Characterization of Microsatellite Loci in the Lichen-Forming Fungus Cetraria aculeata (Parmeliaceae, Ascomycota)

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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	$(GAAG)_n$	214–238	55	FAM	1	100	KU361362
	R: CGGTGGTGAGAAGTGAGAGG							
CA02	F: TGATGTCATCGAAGCCCTGG	$(TTGA)_n$	247–267	53	NED	1	100	KU361368
	R: ATATCCGTTGCGTGGTTGGT							
CA03	F: TAGTAGCGGGCAGTCGAAAC	$(CTCTG)_n$	297–362	53	VIC	1	100	KU361361
	R: TGCTTGAGCTTGCTTCTCGA							
CA04	F: GAAACTGAGGGGAAGTGCCA	$(CTCCAC)_n$	318–344	54	PET	1	100	KU361364
	R: GATGGCTGGTCCCAATGACA							
CA05	F: AACCCCCAACCCACAGAATC	$(CACG)_n$	123–135	54	FAM	2	100	KU361363
	R: GCGAACGAGGATGATTGTGC							
CA06	F: AGAACGGCAGGAAGAAGAGC	$(TTGA)_n$	201–269	55	NED	2	100	KU361367
	R: CCCTGGTCGGCTCCAATATC							
CA07	F: AATGAAACACGTGGCTGTGG	$(GTAG)_n$	205–253	53	VIC	3	100	KU361365
	R: CAGACCGGGTGTGTCATTCA							
CA08	F: AGTGCGGGTGAATGTACGAG	$(AATGA)_n$	221–276	54	FAM	3	100	KU361370
	R: CGGGCCGCTCCATTTGTATA							
CA09	F: ATGAGGCACGCACAGAATGA	$(CCGAGT)_n$	312–380	54	PET	3	100	KU361369
	R: CACTCCCACTCGTGTTACCC							
CA10	F: TGCACTAAGGAGGATGTCGC	$(TCAA)_n$	318–350	53	FAM	3	85.1	KU361366
	R: TTGACTGCTTCCGAGGAGAA							
CA11	F: CCTTTTCACAGCGTCGCTTC	$(ATAC)_n$	364–424	55	PET	2	76.7	KU764488
	R: CCCCTCCTTCTAACTCGCAC							
CA12	F: CGTCTCCGTGTACCATAGCC	$(TTGCTG)_n$	184–284	55	PET	1	60	KU764489
	R: CAGCAGCGTTATCAGCAAGC							
CA13	F: GCTCACCCTCTCAGCAGATC	$(GAAGAG)_n$	265–443	55	VIC	2	76.7	KU764490
	R: TGGCTGCGTTCTCTTACAGG							
CA14	F: GCAACGTGCATGGAAACGTT	$(GTAAA)_n$	219–264	53	PET	2	45	KU764491
	R: TCTCGTTCGGCAGTTGAGAC							
CA15	F: CGCTTGTGATATCGTCCGGA	$(CTCTTG)_n$	188–318	53	FAM	2	70	KU764492
	R: ACATCATCCGCAGCTTCCAA							

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.*

Species	No. of alleles observed														
	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.