGAC	$(TG)_{13}$	FAM	125–156	57	JX437014
Tde05	F: *CTTGATGATATCGCCCCTGT R: CTGCAGCACTTCATCACGTT	$(AAT)_8$	VIC	374–401	57	JX437015
Tde06	F: *AATGCCATCGTCATCAAGGT R: CCTGCAGGCATTCAAACATA	$(TGTC)_9$	NED	214–272	57	JX437016
Tde07	F: *TGGACGCATTGCTTGAGTAG R: CTGGGCAGATGTGATCATTG	$(CAGG)_9$	PET	302–345	57	JX437017
Tde08	F: *GTTTCCCTGCAACCCTGTAA R: AAGCAGCTGTTCCTGTCTCC	$(GA)_{12}$	VIC	395–419	57	JX437018
Tde09	F: *CTCATTTGCACAGCCTTTGA R: CCTCTTTGCTTGCCACATTT	$(AC)_{10}$	PET	363–404	57	JX437019
Tde10	F: *GTCTGCAGCCATCACCTGT R: CCTGTGTTTGAACCCCATGT	$(CA)_{11}$	FAM	165–177	57	JX437020
Tde11	F: *AGGCTGCTCAAGGCAAGTAA R: TGCCTGACATTGATCTCTGC	$(TG)_{12}$	VIC	362–371	57	JX437021
Tde12	F: *TTCCATGATGCCAAAGTCAC R: TTGCAGCCATTCACTACGTC	$(GTCT)_8$	NED	208–256	57	JX437022
Tde13	F: *GTGCAGCTTGTCACTGAAGG R: GCGCCCATACACTTTTGAAT	$(TG)_{12}$	PET	313–321	57	JX437023
Tde14	F: *GGTGACAGAGCACAGCAAGA R: CGTTGCAGGTGTGTTTTCAT	$(AC)_{28}$	FAM	266–307	57	JX437024
Tde15	F: *ATGTGTCCACGGACAGACAA R: ACACCTTTGGTCCTGACAGC	$(TG)_{13}$	VIC	359–409	57	JX437025
Tde16	F: *GTTGCTGTGCGAGTGGTGTA R: CCTGGAATGCCATATGTTTGT	$(TTCG)_9$	NED	120–450	57	JX437026
Tde17	F: *TAGGTGGGCCTCTGAACATT R: TCAGTTGTTGCTCCGCTATG	$(AG)_{13}$	PET	304–386	57	JX437027
Tde18	F: *ATCATGCAACGGAATAAACCA R: CCTGCCTTATGTACTGCCTCA	$(TGTA)_{13}$	VIC	226–386	57	JX437028
Tde19	F: *TGTTTGAGTACGGCCAACAG R: GAACACGATGCACAAGGCTA	$(AC)_{12}$	NED	286–290	57	JX437029
Tde20	F: *TGACCTTCCTCACTCCTGTTC R: AGCACCATGAGAACCAAAGG	$(TTC)_8$	PET	304–316	57	JX437030

Note: T_a = annealing temperature when run individually.

[a] Fragment size range based on 43 samples collected from four subpopulations located in Frankfurt, Germany.

* M13 tail (TGTAAAACGACGGCCAGT).

These 24 loci were tested for variability in 43 samples from four subpopulations of photobionts of the lichen *X. parietina* occurring on branches of four trees of *Juglans regia* L. in Frankfurt, Germany (subpopulation 1: 50°9′9.98″N, 8°45′56.38″E, 110 m a.s.l.; subpopulation 2: 50°9′18.90″N, 8°46′45.41″E, 108 m a.s.l.; subpopulation 3: 50°9′14.73″N, 8°46′25.79″E, 112 m a.s.l.; subpopulation 4: 50°9′27.01″N, 8°46′28.50″E, 158 m a.s.l.). We extracted total genomic DNA using the cetyltrimethylammonium bromide (CTAB) method (Cubero and Crespo, 2002). PCR was carried out as described above, using 0.15 µM of either 6FAM, NED, PET, or VIC–M13-labeled primer in each reaction. Cross-species amplification of all microsatellite loci was performed in five other congeneric species: *T. asymmetrica* Friedl & Gärtner, *T. corticola* (Archibald) Gärtner, *T. gigantea* (Hildreth & Ahmadjian) Gärtner, *T. impressa* Ahmadjian, and *T. simplex* Tscherm.-Woess (Appendix 1). The same PCR conditions were used as described above except that an annealing temperature gradient of 50°C to 57°C was used in the first 30 cycles. For all taxa, DNA quality was confirmed by the successful PCR amplification of algal ITS region with ITS1T and ITS4T primers (Kroken and Taylor, 2000). The PCR reactions (25 µL), containing 0.65 U *Ex Taq* polymerase (TaKaRa Bio Inc., Otsu, Shiga, Japan), 1× reaction buffer, 100 µM of each dNTP, 0.4 µM of each primer, and 1–5 ng of genomic DNA template, were performed with initial denaturation at 95°C for 4 min, followed by 38 cycles of 95°C for 30 s, 50°C for 40 s, 72°C for 1 min, and final elongation at 72°C for 5 min. For microsatellite testing, PCR products were multiplexed: 0.5 µL of each labeled amplicon were added to 98 µL H_2O and were run on a 3730 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) using LIZ-500 as internal size standard. Alleles were sized with Geneious version 5.6 (Drummond et al., 2011). The variability of each microsatellite locus was measured by counting the number of alleles and calculating unbiased haploid diversity using GenAlEx version 6.41 (Peakall and Smouse, 2006).

Cross-species amplification failed in all congeneric species tested, supporting what seems to be a general trend of microsatellite development studies for lichen symbionts, that mycobiont-specific markers have higher intrageneric cross-species transferability than photobiont-specific markers (Dal Grande, 2011; Jones et al., 2012; Dal Grande et al., unpublished data). Four primer pairs did not amplify in the majority of samples tested and were therefore discarded. Twenty loci were polymorphic and consistently amplifiable in all samples of the four subpopulations of *T. decolorans* from the lichen *X. parietina*. Among the 20 microsatellite motifs, 11 were dinucleotide repeats, four were trinucleotide repeats, and five were tetranucleotide repeats. Sequences of the microsatellite loci as they appear in the original sample were deposited in GenBank (Table 1). The microsatellite loci produced three to 15 alleles per locus, and average haploid diversity over loci in four subpopulations varied from 0.636 to 0.821 (Table 2). A total of 36 unique multilocus genotypes were observed in the data set, suggesting that clonal diversity is high in this unicellular alga.

Table 2. Results of initial primer screening in four German subpopulations of *Trebouxia decolorans*.

	Subpopulation 1 ($N = 10$)		Subpopulation 2 ($N = 11$)		Subpopulation 3 ($N = 13$)		Subpopulation 4 ($N = 9$)	
Locus	A	h	A	h	A	h	A	h
Tde01	4	0.71	6	0.91	4	0.78	4	0.69
Tde02	5	0.82	5	0.86	5	0.85	3	0.64
Tde03	7	0.91	6	0.89	8	0.92	4	0.81
Tde04	5	0.8	5	0.84	3	0.71	4	0.81
Tde05	5	0.76	7	0.87	5	0.81	2	0.39
Tde06	5	0.76	6	0.86	6	0.86	4	0.58
Tde07	5	0.76	5	0.78	5	0.85	3	0.56
Tde08	4	0.73	4	0.75	4	0.62	3	0.56
Tde09	6	0.89	7	0.89	5	0.76	5	0.86
Tde10	3	0.64	3	0.71	4	0.68	2	0.22
Tde11	2	0.36	4	0.67	4	0.62	2	0.39
Tde12	5	0.8	5	0.82	6	0.83	3	0.75
Tde13	3	0.73	4	0.78	4	0.81	2	0.5
Tde14	8	0.93	9	0.96	8	0.94	5	0.72
Tde15	3	0.64	5	0.86	3	0.72	2	0.5
Tde16	3	0.38	6	0.84	4	0.68	5	0.81
Tde17	5	0.84	5	0.78	7	0.89	4	0.69
Tde18	6	0.78	7	0.91	6	0.83	5	0.72
Tde19	3	0.6	3	0.66	3	0.71	3	0.75
Tde20	4	0.73	4	0.78	3	0.41	4	0.78

Note: A = number of alleles; h = unbiased haploid diversity; N = sample size for each subpopulation.

CONCLUSIONS

This set of novel polymorphic microsatellite markers can provide insights into fine-scale population structure and transmission mode of the common symbiotic alga *T. decolorans*. They are currently being used to analyze clonal diversity and photobiont selectivity in lichen communities with *X. parietina* and *A. ciliaris*.

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Appendix 1. Voucher information for *Trebouxia* species and lichen samples screened for 20 microsatellite loci.

Trebouxia asymmetrica Friedl & Gärtner strain AB99023C2 ex *Fulgensia fulgida* (Botanische Staatssammlung München, lichen voucher ‘Rambold 6254/2-7’; ITS Sequence GenBank: AF344175).

Trebouxia corticola (Archibald) Gärtner strain UTEX 909 free-living (ITS Sequence GenBank: AJ249566).

Trebouxia decolorans Ahmadjian strain AB05019B2 ex *Xanthoria parietina* (Botanische Staatssammlung München, lichen voucher M-0102151; ITS Sequence GenBank: JF831923).

Trebouxia gigantea (Hildreth & Ahmadjian) Gärtner strain UTEX 2231 ex *Caloplaca cerina* (ITS Sequence GenBank: AJ249577).

Trebouxia simplex Tscherm.-Woess strain AB97017A2 ex *Lecidea silacea* (Botanische Staatssammlung München, lichen voucher M-0039557; ITS Sequence GenBank: AF128270).

Anaptychia ciliaris (L.) Körb. (Botanische Staatssammlung München, lichen voucher M-0102896; ITS Sequence GenBank: JX444960).

Xanthoria parietina (L.) Th. Fr. (Herbarium Senckenbergianum Frankfurt/Main, FR, Germany): Germany, Hesse, Frankfurt, leg. Dal Grande, Singh, Schmitt, 11 July 2012: subpopulation 1: G0101–G0110, subpopulation 2: G0201–G0211, subpopulation 3: G0301–G0313, subpopulation 4: G0401–G0409.

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Primer Note

Characterization of fungus-specific microsatellite markers in the lichen fungus *Usnea subfloridana* (Parmeliaceae)[1]

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- *Premise of the study:* Microsatellite loci were developed for the haploid lichenized fungal species *Usnea subfloridana* to study its population subdivision and the species' response to forest disturbance, fragmentation, and environmental pollution.
- *Methods and Results:* We developed 14 polymorphic microsatellite markers using 454 pyrosequencing data of *U. subfloridana*. The number of alleles per locus ranged from three to 15, and Nei's unbiased gene diversity averaged over nine markers without null alleles ranged from 0.64 to 0.67. Evaluation of the cross-species amplification in *U. glabrescens* and *U. wasmuthii* indicates that these markers are also informative in other *Usnea* species.
- *Conclusions:* These markers will allow us to investigate the effects of forest management and environmental pollution on genetic population structure of *U. subfloridana* and closely related species. Moreover, they will help facilitate phylogeographic studies of *U. subfloridana* across the species' distribution area in Europe.

Key words: Ascomycetes; lichen-forming fungi; microsatellites; population subdivision; *Usnea florida*; *Usnea subfloridana*.

Conspicuous epiphytic lichens like beard lichens (*Usnea* Dill. ex Adans.) are valuable indicators of forest ecosystems, hence contributing to monitoring the conservation value of forest landscapes (Will-Wolf et al., 2002). *Usnea subfloridana* Stirton is a widely distributed species occurring in Europe (Tõrra and Randlane, 2007; Randlane et al., 2009), appearing from the northern boreal to temperate regions (Halonen et al., 1998). The sexually reproducing *U. florida* (L.) Weber ex F. H. Wigg. and *U. subfloridana*, which has a predominantly asexual reproduction with symbiotic propagules, represent a typical species pair, as they share many morphological characters but differ by the characters associated with their dissimilar dispersal strategies (Articus et al., 2002; Randlane et al., 2009). Here, we develop 14 microsatellite markers to study the impact of land use and habitat fragmentation on the lichen's dispersal and population subdivision.

METHODS AND RESULTS

We selected three *U. subfloridana* specimens sampled from Norway, Finland, and Lithuania and two *U. florida* specimens sampled from the United Kingdom (Appendix 1). The central axis, which is of pure fungal origin (haploid), was manually separated and used for DNA extraction with the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocol. Library preparation and whole genome 454 pyrosequencing of pooled DNA samples was performed by Microsynth (Balgach, Switzerland) using a Roche GS FLX sequencer to generate enough random sequences and to isolate a sufficient number of microsatellite loci. Shotgun libraries were prepared using the GS FLX Titanium Rapid Library Preparation Kit (Roche Diagnostics, Basel, Switzerland), and Microsynth provided barcode adapters. We obtained 85,718 reads with an average length of 391 bases and a total of 27,344,042 bases out of a 2/16th run. We screened for all sequence motifs of di-, tri-, tetra-, and pentanucleotide microsatellites in the unassembled reads using MSATCOMMANDER 1.0.2 alpha (Faircloth, 2008). Microsatellites with motifs repeated at least eight times (for di- and trinucleotides) or six times (for all others) were selected. Primer pairs were developed with Primer3 (Rozen and Skaletsky, 2000), implemented in the software MSATCOMMANDER 1.0.2 alpha using the default parameters except for the following: optimal primer size 20 bp, product size 150–450 bp, melting temperature (T_m) 58–65°C. We found 132 primer pairs that fulfilled the specified primer parameters, but 68 pairs were later discarded either because they were duplicates, which were detected after alignment using CLC DNA Workbench 5 (CLC bio, Aarhus, Denmark), or because they contained mononucleotide repeats in the flanking region.

Additionally, we set up axenic algal cell cultures of the photobiont of *U. subfloridana* to assess the symbiont specificity of the newly designed markers. The culture was established under sterile conditions on 1/4 of strength of original *Trebouxia* Organic Nutrient Medium–I according to Ahmadjian (1967). Algal cells were taken from the algal layer of *U. subfloridana* thalli and inoculated on the medium. The cultivation took place under diurnal light (12 h) and darkness (12 h) for four months before the algal culture was harvested and deposited at the Swiss Federal Research Institute WSL (cultures TTA1 and TTA2) at −80°C. Algal cells were disrupted and DNA was extracted with MO BIO PowerPlant DNA Isolation Kit (MO BIO Laboratories, Carlsbad, California, USA) according to the manufacturer's protocol. The three loci that produced positive PCR reactions were excluded from further analyses because

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they were 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 2× 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 using a temperature gradient with one-degree increments from 56–61°C, 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 were run.

Only primers failing to amplify with DNA extracted from the axenic algal culture were considered of fungal origin. These 61 primers were tested for variability under the same conditions as above and using the total DNA of eight specimens of *U. subfloridana* collected from EVO population from southern Estonia (Appendix 1), resulting in 14 loci with satisfactory amplification. Cross-species amplification of two closely related species (Saag et al., 2011) was tested with 12 specimens of *U. glabrescens* (Vainio) Vainio and 14 specimens of *U. wasmuthii* Räsänen, which were collected from the same site. Approximately 50 mg dry weight of each lichen thallus was lyophilized overnight and ground in a Retsch MM2000 mixer mill (Düsseldorf, Germany) for 3 min at 30 Hz, and total DNA was extracted with the same procedure as the algal cells.

To characterize the 14 polymorphic *U. subfloridana* loci (Table 1), we analyzed PCR products of 174 individuals from three populations (Table 2, Appendix 1). Fluorescent forward primers were used for the PCR protocol and the reaction was adjusted to: 5 min at 95°C, followed by 28 cycles of 30 s at 95°C, 90 s at 57°C, and 30 s at 72°C, with a final extension of 80 min at 60°C. PCR products were multiplexed (Table 1) and run on a 3130xl DNA Analyzer with GeneScan 500 LIZ Size Standard (G5 dye set) for fragment analysis (both by Life Technologies). Alleles were determined using GeneMapper version 3.7 (Life Technologies). To characterize the variability of each microsatellite locus, we counted the number of alleles and calculated Nei's unbiased gene diversity using Arlequin version 3.11 (Excoffier et al., 2005).

Sequences of the 14 polymorphic loci were deposited in GenBank as they appear in the original pyrosequencing sample (Table 1). Five loci (Us10–Us14) had more than 10% of null alleles, possibly because of mutations in the primer regions, and were therefore omitted from the population analyses. The nine microsatellite loci that had no null alleles (Us1–Us9) produced three to 15 alleles per locus with a mean of 8.78. Nei's unbiased gene diversity, averaged over nine markers, ranged from 0.64 to 0.67 (Table 2). After PCR optimization for the annealing temperature (Table 1), all nine primers successfully amplified and were polymorphic in *U. subfloridana*, *U. wasmuthii*, and *U. glabrescens*, except marker Us07, which showed no polymorphy in *U. glabrescens*. As is often the case in populations of highly clonal organisms such as lichens (Walser et al., 2004; Dal Grande et al., 2012), significant linkage disequilibrium was found using Arlequin version 3.11 in 41 *U. subfloridana* distinct multilocus genotypes for two pairs of markers (i.e., Us02-Us06 and Us05-Us08).

CONCLUSIONS

The manual separation of the purely fungal central axis of the genus *Usnea* did not provide pure fungal DNA as expected. This preparation led to mixed DNA of the two fungal and algal symbionts and thus symbiont-specificity of genetic markers has to be tested in lichens (Devkota et al., 2014) even if lichens contain purely fungal plectenchyma. The newly developed, highly variable fungus-specific markers are currently being

TABLE 1. Characteristics of 14 microsatellite loci developed for the lichen fungus *Usnea subfloridana*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Fluorescent dye	T_a (°C)	Allele size range (bp)	Primer conc. (μM)	Multiplex[b]	GenBank accession no.
Us01	F: CCCTCCAATCCCATATATAC R: GATCCATCGTCTCCATGATA	$(CA)_{10}$	PET	59	181–303	0.02	1	KC912190
Us02	F: GTTTGCGAGGGATACTTTAC R: GTAGAAAGACAAGGACGCAC	$(TA)_{10}$	NED	57	83–89	0.06	2	KC912191
Us03	F: CCATCTCAAACCCGATAGTC R: CCTCTTATCCTTTGGCAAAT	$(CTT)_{11}$	PET	57	192–218	0.12	2	KC912192
Us04	F: ATTCCTCCGCAGTCAAGTAG R: CTTTACTTTGCGAGCTTCAG	$(CAT)_9$	VIC	57	181–196	0.06	2	KC912193
Us05	F: TCTGGGATGGATGGCATT R: GCGGGAAAGCCACAGATA	$(CA)_9$	FAM	57	161–175	0.20	2	KC912194
Us06	F: CGATGGCGTAATGATCAG R: CAAACATTCGCGTCAGTTAC	$(GAT)_9$	FAM	57	106–127	0.12	2	KC912195
Us07	F: TCCTGAGTTTGAGCCTCTTG R: GTCCAACGCAGCTACTCTCT	$(GTC)_8$	VIC	57	123–135	0.02	3	KC912196
Us08	F: CCTTCACCTTCCCTATACACC R: GAATCCCAGTCAATGCACTC	$(AAC)_{10}$	NED	57	192–217	0.03	3	KC912197
Us09	F: GGAAGGTATGAGGTGAGGTG R: AGAATTTGCAGCACGGGTA	$(GGAT)_6$	FAM	57	341–372	0.30	3	KC912198
Us10	F: ACGAGGGAGGGTAGTTAGG R: GTATGATTGGTTGTGATGCG	$(GA)_9$	PET	59	220–318	1.0	1	KJ446958
Us11	F: AGCATCCCACAACACACTTC R: CACTCTTTATCCTGCTCGC	$(GCA)_9$	NED	59	242–245	1.0	1	KJ446959
Us12	F: CCCTTCTTCCTACTCGGTATC R: CCTACTTCTACCATGCGTCC	$(TTC)_8$	PET	57	281–287	0.2	3	KJ446960
Us13	F: CCATTTACGGCAGAAGACTC R: CGTAGATGGGAGTCCGATAT	$(CTT)_{11}$	FAM	57	122–176	0.1	3	KJ446961
Us14	F: GACCATTAGCCAGGTTAGAG R: CACAGGGTCTCATGATTCTG	$(GCTT)_6$	FAM	59	189–205	0.1	1	KJ446962

Note: T_a = annealing temperature.

[a] See Appendix 1 for specimens used.

[b] Multiplex indicates loci that were mixed in the same capillary electrophoresis run.

Table 2. Characteristics of nine polymorphic microsatellite loci developed for *Usnea subfloridana*[a] and screened in 174 individuals.

	Total ($n = 174$)		EPV ($n = 56$)		EVO ($n = 60$)		EVY ($n = 58$)	
Locus	A	H_e	A	H_e	A	H_e	A	H_e
Us01	15	0.81	9	0.81	14	0.84	9	0.74
Us02	6	0.78	6	0.75	5	0.79	5	0.76
Us03	14	0.78	9	0.73	10	0.77	12	0.83
Us04	3	0.43	3	0.48	3	0.41	3	0.39
Us05	9	0.63	7	0.69	7	0.63	6	0.55
Us06	7	0.71	6	0.72	6	0.72	6	0.71
Us07	6	0.66	4	0.65	6	0.68	4	0.67
Us08	7	0.72	7	0.77	6	0.70	6	0.68
Us09	12	0.42	6	0.43	9	0.43	5	0.41
Average	8.78	0.66	6.33	0.67	7.33	0.66	6.22	0.64

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

[a] See Appendix 1 for populations used in the study.

used to study the genetic differentiation and diversity in *U. subfloridana*, *U. florida*, and related species and will allow us to investigate effects of forest management and environmental pollution on genetic population structure in epiphytic lichens.

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Appendix 1. Location information, number of individuals sampled, and voucher specimens of *Usnea* species used in this study.

Species	Locality	Population	Latitude	Longitude	n	Herbarium[a]	Date
*U. florida**	United Kingdom: Devon, Dartmoor, Lydford	UK	50°33′20″N	04°02′58″W	1	TU-FLO 02	07.10.2006
*U. florida**	United Kingdom: Devon, Morwellham Quay, river Tamar	UK	50°33′20″N	04°02′58″W	1	TU-FLO 03	06.10.2006
U. glabrescens	Estonia: Community of Põlva Vald	EPV_16a	58°06′13.6″N	27°04′29.1″E	1	WSL-21566	29.06.2007
*U. subfloridana**	Norway: Hordaland, Voss, Tvinde	NOR	60°43′43″N	06°29′13″E	1	TU-SUB 01	12.07.2006
*U. subfloridana**	Lithuania: Birzai district, Birzai forest, Buginiai forestry	LIT	56°14′N	24°58′E	1	TU-SUB 05	13.05.2006
*U. subfloridana**	Finland: Northern Savonia, Suonenjoki, Mustolanmäki	FIN	62°44′39″N	27°01′28″E	1	TU-SUB 10	10.07.2007
U. subfloridana	Estonia: Community of Põlva Vald	EPV	58°06′13.6″N	27°04′29.1″E	56	WSL-21521-21583	29.06.2007
U. subfloridana	Estonia: Community of Vastse-Kuuste Vald	EVO	58°07′23.7″N	26°59′22.0″E	60	WSL-21773-21834	03.07.2007
U. subfloridana	Estonia: Community of Vastse-Kuuste Vald	EVY	58°08′29.3″N	27°02′57.9″E	58	WSL-21899-21961	04.07.2007
U. wasmuthii	Estonia: Community of Põlva Vald	EPV_7a	58°06′13.6″N	27°04′29.1″E	1	WSL-21539	29.06.2007

Note: n = number of samples analyzed; Herbarium = Herbarium accession number, Date = collection date.

[a] TU = University of Tartu, WSL = Swiss Federal Research Institute WSL. Specimens stored at WSL are kept frozen at –20°C. All specimens were collected by T.T. and L.M.

*Specimens used for shotgun sequencing.

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Primer Note

Characterization of fungus-specific microsatellite markers in the lichen-forming fungus *Parmelina carporrhizans* (Parmeliaceae)[1]

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- *Premise of the study:* Microsatellite loci were developed to study the lichen-forming fungus *Parmelina* (Parmeliaceae) in different habitats of western Europe and the Mediterranean for baseline studies to understand the effects of climate change on its distribution.
- *Methods and Results:* We cultured *P. carporrhizans* from ascospores for genomic sequencing with Illumina HiSeq. We successfully developed 11 polymorphic microsatellite markers and associated primer sets and assessed them with 30 individuals from two of the Canary Islands. The average number of alleles per locus was 8.8. Nei's unbiased gene diversity of these loci ranged from 0.53 to 0.91 in the tested populations. Amplification in two closely related species (*P. tiliacea*, *P. cryptotiliacea*) yielded only limited success.
- *Conclusions:* The new microsatellite markers will allow the study of genetic diversity and population structure in *P. carporrhizans*. We propose eight markers to combine in two multiplex reactions for further studies on a larger set of populations.

Key words: Ascomycota; lichen-forming fungi; microsatellites; multiplex; *Parmelina carporrhizans*; population genetics.

Parmelina carporrhizans (Taylor) Poelt & Vêzda (Parmeliaceae) is a sexually reproducing foliose lichen species that has long been considered synonymous with the morphologically similar *P. quercina* (Willd.) Hale. Thus, the geographic distribution and degree of conservation of both species are poorly known (Argüello et al., 2007; Clerc and Truong, 2008). These two species are largely allopatric but they occasionally overlap, being apparently parapatric depending on the climatic conditions. Hence they possibly may be used as indicators of climate change. *Parmelina carporrhizans* has an Atlantic-Mediterranean distribution in Europe. It is abundant in the central-western Iberian Peninsula in the humid supra- and mesomediterranean level on deciduous *Quercus* L. vegetation (Argüello et al., 2007; Nuñez-Zapata, 2013). The species also occurs across open forest and in isolated trees above the Canarian monteverde forest in central Macaronesia from 800 to 1500 m and is locally common on Gran Canaria. Further, *P. carporrhizans* is listed as "vulnerable" on the Red Lists of England and Wales (Church et al., 1996; Woods, 2010). Despite these conservation concerns, our knowledge of the population genetics of this species is currently limited.

We developed 11 microsatellite markers for high-resolution population studies in *P. carporrhizans* to provide a better understanding of its genetic diversity, gene flow, and population structure. The enhanced knowledge will allow us to implement an informed conservation plan and investigate potential impacts of climate change on this narrowly distributed species. In addition, we also investigate whether this set of high-resolution microsatellite markers can be applied to other closely related species in the genus *Parmelina* Hale.

METHODS AND RESULTS

We isolated the mycobiont of *P. carporrhizans* from ascospores of two thalli (deposited in the herbarium of the Universidad Complutense de Madrid [MAF], Madrid, Spain: MAF-Lich 19191 and MAF-Lich 19192) collected in Cuevas del Valle, Spain (40°18′28.4″N, 5°00′39.0″W), in October 2012, following the inverted Petri dish method (Ahmadjian, 1993). We germinated spores in Basal Bold Medium (Deason and Bold, 1960), and after two weeks these were transferred to corn meal agar (CMA) and malt yeast (Honegger et al., 2004), where the cultures were grown for four months.

Prior to DNA extraction, we removed secondary metabolites with acetone, and then crushed the samples with pestles in liquid nitrogen and extracted genomic DNA with the DNeasy Plant Kit (QIAGEN, Redwood City, California, USA) according to the manufacturer's instructions.

To confirm the identity of the mycobiont cultures, we amplified the internal transcribed spacer (ITS) region of the nuclear rDNA from the axenic cultured tissues. Genomic DNA (10–25 ng) was used for PCR amplifications. Primers, PCR, and cycle sequencing conditions were the same as described previously

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Table 1. Overview of the microsatellite loci and associated primer sets successfully developed for *Parmelina carporrhizans* and deposited in the National Center for Biotechnology Information (NCBI) database.

Locus	Primer sequences (5′–3′)	Repeat motif	Dye	T_a (°C)	Allele size range (bp)[a]	GenBank accession no.
Pcar1	`F: *CATCAAATCATCCGCTACCA` `R: GGGGAGGTGAGGAGAACAA`	$(AC)_{18}$	FAM	57	124–147	KM875582
Pcar2	`F: *TCACCATGTGGTAGGGTAGC` `R: CTGTATCGAACAAGGCATCG`	$(GTA)_{15}$	NED	57	206–265	KM875583
Pcar3	`F: *TGACCCTGTGACCTCTTGC` `R: GCCTCGGGTCCATACAGAT`	$(AAT)_{17}$	PET	57	109–249	KM875584
Pcar4	`F: *AGGAGGGGGTGAAAAAGAGA` `R: GCTGGTCTTTGCACTCATCA`	$(AAGAG)_{16}$	VIC	57	280–318	KM875585
Pcar5	`F: *GATGCGTATAGCGGTGCAT` `R: TTCTGTGGGATGTATTGCAGA`	$(AG)_{18}$	FAM	57	227–309	KM875586
Pcar6	`F: *GCATTGCATGAGGCTGAAC` `R: TGCAGTGGCAATCAATGTG`	$(CTT)_{15}$	NED	57	203–270	KM875587
Pcar7	`F: *CTGGGGTGGTGATTGTGAG` `R: GCAAGCAGAAAGCAGCAAC`	$(AAG)_{19}$	PET	57	120–223	KM875588
Pcar8	`F: *GCTTGAATTGGAGGGAAGC` `R: GAGGCGTGTATGCCTTAACC`	$(GAT)_{20}$	VIC	57	372–474	KM875589
Pcar9	`F: *GAAACTCCCACCACCGTTC` `R: AAGCATTTTGGTGCATTGG`	$(AG)_{16}$	FAM	57	89–165	KM875590
Pcar10	`F: *GCCCTCCAATGAAGGAGTC` `R: CCTTGGCTGGGATAAGCAT`	$(AC)_{16}$	FAM	57	341–390	KM875591
Pcar11	`F: *CGATAGCGGAGGATTTTCAG` `R: GTCTGCGTCGCCTCTAATTC`	$(ACTC)_{17}$	FAM	57	250–371	KM875592

Note: T_a = annealing temperature.

[a] Size range indicates allele size based on two populations collected in the Canary Islands (see Appendix 1).

* M13 tail: TGTAAAACGACGGCCAGT.

(Argüello et al., 2007). Sequencing was conducted on an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, California, USA) at Centro de Genómica y Proteómica del Parque Científico de Madrid. The identity of the sequences and specimens were confirmed using the MegaBLAST search function in GenBank. ITS sequences were deposited in GenBank (accession numbers KM357892 and KM357893).

From the extracted DNA, approximately 0.5 µg of genomic DNA was used to construct an Illumina library using the Nextera XT multiplex paired-end kit (Illumina, San Diego, California, USA). The library was paired-end sequenced using an Illumina HiSeq 2000 with 100 cycles (version 3 chemistry). Standard Illumina protocols (http://www.illumina.com/) were used to generate the library. Sequencing was carried out at the Stab Vida Laboratory (Madan Parque, Caparica, Portugal). Illumina reads were assembled to contigs using the "De novo assembly" option of the CLC Genomics Workbench version 6.0.4 (CLC bio, Aarhus, Denmark). A total of 38,115,484 reads with an average length of 69.06 bases and a total of 2,632,336,717 bases were recovered. De novo assembly produced 31,035 contigs (N50 = 3615 bp) with an average of approximately 73× coverage, which totaled 36.2 Mbp of genome data.

All the contigs were screened for microsatellites using MSATCOMMANDER 1.0.8 (Faircloth, 2008), accepting di-, tri-, tetra-, penta-, and hexanucleotide repeats of ≥15. We found 63 contigs containing microsatellite sequences with 15 to 20 repeats (29 dinucleotides, 24 trinucleotides, 7 tetranucleotides, 2 pentanucleotides, and 1 hexanucleotide). From these contigs, we designed short primers of 19–21 bp in length with the program Primer3 using default parameters (Rozen and Skaletsky, 2000), expecting some transferability within the genus as reported in other lichen mycobionts (Jones et al., 2012; Devkota et al., 2014). We excluded contigs with short flanking regions, as well as repeated motifs on the flanking region, and selected primer pairs with amplicons between 100 and 400 bp. Finally, an M13 tag (5′-TGTAAAACGACGGCCAGT-3′) was appended to forward primers for subsequent amplification.

Microsatellite PCRs were performed in a 10-µL reaction volume containing ~0.5–5 ng of genomic DNA, 1× Type-it Multiplex Master Mix (QIAGEN, Hilden, Germany), 0.15 µM of reverse primer, 0.01 µM of M13-tailed forward primer, and 0.15 µM of dyer–M13-labeled primer (Schuelke, 2000). PCRs were carried out with an initial 5-min denaturation at 94°C; followed by 35 cycles of 94°C for 30 s, 57°C for 45 s, and 72°C for 45 s; and a final extension of 72°C for 30 min.

We tested the 24 primer pairs with seven accessions of *P. carporrhizans* from different areas of its distribution range and one accession of *P. tiliacea* (Hoffm.) Hale (MAF-Lich 17252); see Appendix 1 for specific localities. Out of these 24 primers, only 12 pairs successfully amplified all of the *P. carporrhizans* samples, and four pairs amplified in *P. tiliacea*. We then tested this subset of 12 primer pairs for variability with 30 samples of *P. carporrhizans* from Gran Canaria and Tenerife (MAF-Lich numbers 19123–19152; Appendix 1), as well as one accession each of *P. tiliacea* and *P. cryptotiliacea* Crespo & Núñez-Zapata (MAF-Lich 19403 and MAF-Lich 19402, respectively). Eight of these primer pairs (Pcar1–Pcar8) amplified all *P. carporrhizans* samples, while the other three (Pcar9–Pcar11) had 3.3–10% missing data. Four of these primer pairs (Pcar3, Pcar5, Pcar7, Pcar9) amplified in *P. tiliacea* and none amplified in *P. cryptotiliacea*. We deposited these 11 primer sequences in GenBank (Table 1); other primer pairs were excluded due to their low amplification rate (<60%). Our limited cross-species amplification results suggest that it may be possible to use some of these markers in other species of the *P. carporrhizans* clade (Nuñez-Zapata, 2013).

Polymorphism within the eight microsatellite loci that amplified across all *P. carporrhizans* samples was determined by counting the number of alleles and calculating Nei's unbiased haploid diversity (Table 2) using GenAlEx version 6.41 (Peakall and Smouse, 2006). The number of alleles ranged from four to 14, and the average unbiased diversity was 0.76, a relatively high number for

Table 2. Number of alleles (*A*) and Nei's unbiased genetic diversity (H_e) of the eight polymorphic microsatellite loci that were amplified with 100% success across 30 samples from the Canary Islands.

	Total		Gran Canaria (n = 20)		Tenerife (n = 10)	
Locus	*A*	H_e	*A*	H_e	*A*	H_e
Pcar1	6	0.55	4	0.56	4	0.53
Pcar2	4	0.64	4	0.73	2	0.56
Pcar3	14	0.89	11	0.87	6	0.91
Pcar4	8	0.82	7	0.78	6	0.87
Pcar5	9	0.78	6	0.68	7	0.87
Pcar6	9	0.78	8	0.87	4	0.71
Pcar7	12	0.89	9	0.90	6	0.89
Pcar8	9	0.75	8	0.89	3	0.60
Average	8.88	0.76	7.13	0.79	4.75	0.74

just 30 individuals from a small geographic area. No identical multilocus genotypes were found among the samples as is expected for a sexually reproducing lichen-forming fungus.

CONCLUSIONS

We developed 11 polymorphic fungus-specific microsatellite markers to facilitate studies of population genetics in *P. carporrhizans*. Eight of the 11 microsatellite primer pairs are being used to analyze *P. carporrhizans* populations. The results from future population genetic studies will help inform us on population responses to global changes, clarify the mechanisms of speciation, as well as define populations of this narrowly distributed species for conservation purposes.

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Appendix 1. Voucher information for specimens of *Parmelina carporrhizans*, *P. cryptotiliacea*, and *P. tiliacea* used in this study. All the specimens are deposited in the Lichen section of MAF herbarium, Faculty of Pharmacy, Universidad Complutense de Madrid, Madrid, Spain (MAF-Lich).[a]

Voucher no.	Species	Locality	Substrate[b]	Geographic coordinates	Elevation (m)	Collectors	Collection date
16476	*P. carporrhizans*	Canakkale (Tr)	*Quercus* sp.	40°06′N 26°55′E	400	A. Crespo, P. K. Divakar & M. Candan	15/06/2007
17252	*P. tiliacea*	Tenerife	Rock	28°07′14″N 16°40′19″W	982	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	19/06/2009
19191	*P. carporrhizans*	Avila (Es)	*Castanea sativa*	40°18′28″N 05°00′39″W	1007	A. Crespo, D. Alors & C. Ruibal	11/10/2012
19404	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19405	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	28°01′50″N 15°37′12″W	1420	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	19/06/2009
19406	*P. carporrhizans*	Gran Canaria	*Prunus* sp.	28°00′01″N 15°32′29″W	954	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	17/06/2009
19407	*P. carporrhizans*	Tetouan (Ma)	Unidentified dead tree	35°20′43″N 05°22′20″W	687	D. Alors & C. G. Boluda	22/10/2013
19408	*P. carporrhizans*	Gran Canaria	*Ulmus* sp.	28°01′29″N 15°35′15″W	1305	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	18/06/2009
19402	*P. cryptotiliacea*	Agadir (Ma)	*Quercus ilex*	30°38′51″N 09°40′34″W	711	D. Alors & C. G. Boluda	23/10/2013
19403	*P. tiliacea*	Azilal (Ma)	*Quercus ilex*	33°25′40″N 05°11′26″W	1439	D. Alors & C. G. Boluda	20/10/2013
19123	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19124	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19125	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19126	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19127	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19128	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19129	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009

Appendix 1. Continued.

Voucher no.	Species	Locality	Substrate[b]	Geographic coordinates	Elevation (m)	Collectors	Collection date
19130	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19131	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19132	*P. carporrhizans*	Gran Canaria	*Pinus radiata*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19133	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19134	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19135	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19136	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19137	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19138	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19139	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19140	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19141	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19142	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19143	*P. carporrhizans*	Gran Canaria	*Castanea sativa*	27°59′21″N 15°35′33″W	1499	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	22/06/2009
19144	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009
19145	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009
19146	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009

APPENDIX 1. Continued.

Voucher no.	Species	Locality	Substrate[b]	Geographic coordinates	Elevation (m)	Collectors	Collection date
19147	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009
19148	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009
19149	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009
19150	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009
19151	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009
19152	*P. carporrhizans*	Tenerife	*Castanea sativa*	28°27′11″N 16°24′55″W	894	A. Crespo, P. Cubas, A. Santo & P. K. Divakar	23/06/2009

Note: Tr = Turkey; Es = Spain; Ma = Morocco.

[a] The first eight samples were tested against all 24 microsatellite primer pairs. The last 32 samples were tested against a subset of 12 microsatellite primer pairs (see Methods and Results).

[b] Scientific authorities for substrate species: *Castanea sativa* Mill., *Pinus radiata* D. Don, *Prunus* L., *Quercus* L., *Quercus ilex* L., *Ulmus* L.

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PRIMER NOTE

CHARACTERIZATION OF MICROSATELLITE LOCI IN THE LICHEN-FORMING FUNGUS *CETRARIA ACULEATA* (PARMELIACEAE, ASCOMYCOTA)[1]

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- *Premise of the study:* Polymorphic microsatellite markers were developed for the lichen species *Cetraria aculeata* (Parmeliaceae) to study fine-scale population diversity and phylogeographic structure.
- *Methods and Results:* Using Illumina HiSeq and MiSeq, 15 fungus-specific microsatellite markers were developed and tested on 81 specimens from four populations from Spain. The number of alleles ranged from four to 13 alleles per locus with a mean of 7.9, and average gene diversities varied from 0.40 to 0.73 over four populations. The amplification rates of 10 markers (CA01–CA10) in populations of *C. aculeata* exceeded 85%. The markers also amplified across a range of closely related species, except for locus CA05, which did not amplify in *C. australiensis* and *C.* "*panamericana*," and locus CA10 which did not amplify in *C. australiensis*.
- *Conclusions:* The identified microsatellite markers will be used to study the genetic diversity and phylogeographic structure in populations of *C. aculeata* in western Eurasia.

Key words: *Cetraria aculeata*; lichen; microsatellites; Parmeliaceae.

In lichens, microsatellite markers have been reported for a number of species and were successfully applied to assess levels of genetic diversity and dispersal patterns (e.g., Walser et al., 2005; Jones et al., 2015). In this paper, we develop microsatellite primers for the lichen species *Cetraria aculeata* (Schreb.) Fr. (Parmeliaceae), a dark brown, fruticose soil lichen characterized by an extremely wide distributional range and ecological niche, which makes it a good model organism to study the impact of environmental factors on population genetic structure and diversity. The species originated in the Northern Hemisphere and dispersed through South America into the Antarctic during the Pleistocene (Fernández-Mendoza and Printzen, 2013), establishing one of its centers of diversity in the Mediterranean region (Fernández-Mendoza et al., 2011; Printzen et al., 2013). Mediterranean haplotypes of *C. aculeata* mycobionts are similar to those from the Afro-alpine mountain ranges (Lutsak et al., 2015), while central and northern European haplotypes differ more strongly (Fernández-Mendoza et al., 2011). The fine-scale structure of both symbionts is not sufficiently resolved by DNA sequence data. Therefore, microsatellite markers are of high importance to study the phylogeographic structure and genetic diversity of *C. aculeata* across western Eurasia.

METHODS AND RESULTS

Total genomic DNA was extracted from one thallus of *C. aculeata* (isolate 3041, Ukrainian Nature Steppe Reserve, FR-0261072) in four single reactions with the DNeasy 96 Plant Kit (QIAGEN, Hilden, Germany). The samples were frozen in liquid nitrogen, ground, and incubated overnight at 37°C in a mixture of enzymes: 400 μL of Glusulase (PerkinElmer, Boston, Massachusetts, USA) and 500 μL of 0.1 mg mL^{-1} Zymolyase 20T (Seikagaku Biobusiness, Tokyo, Japan) in 500 μL of phosphate buffer (pH 7.4). The DNA extracts were pooled, and libraries for the Illumina HiSeq and MiSeq sequencing platforms were created. A total of 25,727,973 paired-end reads with an average length of 100 bp and 15,120,929 paired-end reads with an average length of 250 bp were obtained from the HiSeq and MiSeq runs, respectively. Illumina adapters and primers were trimmed and quality filtering was performed using Trimmomatic software version 0.27 (Bolger et al., 2014). The lichen metagenome was assembled using Velvet software version 1.2.10 (Zerbino and Birney, 2008). Using the BLAST tool v2.2.25+, algal and bacterial contigs were filtered out. The latest available National Center for Biotechnology Information (NCBI) nucleotide database (NT Database, 24 May 2013) was used for aligning the assembled contigs. The alignment file was processed by using MEGAN5 version 4.70.4 software under default settings (Huson et al., 2007). Plots generated by MEGAN were used for assessing the bacterial, fungal, and algal contigs, which served as a reference for mapping the raw reads in the second round of genome assembly. The inferred contigs were screened for tri-, tetra-, penta-, and hexanucleotide repeats with at

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TABLE 1. Overview of the microsatellite loci developed for the mycobiont of *Cetraria aculeata*.

Locus	Primer sequences (5′–3′)	Repeat motif[a]	Allele size range (bp)	T_a (°C)	Fluorescent dye	PCR multiplex	Amplification rate (%)	GenBank accession no.
CA01	F: GTATGGTGGTGTCACGGGTT R: CGGTGGTGAGAAGTGAGAGG	$(GAAG)_n$	214–238	55	FAM	1	100	KU361362
CA02	F: TGATGTCATCGAAGCCCTGG R: ATATCCGTTGCGTGGTTGGT	$(TTGA)_n$	247–267	53	NED	1	100	KU361368
CA03	F: TAGTAGCGGGCAGTCGAAAC R: TGCTTGAGCTTGCTTCTCGA	$(CTCTG)_n$	297–362	53	VIC	1	100	KU361361
CA04	F: GAAACTGAGGGGAAGTGCCA R: GATGGCTGGTCCCAATGACA	$(CTCCAC)_n$	318–344	54	PET	1	100	KU361364
CA05	F: AACCCCCAACCCACAGAATC R: GCGAACGAGGATGATTGTGC	$(CACG)_n$	123–135	54	FAM	2	100	KU361363
CA06	F: AGAACGGCAGGAAGAAGAGC R: CCCTGGTCGGCTCCAATATC	$(TTGA)_n$	201–269	55	NED	2	100	KU361367
CA07	F: AATGAAACACGTGGCTGTGG R: CAGACCGGGTGTGTCATTCA	$(GTAG)_n$	205–253	53	VIC	3	100	KU361365
CA08	F: AGTGCGGGTGAATGTACGAG R: CGGGCCGCTCCATTTGTATA	$(AATGA)_n$	221–276	54	FAM	3	100	KU361370
CA09	F: ATGAGGCACGCACAGAATGA R: CACTCCCACTCGTGTTACCC	$(CCGAGT)_n$	312–380	54	PET	3	100	KU361369
CA10	F: TGCACTAAGGAGGATGTCGC R: TTGACTGCTTCCGAGGAGAA	$(TCAA)_n$	318–350	53	FAM	3	85.1	KU361366
CA11	F: CCTTTTCACAGCGTCGCTTC R: CCCCTCCTTCTAACTCGCAC	$(ATAC)_n$	364–424	55	PET	2	76.7	KU764488
CA12	F: CGTCTCCGTGTACCATAGCC R: CAGCAGCGTTATCAGCAAGC	$(TTGCTG)_n$	184–284	55	PET	1	60	KU764489
CA13	F: GCTCACCCTCTCAGCAGATC R: TGGCTGCGTTCTCTTACAGG	$(GAAGAG)_n$	265–443	55	VIC	2	76.7	KU764490
CA14	F: GCAACGTGCATGGAAACGTT R: TCTCGTTCGGCAGTTGAGAC	$(GTAAA)_n$	219–264	53	PET	2	45	KU764491
CA15	F: CGCTTGTGATATCGTCCGGA R: ACATCATCCGCAGCTTCCAA	$(CTCTTG)_n$	188–318	53	FAM	2	70	KU764492

Note: T_a = annealing temperature.
[a] n stands for numerous repeats.

least five repeats using the MIcroSAtellite identification tool (MISA; Thiel et al., 2003; http://pgrc.ipk-gatersleben.de/misa/misa.html). We selected 40 loci with perfect (uninterrupted) microsatellites and a repeat size of four to six base pairs for testing. Primers were designed using the Primer3 plugin in Geneious R7.1.9 (created by BioMatters, http://www.geneious.com/).

On a subset of eight specimens of *C. aculeata*, 24 primer pairs amplified successfully and showed significant variability. Single PCR reactions were performed using illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare, Little Chalfont, Buckinghamshire, England) according to the manufacturer's protocol with the following conditions: denaturation for 5 min at 95°C; followed by 10 cycles of 1 min at 94°C, 1 min at 65–57°C (touchdown of −1° per cycle), and 1 min at 72°C; followed by 20 cycles of 1 min at 94°C and 1 min at 54, 53, or 52°C (depending on primer pair); and a final extension of 10 min at 72°C. The 24 primer pairs were also tested in PCR reactions with three axenic cultures of *Trebouxia jamesii* (Hildreth & Ahmadjian) Gärtner, a photobiont of *C. aculeata*, isolated from specimens from Antarctica, Spain, and Germany. Eight primer pairs produced PCR products with at least one of the cultures and were discarded. The remaining 16 primer pairs were assumed to be fungal specific. Forward primers were labeled with fluorescent dyes and used in multiplex PCRs with the Type-It Multiplex Kit (QIAGEN). PCR reactions were performed in a total volume of 25 μL, which contained 12.5 μL of Type-It Multiplex PCR Master Mix, 2.5 μL of primer mix, 5 μL of RNA-free water, and 5 μL of sample DNA. The PCR conditions were set to: denaturation for 5 min at 95°C; followed by five cycles of 30 s at 95°C, 90 s at 57°C, and 30 s at 72°C; then 15 cycles of

TABLE 2. Characteristics of microsatellite loci CA01–CA10 in populations of *Cetraria aculeata* from Spain.[a]

	Total		Spa1 (n = 10)		Spa2 (n = 23)		Spa3 (n = 24)		Spa4 (n = 24)	
Locus	A	H_e	A	H_e	A	H_e	A	H_e	A	H_e
CA01	6	0.423	3	0.511	3	0.466	3	0.409	3	0.304
CA02	5	0.404	2	0.356	4	0.704	3	0.475	2	0.083
CA03	10	0.713	5	0.667	4	0.668	8	0.812	4	0.707
CA04	5	0.501	3	0.711	3	0.372	3	0.359	3	0.562
CA05	4	0.524	3	0.622	3	0.502	3	0.627	2	0.344
CA06	13	0.726	5	0.822	6	0.771	8	0.804	2	0.507
CA07	9	0.564	4	0.733	6	0.613	5	0.486	4	0.424
CA08	10	0.679	4	0.778	8	0.834	6	0.746	3	0.359
CA09	9	0.674	3	0.644	5	0.708	4	0.634	4	0.710
CA10	8	0.522	4	0.711	4	0.575	1	0.000	6	0.801
Mean	7.9	0.573	3.6	0.656	4.6	0.621	4.4	0.535	3.3	0.480

Note: A = number of alleles; H_e = Nei's unbiased gene diversity; n = total number of samples per population analyzed.
[a] Population locality and voucher information are provided in Appendix 1.

Table 3. Cross-amplification of *Cetraria aculeata* microsatellite markers with related species of the genus *Cetraria.*

	No. of alleles observed														
Species	CA01	CA02	CA03	CA04	CA05	CA06	CA07	CA08	CA09	CA10	CA11	CA12	CA13	CA14	CA15
C. australiensis	1	1	1	1	0	1	2	1	2	0	0	0	0	0	0
C. crespoae	2	1	1	2	2	2	2	2	2	2	0	0	2	0	0
C. muricata	1	2	1	2	2	1	2	2	2	2	1	1	1	0	0
C. odontella	2	1	1	2	1	1	1	1	1	1	1	0	0	0	0
C. “*panamericana*”	1	1	1	2	0	1	1	1	1	1	0	0	1	0	0

Note: 0 = locus did not amplify; 1 = locus amplified in a single specimen; 2 = locus amplified in two specimens tested.

30 s at 95°C, 90 s at 55°C, and 30 s at 72°C; then 10 cycles of 30 s at 95°C, 90 s at 54°C, and 30 s at 72°C; and 10 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. One more primer pair was eliminated, as it yielded products of inconsistent size.

A data set of 81 specimens of *C. aculeata* was used to test the amplification rates and the variability of selected microsatellite loci. The data set consisted of four populations of 10 to 24 individuals collected in Spain (Appendix 1). Sequences and primers were submitted to GenBank (Table 1). Alleles were sized using GeneMarker version 1.90 (SoftGenetics, State College, Pennsylvania, USA). The variability of the microsatellite loci was measured by counting the number of alleles and calculating Nei’s unbiased gene diversity using GenAlEx 6.5 (Peakall and Smouse, 2012).

The microsatellite markers CA01–CA09 showed an amplification rate of 100%. CA10 amplified in 85% of the samples (Table 1). Tetranucleotide microsatellites (six out of 10) were the most common microsatellite motif. The microsatellite loci showed between four (CA05) and 13 (CA06) alleles per locus, with a mean of 7.9 and average gene diversities between 0.40 and 0.73 across four populations (Table 2). The other five markers (CA11–CA15) were not considered for the statistical analysis due to lower amplification rates. The primers developed were also tested for cross-species amplification with specimens of *C. muricata* (Ach.) Eckfeldt, *C. crespoae* (Barreno & Vázquez) Kärnefelt, *C. odontella* (Ach.) Ach., *C. australiensis* W. A. Weber ex Kärnefelt, and the still undescribed *C.* “*panamericana*” (listed in Appendix 1). The polymorphic markers CA01–CA10 successfully amplified in all the species, except for locus CA05 in *C. australiensis* and *C.* “*panamericana*,” and locus CA10 in *C. australiensis* (Table 3). Linkage disequilibrium in pairwise combinations of the loci within populations and across the total sample was tested using GENEPOP 4.2 (Raymond and Rousset, 1995). Holm-Bonferroni sequential correction of P values (Holm, 1979) using the implementation by Gaetano (2013) and a significance threshold of 0.05 resulted in nine significant deviations from linkage equilibrium out of 45 pairwise comparisons across all populations. The deviations involved all loci except CA06 and CA10. Only five out of 171 pairwise comparisons within populations showed significant deviation from linkage equilibrium. These deviations are more likely explained by the predominantly clonal reproduction of *C. aculeata* or population structure than by physical linkage of microsatellite loci.

CONCLUSIONS

The microsatellite markers developed here will facilitate the studies of genetic diversity and population structure of *C. aculeata* along geographic and bioclimatic gradients. These studies will increase our understanding of dispersal patterns and local adaptation in lichens. The fact that primers amplify across a broader range of species may also contribute to a better understanding of species boundaries and speciation within the genus.

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Appendix 1. Localities and collection data for *Cetraria* species used in this study.

Species	*n*	Geographic coordinates	Locality data	Collector and collection year	Voucher no.[a]
C. aculeata (Schreb.) Fr.	10	40°31′26.3568″N, 3°27′45.8706″W	Spain (Spa1): Provincia de Madrid, Canencia	Fernández-Mendoza, 2014	FR-0261059
C. aculeata	23	43°6′41.58″N, 5°33′8.9316″W	Spain (Spa2): Provincia de Asturias, Gamoniteiro	Fernández-Mendoza, 2014	FR-0261060
C. aculeata	24	41°2′5.172″N, 3°10′20.5674″W	Spain (Spa3): Provincia de Jaén, Despeñaperros	Fernández-Mendoza, 2011	FR-0261062
C. aculeata	24	39°47′47.4612″N, 1°16′26.3316″W	Spain (Spa4): Provincia de Cuenca, Casillas de Ranera	Fernández-Mendoza, 2014	FR-0261063
C. australiensis W. A. Weber ex Kärnefelt	2	36°27′26.5278″S, 148°16′4.962″E	Australia: New South Wales, Mount Kosciuszko	Fernández-Mendoza & de Miquel, 2009	FR-0261079, FR-0261080
C. crespoae (Barreno & Vázquez) Kärnefelt	2	39°31′58.0008″N, 4°57′7.9986″W	Spain: Toledo, National Park de Cabañeros	Pérez-Ortega, 2010	FR-0261081, FR-0261082
C. muricata (Ach.) Eckfeldt	1	49°17′00″N, 87°53′00.66″E	Russian Federation: Altai Republic, Altai Mts.	Lustyk & Kočí, 2011	hb. P. Resl
C. muricata	1	56°35′56.997″N, 4°44′50.5206″W	United Kingdom: Scotland, Rannoch Moor	Fernández-Mendoza, 2010	FR-0261084
C. odontella (Ach.) Ach.	1	68°32′59.9994″N, 27°22′30″E	Finland: Lake Inari	Westberg	LD1188229
C. odontella	1	44°57′56.7504″N, 6°36′19.1478″E	France: Granon	Fernández-Mendoza, 2013	FR-0261085
C. “*panamericana*”	2	51°10′0.0006″N, 117°24′0″W	Canada: British Columbia, Glacier National Park	Goward, 2005	FR-0261086, FR-0261087

Note: *n* = number of individuals.

[a] Vouchers are deposited in Herbarium Senckenbergianum Frankfurt (FR), Lund University Herbarium (LD), and the private herbarium of Philipp Resl.