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Research Article

Detecting the Early Genetic Effects of Habitat Degradation in Small Size Remnant Populations of *Machilus thunbergii* Sieb. et Zucc. (Lauraceae)

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Habitat degradation caused by human activities has reduced the sizes of many plant populations worldwide, generally with negative genetic impacts. However, detecting such impacts in tree species is not easy because trees have long life spans. *Machilus thunbergii* Sieb. et Zucc. (Lauraceae) is a dominant tree species of broad-leaved evergreen forests distributed primarily along the Japanese coast. Inland habitats for this species have become degraded by human activities. To investigate the effects of habitat degradation on genetic structure, we compared the genetic diversities of mature and juvenile trees of five *M. thunbergii* populations around Lake Biwa in Japan. Allelic diversity was influenced by past lineage admixture events, but the effects of forest size were not clear. On the other hand, the inbreeding coefficient of the juvenile stage was higher in small populations, whereas large populations maintained panmictic breeding. Also, the extent of genetic differentiation was greater in juveniles than in mature trees. We detected the early genetic effects of habitat degradation in small, isolated *M. thunbergii* populations, indicating that habitat degradation increases inbreeding and genetic differentiation between populations.

1. Introduction

Warm-temperate evergreen forests in East Asia occur primarily at low elevations and feature the dominant genera *Machilus (Persea)*, *Castanopsis*, *Quercus*, *Lithocarpus*, *Cinnamomum,* and *Neolitsea* [1]. However, in the main island of Japan, such forests are highly degraded because of human disturbance. Generally, habitat degradation caused by humans has reduced the sizes and increased the spatial isolation of many plant populations worldwide. Small population size and increased isolation can cause genetic erosion, increased genetic divergence via random drift, increased inbreeding, reduced gene flow, disrupted pollination, and increased probability of local extinction [2–4]. Genetic erosion caused by habitat fragmentation can be of immediate concern if genetic changes directly influence individual fitness and the short-term viability of remnant populations. One long-term evolutionary consequence of genetic erosion is limitation of the ability to respond to environmental changes, which is expected to increase the probability of extinction [2, 4]. Recent meta-analyses suggested that plant species generally exhibit negative genetic responses to habitat fragmentation [5, 6]. However, detecting the impact of habitat fragmentation on the genetic structures of tree species is not easy [7]. Many tree species may be buffered from the effects of such fragmentation by individual longevity, high intrapopulation genetic diversity, and the potential for long-distance pollen flow. Thus, several earlier studies of trees found no evidence that fragmentation influenced genetic parameters (e.g., [8, 9]). In addition, any reduction in genetic diversity or increase in the level of inbreeding may not be immediately evident after forest fragmentation because the older trees remaining after

habitat isolation often have the same extent of genetic diversity as that observed prior to fragmentation [7]. Therefore, assessment of only mature members of a population may yield no information on the contemporary genetic effects of isolation. One possible approach to study of the impact of forest fragmentation on the genetic diversity of tree species is to compare the genetic diversity and structure between predisturbance adult populations and postdisturbance generations cohorts [10]. Such comparisons can reveal whether significant, potentially deleterious genetic changes are occurring in the present generation (e.g., [10–13]).

Lake Biwa is the largest freshwater lake in Japan and is located in the west-central region of the country. The lakeside hosts many coastal plants, including *Calystegia soldanella* (L.) R. Br. (Convolvulaceae), *Vitex trifolia* subsp. *litoralis* Steenis, *Lathyrus japonicus* Willd. (Leguminosae), *Arabidopsis kamchatica* subsp. *kawasakiana* (Makino) K. Shimizu & Kudoh (Brassicaceae), *Raphanus raphanistrum* subsp. *sativus* (L.) Domin, *Dianthus japonicus* Thunb. (Caryophyllaceae), and *Pinus thunbergii* Parl. (Pinaceae) [14]. *Machilus thunbergii* Sieb. et Zucc. (Lauraceae) is a broad-leaved evergreen tree species in warm-temperate forests of Japan and also distributed around Lake Biwa. These populations are important because this species is rarely found inland and regarded as a flagship species around Lake Biwa [15]. However, these *M. thunbergii* populations are heavily degraded because their distribution overlaps areas of human activity [16]. As a result of such disturbance, *M. thunbergii* forests around Lake Biwa now occur almost exclusively around Shinto shrines or on islands in the lake. Therefore, some *M. thunbergii* populations around the lake are expected to exhibit low levels of genetic diversity, high levels of inbreeding, and high extents of genetic differentiation.

The objectives of the present study were to analyze and to assess the effects of habitat degradation on local genetic diversity and differentiation of *M. thunbergii* populations that persist around Lake Biwa. We compared the genetic diversities of mature and juvenile *M. thunbergii* in each population, using seven microsatellite markers and evaluated the extent of genetic divergence among populations.

2. Materials and Methods

2.1. Study Species. *Machilus thunbergii* Sieb. et Zucc. is a representative dominant tree species of warm-temperate evergreen forests in Japan. It is distributed mainly around the sea coast, and over 90% of all trees are distributed along the shore [17, 18]. The tree grows to approximately 15 m and blooms from April to June. Bees, flies, and beetles visit flowers and disperse pollen [19]. *Machilus thunbergii* is a heterodichogamous species, consisting of two types of protogynous and bisexual flowers at the individual-tree level: a morning female-afternoon male morph and a morning male-afternoon female morph [20]. Seeds generally mature from June to August [21, 22] and are dispersed by birds, mainly the brown-eared bulbul (*Hypsipetes amaurotis* Temminck) and the white-cheeked starling (*Sturnus cineraceus* Temminck).

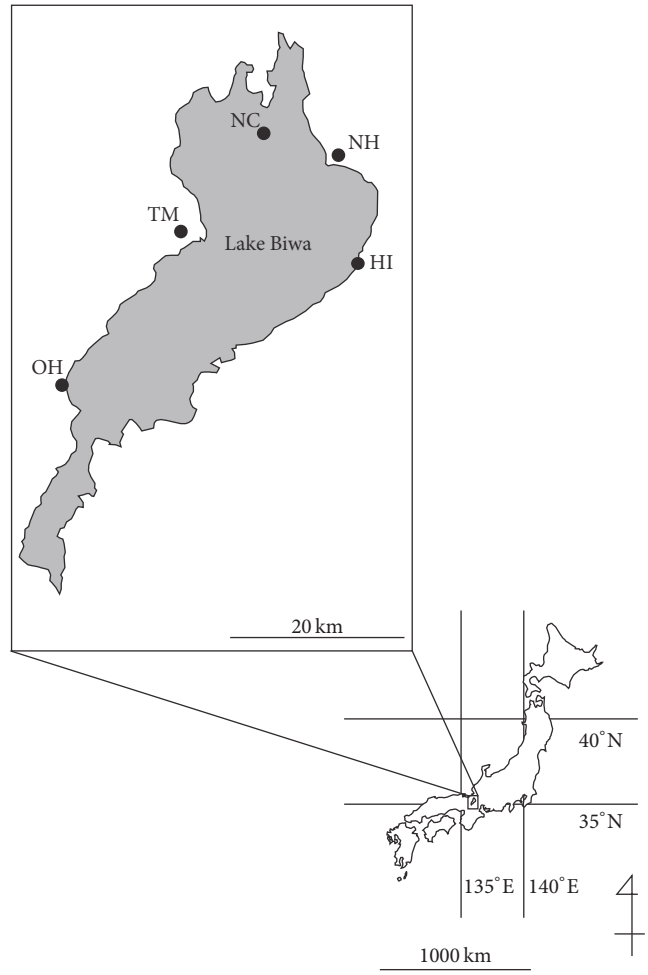

Figure 1: Location of the study sites around Lake Biwa, Japan.

2.2. Study Site. We worked in five warm-temperate evergreen forests around Lake Biwa (Figure 1; Table 1). Lake Biwa is the largest freshwater lake in Japan and is located in the west-central region of the country.

Chikubushima Island (NC; Nagahama, Shiga, Japan), which is 2 km away from the shoreline, is uninhabited, and the level of conservation is high. The forest canopy of the island is dominated by evergreen broad-leaved species including *M. thunbergii*, *Quercus myrsinifolia* Blume, and *Ilex integra* Thunb. This forest has maintained almost the same primeval state since 15th century. This forest contained more than 100 individuals of *M. thunbergii*. The Hikitari shrine site (NH; Nagahama, Shiga, Japan) is a shrine forest dominated by *M. thunbergii, Q. myrsinifolia,* and *Zelkova serrata* (Thunb.) Makino. This forest is surrounded by agricultural area and highly fragmented. This forest contained 15 individuals of *M. thunbergii*. The Inukami River site (HI; Hikone, Shiga, Japan) is a riverside forest located at the outlet of the Inukami River and has a canopy dominated by *M. thunbergii*, *Aphananthe aspera* (Thunb.) Plach., *Quercus aliena* Blume, and *Celtis sinensis* Pers. Area of this forest is decreasing since 1960s owing to river improvement but relatively large size forest

Table 1: Location and size of each *M. thunbergii* study site.

Study site	Locality	Abbreviation	Latitude	Longitude	Forest area (ha)
Hassho—shrine	Otsu, Shiga	OH	35°10′N	135°54′E	1.46
Myounji—temple	Takashima, Shiga	TM	35°19′N	136°04′E	0.13
Chikubushima—island	Nagahama, Shiga	NC	35°25′N	136°08′E	14.17
Hikitari—shrine	Nagahama, Shiga	NH	35°26′N	136°12′E	0.88
Inukami—river	Hikone, Shiga	HI	35°15′N	136°13′E	9.41

still maintained. This forest contained about 60 individuals of *M. thunbergii*. The Hassho shrine site (OH; Otsu, Shiga, Japan) is another shrine forest, with a canopy dominated by *M. thunbergii*, *Castanopsis cuspidata* (Thunb.) Schottky, and *Cryptomeria japonica* (Thunb. ex L.f.) D. Don. This forest is surrounded by urban area and highly fragmented. This forest contained about 24 individuals of *M. thunbergii*.

The Myounji temple site (TM; Takashima, Shiga, Japan) is a temple forest with a canopy dominated by *M. thunbergii*, *C. cuspidata*, *Q. myrsinifolia*, and *Z. serrata*. This forest is surrounded by paddy fields and highly fragmented. This forest contained 9 individuals of *M. thunbergii*. In this paper, we used areas of forest (ha) as an indicator of population size because we could not evaluate exactly the number of individuals in HI and NC population.

2.3. Sample Collection. We collected leaf of mature *M. thunbergii* trees. The average sample size per population was 29 (range: 9–55), and 147 individual trees were sampled in total. In this study, a mature tree was defined as an individual >130 cm in height and >5 cm in diameter at breast height. Samples from mature trees were randomly collected at HI and NC, whereas all individuals within a population were sampled at OH, TM, and NH. In the TM population, we sampled just nine mature trees because there were only nine mature trees.

We also collected leaf samples from juvenile trees, including current-year seedlings and small saplings (>1 year old) to eliminate year-specific effects such as differences in flower or seed mass. The average sample size per population was 34 (range: 18–55), and 172 individuals were sampled in total. Juvenile samples were randomly collected from HI in 2006, OH in 2010, TM and NH in 2014, and NC in 2015. Leaf samples were stored at 4°C with silica gel prior to DNA extraction using a modified cetyltrimethylammonium bromide method [23].

2.4. Microsatellite Typing. Seven loci, corresponding to the microsatellite markers Mt03, Mt04, Mt05, Mt13, Mt14, Mt16, and Mt20 identified by Kaneko et al. [24], were used in the analysis. We scored the genotypes of these microsatellite loci in each DNA sample. We performed PCR amplification using a DNA thermal cycler (Eppendorf, Mastercycler ep gradient S) under the following conditions: initial denaturation at 94°C for 9 min, followed by 40 cycles of denaturation at 94°C for 30 s and 1 min annealing at 72°C with a final extension step of 72°C for 5 min. The reaction mixtures (5 μl) contained 2 μl PCR Master Mix (Qiagen Multiplex PCR Buffer, pH 8.7, consisting of dNTPs, Qiagen HotStarTaq DNA Polymerase, and $MgCl_2$ to a final concentration of 3 mM) and 10 ng template DNA. The sizes of the PCR products were determined by automated fluorescence scanning using a 3130xl Genetic Analyzer (Applied Biosystems) running the GeneMapper software (Applied Biosystems).

2.5. Genetic Diversity and Genetic Differentiation. For each population, the genetic diversity was evaluated by calculating allelic richness (A_R; [25]), expected heterozygosity (H_E), and observed heterozygosity (H_O) values and the fixation index (F). All parameters were calculated using version 2.9.3 of the FSTAT software [26]. Deviations from Hardy–Weinberg equilibrium (HWE) were assessed using FSTAT. The null alleles affect population parameters which are estimated based on the proportion of heterozygotes. So, we also estimated frequency of null allele for mature and juvenile population of each site and posterior distribution of $F_{(\text{null})}$ values. $F_{(\text{null})}$ is inbreeding coefficient which is estimated in consideration of the presence of null alleles. Estimations of $F_{(\text{null})}$ and the average null allele frequency were conducted under an individual inbreeding model (IIM) using INEst 2.0 (Table 2) [27].

To investigate the relationships between genetic diversity (A_R, H_E, F) and forest area (ha), we applied a generalized linear model with an identity link function and Gaussian distribution using the R 2.11.1 software [28]. Nei's estimates [29] of heterozygosity (H_S, H_T) were calculated for multiple loci using FSTAT. To estimate the population size reduction at the mature stage, recent population bottlenecks were evaluated using version 1.2.02 of the BOTTLENECK software [30]. We simulated equilibrium conditions (10000 replications) using both an infinite alleles model (IAM) and a two-phase model (TPM). We used the Wilcoxon signed-rank test to determine if significant excess heterozygosity existed. The TM population was excluded from this analysis due to its small sample size.

To estimate genetic differentiation among populations at both the mature and the juvenile stages, F_{ST} [31], R_{ST} [32], G'_{ST} [33] values were calculated. F_{ST}, R_{ST}, H_S, H_T were computed using FSTAT, and G'_{ST} values were computed using GenAlEx 6.5 [34].

3. Results

3.1. Within-Population Genetic Diversity. A_R ranged from 2.67 to 4.11 for mature trees and from 2.58 to 4.20 for juveniles (Table 2). A_R values of mature trees were highest in NC and

Table 2: Genetic diversity of five *M. thunbergii* populations around Lake Biwa.

Site	Population	N	A_R	H_O	H_E	F	$F_{(\text{null})}$	*Null allele*
OH	Mature	24	2.67	0.60	0.51	−0.17	0.02	0.02
	Juvenile	39	2.58	0.41	0.50	0.19*	0.05	0.12
TM	Mature	9	3.39	0.41	0.49	0.23	0.10	0.19
	Juvenile	20	3.05	0.41	0.51	0.23*	0.16	0.17
NC	Mature	55	4.11	0.58	0.62	0.08	0.05	0.05
	Juvenile	40	4.20	0.58	0.64	0.11	0.08	0.07
NH	Mature	15	3.84	0.61	0.62	0.06	0.08	0.04
	Juvenile	18	3.79	0.51	0.62	0.2*	0.16	0.09
HI	Mature	44	3.58	0.66	0.64	−0.009	0.02	0.01
	Juvenile	55	3.43	0.52	0.58	0.12	0.07	0.06

N, number of samples; A_R, allelic richness (calculated from 14 gene copies); H_O, observed heterozygosity; H_E, expected heterozygosity; F, inbreeding coefficient for juvenile and mature life stages of *M. thunbergii*; * population significantly deviated from Hardy–Weinberg equilibrium ($P \leq 0.05$); $F_{(\text{null})}$, Bayesian estimated posterior mean value of F in consideration of presence of null alleles; *null allele*, the average null allele frequency.

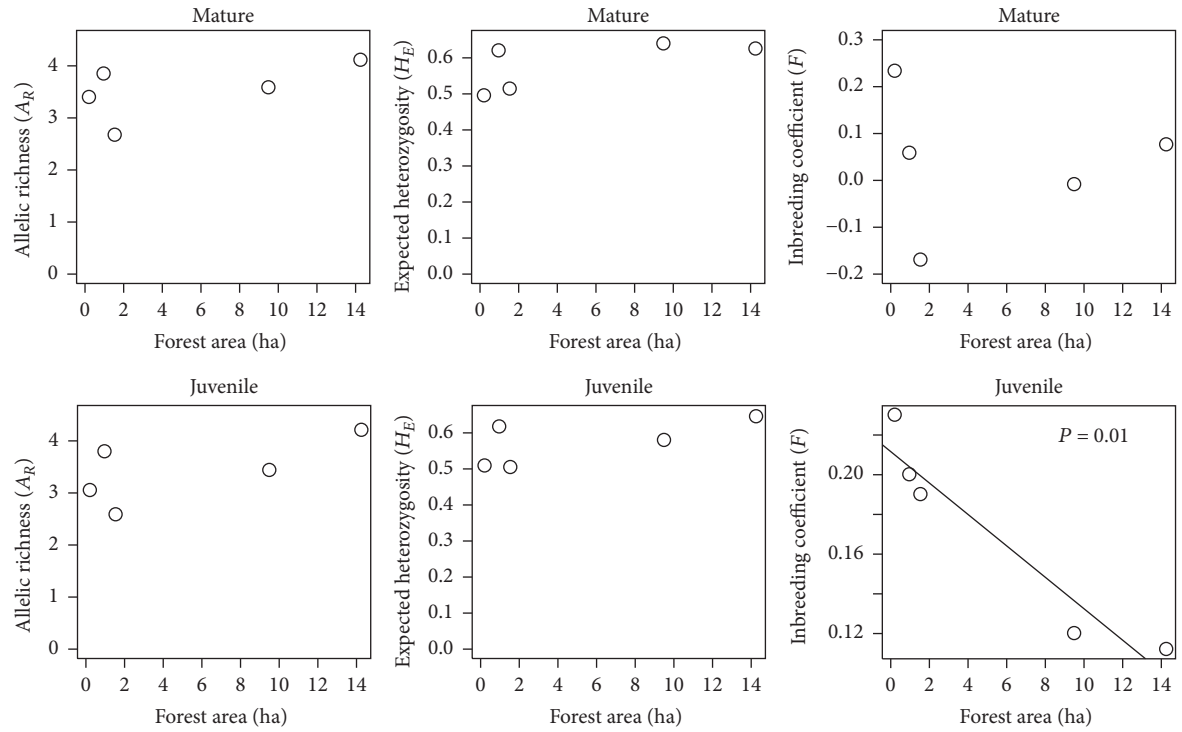

Figure 2: Relationships between the genetic diversity parameters (A_R, H_E, F) and forest area in mature and juvenile *Machilus thunbergii* trees.

lowest in OH. H_O and H_E values of mature trees ranged from 0.41 to 0.66 and 0.49 to 0.64, respectively, and those of juveniles ranged from 0.41 to 0.58 and 0.5 to 0.64. F values ranged from −0.17 to 0.23 for mature trees and from 0.11 to 0.23 for juveniles. F values of mature trees were highest at TM and lowest at OH, whereas those of juvenile trees were highest at TM and lowest at NC. No mature tree population deviated significantly from HWE, but deviation from HWE was observed in juvenile populations at OH, TM, and NH ($P < 0.05$). The posterior mean values of $F_{(\text{null})}$ ranged from 0.02 to 0.10 for mature trees and from 0.05 to 0.16 for juveniles. Frequency of null allele ranged from 0.01 to 0.19 for mature trees and from 0.04 to 0.17 for juveniles.

Genetic variation within populations, as revealed by H_S, was higher for mature trees (0.60) than for juveniles (0.58) (Table 3). Values of H_T were the same in mature trees and juveniles (0.67). Genetic variation among populations was higher among juvenile compared to adult trees (F_{ST} = 0.17 and 0.13; R_{ST} = 0.27 and 0.21; G'_{ST} = 0.35 and 0.30, resp., Table 3).

3.2. Relationships between Forest Area and Genetic Diversity. Statistical analyses revealed that F values of juvenile trees were significantly negatively correlated with forest area (P = 0.01, Figure 2). F values of mature trees and A_R and H_E values of both mature and juvenile trees were not significantly correlated with forest area.

Table 3: Genetic variation within and among populations of *M. thunbergii*.

Populations	H_S	H_T	G'_{ST}	F_{ST}	R_{ST}
Among mature trees	0.60	0.67	0.30	0.13	0.21
Among juveniles	0.58	0.67	0.35	0.17	0.27

H_S, expected genetic diversity within subpopulations; H_T, total genetic diversity of overall population; G'_{ST}, genetic differentiation index of Hedrick (2005); F_{ST}, genetic differentiation index of Weir and Cockerham (1984); R_{ST}, genetic differentiation index of Rousset (1996).

3.3. Bottleneck Test. The BOTTLENECK analysis (using Wilcoxon's signed-rank test) indicated that recent population bottlenecks had occurred. When the Wilcoxon test was run under the IAM and TPM conditions, significant excess heterozygosity was detected in the OH, NH, and HI populations (Table 4). When the Sign test was run under the IAM conditions, significant excess heterozygosity was detected in the NH and HI populations (Table 4).

4. Discussion

4.1. Genetic Diversity. Previous meta-analysis indicated that the number of alleles is generally correlated with population size and rapidly declines with population size reduction [35]. However, in our result, the relationship between A_R value and forest area was not significant (Figure 2). We suppose that population history may play an important role in A_R value. In the prior study, we have revealed that populations on the western and eastern sides of Lake Biwa have different lineages, and these two lineages were admixed on the northern side of the lake from the phylogenetic analysis of *M. thunbergii* in the Kinki region [36]. Admixture of genetically differentiated populations increases the genetic heterogeneity of the newly established population [37]. In our results, NH and TM that locate at north side of Lake Biwa maintained relatively high levels of A_R value (Table 3) although these population sizes are small. These facts suggest that the effect of past lineage admixture events is grater than that of current population size on A_R value for *M. thunbergii* populations around Lake Biwa.

Values of H_E were not clearly different among these populations (Table 2). Generally, allelic diversity declines more rapidly than heterozygosity when the population size decreases, and a change in heterozygosity may take several generations to become apparent [2, 7]. Aguilar et al. [5] reported significant correlations between the number of generations and the extent of reduction of H_E. We suspect that because the reduction in population size of *M. thunbergii* around Lake Biwa occurred recently, values of H_E reflect the situation before the population size declined. Bottleneck testing supported this speculation and demonstrated significant excess heterozygosity in the OH, NH, and HI populations (Table 4, TM population was not analyzed). Bottleneck testing assumes that the reduction of allele number is faster than that of H_E [38], and it is effective for assessing recently experienced population size reduction [30]. Thus, our results indicate that the NC population has not experienced recent population size reduction and maintained current population size for a long time, whereas the OH, NH, and HI populations experienced a population reduction within the past few generations.

4.2. Inbreeding Coefficient. Our analyses indicated that HWE is maintained in all populations at the mature stage (Table 2), although the statistical significance of the trend in the TM population needs further consideration owing to the small population size. Selection against homozygotes during recruitment could have affected the maintenance of HWE at the mature stage, as tree species generally experience high levels of inbreeding depression early in the life cycle [39]. *Machilus thunbergii* is a heterodichogamous tree, and this breeding system promotes outcrossing and regulates selfing [20]. The magnitude of inbreeding depression is strong in naturally outbreeding species [40], and the effects of inbreeding depression occur successively at different developmental stages, suggesting reduced levels of inbreeding at later stages due to enhanced mortality caused by inbreeding depression. Thus, selective death of homozygote individuals during growth from the juvenile to the mature stage may have reduced F values of mature trees.

We detected significant deviation from HWE at the juvenile stage in small and disturbed populations (OH, TM, and NH) and maintenance of HWE in relatively large populations (HI and NC; Table 2). In addition, F values of the juvenile stage were significantly correlated with forest area (Figure 2). The most likely explanation for these results is that human exploitation and habitat degradation have reduced the effective population size of adult trees in remnant populations, thus decreasing pollen availability and increasing the level of either self-fertilization or biparental inbreeding in the small, disturbed populations. Some previous researches indicate habitat degradation increases mating with relatives and genetic differentiation of future generations [7, 41–43]. On the other hand, result of $F_{(\text{null})}$ and frequency of null allele indicate some populations contain substantial proportion of null alleles and this also contributed to the excessive homozygosity. Thus, the values of F of TM and OH population need further consideration.

Pollen flow among *M. thunbergii* trees occurs primarily within 200 m (Watanabe et al., unpublished data). In addition, insect pollinators often stay within a habitat fragment, rather than moving among fragments (e.g., [44, 45]). Moreover, habitat degradation has a strong influence on animal pollinators because it affects their population size and foraging behavior [46].

In this study, we were not able to evaluate contemporary gene flow, but results of inbreeding coefficient suggest that the chance of outside gene flow of *M. thunbergii* is possibly diminished by forest degradation in the OH, TM, and NH populations, whereas panmictic breeding is maintained in relatively large populations such as NC.

The genetic differentiation index values may also reflect an increase of inbreeding at the juvenile stage in these populations. The values were higher among juveniles than among mature trees (Table 3). When contemporary gene flow is restricted by habitat degradation, we would expect to find

Table 4: Probability of a bottleneck estimated using the program BOTTLENECK.

Population	Sign		Wilcoxon	
	IAM	TPM	IAM	TPM
OH	0.069	0.089	0.008*	0.027*
TM	NA	NA	NA	NA
NC	0.579	0.105	0.188	0.766
NH	0.018*	0.403	0.004*	0.039*
HI	0.015*	0.122	0.004*	0.008*

The probabilities of significant heterozygosity excess (evidence of bottleneck) for two-tailed Sign and Wilcoxon tests under the IAM and the TPM are marked with an asterisk ($^{*}P < 0.05$). NA, population that was not analyzed.

greater differentiation between seedlings (which are experiencing contemporary, limited gene flow) compared to adults (which experienced historic gene flow).

4.3. Conclusions. Our data suggest that the patterns of allelic diversity were more influenced by the past lineage admixture events than by the current population sizes for *M. thunbergii* populations around Lake Biwa. The extent of inbreeding at the juvenile stage was influenced by population size, and panmictic breeding was maintained in a large population. Our findings suggest that a recent population size decline will modify the mating pattern and increase inbreeding in these *M. thunbergii* populations.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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