Applications in Plant Sciences

PRIMER NOTE

Development of 20 chloroplast microsatellite primers in wuyao (*Lindera aggregata*, Lauraceae)

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PREMISE OF THE STUDY: To investigate the population genetics and evolutionary history of wuyao (*Lindera aggregata*, Lauraceae) and other *Lindera* species, polymorphic chloroplast microsatellite (cpSSR) primers were developed.

METHODS AND RESULTS: The complete chloroplast genome of *L. glauca* was used to design 74 primers for *L. aggregata*. Agarose gel electrophoresis and Sanger sequencing were used to select 20 polymorphic primers. These primers were then tested in 70 individuals from three populations of *L. aggregata*. The number of alleles ranged from two to four, and the unbiased haploid diversity index ranged from 0.457 to 0.685. Up to 17 primers successfully amplified in four other *Lindera* species: *L. prattii*, *L. chunii*, *L. lungshengensis*, and *L. pulcherrima* var. *hemsleyana*.

CONCLUSIONS: The 20 primers reported here can potentially be used for future genetic studies of *L. aggregata* and other *Lindera* species.

KEY WORDS chloroplast microsatellite; Lauraceae; *Lindera aggregata*; wuyao.

Wuyao, *Lindera aggregata* (Sims) Kosterm. (Lauraceae), is a plant species whose use in traditional Chinese medicine can be traced back to the Zhou Dynasty (1046–256 BC). Wuyao has complex pharmacological effects, including anti-inflammatory, antiviral, antibacterial, antioxidant, and anti-fatigue effects (Chen and Yu, 2011). Since 2005, wuyao has been protected as a medicinal remedy in Tiantai County, Zhejiang Province, China (between 28.95–29.34°N and 120.69–121.26°E). Because *L. aggregata* is a dominant species in evergreen broad-leaved forests and is widely distributed in subtropical China (Wang, 2006), its wild genetic diversity is an excellent source for genetic breeding.

DNA polymorphisms in the form of microsatellite (or simple sequence repeat [SSR]) markers are a powerful source for population genetic studies (Kalia et al., 2011). SSRs are present in both nuclear and organelle (such as chloroplast) genomes. Nuclear microsatellites (nSSRs) are codominant, biparentally inherited, and highly polymorphic, making them suitable to investigate distributions of genetic diversity and recent demographic histories. Chloroplast microsatellites (cpSSRs) also possess some unique characteristics, such as haploidy, nonrecombination, and uniparental inheritance (Ebert and Peakall, 2009). In particular, the chloroplast genome has a low nucleotide substitution rate, and therefore cpSSRs can provide unique insights into ancient evolutionary processes (Provan et al., 2001). Abundant nSSR primers have been developed in different *Lindera* Thunb. species, such as *L. melissifolia* (Walter) Blume (Echt et al., 2006), *L. benzoin* (L.) Blume (Edwards and Niesenbaum, 2007), and *L. glauca* (Siebold & Zucc.) Blume (Zhu et al., 2016), and these nSSRs have been used to investigate evolutionary histories of *Lindera* species (such as *L. obtusiloba* Blume [Ye et al., 2017]). However, no specific cpSSR primers have been developed in the *Lindera* genus. As complete chloroplast genomes have been widely used for cpSSR primer design (Deng et al., 2017), an existing chloroplast genome of *L. glauca* was used to develop novel cpSSR primers for *L. aggregata*.

METHODS AND RESULTS

We collected 70 *L. aggregata* individuals from three populations: Wuyunjie (WYJ) in Hunan Province, Tianmu Mountain (TMSH) in Zhejiang Province, and Nanning (NAN) in Guangxi Province (Appendix 1). Total genomic DNA was extracted from dried leaves using the Plant Genomic DNA Kit (DP305-03; Tiangen, Beijing, China).

From the complete chloroplast genome of *L. glauca* (GenBank accession number MF188124.1), 74 cpSSR loci were identified using SSRHunter 1.3 (Li and Wan, 2005) with the repeat threshold settings of 10 repeat units for mononucleotide and five, four, three, or three repeats for di-, tri-, tetra-, and pentanucleotide cpSSRs, respectively. Based on their flanking regions,

TABLE 1. Characteristics of 20 chloroplast microsatellite markers of *Lindera aggregata* and locus position in the *L. glauca* chloroplast genome.

Locus	Primer sequences (5′–3′)	Allele size range (bp)	T_a (°C)	Repeat motif	Fluorescent label[a]	GenBank accession no.	Position
LAG20	F: TGGCCGTTGTTCCTTATTTC R: CAACCCAATCCTTGTTTTGC	203–205	60	$(A)_{10}$	HEX	MH569843	*atpB*/*rbcL* (57972–58176)
LAG5	F: GCAGAAGTCCCAACCCTATTG R: TTGAAAAATGGTAACTTCTTCATTTG	200–202	60	$(A)_{16}$	6-FAM	MH569849	*ndhF*/*rpl32* (116030–116227)
LAG9	F: GGAAGCGGCAGAAATCAAT R: CAAAGACTCCACGGATAGGAA	226–227	60	$(A)_{14}$	TAMRA	MH569848	*atpH*/*atpI* (15049–15270)
LAG11	F: TAGTGGGAGTGACACGGATT R: GGCGATATGTCTACGCTGGT	201–205	60	$(A)_{13}$	HEX	MH569847	*trnD*-GUC/*trnY*-GUA (32657–32864)
LAG12	F: ATTCTTCCCATCCCAAAACC R: CGGGGTAGAGCAGTTTGGTA	161–162	60	$(A)_{16}$	6-FAM	MH569846	*trnG*-UCC/*trnfM*-CAU (38941–39103)
LAG15	F: TACCGGAAAGAGTGGAAAGG R: TTCCCGTCAGACTTGAACCT	175–178	60	$(A)_{16}$	TAMRA	MH569845	*trnS*-GGA/*rps4* (47813–47983)
LAG19	F: TTCTCGATGAAGTCGGTTGA R: AAGAAAAAGCCCGCTACGA	154–158	60	$(A)_{12}$	HEX	MH569844	*clpP* (74861–75018)
LAG21	F: CCGTGTGAGAGCAACAATGA R: ATTTCAAAGAAGGCGGAGGT	165–166	60	$(C)_{11}(T)_{11}$	6-FAM	MH569842	*matK*/*rps16* (5095–5264)
LAG23	F: AAACGATTGATTCCCCTGTG R: TGGAGCTCGAGCATAAAGAA	214–215	60	$(T)_{13}$	TAMRA	MH569841	*rps16* (5907–6117)
LAG24	F: TGCATCATGTGAGAATCCAAA R: TCACAAACAAACGGATCGAG	226–228	60	$(T)_{13}$	HEX	MH569840	*rps16*/*trnQ*-UUG (7843–8067)
LAG26	F: AAGTCATTTGGGGTGCATTC R: TTCATTCGGCTCCTTTATGG	178–180	60	$(T)_{17}$	6-FAM	MH569839	*trnG*-UCC (10893–11071)
LAG28	F: GATTGGCTTAGAGCCCTTACA R: GTGAATCCATGGAGGGTCAT	182–183	60	$(T)_{10}$	TAMRA	MH569838	*atpH*/*atpI* (15585–15764)
LAG29	F: ATGGCCAAAATGAACTCCTG R: CGGTCAATCTCCGGTAGAAG	159–164	60	$(T)_{10}$	HEX	MH569837	*rps2*/*rpoC2* (17350–17507)
LAG31	F: GGCTCCTGTAACCGTGTCAT R: GATGCCCCTGACTCTGACAT	227–231	60	$(T)_{13}$	6-FAM	MH569836	*rpoC1* (23959–24184)
LAG32	F: GTAACCCCGCCAAGAATGTA R: ATACACAGTTGCCCCTTGGA	235–237	60	$(T)_{10}$	TAMRA	MH569835	*trnC*-GCA/*petN* (29753–29989)
LAG33	F: AGGGATACACATGATGGGAAA R: GGTTCCCTCTTTGAACAGCA	165–167	60	$(T)_{12}$	HEX	MH569834	*petN*/*psbM* (30459–30623)
LAG38	F: CGGGAGTCATTGGTTCAAA R: CATATTTGGATTCCGCCAAT	225–226	60	$(T)_{12}$	6-FAM	MH569833	*trnM*-CAU/*atpE* (55474–55692)
LAG41	F: TGTGATTCAGCAATCCCAAA R: ATGTATCGGGGTCCATTTCA	168–170	60	$(T)_{12}$	TAMRA	MH569832	*clpP* (74203–74372)
LAG48	F: CACCCCCAGACCTCCTAATA R: GCGGGATTGATCCAATAACT	245–248	60	$(T)_{10}$	HEX	MH569831	*ndhG*/*ndhI* (123356–123598)
LAG49	F: TGAAATTCAACAGACCCTTTG R: TCCAGATTATTGGGTGTATCAGA	182–183	56	$(T)_{12}$	6-FAM	MH569830	*rps15*/*ycf1* (128090–128268)

Note: T_a = annealing temperature.
[a]Forward primers were modified at the 5′ end with fluorescent labels: HEX (green), 6-FAM (blue), or TAMRA (yellow).

we designed 74 cpSSR primers using Primer3Plus (Untergasser et al., 2007) with the following settings: primer size 18–27 bp, product length 150–300 bp, annealing temperature 57.0–63.0°C, and GC 20.0–80.0%.

Using the 74 cpSSR primers, PCR assays were performed in individual 40-μL reaction mixes containing 10–20 ng of template DNA, 1× buffer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 mM each dNTP, 2.0 mM $MgCl_2$, 0.1 mM each primer, and two units of *Taq* (TaKaRa Biotechnology Co., Dalian, China). PCR amplifications were performed as follows: an initial denaturation step at 94°C for 5 min; followed by 30 cycles of 50 s at 94°C, 50 s at the annealing temperature (Table 1), and 50 s at 72°C; with a final extension step at 72°C for 10 min. Agarose gel electrophoresis was used to select primers that generated a single clear band, and these primers were used to amplify cpSSRs in one individual each from the WYJ, TMSH, and NAN populations (Appendix 1). The amplicons were sequenced and then read in CodonCode Aligner 3.6.1 (CodonCode Corporation, Centerville, Massachusetts, USA) to screen for polymorphic loci.

To determine the allelic size range of the polymorphic cpSSR loci, a fluorescent label was added to each forward primer (Table 1) and PCR was then performed in all 70 individuals following the procedure mentioned above (Appendix 1). The amplicons were resolved on an ABI 3500XL automated DNA sequencer with the GeneScan 500 ROX Size Standard (Applied Biosystems, Foster City, California, USA). The microsatellite marker profiles were analyzed using GeneMarker version 1.80 (Holland and Parson, 2011). To reduce score error, two people independently read all alleles, and any disputes were decided by a third person. For each locus, the number of alleles and unbiased haploid diversity index were calculated using GenAlEx 6.5 (Peakall and Smouse, 2012). The polymorphic cpSSR primers were further cross-amplified in

TABLE 2. Genetic diversity of 20 chloroplast microsatellites in three populations of *Lindera aggregata* and cross-amplification in four other *Lindera* species.[a]

	Lindera aggregata											
	WYJ (*n* = 22)		TMSH (*n* = 24)		NAN (*n* = 24)		All (*n* = 70)		*L. prattii* (*n* = 1)	*L. chunii* (*n* = 1)	*L. lungshengensis* (*n* = 1)	*L. pulcherrima* var. *hemsleyana* (Allele size range) (*n* = 8)
Locus	*A*	*h*	*A*	*h*	*A*	*h*	*A*	*h*				
LAG20	1	0.000	1	0.000	1	0.000	2	0.457	+	+	+	+ (208)
LAG5	1	0.000	1	0.000	1	0.000	3	0.676	+	+	+	+ (202–205)
LAG9	1	0.000	1	0.000	1	0.000	2	0.457	—	—	—	+ (235–237)
LAG11	1	0.000	2	0.344	2	0.083	4	0.552	+	+	+	+ (203–204)
LAG12	1	0.000	2	0.083	1	0.000	2	0.466	+	+	+	+ (160)
LAG15	1	0.000	2	0.344	1	0.000	3	0.542	—	—	—	+ (170–174)
LAG19	1	0.000	3	0.409	1	0.000	4	0.677	+	+	+	+ (154–155)
LAG21	1	0.000	2	0.344	1	0.000	2	0.492	—	—	—	+ (167–168)
LAG23	1	0.000	1	0.000	1	0.000	2	0.457	—	—	—	—
LAG24	1	0.000	2	0.344	1	0.000	3	0.670	+	+	+	+ (226–228)
LAG26	1	0.000	1	0.000	1	0.000	3	0.676	+	+	+	+ (181)
LAG28	1	0.000	1	0.000	1	0.000	2	0.457	—	—	—	—
LAG29	1	0.000	2	0.083	1	0.000	4	0.685	+	+	+	+ (156)
LAG31	1	0.000	2	0.083	1	0.000	4	0.685	+	+	+	+ (227)
LAG32	1	0.000	1	0.000	1	0.000	3	0.676	+	+	+	+ (234)
LAG33	2	0.519	1	0.000	1	0.000	3	0.626	+	+	+	+ (164–167)
LAG38	1	0.000	1	0.000	1	0.000	2	0.457	+	+	+	+ (222–223)
LAG41	1	0.000	1	0.000	1	0.000	3	0.676	+	+	+	+ (168)
LAG48	1	0.000	1	0.000	1	0.000	2	0.457	+	+	+	+ (242–243)
LAG49	1	0.000	1	0.000	1	0.000	2	0.457	—	—	—	—

Note: + = successful amplification; — = unsuccessful amplification; *A* = number of alleles; *h* = unbiased haploid diversity; *n* = number of individuals.
[a]Voucher and locality information are provided in Appendix 1.

L. prattii Gamble, *L. chunii* Merr., and *L. lungshengensis* S. Lee using one individual in each species, and in *L. pulcherrima* (Nees) Benth. ex Hook. f. var. *hemsleyana* (Diels) H. P. Tsui using eight individuals (Appendix 1).

Among the 74 cpSSR primers developed in *L. aggregata*, five primers could not be successfully amplified, 49 primers were monomorphic, and the remaining 20 primers were polymorphic (Table 1). In all 70 individuals, the number of alleles ranged from two to four, and the unbiased haploid diversity index ranged from 0.457 to 0.685. In the three populations of *L. aggregata*, low genetic diversity was evident because most loci were fixed with one allele, and the TMSH population had relatively higher genetic diversity (Table 2). Up to 17 cpSSR primers were successfully amplified in *L. prattii*, *L. chunii*, and *L. lungshengensis*, and similar cpSSR range sizes were found in *L. pulcherrima* var. *hemsleyana* (Table 2).

CONCLUSIONS

Using an existing chloroplast genome of *L. glauca*, we successfully developed and characterized 20 polymorphic *L. aggregata* chloroplast microsatellite markers. A limited number of alleles were found in all 20 loci, indicating a very low evolutionary rate in the chloroplast genome or a shallow history of *L. aggregata*. Low intrapopulation and high interpopulation diversity found in the three populations indicate these populations may have experienced long-term isolation (Avise, 2000). Due to the high rate of cross-amplification (70–85%), the 20 polymorphic cpSSR primers will likely be useful in intra- or interspecific genetic studies in *Lindera*.

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DATA ACCESSIBILITY

All sequences have been deposited to the National Center for Biotechnology Information GenBank database, and accession numbers are provided in Table 1.

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APPENDIX 1. Location and voucher information for *Lindera* species used in this study.

Taxon	Population	Location	*n*	Latitude	Longitude	Voucher no.[a]
Lindera aggregata (Sims) Kosterm.	TMSH	Mt. Tianmu, Zhejiang, China	24	30.42	119.41	SHM22266
	WYJ	Wuyunjie, Hunan, China	22	28.62	111.49	SHM23283
	NAN	Nanning, Guangxi, China	24	22.73	108.30	SHM23287
L. prattii Gamble	EM	Mt. Emei, Sichuan, China	1	29.55	103.37	SHM23285
L. pulcherrima (Nees) Benth. ex Hook. f. var. *hemsleyana* (Diels) H. P. Tsui	CHD	Mt. Jinfo, Chongqing, China	8	29.08	107.16	SHM23295
L. chunii Merr.	DHS	Mt. Dinghu, Guangdong, China	1	23.17	112.55	SHM23299
L. lungshengensis S. Lee	LSH	Longsheng County, Guangxi, China	1	25.79	110.01	SHM23280

Note: n = number of individuals.
[a]Voucher specimens were deposited in the Shanghai Museum of Natural History (SHM), Shanghai, China.