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AJB Primer Notes & Protocols in the Plant Sciences

Isolation and characterization of microsatellites in the lichen *Buellia frigida* (Physciaceae), an Antarctic endemic[1]

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- *Premise of the study:* Microsatellite markers were characterized for an Antarctic endemic, *Buellia frigida*, to investigate population structure and origin of Antarctic lichens.
- *Methods and Results:* Five primer sets were characterized. All loci were polymorphic with eight to 16 alleles per locus in a sample of 59 lichens.
- *Conclusions:* The microsatellite markers potentially provide insight into population structure and gene flow of *B. frigida*.

Key words: Antarctica; *Buellia frigida*; microsatellites; Physciaceae; population structure.

Antarctica has a predominantly cryptogamic flora dominated by lichens (roughly 400 species of lichens, 50 liverworts, 100 mosses); only two species of higher plants occur and only on the warmer Antarctic Peninsula (Romeike et al., 2002). There is rapid decline in species numbers with increasing latitude, and on the main Antarctic continent there are approximately 100 species of lichen, 20 species of moss, and no vascular plants recorded (Green et al., 2007). At the Last Glacial Maximum, Antarctic ice sheets were substantially larger than at present, and there is growing interest in whether the present-day flora survived glaciation in refugia or represent the result of recent long-distance dispersal (Convey et al., 2008). Previous molecular studies of lichens in Antarctica have used markers such as ITS (Dyer and Murtagh, 2001; Romeike et al., 2002), which provide little information on population structure. Microsatellites are more rapidly evolving, are characterized by high levels of polymorphism, and are highly specific, making them ideal to use with symbiotic organisms. Here, we describe microsatellite markers for a common Antarctic endemic, *Buellia frigida* Darb. (Physciaceae), found in coastal and mountainous locations. Its widespread distribution within Antarctica makes it ideal for studies of population structure, which can provide insights into population connectivity and the evolutionary history of Antarctica.

METHODS AND RESULTS

Fungal genomic DNA was extracted from 35 apothecia, cut from a single dried thallus of *B. frigida* collected from Cape Hallett, Ross Dependency, Antarctica (72°19′23″S, 170°13′36″E).* Apothecia were chosen because this area is relatively free of algal material. DNA was isolated using a DNeasy mini plant extraction kit (QIAGEN, Valencia, California, USA) following the manufacturer's protocol with the addition of being shaken overnight at 55°C in lysis buffer. DNA concentration was estimated by comparison with a 100 bp ladder (Invitrogen Life Science, Auckland, New Zealand) on a 1% agarose gel. DNA purity was assessed by PCR amplification using universal ITS primers (ITS1/ITS4; White et al., 1990) to check for contaminants. A microsatellite library was constructed according to Edwards et al. (1996) and Karagyozov et al. (1993), with DNA digested by *Rsa*I and *Hae*III (separately) for 1 h at 37°C (consisting of 1 μg template DNA, 20 U of enzyme, and 1× buffer), followed by ligation to the *Eco*R1 adapter used by Karagyozov et al. (1993) (the 21-mer 5′-CTCTTGCTTGAATTCGGACTA-3′, and a phosphorylated 25-mer 5′-pTAGTCCGAATTCAAGCAAGAGCACA-3′). The ligated fragments were amplified using PCR in 50 μL reactions using the following reagents: the 21-mer 5′-CTCTTGCTTGAATTCGGACTA-3′ at 2 μM, 50 ng fragments, 200 μM dNTPs, 1× J buffer (Jeffreys et al., 1988), and 1 U *Taq* polymerase (Invitrogen). A PTC-100 thermocycler (MJ Research, Ramsey, Minnesota, USA) was used with the following cycling conditions: 30 cycles of 30 s each at 94°C, 60°C, and 72°C. The product was denatured, then incubated in 1 M sodium phosphate with the microsatellite oligonucleotides ($(GT)_{15}$ and $(GA)_{15}$) previously bound to nylon membranes as in the enrichment method described by Edwards et al. (1996). Hybridized DNA fragments were eluted from the membranes by boiling at 100°C for 5 min, precipitated by addition of 2.5 volumes of ethanol and 5 μL of 5% linear acrylamide, then centrifuged at 16 000 × g for 20 min. The supernatant was discarded, the pellet washed with 70% ethanol, and the fragments resuspended in 25 μL TE buffer. This was followed by a second PCR (as above) using 1 μL of the ligated fragments and a second round of enrichment. The DNA was then digested with *Eco*RI, ligated into pGEM-T vector, and transformed in *Escherichia coli*. Positive colonies were plated and 26 of 110 clones were chosen for sequencing using primer M13F (Invitrogen) on an ABI 3130XL automated DNA sequencer (Applied Biosystems, Carlsbad, California, USA). Of these, only five had perfect repeats and contained suitable sequences for primer development. Primers for

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*Vouchers of representative samples collected by Tracey Jones are deposited in the University of Waikato Herbarium and include latitude, longitude, altitude, and substrate type (accession numbers WAIK22460, WAIK22459, WAIK22458, WAIK22457, WAIK22456, WAIK22455). A voucher of the sample the microsatellites were developed from was collected by Catherine Beard and is held in the collection of Prof. Rod Seppelt, Australian Antarctic Division, and includes latitude, longitude, altitude, and substrate type (accession number ADT no. 25347).

TABLE 1. Characterization of five microsatellite loci in the mycobiont of *Buellia frigida*.

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Size (bp)	*A*	GenBank accession no.
Buef1	F: AGCAGGTATCCTCGTGCTGT R: ACCTCAATTCAGGCATGCAG	$(GT)_{21}$	60.5	189	16	JN205305
Buef2	F: ACTGCAGAGCATGTGGTGAC R: CCCATCTCAGCCAGAAGGTA	$(CA)_{15}$	60	186	10	JN205306
Buef3	F: GCATGGTGTACAACTCCCTGT R: CTTTGCCTCTGCAAACCAGT	$(GT)_{14}$	60.2	168	11	JN205307
Buef4	F: AAGCTGCCTGTCTCCAAGAA R: ACGTGAGTCCTCACCCTTGT	$(CA)_{15}$	59.9	151	8	JN205308
Buef5	F: GGGGACAAACCCGTAGAGA R: AGCAGGTATCCTCGTGCTGT	$(CA)_{21}$	59.9	164	13	JN205309

Note: *A* = number of alleles; T_a = annealing temperature.

microsatellite analysis were chosen using the online Primer3 (Rozen and Skaletsky, 2000; http://www.ncbi.nlm.nih.gov/tools/primer-blast) with the following conditions: optimal melting temperature (T_m) 60°C, optimal fragment size 200 bp, GC content between 20–80%.

To avoid bias toward only haploid tissue (e.g., thalli), DNA was extracted from whole samples (N = 59), including apothecia from Gondwana, Antarctica (74°35′23″S–74°46′30″S, 164°00′53″E–164°13′12″E), using a high alkaline PEG reagent DNA extraction protocol (Chomczynski and Rymaszewski, 2004), with the following modifications: the sample was ground with liquid nitrogen before addition of 20–40 μL of PEG reagent, shaken at 55°C for 1 h, the lysate was spun at 4000 rpm for 20 s, and an aliquot of 2 μL supernatant added to 58 μL of H_2O. A modified dialysis technique based on Freifelder and Better (1982) was used to reduce inhibitors by preferentially soaking them into agarose. The elute from PEG extraction was soaked into the top of 1.2% agarose (in 1 : 10 TE) in 0.2 mL thin-walled tubes with a pinhole-pierced bottom in a 1.5 mL Eppendorf tube overnight or spun for 10 min at 4000 rpm; 20–40 μL of 1 : 10 TE was added and nonabsorbed DNA removed from the surface after vortexing. This wash was spun at 4000 rpm for 15 s and the supernatant recovered. To assess the purity of the whole lichen DNA extract, universal ITS primers (ITS1/ITS4; White et al., 1990) were used in PCR. The resultant products were of similar size to published ITS sequences of the mycobiont (≈580 bp) and photobiont (≈650 bp), and were sequenced to confirm they corresponded with recognized species in GenBank.

Radioactive microsatellite products were analyzed on sequencing gels. A ^{33}P-γATP–labeled forward primer (0.1 μM) was used in 10 μL PCR reactions containing 1 μL of DNA (≈20 ng), 1× Platinum *Taq* buffer, 1.5 mM $MgCl_2$, 1 μM reverse primer, 200 μM dNTPs, and 0.5 U Platinum *Taq* DNA polymerase (Invitrogen) in a PTC-100 thermocycler (MJ Research) with initial denaturing at 94°C for 2 min, then 29 cycles of 94°C for 15 s, 64°C for 15 s, and 72°C for 1 min. The products were denatured at 90°C in a formamide-based loading buffer before being run on 6% denaturing polyacrylamide gels at 55 W for ≈3 h. The gel was then transferred to 3 mm filter paper, vacuum dried for 2 h, and exposed to Kodak XAR film for 1–3 d. All samples were run with a 10 bp ^{33}P-labeled ladder (Invitrogen) for reference.

Primers (Table 1) were tested on three lichen species: *B. frigida* (N = 59), *Umbilicaria aprina* Nyl. (N = 79), *U. decussata* (Vill.) Zahlbr. (N = 40), and a photobiont species present in lichens, *Trebouxia jamesii* (Hildreth & Ahmadjian) Gärtner. Primers produced a product in *B. frigida* and *U. aprina* (with ≈30% success; locus range in Table 2), but not in *U. decussata* or the photobiont *T. jamesii*. Of the original 59 *B. frigida* samples, 39 provided a product of four or more of the five alleles and were used in further analyses. Expected heterozygosity was calculated in GenAlEx 6.4 (Peakall and Smouse, 2006), and all loci were polymorphic, with a high mean expected heterozygosity (H_e = 0.833). Tests were performed for linkage disequilibrium (P > 0.05 in all cases, corrected for multiple comparisons), and observed heterozygosity departed significantly from expected heterozygosity under Hardy–Weinberg equilibrium (P < 0.0001 in all cases) using GENEPOP version 4.0 (Raymond and Rousset, 1995). It is possible that this excess of homozygotes indicates *B. frigida* are selfing as in other Antarctic species (Dyer and Murtagh, 2001) either with heterogeneous origins (>1 propagation event) or mutation events leading to heterozygotes with null alleles. Alternatively, the excess of homozygotes could have resulted from PCR bias toward haploid thallus tissue.

TABLE 2. Results of initial primer screening in one population (N = 59) of *Buellia frigida*.

Population (N = 39)	Size (bp)[a]	H_o	H_e	HWE[b]	Size (bp)[c]
Buef1	172–220	0.121	0.899	<0.0001*	178–204
Buef2	174–200	0.091	0.719	<0.0001*	174–208
Buef3	164–194	0.257	0.867	<0.0001*	158–186
Buef4	140–222	0.091	0.799	<0.0001*	140–172
Buef5	146–188	0.071	0.879	<0.0001*	148–176

Note: H_e = expected heterozygosity; H_o = observed heterozygosity.
[a] Ranges of repeat size.
[b] P values for Hardy–Weinberg equilibrium (exact probability method).
[c] Indicates *Umbilicaria aprina* allele range.
* Indicates H_o departed significantly from H_e under HWE (P < 0.01).

CONCLUSIONS

The initial data for these microsatellites suggest they are suitable for individual genotyping and characterization of *B. frigida* population structure and are mycobiont specific with a high degree of polymorphism. We conclude that the markers can potentially provide insight into population structure and gene flow, and they are currently being used to analyze population genetic structure in *B. frigida* across a latitudinal gradient in the Ross Sea sector of Antarctica.

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