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PRIMER NOTE

Microsatellites for *Lindera* species

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Abstract

Microsatellite markers were developed for conservation genetic studies of *Lindera melissifolia* (pondberry), a federally endangered shrub of southern bottomland ecosystems. Microsatellite sequences were obtained from DNA libraries that were enriched for the $(AC)_n$ simple sequence repeat motif. From 35 clone sequences, 20 primer pairs were designed and evaluated. Eleven primer pairs amplified polymorphic marker loci in pondberry while two did so in *Lindera benzoin* (spicebush). In 46 samples from a single pondberry site the number of microsatellite alleles ranged from two to 11 per locus with observed heterozygosity values of 0.07–0.91.

Keywords: endangered species, pondberry, spicebush

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Lindera melissifolia (Walt.) Blume is an aromatic, rhizomatous, dioecious shrub in the Lauraceae that grows in seasonally flooded forests, on the edges of sinks and ponds, or on rises adjacent to such areas (Devall *et al*. 2001). Common names are pondberry, southern spicebush and Jove's fruit. It is found often in clonal patches at disjunct sites in Alabama, Arkansas, Georgia, North Carolina, South Carolina, Mississippi and Missouri. Since 1986 pondberry has been federally listed as endangered (Federal Register 1986) and has a global conservation priority rank of G2 (www.natureserve.org/explorer/). During this past century, altered land-use patterns have fragmented potential habitat across its range (Devall *et al*. 2001). The current study began because little information exists about environmental and genetic factors required to sustain sexually or vegetatively reproducing populations.

An isozyme diversity survey found the highest genetic diversity for pondberry to be in the Delta National Forest of western Mississippi (Godt & Hamrick 1996). It is not known whether that diversity is declining and there is little information on gene flow, inbreeding and genetic structure within and among pondberry sites. Such data if available could be used for classification of the self-sustaining populations that are required under the US Fish and Wildlife Service Recovery Plan (USFWS 1993). The allelic diversity that is characteristic of microsatellite loci can provide the detailed population genetic information needed to establish best conservation practices.

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DNA from pondberry was used to construct libraries that were enriched for the $(AC)_n$ simple sequence repeat motif. Prior to enrichment cloning as described by Khasa *et al*. (2000), DNA was digested with restriction enzymes *Hae*III, *Rsa*I and *Alu*I in the presence of T4 DNA ligase, restriction enzyme *Psh*AI, and the blunt-end adapters M28 (5′-CTCTTGCTTGAATTCGGACTA) and M29 (5′-pTAGTCCGAATTCAAGCAAGAGCACA). Bacterial colonies containing cloned inserts of interest were identified by colony-lift hybridization with ^{32}P-labelled $(AC)_{12}$ probes. Cloned inserts were sequenced by ^{33}P-labelled cycle dideoxy termination (Amersham) using M13 forward and reverse universal sequencing primers. DNA sequencing products were run on 6% polyacrylamide sequencing gels and sequences of microsatellite regions and flanking polymerase chain reaction (PCR) primer targets were read manually from the autoradiograms. Twenty primer pairs were selected and synthesized from 29 unique sequences. Forward primers were variously 5′-end labelled with one of the following fluorophores: 6-FAM, VIC, PET, or NED. All reverse primers were PIG-tailed with the sequence GTTTCTT on the 5′ end to favour 3′ adenylation of the forward amplified strand (Brownstein *et al*. 1996).

We first evaluated the primer pairs in eight pondberry plants using the following PCR protocol: 3 ng total DNA, 100 μM of each primer, 200 μM of each dNTP, 0.1 U/μL *Taq* DNA polymerase, 2.0 mM $MgCl_2$, 50 mM KCl, and 20 mM Tris-HCl, pH 8.4, in a 12-μL reaction volume. PCRs were completed using the following touchdown protocol on

Table 1 Characterization in two *Lindera* species of 11 microsatellite loci developed from *L. melissifolia* DNA. Allele, heterozygosity and *F* values were from 46 genotypes in each of *L. melissifolia* (*Lm* — data in bold) and *L. benzoin* (*Lb*). PCR amplification failure rate is noted for *Lb*; all *Lm* samples amplified. H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient; *P*, exact test *P* value for Hardy–Weinberg equilibrium using the F_{IS} statistic

Locus	Sequenced repeat	Primer sequences (5′–3′)	Species	Size range (bp)	No. alleles (% failed PCR)	H_O	H_E	F_{IS}	*P*
LmSI001	$(AC)_{18}$	F: ATTAAATAAGGGTAAGAACTCTC	***Lm***	**193–201**	**5**	**0.63**	**0.64**	**0.02**	**0.018**
		R: AAAATCATTGGTTTGCCCTC	*Lb*	na	0 (100)	—	—	—	—
LmSI002	$(AC)_{27}$	F: ATAATGCCTCTTGCTTAGGC	***Lm***	**136–197**	**11**	**0.91**	**0.87**	**–0.05**	**0.906**
		R: TTCAACTTGATTGAGGACATC	*Lb*	116–130	multiple	—	—	—	—
LmSI004	$(AC)_{10}(A)_9$	F: CCACTTGTGAATAATCCAATC	***Lm***	**165–167**	**2**	**0.07**	**0.06**	**–0.02**	**1**
		R: GTGACAAACTAAGAATATTGCAC	*Lb*	172	1 (0)	0 .00	0.00	—	—
LmSI013	$(TC)_{27}(CA)_8$	F: TAGCTCAGTGGTGAGGCACC	***Lm***	**177–220**	**9**	**0.80**	**0.80**	**0.00**	**0.804**
		R: ACTGGTAGCTCAGTGGTGA	*Lb*	133–185	10 (80)	0.67	0.80	0.28	0.00
LmSI027	$(AC)_{13}$	F: CTAAGAAGTGTCCAAGTTAGTC	***Lm***	**234–236**	**2**	**0.24**	**0.21**	**–0.12**	**1**
		R: CCATGGATACTCCATTGGTA	*Lb*	na	0 (100)	—	—	—	—
LmSI035	$(TG)_{15}(AG)_{10}$	F: CAGTCTCATTATCCCAAGGC	***Lm***	**226–254**	**5**	**0.78**	**0.72**	**–0.09**	**0.895**
		R: TTGACTCCTCAAGTCTGATC	*Lb*	193–217	8 (0)	0.74	0.72	0.0	0.561
LmSI047	$(AC)_{16}$	F: TAGCTTAGGAGAGTCCCC	***Lm***	**292–298**	**4**	**0.46**	**0.62**	**0.26**	**0.009**
		R: GGATGGATATTGTTTTTGATGC	*Lb*	na	0 (100)	—	—	—	—
LmSI049	$(TG)_6(AG)_{10}$	F: GTTGGCATAAACTGGTGGTTTGA	***Lm***	**164–172**	**5**	**0.56**	**0.59**	**–0.04**	**0.218**
		R: TAGTAAACTACACTAGCAATGAC	*Lb*	160–174	5 (22)	0.44	0.48	0.11	0.145
LmSI050	$(AC)_{22}$	F: CCATTATTTCAATCACTGGAGA	***Lm***	**336–380**	**8**	**0.54**	**0.83**	**0.35**	**0.000**
		R: GATTCACTCCAATAGGCAAAC	*Lb*	350–362	3 (0)	0.21	0.18	–0.08	1
LmSI056	$(TA)_7(TG)_{14}$	F: TTGGCTATTAGATAGGGGCA	***Lm***	**223–235**	**6**	**0.63**	**0.57**	**–0.11**	**0.299**
		R: AAAACACTAACAAATCATTGGC	*Lb*	219–237	8 (54)	0.29	0.78	0.66	0.00
LmSI061	$(GT)_{18}(GA)_{29}$	F: TTGTGCCATGGGCTCTTGTC	***Lm***	**405–441**	**9**	**0.80**	**0.74**	**–0.09**	**0.842**
		R: CTCTAGGAATGGGTACTCC	*Lb*	335	1 (0)	0.00	0.00	—	—

na, no amplification; GenBank Accession nos are BV681306–BV681316 for the clone sequences.

PTC-200 thermal cyclers (MJ Research): 2 min at 94 °C; followed by 20 cycles of 30 s at 94 °C, 30 s at 60 °C minus 0.5 °C per cycle, and 30 s at 72 °C; followed by 15 cycles of 30 s at 92 °C, 30 s at 50 °C, 1 min at 72 °C; followed by a 15-min extension at 72 °C. Reactions were held at 4 °C until the products were prepared for analysis. The amplification quality and fragment size polymorphism of PCR products were evaluated by electrophoresis on 3% agarose horizontal slab gels. All 20 primer pairs amplified a fragment, or fragments, at or near the expected size (data not shown).

The markers were then used to determine the genotypes of 46 samples each for pondberry and spicebush, *Lindera benzoin* (L.) Blume. Pondberry was collected from one site in the Delta National Forest and spicebush from one site in the Dahomey National Wildlife Refuge of western Mississippi. Fragment analyses for these samples were performed with an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) fitted with a 36-cm capillary array. Allele sizes were determined with ABI PRISM GS (500-250) LIZ internal size standards using the third order least-squares algorithm implemented by ABI PRISM GENEMAPPER version 3.7 software. Based on these results 11 primer pairs were deemed of value for further genotyping (Table 1).

The 11 polymorphic loci in pondberry had from two to 11 alleles (mean = 6.0) that gave a mean observed heterozygosity of 0.584, a mean expected heterozygosity of 0.604, and a mean inbreeding coefficient (F_{IS}) of 0.032. A global exact test for a heterozygote deficit (using GENEPOP version 3.4, Raymond & Rousset 1995) confirmed that this F_{IS} value exceeded Hardy–Weinberg expectations ($P = 0.0034$) after Bonferroni correction at the 5% nominal level of $P < 0.0045$. The test for individual loci showed that only locus LmSI050 exceeded Hardy–Weinberg equilibrium ($P = 0.0040$). There was evidence of null alleles LmSI050 that may explain the observed heterozygote deficit at that locus (van Oosterhout *et al*. 2004). Given that, the population may have been at Hardy–Weinberg equilibrium and not subjected to inbreeding. None of the locus pairs exhibited linkage disequilibrium after Bonferroni correction at the 5% nominal level of $P < 0.00091$ (Raymond & Rousset 1995).

For the congener spicebush, eight primer pairs worked with various degrees of success but only two (LmSI035 and LmSI050) reliably amplified informative loci (Table 1). The range of allele sizes between the species did not overlap for LmSI035, while for LmSI050 they did. Monomorphic spicebush loci LmSI004 and LmSI061 each had alleles that were not found in pondberry. Thus, species-specific alleles

potentially exist at loci LmSI004, LmSI035 and LmSI061. The high PCR failure rate in spicebush seen for eight of 12 primer pairs developed from pondberry suggests significant sequence divergence between these *Lindera* species.

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