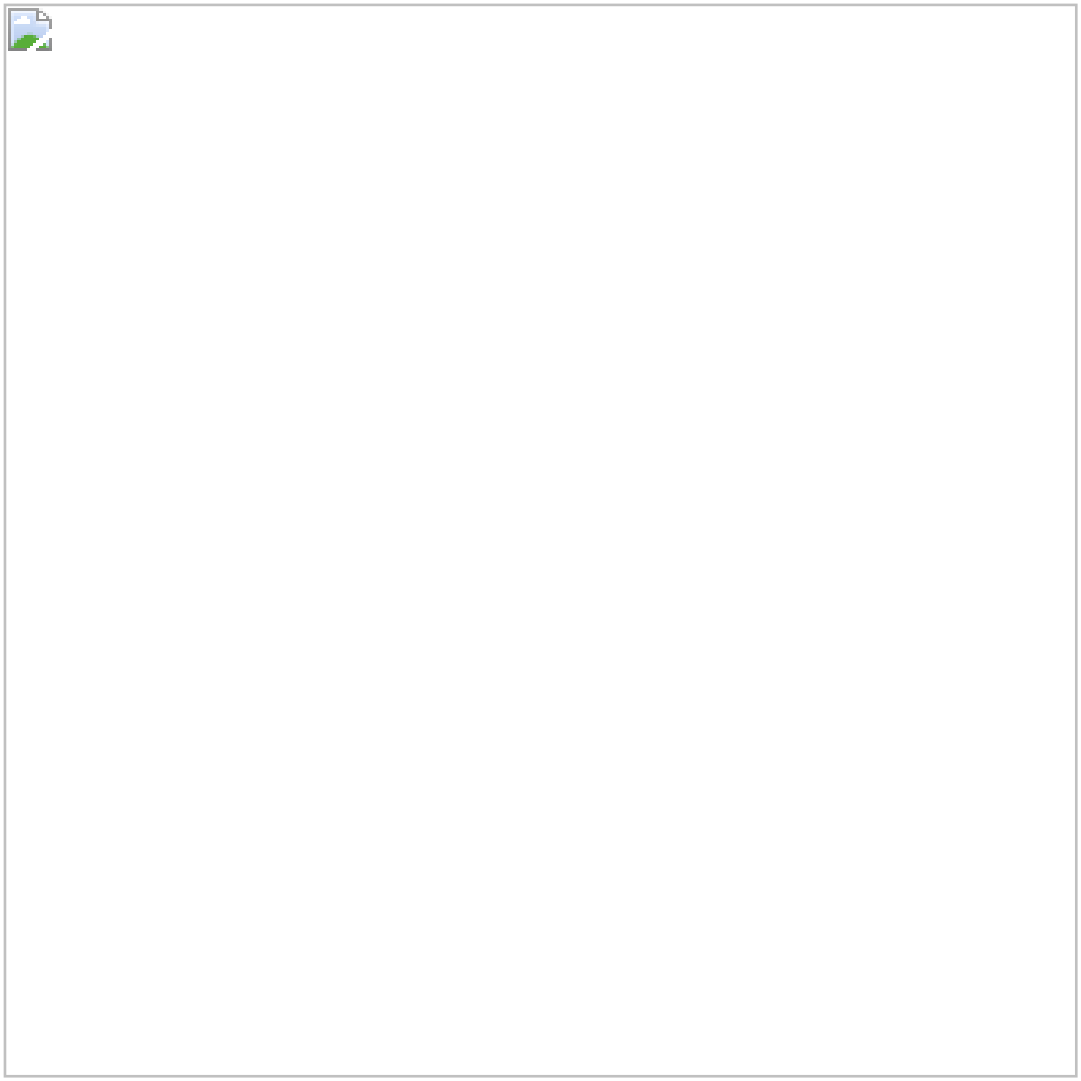

Twenty-Seven Low-Copy Nuclear Primers for Lindera obtusiloba (Lauraceae): A Tertiary Relict Species in East Asia

Authors: Ye, Jun-Wei, Li, Qin, Tian, Xiang-Yu, Bao, Lei, Wang, Hong-Fang, et al.

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PRIMER NOTE

TWENTY-SEVEN LOW-COPY NUCLEAR PRIMERS FOR *LINDERA OBTUSILOBA* (LAURACEAE): A TERTIARY RELICT SPECIES IN EAST ASIA[1]

JUN-WEI YE[2,3], QIN LI[4,5], XIANG-YU TIAN[3], LEI BAO[3,6], HONG-FANG WANG[3], AND JIAN-PING GE[3]

[2]Natural History Research Center of Shanghai Natural History Museum, Shanghai Science and Technology Museum, Shanghai 200127, People's Republic of China; [3]State Key Laboratory of Earth Surface Processes and Resource Ecology and Ministry of Education Key Laboratory for Biodiversity Science and Ecological Engineering, College of Life Sciences, Beijing Normal University, Beijing 100875, People's Republic of China; [4]Center for Watershed Ecology, Nanchang University, Nanchang 330031, People's Republic of China; and [5]Institute of Biodiversity Science, Fudan University, Shanghai 200438, People's Republic of China

- *Premise of the study:* To investigate a more detailed evolutionary history of *Lindera obtusiloba* (Lauraceae) and other *Lindera* species, polymorphic low-copy nuclear primers were developed.
- *Methods and Results:* Unigenes of the *L. obtusiloba* transcriptome greater than 800 bp in length were randomly chosen for initial design of 168 primers. Agarose gel electrophoresis and Sanger sequencing were used to select low-copy nuclear genes. Twenty-seven primers were obtained and were used to investigate genetic diversity in 90 individuals from 24 populations. The nucleotide diversity ranged from 2.11×10^{-3} to 8.99×10^{-3}, and haplotype diversity ranged from 0.57 to 0.97. These primers were also cross-amplified in *L. aggregata*, *L. chunii*, *L. erythrocarpa*, and *L. glauca*; up to 15 primers were successfully amplified in these related species.
- *Conclusions:* This methodology is effective for development of low-copy nuclear primers. The 27 primers developed here will be useful for evolutionary studies of *L. obtusiloba* and other *Lindera* species.

Key words: Lauraceae; *Lindera*; *Lindera obtusiloba*; low-copy nuclear gene; transcriptome.

Lindera obtusiloba Blume (Lauraceae) is a deciduous plant distributed in both northern and southern floral regions of the Tertiary relict flora in East Asia (Donoghue et al., 2001; Milne and Abbott, 2002). These two regions harbor two distinct *L. obtusiloba* genealogies that were probably triggered by the intermediate arid belt (Ye et al., 2017), providing a perfect system to investigate the floral subdivision of the East Asian Tertiary relict flora and the effect of the west-east–oriented arid belt. Only four chloroplast fragments and six nuclear microsatellites were used in Ye et al. (2017), limiting a detailed evolutionary history inference within each floral region. The nuclear microsatellites used in Ye et al. (2017) were designed for *L. melissifolia* (Walter) Blume (Echt et al., 2006) or *L. benzoin* (L.) Blume (Edwards and Niesenbaum, 2007); therefore, in this study, we aimed to design species-specific low-copy nuclear primers for *L. obtusiloba*.

Transcriptome sequences are widely used in studies of plant evolutionary history (e.g., Ai et al., 2015) and can be used for development of low-copy nuclear primers (Bai and Zhang, 2014). For example, Higashi et al. (2015) developed eight primers using 100 expressed sequence tag (EST) markers of Ericaceae, and the phylogeny of *Shortia* Raf. was inferred through these primers. In this study, the transcriptome data of *L. obtusiloba* were used to develop low-copy nuclear primers, and these primers were cross-amplified in other *Lindera* Thunb. species.

METHODS AND RESULTS

Two *L. obtusiloba* leaves were collected in the populations XRD and TMSH (Appendix 1) and used for transcriptome sequencing. Total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), and the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, Massachusetts, USA) was used to generate sequencing libraries. An index code was added to each sample. TruSeq PE Cluster Kit v3-cBot-HS (Illumina, San Diego, California, USA) on a cBot Cluster Generation System was used to cluster the index-coded samples. The Illumina HiSeq 2500 platform was used to sequence the libraries and generate paired-end

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[6]Author for correspondence: baolei@bnu.edu.cn

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TABLE 1. Characteristics of the 27 *Lindera obtusiloba* low-copy nuclear loci.

Locus	Primer sequences (5′–3′)	Length (bp)	T_a (°C)	GenBank accession no.	Exon (bp)	Intron (bp)	Putative function	Closest species	*E*-value
2AP	R: ACTGGGTTTCATTTGTTG F: GCTGTTGGCTTTGTTCC	810	56	MF152421	1–181; 778–810	182–777	CSC1-like protein ERD4 (LOC104588785)	*Nelumbo nucifera*	0
2DA	R: CAAGCAAAGGGCTCAATG F: GCCTCGCCTCTTCAGTAA	581	60	MF152429	405–581	1–404	Uncharacterized LOC103717698 (LOC103717698)	*Phoenix dactylifera*	3E-35
ACY	R: TCGCTTTGGCAATGTTTC F: GATCTCGCAGATGGCTTT	944	52	MF152435	1–451; 902–944	452–901	Acyl-coenzyme A oxidase 2, peroxisomal (LOC109009398)	*Juglans regia*	0
BAE	R: TGGCAGCAGATTGGTAGT F: GTCCTTTGGGTAGAAGTCAT	629	60	MF152452	141–551	1–140; 552–629	Sucrose galactosyltransferase 2 (LOC104597400)	*Nelumbo nucifera*	0
COD1	R: TGGTGGCAAGACCTGGAT F: GCTGGGTTCTGGAATGTAG	154	56	MF152461	1–154		Flavanone-3-hydroxylase (F3H) gene	*Persea americana*	0
FASP	R: GACTGGTACTGCGGTGAC F: CCTCTTCCCTCCAAACA	626	56	MF152466	71–225	1–70; 226–627	Omega-6 fatty acid desaturase, chloroplastic-like (LOC104603306)	*Nelumbo nucifera*	0
GPN	R: AAAGCCAGTCAGAATAACC F: TCTGCTAAATCAGCCACA	516	48	MF152474	1–23; 203–366; 457–516	24–202; 367–456	hsp70 nucleotide exchange factor FES1 (LOC100266149)	*Vitis vinifera*	0
HET	R: GGGCAGACCCTAAGAAT F: CAGGTTTAGCAGGAGGTA	612	56	MF152477	580–612	1–579	Heterogeneous nuclear ribonucleoprotein R (LOC109822129)	*Asparagus officinalis*	2E-114
HIST	R: ATTGAACCTGCCCTTAC F: AGATTGTATCCACCTTCC	228	56	MF152485	128–228	1–127	Histone deacetylase 14 (LOC103720526)	*Phoenix dactylifera*	0
HPT	R: CTCATCCGTTCTCCTTTT F: GGTCTTAGCAAACCTTCC	394	52	MF152490	1–67	68–394	Uncharacterized LOC104604799 (LOC104604799)	*Nelumbo nucifera*	7E-126
HYPO	R: TCAGCATCCATCCTACGG F: CCAGGCAAAACAATACCC	305	52	MF152499		1–305	Vesicle-associated protein 1-3-like (LOC103961248)	*Pyrus ×bretschneideri*	4E-103
INTE	R: TTGAAGGAACAAGGGAG F: ATTCATTCTTGGTGTCATA	324	48	MF152505	1–47; 169–324	48–168	Proton pump-interactor 1-like (LOC109013106)	*Juglans regia*	4E-85
ISOM	R: AAGAAGGCTAAATCCGTT F: CGTAGGGTATCCTGTGAC	341	48	MF152511	313–341	1–312	Protein disulfide-isomerase A6 (LOC105052579)	*Elaeis guineensis*	0
LEP2	R: GTTCAAGATGGCTGGGTA F: GACAGCAAAGATGACCCT	391	56	MF152517	1–53; 144–275; 369–391	54–143; 276–368	F-box/LRR-repeat protein 14 (LOC100243795)	*Vitis vinifera*	0
LG3	R: GGGTGGTTGAGGATGTTA F: CAGGACGTTGTCTTCGTT	472	56	MF152522	191–462	1–190; 463–472	Transcinnamate 4-monooxygenase (LOC104593756)	*Nelumbo nucifera*	0
LPD	R: GCGGCCAGGTTTAAGAAA F: TCGGAGGTCGTAGGGTGA	187	56	MF152530	1–187		N-succinyldiaminopimelate aminotransferase DapC (LOC104882468)	*Vitis vinifera*	0
MALA	R: GGTTGGACGAGAATAGAGC F: TCGCAGGTATCCCACTGAT	328	56	MF152532	93–172; 296–328	1–92; 173–295	Malate dehydrogenase [NADP], chloroplastic-like (LOC104587331)	*Nelumbo nucifera*	0
MPD	R: CCCCAGCATAAAGGAACT F: CTCGACATCACCGACACT	622	48	MF152537	471–621	1–470; 622	Ankyrin repeat domain-containing protein 2A-like (LOC104592662)	*Nelumbo nucifera*	0
PENT	R: TTGATGCGTATAACACTTTG F: ATGATTTCGTTGGCTTTG	456	56	MF152540	1–37; 288–456	38–287	Tetratricopeptide repeat-like superfamily protein	*Cinnamomum camphora*	0
PORI	R: CTGCAAACCCTGTCGTTA F: CCTGGTGTCTACTTCTCCC	417	56	MF152544	1–102; 215–417	103–219	Mitochondrial outer membrane protein porin of 34 kDa (LOC108992813)	*Juglans regia*	3E-159
PRUP	R: GCACAGACCTCGTGTCGT F: TGCCCAGCCATTCATAAC	586	60	MF152547	1–64; 154–184; 531–586	65–153;185–530	Peroxiredoxin-2F, mitochondrial (LOC18792220)	*Prunus persica*	5E-96
SPT2	R: GTATTGTATGAGATGGGGTCT F: AACTCGGAGGGAGTGTTC	497	56	MF152552	1–62; 460–497	63–459	F-box protein SKIP31-like (LOC104610293)	*Nelumbo nucifera*	3E-175
STOP	R: GGCGGCTCAACAAGAAG F: CTGCCAAGAGTCTCACCAC	600	60	MF152560	569–600	1–568	Phosphatidylserine decarboxylase proenzyme 2-like (LOC104599579)	*Nelumbo nucifera*	0
STP	R: TGTTGCGGTTAAGATATTGG F: GTTCCTGTCTCGGGTGTC	386	56	MF152569	1–386		Serine/threonine-protein kinase HT1 (LOC104590294)	*Nelumbo nucifera*	0
TDM	R: GCATCTTTGCCCTCCTCT F: CTTCGGTCTTCAATCCCT	686	56	MF159113	643–677	1–642; 678–686	F-box protein PP2-A15 (LOC104612530)	*Nelumbo nucifera*	1E-168

TABLE 1. Continued.

Locus	Primer sequences (5′–3′)	Length (bp)	T_a (°C)	GenBank accession no.	Exon (bp)	Intron (bp)	Putative function	Closest species	*E*-value
TPP	R: AATGGTCCAGGTGGTGAT F: TTTGCAGCCAGTTCTTTG	614	56	MF152574	140–257; 385–458; 569–614	1–139; 258–384; 459–568;	ATP-dependent Clp protease proteolytic subunit-related protein 2, chloroplastic (LOC104611846)	*Nelumbo nucifera*	0
VEST	R: GGTCGAAACAACCCAGAT F: TGAAGAGCCCAGCAAAT	592	60	MF152583	47–331; 440–592	1-46; 332–439	Putative glucuronosyltransferase PGSIP8 (LOC105059517)	*Elaeis guineensis*	1E-165

Note: T_a = annealing temperature.

reads. The raw reads were cleaned by removing reads containing adapters, reads including more than 10% unknown base information, and reads with low quality. All clean reads were assembled by Trinity (v2012-10-05) (Grabherr et al., 2011). The transcriptome data can be accessed in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) (NCBI Resource Coordinators, 2017) under accession numbers SRR5888830 and SRR5892454. In total, 191,545 unigenes were obtained, and unigenes greater than 800 bp in length were randomly chosen for initial design of 168 primers. We BLASTed these unigenes in nucleotide collection (nr/nt) database using MEGABLAST (optimized for highly similar sequences) in the NCBI database. The exon position, intron length, and putative function were justified by the gene information of the closest gene in the NCBI database. Primer pairs were designed in separate exon regions using Primer Premier 5 (PREMIER Biosoft International, Palo Alto, California, USA) under the following criteria: (i) size of primers 17–23 bp, (ii) annealing temperature (T_a) 45–64°C, (iii) T_a difference between primer pairs less than 4°C, (iv) primer pair score greater than 90, and (v) putative amplified product length less than 1200 bp.

PCRs were performed following the procedure in Ye et al. (2017) with adjusted annealing temperatures (Table 1). Agarose gel electrophoresis was used to select primers that generated only one clear band, and these primers were amplified in eight individuals. The amplicons were sequenced and then read in CodonCode Aligner 3.6.1 (CodonCode Corporation, Centerville, Massachusetts, USA; http://www.codoncode.com/aligner/). The loci with all nucleotide sites that exhibit fewer than two types of nucleotide variants were treated as low-copy nuclear loci. Low-copy nuclear loci were tested in 90 individuals sampled from 24 populations of *L. obtusiloba* (Appendix 2). After reading in CodonCode Aligner 3.6.1, PHASE function in DnaSP 5.10.01 (Rozas et al., 2003) was used to determine heterozygous and polymorphic sites, determine haplotypes, and to calculate genetic diversities, including nucleotide diversity (π) and haplotype diversity (H_d), of each locus. SPADS 1.0 (Dellicour and Mardulyn, 2014) was used to calculate haplotypes, π, and allelic richness in 24 populations. Genotypic disequilibrium was assessed using all locus pairs in all populations by randomization using FSTAT 2.9.3 with Bonferroni correction (Goudet, 2001). Local BLAST function in BioEdit 7.1.9 (Hall, 1999) was used to determine the intron and exon positions of all low-copy nuclear loci, and the unigenes for primer design were used as database (Appendix S1). Low-copy nuclear genes were cross-amplified in two individuals of four other *Lindera* species, including *L. aggregata* (Sims) Kosterm., *L. chunii* Merr., *L. erythrocarpa* Makino, and *L. glauca* (Siebold & Zucc.) Blume (Appendix 1).

Ninety-six of the 168 tested primers did not amplify or generated multiple bands, 45 produced messy sequences, and 27 produced clear sequences (Table 1). The product length of the 27 loci ranged from 154 to 944 bp. The number of polymorphic sites and haplotypes ranged from six to 71 and five to 49, respectively, with a mean of 27 and 19, respectively. In addition, π ranged from 2.11×10^{-3} to 8.99×10^{-3} with a mean of 6.06×10^{-3}, and H_d ranged from 0.57 to 0.97, with a mean of 0.77 (Table 2). In the 24 populations, the number of haplotypes ranged from 39 to 76, π ranged from 0.76×10^{-3} to 1.80×10^{-3}, and allelic richness ranged from 1.43 to 1.94 (Appendix 2). No significant genotypic disequilibrium was observed among 351 locus pairs. Fifteen primers were successfully amplified in *L. aggregata* and *L. erythrocarpa*, and 14 primers were successfully amplified in *L. chunii* and *L. glauca* (Table 2).

CONCLUSIONS

Given that information regarding exon position and intron sequence are not included in transcriptome sequencing, the success rate of primer development using transcriptome data would be expected to be low (Bai and Zhang, 2014). In this study, we developed 27 polymorphic primers out of a set of 168 primers, with a ratio of approximately 16%. The success rate is increased twofold compared with that of Higashi et al. (2015). This methodology provides an effective approach for the development of new low-copy nuclear primers.

Twenty-seven novel polymorphic low-copy nuclear primers were developed using transcriptome data from *L. obtusiloba*. These primers can be used to investigate the evolutionary history of *L. obtusiloba* and other *Lindera* species.

TABLE 2. Genetic diversity and cross-amplification of the 27 *Lindera obtusiloba* low-copy nuclear loci.

Locus	*n*	*V*	*S*	*P*	*H*	H_d	π (×10^{-3})	*Lindera aggregata*	*Lindera erythrocarpa*	*Lindera chunii*	*Lindera glauca*
2AP	82	41	11	30	31	0.88	8.04	—	—	—	—
2DA	84	44	4	40	19	0.88	8.99	+	+	+	+
ACY	85	71	14	57	49	0.97	8.53	+	—	+	+
BAE	86	36	11	25	28	0.84	6.86	—	+	—	—
COD1	87	10	2	8	13	0.73	8.55	+	—	—	—
FASP	88	44	18	26	23	0.80	6.72	+	+	+	+
GPN	88	28	11	17	17	0.73	4.79	+	+	+	+
HET	87	22	4	18	15	0.77	6.27	+	—	+	+
HIST	85	20	7	13	14	0.79	7.39	—	+	—	—
HPT	86	32	9	23	24	0.84	5.46	—	+	—	—
HYPO	87	16	4	12	15	0.72	6.65	—	—	—	—
INTE	88	23	8	15	15	0.71	8.78	+	+	+	+
ISOM	87	16	5	11	13	0.75	5.39	+	—	+	+
LEP2	81	20	10	10	18	0.68	5.16	+	—	+	+
LG3	86	16	4	12	16	0.84	3.55	+	—	—	—
LPD	89	6	0	6	5	0.57	3.71	—	+	+	+
MALA	88	16	0	16	12	0.71	6.16	+	+	+	+
MPD	86	20	2	18	16	0.82	6.27	+	—	+	+
PENT	88	20	2	18	13	0.57	2.11	—	+	—	—
PORI	88	27	11	19	12	0.68	6.75	+	+	—	—
PRUP	88	22	4	18	21	0.85	4.75	+	—	—	—
SPT2	87	30	8	22	22	0.81	5.24	—	+	+	+
STOP	89	30	9	21	23	0.80	8.56	—	—	—	—
STP	89	12	2	10	15	0.73	3.31	—	+	+	+
TDM	87	40	11	29	21	0.80	5.29	—	+	—	—
TPP	88	35	12	23	21	0.76	6.03	—	+	—	—
VEST	88	28	12	16	28	0.88	4.22	+	—	+	+

Note: — = unsuccessful amplification; + = successful amplification; *H* = haplotypes; H_d = haplotype diversity; π = nucleotide diversity; *P* = parsimony informative sites; *S* = singleton variable sites; *V* = variable sites.

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

Species	Population	Location	Latitude	Longitude	Voucher no.[a]
Lindera obtusiloba Blume	ANZH	Anzihe Nature Reserve, Sichuan, China	30.81	103.13	SHM23259
	BDGS	Mt. Badagong, Hunan, China	29.69	109.79	SHM23260
	BHSH	Bukhansan National Park, Seoul City, Korea	37.65	126.99	SHM23261
	BM	Bomi, Xizang, China	29.87	95.73	SHM23262
	DAL	Dalian, Liaoning, China	38.90	121.46	SHM23263
	DBSH	Mt. Daba, Anhui, China	31.01	116.11	SHM23264
	JAP	Tokyo, Japan	35.95	139.30	SHM23074
	JWS	Gariwangsan, Gangwon Province, Korea	37.43	128.56	SHM23265
	KI	Mt. Iizuna, Japan	36.72	138.15	SHM23266
	KYSH	Mt. Kunyu, Shandong, China	37.26	121.73	SHM23267
	LAJ	Lajing, Yunnan, China	26.49	99.28	SHM23073
	LISH	Mt. Li, Shanxi, China	35.43	111.98	SHM23268
	MCSH	Mt. Micang, Shannxi, China	32.69	107.53	SHM23269
	NI	Nikko, Japan	36.75	139.42	SHM23270
	PMA	Pianma, Yunnan, China	25.99	98.66	SHM23271
	TMSH	Mt. Tianmu, Zhejiang, China	30.42	119.41	SHM23070
	UH	Masuda, Japan	34.55	132.04	SHM23272
	WEIX	Weixi, Yunnan, China	27.18	99.29	SHM23273
	WYSH	Mt. Wuyi, Jiangxi, China	27.93	117.69	SHM23274
	XRD	Zhuanghe, Liaoning, China	40.02	122.96	SHM23071
	XYSH	Seoraksan National Park, Gangwon Province, Korea	38.17	128.49	SHM23275
	XZD	Xiaozhongdian, Yunnan, China	27.34	99.84	SHM23276
	YTSH	Mt. Yuntai, Jiangsu, China	34.72	119.44	SHM23277
	ZYSH	Mt. Jiri, South Gyeongsang Province, Korea	35.29	127.49	SHM23278
Lindera aggregata (Sims) Kosterm.	TMSH	Mt. Tianmu, Zhejiang, China	30.42	119.41	SHM22266
Lindera chunii Merr.	DHS	Mt. Dinghu, Guangdong, China	23.17	112.55	SHM23280
Lindera erythrocarpa Makino	KYSH	Mt. Kunyu, Shandong, China	37.26	121.73	SHM23279
Lindera glauca (Siebold & Zucc.) Blume	TMSH	Mt. Tianmu, Zhejiang, China	30.42	119.41	SHM23281

[a]Voucher specimens were deposited in Shanghai Natural History Museum (SHM), Shanghai, China.

Appendix 2. Genetic diversity in 24 populations of the 27 low-copy nuclear loci in *Lindera obtusiloba*.

Population	*n*	No. of haplotypes	π ($\times 10^{-3}$)	Allelic richness
ANZH	5	55	0.77	1.58
BDGS	3	61	1.50	1.94
BHSH	3	56	1.23	1.79
BM	3	51	1.08	1.64
DAL	3	41	1.14	1.43
DBSH	5	60	1.25	1.71
JAP	6	76	1.80	1.89
JWS	5	53	1.09	1.51
KI	3	59	1.80	1.92
KYSH	3	52	1.39	1.70
LAJ	5	70	1.48	1.83
LISH	3	50	0.87	1.61
MCSH	3	65	1.44	1.90
NI	2	39	0.76	1.44
PMA	4	56	1.29	1.69
TMSH	5	55	1.25	1.72
UH	6	68	1.10	1.68
WEIX	3	59	1.60	1.85
WYSH	3	49	1.13	1.63
XRD	5	55	1.49	1.66
XYSH	3	47	1.47	1.57
XZD	3	55	1.26	1.73
YTSH	3	47	1.00	1.58
ZYSH	3	51	1.49	1.71

Note: *n* = number of individuals; π = nucleotide diversity.