

Genetic Diversity and Structure of Apomictic and Sexually Reproducing *Lindera* Species (Lauraceae) in Japan

Mizuho Nakamura, Satoshi Namami, Seiya Okuno, Shun K. Hirota, Ayumi Matsuo, Yoshihisa Suyama, Hayato Tokumoto, Shizue Yoshinara, Akira Itoh

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本研究のポイント	◇ ヤマコウバシがたった 1 本の雌株から生じた巨大なクローンであることを解明。 ◇ 生物の世界に性というシステムが広く存在する理由の解明につながる可能性を示唆。
概要	研究グループは、日本の山野に生える 4 種の雌雄異株樹種、ヤマコウバシ、アブラチャン、クロモジ、ダンコウバイの DNA 情報から、これらの樹種が雌雄異株性の不利をいかに克服して集団を維持しているのか、その仕組みを探りました。その結果、アブラチャン、クロモジ、ダンコウバイの 3 種は、自家受精や単為生殖を行わず、必ず雌雄の交配によって近交弱勢を避けていることが示されたものの、ヤマコウバシは雌株が単独で種子を生産することで雌雄異株性の不利を克服し、さらに日本のヤマコウバシがたった 1 本の雌株から生じた巨大なクローンであることが分かりました。この巨大クローンの分布の範囲は距離にして 1000 km を超える、世界的にも極めて珍しい大規模なものです。
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<p>研究の背景</p>	<p>雌雄異株の落葉樹であるヤマコウバシは、大陸には雄株と雌株が生育しています。しかし日本では雌株しか見つかっておらず、雌株が花粉を受け取ることなく、単為生殖で種子を生産します。作られた種子は原則的には母樹と同じ遺伝子をもつため、集団内に遺伝的多様性は生じないと考えられていました。しかし、単為生殖を行う植物の中には、種子生産の段階で遺伝的な変異を生み、ある程度の遺伝的多様性が見られたり、数百 km の範囲を調べると、遺伝的に異なる複数の集団が見られる種もあることが分かっていました。そこで我々は、市民や研究者からも高い関心がもたれている日本のヤマコウバシについて、これがどのタイプに当てはまるのか、またどのように集団を維持しているのかを明らかにすべく研究に着手しました。</p>
<p>研究の内容</p>	<p>我々のチームは、ヤマコウバシの親木や種子のサンプルを東北（宮城）から九州（熊本）にかけて集め、東北大学と大阪府立大学で所有する遺伝子の塩基配列を高速に読み出せる装置である次世代シーケンサーを駆使することで、DNA 配列の多数の 1 塩基多型 (SNPs) を検出しました。塩基配列をサンプル間で比較したところ、種子の遺伝子型は母樹のものとの正確なコピーであることだけでなく、日本中のサンプル間で変異がほとんど見られないことが明らかになりました。これは、直線距離にして 1000 km を超える範囲に分布するヤマコウバシが、たった 1 本の雌株から生じた巨大なクローンであることを意味します。これほど大規模なクローンは世界的にも珍しく、驚くべき結果です。</p> <p>さらに我々は、ヤマコウバシと近縁の雌雄異株樹種であるアブラチャン、クロモジ、ダンコウバイの 3 種についても同様の調査を行いました。これら 3 種については、日本に雌雄両方の株が生育しています。解析の結果、これらの樹種では自家受精や単為生殖を行わず、近交弱勢が防がれていることが分かりました。このことは、動いて交配相手を探すことができないという雌雄異株植物の不利を補っていると考えられます。</p>
<p>今後の展開と応用について</p>	<p>植物種の多くは 1 株の中におしべとめしべを兼ね備える両性株の生物ですが、中には雄株と雌株に分かれた雌雄異株の種もあります。動いて交配相手を探すことができず不利を抱えているはずの雌雄異株植物が、いかに集団を維持しているのかについては未解明の点が多く残されています。今回の結果から、アブラチャン、クロモジ、ダンコウバイのように、近交弱勢を避けることで不利を補っている植物もあれば、ヤマコウバシのように、雌株が単独で種子生産する有利性を獲得した種もあり、植物の生き様が多様性に富んでいることが示されました。さらに研究を進めることで、生物の世界に性というシステムが広く存在する理由の解明につながる可能性があると考えています。</p> <p>最後に、ヤマコウバシのように遺伝的な多様性が低い集団は、地球温暖化などの環境の変化によって一網打尽にされ絶滅する危険があるため、その生育状況を注意深く見守る必要があります。</p>

‘ヤマコウバシがたった 1 本の雌株から生じた巨大なクローンであることを発見！’. 大阪市立大学.
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Article

Genetic Diversity and Structure of Apomictic and Sexually Reproducing *Lindera* Species (Lauraceae) in Japan

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Abstract: Research Highlights: genetic diversity in populations were compared among related shrub species with different reproductive systems. Background and Objectives: *Lindera* species are dioecious trees or shrubs that produce seeds by mating of males and females. To evaluate the importance of genetic diversity for the persistence of natural populations, we compared genetic information among four *Lindera* species in Japan. Three are dioecious shrubs (*Lindera praecox*, *Lindera umbellata*, and *Lindera obtusiloba*) that produce seeds by sexual reproduction. The remaining species, *Lindera glauca*, reproduces by apomixis; only female plants are found in Japan. Materials and Methods: all four species were sampled across a wide geographic area, from Tohoku to Kyushu, Japan. Single nucleotide polymorphisms (SNPs) were detected by multiplexed ISSR genotyping by sequencing (MIG-seq) and the resulting genetic diversity parameters were compared among populations. Results: in all sexually reproducing species, the values of observed heterozygosity were close to the expected ones and the inbreeding coefficients were nearly 0. These results were supposed to be caused by their obligate outcrossing. The genetic difference increased, in ascending order, between a mother plant and its seeds, within populations, and across geographic space. We observed a substantial geographic component in the genetic structure of these species. For *L. glauca*, the genetic difference between a mother and its seeds, within populations, and across space were not significantly different from what would be expected from PCR errors. Genetic diversity within and among populations of *L. glauca* was extremely low. Conclusions: apomixis has the advantage of being able to found populations from a single individual, without mating, which may outweigh the disadvantages associated with the extremely low genetic diversity of *L. glauca*. This may explain why this species is so widely distributed in Japan. Provided that the current genotypes remain suited to environmental conditions, *L. glauca* may not be constrained by its limited genetic diversity.

Keywords: apomixis; clone; dioecy; geographic scale; MIG-seq; shrub; SNP



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1. Introduction

Genetic diversity allows for environmental adaptation [1,2]. In plants, genetic diversity depends in part on the reproductive system of a given species; outcrossing species tend to be more genetically diverse [3]. Genetic diversity has been found to be higher in dioecious species, which are obligate outcrossers, compared to related cosexual species [4]. However, dioecious plants may have two reproductive disadvantages: pollination limitation because two individuals of opposite sexes are required to reproduce sexually and low colonization ability, because only half of the population contributes to seed dispersal. The seed-shadow handicap, related to reduced dispersal around female plants, typically results in a clumped

distribution at the local scale [5], and potentially also at greater geographic scales. These advantages and disadvantages are reversed in apomictic plants.

Apomixis, the asexual formation of seeds, occurs in over 400 angiosperm genera [6]. Apomixis is considered to be advantageous for population growth and expansion, because populations can be founded by a single individual and there are no pollination limitations [7]. However, because apomictic seeds are produced without meiosis or fertilization, each seed is a genetic replica of the maternal plant unless somatic mutation occurs. Therefore, apomictic plants may have reduced disease resistance and adaptability to environmental changes.

Lindera species (Lauraceae) are dioecious trees or shrubs that produce seeds by mating of males and females. There are over 100 species of *Lindera* in the world, seven of which are native to Japan [8]. Of Japanese *Lindera* species, *Lindera glauca* Bl. occurs in Japan as strictly female plants that reproduce by apomixis. There are remaining questions as to how populations of this species are maintained in Japan. If genetic diversity is exceptionally low, this may support the commonly accepted theory that apomixis is advantageous in the short term due to enhanced colonization ability. However, if *L. glauca* has a similar level of genetic diversity to sexually reproducing species, this would suggest that the trade-off for enhanced colonization is too high, and that there is high pressure to maintain genetic diversity even in apomictic species. Previous research on *Taraxacum officinale* [9] and *Miconia albicans* [7] suggested that different genotypes were maintained in apomictic populations through somatic mutation or meiosis.

The purpose of this study was to evaluate the importance of genetic diversity for the persistence of wild plant populations. For the purpose, we compared the genetic diversity and geographic genetic structure of *L. glauca* to three sexually reproducing congeners, *Lindera praecox* Bl., *Lindera umbellata* Thunb., and *Lindera obtusiloba* Bl., to determine the extent of variation in terms of genotype coincidence between mother plants and seeds, within populations and across geographic space.

2. Materials and Methods

2.1. Focal Species

All four species are dioecious, deciduous forest understory shrubs. *Lindera praecox* is endemic to Japan, while the remaining three species are found on the Asian Continent and the Japanese Archipelago. Pliocene fossils of *L. praecox*, *L. umbellata*, and *L. obtusiloba* have been found in Japan [10], and Pleistocene fossils of *L. glauca* have been found [11]. The species flower in spring, from late February to early May, with each species flowering for 2–4 weeks [8,12]. The flowers, which are borne in pseudo-umbel-shaped inflorescences, are small (4–9 mm in diameter), dish-shaped, yellow or green in color, and unisexual, with sterile rudiments of the other sex [12].

In the past, the seeds of *L. praecox* were used to press oil for lanterns [13]. *Lindera umbellata* extracts had the effects of prevention of onset and progression of nephropathy [14]. *Lindera obtusiloba* has been widely used in a traditional medicine as the anti-allergic inflammatory effect [15]. The fruits of *L. glauca* contain some aromatic essential oils, alkaloids, terpenoids, and flavonoids, which are widely employed in the essence, medicine, and chemical industries [16].

The sex ratio of populations of *L. praecox*, *L. umbellata*, and *L. obtusiloba* is approximately 1:1 in forests in western Japan [12]. Both male and female flowers attract insect pollinators, primarily Diptera and Coleoptera [8]. Regarding *L. glauca*, only female plants are found in Japan, but both males and females are found on the Asian Continent [17,18]. The sex ratio of *L. glauca* in the wild in mainland China is estimated to be predominantly female [19]. Dupont [17] conducted bagging experiments to clarify whether or not pollination was necessary for seed set of *Lindera* species. She covered flower buds on branches of female plants of *L. umbellata*, *L. obtusiloba*, and *L. glauca* with nylon meshes to keep pollen out. The results for *L. umbellata* and *L. obtusiloba* showed that pollination was essential for their seed set. On the other hand, females of *L. glauca* produced seeds without pollination

or male flowers, indicating apomixis. For some woody plants, clonal growth arises by wide-ranging roots that produce new shoots, termed stolon. However, the four *Lindera* species do not reproduce laterally by stolons. Hence, stems derived from an individual can easily be identified.

2.2. Field Collections and DNA Extraction

We collected leaves from 105 individuals of *L. glauca* in natural vegetations at 29 locations, representing the entirety of its distribution in Japan (Supplementary Material Table S1). We tried to collect up to five individuals from one location, but in some locations, we could only find one. As a result, leaves were taken from one to five individuals per location. From six locations among the 29 locations, i.e., Takaragaike, Kasugayama, Myokensan, Shigisan, Okayama-shi, and Umamiyama, five seeds from each selected mature individual were taken (Supplementary Material Table S2). For *L. praecox*, we collected leaves from 45 individuals (one to five individuals per location) in natural vegetations at 11 locations (Table S1). From two locations, among the 11 locations, i.e., Mitarai Valley and Tsuno, six to twelve seeds were collected from each of six mature individuals (Table S2). For *L. umbellata*, we collected leaves from 27 individuals at six locations on Honshu Island (two–five individuals per location) (Table S1). From a mature individual among six locations, i.e., Mitarai Valley, five seeds were collected (Table S2). For *L. obtusiloba*, we collected leaves from 39 individuals at eight locations on Honshu, Shikoku, and Tsushima Islands (four–five individuals per location) (Table S1). From two individuals at Mitarai Valley, 11 and 12 seeds were collected (Table S2). We recorded the geographic location, i.e., latitude and longitude, and diameter at breast height (DBH; 1.3 m) of each sampled individual (Table S1).

DNA was extracted from leaves and seed cotyledons for each species using a DNA extraction kit (DNeasy Plant Mini Kit; QIAGEN, Tokyo, Japan) or a DNA extraction buffer (UniversAll™ Extraction Buffer II; Yeastern Biotech, New Taipei City, Taiwan).

2.3. Multiplexed ISSR Genotyping by Sequencing (MIG-Seq) Analysis

DNA sequences were subjected to MIG-seq analysis [20] and single nucleotide polymorphisms (SNPs) were identified. In MIG-seq analysis, the region between single sequence repeats (SSRs), i.e., the inter-SSR (ISSR), is amplified by polymerase chain reaction (PCR). Approximately 1.5 ng/μL of DNA was used for the first PCR, as template DNA. The concentration of the DNA solution was measured using a Quantus™ Fluorometer (Promega, Madison, WI, USA), and the concentration of the template DNA solution was adjusted by adding sterile water.

For the first PCR, a mixture of primers was prepared from eight types of forward and reverse primers intended to amplify the ISSR region. The fragments were amplified with a Multiplex PCR Assay Kit Ver. 2 (TaKaRa Bio Inc., Shiga, Japan) using 7 μL reaction volumes containing 1.0 μL of template DNA, 1.4 μL of PCR primers (mix of primers, each at a concentration of 2.0 μM), 3.5 μL of 2 × Multiplex PCR Buffer, 0.035 μL of Multiplex PCR Enzyme Mix and 1.065 μL of water. The PCR cycling conditions used for the Verti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) were as follows: 94 °C for 1 min followed by 27 cycles of 94 °C for 30 s, 38 °C for 1 min, 72 °C for 1 min, and finally 72 °C for 10 min.

The second PCR used the products of the first PCR diluted to a concentration of 1:50 by adding sterile water. The second PCR was performed to add complementary sequences as an index to both ends of the first PCR products to identify individuals. The fragments were amplified with PrimeSTAR GXL DNA polymerase (TaKaRa Bio Inc., Shiga, Japan) using 12 μL reaction volumes containing 2.5 μL of diluted first PCR products as template DNA, 2.4 μL of second PCR primers (mix of primers, each at a concentration of 2.0 μM), 2.4 μL of 5 × PrimeSTAR Buffer, 0.96 μL of dNTP mixture, 0.24 μL of PrimeSTAR GXL polymerase, and 3.5 μL of water. The PCR cycling conditions were as follows: 12 cycles of 98 °C for 10 s, 54 °C for 15 s, and 68 °C for 1 min. After purification of the second

PCR products with AMPure XP magnetic beads, only 300–800 bp fragments with a size suitable for sequencing were selected by the Blue Pippin DNA Size Selection System (Sage Science, Beverly, MA, USA). Tape Station 4200 (Agilent Technologies, Santa Clara, CA, USA) and Qubit fluorometer (Life Technology, Carlsbad, CA, USA) were used to measure the frequency distribution of fragment lengths and the amount of fragments. Prepared to a concentration of 12 pM, the DNA library was used for sequencing on an Illumina MiSeq sequencing platform using a MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA). The sequencing results were input into Stacks v.1.47 [21] to identify loci and detect SNPs. Following Catchen et al. [22], we used “ustacks” for building loci, “cstacks” for creating the loci catalogue for all samples, and “sstacks” for checking against the catalog. Finally, the “population” module was used to estimate a “population genetics statistic” from a population of individual samples.

2.4. Data Analyses

Only loci with more than 50% of the population and less than 60% heterozygosity were extracted. For each species, the number of alleles (N_a), number of effective alleles (N_e), observed heterozygosity (H_o), expected heterozygosity (H_e), and an inbreeding coefficient (F_{IS}) were estimated using GenAlEx v.6.5 [23]. Genotypes, including those from seed samples, were compared to identify clones and to assess genotype diversity for each species.

We calculated the proportion of different alleles on each SNP loci as follows: if individuals shared an identical genotype (e.g., A/T and A/T), the proportion of different alleles was 0; if one allele differed between individuals (e.g., A/T and A/G), it was 0.5; and if the genotypes differed completely (e.g., A/T and G/C), it was 1. Once all loci had been assessed, the mean proportion of different alleles was defined as the genetic difference between individuals. To investigate the extent to which genetic variation occurs in apomictic seed production relative to sexual reproduction, the genetic difference between mother plants and their seeds was determined. We then determined the genetic difference between grown-up individuals to assess genotype diversity within a population by pairings limited to individuals growing in the same location. For the assessment the genotype diversity across the entire study area, the genetic difference was calculated for all pairs of grown-up individuals after combining all individuals sampled from all locations into a single pool. We estimated the possibility that pseudo-variant loci occurred originating from PCR errors using the genotype data from pairs of replicates of the same individual. We performed PCR amplification and genotyping twice for seven individuals of *L. glauca* and six individuals of *L. praecox*, *L. umbellata*, and *L. obtusifolia*. We defined the pseudo-variant rate as the genetic difference between replicates of the same individual.

To examine the distribution of genetic variation within and among studied locations and to explore genetic relationships among individuals and geographic genetic structure, pairwise individual-by-individual genetic distance matrixes were calculated for codominant data [24] using GenAlEx v.6.5. To deal with missing data, we selected the option “interpolate missing”. Based on the matrixes, we conducted analysis of molecular variance (AMOVA) using the R package “pegas” v.0.14 [25] and a principal coordinate analysis (PCoA) to understand the genetic relationships among individuals and among locations in detail using the R package “vegan” v.2.5 [26]. To evaluate the relationship between geographic distance and genetic divergence, we calculated a fixation index (F_{ST}) value between populations, and assessed the correlation between F_{ST} and the geographic distance between populations using the R package “ape” v.5.4 [27].

3. Results

3.1. Genetic Diversity Parameters

For *L. glauca*, we obtained 246 SNP loci from 147 samples, including 105 individuals, replicates of seven individuals and 35 seeds, taken from 29 populations. The mean N_a and N_e were 0.903 and 0.894, respectively. H_e varied from 0.000 to 0.018, whereas H_o varied

from 0.000 to 0.027. F_{IS} was negative excluding Hagi (HG) (range: -0.760 to 0.429 ; mean = -0.441) (Table 1).

Table 1. Genetic diversity parameters of the 29 populations of *Lindera glauca* (Lauraceae) in Japan based on 246 single nucleotide polymorphism (SNP) loci. Letters in parentheses are abbreviations of sampling location.

Sampling Location	<i>N</i>	<i>N_a</i>	<i>N_e</i>	<i>H_e</i>	<i>H_o</i>	<i>F_{IS}</i>
Sendai, Miyagi (SD)	1	0.687	0.687	0.004	0.008	-
Hachioji, Tokyo (HT)	2	0.796	0.792	0.012	0.021	-0.760
Sagamihara, Kanagawa (SH)	2	0.936	0.927	0.012	0.019	-0.538
Kawasaki, Kanagawa (KW)	3	0.909	0.896	0.01	0.014	-0.401
Nagano, Nagano (NG)	5	1.023	1.005	0.014	0.021	-0.504
Kasugai, Aichi (I)	5	1.023	1.009	0.012	0.018	-0.461
Aioiyama, Aichi (A)	5	1.034	1.021	0.014	0.02	-0.381
Hino, Shiga (HN)	3	0.002	0.003	0.01	0.018	-0.749
Takaragaike, Kyoto (T)	5	1.023	1.017	0.013	0.018	-0.328
Kasugayama, Nara (K)	5	1.019	1.008	0.011	0.018	-0.598
Myokensan, Osaka (M)	5	1.038	1.029	0.018	0.027	-0.499
Shigisan, Osaka (S)	5	1.026	1.020	0.011	0.016	-0.434
Takedao, Hyogo (TK)	4	0.996	0.981	0.014	0.02	-0.496
Okayamashi, Okayama (O)	5	1.000	0.995	0.008	0.012	-0.578
Hiroshimashi, Hiroshima (H)	4	0.909	0.900	0.01	0.016	-0.665
Masuda, Shimane (MS)	5	1.038	1.022	0.016	0.019	-0.222
Tsuwano, Shimane (TW)	2	0.977	0.972	0.006	0.009	-0.527
Izumo, Shimane (IZ)	5	1.038	1.023	0.013	0.017	-0.263
Yamaguchishi, Yamaguchi (YG)	5	1.023	1.012	0.007	0.01	-0.360
Hagi, Yamaguchi (HG)	2	0.962	0.961	0.003	0.002	0.429
Bizan, Tokushima (B)	3	1.019	1.015	0.012	0.019	-0.565
Iinoyama, Kagawa (IN)	5	0.985	0.961	0.014	0.019	-0.391
Shiunzan, Kagawa (SU)	5	1.049	1.021	0.018	0.024	-0.306
Tsuno, Kochi (TN)	3	0.887	0.881	0.009	0.015	-0.618
Sameura, Kochi (SM)	5	1.038	1.018	0.011	0.016	-0.377
Tsushima, Nagasaki (TS)	1	0.596	0.596	0.008	0.015	-
Umamiyama, Fukuoka (UM)	3	0.898	0.886	0.011	0.016	-0.435
Sasga, Saga (SA)	1	0.811	0.811	0.008	0.015	-
Sannotake, Kumamoto (SN)	1	0.457	0.457	0	0	-
Regional mean		0.903	0.894	0.011	0.016	-0.441

N, number of individuals; *N_a*, number of observed alleles; *N_e*, effective number of alleles; *H_e*, expected heterozygosity; *H_o*, observed heterozygosity; *F_{IS}*: inbreeding coefficient.

For *L. praecox*, we obtained 355 SNP loci from 114 samples, including 45 individuals, replicates of six individuals and 63 seeds, taken from 11 populations. The mean *N_a* and *N_e* were 1.025 and 0.968, respectively. *H_e* ranged from 0.008 to 0.075, whereas *H_o* ranged from 0.016 to 0.070. *F_{IS}* was positive in all populations excluding Hiroshima (H) (range: -0.223 to 0.151 ; mean = 0.057) (Table 2).

For *L. umbellata*, we obtained 280 SNP loci from 38 samples, including 27 individuals, replicates of six individuals and five seeds, taken from six populations. The mean *N_a* and *N_e* were 1.210 and 1.115, respectively. *H_e* ranged from 0.056 to 0.096, whereas *H_o* ranged from 0.075 to 0.114. *F_{IS}* was negative in all populations excluding Hino (HN) and Myokensan (M) (range: -0.356 to 0.020 ; mean = -0.142) (Table 3).

Table 2. Genetic diversity parameters of the 11 populations of *Lindera praecox* (Lauraceae) in Japan based on 355 SNP loci. Letters in parentheses are abbreviations of sampling location.

Sampling Location	N	Na	Ne	He	Ho	F _{IS}
Sendai, Miyagi (SD)	1	0.402	0.402	0.008	0.016	-
Tsukubasan, Ibaraki (TB)	5	1.136	1.064	0.063	0.056	0.108
Hachioji, Tokyo (HT)	1	0.786	0.786	0.022	0.043	-
Nagano, Nagano (NG)	5	1.125	1.059	0.059	0.054	0.094
Hino, Shiga (HN)	5	1.146	1.073	0.070	0.070	0.010
Mitarai, Nara (MT)	5	1.164	1.083	0.061	0.057	0.072
Kongosan, Osaka (KG)	5	1.195	1.114	0.075	0.068	0.094
Takedao, Hyogo (TK)	5	1.160	1.072	0.062	0.052	0.151
Hiroshimashi, Hiroshima (H)	3	0.996	0.960	0.043	0.052	-0.223
Tsuno, Kochi (TN)	5	1.094	1.031	0.055	0.047	0.140
Umamiyama, Fukuoka (UM)	5	1.070	1.009	0.054	0.050	0.071
Regional mean		1.025	0.968	0.052	0.051	0.057

N, number of individuals; Na, number of observed alleles; Ne, effective number of alleles; He, expected heterozygosity; Ho, observed heterozygosity; F_{IS}: inbreeding coefficient.

Table 3. Genetic diversity parameters of the six populations of *Lindera umbellata* (Lauraceae) in Japan based on 280 SNP loci. Letters in parentheses are abbreviations of sampling location.

Sampling Location	N	Na	Ne	He	Ho	F _{IS}
Sendai, Miyagi (SD)	5	1.120	1.102	0.071	0.089	-0.252
Tsukubasan, Ibaraki (TB)	5	1.234	1.142	0.096	0.114	-0.183
Hino, Shiga (HN)	5	1.268	1.133	0.090	0.088	0.020
Mitarai, Nara (MT)	5	1.204	1.109	0.080	0.087	-0.089
Myokensan, Osaka (M)	5	1.257	1.138	0.084	0.084	0.006
Takedao, Hyogo (TK)	2	1.102	1.069	0.056	0.075	-0.356
Regional mean		1.210	1.115	0.079	0.089	-0.142

N, number of individuals; Na, number of observed alleles; Ne, effective number of alleles; He, expected heterozygosity; Ho, observed heterozygosity; F_{IS}: inbreeding coefficient.

For *L. obtusiloba*, 688 SNP loci were obtained from 68 samples, including 39 individuals, replicates of six individuals and 23 seeds, taken from eight populations. The mean Na and Ne were 1.227 and 1.124, respectively. He ranged from 0.071 to 0.097, whereas Ho ranged from 0.073 to 0.084. F_{IS} was close to zero in all population (range: -0.087 to 0.149; mean = 0.022) (Table 4).

Table 4. Genetic diversity parameters of the eight populations of *Lindera obtusiloba* (Lauraceae) in Japan based on 688 SNP loci. Letters in parentheses are abbreviations of sampling location.

Sampling Location	N	Na	Ne	He	Ho	F _{IS}
Nagano, Nagano (NG)	5	1.268	1.14	0.097	0.083	0.149
Hino, Shiga (HN)	5	1.233	1.133	0.085	0.083	0.027
Mitarai, Nara (MT)	5	1.203	1.108	0.078	0.084	-0.078
Myokensan, Osaka (M)	5	1.248	1.134	0.084	0.073	0.133
Kongosan, Osaka (KG)	5	1.264	1.145	0.093	0.084	0.095
Takedao, Hyogo (TK)	5	1.249	1.138	0.085	0.084	0.008
Iinoyama, Kagawa (IN)	4	1.177	1.103	0.072	0.079	-0.087
Tsushima, Nagasaki (TS)	5	1.172	1.095	0.071	0.076	-0.070
Regional mean		1.227	1.124	0.083	0.081	0.022

N, number of individuals; Na, number of observed alleles; Ne, effective number of alleles; He, expected heterozygosity; Ho, observed heterozygosity; F_{IS}: inbreeding coefficient.

3.2. Clone Identification and Genotype Diversity

For *L. praecox*, *L. umbellata*, and *L. obtusiloba*, pseudo-variant rates ranged from 0.005 to 0.013, 0.005–0.012, and 0.004–0.070, respectively (Figure 1a–c). Pseudo-variant rates in the seven pairs of replicated *L. glauca* samples ranged from 0.004 to 0.017 (Figure 1d).

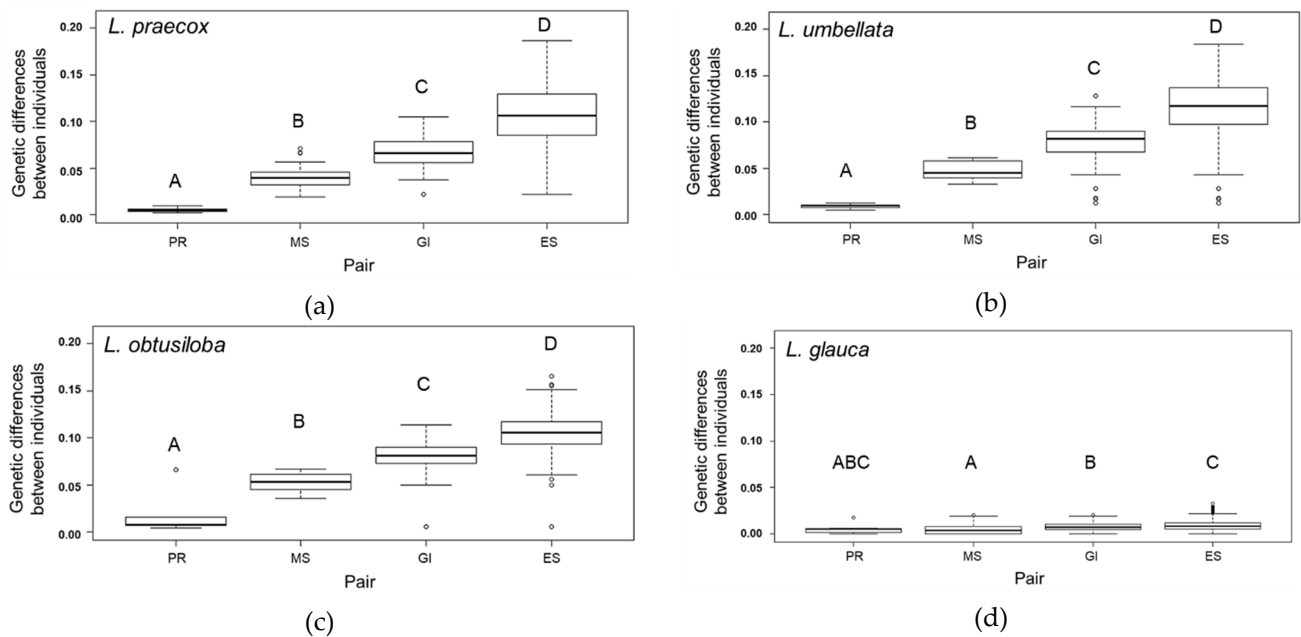


Figure 1. Boxplot of the genetic difference between individuals of (a) *Lindera praecox*, (b) *L. umbellata*, (c) *L. obtusiloba*, and (d) *L. glauca* (Lauraceae). The box represents the interquartile range (25th–75th percentiles); while the line across the box indicates the median. Whiskers extend to the 1.5-fold of the interquartile range. The circles indicate outliers. Values denoted by the same letters are not significantly different from each other at $p < 0.05$ (Kruskal–Wallis test and Wilcoxon test with sequential Bonferroni correction). PR; the possibility that pseudo-variant loci occurred originating from PCR errors, MS; the genetic difference between mother plants and their seeds, GI; the genetic difference between grown-up individuals by pairings individuals growing in the same location, and ES; the genetic difference calculated for all pairs of grown-up individuals after combining all individuals sampled from all locations into a single pool.

For *L. praecox*, *L. umbellata*, and *L. obtusiloba*, the genetic difference between a mother plant and its seeds, the genetic difference within populations, and the genetic difference across the entire study area were significantly greater than would be expected from pseudo-variant rate ($p < 0.05$; Kruskal–Wallis test and Wilcoxon test with sequential Bonferroni correction; Figure 1). Additionally, the genetic differences were significantly different among these three scales ($p < 0.05$; Wilcoxon test with sequential Bonferroni correction; Figure 1a–c). For *L. glauca*, the genetic difference at these three scales was not significantly different than what would be expected from pseudo-variant rates ($p > 0.05$, Kruskal–Wallis test and Wilcoxon test with sequential Bonferroni correction; Figure 1d). However, the genetic difference among the three scales was significantly different from each other ($p < 0.05$; Wilcoxon test with sequential Bonferroni correction; Figure 1d).

3.3. Genetic Structure

Results of AMOVA indicated that genetic variation within populations was greater than among populations for *L. praecox*, at 56% and 44%, respectively (Table 5). For *L. umbellata*, genetic variation was greater within populations (53%), followed by among population (47%). Genetic variation among populations of *L. obtusiloba* (76%) was substantially larger than within populations (24%). For *L. glauca*, geographic differences contributed to only 9% of the genetic variation within this species.

Table 5. Analysis of Molecular Variance (AMOVA) results for the studied four *Lindera* populations.

	Source of Variation	df	Sum of Squares	Variance Components	%
(A)	<i>L. praecox</i>				
	Among population	10	427,635.500	8034.000	44
	Within population	34	352,842.900	10,378.000	56
	Total	44	780,478.400	18,412.000	
(B)	<i>L. umbellata</i>				
	Among population	5	57,977.820	2084.700	47
	Within population	21	48,930.350	2330.000	53
	Total	26	106,908.170	4414.700	
(C)	<i>L. obtusiloba</i>				
	Among population	7	477,950.100	9319.100	76
	Within population	31	709,215.400	2877.900	24
	Total	38	1,187,165.500	12,197.000	
(D)	<i>L. glauca</i>				
	Among population	28	1005.172	2.706	9
	Within population	76	2007.043	26.066	91
	Total	104	3012.215	28.772	

Pairwise Mantel tests for *L. praecox* (Figure 2a), F_{ST} and distance were positively correlated ($p = 0.025$). For *L. umbellata* (Figure 2b) indicated no statistically significant correlation between F_{ST} and the geographic distance between populations ($p = 0.897$). *Lindera obtusiloba* and *L. glauca* (Figure 2c,d) also showed F_{ST} and distance were positively correlated ($p = 0.023$ and 0.010 , respectively).

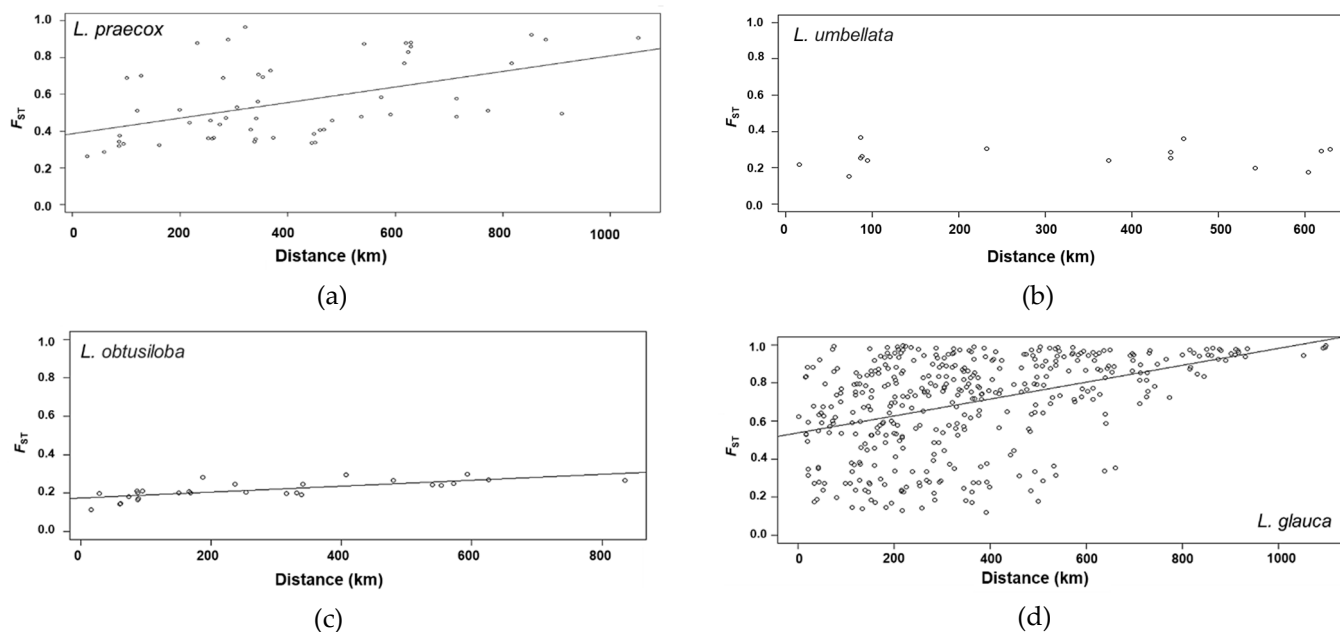


Figure 2. Mantel test for the correlation between F_{ST} and geographic distance among populations of (a) *L. praecox* ($p = 0.025$), (b) *L. umbellata* ($p = 0.897$), (c) *L. obtusiloba* ($p = 0.023$), and (d) *L. glauca* ($p = 0.010$).

The PCoA of *L. praecox* and *L. obtusiloba* appears to have genetically distinct variation within their range from east to west (Figure 3a,c), and geographic genetic differentiation of *L. umbellata* was clear but its pattern was complex (Figure 3b). The PCoA plot of *L. glauca* showed no clear differentiation in genetic composition throughout its geographic range in Japan (Figure 3d).

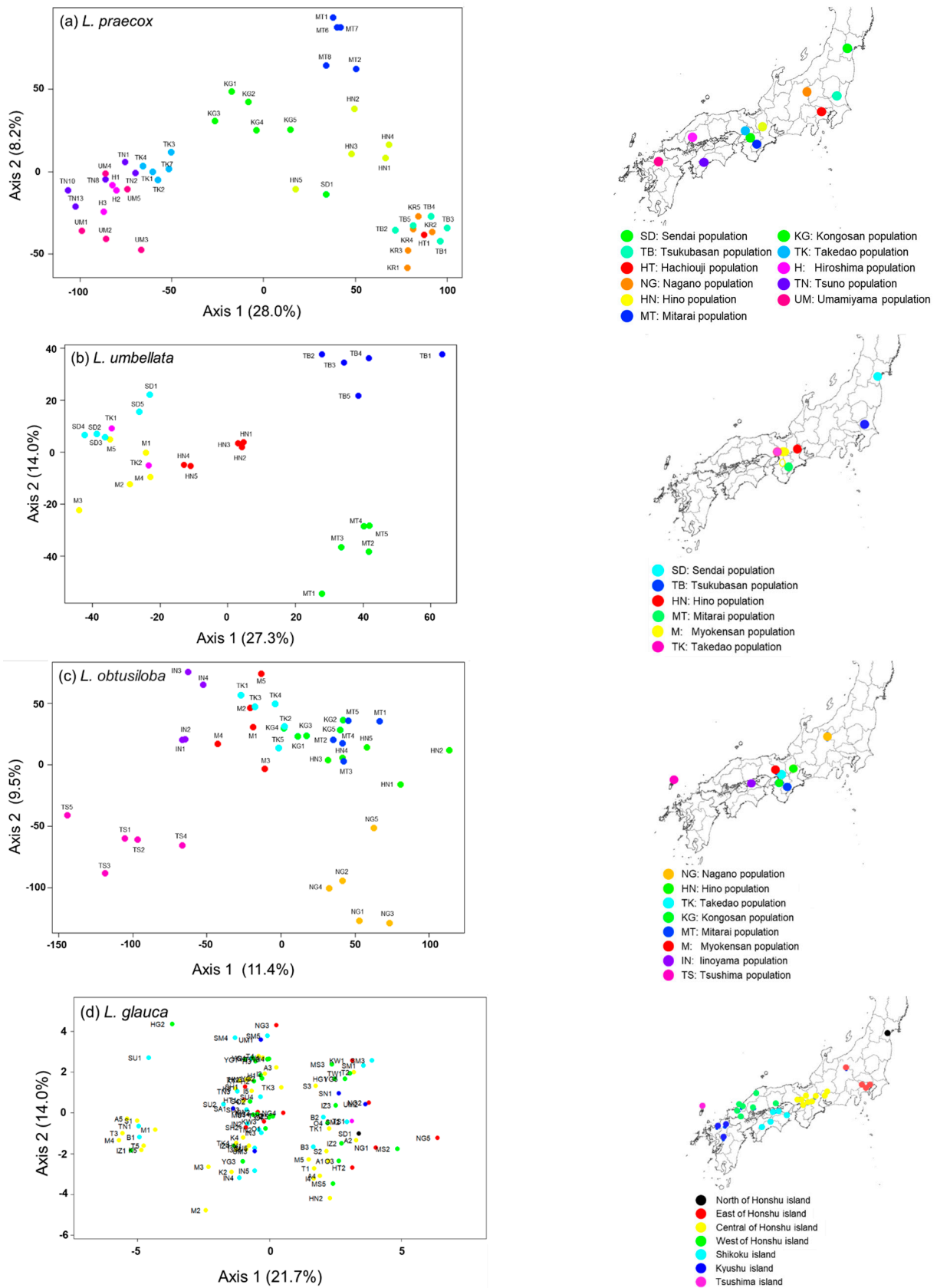


Figure 3. Principal coordinate analysis (PCoA) plot of (a) 45 individuals of *L. praecox*, (b) 27 individuals of *L. umbellata*, (c) 39 individuals of *L. obtusiloba*, and (d) 105 individuals of *L. glauca*. Percentages of total variation explained by each coordinate are shown in parentheses. The marks on the map indicate the location of each population.

4. Discussion

A key advantage of MIG-seq is that it can provide putative neutral loci [28], which are useful for analyses of genetic structure. Therefore, this approach has been used recently in population genetics (e.g., [29,30]).

Obligate outcrossing species is expected to have the values of H_o close to those of H_e as reported by Ye et al. [31] for *L. obtusiloba*. For the genetic diversity of the dioecious tree species *Phoebe zhennan* (Lauraceae), H_o was higher than H_e ($H_o = 0.243$, $H_e = 0.145$), indicating low inbreeding [32]. Our results are similar to those of previous studies. This may be an advantage of dioecy, which avoids inbreeding. Our study results also suggest that dioecious plants have greater heterozygosity than cosexual plants, based on a comparison of our F_{IS} values with previously published estimates based on the genome-wide SNPs data (Tables 2–4), 0.035–0.091 (*Rhododendron japonoheptamerum*) [29]; 0.05–0.19 (*Platycladus orientalis*) [33]; 0.05 (*Thuja koraiensis*) [33]; 0.051–0.754 (*Tectona grandis*) [30]). However, the level of heterozygosity is influenced not only by reproductive system of the focal species, but also other factors, e.g., population size [34], age of individuals [35]. Thus, careful verification is needed.

The all-female populations of *L. glauca* assessed here showed higher heterozygosity than the other three *Lindera* species, on average, and exhibited negative F_{IS} values at all sites except for one site. These results are similar to a previous study that employed nuclear microsatellite analyses [18]. Zhu et al. [18] suggested that seven Japanese populations of *L. glauca* exhibited greater heterozygosity than what was observed across a large range of populations in China. For an apomictic lineage, the high and well-preserved heterozygosity in *L. glauca* populations in Japan may attribute to asexual (apomictic) reproduction as reported for *Prunus avium* [36], due to the absence of sexual recombination and/or the lack of accumulation of somatic mutations over time.

We found evidence of genetic differentiation among populations of *L. praecox*, *L. umbellata*, and *L. obtusiloba* as has often been reported for other plant species [37–39]. First, AMOVA results indicated that regional variation explained a large proportion of the total variation (Table 5). Second, the PCoA results indicated geographic divisions in populations. Finally, we found that populations that were further apart in space were more genetically different in *L. praecox* and *L. obtusiloba* (Figure 2a,c). Collectively, these results reflect geographically limited gene flow, a varied history of population expansion, or strong adaptation to local environmental conditions.

By contrast, populations of *L. glauca* had substantially lower genetic variation than these three species. Even in apomictic plants, genetic diversity can occur in the process of seed production by incomplete meiosis and/or recombination [40]. In the case of *L. glauca* in Japan, the comparison of multi-level genetic distance and PCR error rates indicated no significant differences for any level, which suggests that seeds were exact copies of their mothers, as the genetic difference between mothers and seeds were within the range of PCR errors (Figure 1d). A seed production process that does not produce mutations would lead genetically uniform populations in Japan.

Here, we compare our own results in Japan with available results from China. *Lindera obtusiloba* produces seeds by sexual mating both in China and Japan. Genetically distinct populations in different regions were observed in China [31]. Our results are similar to those of the previous study. On the other hand, the situation is different for *L. glauca*. Both females and males of *Lindera glauca* exist in China, and they reproduce sexually. A variety of genotypes existed in different regions and geographic genetic structure was observed in China [18]. While in Japan, seed production by apomixis resulted in an extremely uniform genetic structure. These results of comparisons suggest that reproductive system has a strong influence on genetic structure of plant populations.

We were surprised that virtually no genetic variation was observed in *L. glauca* across its entire geographic range in Japan. The genetic difference between individuals in the whole range of Japan was not significantly different from PCR errors (Figure 1d). In addition, we found significant but weak correlation between genetic and geographic

distance (Figure 2d), and the PCoA results indicated unclear geographic distinctions among populations (Figure 3d). These are contrast to the previous studies which detected a clear geographic genetic structure of apomictic plants, *Miconia albicans* [7] and *Taraxacum officinale* [9]. These results suggested that a population of *L. glauca* in Japan might be a huge clone. Bricker et al. [41] reported that a dioecious aquatic plant, *Thalassia testudinum*, formed a “mega-clone” that spread over nearly 50 km by vegetative fragments. The European populations of *Gagea spathacea* (Liliaceae) were dominated by a single “mega-clone” spreading all over Central Europe [42]. Similarly, the *L. glauca* population in Japan could also be considered as a “mega-clone” that spread out over at least 1100 km in distance from Sendai to Kumamoto. There are two possible reasons for the formation of genetically uniform populations. First, Japanese populations of *L. glauca* might be founded by one asexually reproducing female as suggested by Dupont [17]. Second, population of *L. glauca* in Japan may still be young on the geological time scale. The population of *L. glauca* has been in Japan since prehistoric times because Pleistocene fossils of *L. glauca* have been found [11]. Genetic diversification via accumulated mutations has not yet occurred, although it may occur in the future. However, there were significant dissimilarities in the degree of the genetic difference between a mother plant and its seeds, between individuals in a population, and between individuals in the entire study area (Figure 1d). The result suggested that slight mutations may have occurred and accumulated.

Although *L. glauca* has been present in Japan since the Pleistocene (0.01–2.58 million years ago (Ma)) [11], the genetic diversity of this species has remained at a fairly low level until now. According to Xiong et al. [19], wild *L. glauca* in mainland China have a highly skewed sex ratio with females predominating, and their genetic diversity is thought to be maintained by sexual reproduction with a small number of male plants. It is not clear whether Japanese *L. glauca* can cross with the Chinese males. However, if they can, immigration of males may increase genetic diversity as well as the Chinese *L. glauca*.

Finally, the loss of genetic diversity relates to an increase in extinction risk [43]. Despite this, populations with extremely low genetic diversity have been reported worldwide [42,44]. In the apomictic *L. glauca*, the advantages of population establishment or expansion by a single individual, without mating, may outweigh the disadvantages associated with the extremely low genetic diversity. If environmental conditions remain suitable for the extant genotype, genetic diversity may not be required to maintain these populations, at least in the short term.

5. Conclusions

The three species that produce seeds by sexual reproduction, *Lindera praecox*, *L. umbellata*, and *L. obtusiloba* had lower values for inbreeding coefficient, indicating that there is indeed an effect of dioecy. Genetic variations were also found both at the local scale and the geographic scale. It is possible that they have higher genetic diversity than cosexual plants, and the effect of inbreeding depression may be suppressed. On the other hand, in *L. glauca*, the advantage of being able to found populations from single individual, without mating, may outweigh the disadvantages associated with extremely low genetic diversity. This may explain why this species is so widely distributed in Japan. Provided that the current genotypes remain suited to environmental conditions, *L. glauca* may not be constrained by its limited genetic diversity.

Supplementary Materials: The following are available online at <https://www.mdpi.com/1999-4907/12/2/227/s1>, Table S1; sampling locations and sample size of leaf collection of the four *Lindera* species, Table S2; sampling locations and sample size of seeds of the four *Lindera* species.

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