

1 **Pollen dispersal patterns and population persistence in a small isolated population of**

2 ***Fagus crenata***

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1    **Abstract**

2    The potential of long-distance pollen dispersal and the effects of small population size and  
3    population isolation on persistence of *Fagus crenata* populations were investigated in a small,  
4    severely isolated population (the Gofuku-ji population) and two other populations located  
5    within 7 km of this population (including 87 adult trees in total). Parentage analysis using 13  
6    microsatellite loci showed that 94 of 100 seedlings derived from seeds collected from the  
7    Gofuku-ji population had parent pairs within this population, six had one parent within the  
8    population, and four of the six seedlings had alleles that were not detected in any of the three  
9    populations, indicating that some pollen is dispersed over distances exceeding 7 km. The  
10   estimated expected heterozygosity and effective population size were lower in the Gofuku-ji  
11   population than in previously examined large continuous populations. Therefore, levels of  
12   genetic diversity within the population may have been reduced by strong genetic drift and  
13   limitations of pollen- and seed-mediated gene flow associated with the small size and severe  
14   isolation. The contemporary mating pattern estimated at the seedling stage was biased toward  
15   outbreeding, which may be explained by possible processes: the level of inbreeding in the adult  
16   trees is increased, then inbreeding frequently occurs but is rarely successful, while outbreeding  
17   successfully produces offspring. Additionally, high levels of significant linkage disequilibrium

1 and higher numbers of alleles than expected under mutation-drift equilibrium from analyses of  
2 the populations' evolutionary history suggest that the Gofuku-ji population may have  
3 experienced admixture before its severe isolation. Therefore, the persistence of the Gofuku-ji  
4 population is being adversely affected by the decrease in population size and severe isolation.  
5 Further studies of gene flow via pollen in other populations with various degrees of isolation  
6 could enhance our understanding of the effects of population isolation and long-distance pollen  
7 dispersal in *F. crenata* and similar species.

8 Keywords: genetic drift, habitat fragmentation, inbreeding, long-distance pollen dispersal,  
9 pollen limitation, population admixture

## 1 **Introduction**

2 The fragmentation of forests has been increased by growing demands for food and changes in  
3 land use in many parts of the world (Millennium Ecosystem Assessment 2005). Consequently,  
4 there have been increases in the occurrence of small and spatially isolated forest patches in  
5 matrices of agricultural or urban land. When a population becomes small, its persistence is  
6 increasingly affected by not only demographic and environmental stochasticity but also genetic  
7 factors: the decrease in population size may lead to a loss of genetic diversity due to genetic  
8 drift and inbreeding, which may cause an immediate loss of fitness (inbreeding depression) and  
9 reduce evolutionary potential to cope with changing environments (Frankham 2005; Ouborg et  
10 al. 2006). Although the loss of genetic diversity and inbreeding can be mitigated by gene flow  
11 from other populations (Richards 2000), gene flow via pollen and seeds may be reduced by  
12 severe isolation of populations. As levels of gene flow via pollen in many plant species are  
13 generally at least an order of magnitude greater than that via seeds at the range-wide scale, gene  
14 flow in plant species may rely mainly on pollen dispersal (Petit et al. 2005). Therefore, pollen  
15 dispersal among populations may be very important for the persistence of isolated populations  
16 of plant species (Richards 2000).

17 Fragmented forests often suffer, in comparison with continuous forest, from

1 fragmentation-related declines in pollen availability, pollen diversity and proportions of  
2 outcrossing, fruit set and seed set (Aizen and Feinsinger 1994; Nason and Hamrick 1997;  
3 Aguilar and Galetto 2004; Llorens et al. 2012). These effects of habitat fragmentation may have  
4 serious consequences for seed production, offspring fitness, genetic diversity and ultimately  
5 population persistence (Young et al. 1996; Leimu et al. 2006). Therefore, determining the  
6 genetic composition of pollen donors of seeds or seedlings is highly important for estimating  
7 short-term effects of increases in frequencies of inbreeding (inbreeding depression) and  
8 long-term effects of reductions in genetic diversity on population viability, evaluating the  
9 sustainability of isolated small populations, and formulating effective strategies for conserving  
10 fragmented habitats and the populations they host.

11 However, gene flow via pollen in wind-pollinated tree species can be extensive and thus may  
12 mitigate losses of genetic diversity due to genetic drift and inbreeding following habitat  
13 fragmentation (Hamrick 2004). For example, Gerber et al. (2014) found that pollen from outside  
14 the stand was responsible for 60% of pollinations in eight stands of *Quercus* species distributed  
15 across Europe, and suggested that geographically remote stands are unlikely to be genetically  
16 isolated (although the cited study found large variation in immigration rates of pollen among  
17 stands and did not include any extreme cases of small, severely isolated populations). In

1 addition, Ortego et al. (2014) found that the genetic diversity of seedlings and proportions of  
2 self-fertilization in an extremely fragmented stand did not differ from those in a continuous  
3 stand of *Q. ilex*. Therefore, the outcrossing mating system and extensive gene flow of  
4 wind-pollinated tree species may enable them to tolerate habitat fragmentation, and effects of  
5 fragmentation have been relatively difficult to detect in populations of such species (but see  
6 O'Connell et al. 2006). However, difficulties in detecting fragmentation effects may be at least  
7 partly due to the misapplication of ecological concepts and associated evolutionary assumptions,  
8 i.e., fragment boundaries often do not represent boundaries for mating populations of forest  
9 trees because long-distance pollination and sometimes seed dispersal mitigate genetic isolation  
10 in many species (Kramer et al. 2008). Nevertheless, effects of severe habitat fragmentation have  
11 been detected, even in wind-pollinated tree species, by highly accurate genetic analyses and  
12 careful selection of study sites in recent years. For example, Jump and Peñuelas (2006) showed  
13 that habitat fragmentation has led to genetic bottlenecks in populations of *F. sylvatica* and the  
14 breakup of the species' panmictic breeding, resulting in significantly elevated levels of  
15 inbreeding, population divergence, and reduced genetic diversity within populations.  
16 Furthermore, in a meta-analysis of data on 97 woody plant species from 98 studies of habitat  
17 fragmentation, Vranckx et al. (2012) found that wind-pollinated trees and shrubs appeared to

1 lose genetic diversity through habitat fragmentation. Lowe et al. (2015) have pointed out, based  
2 on the findings from the recent studies about forest fragmentation genetics, that forest tree  
3 species are not completely resilient to the population genetic consequences of forest  
4 fragmentation, and that the impacts are not necessarily simple losses of genetic diversity and  
5 increased differentiation among populations but rather vary by species with different mating and  
6 breeding systems, landscape context (e.g. scale and land-use type), and the nature of impacts  
7 (e.g. fragmentation versus logging).

8 *Fagus crenata* is a monoecious, long-lived woody angiosperm, with an outcrossing breeding  
9 system relying on wind pollination. Although the genus *Fagus* has high potential for  
10 long-distance pollen dispersal (Oddou-Muratorio et al. 2010; Piotti et al. 2012; Inanaga et al.  
11 2014), empirical evidence of such dispersal is rare. In this study, the potential of long-distance  
12 pollen dispersal and the effects of small population size and population isolation on persistence  
13 of *Fagus crenata* populations were investigated in a small, severely isolated population and two  
14 other populations located within a 7-km radius from this population.

15

## 16 **Materials and methods**

### 17 *Study site and field methods*

18 A small population of *F. crenata* is located at Gofuku-ji Temple (36°09'56"N, 138°01'10"E) at



1 altitudes 990-1050 m above sea level (a.s.l.) in Matsumoto City, Nagano Prefecture, Japan  
2 (Figs. 1, 2). This population, hereafter called the Gofuku-ji population, constitutes a mixed  
3 natural forest (ca. 1.7 ha) with *Pinus densiflora* and *Quercus serrata*, with the dominance of *F.*  
4 *crenata* trees [density of stems with a diameter at breast height (DBH)  $\geq$  5 cm, ca. 57.1 ha<sup>-1</sup>;  
5 canopy coverage,  $\geq$  50%], and has been isolated because of surrounding planted forests of  
6 *Cryptomeria japonica*, *Chamaecyparis obtusa*, *Larix kaempferi* and *P. densiflora*, secondary  
7 forests of deciduous broad-leaved trees (not including *F. crenata*), farm lands, and residential  
8 areas. In an exhaustive survey of the surroundings, we found that only two stands of *F. crenata*  
9 (at Bunano-ki Gongen, 36°09'39"N, 138°02'28"E; and Mt. Futatsu-yama, 36°09'31"N,  
10 138°05'03"E) were located within a 7-km radius from the Gofuku-ji population, but other small  
11 stands were scattered at further distances (Fig. 1). No *F. crenata* trees have been observed in  
12 residential areas of Matsumoto City, including fragmented forests around shrines and temples  
13 (Tsuchida 1986; Matsumoto City Government 1996).

14 We operationally defined adult trees as those with DBH  $\geq$  5 cm, which should include all  
15 flowering individuals, as field observations in another *F. crenata* population by Y. Asuka and N.  
16 Tomaru (unpublished data) indicate that the minimum DBH of flowering individuals is about 12  
17 cm. All *F. crenata* trees meeting the  $\geq$  5 cm DBH criterion in the Gofuku-ji, Bunano-ki Gongen

1 (two trees), and Mt. Futatsu-yama (six trees) populations were mapped using a hand-held GPS  
2 unit (eTrex 20J, GARMIN), and their DBH was measured. In the Gofuku-ji population, many of  
3 the adult trees have formed spatially tight clusters of multiple stems, so we recorded and  
4 measured the DBH of all stems (with a  $DBH \geq 5$  cm) of adult trees, and mapped the central  
5 points of clusters. Sixty-three single-stemmed trees and 15 clusters including 34 stems (i.e. 97  
6 stems in total) of *F. crenata* were found in the Gofuku-ji population, and leaves from all the  
7 stems were collected in September 2013. We also collected leaves from the two and six adult  
8 trees of the Bunano-ki Gongen and Mt. Futatsu-yama populations, respectively. Therefore, we  
9 sampled leaves from a total of 105 stems of adult trees.

10 In a previous study about seed production and germination rates in *F. crenata* (Koyama and  
11 Ida 2013), 11 seed traps were set in the Gofuku-ji population in 2011, when *F. crenata*  
12 populations throughout Nagano Prefecture produced good crops. The traps were placed on the  
13 lower part of a slope, at 990–1020 m a.s.l., in the vicinity of adult trees with a large DBH (Fig.  
14 2). Data for two traps (one of which caught no seeds while the other caught only one) were  
15 excluded from analysis. We recalculated means and standard deviations of the proportion of  
16 empty seeds and germination rate of sound seeds caught in nine traps, using the raw data  
17 collected by Koyama and Ida (2013), and found them to be  $0.693 \pm 0.291$  and  $0.399 \pm 0.322$ ,

1 respectively (Table S1). After the germination test, 236 germinated seedlings were immediately  
2 planted randomly in nursery beds in a greenhouse in the spring of 2012. In August 2013, there  
3 were 154 survivors of these seedlings, and leaves were collected from 100 randomly selected  
4 seedlings. All samples were stored at  $-30\text{ }^{\circ}\text{C}$  until DNA was extracted.

5

#### 6 *DNA extraction and microsatellite genotyping*

7 Total genomic DNA was extracted from the sampled leaves using the  
8 hexadecyltrimethylammoniumbromide (CTAB) method (Murray and Thompson 1980) with  
9 minor modifications. We amplified microsatellite DNA at 13 loci: *FS1-03* and *FS4-46*  
10 developed for *F. sylvatica* (Pastorelli et al. 2003), *F115* for *F. longipetiolata* (Liu et al. 2009),  
11 and *sfc0007-2*, *sfc0018*, *sfc0036*, *sfc0161*, *sfc0195-2*, *sfc0305*, *sfc0360-2*, *sfc1063*, *sfc1105*, and  
12 *sfc1143*, developed for *F. crenata* (Asuka et al. 2004). Polymerase chain reactions (PCRs) were  
13 carried out in 6.0- $\mu\text{l}$  mixtures containing 50 ng of genomic DNA, 0.2  $\mu\text{M}$  of each primer (the  
14 forward primer in each pair was labelled with a fluorescent dye), and 3.0  $\mu\text{l}$  of  $2 \times$  Qiagen  
15 Multiplex Master Mix (QIAGEN, Venlo, the Netherlands) using a GeneAmp PCR System 9700  
16 thermal cycler (Applied Biosystems, Foster City, United States). The thermal program consisted  
17 of initial denaturation at  $95\text{ }^{\circ}\text{C}$  for 0.5 min, followed by 35 cycles of denaturation at  $94\text{ }^{\circ}\text{C}$  for

1 0.5 min, annealing at 57 °C for 1.5 min, and extension at 72 °C for 1 min, then a final extension  
2 at 60 °C for 30 min. PCR products were separated by capillary electrophoresis using a 3130  
3 Genetic Analyzer (Applied Biosystems) and 11.15- $\mu$ l mixtures containing 1.0  $\mu$ l of PCR  
4 products, 10.0  $\mu$ l of Hi-Di Formamide, and 0.15  $\mu$ l of DNA size marker (500 LIZ size standard;  
5 Life Technologies, Foster City, CA, USA). Genotypes were scored with GeneMapper ver. 4.1  
6 (Applied Biosystems).

#### 7 8 *Data analyses*

9 We first examined whether the multiple stems within each cluster in the Gofuku-ji population  
10 shared the same multilocus genotype. When all the stems within a cluster shared an identical  
11 multilocus genotype with no mismatched locus, the cluster was considered to consist of a single  
12 genet. The adult trees with different multilocus genotypes were then used for the following  
13 analyses. The total probability of identity for the Gofuku-ji population and the total exclusion  
14 probabilities for single parents and parent pairs across all three populations were calculated  
15 across all loci using the program CERVUS ver. 3.0.3 (Kalinowski et al. 2007).

16 To evaluate the genetic diversity of adult trees in the Gofuku-ji population, we estimated the  
17 number of alleles ( $A$ ), observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosity, and inbreeding

1 coefficient ( $F_{IS}$ ; Weir and Cockerham 1984) at each locus and across all loci. Deviations from  
2 Hardy–Weinberg equilibrium (HWE) at each locus and across all loci were evaluated by the  
3 exact test. To evaluate the associations between loci in adult trees in the Gofuku-ji population,  
4 we conducted the exact test for linkage disequilibrium. Bonferroni correction was used to  
5 determine significance in the above two multiple tests. These calculations were performed using  
6 the program GENEPOP ver.4.1.3 (Rousset 2008). Mean values of  $H_E$  across seven loci used in  
7 this study (*sfc0018*, *sfc0036*, *sfc0161*, *sfc0305*, *sfc1063*, *sfc1105*, and *sfc1143*) for two  
8 continuous large populations, at Hatomachi Pass and Mt. Haku-san, calculated using data from  
9 Hiraoka and Tomaru (2009) were compared with the corresponding value for the Gofuku-ji  
10 population using a Wilcoxon signed rank test with Bonferroni correction. The two populations  
11 considered for comparison are the geographically closest to the Gofuku-ji population among  
12 those investigated by Hiraoka and Tomaru (2009).

13 To examine the current patterns of pollen dispersal and mating in the Gofuku-ji population,  
14 we conducted the following three analyses. First, we conducted parentage analysis considering  
15 all adult trees in the Gofuku-ji, Bunano-ki Gongen and Mt. Futatsu-yama populations as  
16 potential parents for seedlings in the Gofuku-ji population, by simple exclusion methodology  
17 (Jones and Ardren 2003) based on multilocus genotypes using the program CERVUS ver. 3.0.3

1 (Kalinowski et al. 2007). We considered selfing and allowed one mismatched locus between  
2 seedlings and their parents to account for possible genotyping errors in the parentage analysis. If  
3 an adult tree could produce a haplotype shared with a seedling's genotype, it was considered a  
4 candidate parent of the seedling, and if a pair of adult trees could produce a combination of  
5 haplotypes identical to the genotype of a seedling, it was considered a candidate parent pair. If a  
6 seedling had no candidate parent within the three populations, it was regarded as having no  
7 parent within the populations (indicating seed dispersal from outside the populations). If a  
8 seedling had only one candidate parent or parent pair, that candidate parent or parent pair was  
9 regarded as its true parent or parent pair. If a seedling had multiple candidate parents or parent  
10 pairs, it was regarded as having a parent or parent pair within the populations, but its parentage  
11 could not be assigned. If a seedling had one or more candidate parents but did not have any  
12 candidate parent pair, it was regarded as having a seed parent within the populations but pollen  
13 parent outside the populations (indicating pollen dispersal from outside the populations),  
14 because *F. crenata* seeds are primarily dispersed by gravity and over short distances up to  
15 20–30 m (Hashizume et al. 1984; Oddou-Muratorio et al. 2010).

16 Second, we estimated  $F_{IS}$  values at each locus and across all loci for seedlings in the  
17 Gofukuji-population, and deviations from HWE at each locus and across all loci were evaluated

1 by the exact test, as in the analysis of adult trees.

2 Third, using the parentage analysis results, we examined whether the mean value of the  
3 kinship coefficient  $F_{ij}$  (coancestry; Loiselle et al. 1995) between mating pairs of parents  
4 producing seedlings within the Gofuku-ji population differed significantly from that expected as  
5 a result of random mating. We compared the empirical data with the distribution of mean values  
6 generated by a randomization procedure repeated 1000 times (Nakanishi et al. 2004). The  
7 randomization procedure was conducted as follows. If  $n$  seedlings could be assigned to parent  
8 pairs,  $n$  pairs of adult trees were randomly chosen from 55 adult trees with  $DBH \geq 12$  cm (see  
9 *Study site and field methods*) in the Gofuku-ji population, with replacement, and a mean  $F_{ij}$   
10 value for the  $n$  pairs was calculated. The  $F_{ij}$  values were calculated using the program SPAGeDi  
11 ver. 1.4c (Hardy and Vekemans 2002).

12 Subsequently, we conducted the following four analyses to examine historical effects of  
13 small population size and population isolation on the Gofuku-ji population.

14 First, effective population sizes ( $N_e$ ) for adult trees in the Gofuku-ji population were  
15 estimated using linkage disequilibrium (LD), heterozygote excess (HE) and molecular  
16 co-ancestry (Co-ancestry) methods by the program NeEstimator ver. 2.01 (Do et al. 2014). The  
17 minimum frequency of alleles used for the LD and HE methods was 0.01 in order to avoid the

1 influence of rare alleles. We obtained 95% confidence intervals (CI) for  $N_e$  estimates based on  
2 the LD and Co-ancestry methods by the jackknife method (Waples and Do 2008; Do et al.  
3 2014) and for those based on the HE method by the parametric method (Zhdanova and  
4 Pudovkin 2008). To evaluate values of  $N_e$  for the Gofuku-ji population, we compared them to  
5 those calculated for the two large continuous populations at the Hatomachi Pass and Mt.  
6 Haku-san mentioned above (Hiraoka and Tomaru 2009).

7 Second, we examined the likelihood of a population bottleneck for the Gofuku-ji population  
8 using the two-phase model with 95% single-step mutations and 5% multi-step mutations  
9 implemented in the program BOTTLENECK ver. 1.2.02 (Piry et al. 1999). The one-tailed  
10 Wilcoxon test was used to detect not only excess but also deficiency of expected heterozygosity  
11 ( $H_E$ ) compared to that expected under mutation-drift equilibrium ( $H_{EQ}$ ).

12 Third, to evaluate the spatial genetic structure (SGS) among the adult trees in the Gofuku-ji  
13 population, we determined a  $S_p$  statistic (Vekemans and Hardy 2004), as follows. The mean  $F_{ij}$   
14 value was calculated for each of eight continuous distance classes of 10-m intervals, from 0–10  
15 m to 70–80 m, in the population using the program SPAGeDi, and the  $S_p$  value was calculated  
16 using the formula  $S_p = -b_F/(1 - F_1)$  (Vekemans and Hardy 2004), where  $b_F$  is the regression  
17 slope calculated by regressing the mean  $F_{ij}$  values against the logarithm of the distance between



1 adult trees, and  $F_1$  is the mean  $F_{ij}$  value for the first distance class (0–10 m). The significance of  
2 the mean  $F_{ij}$  and  $b_F$  values was then assessed by permutation tests with 1000 permutations. The  
3 standard errors of  $b_F$  and  $Sp$  were calculated by jack-knifing over loci. We considered the  
4 presence of SGS by testing the significance of the  $b_F$  value rather than the mean  $F_{ij}$  values,  
5 following Vekemans and Hardy (2004).

6 Fourth, we examined the genetic relationships among the adult trees of the Gofuku-ji  
7 population using the program COLONY ver. 2.0.6.1 (Jones and Wang 2010), assuming a 0.01  
8 genotyping error rate, absence of selfing, and candidate male and female genotypes including  
9 adult trees with  $DBH \geq 12$  cm (see *Study site and field methods*). We computed the likelihood  
10 of each pair of adult trees being full-sibs, half-sibs, or parent-and-offspring ten times, and the  
11 pair was identified as such if the corresponding mean likelihood exceeded 0.75, otherwise they  
12 were classified as unrelated, following Till-Bottraud et al. (2012).

13

## 14 **Results**

15 Stems within 14 of the 15 clusters in the Gofuku-ji population shared the same multilocus  
16 genotypes, indicating that each cluster represented a single genet. The other cluster was divided  
17 into two genets because its stems had two different multilocus genotypes (12 mismatching loci).

1 Therefore, there were 79 adult trees (genets) in the Gofuku-ji population (16 trees in the 15  
2 clusters and 63 trees with single stems). Including eight adult trees from the two populations  
3 within 7 km of the Gofuku-ji population, Bunano-ki Gongen (two trees) and Mt. Futatsu-yama  
4 (six trees), we used genotype data from 87 adult trees in the following parentage analysis.

5 For adult trees in the Gofuku-ji population, 4–29 alleles per locus (mean = 14.2) were  
6 detected, and the values of  $H_O$  and  $H_E$  ranged from 0.203 to 0.949 (0.804) and 0.188 to 0.862  
7 (0.728), respectively (Table 1).  $F_{IS}$  values at each locus and over all loci were all negative, and  
8 deviation from HWE was significant at *FSI-03* ( $P < 0.05$ ) and across all loci ( $P < 0.001$ ). The  
9 total probability of identity and the exclusion probabilities for single parent and parent pairs  
10 across all loci were 0.000000, 0.998830, and 0.999999, respectively. These results strongly  
11 indicate that the 13 loci provided sufficient resolution for robust clonal and parentage analyses.  
12 We found that 29 (37.2%) out of the 78 locus pairs in the Gofuku-ji population showed  
13 significant genotypic linkage disequilibrium ( $P < 0.05$ ). The mean value (0.728) of  $H_E$  across  
14 the seven comparable loci for adult trees in the Gofuku-ji population was significantly lower  
15 than that in the two continuous large populations at Hatomachi Pass (0.875) and Mt. Haku-san  
16 (0.863) ( $P < 0.05$ ).

17 We obtained the following results from the three analyses of the current patterns of pollen

1 dispersal and mating in the Gofuku-ji population. First, 94 seedlings (of 100 examined) had  
2 only one candidate parent pair within the Gofuku-ji population, which could thus be defined as  
3 true parent pairs (Table 2). The remaining six seedlings had a single parent (identification  
4 number 671) within the Gofuku-ji population but did not have any other candidate parent within  
5 the Gofuku-ji, Bunano-ki Gongen or Mt. Futatsu-yama populations. In addition, two alleles  
6 (167 and 186 at locus *sfc305*), which were not detected in samples from adult trees within the  
7 three populations, were found in three and one of the six seedlings, respectively. The six  
8 seedlings were regarded as having a seed parent within the Gofuku-ji population but pollen  
9 parents outside all three of the Gofuku-ji, Bunano-ki Gongen and Mt. Futatsu-yama populations  
10 (indicating pollen dispersal from outside the populations). A few large adult trees (with DBH  $\geq$   
11 40 cm) were found to have largely contributed to the seedlings as parents: one (identification  
12 number 671) as a parent for most of the seedlings (96) and another four (identification numbers  
13 648, 674, 679 and 689) for 24, 23, 30, and 9 seedlings, respectively. Second,  $F_{IS}$  values at each  
14 locus and over all loci for seedlings were all negative, and deviation from HWE was significant  
15 at eight out of the 13 loci (61.5%) and across all loci ( $P < 0.05$  or  $0.001$ ; Table 3). Third, the  
16 mean  $F_{ij}$  value between parent pairs producing the 94 seedlings within the Gofuku-ji population  
17 was  $-0.043$  and significantly lower than that generated by the permutation procedure under

1 random mating (mean  $\pm$  standard deviation =  $0.000 \pm 0.007$ ,  $P < 0.01$ ).

2 Subsequently, the four analyses of historical effects of small population size and population  
3 isolation on the Gofuku-ji population provided the following results. First, the estimated values  
4 of  $N_e$  (and 95% CIs) for adult trees in the Gofuku-ji population obtained using LD, HE and  
5 Co-ancestry methods were 60.7 (52.3–71.2), 11.7 (8.9–17.3), and 5.4 (4.2–6.7), respectively  
6 (Table S2). These values were significantly lower than corresponding values for both the  
7 Hatomachi Pass and Mt. Haku-san populations except for the value estimated using Co-ancestry  
8 method for Mt. Haku-san population (Table S2). Second, we found no evidence of a recent  
9 bottleneck (i.e. no  $H_E$  excess compared to  $H_{EQ}$ ) in genotypes of adult trees in the Gofuku-ji  
10 population, but there was significant  $H_E$  deficiency ( $P < 0.001$ ). Third, in the correlogram of  
11 mean  $F_{ij}$  for adult trees, the values were not significant at any distance class (Fig. S1), and the  
12 estimated value of  $S_p$  was low [ $0.002312 \pm 0.000597$  (standard error)]. However, the estimated  
13 slope of regression [ $b_F = -0.002304 \pm 0.000592$  (standard error)] was significant (permutation  
14 test,  $P < 0.01$ ; indicating that spatial genetic structure was present but very weak, and that  
15 regeneration has occurred through spatially limited seed dispersal). Fourth, the COLONY  
16 analysis identified 8 (0.2%), 22 (0.7%), and 0 (0%) of the 3081 possible pairs of adult trees as  
17 full-sibs, half-sibs, and parent-and-offspring, respectively. The true parent pairs of the seedlings

1 were not inferred to be full-sibs, half-sibs, or parent-and-offspring.

2

### 3 **Discussion**

#### 4 *Current patterns of pollen dispersal and mating in the Gofuku-ji population*

5 The parentage analysis showed that 94 of the 100 seedlings analyzed had parent pairs within the  
6 Gofuku-ji population, indicating a high percentage of mating within the population (94.0 %),  
7 while six seedlings had only one parent within the Gofuku-ji population and also did not have  
8 any other parent within the Bunano-ki Gongen or Mt. Futatsu-yama populations. Four of these  
9 six seedlings had alleles (two in total) that were not detected in any sampled adult trees.  
10 Because gravity-dispersed seeds of *F. crenata* generally move over distances shorter than 30 m  
11 (Hashizume et al. 1984; Oddou-Muratorio et al. 2010), pollen dispersal by wind presumably  
12 mediates most immigration of genes into the population. Immigration of seeds from parents in  
13 distant populations fertilized by pollen that travelled long distances from parents in the  
14 Gofuku-ji population is possible, but highly improbable. Thus, our results suggest that some  
15 pollen is dispersed from outside the Gofuku-ji population over distances exceeding 7 km.  
16 Because there are ten *F. crenata* populations proximately outside the 7-km radius from the  
17 Gofuku-ji population (Fig. 1), pollen dispersal may have been from some of these populations.

1 Several previous studies have also provided evidence of long-distance dispersal of *Fagus* pollen.  
2 For example, Kitamura et al. (2008) detected four allozyme alleles in seeds that were not  
3 present in mature trees in small remnant *F. crenata* populations, suggesting long-distance pollen  
4 dispersal. Furthermore, the pollen dispersal kernel of the genus *Fagus* is generally fat-tailed  
5 (Oddou-Muratorio et al. 2010; Piotti et al. 2012; Inanaga et al. 2014), corroborating its potential  
6 capacity for long-distance pollen dispersal. However, this study provides the first clear genetic  
7 (parentage-based) evidence of contemporary pollen dispersal over a distance of several  
8 kilometers in *F. crenata* populations.

9  $F_{IS}$  values for seedlings in the Gofuku-ji population were all negative and significantly  
10 deviated from HWE at many loci. The mean value of  $F_{ij}$  between mating pairs of parents within  
11 the population was significantly lower than that under random mating. These results indicate  
12 that the contemporary mating pattern estimated at the seedling stage in the population is biased  
13 towards outbreeding, compared to random mating within the population. If a small population  
14 persists through many generations, every member may become related to some degree to every  
15 other member of the population, and subsequently mating pairs in the population must be  
16 related to some degree even if mating is random (remote inbreeding; Hartl 2000). Terazawa  
17 (1997) observed that only 2.2 to 10.4% of seeds produced by *F. crenata* in manual

1 self-pollination experiments were viable, and 79.9 to 96.6% were empty, indicating that *F.*  
2 *crenata* has imperfect self-incompatibility, allowing partial selfing, and/or inbreeding  
3 depression resulting in the abortion of embryos. Furthermore, reanalysis of data collected in a  
4 previous study (Koyama and Ida 2013) revealed that the proportion of empty seeds was higher,  
5 and the germination rate lower, in the Gofuku-ji population than corresponding values for four  
6 other populations (isolated or continuous; Table S1). Therefore, the bias towards outbreeding  
7 detected at the seedling stage may be explained by the level of inbreeding in the adult trees  
8 being increased. Pollinations may frequently occur between close relatives, even if mating is  
9 random, due to the population's high level of inbreeding, but may be rarely successful because  
10 of fertilization failure (due to the imperfect self-incompatibility) or failure of the offspring to  
11 survive (due to inbreeding depression). In contrast, pollinations between less closely related  
12 trees may result in offspring that successfully develop into seedlings. As a consequence, the  
13 mating producing seedlings is biased to outbreeding. This explanation is indirectly supported by  
14 the absence of seeds originating from selfing and a very low rate of biparental inbreeding  
15 (2.7%) previously observed in a large, continuous *F. crenata* population (Inanaga et al. 2014),  
16 which may have been partly due to post-pollination mechanisms (imperfect self-incompatibility  
17 and/or inbreeding depression).

1 Another possible factor to increase the proportion of empty seeds in the Gofuku-ji population  
2 is pollen limitation (Wilcock and Neiland 2002). Small population size and severe isolation of  
3 the population may increase pollen shortage through the associated scarcity of potential pollen  
4 parents and pollen immigration from outside populations. Wang (2003) found that the  
5 proportion of empty seeds was significantly negatively correlated with outcrossing rates in *F.*  
6 *sylvatica*, and pollen limitation generally increases selfing rates of plant species (Larson and  
7 Barrett 2000). Thus, pollen limitation–selfing interactions may also partially explain the high  
8 proportion of empty seeds.

9 Only 12 adult trees contributed as parents to the examined seedlings (21.8% of 55 candidate  
10 parents with  $DBH \geq 12$  cm) and there were only 12 parent pairs of seedlings. All of these 12  
11 adult trees had a relatively large DBH (mostly  $\geq 40$  cm), in accordance with expectations as  
12 male and female reproductive success of forest trees is generally related to size variables such as  
13 DBH (Schnabel et al. 1998; Oddou-Muratorio et al. 2005). The biased contribution of adult  
14 trees to seedlings may be associated with the placement of all the seed traps on the lower part of  
15 a single slope, which is the location of most adult trees producing seeds according to field  
16 observations (Y. Koyama, personal communication). Therefore, limited and biased  
17 combinations of matings between adult trees could also contribute to the bias towards



1    outbreeding detected at the seedling stage in the Gofuku-ji population.

2

3    *Historical effects of small population size, isolation, and admixture of populations on the*  
4    *Gofuku-ji population*

5        The mean  $H_E$  value in the Gofuku-ji population was lower than the range of mean  $H_E$  values  
6    in *F. crenata* populations sampled throughout the entire geographic range of the species  
7    reported by Hiraoka and Tomaru (2009). In the present study, our comparison showed that the  
8    mean value of  $H_E$  over the same seven loci was lower in the Gofuku-ji population than in two  
9    other large, continuous populations at Hatomachi Pass and Mt. Haku-san. Correspondingly, the  
10   three effective population sizes ( $N_e$ ) estimated for the Gofuku-ji population were smaller than  
11   those for the two continuous large populations. Furthermore, as already discussed, the level of  
12   inbreeding within the population may have been increased. Generally, fragmented populations  
13   of long-lived woody species show little or no loss of genetic diversity compared to that in  
14   continuous populations, because most forest fragmentation has occurred recently, in the last  
15   20–200 years, thus most fragmented populations may have passed through only one or two  
16   generations since their isolation from continuous forests (Kramer et al. 2008). The longevity of  
17   forest trees, combined with effective pollen and seed dispersal, can enhance their resistance to

1 adverse effects of habitat fragmentation (Hamrick 2004; Lowe et al. 2015). Our results suggest  
2 that levels of genetic diversity and inbreeding in the Gofuku-ji population have been historically  
3 reduced and increased, respectively, due to its small size and limitations of pollen and seed  
4 immigration, indicating that the population may have been isolated for a relatively long time.  
5 However, the population's  $H_E$  values were still high (although lower than those of the large  
6 continuous Hatomachi Pass and Mt. Haku-san populations), and no evidence of a recent  
7 bottleneck (i.e. no  $H_E$  excess) was observed in genotypes of adult trees, but there was significant  
8  $H_E$  deficiency. These results suggest that other factors, such as evolutionary history, have also  
9 influenced the population's genetic composition.

10 Significant linkage disequilibrium (LD) was found in 37.2% of all possible locus-pairs in the  
11 Gofuku-ji population. Low effective population size is generally one of the important causes for  
12 LD (Flint-Garcia et al. 2003). Low effective population size may increase the level of  
13 inbreeding, and consequent increases in frequencies of homozygotes may reduce frequencies of  
14 effective recombination. However, the Gofuku-ji population showed an excess of  
15 heterozygote frequencies rather than an excess of in homozygote frequencies. Therefore, the  
16 high degree of LD may be due to other mechanisms, involving admixture of populations with  
17 differing allele frequencies (Hartl and Clark 2007). Genetic divergence between *F. crenata*

1 populations on the Japan Sea and Pacific sides of the Japanese archipelago has been detected in  
2 both nuclear microsatellite loci (Hiraoka and Tomaru 2009) and chloroplast DNA (Fujii et al.  
3 2002). The chloroplast DNA haplotype (E) detected in the Gofuku-ji population is mainly found  
4 in populations of the Pacific side, but the population is located at the boundary of the two sides  
5 (Koyama et al. 2012). Two distinct clusters of nuclear DNA are geographically structured  
6 between populations on the Japan Sea and Pacific sides (Hiraoka and Tomaru 2009), and there  
7 is a clinal admixture of the two clusters in the inland area where the Gofuku-ji population is  
8 located (Koyama 2012; revealed by STRUCTURE analysis, Pritchard et al. 2000). Therefore,  
9 the significant LD in the Gofuku-ji population may have resulted from admixture of Japan Sea  
10 and Pacific side populations, probably mediated by immigration of nuclear genes via pollen  
11 dispersal from adjacent populations of the Japan Sea side.

12 The analysis using the BOTTLENECK program showed that the  $H_E$  value for the Gofuku-ji  
13 population was significantly lower than the  $H_{eq}$  value expected under mutation–drift equilibrium.  
14 This indicates that the population has an excess number of alleles when compared with the  
15 number expected in a population having the same value of  $H_E$  under mutation–drift equilibrium  
16 (Maruyama and Fuerst 1985). This excess number of alleles may be due to substantial historical  
17 gene flow via pollen from Japan Sea side population to the Gofuku-ji population, which may

1 have increased numbers of alleles to greater than expected levels in the population. Moreover,  
2 population admixture could also explain the negative  $F_{IS}$  values for adult trees in the Gofuku-ji  
3 population, which may have resulted from the increase in heterozygotes due to historical gene  
4 flow between populations with differing allele frequencies (the Wahlund principle; Hartl and  
5 Clark 2007). Thus there may have been substantial pollen immigration from Japan Sea side  
6 populations to the Gofuku-ji population before the population became severely isolated, as  
7 already described. Following isolation, pollen immigration would have fallen to low  
8 contemporary rates, while the level of inbreeding within the population has risen. For all of  
9 these reasons, historical population admixture probably accounts for much of the significant LD,  
10 negative  $F_{IS}$ ,  $H_E$  deficiency compared to  $H_{EQ}$  and relatively high  $H_E$  values (despite the limited  
11 population size) detected in the Gofuku-ji population.

12

### 13 *Conclusions*

14 The severe isolation in the Gofuku-ji population could prevent contemporary pollen  
15 immigration, although some pollen dispersal from outside the Gofuku-ji population occurred  
16 over greater distances than 7 km. There are also indications of substantial previous admixture  
17 with other populations, but the population's small size, severe isolation, and consequently high

1 level of inbreeding, may cause declines in its seed production and genetic diversity. These  
2 effects could have serious consequences for the persistence of the Gofuku-ji population. In the  
3 present study, we focused on only one population. Further studies of gene flow via pollen in  
4 other populations with various degrees of isolation are needed to enhance our understanding of  
5 the effects of population isolation and long-distance pollen dispersal in order to develop  
6 effective conservation strategies for small isolated populations of *F. crenata* and similar species.

7

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15

#### 16 **Data Archiving Statement**

17 All genotype data in this study will be deposited in the Dryad Digital Repository.

1

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1 **Table 1** Genetic diversity estimates at the 13 microsatellite loci for 79 adult trees of *Fagus*  
 2 *crenata* in the Gofuku-ji population

Locus	Dye	<i>A</i>	$H_O$	$H_E$	$F_{IS}^a$
<i>FS1-03</i>	FAM	11	0.949	0.796	-0.193 *
<i>FS4-46</i>	PET	10	0.747	0.746	-0.001
<i>Fl15</i>	FAM	19	0.924	0.839	-0.102
<i>sfc0007-2</i>	VIC	14	0.848	0.712	-0.191
<i>sfc0018</i>	NED	12	0.924	0.765	-0.208
<i>sfc0036</i>	NED	14	0.873	0.828	-0.055
<i>sfc0161</i>	FAM	29	0.911	0.862	-0.058
<i>sfc0195-2</i>	PET	6	0.595	0.548	-0.086
<i>sfc0305</i>	NED	18	0.924	0.842	-0.097
<i>sfc0360-2</i>	PET	4	0.203	0.188	-0.075
<i>sfc1063</i>	NED	12	0.937	0.838	-0.118
<i>sfc1105</i>	FAM	19	0.726	0.662	-0.091
<i>sfc1143</i>	VIC	16	0.886	0.833	-0.064
Mean		14.2	0.804	0.728	-0.104 ***

3 Dye, fluorescent dye used to label the forward primer of each pair; *A*, number of alleles  
 4 detected;  $H_O$ , observed heterozygosity;  $H_E$ , expected heterozygosity;  $F_{IS}$ , inbreeding coefficient.  
 5 <sup>a</sup>Departure from Hardy–Weinberg equilibrium at each locus and across loci was evaluated by  
 6 the exact test. Bonferroni correction was used to determine significance in the multiple tests. \**P*  
 7 < 0.05, \*\*\**P*<0.001.

8

- 1 **Table 2** Parent pairs (represented by the identification number of adult trees) of 100 *Fagus*  
 2 *crenata* seedlings inferred from the parentage analysis

Parent pair		No. of seedlings
671	648	24
671	649	1
671	654	2
671	664	4
671	674	22
671	679	28
671	689	7
671	718	1
671	731	1
674	686	1
679	689	2
686	700	1
671	-	6

- 3 All candidate parents for the seedlings analyzed were present in the Gofuku-ji population.  
 4 The hyphen indicates seedlings that had one candidate parent (671), but not the other, within the  
 5 Gofuku-ji, Bunano-ki Gongen, and Mt. Futatsu-yama populations (indicating pollen dispersal  
 6 from outside the populations).

7

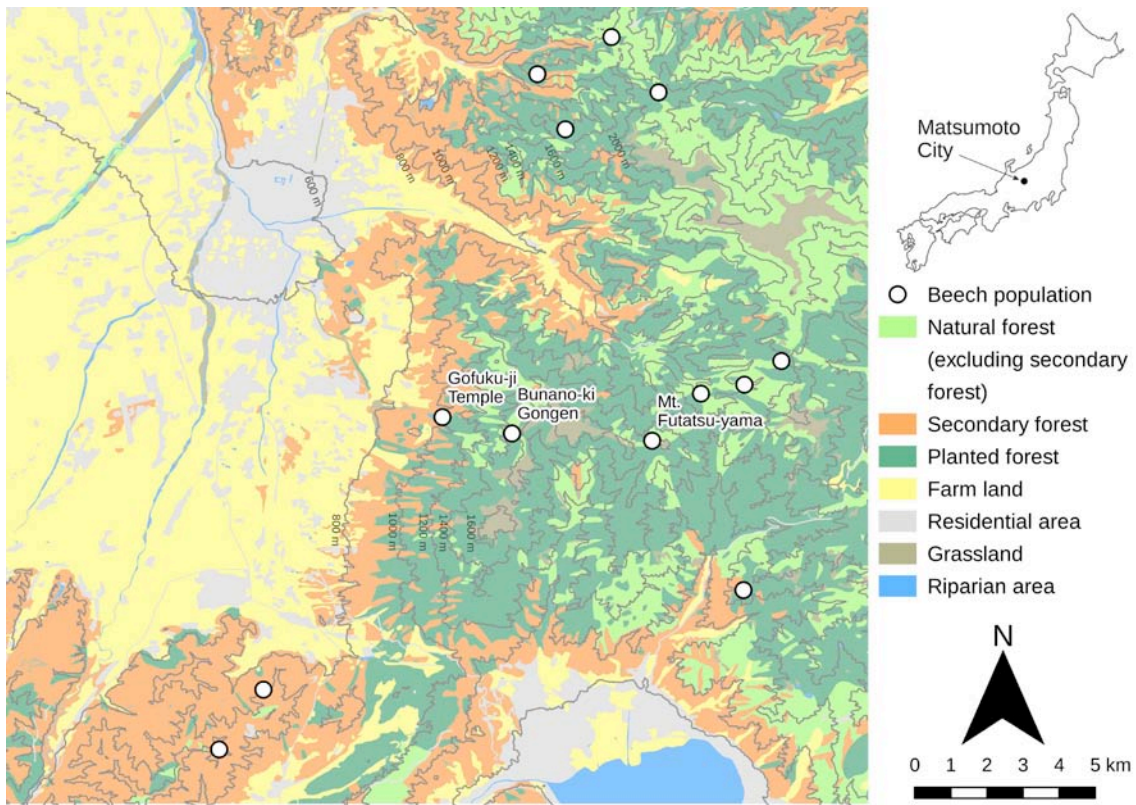


1 **Table 3** Inbreeding coefficients at the 13 microsatellite loci for 100 *Fagus crenata* seedlings in  
 2 the Gofuku-ji population

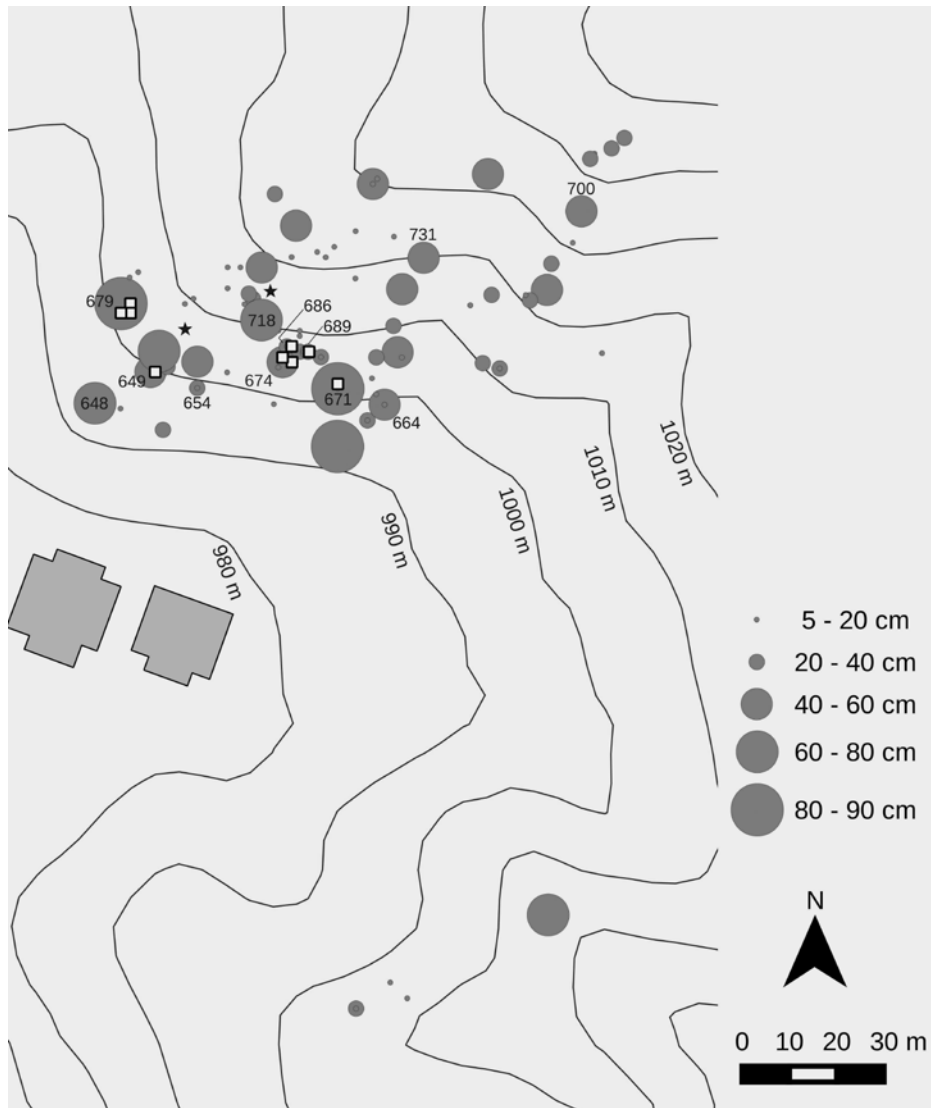
Locus	$F_{IS}^a$
<i>FS1-03</i>	-0.017
<i>FS4-46</i>	-0.345 ***
<i>F115</i>	-0.219 ***
<i>sfc0007-2</i>	-0.150
<i>sfc0018</i>	-0.221 ***
<i>sfc0036</i>	-0.052
<i>sfc0161</i>	-0.156 ***
<i>sfc0195-2</i>	-0.174
<i>sfc0305</i>	-0.188 ***
<i>sfc0360-2</i>	-0.023
<i>sfc1063</i>	-0.278 ***
<i>sfc1105</i>	-0.162 *
<i>sfc1143</i>	-0.204 ***
All loci	-0.180 ***

3 <sup>a</sup>Departure from Hardy–Weinberg equilibrium at each locus and across loci was evaluated by  
 4 the exact test. Bonferroni correction was used to determine significance in the multiple tests. \**P*  
 5 < 0.05, \*\*\**P*<0.001.

6



1 **Fig. 1** Map of land cover around the *Fagus crenata* population at Gofuku-ji Temple in  
 2 Matsumoto City, Nagano Prefecture, Japan, generated from vegetation maps from the Japanese  
 3 Ministry of the Environment (<http://www.vegetation.biodic.go.jp/index.html>) and Fundamental  
 4 Geospatial Data from the Geospatial Information Authority of Japan  
 5 (<http://www.gsi.go.jp/kiban/index.html>). Only two beech populations were located within a  
 6 7-km radius from the Gofuku-ji population, at Bunano-ki Gongen and Mt. Futatsu-yama, but  
 7 other small isolated populations were scattered at further distances.  
 8



1

2 **Fig. 2** Map of the Gofuku-ji population with 10-m contours, showing locations of the adult  
 3 *Fagus crenata* trees (filled circles with size ranges indicating diameters at breast height and  
 4 integers indicating identification numbers of the parents of seedlings inferred from the  
 5 parentage analysis; see Results), the nine seed traps (open squares) included in the analysis, and  
 6 the two seed traps excluded from the analysis (black stars).

7

1 **Electronic supplementary materials**

2 **Table S1** Locations of five *Fagus crenata* populations studied by Koyama and Ida (2013), numbers of seed traps set in each population, and means

3  $\pm$  standard errors of the proportion of empty seeds and germination rate of sound seeds among seed traps in each population.

Population	Latitude	Longitude	Altitude (m)	Number of seed traps	Proportion of empty seeds	Germination rate
Continuous populations						
Nabekura	36°58'38"	138°23'31"	1000	5	0.222 $\pm$ 0.129 <sup>a</sup>	0.646 $\pm$ 0.062 <sup>a</sup>
Kayano-daira	36°50'18"	138°30'00"	1500	7	0.483 $\pm$ 0.067 <sup>b</sup>	0.709 $\pm$ 0.067 <sup>a</sup>
Isolated populations						
Ohbora	36°30'11"	138°19'42"	1360	5	0.323 $\pm$ 0.079 <sup>c</sup>	0.642 $\pm$ 0.163 <sup>a</sup>
Hijiri-yama	36°29'33"	138°01'13"	1180	5	0.549 $\pm$ 0.091 <sup>b</sup>	0.674 $\pm$ 0.094 <sup>a</sup>
Gofuku-ji	36°09'56"	138°01'10"	1020	11 (2 traps were discarded)	0.693 $\pm$ 0.291 <sup>d</sup>	0.399 $\pm$ 0.322 <sup>b</sup>

4 Note: Koyama and Ida (2013) examined *Fagus crenata* seed production in 2011, when *F. crenata* populations throughout Nagano Prefecture

5 produced good crops. They set 5-11 seed traps in each of five *F. crenata* populations, of which two and three (including the Gofuku-ji population)

6 were continuous and isolated, respectively. They counted sound, empty and insect-damaged seeds caught in each trap and tested the germination

1 rates of the sound seeds. In the present study, we recalculated means and standard deviations of the proportion of empty seeds (after excluding the  
2 insect-damaged seeds) and the germination rate of the sound seeds, among traps in each population, using the raw data they collected. We then  
3 compared the proportion of empty seeds and the germination rate obtained for the Gofuku-ji population with corresponding values for the other  
4 two isolated and two continuous populations using a generalized linear mixed model (GLMM) and a likelihood ratio test. Two GLMM models  
5 were constructed: one explaining the proportions of empty seeds and the other explaining the germination rates. We used a binomial error  
6 distribution and a logit-link function for the response variables of sound or empty seeds (0/1) and germinated or not (0/1), “seed trap” as a random  
7 effect variable, and “all possible groupings of populations” [46 sets, e.g. (Nabekura, Kayano-daira) and (Ohbora, Hijiri-yama, Gofuku-ji)] as fixed  
8 effect variables. In each GLMM model, the best model with one set of population grouping as a fixed effect variable was then selected using  
9 Akaike’s Information Criterion. Once the best model was selected, we performed a likelihood ratio test (the best model vs. the model with “seed  
10 trap” as a random effect variable but without any fixed effect variables) to examine the significance of the population grouping. The groupings of  
11 populations in the best models explaining the proportions of empty seeds and the germination rates (indicated with the same superscripts in Table

- 1 S1) were both significant at  $P < 0.001$  according to the likelihood ratio tests. Therefore, the proportion of empty seeds and the germination rate
- 2 were found to be significantly higher and lower for the Gofuku-ji population than for the other four populations, respectively.