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Research Article

Analysis of the genetic diversity and population structure of *Lindera obtusiloba* (Lauraceae), a dioecious tree in Korea

Ho Bang Kim · Hye-Young Lee · Mi Sun Lee · Yi Lee · Youngtae Choi · Sung-Yeol Kim · Jaeyong Choi

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Abstract *Lindera obtusiloba* (Lauraceae) is a dioecious tree that is widely distributed in the low-altitude montane forests of East Asia, including Korea. Despite its various pharmacological properties and ornamental value, the genetic diversity and population structure of this species in Korea have not been explored. In this study, we selected 6 nuclear and 6 chloroplast microsatellite markers with polymorphism or clean cross-amplification and used these markers to perform genetic diversity and population structure analyses of *L. obtusiloba* samples collected from 20 geographical regions. Using these 12 markers, we identified a total of 44 alleles, ranging from 1 to 8 per locus, and the average observed and expected heterozygosity values were 0.11 and 0.44, respectively. The average polymorphism information content was 0.39. Genetic relationship and population structure analyses revealed that the natural *L. obtusiloba* population in Korea is composed of 2 clusters, possibly due to two different plastid genotypes. The same clustering patterns have also been observed in *Lindera* species in mainland China and Japan.

Keywords Dioecy, Genetic diversity, *Lindera obtusiloba*, Microsatellites, Population structure

H. B. Kim · H.-Y. Lee
Life Sciences Research Institute, Biomedic Co., Ltd., Bucheon 14548, Republic of Korea

H. B. Kim
Department of Environmental Horticulture, School of Equine Science and Horticulture, Cheju Halla University, Jeju 63092, Republic of Korea

M. S. Lee · Y. Lee
Department of Industrial Plant Science and Technology, Chungbuk National University, Cheongju 28644, Republic of Korea

Y. Choi
Division of Forest Diversity, Korea National Arboretum, Pocheon 11186, Republic of Korea

S.-Y. Kim
Enfield Co., Chungnam National University, Daejeon 34134, Republic of Korea

J. Choi (✉)
Department of Environment and Forest Resources, Chungnam National University, Daejeon 34134, Republic of Korea
e-mail: jaychoi@cnu.ac.kr

Introduction

Lindera obtusiloba Blume belongs to the family Lauraceae and is called blunt-lobed spice bush (or spicebush) due to its spicy odor. A dioecious and deciduous forest understory shrub, *L. obtusiloba* has a broad distribution across the forests of East Asia, including Korea. There are over 100 species of the genus *Lindera* globally, eight of which are native to Korea: *L. obtusiloba* Blume, *L. obtusiloba* for. *quinquelobum* Uyeki, *L. obtusiloba* for. *villosum* Nakai, *L. obtusiloba* for. *ovata* T. Lee, *L. glauca* (Siebold & Zucc.) Blume, *L. angustifolia* W.C. Cheng, *L. erythrocarpa* Makino, *L. sericea* (Siebold & Zucc.) Blume. Some *Lindera* species such as *L. obtusiloba* and *L. angustifolia* are sensitive to aridity (Ye et al. 2017).

Tissue extracts of *L. obtusiloba* have diverse pharmacological effects, including antioxidant, anti-inflammatory, antiplatelet, antithrombotic, antimicrobial, antitumor, and anti-metastatic activities, sedative and antinociceptive properties, antidepressant-like effects, and inhibitory effects of allergenic inflammation (Hong 2013; Kim et al. 2009, 2014, 2016b; Lee et al. 2012; Lim et al. 2016; Park et al. 2021; Yun et al. 2012). Kim et al. (2015) reported that methanolic extract of *L. obtusiloba* had a lifespan-extending effect in a model organism, *Caenorhabditis elegans*, by increasing antioxidant enzyme activities, thereby reducing intracellular reactive oxygen species levels. Many efforts also have been made to use *L. obtusiloba* extracts as functional cosmetic ingredients

(Bang et al. 2008; Park et al. 2009; Won et al. 2012; Yu and Kim 2017).

L. obtusiloba also has high value as a landscaping tree due to its deep yellow flowers in early spring and beautiful foliage in autumn. In the past, the seeds were pressed for hair oil. Young leaves were often consumed as tea or fried as a foodstuff. Dried stem and bark were also used as traditional medicine (http://www.nature.go.kr/main/Main.do).

Microsatellites, also often referred to as simple sequence repeats (SSRs) and short tandem repeats (STRs), are highly informative DNA markers due to their high degree of polymorphism, codominant mode of inheritance, and their wide distribution on nuclear/organelle genomes. Microsatellite markers have been widely used for various molecular genetic analyses, including genetic diversity studies and population structure analysis (Ahn et al. 2021; Chung et al. 2019; Kim et al. 2016a). Polymorphic nuclear or chloroplast microsatellite loci have already been reported for *L. benzoin* and *L. aggregata* (Edwards and Niesenbaum 2007; Ye and Li 2019). Molecular genetic studies including genetic diversity, population structure and phytogeography of *Lindera* species have been performed using nuclear and chloroplast microsatellites by research groups in China and Japan (Nakamura et al. 2021; Ye et al. 2017; Zhu et al. 2020).

Although *L. obtusiloba* is native to Korea and has a plethora of biological activities and ornamental uses, the genetic diversity and population structure of *L. obtusiloba* in Korea have not been explored. To perform these studies, we collected *L. obtusiloba* accessions from natural vegetation at 20 collection sites in Korea. We then performed genetic diversity and population structure analysis using nuclear and chloroplast microsatellite markers.

Materials and Methods

Plant materials and genomic DNA isolation

Leaf tissues of *L. obtusiloba* used in this study were collected from 100 individuals (5 individuals per location) of natural vegetation at 20 collection sites in the Republic of Korea (Fig. 1 and Supplementary Table 1). The harvested leaf tissues were moved to the laboratory, rinsed with running tap water and then stored at 80°C until use. Genomic DNA (gDNA) was isolated from the leaf tissue using a Biomedic® Plant gDNA Extraction Kit (Biomedic Co., Ltd., Bucheon, Korea) according to the manufacturer's protocol. The purified gDNA was quantified and qualified using a DeNovix DS-11+ spectrophotometer (DeNovix, Wilmington, DE, USA) and 1% agarose gel electrophoresis, respectively.

Polymorphism analysis of microsatellite markers

Nuclear and chloroplast microsatellite markers were previously developed from *L. benzoin* and *L. aggregata*, respectively (Edwards and Nissenbaum 2007; Ye and Li 2019). To check cross-amplification, PCR efficiency and polymorphism, gDNAs of 1 representative individual from each of 8 populations were subjected to routine PCR as described previously (Woo et al. 2019). The PCR reaction and cycling conditions were described in our previous report (Woo et al. 2019). PCR products were separated on a 2.5% (w/v) agarose gel to confirm PCR amplification and polymorphism among

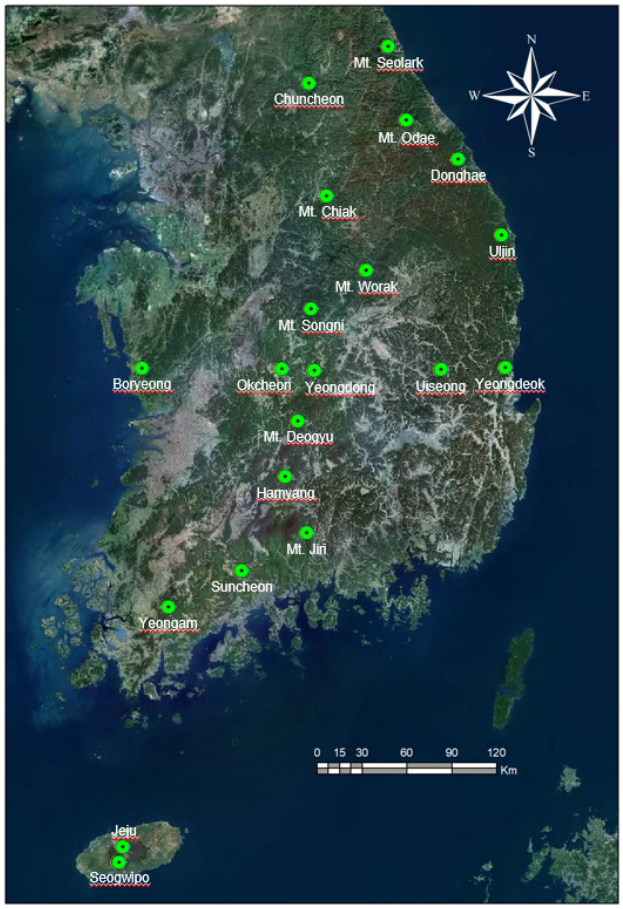

Fig. 1 *L. obtusiloba* sample collection areas for the genetic diversity and population structure analyses. The collection areas are as follows: Boryeong (Chungcheongnam-do), Chuncheon (Gangwon-do), Mt. Chiak, Mt. Worak, Mt. Songni, Okcheon (Chungcheongbuk-do), Yeongdong (Chungcheongbuk-do), Mt. Deogyu, Hamyang (Gyeongsangnam-do), Mt. Jiri, Suncheon (Jeollanam-do), Yeongam (Jeollanam-do), Mt. Seolark, Mt. Odae, Donghae (Gangwon-do), Uljin (Gyeongsangbuk-do), Uiseong (Gyeongsangbuk-do), Yeongdeok (Gyeongsangbuk-do), Jeju-si (Jeju-do), and Seogwipo-si (Jeju-do)

the tested accessions.

Fragment analysis for genotyping

To determine genotypes of the selected polymorphic microsatellite loci, forward primers were labelled with a virtual dye (6-FAM; Applied Biosystems, Waltham, MA, USA). The PCR reaction and cycling conditions were the same as those reported previously (Kim et al. 2016a). Fragment analysis of the PCR products followed the previous report (Kim et al. 2016a). Briefly, 0.2 μL of PCR product was mixed with 9.8 μL Hi-Di formamide (Applied Biosystems) and 0.2 μL of the GeneScan™ 500 LIZ® size standard (Applied Biosystems). The mixture was denatured at 95°C for 5 min and placed on ice. The amplified fragments were separated by capillary electrophoresis on an ABI 3730 DNA analyzer (Applied Biosystems) using a 50-cm capillary with the pre-installed DS-33 dye set. Allele size and number were called using the GeneMapper software (ver. 4.0; Applied Biosystems).

Data analysis

Genetic parameters such as major allele frequency (M_{AF}), number of alleles (NA), genetic diversity (GD, often referred to as expected heterozygosity), observed heterozygosity (HO), and polymorphic information content (PIC) were measured by calculating the shared allele frequencies using the PowerMarker software (v. 3.25) (Liu and Muse 2005). An unweighted pair group method with arithmetic mean (UPGMA) dendrogram was created using MEGA software (v. 7.0) (Kumar et al. 2016), which is embedded in PowerMarker, using the UPGMA algorithm.

Model-based methods of the STRUCTURE software (v. 2.3.4; Pritchard et al. 2000) were used to analyze the population structure of the collection in this study. The input data of PowerMarker software were appropriately converted into the input data form of the STRUCTURE software using the CONVERT software (v. 1.31). For population structure analysis, number of the sub-population labeled with *K* was identified with the following parameters: Run Length of Burning Period = 100,000; Number of Markov Chain Monte Carlo (MCMC) Reps after Burning = 100,000. Each *K* value was run 3 times with a *K* value varying from 1 to 12. The optimum *K* value was determined by plotting the mean estimate of the log-likelihood of the data (LnP(D)) against the given *K* value. The true number of sub-populations was determined using the maximal ΔK value.

Results

Selection of polymorphic microsatellite markers

To select microsatellite loci with polymorphism, cross-amplification, clean PCR amplicons, and high PCR efficiency, we applied 17 marker candidates (10 nuclear markers and 7 chloroplast markers) to 1 representative individual from each of 8 different populations (21LO11-1, 21LO12-1, 21LO13-1, 21LO14-1, 21LO15-1, 21LO16-1, 21LO21-1, and 21LO22-1) (data not shown). Of 10 nuclear markers that were developed from *L. benzoin*, 9 markers resulted in cross-amplification in *L. obtusiloba*. Of 7 chloroplast markers that were developed from *L. obtusiloba*, 6 markers showed clean amplification with polymorphism. From the marker screening, we finally obtained 12 markers, 6 nuclear and 6 chloroplast markers, that fulfilled the selection requirements mentioned previously (Table 1); we applied them to down-stream studies such as genetic diversity and population structure analysis.

Evaluation of genetic diversity

To evaluate the genetic diversity of the natural population of *L. obtusiloba* in Korea, the 12 selected polymorphic markers were applied to 100 individuals from 20 natural populations (Supplementary Table 2). The genetic characteristics of microsatellite loci based on the genotypes of 100 accessions used in this study are summarized in Table 2. A total of 44 alleles, ranging from 1 (BbB105 and LbD6) to 8 (LbA7) per locus, were identified among the 100 *L. obtusiloba* accessions from 20 natural populations, with an average of 3.67 alleles per locus. M_{AF} varied from 0.39 (LbA7) to 1 (BbB105 and LbD6). The average GD value was 0.44, ranging from 0 (BbB105 and LbD6) to 0.71 (LbA7), and the average PIC value was 0.39, from 0 (BbB105 and LbD6) to 0.67 (LbA7). The average HO was 0.11, with the lowest value in two nuclear markers (BbB105 and LbD6) and all of the chloroplast markers (0), and the highest value in LbA7 (0.66). Therefore, two nuclear markers, BbB105 and LbD6, showed no polymorphism in the Korean *L. obtusiloba* population analyzed in this study.

A total of 44 alleles derived from the 12 microsatellite loci were used to evaluate genetic relationships among the accessions from 20 populations. A UPGMA dendrogram was constructed based on the genetic similarity matrices among the accessions. Fig. 2 illustrates the results of the cluster analysis based on nuclear and chloroplast micro-

Table 1 Nuclear and chloroplast microsatellite markers used for genotyping of *L. obtusiloba*

Locus name	Primer name	Primer sequence (5' to 3')	Repeat motif	GenBank acc. no.	Source species	Location	References
LbA7	LbA7-F	AAAACGGATCAGATACTCCC	(AC)13	EF193199	*L. benzoin*	Nuclear	Edwards and Nissenbaum 2007
	LbA7-R	GCAGCATTATTGGGTTAGTG					
LbB105	LbB105-F	ACAGGTCTTGACTTTGGGATAT	(GA)11	EF193201			
	LbB105-R	GGATGGCTTATGGAGTGG					
LbB122	LbB122-F	TGCTCAAGGAGAGATTCAAC	(AG)17	EF193202			
	LbB122-R	CTCAGCCGAGTCTACTATCG					
LbC10	LbC10-F	TTCCTAAACCCTGTTGTAAAAC	(AAG)15	EF193204			
	LbC10-R	GCCAATCATGTGACTATTGTC					
LbC101	LbC101-F	GCCTGATTCCACATAAATTG	(AAG)8	EF193205			
	LbC101-R	AGAAACCAGTGGTCGAAATAC					
LbD6	LbD6-F	CGTTAGGATACAAAGACCAGAG	(ATG)11	EF193208			
	LbD6-R	ATCACACCCTCAAATCATAGTC					
LAG20	LAG20-F	TGGCCGTTGTTCCTTATTTC	(A)12	Cp genome NC_045262.1	*L. obtusiloba*	*atpB/rbcL*	Ye and Li 2019
	LAG20-R	CAACCCAATCCTTGTTTTGC					
LAG9	LAG9-F	GGAAGCGGCAGAAATCAAT	(A)11			*atpH/atpI*	
	LAG9-R	CAAAGACTCCACGGATAGGAA					
LAG24	LAG24-F	TGCATCATGTGAGAATCCAAA	(T)15			*rps16/trnQ-UUG*	
	LAG24-R	TCACAAACAAACGGATCGAG					
LAG29	LAG29-F	ATGGCCAAAATGAACTCCTG	(T)15			*rps2/rpoC2*	
	LAG29-R	CGGTCAATCTCCGGTAGAAG					
LAG31	LAG31-F	GGCTCCTGTAACCGTGTCAT	(T)11			*rpoC1 intron*	
	LAG31-R	GATGCCCCTGACTCTGACAT					
LAG32	LAG32-F	GTAACCCCGCCAAGAATGTA	(T)9			*trnC-GCA/petN*	
	LAG32-R	ATACACAGTTGCCCCTTGGA					

Table 2 Characteristics of the 12 polymorphic microsatellite loci in the collected *L. obtusiloba* accessions

Locus	SS	NOBS	Availability	NG	MAF	NA	GD	Heterozygosity	PIC
LbA7	100	98.00	0.98	17.00	0.39	8.00	0.71	0.66	0.67
LbB105	100	100.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00
LbB122	100	100.00	1.00	3.00	0.61	2.00	0.48	0.49	0.36
LbC10	100	99.00	0.99	4.00	0.92	3.00	0.14	0.13	0.13
LbC101	100	100.00	1.00	3.00	0.96	2.00	0.09	0.07	0.08
LbD6	100	100.00	1.00	1.00	1.00	1.00	0.00	0.00	0.00
LAG20	100	100.00	1.00	3.00	0.53	3.00	0.60	0.00	0.53
LAG9	100	100.00	1.00	3.00	0.47	3.00	0.58	0.00	0.49
LAG24	100	100.00	1.00	6.00	0.50	6.00	0.68	0.00	0.64
LAG29	100	100.00	1.00	4.00	0.51	4.00	0.63	0.00	0.57
LAG31	100	100.00	1.00	5.00	0.42	5.00	0.65	0.00	0.59
LAG32	100	100.00	1.00	6.00	0.45	6.00	0.67	0.00	0.61
Mean	100	99.75	1.00	4.67	0.65	3.67	0.44	0.11	0.39

SS, sample size; N_{OBS}, number of observations; Availability is defined as 1-OBS/n, where OBS is the number of observations and n is the number of individuals sampled; N_G, genotype number; M_{AF}, major allele frequency; N_A, number of alleles; GD, genetic diversity (often referred to as expected heterozygosity), defined as the probability that two randomly chosen alleles from the population are different; Heterozygosity is the simple proportion of heterozygous individuals in the population; PIC, polymorphism information content.

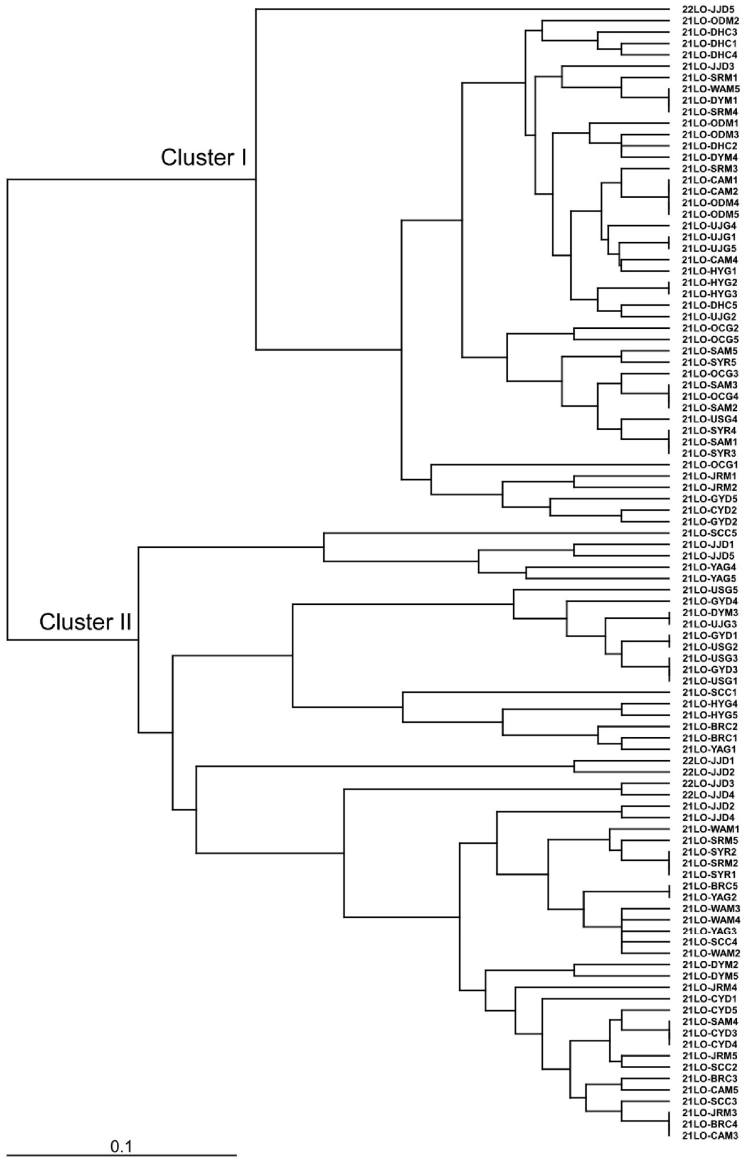

Fig. 2 A UPGMA tree based on the genetic distances among 100 accessions (5 individuals per location) from the 20 different collection areas using 12 polymorphic microsatellite markers consisting of 6 nuclear and 6 chloroplast markers. The 20 natural populations used in this study are abbreviated as follows: BRC (Boryeong-si, Chungcheongnam-do), SYR (Chuncheon-si, Gangwon-do), CAM (Mt. Chiak), WAM (Mt. Worak), SRM (Mt. Songni), OCG (Okcheon-gun, Chungcheongbuk-do), CYD (Yeongdong-gun, Chungcheongbuk-do), DYM (Mt. Deogyu), HYG (Hamyang-gun, Gyeongsangnam-do), JRM (Mt. Jiri), SCC (Suncheon-si, Jeollanam-do), YAG (Yeongam-gun, Jeollanam-do), SAM (Mt. Seolark), ODM (Mt. Odae), DHC (Donghae-si, Gangwon-do), UJG (Uljin-gun, Gyeongsangbuk-do), USG (Uiseong-gun, Gyeongsangbuk-do), GYD (Yeongdeok, Gyeongsangbuk-do), 21LO-JJD (Jeju-si, Jeju-do), and 22LO-JJD (Seogwipo-si, Jeju-do)

satellite data. The resulting tree reveals that the Korean *L. obtusiloba* population can be distinctly classified into 2 clusters. Cluster 1 and Cluster 2 consist of 46 and 54 accessions, respectively.

Analysis of population structure

The population structure of 100 accessions from 20 natural vegetation samples based on 12 microsatellite markers was inferred from the Bayesian approach using the software STRUCTURE (v. 2.3.4). Individual proportions of membership in each group were estimated using a multi-allele data set and the results revealed the existence of several population structures. The distribution of L(K) did not show a clear mode for the true *K* (Supplementary Fig. 1A). An ad hoc quantity ($\triangle K$) was used to overcome the difficulty of interpreting the real *K* values (Evanno et al. 2005). The true value of *K* was determined by illustration of the peak

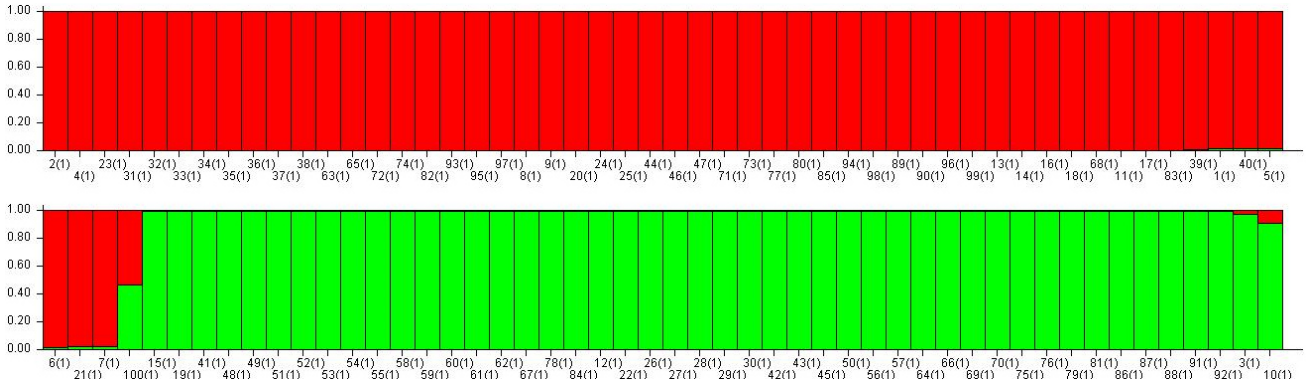

Fig. 3 Estimated population structure of the 100 *L. obtusiloba* accessions collected from 20 populations, generated using STRUCTURE (K=2) based on the 12 selected microsatellite markers. The numbers on the x-axis represent each accession number in Supplementary Table 1

based on ΔK. The highest peak of ΔK was found to be K=2 (Supplementary Fig. 1B), suggesting that the entire population could be grouped into two subpopulations, Pop1 and Pop2. Based on membership fractions, accessions with a probability of $\geq$ 99.4% were assigned to the corresponding subpopulation (Pop1 or Pop2), whereas the others were categorized as admixture (Fig. 3). The Pop1, Pop2 and admixture categories included 46, 44, and 10 accessions, respectively. The 10 accessions (sampling site) that were grouped into the admixture category were 1, 3, 5 (Jeju-si, Jeju-do), 6, 7, 10 (Seogwipo-si, Jeju-do), 21 (Yeongam-gun, Jeollanam-do), 39, 40 (Mt. Chiak) and 100 (Suncheon-si, Jeollanam-do).

Discussion

According to fossil studies, *L. obtusiloba* has a very close relationship to fossil species, *L. paraobtusiloba*, with which it shares unique leaf characteristics (oval, trifid and trinerved), suggesting that it is a relict of the Tertiary Period (i.e. 65-15 million years ago) (Ye et al. 2017). Although *L. obtusiloba* has potential industrial utilities due to its various biological activities, the genetic diversity of Korean populations has not been explored on a large scale. Therefore, its genetic diversity and population structure were evaluated using nuclear and chloroplastic microsatellite markers in this study.

Sexual reproduction is predominant in angiosperms. However, some groups of sexual plants can reproduce asexually via apomixis, which produces exact genetic replicas of maternal plants (Xu et al. 2022). *Lindera* species have been found to harbor both obligate sexual and asexual reproduction systems. Chinese *L. glauca* was found to have both apomixis and a sexual reproduction system (Xiong et al. 2020; Zhu et al. 2020), whereas its Japanese female plants can only asexually reproduce *via* apomixis (Nakamura et al. 2021). Most *Lindera* species, including *L. obtusiloba*, produce seeds by obligate sexual reproduction (Nakamura et al. 2021). In all sexually reproducing species, values of observed heterozygosity were close to the expected ones. This was observed in our study using nuclear microsatellite markers (Table 2) and *L. obtusiloba* populations in China and Japan (Nakamura et al. 2021; Ye et al. 2017), indicating obligate outcrossing in this *Lindera* species.

Analyses of genetic diversity and population structure using microsatellite markers revealed that the natural population of *L. obtusiloba* in Korea is composed of 2 clusters (Figs. 2 and 3). This genetic clustering pattern was also observed in a Tertiary relict species, *L. obtusiloba* in East Asia (Ye et al. 2017) and natural *L. glauca* populations in mainland China (Xiong et al. 2020). Studies by Ye et al. (2017) showed that *L. obtusiloba* in East Asia can be subdivided into a northern region (NEA) and a southern region (SEA). All *L. obtusiloba* accessions from a limited number of regions in Korea and Japan were grouped into NEA (Ye et al. 2017). To further validate if Korean *L. obtusiloba* populations used in this study also reflect two genetic lineages, NEA and SEA, large-scale studies including populations from mainland China and Japan need to be performed in the future.

It is widely believed that plastid genomes are inherited from the maternal parent (Bock 2007). Although the inheritance mode of plastid DNA in *L. obtusiloba* has not been studied yet, the organelle genome of *L. glauca* is assumed to be maternal inheritance (Xiong et al. 2020). A UPGMA dendrogram based on the genetic distances using chloroplast markers only showed that Korean *L. obtusiloba* populations are composed of 2 clusters (Supplementary Fig. 2), as observed in the dendrogram constructed using

both nuclear and chloroplast markers. These results suggest that Korean *L. obtusiloba* harbors two genotypes of the chloroplast genome. *L. obtusiloba* has unique leaf shape; broad leaves mostly three-lobed at the tip. The trifid leaf morphology was not described in its closely related *Lindera* species such as *L. glauca, L. sericea* and *L. benizoin* (https://www.rhs.org.uk/). Genetic diversity analysis using nuclear and chloroplast microsatellite markers revealed that Korean *L. obtusiloba* populations were divided into 2 clusters. However, there were no distinguishable differences on leaf shape between 2 groups (Supplementary Fig. 3).

Although 10 nuclear markers were developed from *L. benzoin* (Edwards and Nissenbaum 2007), most of the markers usedp in this study resulted in cross-amplification in *L. obtusiloba*, except for marker Lbc102. This result indicates that the nuclear and chloroplast markers applied in this study could be applicable to the genetic diversity analysis of the subtribe Lauriineae (Laureae, Lauraceae), which includes the genus *Lindera*, the genus *Listea*, and the genus *Parasassafras*, among others (Liu et al. 2022). Chloroplast microsatellite primers derived from *L. aggregata* were successfully amplified in four other *Lindera* species: *L. prattii*, *L. chunii*, *L. lungshengensis*, and *L. pulcherrima* var. *hemsleyana* (Ye and Li 2019). Ye et al. (2017) also developed low-copy nuclear primers from the unigenes of the *L. obtusiloba* transcriptome, which were successfully cross-amplified among four *Lindera* species that were positioned at different clades in the phylogenetic tree of the family Lauraceae (Liu et al. 2022; Tian et al. 2019). Cross-taxa transferability of microsatellite markers has been widely reported in many species due to recent developments in transcriptome/genome sequencing technologies (Biswas et al. 2020; Singh et al. 2019).

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