American Journal of Botany

American Journal of Botany: e102–e104. 2010.

AJB Primer Notes & Protocols in the Plant Sciences

Microsatellite primers in the *Peltigera dolichorhiza* complex (lichenized ascomycete, Peltigerales)[1]

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- *Premise of the study:* Microsatellite primers were developed for the lichen-forming fungus *Peltigera dolichorhiza* to investigate partitioning of genetic variation in a widespread, morphologically and chemically variable taxon likely to represent a complex of cryptic lineages, including *P. neopolydactyla*.
- *Methods and Results:* Using next generation shotgun sequence reads, 331 primer pairs were designed to amplify microsatellite sequences from an African accession of *P. dolichorhiza*. Eleven primer pairs representing the longest repeat units identified were tested on 15 *P. dolichorhiza* accessions from Africa (incl. Réunion), South America, Papua New Guinea, and on two accessions of *P. neopolydactyla* from North America. The primers amplified di-, tri, tetra-, and pentanucelotide repeats with 3–8 alleles per locus. All individuals represent distinct multiloci genotypes.
- *Conclusions:* These results indicate the utility of the new microsatellite primers for testing genetic differentiation within the widespread complex of *P. dolichorhiza*.

Key words: lichen; microsatellite; *Peltigera*.

The development of microsatellites using enrichment or selective hybridization protocols can be time-consuming (Zane et al., 2002); furthermore, enrichment-based protocols can lead to bias due to targeting only certain microsatellite loci (Castoe et al., 2010). Next generation sequencing data provide a rapid, potentially unbiased, and powerful tool for microsatellite primer development (Castoe et al., 2010; Gompert et al., 2010).

The lichen-forming ascomycete *Peltigera dolichorhiza* (Nyl.) Nyl. is considered a pantropical species extending to the temperate regions of the Southern Hemisphere. The similar *P. neopolydactyla* (Gyeln.) Gyeln. occurs primarily in northern temperate and boreal forests. Both species are morphologically and chemically variable, and their differentiation is ambiguous. A study of *P. dolichorhiza* in Papua New Guinea (Sérusiaux et al., 2009) reveals variation in the ITS sequence among populations and cryptic speciation, with at least two lineages that are morphologically and chemically similar. Furthermore, phylogenetic analyses including the only publically available sequence for *P. neopolydactyla* suggest that it is nested with *P. dolichorhiza.* Broadening the sampling to populations from throughout the range further indicates that neither species as currently defined is monophyletic, but that together they compose a complex of discrete lineages (Sérusiaux et al., unpublished).

METHODS AND RESULTS

Total genomic DNA was extracted from 20 mg of *Peltigera dolichorhiza* from Réunion using the CTAB protocol from McNeal et al. (2006), but with an ethanol precipitation. The DNA was resuspended in 120 μL 5mM Tris/HCl, pH 8.5. DNA quality was checked by agarose gel electrophoresis, optical density, and fluorometry. The sample was sent to the IGSP Sequencing Core Facility, Duke University, for Roche FLX 454 RL shotgun library preparation using the Titanium system. The library was pooled with five other Multiplex Identifier(MID)-tagged libraries and run on a PicoTitre Plate on a Genome Sequencer FLX Instrument. The 467 027 *Peltigera* sequence reads (185 606 677 bases) were trimmed of adaptor and low-quality regions and assembled into contigs using Roche GS de novo Assembler on the UConn Biotechnology computer cluster, using default parameters (e.g., 40 bases minimum overlap and 100 bases minimum contig length) for a large or complex genome, except the minimum overlap identity percentage was set at 95%. 64 460 contigs were generated, varying in length from 250 to 10 186 bases. The assembly used 367 378 of the input reads, comprising 140 646 606 bases (c. 80%), with an inferred read error of 0.7%. A custom perl script was used to extract 99 649 singleton sequences (that did not assemble into contigs), which could also be screened for microsatellite regions.

To identify microsatellite regions, contig FASTA files were run through msatCommander 0.8.2 (Faircloth, 2008), set to automatically design primers using primer3 (Rozen & Skaletsky, 2000) as its primer design engine, accepting dinucleotide repeats of ≥9, trinucleotide repeats of ≥7, tetranucleotide repeats of ≥6, pentanucleotide repeats of ≥5 and hexanucleotide repeats of ≥5. A total of 653 repeats were found in the 64 460 contigs screened, comprising 291 dinucleotide, 272 trinucleotide, 43 tetranucleotide, 15 pentanucleotide, and 32 hexanucleotide repeats. Primers were successfully designed for 331 repeats. Primer pairs, representing the longest di-, tri, and tetranucleotide repeats for which primers were successfully developed, were selected for initial screening. The blast algorithm was used to query the NCBI nucleotide collection to identify non-*Peltigera* sequences. Twenty pairs were tested first by PCR

[1] Manuscript received 29 July 2010; revision accepted 23 August 2010.

The authors thank J. C. Villarreal A. for assistance with microsatellite amplification and sequencing, P. Lapierre for bioinformatic support, and B. F. Lang for help identifying mitochondrial regions. The UConn Bioinformatics Facility provided computing resources for 454 sequence assembly. This study was made possible through financial support from the Belgian Scientific Research Fund, the University of Liège, and the University of Connecticut.

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doi:10.3732/ajb.1000283

TABLE 1. Microsatellite loci identified and screened for populations of *Peltigera dolichorhiza.* Shown for each locus are the primer pairs, the repeat type and its variation within the sampling, size of original fragment (bp), number of individuals scored (n), number of alleles observed, Nei's gene diversity (H_e) and GenBank accession number for the locus as it occurs in the sample from La Réunion.

	Primers forward/reverse	Repeat	Size	n	Alleles	He	GenBank Accession No.
Peltdol 1	F: AGTGGCATAGTAGTTCTCGAATTTATC	$(ACAT)_{5-11}$	184	17	4	0.394	HM542469
	R: GAGGTAGTGGGTCAGCGG						
Peltdol 2	F: AAATCGGTGCATGCCAAGG	$(AATG)_{3-8}$	371	16	5	0.641	HM542470
	R: TGAGCCATGTCCGGTAAGC						
Peltdol 5	F: CCGCTGCCGTTCCATTTG	$(CTT)_{6-15}$	350	17	6	0.768	HM542471
	R: ACCGACGAACCTCTGAACG						
Peltdol 7	F: CTGCCGTTTACCACCGAAG	$(GTT)_{4-19}$	301	16	6	0.734	HM542472
	R: AGATCTCAATCGGGAGAAGGC						
Peltdol 10	F: TTCCGCCAACCAATCTTCG	$(AAC)_{7-20}$	326	16	8	0.805	HM542473
	R: GGTTTCTGCTTGAGGTCGC						
Peltdol 9	F: TCTCCCTGCTTGGCTAGTG	$(GTT)_{8-31}$	273	17	8	0.803	HM542474
	R: CTGCATGAGGGCGAAAGTC						
Peltdol 12	F: GTCGTTCGCCGAGTATTATGG	$(AAGTAC)_{1-7}$	145	15	5	0.658	HM542475
	R: GAAAGAGCACACAGCTCCG						
Peltdol 15	F: ATCGTCCGGCATCTACTGC	$(GGCATT)_{3-6}$	321	17	3	0.547	HM542476
	R: TGACTGGGATAACATTAAGCAAGG						
Peltdol 17	F: TAAGCATCCAGCCTGACGG	$(ATGT)_{7-13}$	392	14	4	0.541	HM542477
	R: GCTTCTTGCCTTGGCACC						
Peltdol 19	F: AGTCGCGTCAACAAACTGC	$(AAC)_{4-10}$	387	16	3	0.508	HM542478
	R: CTGTTGGCATATGACCTGGG						
Peltdol 20	F: GGAAGTTGGTGTGAATTGTAGC	$(AAG)_{3-9}$	123	17	4	0.561	HM542479
	R: GAGCGACTCAAACCCTTCG						

amplification of the *Peltigera* DNA extract used to generate the 454 library, then by amplification of 16 further herbarium accessions sampled across geographic regions (Appendix 1). PCR amplifications were performed in a 20-μL final volume containing c. 30 ng of template DNA, 1× HotMaster *taq* buffer (containing 2.5 mM Mg^{2+}), 0.2 μM of each primer, 0.2 mM of each dNTP, and 1 unit HotMaster *taq* DNA polymerase (5 Prime GmbH, Hamburg, Germany). Thermal cycling on a BioRad S1000 involved an initial 2 min denaturation at 94°C; 5 cycles at 94°C for 30 s, annealing at 55°C for 45 s and extension at 65°C for 2 min 30 s; 35 cycles at 94°C for 45 s, annealing at 58°C for 45 s and extension at 60°C for 3 min. Variation in the regions was first assessed on 1% agarose TBE gels stained with SYBRsafe, and then by Sanger sequencing on an ABI 3130*xl* Genetic Analyzer (Applied Biosystems, Foster City, CA). The assembly of a near-complete sequenced mitochondrial genome for *Peltigera* (Goffinet et al., unpublished) allowed us to identify the microsatellite loci that were mitochondrial, and by default, which were nuclear. We targeted only nuclear loci. Sequences of the microsatellites as they appear in the original sample from Réunion are deposited in GenBank (Table 1). Eleven nuclear loci were routinely amplified for most samples. Microsatellites amplified for the majority of samples, with no geographic pattern emerging in the distribution of PCR failure, suggesting that the quality of DNA obtained from herbarium samples may account for negative PCR. Microsatellite alleles were scored by counting the number of repeats identified by sequencing. The number of alleles per locus varied between three and eight, and degrees of gene diversity (H_e; Table 1) estimated according to Nei (1978). Furthermore, each individual holds a unique set of alleles such that the number of haplotypes matches the number of individuals screened.

CONCLUSIONS

Microsatellite markers compose a preferred source of genetic loci for assessing the genetic structure of populations due to their diversity and the ability to screen these from preserved tissues. Although commonly used across vascular plant and vertebrate lineages, they appeared both harder to isolate and to exhibit lower polymorphism in fungi (Dutech et al., 2007). Microsatellites have only been developed for one species of lichen-forming ascomycetes, namely *Lobaria pulmonaria* (L.) Hoffm. (Widmer et al., 2010). Using a large set of randomly sequenced genomic fragments, we identified 331 potential microsatellite loci, designed primers for 11 selected tri- or higher nucleotide repeats, and successfully amplified and sequenced these across multiple populations of the *Peltigera dolichorhiza* complex, providing the first set of microsatellites for any lichenized fungus based on 454 data, for any species of *Peltigera*, and for use in a phylogeographic study of a putative set of cryptic species.

Using assembled next-gen sequences rather than raw reads in microsatellite development offers more possibilities for primer design, as contigs tend to be longer than individual reads. Reads from contaminants may also be less liable to assemble, as it is expected that their coverage will be lower than that of the study organism and thus using contigged reads reduces the chance of designing primers on, e.g., human or bacterial templates. That said, the largest contigs are assembled from organellar genome reads (the mitochondria, and in plants, the chloroplast), where the genome is present in the cell in multiple copies, meaning that mining only the contig files for microsatellites can lead to reduced discovery of nuclear markers. Also, in organisms with low numbers of microsatellites, the singleton data set provides a valuable set of further markers to test.

All loci screened exhibit variation among individuals, even among locally sympatric thalli, and each individual represents a distinct haplotype. Allelic diversity and frequencies are high suggesting extensive gene flow among populations. *Peltigera dolichorhiza* reproduces sexually and typically bears numerous apothecia per thalli. Whether this species is homothallic remains unknown but can now be assessed by screening microsatellites from single spore isolates.

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APPENDIX 1. Voucher information for samples of the *Peltigera dolichorhiza* complex screened for 11 microsatellites. Vouchers are housed in LG unless otherwise indicated. All specimens are identified following Sérusiaux et al. (2009) and confirmed to belong to the complex based on ITS sequences. * identifies sample used for developing microsatellites primers.

Species—Country and locality, collection

Peltigera dolichorhiza *La Réunion (Indian Ocean), *Magain & Sérusiaux* Nov. 2009; Rwanda, Nyungwe Nat. Park, *Sérusiaux* Sept. 2006; Rwanda, Nyungwe Nat. Park, *Sérusiaux* Sept. 2006 (CONN); Rwanda, Volcanoes Nat. Park, *Sérusiaux* Apr. 2005; Rwanda, Nyungwe Nat. Park, *Sérusiaux* Apr. 2005; Rwanda, Volcanoes Nat. Park, *Sérusiaux* Sept. 2006; Rwanda, Volcanoes Nat. Park, *Sérusiaux* Sept. 2006 (CONN); Rwanda, Volcanoes Nat. Park, *Sérusiaux* Sept. 2006; Rwanda, Nyungwe Nat. Park, *Sérusiaux* Sept. 2006; Papua New Guinea, Gahavisuka, *Sérusiaux* Oct. 1995; Papua New Guinea, Myola, *Sipman 38261* (B); Papua New Guinea, Gahavisuka, *Sipman 22199* (B); Papua New Guinea, Myola, *Sérusiaux* Oct. 1995; Papua New Guinea, Teptep, *Koponen 34738* (H); Chile, XII region, *Goffinet 8493* (CONN).

Peltigera neopolydactyla Canada, British Columbia, *Goffinet 3568* (CONN); USA, Alaska, *Goffinet 9381* (CONN).