Genetic consequences of rapid population decline and restoration of the critically endangered herb *Polemonium kiushianum*

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Abstract

Many critically endangered species require not only in situ but also ex situ conservation to reduce extinction risk. In this study, all five known wild populations and two artificially managed ex situ populations outside the species' native range of *Polemonium kiushianum*, a critically endangered herb species in Japan, were studied, using 10 polymorphic microsatellite markers to assess the genetic consequences of habitat degradation on the wild populations and the establishment of ex situ populations. Levels of genetic diversity among the wild populations were similar to each other, whereas genetic diversity in the ex situ populations was considerably lower than that of the wild populations. A significant level of genetic differentiation was associated with a recent bottleneck and genetic drift in the wild populations. The STRUCTURE

analysis revealed that the genetic composition of the two ex situ populations differed from that of the five wild populations. The low genetic diversity observed in the ex situ populations and different genetic composition between wild and ex situ populations may be due to genetic drift with few founders for the ex situ populations and the management strategy used for the ex situ populations. Seed transfer simulations using simulated genotypes generated on computer programs indicated that 1,000 or more seeds were needed to enhance genetic diversity and recover the genetic composition of the present ex situ populations. These simulations used to predict the genetic consequences of immigration represent a powerful tool for conservation management of critically endangered species based on genetic data.

Keyword: ex situ conservation, genetic drift, genetic structure, microsatellite, *Polemonium kiushianum*, seed transfer simulation

1. Introduction

Many plant species throughout the world are threatened with extinction due to habitat destruction and fragmentation as a result of human activities. Populations of endangered species in their natural habitat are vulnerable to loss of genetic diversity due to a decline and/or fluctuation in population sizes, resulting in reduced gene flow among remnant populations, inbreeding, and genetic drift (Lowe et al., 2005; Frankham et al., 2010). Empirical and experimental studies also indicate that demographic decline and population bottlenecks cause or contribute to a loss of genetic diversity (Cornuet and Luikart, 1996; Young et al., 1996). The loss of genetic diversity associated with demographic decline can affect the viability of the population in short term (Keller and Waller 2002) and limitations in the species' ability to respond to the changing environment in long term (Young et al 1996).

Although preservation of the natural habitat is critical for the conservation of ecological interactions, ex situ measures may be appropriate for safeguarding individual species against extinction in the wild (Russello and Amato, 2007). The primary purpose of ex situ conservation is to maintain wild species outside their natural habitat so that species recovery and reintroduction can be attempted in the case of severe decline or extinction of wild populations (Husband and Campbell, 2004). However, despite the significance of ex situ conservation in endangered species management, these populations are exposed to forces similar to those encountered by in situ populations, which may decrease genetic diversity. For example, ex situ populations usually have a restricted founder source and small population size and are susceptible to inbreeding and inbreeding depression (Frankel and Soulé, 1981; Williams et al., 2002). Low genetic diversity in ex situ populations is likely to increase extinction risk to an equal extent as seen in wild populations.

In cases of low genetic diversity in ex situ populations, restoration of genetic diversity is achieved by the immigration of additional breeding stock from wild populations. Moreover, information regarding the number of these stocks needed to enhance the genetic diversity of ex situ populations is an important consideration. However, immigration from wild to ex situ populations increases the short-term risk of extinction of wild populations by removing individuals (Menges et al., 2004). Thus, prior prediction of the effects of immigration of additional breeding stocks by simulation approaches prior to their removal from wild populations will provide useful information regarding the quantities of additional breeding stocks that are required for recovery of genetic diversity in ex situ populations.

Polemonium kiushianum Kitam. (Polemoniaceae) is an endangered perennial herb endemic to the semi-natural grasslands of the Aso region of Kyushu, Japan (Environment Agency of Japan, 2000). This species has experienced rapid population decline due to habitat

loss as a result of conifer plantation establishment and the abandonment of traditional grassland management (Sei, 2006). Only a few hundred individuals and five populations of this species remain, and most subpopulations have become locally extinct in the last three decades. As a result, *P. kiushianum* has been categorized as 'critically endangered' (CR) in the Japanese Red Data Book, and is now protected by the 'Law for the Conservation of Endangered Species of Wild Fauna and Flora' in Japan. In response to this situation, ex situ populations outside the species' native range were founded to provide for the degradation of the wild populations. They show promise as a temporary pool of genetic diversity that can be used to bolster wild populations. *Polemonium kiushianum* provides an excellent model for comparing the impact on genetic diversity that population decline of wild populations and the recent establishment of ex situ populations has made, and allows for an examination of the potential use of simulations to predict the impact of seed transfer for enhancing the genetic diversity of ex situ populations. In the present study, the genetic status of all remaining wild populations of *P. kiushianum* and its ex situ populations were assessed using polymorphic microsatellite markers. The study aimed to evaluate the following: (1) the genetic diversity, genetic structure, and recent demographic history in wild populations; (2) the genetic consequences of the foundation of ex situ populations; and (3) the possibility of recovering genetic diversity in ex situ populations by seed transfer from the remaining wild populations using simulated genotypes generated on a computer program.

2. Material and methods

2.1. Study site and species

The Aso region is located in central Kyushu, south-western Japan (Fig. 1), and consists

of central volcanic mountains (up to 1,592 m above sea level [a.s.l.]) and surrounding somma (800–1,100 m a.s.l.). The mean annual temperature and annual precipitation between 1992 and 2011 were 13.4°C and 2,386 mm, respectively (Takamori Meteorogical Observation Station, at 555 m a.s.l.). In the Aso region, vast semi-natural grasslands have been maintained by human-related influences such as hand mowing, artificial burning, and livestock grazing (Takahashi, 2009). Phytolith and microscopic charcoal analyses have shown that grasslands in this region have been established for over 10,000 years, and formation of these grasslands has been attributed to burning as a consequence of anthropogenic activities (Miyabuchi et al., 2012). Since vast grassland vegetation has been maintained, the Aso region provides a habitat for rare grassland herbs such as *Echinops setifer*, *Viola orientalis*, and *Campanula glomerata* var. *dahurica* (Hotta, 1974), and has high grassland plant species diversity. However, due to abandonment of the traditional management of grasslands and land-use change from grasslands to conifer plantations or pastures, the area of the semi-natural grasslands has decreased dramatically in recent years and, the populations of many grassland plant species have undergone a severe decline (Takahashi, 2009).

Polemonium kiushianum is an endangered plant of eastern somma of the Aso region. This species is a diploid (2n = 18, Inaba et al., 2010) perennial herb that occurs in sunny meadows maintained by mowing at altitudes of approximately 700-900 m. This species is not a clonal plant and the individual longevity might be 3–4 years, with a maximum of up to 10 years (Yokogawa et al., unpublished results). Pollinators of this species are bumblebees or small solitary bees (Yokogawa et al., unpublished results) and seeds are dispersed by gravity. Whereas 46 wild populations were identified from 1988 to 1993 (Sei, 2006), only seven wild populations were found in 2004 (Matoba et al., 2011). This number had declined to only five wild populations by 2009 (Fig. 1; Table 1). The habitats of three of the remaining five wild

121 populations have been degraded by artificial conifer plantations (mainly Japanese cedar,
122 *Cryptomeria japonica* (L.f.) D. Don), and thus the population size is very small (Table 1;
123 population W3, W4, and W5). In contrast, the other two populations that remain in mowed
124 grassland have relatively large population sizes (Table 1; population W1 and W2). However, the
125 sizes of these populations were very small 10 years ago due to the abandonment of mowing.
126 After the population decline, conservation activities such as the restart of mowing were
127 conducted, and the population sizes subsequently recovered. The two ex situ populations were
128 established outside the species' native range, and from seeds collected from the W2 and W3
129 populations in 1999-2001 (Table 1; population E6 and E7). The seed sources of each ex situ
130 population, whether from one seed source population (W2 or W3) or both seed source
131 populations (W2 and W3), could not be clearly distinguished. Populations E6 and E7 are about
132 5 km and 25 km away from the edge of the species' native range, respectively. The growing
133 environment of *P. kiushianum* in E6 is the deciduous forest floor with mowing in autumn and
134 that of E7 is sunny grasslands with mowing in autumn.
135
136 *2.2. Sampling and microsatellite analysis*
137 In 2008 and 2009, leaf samples of 182 individuals of *P. kiushianum* were collected
138 from the five wild populations (Fig. 1) and the two ex situ populations. In large populations
139 without sampling restriction, we randomly selected over 20 individuals spaced at least 2 m apart,
140 or all individuals if less than 20 were present (in population W3). The population size and
141 sample size of each population are indicated in Table 1 and Table 2, respectively. Genomic DNA
142 was extracted using a modified CTAB method (Milligan, 1992).
143 The genotypes of each individual were characterized at 10 microsatellite loci. Seven
144 out of the 10 loci were developed by Yokogawa et al. (2009): *Pkiu006*, *Pkiu059*, *Pkiu129*,

Pkiu135, *Pkiu208*, *Pkiu212*, and *Pkiu227*. We designed three additional microsatellite primer pairs, *Pkiu593*, *Pkiu627*, and *Pkiu965* (Table A.1), using the same protocol as Yokogawa et al. (2009). The PCR amplifications were performed following the standard protocol of the Qiagen Multiplex PCR kit (Qiagen), in a final volume of 6 μL, which contained 5 ng of extracted DNA, 3 μL of 2× Multiplex PCR Master Mix, and 0.2 mmol/L of each multiplexed primer. The PCR amplifications were carried out with a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems), using the following conditions: initial denaturation at 95°C for 15 min, followed by 28 cycles of denaturation at 94°C for 30 s, annealing of the designed specific primers at the designated temperatures for 1 min 30 s, extension at 72°C for 1 min, and final extension at 60°C for 30 min. The sizes of the PCR products were measured using an ABI PRISM 3100 Genetic Analyser and Genotyper software (Applied Biosystems).

2.3. Statistical analysis of genetic diversity and structure

For each population, the genetic diversity was evaluated in terms of Nei's unbiased expected heterozygosity (H_E; Nei, 1987) and observed heterozygosity (H_O), the average number of alleles per locus (*A*), allelic richness (*AR*; El Mousadik and Petit, 1996), the summed number of rare alleles with frequencies less than 5% among the total population (*RA*), the summed number of private alleles that are only present in a single population (*Pr*), and the fixation index (F_{IS}). With the exception of the numbers of rare and private alleles, all of these parameters were calculated using FSTAT ver. 2.9.3 software (Goudet, 2001). Deviation from Hardy-Weinberg equilibrium was determined using FSTAT.

Recent bottlenecks in the populations were evaluated by BOTTLENECK ver. 1.2.02 (Piry et al., 1999). We simulated equilibrium conditions (10,000 replications) assuming the infinite allele mutation model (IAM) and the two-phase model (TPM, mutations with 95%

single-step mutations and 5% multistep mutations, with a variance among multiple steps of approximately 12). We used the Wilcoxon signed rank test to determine a significant excess of heterozygosity. Population W3 was excluded from this analysis due to its small sample size.

To estimate the genetic differentiation among populations, F_{ST} values (Weir and Cockerham, 1984) were calculated. The significance of F_{ST} values was tested by comparison to the 95% confidence intervals derived from 1,000 bootstrap permutations. Pairwise F_{ST} values were calculated by randomizing multilocus genotypes between two populations with Bonferroni corrections. We also calculated standardized values of G'_{ST} (Hedrick, 2005) using averaged values of heterozygosity within populations at Hardy-Weinberg equilibrium (H_S), the expected heterozygosity of all populations pooled (H_T), and genetic differentiation among populations (G_{ST}).

We evaluated genetic relationships among populations using Bayesian clustering STRUCTURE ver. 2.3 (Prichard et al., 2000), which assigns individuals into K clusters. Population structure was simulated with values of K = 1–10 under an admixture model, the correlated allele frequencies model (Falush et al., 2003), and the LOCPRIOR model (Hubisz et al., 2009). All runs involved 1,000,000 Markov chain Monte Carlo generations, after a burn-in period of 100,000 iterations. Twenty runs were performed for each value of K. The number of clusters was determined by comparing mean values and variability of log likelihoods in each run. To select the optimal value of K, we also used the ΔK method (Evanno et al., 2005). The F value, the amount of genetic drift between each cluster and a common ancestral population, and the expected heterozygosity were calculated for each cluster.

2.4. The Optimization method and Simulation analysis of seed transfer from wild to ex situ populations

First, to evaluate the necessity of each wild population as seed sources to restore genetic diversity in ex situ populations, we carried out optimization method with simulated annealing algorithm (Possingham et al. 2000). Although the presence-absence matrix of species was used for optimization analysis in the original paper, an allele matrix by population was used as being directly analogous to species in the present study. We defined alleles as our conservation goal and the optimization problem was to find the smallest number of wild populations that better complement the allele composition already preserved in ex situ populations (see Diniz-Filho et al. 2012). We used the selection frequency at which each population appeared in 100 analyzed solutions as an indicator of the importance of the seed source. We also assumed that the ex situ populations were already protected. These analyses were conducted using the Marxan software (Ball et al. 2009).

Second, to evaluate the genetic effects of transferring seeds from wild to ex situ populations, simulation analyses of seed translocation were conducted using the current data of both wild and ex situ populations. We generated seed genotypes based on the actual genotypes of wild populations using HYBLIDLAB (Nielsen et al., 2006) and simulated the possible changes in genetic diversity and genetic composition of the ex situ populations after the addition of these seed genotypes. This analysis was undertaken using a 2-stage process. Firstly, 20 seed genotypes per seed parent were generated assuming random mating using HYBRIDLAB. Each seed genotype was generated from genotype data of the seed parent source population. This process was undertaken for 10 independent runs, where the number of wild individuals from which seeds were artificially collected ranged from 10 (200 generated genotypes) to 100 (2000 generated genotypes), with the sample size for each run differing by increments of 10. Of the total number of seeds generated for each run, only 20% of seeds were retained for analysis (with the remaining 80% randomly deleted), a strategy simulating the 80% mortality rate of *P*.

kiushianum observed by Otaki (2000). The second step involved crossing of the individuals in the ex situ population with the generated seed genotypes using HYBRIDLAB. For each of the 10 runs, 30 F_1 seeds were generated and used for the analysis of genetic diversity. The genetic diversity of each run was evaluated in terms of expected heterozygosity and allelic richness. Simulated genotypes were analyzed for genetic structure using STRUCTURE ver. 2.3 with genotypes of the wild and ex situ populations. The same procedures described above for the STRUCTURE analysis were performed with $K = 2$.

3. Results

3.1 Genetic diversity and population bottlenecks

Levels of genetic diversity among wild populations were similar to each other, and genetic diversity in the ex situ populations was considerably lower than that in the wild populations. For the 10 polymorphic loci genotyped, a total of 57 alleles were observed among 182 individuals of *Polemoniun kiushianum.* The number of alleles per locus (*A*) ranged from 2 to 13, with an average of 5.7. The allelic richness within each population (*AR*) ranged from 2.45 to 2.94, with an average of 2.70. The average observed heterozygosity (H_O) and expected heterozygosity (H_E) within each population ranged from 0.34 to 0.47, with an average of 0.39, and from 0.37 to 0.47, with an average of 0.40, respectively (Table 2). The fixation index (F_{IS}) value did not deviate significantly from zero in any population. Twenty-eight alleles (49%) were rare alleles (*RA*) with frequencies of less than 5%, and 16 alleles (28%) were private alleles (*Pr*) that were only present in a single population. Many rare or private alleles were found in wild populations, whereas only one rare and one private allele were found in the ex situ populations (Table 2). BOTTLENECK (tested by Wilcoxon's signed rank test) analysis indicated recent

population bottlenecks in all analysed wild populations. Under the IAM, a significant excess of heterozygosity was detected in all analysed wild populations, which was not detected in the two ex situ populations (Table 2). There was no excess of heterozygosity in any of the six populations under the TPM (Table 2).

3.2 Genetic differentiation and structure of wild and ex situ populations

Significant genetic differentiation was observed among the populations. The F_{ST} value was 0.100 with 95% confidence intervals of 0.073 to 0.124 across all seven populations, and 0.092 with 95% confidence intervals of 0.63 to 0.126 across the five wild populations, respectively. Pairwise F_{ST} estimates ranged from 0.03 to 0.21 (Table A.2) and all of these were significantly larger than 0 despite the small geographic distance between the wild populations. The G'_{ST} value was 0.180 across all seven populations and 0.166 across the five wild populations.

STRUCTURE analysis indicated that populations of *P. kiushianum* are divided into distinct genetic clusters (Fig. 2). The ΔK value representing the hierarchical approach for STRUCTURE analysis was clearly the highest at $K = 3$ (Fig. 2b). Thus, $K = 3$ was the uppermost hierarchical level of genetic structure. Meanwhile, although the variance of log likelihood among runs was high, and the results of membership analyses were unstable and multimodal among runs at $K \geq 5$ (Fig. 2a), the variance of log likelihood among runs was low and no multimodalities were detected at $K = 4$. Therefore, $K = 4$ also yielded meaningful results. Consequently, the results obtained with $K = 3$ and $K = 4$ are shown herein (Fig. 2c). When $K = 3$, individuals were clearly divided into 3 clusters. Wild populations W1, W2, and W3, wild populations W4 and W5, and ex situ populations E6 and E7 were assigned to cluster I, cluster II, and cluster III, respectively (Fig. 2). The *F* values of clusters I and II were lower than that of

cluster III, and the expected heterozygosity of cluster I, II, and III was 0.46, 0.43, and 0.38, respectively (Fig. 2). These results suggested that ex situ populations have undergone larger genetic drift compared to the wild populations. When $K = 4$, cluster II at $K = 3$ was divided into 2 clusters, cluster II-a, and II-b (Fig 2). The F value of cluster I was low, cluster II-a was moderate, and that of cluster II-b and III was high. The expected heterozygosity of cluster I (0.45) was the highest, cluster II-b (0.42) was moderate, and that of cluster II-a (0.39) and III (0.38) was the lowest.

3.3 Simulation of genetic diversity and composition of seed transfers to the ex situ population

According to the results of the optimization method with simulated annealing algorithm (Possingham et al. 2000), the solution frequencies of all wild populations were 100% (i.e., they were found in 100% of the solutions). Thus, all five wild populations were necessary as seed source for ex situ populations to preserve all alleles in ex situ populations. Seed transfer simulations indicated that random seed transfer from wild to ex situ populations required a substantially large number of seeds in order to recover the genetic diversity and genetic composition of the ex situ population. When the number of seeds transferred from wild to ex situ populations was between 600 and 1000, the allelic richness and expected heterozygosity of the simulated ex situ populations reached a plateau (Fig. 3), although the number of individuals in each of the two ex situ populations was approximately 100. The assignment probability of wild clusters of simulated ex situ populations peaked when the number of seeds transferred from wild to ex situ populations was 1600 (Fig. 3). To achieve ex situ population genetic compositions that closely approximated those of the wild populations, an abundance of seeds from the wild populations was needed.

4. Discussion

4.1 Genetic characteristics and demographic history of wild populations

Although population sizes of the large wild populations (W1 and W2) of *Polemonium kiushianum* were more than 10 times larger than those of the small wild populations (W3, W4, and W5), the levels of allelic diversity and heterozygosity among the wild populations were similar to each other. In general, allelic diversity and heterozygosity are positively correlated with population size (e.g. Leimu et al., 2006). However, in the presence of a population undergoing size fluctuations, genetic diversity is most strongly influenced by the generation of a minimum population size (Frankham et al., 2010). The relatively low genetic diversity in large wild populations W1 and W2 may be explained by fluctuations in the sizes of these populations. Small W1 and W2 population sizes were observed 10 years prior to this study due to the abandonment of mowing and short-term population bottleneck effects were also indicated by the BOTTLENECK analysis under IAM in the present study. Therefore, the genetic diversity of the large wild populations may reflect their small size observed 10 years prior to this study. On the other hand, population W3 has high genetic diversity with 2 private alleles compared to the other populations (Table 2) despite having the smallest population size (Table 1). This population had a large population size 20 years ago (Otaki 2000); this suggests that population W3 is likely to harbor past genetic diversity.

Despite the small geographic range of *P. kiushianum*, there was significant genetic differentiation among the wild populations. The wild populations of this species experienced genetic drift and population bottleneck. Given the short longevity of this species (3-4 years and up to 10 years; Yokogawa et al. unpublished results) and the drastic decrease in the semi-natural grassland, including the habitat of this species, over the past century in the study area (Shoji, 2006), this genetic differentiation and drift are likely to have occurred in association with

habitat fragmentation. Other studies on endangered plants in fragmented grassland on similar geographic scales (10–20 km) suggest that genetic drift and bottlenecks can lead to population genetic differences after fragmentation of grassland areas (Honnay et al., 2006; Jacquemyn et al., 2010). Population differentiation and the effects of genetic drift in *P. kiushianum* may indicate that gene flow has been disrupted by population fragmentation. Given that pollinators of this species are bumblebees and small solitary bees (Yokogawa et al. unpublished data) and their maximum foraging distance are several hundred meter and up to 1.5 km (Knight et al., 2003; Zurbuchen et al., 2010), pollinator-mediated gene flow between remnant populations (minimum pairwise geographic distance is 1.4 km; Table A2) is unlikely to occur. Thus, it is important to restore local extinct populations as stepping-stones to increase gene flow between the remnant wild populations.

4.2 Genetic diversity and composition of ex situ populations

The genetic diversity of the ex situ populations of *P. kiushianum* was generally lower than that of the wild populations, particularly for rare and private alleles. These results indicate that the genetic diversity in ex situ populations may not be sufficient to maintain the genetic diversity of the species in the case of extinction of wild populations. In plant population genetic studies comparing genetic diversity between ex situ populations and wild populations, similar observed levels of genetic diversity in wild and ex situ populations have been found in some of the populations that were studied, for example the short-lived herb *Cynoglossum officinale* (Enßlin et al., 2011) and the endangered Chinese tree *Vatica guangxiensis* (Li et al., 2002). The high genetic diversity observed in ex situ populations could be due to mating among plants from several populations of different origin (Enßlin et al., 2011). In contrast, genetic diversity in ex situ populations was lower than that of natural populations of transplanted eelgrass *Zostera*

337 *marina* (Williams and Davis, 1996), fruit tree *Inga edulis* (Hollingsworth et al., 2005), and
338 evergreen oak *Cyclobalanopsis myrsinaefolia* (Liu et al., 2008). The low genetic diversity in ex
339 situ or planted populations could be due to founder effects associated with the establishment of
340 these populations. The ex situ populations of *P. kiushianum* would also have lost genetic
341 diversity during establishment.
342 The results of the STRUCTURE analyses indicated not only the reduction of genetic
343 diversity but also changes in genetic composition in the ex situ populations compared with those
344 of the wild populations. These results may have been caused by founder effects that occurred
345 when the ex situ populations were established and/or genetic drift after establishment of the ex
346 situ populations associated with small population size and management strategies. Genetic
347 differences between wild and artificial populations caused by insufficient sampling of founders
348 for artificial populations have been reported in other plant species (Li et al., 2005). Many
349 individuals in restored populations of *P. kiushianum* have been regenerated every year to obtain
350 nursery stock for markets, and alternation of generations in ex situ populations would be more
351 rapid than that in wild populations. These management strategies could drive genetic drift after
352 the establishment of ex situ populations. Population bottlenecks in ex situ populations were not
353 detected by BOTTLENECK analysis, as the ex situ populations achieved near mutation-drift
354 equilibrium (see Priy, 1999) as a consequence of management for faster alternation of
355 generations, while *F* values (as indicators of genetic drift in the STRUCTURE analysis) were
356 high in the ex situ populations. The F_{ST} values for both wild and ex situ populations were higher
357 than the F_{ST} values for wild populations. Moreover, although the ex situ populations E6 and E7
358 were established using seeds collected from populations W2 and W3, these populations were
359 not clustered together in the STRUCTURE analysis (Fig 2). These results also suggest that two
360 ex situ populations experienced severe genetic drift, and that the genetic composition of these

populations differed substantially compared to the wild populations. The low genetic diversity and different genetic composition observed in ex situ populations may indicate genetic deterioration in ex situ populations through the process of establishment and management of these populations.

4.3 Restoration of genetic diversity in ex situ populations and effectiveness of seed transfer simulation

The genetic deterioration in ex situ populations used as reintroduction sources leads directly to the success or failure of reintroduction, and individuals used for reintroduction should have high genetic diversity (Frankham et al. 2010). Optimization analysis revealed that additional seed sources for ex situ populations of *Polemonium kiushianum* have to be collected from all five wild populations to preserve all alleles. Moreover, the simulation of seed transfer from wild to ex situ populations demonstrated that more than 1,000 seeds were needed to achieve ex situ population genetic composition that closely approximated that of the wild populations. These results indicated that the transfer of many seeds from wild to ex situ populations could be useful for enhancing genetic diversity in ex situ populations.

However, seed transfer from wild to ex situ population could negatively affect population demographics by seed removal. Demographic models of the effects of seed collection on extinction risk of 22 perennial species revealed that harvesting 10% of seeds typically does not increase their extinction risks (Menges et al., 2004). The estimated mean value and standard deviation of the number of seeds per individual of *P. kiushianum* was 412 ± 430 (Yokogawa et al., unpublished data). Collecting less than 40 seeds from each wild individual would result in less than 10% of seeds being collected, which may have little impact on population viability. Additionally, the transfer of a small number of seeds from wild to ex situ

populations every year would be a useful approach for enhancing the genetic diversity of ex situ populations, while at the same time minimally impacting the viability of wild populations. This constant immigration from wild to ex situ population would also reduce the rate of genetic adaptation to the ex situ environment (Woodworth et al., 2002).

In general, using local seed sources to maximize local adaptation and prevent out breeding depression is recommended in the restoration of endangered species (Mijnsbrugge et al., 2010, Aavik et al. 2012). However, strict use of local seed sources can decrease the availability of high-quality seeds for restoration in highly modified landscapes (Broadhurst et al., 2008). We conducted seed transfer simulation with random seed collection from all wild populations; in other words, each population was not treated separately because the genetic differentiation observed in *P. kiushianum* is likely to be caused by habitat fragmentation with highly fragmented grassland landscapes. However, contributing factors to genetic differentiation vary among different endangered species. The management and conservation strategy of genetic diversity in ex situ populations have to be determined based on the genetic data of the target species and its surrounding landscape.

Given the extinction crisis that is occurring throughout the world, ensuring the maintenance of genetically viable ex situ populations of endangered species is crucial. This study shows that the simulation method using simulated genotypes can be used to aid conservation programs for critically endangered species based on genotype data. Most importantly, the effectiveness of any transfer of individuals or seeds can be simulated before the removal of seeds from wild populations is undertaken. These conservation approaches could provide a means to ensure efficient genetic management of ex situ populations.

5. Conclusions

409 The findings of the present study have important implications for the conservation
410 management of *Polemonium kiushianum* and other critically endangered species both in situ and
411 ex situ. Our results show that despite the differences in population sizes, the levels of genetic
412 diversity in all the remaining wild populations were similar to each other. Moreover, these
413 populations underwent genetic differentiation and severe drift associated with habitat
414 degradation, and show decreased gene flow between the remnant populations. The removal of
415 barriers to gene flow and improvement of connectivity between remnant populations will be the
416 priority for the conservation of this species. In other words, it is important to restore grasslands
417 including the habitat of this species, by restarting the management of grasslands and removing
418 artificial conifer plantations that have fragmented grasslands in the study area.
419 While the ex situ populations of *P. kiushianum* have a lower genetic diversity and
420 different genetic composition compared with those of the wild populations, seed transfer
421 simulations revealed that seed transfer from wild populations would be a useful approach to
422 enhance the genetic diversity of ex situ populations. These results also indicated that more than
423 1,000 seeds were needed to achieve the desired genetic composition in ex situ populations. We
424 recommend the constant immigration of seeds from all wild populations to ex situ populations
425 to maintain genetic diversity and ability of ex situ populations to serve as reintroduction sources.
426 Seed source collections without genetic information cause loss of genetic diversity and/or
427 changes in genetic composition in ex situ conservation (Li et al. 2005; Enßlin et al. 2011).
428 Before seed transfer management or foundation of ex situ populations, it is preferable to predict
429 their effects to preserve the genetic diversity of endangered species in ex situ conservation.
430
431 **Appendix A. Supplementary material**
432 Characteristics of three new microsatellite loci for *Polemonium kiushianum* (Table

A.1) and pairwise F_{ST} values and geographic distance among populations (Table A. 2).

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Reference

Aavik T., Edwards, P.J., Holderegger, R., Graf, R., Billeter, R., 2012. Genetic consequences of using seed mixtures in restoration: A case study of a wetland plant *Lychnis flos-cuculi*. Biological Conservation 145, 195-204.

Ball, I.R., H.P. Possingham, and M. Watts. 2009. Marxan and relatives: Software for spatial conservation prioritisation. In: Moilanen, A., Wilson, K.A., Possingham, H.P., (eds) Spatial conservation prioritisation: Quantitative methods and computational tools. Oxford University Press, Oxford, pp. 185-195.

Broadhurst, L.M, Lowe, A., Coates, D.J., Cunningham, S.A., McDonald, M., Vesk, P.A., Yates, C., 2008. Seed supply for broadscale restoration: maximizing evolutionary potential. Evolutionary Applications 1, 587-597.

Cornuet, J.M., Luikart, G,. 1996. Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. Genetics 144, 2001-2014.

Diniz-Filho, J.A.J., Melo, D.B., Oliveira, G.de, Collevatti, R.G., Soares, T.N., Nabout, J.C.,

Lima, J.de.S., Dobrovolski, R., Chaves, L.J., Naves, R.V., Loyola, R.D., Telles, M.P.C., 2012. Planning for optimal conservation of geographical genetic variability within species. Conservation Genetics 13, 1085-1093.

El Mousadik, A., Petit, R.J., 1996. High level of genetic differentiation for allelic richness among populations of the argan tree [*Argania spinosa* (L.) Skeels] endemic to Morocco. Theoretical and Applied Genetics 92, 832-839.

Enßlin, A., Sandner, T.M., Matthies, D., 2011. Consequences of ex situ cultivation of plants: Genetic diversity, fitness and adaptation of the monocarpic *Cynoglossum officinale* L. in botanic gardens. Biological Conservation 144, 272-278.

Environment Agency of Japan, 2000. Threatened wildlife of Japan –-–– Red Data Book – , Vascular plants, second ed. Japan Wildlife Research Center, Tokyo (in Japanese).

Evanno, G., Regnaut, S., Goudet, J., 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology 14, 2611-2620.

Falush, D., Stephens, M., Pritchard, J.K., 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. Genetics 164, 1567-1678.

Frankel, O.H., Soulé. M.E., 1981. Conservation and Evolustion. Cambridge University Press, Cambridge.

Frankham, R., Ballou, J.D., Briscoe, D.A., 2010. Introduction to conservation genetics, second ed. Cambridge Universtiy Press, Cambridge.

Goudet, J., 2001. FSTAT; a program to estimate and test gene diversities and fixation indices version 2.9.3. <http://www2.unil.ch/popgen/softwares/fstat.htm> (accessed 12.01.12).

Hedrick, P.W., 2005. A standardized genetic differentiation measure. Evolution 59, 1633-1638.

Honnay, O., Adriaens, D., Coart, E., Jacquemyn, H., Roldan-Ruiz, I., 2007. Genetic diversity

within and between remnant populations of the endangered calcareous grassland plant *Globularia bisnagarica* L. Conservation Genetics 8, 293-303.

Hotta, M. 1974. Evolutionary Biology in Plants: History and Geography of Plants. Sanseido, Tokyo (in Japanese).

Hollingsworth, P.M., Dawson, I.K., Goodall-Copestake, P., Richardson, J.E., Weber, J.C., Sotelo Montes, C., Pennington, T., 2005. Do farmers reduce genetic diversity when they domesticate tropical trees? A case study from Amazonia. Molecular Ecology 14, 497-501.

Hubisz, M.J., Falush, D., Stephens, M., Pritchard, J.K., 2009. Inferring weak population structure with the assistance of sample group information. Molecular Ecology Resources 9, 1322-1332.

Husband, B.C., Campbell, L.G., 2004. Population responses to novel environmental: implications for ex situ plant conservation. In: Guerrant, E.O., Havens, K., Maunder, M. (Eds.), Ex Situ Plant Conservation: Supporting Species Survival in the Wild. Island Press, Washington, pp. 231-266.

Inaba, K., Matoba, H., Nagano, K., Uchiyama, H., 2010. Cytological studies of the critically endangered plants in Japan (1) *Polemonium kiushianum* (Polemoniaceae). Journal of Japanese Botany 85,118-121.

Jacquemyn, H., Roldan-Ruiz, I., Honnay, O., 2010. Evidence for demographic bottlenecks and limited gene flow leading to low genetic diversity in a rare thistle. Conservation Genetics 11, 1979-1987.

Keller, L.F., Waller, D.M., 2002. Inbreeding effects in wild populations. Trends in Ecology Evolution 17, 230-241.

Knight, M.E., Martin, A.P., Bishop, S., Osborne, J.L., Hale, R.J., Sanderson, A., Goulson, D.,

2005. An interspecific comparison of foraging range and nest density of four bumblebee (*Bombus*) species. Molecular Ecology 14, 1811-1820.

Leimu, R., Mutikainen, P., Koricheva, J., Fischer, M., 2006. How general are positive relationships between plant population size, fitness and genetic variation? Journal of Ecology 94, 942-952.

Li, Q., Zu, Z., He, T., 2002. Ex situ genetic conservation of endangered *Vatica guangxiensis* (Dipterocarpaceae) in China. Biological conservation 106, 151-156.

Li, Y.Y., Chen, X.Y., Zhang, X., Wu, T.Y., Lu, H.P., Cai, Y.W., 2005. Genetic differences between wild and artificial populations of *Metasequoia glyptostroboides*: Implications for species recovery. Conservation Biology 19, 224-231.

Liu, M.H., Chen, X.Y., Zhang, X., Shen, D.W., 2008. A population genetic evaluation of ecological restoration with the case study on *Cyclobalanopsis myrsinaefolia* (Fagaceae). Plant Ecology 197, 31-41.

Lowe, A.J., Boshier, D., Ward, M., Bacles, C.F.E., Navarro, C., 2005. Genetic resource impacts of habitat loss and degradation; reconciling empirical evidence and predicted theory for neotropical trees. Heredity 95, 255-273.

Matoba, H., Inaba, K., Nagano, K., Uchiyama, H., 2011. Use of RAPD analysis to assess the threat of interspecific hybridization to the critically endangered *Polemonium kiushianum* in Japan. Journal of Plant Research 124, 125-130.

Menges E.S., Guerrant, E.O., Hamze S., 2004. Effects of seed collection on the extinction risk of perennial plants. In: Guerrant, E.O., Havens, K., Maunder, M. (Eds.), Ex Situ Plant Conservation: Supporting Species Survival in the Wild. Island Press, Washington, pp. 305-324.

Mijnsbrugge, K.V., Bischoff, A., Smith, B. 2010. A question of origin: Where and how to collect

seed for ecological restoration. Basic and Applied Ecology 11, 300-311.

Milligan, B., 1992. Plant DNA isolation. In: Hoelzel A. R. (Eds.). Molecular Genetic Analysis of Populations: A Practical Approach. IRL Press, Oxford, pp. 59–88

Miyabuchi, Y., Sugiyama, S., Nagaoka, Y., 2012. Vegetation and fire history during the last 30,000 years based on phytolith and macroscopic charcoal records in the eastern of Aso Volcano, Japan. Quaternary international 254, 28-35.

Nei, M., 1987. Molecular evolutionary genetics. Columbia University Press, New York.

Nielsen, E.E., Bach, L.A., Kotlichi, P., 2006. HYBRIDLAB (version 1.0): a program for generating simulated hybrids from population samples. Molecular Ecology Notes 6, 971-973.

Otaki, N, 2000. Germination of buried seeds of *Polemonium kiushianum*. Botany 50, 82-84. (in Japanese)

Piry, S., Luikart, G., Cornuet, J.M., 1999. BOTTLENECK: A computer program for detecting recent reductions in the effective population size using allele frequency data. Journal of Heredity 90, 502-503.

Possingham, H., Ball, I., Andelman, S., 2000. Mathematical methods for identifying representative reserve networks. In: Ferson, S., Burgman, M., (eds) Quantitative methods for conservation biology . Springer, New York, pp. 291-305.

Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of population structure using multilocus genotype data. Genetics 155, 945-959.

Russello, M.A., Amato, G., 2007. On the horns of a dilemma: molecular approaches refine ex situ conservation in crisis. Molecular Ecology 16, 2405-2406.

Sei, S., 2006. Current status of grassland plants. In: Botanical society of Japan (Eds.). Crisis of Plants in Kyushu region: Public Symposium of the 70th Plenary Meeting of the

553 Botanical Society of Japan, Kumamoto, pp. 13-20. (in Japanese)
554 Shoji, A., 2006. Conservation of vast grasslands in Aso, Kyushu region. Ecosophia 18, 22-27.
555 (in Japanese)
556 Takahashi, Y., 2009. Management and restoration of grassland landscape for species
557 conservation: a case of Aso Grassland. The Japanese Institute of Landscape
558 Architecture 72, 394-398. (in Japanese with English summary).
559 Weir, B.S., Cockerham, C.C., 1984. Estimating f-statistics for the analysis of population
560 structure. Evolution 38, 1358-1370.
561 Williams, L.W., Serfass, T.L., Cogan, R., Rhodes, O.E., 2002. Microsatellite variation in the
562 reintroduced Pennsylvania elk herd. Molecular Ecology 11, 1299-1310.
563 Williams, S.L., Davis, C.A., 1996. Population genetic analyses of transplanted eelgrass (*Zostera*
564 *marina*) beds reveal reduced genetic diversity in southern California. Restoration
565 Ecology 4, 163-180.
566 Woodworth, L,M., Montgomery, M.,E., Briscoe, D.,A. Frankham, R., 2002. Rapid genetic
567 deterioration in captive populations: Causes and conservation implications.
568 Conservation Genetics 3, 277-288.
569 Yokogawa, M., Kaneko, S., Isagi, Y., 2009. Development of microsatellite markers for
570 *Polemonium kiushianum* (Polemoniaceae), a critically endangered grassland plant
571 species in Japan. Conservation Genetics 10, 1445-1447.
572 Young, A., Boyle, T., Brown, T., 1996. The population genetic consequences of habitat
573 fragmentation for plants. Trends in Ecology Evolution 11, 413-418.
574 Zurbuchen, A., Landert, L., Klaiber, J., Müller, A., Hein, S., Dorn, S., 2010. Maximum foraging
575 ranges in solitary bees: only few individuals have the capability to cover long foraging
576 distance. Biological Conservation 143, 669-676.

577 **Table 1.** Population characteristics of *Polemonium kiushianum* examined using microsatellite
578 markers.
579
580
581
582

583 **Table 2.** Genetic diversity measurements of each sampled population of *Polemonium*
584 *kiushianum*. *N*, numbers of samples; *A*, numbers of alleles per locus; *AR*, allelic richness; *RA*,
585 summed number of rare alleles; *Pr*, summed number of private alleles; H_O; expected
586 heterozygosity; H_E, observed heterozygosity; F_{IS}, fixation index; IAM, infinate allele model;
587 TPM two phase model; N.A., population that was not analysed.
588

589 **Fig 1.** (a) Location of the study site, the Aso region. (b) Relative location of the wild
590 populations analyzed in this study. To prevent illegal digging, the precise latitude and longitude,
591 cardinal direction, and topography are not shown in (b).
592
593
594

Fig 2. Results of Bayesian clustering in STRUCTURE analysis (Prichard et al., 2000). (a) Value of ln *P* (*X*/*K*) for *K* = 1 through *K* = 10 ± SE averaged across 20 runs from the simulation in the STRUCTURE (Prichard et al., 2000). (b) Δ*K* based on the rate of change in the log probability of data between successive *K* values (Evanno et al., 2005). (c) The proportion of the membership coefficient of 182 individuals in seven populations for each of the inferred clusters for *K* = 3 and *K* = 4. Each column represents an individual.

Fig 3. Relationship between the number of seeds transferred from wild to ex situ populations and allelic richness (a), expected heterozygosity (b), and inferred cluster of wild populations defined using STRUCTURE analysis (c) for assumed seed transfer populations. The dashed line indicates the value for the total wild population. Genotype data of ex situ population E6 was used.

611 **Table A.1.** Characteristics of three new microsatellite loci for *Polemonium kiushianum* and their
612 variability. Deviation from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium
613 between loci were tested with FSTAT ver 2.9.3 software (Goudet 2001) using genotype data of
614 population W2. Significance levels were adjusted using Bonferroni correction for multiple
615 testing. Although significant deviations ($P < 0.05$) from HWE were detected for Pkiu965, no
616 significant deviations from HWE were detected for other two loci. There was no evidence of
617 significant linkage disequilibrium between any two of the loci.
618

Table A. 2. Pairwise F_{ST} values (above diagonal) and pairwise geographic distance (below diagonal) between *Polemonium kiushianum* populations. The significances were indicated by asterisks (* $P < 0.05$, ** $P < 0.01$).

Table 1.

Population	Wild or Ex situ	Number of flowering individuals	Habitat type	Maintenance
W1	wild	ca. 400	grasslands	mowing in autumn
W2	wild	ca. 250	grasslands	mowing in autumn
W3	wild	3	edge of conifer plantation	abandonment
W4	wild	50	gap of conifer plantation	mowing in autumn
W5	wild	21	gap of conifer plantation	abandonment
E6	ex situ	ca. 100	broad-leaved deciduous forest	mowing in autumn
E7	ex situ	ca. 100	grassland with deciduous trees	mowing in autumn

Table 2.

Population	*N*	*A*	*AR*	*RA*	*Pr*	H_O	H_E	F_{IS}	*P* value of Bottleneck analysis	
									IAM	TPM
Wild populations										
W1	34	3.80	2.68	4	1	0.38	0.39	0.01	0.01	0.58
W2	32	3.80	2.94	4	5	0.47	0.47	0.04	0.04	0.46
W3	7	2.90	2.90	2	2	0.34	0.42	0.26	N.A.	N.A.
W4	32	3.70	2.71	6	5	0.36	0.38	0.06	0.02	0.47
W5	21	3.20	2.56	4	2	0.46	0.41	-0.07	0.01	0.28
Ex situ populations										
E6	32	3.10	2.45	1	1	0.34	0.39	0.08	0.10	0.67
E7	24	3.10	2.66	0	0	0.35	0.37	0.06	0.15	0.71
	average	3.37	2.70	3.0	2.3	0.39	0.40			

Fig 1.

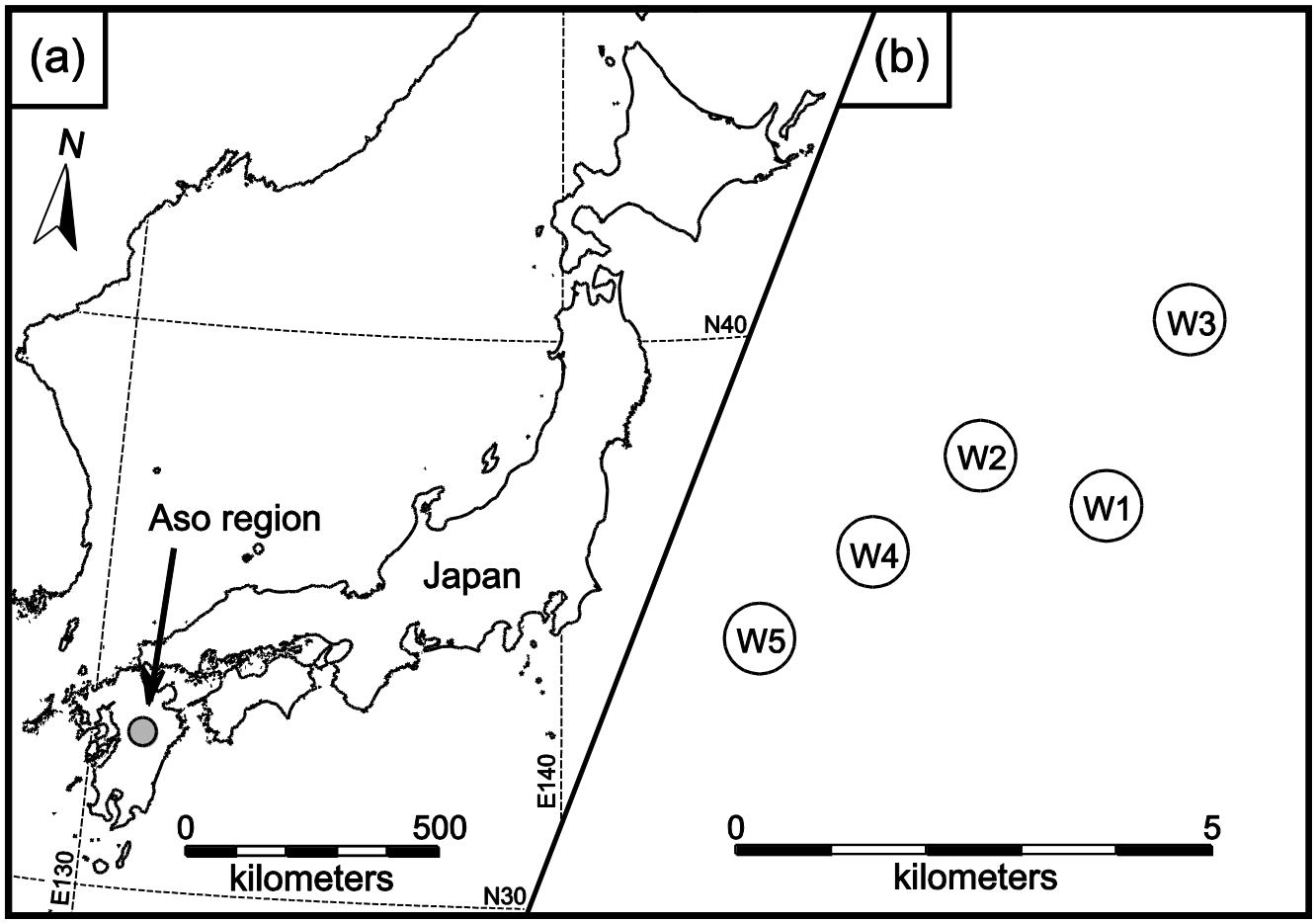
(a)
N
N40
Aso region
Japan
E140
E130
N30
0
500
kilometers
(b)
W3
W2
W1
W4
W5
0
5
kilometers

Fig 2.

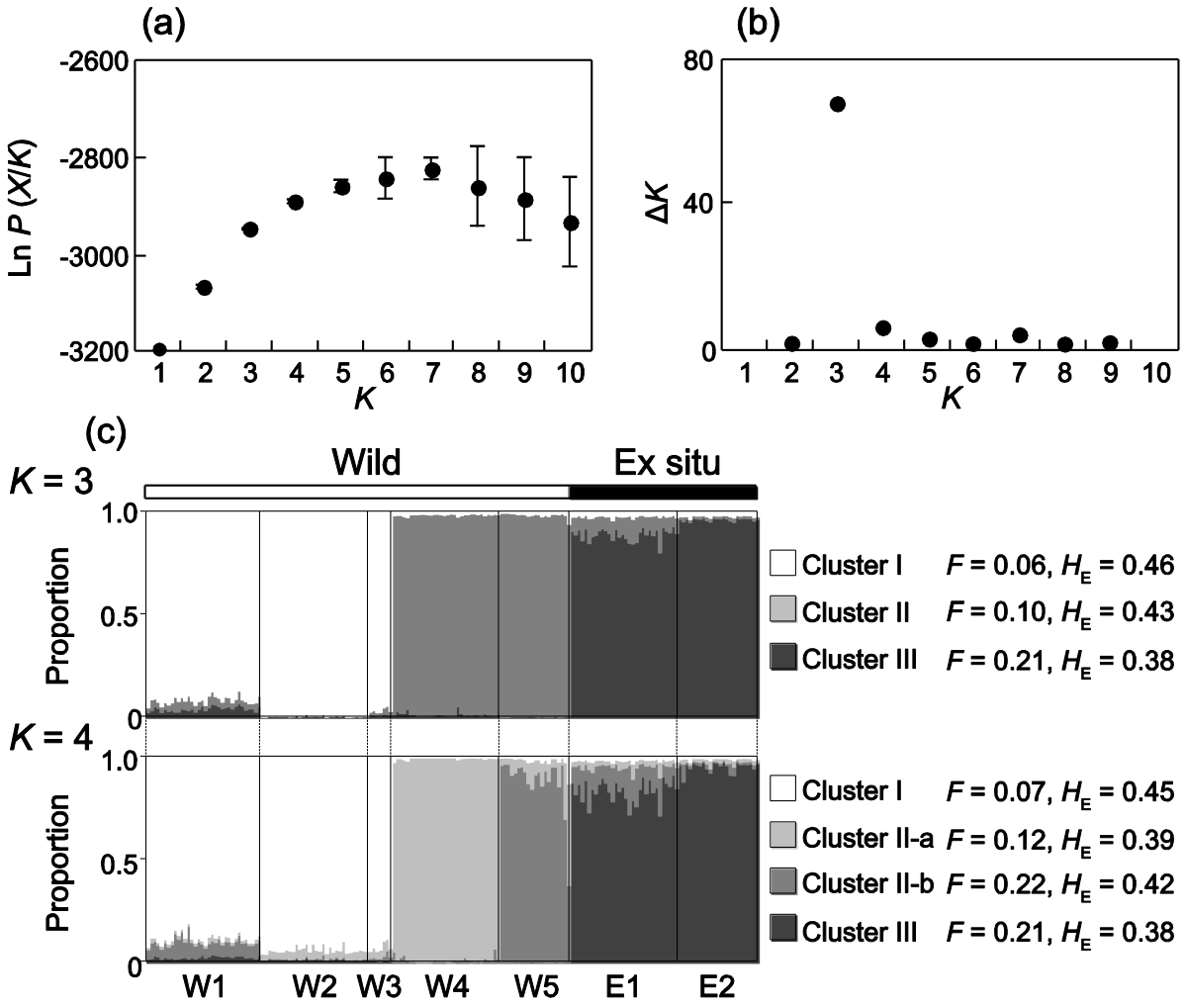
(a)
Ln P (X/K)
-2600
-2800
-3000
-3200
1 2 3 4 5 6 7 8 9 10
K
(b)
ΔK
80
40
0
1 2 3 4 5 6 7 8 9 10
K
(c)
K = 3
Wild
Ex situ
1.0
0.5
0
Proportion
Cluster I F = 0.06, H_E = 0.46
Cluster II F = 0.10, H_E = 0.43
Cluster III F = 0.21, H_E = 0.38
K = 4
1.0
0.5
0
Proportion
Cluster I F = 0.07, H_E = 0.45
Cluster II-a F = 0.12, H_E = 0.39
Cluster II-b F = 0.22, H_E = 0.42
Cluster III F = 0.21, H_E = 0.38
W1 W2 W3 W4 W5 E1 E2

Fig 3.

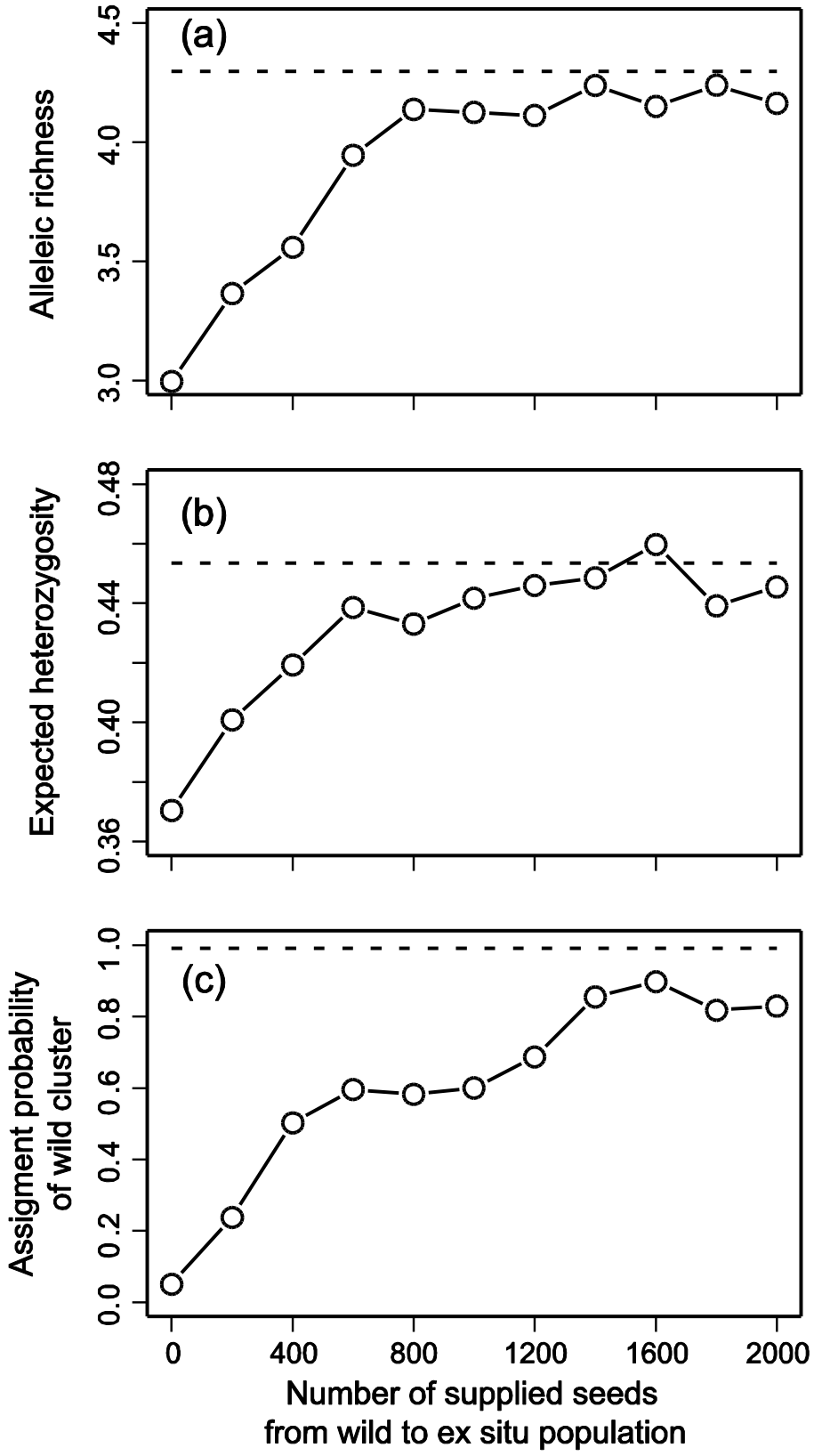
(a)
Alleleic richness
3.0
3.5
4.0
4.5
(b)
Expected heterozygosity
0.36
0.40
0.44
0.48
(c)
Assigment probability
of wild cluster
0.0
0.2
0.4
0.6
0.8
1.0
0
400
800
1200
1600
2000
Number of supplied seeds
from wild to ex situ population

Table A. 1.

Locus	Repeat motif	Primer sequence (5'-3')	*T*a (°C)	Size range (bp)	*A*	H_O	H_E	Accession number
Pkiu593	$(AG)_6(AC)_{11}$	AGAGAGAGAGAGACACACACAC	57	189-204	4	0.688	0.604	AB721308
		CAGACAACTCCATGTTTGAGAT						
Pkiu627	$(AC)_6(TC)_7$	ACACACACACACTCTCTCTCTC	57	257-275	3	0.129	0.177	AB721309
		GAGGGACAGAGAGATCAAGAAC						
Pkiu965	$(AG)_6(AC)_{10}$	AGAGAGAGAGAGACACAC	45	154-174	4	0.483*	0.699	AB721310
		TAATAGTCATAAAATAAGAGGT						

Table A. 2. Pairwise F_{ST} values (above diagonal) and pairwise geographic distance (below diagonal) between *Polemonium kiushianum* populations.

The significances were indicated by asterisks (* $P < 0.05$, ** $P < 0.01$).

	Wild					Restored	
Population	W1	W2	W3	W4	W5	R6	R7
W1	---	0.03*	0.07**	0.09**	0.12**	0.10**	0.10**
W2	1.4	---	0.08**	0.08**	0.11**	0.10**	0.09**
W3	2.1	2.7	---	0.17**	0.18**	0.21**	0.18**
W4	2.5	1.5	4.2	---	0.13**	0.09**	0.08**
W5	4.0	3.0	5.7	1.51	---	0.14**	0.16**
R6	6.9	6.4	5.3	7.45	8.49	---	0.08**
R7	32.2	33.6	31.8	34.5	35.6	35.5	---