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DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE LOCI FOR *LINDERA GLAUCA* (LAURACEAE)¹

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- **Premise of the study:** Microsatellite primers were developed to investigate population genetic structure in *Lindera glauca* (Lauraceae).
- **Methods and Results:** Twenty-five microsatellite primers were developed and optimized for *L. glauca* using Illumina's Solexa sequencing technology. These novel primers were found to be polymorphic in nine wild *L. glauca* populations with 81 total alleles confirmed and genotyped via capillary gel electrophoresis. The total number of alleles, observed heterozygosity, and expected heterozygosity for each population ranged from one to four, from 0.00 to 0.90, and from 0.00 to 0.79, respectively. In addition, the 25 primers were tested in 10 additional individuals of the related species *L. communis*, and all but four markers showed good amplification results.
- **Conclusions:** This set of microsatellite primers is the first specifically developed for *L. glauca* and will facilitate studies of genetic diversity and evolution among populations of this species.

Key words: genetic diversity; Lauraceae; *Lindera glauca*; microsatellite; polymorphism.

Lindera glauca Blume is a deciduous shrub or small tree that belongs to the family Lauraceae. It is extensively distributed in mountainous regions at low altitudes in central and southern China and is also found in Japan, Korea, and Taiwan. It is of potentially great economic value and ecological importance owing to its various valuable properties, including its natural abundance, the medicinal value of its leaves and roots, its high-quality wood, and the wide applications of its volatile oil in the biochemical and medicinal industries (Liu et al., 1992; Seki et al., 1994; Wang et al., 1994, 2011; Sun et al., 2011; Huh et al., 2014). However, few studies have investigated its population genetic diversity and genetic relationships among germplasm and breeding populations. Male individuals of *L. glauca* trees are very rare in China, and only female individuals are found in Japan (Dupont, 2002), although male individuals have been known from continental Asia in the past several decades (Wang, 1972; Li, 1982). Consequently, understanding the genetic diversity of this species is relevant to the utilization and conservation of its germplasm resources, to population genetic studies, and to the evolution of apomixis in this dioecious species.

Microsatellites, or simple sequence repeats (SSRs), have been widely used as genetic markers owing to their multiallelic nature, codominant inheritance, and thorough genome coverage (Powell et al., 1996). They are a powerful tool and an effective

way to analyze population genetic structure, marker-assisted breeding, gene flow, levels of inbreeding, and germplasm identification (Varshney et al., 2005). However, no studies have previously published SSR markers for this species. Therefore, we used a next-generation transcriptome sequencing approach (Illumina's Solexa sequencing technology) to develop microsatellites specifically for *L. glauca*.

METHODS AND RESULTS

Plant materials and DNA extraction—Leaves and fruits of wild *L. glauca* were collected from nine locations in China in 2014 and 2015 (Appendix 1). Genomic DNA was extracted from the leaves of one individual from each of nine total populations using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987).

Development of SSRs and primer design—In this study, we used transcriptome data from Niu et al. (2015) to develop microsatellite markers. We used the 163,427 unigenes from the transcriptome data for SSR exploitation using QDD version 3.1 software (Megléczy et al., 2014) with at least five, five, four, four, three, and two SSR motif repeat units for di-, tri-, tetra-, penta-, hexa-, and heptanucleotide and higher-order nucleotides, respectively. A total of 8969 putative SSRs (excluding mononucleotide repeats) were detected, with the majority of repeats being dinucleotide (66.83%), followed by trinucleotide (33.77%), tetranucleotide (1.87%), pentanucleotide (0.50%), and hexanucleotide (1.04%). With this detailed information, the program PRIMER 5 (PRIMER-E, Auckland, New Zealand) was then used to design 27,350 primer pairs with primer lengths of 18–25 bp, amplification product sizes of 100–400 bp, GC contents from 40% to 60%, and annealing temperatures ranging from 55°C to 65°C.

PCR amplification and fragment analysis—An initial polymorphism screening of 120 primer pairs, including 50 primer pairs for dinucleotide motifs, 40 for trinucleotide motifs, 15 for tetranucleotide motifs, 10 for pentanucleotide motifs, and five for hexanucleotide motifs, was performed using

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TABLE 1. Characteristics of 25 microsatellite loci developed for *Lindera glauca*.

Locus	Primer sequences (5'–3')	Fluorescent dye	Repeat motif	Allele size (bp)	T_a (°C)	GenBank accession no.
XBLG-013	F: CGAGGGAGAGATCGACGC R: ATGGCACCACGAAGTGTGTT	FAM	(AG) ₅	190	58	KX545436
XBLG-033	F: CGGGATGACAATTTGCATGT R: TGGAGCAGATTATGGTTTCCA	HEX	(AG) ₅	259	58	KX545437
XBLG-036	F: CATCACCTCCCTCAAATCCC R: GTTCCGAAATTCGAGGC	FAM	(AG) ₇	263	58	KX545438
XBLG-049	F: TTTACAACCAGGTGGCTA R: CACTGGGACTAAGACACGGC	TAM	(AC) ₆	191	58	KX545439
XBLG-051	F: CAAACAGAACCAAGACATCCAA R: ATGGAGGAGCATGATTCGAG	HEX	(ATAC) ₆	148	55	KX545440
XBLG-053	F: TCCTCTTATCTCTTCCCTTCTGA R: TCAGACCAACAGGAACATGC	TAM	(AT) ₇	268	55	KX545441
XBLG-055	F: CCTCTTCAACCAACCTCC R: CTGCAACTCCATGTGAGGG	FAM	(AAG) ₅	236	55	KX545442
XBLG-056	F: CAACTGTACGTGGTGGGT R: AGCCACACCAGATCTTCAC	ROX	(AG) ₈	283	55	KX545443
XBLG-058	F: AGTCCAGGTAACAGACTCC R: CCCAGTTTCCAGGTAAGAA	HEX	(AAC) ₆	277	55	KX545444
XBLG-060	F: ATTCACCCATCTCCTTCTT R: GATTCTAAGAAGAAGAAGAAAGTACCC	FAM	(AAG) ₆	197	55	KX545445
XBLG-062	F: AACATCATCCCTCCATCCA R: CCAGCCAGTTAGGGTTTCAC	ROX	(AATCC) ₅	192	55	KX545446
XBLG-063	F: CATGGCAACGCAAAATCCTAT R: CTAGATCCTTGGCCATGTTT	TAM	(ATC) ₆	196	55	KX545447
XBLG-066	F: GTCGACGAGGACGAGGAC R: TCGAATGAGGAAAGTTGGC	HEX	(CCG) ₅	187	55	KX545448
XBLG-073	F: ACCACAAGATAAGCTACAATGC R: GGGCCTTAATGTCTATGGCA	FAM	(ACGC) ₅	219	55	KX545449
XBLG-076	F: GGATGCTCTAAGGTGCTTGC R: GGAATCGCCATTCTCCCT	ROX	(AG) ₇	182	55	KX545450
XBLG-082	F: TGTGGAAACAGATCCCAATGA R: ACAAAGCAGAGCTGCTGACA	TAM	(AGC) ₅	168	55	KX545451
XBLG-083	F: CTCCTCATCGATCCACCG R: AAACCAACACTGTACAACCTAAA	HEX	(AAG) ₅	186	55	KX545452
XBLG-084	F: AAGTGAGGCGATACGATTGG R: ACATGACCATAACATGGGTGA	FAM	(AGG) ₅	144	55	KX545453
XBLG-086	F: TTGGACTAGGCTTTGATCG R: CCCATCATCAATGTGGTTATAGA	TAM	(ACC) ₆	190	55	KX545454
XBLG-089	F: TGCTTGTGATCGAAATCAGG R: ACTTCAGAGGCATTCCAGCA	FAM	(AG) ₇	177	55	KX545455
XBLG-092	F: CTCAGCCGATTGATGATCC R: TCATAACATGTACATTCAAAGGA	TAM	(AG) ₈	144	55	KX545456
XBLG-097	F: TTTGGGAAAGTCCCATGAAA R: GGGTACAAGTGATACAATGAGG	TAM	(ATC) ₆	193	55	KX545457
XBLG-099	F: TGCAAGGGTACATGCCATAG R: CCAAACATTTGCCCACTTCT	ROX	(AC) ₇	165	55	KX545458
XBLG-111	F: GAGAGGTACAACCCACG R: GCCCGAAGTTAAGTAAATGGAT	HEX	(ACT) ₆	192	58	KX545459
XBLG-119	F: GCATGGTGTGTTTGGTCAAG R: TCTCAACAGACCCTCGTCC	ROX	(AAG) ₅	350	58	KX545460

Note: T_a = annealing temperature.

polyacrylamide gel electrophoresis. We hand-selected 120 loci based on desired criteria (representative loci with different repeat unit lengths), of which 25 (20.83%) were successfully amplified and found to be polymorphic in the nine wild *L. glauca* populations (Appendix 1, Table 1), while 71 (59.17%) primer pairs produced no product, 21 (17.50%) amplified monomorphic markers or identical heterozygotic genotypes, and three (2.50%) produced larger or smaller products than the expected size. Forward primers of the 25 primer pairs were further labeled with fluorescently labeled nucleotides (M13: 5'-TGTAACAGGACCGCCAGT-3'). PCR reactions were performed in a total reaction volume of 15 μ L, which contained 7.5 μ L of 2 \times *Taq* PCR MasterMix (Aidlab, Beijing, China), 1.0 μ L of 30 ng/ μ L DNA, 5.5 μ L of ddH₂O, 0.5 μ L of 10 μ M reverse primer, 0.2 μ L of 10 μ M forward primer, and 0.3 μ L of 10 μ M fluorescent dyes (M13F-FAM, M13F-HEX, M13F-TAM, and M13F-ROX). Thermocycling program conditions included a 5-min melting step of 94°C; then 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 35 s; and a final extension step of 72°C for 10 min. Ten microliters each of all M13F-labeled PCR products were sent to the

Ruibo Biotechnology Center DNA Sequencing Facility (Beijing, China) for fragment analysis using an ABI 3730XL DNA Analyzer with a GeneScan 500 LIZ Size Standard (Applied Biosystems, Changping, Beijing, China). Allele genotyping was performed using GeneMarker version 2.2.0 software (Soft-Genetics, State College, Pennsylvania, USA).

Detection of SSR polymorphism and data analysis—The 25 novel polymorphic SSRs yielded 81 total alleles confirmed and genotyped via capillary gel electrophoresis. Using GENEPOP 3.2 software (Rousset, 2008) for each population, the resulting genotypic data from the capillary gel electrophoresis were analyzed to obtain standard descriptive statistics and to test for deviations from Hardy–Weinberg equilibrium (HWE) assumptions (Table 2). The total number of alleles ranged from one to four with a mean of 3.240. The observed and expected heterozygosity ranged from 0.00 to 0.90 and from 0.00 to 0.79 with averages of 0.201 and 0.479, respectively. HWE and linkage disequilibrium using Bonferroni correction were tested for every locus. Less than half of the loci (five,

TABLE 2. Descriptive statistics of the 25 newly developed polymorphic microsatellites of *Lindera glauca*.^a

Locus	ATM (<i>n</i> = 10)			JGS (<i>n</i> = 10)			LDZ (<i>n</i> = 10)			SIG (<i>n</i> = 10)			NTB (<i>n</i> = 10)			YTH (<i>n</i> = 10)			DBS (<i>n</i> = 10)			HMF (<i>n</i> = 10)			TMS (<i>n</i> = 10)											
	A	H _o	H _e	A	H _o	H _e	A	H _o	H _e	A	H _o	H _e	A	H _o	H _e	A	H _o	H _e	A	H _o	H _e	A	H _o	H _e	A	H _o	H _e									
XBLG-013	3	0.30	0.54	**	1	0.00	0.00	M	2	0.00	0.19	***	4	0.00	0.69	***	4	0.00	0.69	***	4	0.20	0.67	**	2	0.00	0.51	***	2	0.00	0.34	***	4	0.00	0.61	***
XBLG-033	1	0.00	0.00	M	3	0.00	0.65	***	2	0.40	0.51	***	1	0.00	0.00	M	1	0.00	0.00	M	2	0.00	0.00	M	2	0.10	0.39	**	2	0.10	0.39	**	2	0.20	0.19	n.s.
XBLG-036	2	0.30	0.52	n.s.	2	0.20	0.19	n.s.	2	0.00	0.19	***	3	0.00	0.59	***	3	0.00	0.59	***	3	0.00	0.19	***	2	0.00	0.19	n.s.	2	0.00	0.19	n.s.	3	0.10	0.68	***
XBLG-049	3	0.20	0.56	n.s.	3	0.40	0.69	n.s.	3	0.10	0.65	***	3	0.10	0.53	***	2	0.20	0.19	n.s.	2	0.10	0.27	*	3	0.50	0.68	n.s.	3	0.10	0.64	**	2	0.00	0.51	***
XBLG-051	3	0.20	0.61	***	2	0.70	0.48	n.s.	3	0.90	0.65	**	3	0.30	0.27	n.s.	3	0.30	0.43	n.s.	3	0.30	0.54	n.s.	3	0.00	0.36	***	2	0.20	0.34	n.s.	3	0.10	0.68	n.s.
XBLG-053	3	0.40	0.47	n.s.	2	0.40	0.34	n.s.	3	0.60	0.69	n.s.	2	0.00	0.51	***	2	0.00	0.44	***	2	0.10	0.62	***	3	0.10	0.62	n.s.	3	0.10	0.59	***	3	0.50	0.43	n.s.
XBLG-055	3	0.30	0.69	*	3	0.60	0.57	***	2	0.00	0.19	***	2	0.00	0.19	***	2	0.00	0.19	***	2	0.00	0.48	***	3	0.30	0.54	**	3	0.00	0.65	***	3	0.80	0.69	*
XBLG-056	1	0.00	0.00	M	1	0.00	0.00	M	2	0.00	0.19	***	3	0.20	0.35	n.s.	2	0.00	0.19	***	2	0.30	0.48	n.s.	2	0.00	0.19	***	2	0.00	0.34	***	2	0.00	0.34	***
XBLG-058	3	0.30	0.53	***	4	0.60	0.50	n.s.	2	0.70	0.48	n.s.	4	0.60	0.66	n.s.	4	0.60	0.66	n.s.	4	0.30	0.72	***	4	0.40	0.69	**	3	0.20	0.61	**	4	0.20	0.65	**
XBLG-060	3	0.30	0.43	***	1	0.00	0.00	M	4	0.20	0.36	**	4	0.70	0.79	n.s.	3	0.20	0.57	***	4	0.30	0.62	***	4	0.20	0.34	n.s.	2	0.20	0.34	n.s.	3	0.20	0.61	**
XBLG-062	1	0.00	0.00	M	1	0.00	0.00	M	1	0.00	0.00	M	4	0.30	0.28	n.s.	2	0.10	0.10	n.s.	4	0.30	0.44	*	1	0.00	0.00	M	1	0.00	0.00	M	3	0.20	0.19	n.s.
XBLG-063	2	0.20	0.44	n.s.	3	0.10	0.43	***	2	0.00	0.19	***	2	0.20	0.21	*	3	0.30	0.59	***	3	0.30	0.56	n.s.	3	0.30	0.56	n.s.	3	0.20	0.35	n.s.	2	0.00	0.51	***
XBLG-066	1	0.00	0.00	M	1	0.00	0.00	M	2	0.00	0.19	***	2	0.10	0.10	n.s.	1	0.00	0.00	M	2	0.10	0.10	n.s.	2	0.10	0.10	n.s.	2	0.50	0.39	n.s.	2	0.50	0.39	n.s.
XBLG-073	1	0.00	0.00	M	2	0.20	0.19	n.s.	2	0.00	0.19	***	1	0.00	0.00	M	1	0.00	0.00	M	1	0.00	0.00	M	2	0.10	0.27	n.s.	1	0.00	0.00	M	2	0.30	0.27	n.s.
XBLG-076	1	0.00	0.00	M	1	0.00	0.00	M	2	0.60	0.44	n.s.	2	0.50	0.39	n.s.	2	0.30	0.27	n.s.	2	0.40	0.34	n.s.	3	0.40	0.35	n.s.	2	0.50	0.39	n.s.	3	0.20	0.54	n.s.
XBLG-082	3	0.20	0.19	n.s.	3	0.20	0.19	n.s.	3	0.30	0.42	n.s.	3	0.50	0.54	n.s.	3	0.30	0.62	*	3	0.30	0.59	***	3	0.30	0.59	***	3	0.30	0.62	**	3	0.30	0.62	**
XBLG-083	3	0.30	0.54	n.s.	2	0.30	0.27	n.s.	3	0.00	0.36	***	3	0.30	0.58	*	3	0.10	0.42	**	3	0.10	0.56	*	2	0.00	0.19	***	2	0.10	0.10	n.s.	2	0.00	0.19	***
XBLG-084	2	0.10	0.52	*	2	0.00	0.44	***	2	0.00	0.53	***	3	0.00	0.61	***	3	0.40	0.51	n.s.	2	0.30	0.48	n.s.	2	0.30	0.48	n.s.	2	0.00	0.34	***	3	0.10	0.53	***
XBLG-086	1	0.00	0.00	M	1	0.00	0.00	M	2	0.20	0.34	n.s.	2	0.10	0.27	*	2	0.20	0.34	n.s.	2	0.40	0.51	n.s.	2	0.00	0.19	***	3	0.20	0.54	*	3	0.20	0.54	n.s.
XBLG-089	2	0.30	0.27	n.s.	1	0.00	0.00	M	1	0.00	0.00	M	1	0.00	0.00	M	2	0.40	0.51	n.s.	2	0.00	0.00	M	1	0.00	0.00	M	2	0.60	0.44	n.s.	2	0.60	0.44	n.s.
XBLG-092	2	0.40	0.51	n.s.	3	0.80	0.57	n.s.	2	0.00	0.19	***	4	0.40	0.76	*	2	0.10	0.39	**	2	0.20	0.34	n.s.	3	0.50	0.67	*	2	0.20	0.34	n.s.	3	0.10	0.51	**
XBLG-097	3	0.60	0.68	*	3	0.90	0.59	n.s.	3	0.10	0.28	***	3	0.40	0.48	***	3	0.20	0.57	n.s.	3	0.30	0.60	n.s.	4	0.30	0.60	n.s.	3	0.20	0.19	n.s.	3	0.20	0.19	n.s.
XBLG-099	3	0.20	0.57	***	3	0.20	0.56	n.s.	2	0.10	0.10	n.s.	2	0.00	0.51	***	3	0.00	0.65	***	3	0.10	0.69	***	3	0.10	0.69	***	3	0.50	0.47	n.s.	4	0.70	0.66	n.s.
XBLG-111	3	0.20	0.36	***	3	0.10	0.43	***	3	0.60	0.65	**	3	0.20	0.62	**	2	0.00	0.19	***	2	0.20	0.53	**	2	0.20	0.53	**	3	0.20	0.28	*	2	0.30	0.52	n.s.
XBLG-119	2	0.30	0.48	n.s.	2	0.10	0.39	**	2	0.10	0.48	**	2	0.30	0.39	n.s.	2	0.10	0.52	**	2	0.20	0.53	*	2	0.20	0.53	*	2	0.10	0.52	**	2	0.00	0.19	***
Mean	2.20	0.20	0.36		2.00	0.23	0.30		2.14	0.20	0.34		2.64	0.21	0.46		2.32	0.14	0.37		2.64	0.22	0.44		2.24	0.16	0.34		2.40	0.18	0.40		2.72	0.27	0.47	

Note: A = total number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; n = number of individuals sampled.

^a Locality and voucher information are provided in Appendix 1.

^b Asterisks indicate significant deviation from Hardy–Weinberg equilibrium (*P < 0.05, **P < 0.01, ***P < 0.001); M = monomorphic; n.s. = not significant.

TABLE 3. Cross-amplification results for the 25 polymorphic cDNA-SSR loci developed for *Lindera glauca* in 10 individuals of *L. communis*.^a

Locus	LC001	LC002	LC004	LC005	LC009	LC010	LC011	LC019	LC021	LC022
XBLG-013	0	0	1	0	0	0	1	0	1	0
XBLG-033	0	1	1	0	1	0	0	0	0	0
XBLG-036	1	0	0	1	0	1	1	0	0	0
XBLG-049	1	1	1	1	0	1	1	1	0	1
XBLG-051	1	1	1	1	1	1	1	1	1	1
XBLG-053	1	1	1	1	1	1	1	1	1	1
XBLG-055	1	1	1	1	1	1	1	0	1	1
XBLG-056	1	1	1	1	0	1	1	1	1	1
XBLG-058	0	0	1	1	1	1	1	1	1	1
XBLG-060	1	1	1	1	0	1	1	1	1	1
XBLG-062	1	1	1	1	1	1	1	1	1	1
XBLG-063	1	1	1	1	1	1	1	1	1	1
XBLG-066	1	1	1	1	1	1	0	1	1	0
XBLG-073	1	1	1	0	1	1	1	1	0	1
XBLG-076	1	1	1	1	1	1	0	1	1	1
XBLG-082	1	0	1	1	1	1	1	1	1	1
XBLG-083	1	1	1	0	0	1	0	1	1	1
XBLG-084	1	0	1	1	1	0	1	1	1	0
XBLG-086	1	1	1	1	0	1	1	0	1	1
XBLG-089	1	1	1	1	0	1	1	1	1	1
XBLG-092	0	1	1	1	1	0	1	1	1	1
XBLG-097	1	0	1	0	1	1	1	1	1	1
XBLG-099	0	1	1	1	1	1	1	1	1	1
XBLG-111	1	0	1	0	1	1	1	0	1	0
XBLG-119	0	0	1	0	0	0	1	0	0	0

Note: 1 = successful amplification; 0 = failed amplification.

^aLC = population names of *Lindera communis*. Samples were collected in Longjiang County, Yunnan Province, China (geographic coordinates: 24°46'33"N, 98°39'25"E; elevation: 1219 m) and identification codes are kept at the Laboratory of Systematic Evolution and Biogeography of Woody Plants, School of Nature Conservation, Beijing Forestry University, Beijing, China.

five, 12, nine, eight, seven, eight, seven, and seven loci in populations ATM, JGS, LDZ, SJG, NTB, YTH, DBS, HMF, and TMS, respectively) showed significant departure from HW ($P < 0.001$). Significant linkage disequilibrium was not detected between any pair of loci ($P < 0.001$).

Cross-species amplifications—The 25 primers were tested in 10 individuals of *L. communis* Hemsl. under the same PCR conditions as above. All 25 were found to amplify in at least 21 of the species (Table 3).

CONCLUSIONS

In the current study, we developed 25 novel cDNA-SSR markers that were highly polymorphic in *L. glauca* and used these markers to successfully investigate genetic distances within nine wild populations of *L. glauca*. The collection of SSRs presented herein provide a means to assess genetic diversity and to further investigate large-scale and fine-scale population genetic structure in *L. glauca*. These markers may also be useful for germplasm identification and breeding programs in both this species and other species in the genus *Lindera* Thunb.

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APPENDIX 1. Location and sampling information for *Lindera glauca* individuals used in this study.^a

Population	Sample accession no.	Geographic coordinates		Elevation (m)	Province in China	County	<i>n</i>
		Latitude	Longitude				
ATM	A14-10	31°13'30"N	115°51'35"E	646–834	Anhui	Jinzhai	10
JGS	J13-09	31°52'15"N	114°05'13"E	203–317	Henan	Xinyang	10
LDZ	L14-04	31°56'47"N	114°15'26"E	154–261	Henan	Dongzhai	10
SJG	S14-10	31°44'58"N	115°32'29"E	243–476	Henan	Shangcheng	10
NTB	N14-04	32°19'45"N	113°25'24"E	241–256	Henan	Tongbai	10
YTH	Y14-04	31°03'24"N	115°51'54"E	647–734	Hubei	Yingshan	10
DBS	D14-09	31°00'32"N	115°50'12"E	834–1003	Hubei	Yingshan	10
HMF	H14-09	28°26'51"N	113°00'22"E	224–257	Hunan	Wangcheng	10
TMS	T14-09	30°19'28"N	119°26'56"E	359–432	Zhejiang	Linan	10

Note: *n* = number of individuals sampled.

^aSample accession numbers refer to voucher specimens deposited in the Herbarium of the Beijing Forestry University (BJFC), Beijing, China; geographic coordinates and elevation were obtained with a portable GPS receiver.