Fine-scale genetic differentiation of a temperate herb: relevance of local environments and demographic change

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ABSTRACT

The genetic structure of a plant species is shaped by environmental adaptation and demographic factors, but their relative contributions are still unknown. To examine the environment- or geography-related differentiation, we quantified genetic variation among 41 populations of a temperate herb, *Arabidopsis halleri* subsp. *gemmifera* (Brassicaceae). We analysed 19 microsatellite loci, which showed a significant population differentiation and a moderate within-population genetic diversity (global $G_{st} = 0.42$ and $H_s = 0.19$). Our structure analysis and phylogenetic network did not detect more than two genetic groups across the Japanese mainland but found fine-scale genetic differentiations and admixed patterns around the central area. Across the Japanese mainland, we found significant evidence for isolation-by-distance but not for isolation-by-environments. However, at least within the central area, the magnitude of genetic differentiation tended to increase with microhabitat dissimilarity in light conditions and water availability. Furthermore, most populations have been estimated to experience a recent decline in the effective population size, indicating a possibility of bottleneck effects on the pattern of genetic variation. These findings highlight a potential influence of the microhabitat conditions and demographic change on the local-scale genetic differentiation among natural plant populations.

Keywords: *Arabidopsis halleri* subsp. *gemmifera*; Brassicaceae; genetic structure; isolation-by-distance; isolation-by-adaptation; Japan.
INTRODUCTION

Current patterns of genetic structure within a single plant species are shaped by demographic (e.g. genetic drift or gene flow) and ecological (e.g. species’ life-history traits or adaptation to local environments) factors (Sharbel et al. 2000; Platt et al. 2010; Willi and Määttänen 2011). At a regional scale where current migrations are probably limited (e.g. on a several hundred-kilometre scale), many phylogeographical studies have revealed historical influences on the genetic structure of natural populations (e.g. Fujii and Senni 2006; Meeus et al. 2012; Garrido et al. 2012). For example, impacts of glacial periods upon temperate flora were prominent in the regional patterns of genetic differentiation, since they have been relevant to latitudinal or altitudinal migrations, population extinction, range expansion, and fragmentation (Hewitt 2000). On the other hand, at a local scale where ongoing migrations and drifts are possible, landscape genetic studies have reported that habitat fragmentations and life-history traits of each plant species can be determinants of within-population genetic diversity and genetic differentiation among proximate populations, because they are involved in limited seed or pollen dispersal and genetic drifts (e.g. Kudoh and Whigham 1997; Vekemans and Hardy 2004; Bomblies et al. 2010).

In addition to ample evidence about the genetic structure of plant populations from phylogeographical and landscape studies, there has also been increasing evidence that local environment and geographic scale exert mutually non-exclusive influences on the pattern of genetic structure (Nosil et al. 2009; Sobel et al. 2010; Lee and Mitchell-Olds 2011). For example, environmental factors may contribute to genetic differentiation by mediating local adaptation that can reduce the fitness of immigrants or promote reproductive isolation. These conceptual frameworks, which are termed ‘ecological speciation’ (Sobel et al. 2010) or ‘isolation-by-adaptation’ (Nosil et al. 2009), have been recognised as important. Thus, for a comprehensive understanding of an evolutionary trajectory of plant populations, it is necessary to consider the interaction between ecological and demographic processes.
The Japanese archipelago consists of four main islands extending over 2,000 km in the southwest-northeast direction and encompassing a wide range of climatic zones, including many floral or vegetation types from warm-temperate, evergreen, and broadleaf forests to temperate, deciduous and sub-boreal coniferous forests (Numata 1974). No major glaciers were present in Japan during the last glacial period, but the temperature and precipitation were significantly lower than at present (Tsukada 1988). Reconstruction of past vegetation from palaeoecological (Takahara et al. 2000) and phylogeographical data (Fujii and Senni 2006) indicates that the main vegetation zones were displaced towards the south and lower altitudes (see Dobson 1994). In the temperate zone of Japan in particular, the genetic structure of deciduous broadleaved tree species showed a regional genetic differentiation between the coastal areas of the Sea of Japan and Pacific Ocean (Hiraoka and Tomaru 2009α; Iwasaki et al. 2012). These regional-scale investigations postulate a phylogeographically unique scenario of recent range expansions from the central region along coastal areas of the Japanese mainland. Accumulated phylogeographical evidence also suggests that historical climate changes have shifted the distribution range, and consequently altered genetic diversity of Japanese vegetation (e.g. Sakaguchi et al. 2010, 2011). However, local-scale analyses are still limited regarding the pattern of genetic differentiation and its association with local environments.

*Arabidopsis halleri* subsp. *gemmifera* (syn. *Arabis gemmifera*) is distributed throughout Japan and the Russian Far East (Hoffman 2005), where regional scales for this subspecies can be considered to be several hundred kilometres or more. *Arabidopsis halleri* subsp. *gemmifera* can reproduce clonally: After flowering, plants produce new rosettes on the main and lateral meristems of flowering stems (Aikawa et al. 2010), and these rosettes often establish as clonal offspring once they have rooted and attached themselves to the ground. The flowers are self-incompatible and produce fruits through cross-pollination (Tsuchimatsu et al. 2012). The pollinators are small solitary bees and flower flies, and the seeds have no specific structure for long-distance dispersal. This subspecies is often found in somewhat isolated habitats near open gravel sites along valleys or in gaps within vegetation (Ihara 1976). The sporadic distribution, together with the lack of long-
distance dispersal, may therefore enhance the genetic structure formed at a local scale (<10 to a few hundred kilometres). Furthermore, *A. halleri* subsp. *gemmifera* often occurs nearby human-disturbed habitats such as forest pathways and abandoned mines, whereby artificial dispersals may homogenize the local genetic structure.

In this study, we examined genetic differentiation among natural populations of *A. halleri* subsp. *gemmifera*. In particular, we conducted a fine-scale sampling around the Kinki area, because private genetic groups or admixed populations have often been reported for its coastal regions in temperate plants of Japan (Hiraoka and Tomaru 2009b; Iwasaki *et al.* 2010). We used cross-species microsatellite markers developed for a close relative, *A. thaliana*, to detect genetic structures at a fine resolution. Microsatellite loci are known to be highly polymorphic (e.g. Emanuelli *et al.* 2013) and consequently provide us with suitable levels of variation for quantifying genetic differentiation at a fine scale. Three specific questions were addressed in this study. Is there any distinct genetic structure among *A. halleri* subsp. *gemmifera* populations? Is the magnitude of genetic differentiation associated with environmental gradients and/or geographic distance? Are demographic processes involved in the genetic differentiation? To answer these questions, we initially generated a Bayesian clustering and phylogenetic network to determine the patterns of genetic differentiation. Secondly, we analysed the isolation-by-distance or -by-environmental differences. Finally, to estimate the demographic processes of each population, declines/expansions of effective population sizes were tested using a coalescence-based method.
METHODS

Study sites, plant materials, and DNA extraction

We studied 41 populations in Japan; i.e. 30 sites located within the Kinki area and its surroundings (referred to hereafter as the Kinki area) and 11 sites far from the Kinki area (hereafter, as outside the Kinki area) (Table 1; Fig. 1A, B). *Arabidopsis halleri* subsp. *gemmafera* often inhabits habitats along valleys or forest margins, where the size of study sites roughly ranged from hundreds to thousands meters in transect length [see SUPPORTING INFORMATION, Fig. S1]. We sampled fresh, young leaves from four to 14 individuals for each sampling site (Table 1). Sampled plants were at least 1 m apart from each other to minimize the possibility of multiple sampling from single clonal lineages. Total DNA was extracted using a DNeasy Plant Mini Kit (Qiagen), CTAB (Doyle and Doyle 1987) or miniscale DNA extraction method (Goto 2005) and stored at –20°C until use.

Microsatellite analysis

A preliminary investigation used 40 primers previously reported for *Arabidopsis* species (Bell and Ecker 1994; Loridon et al. 1998; Clauss et al. 2002; Symond and Lloyd 2003; Mable and Adam 2007; Llaurens et al. 2008). We selected 19 markers that were successfully amplified, regardless of the amount of polymorphism. Three primer set combinations (loci 8, 4, and 7) were prepared for multiplex PCR with fluorescent dyes (Table 2) so that similar fragment sizes could be distinguished by different dyes. For each sample, we determined the genotypes of 19 loci based on the distinct electrophoretic mobility of amplified fragments shorter than 50 bp.

The primer concentration was 10 μM. The multiplex PCR reaction was performed in a volume of 10 μL, containing 0.5 μL DNA, 5 μL Multiplex PCR Master Mix (QIAGEN), and 1 pmol of each primer. The PCR reaction proceeded as follows: 15 min at 95°C; 30 cycles of 30 s at 94°C, 90 s at 53°C, 1 min at 72°C for multiplex set 1, or 35 cycles of 30 s at 94°C, 90 s at 58°C, and 1 min at 72°C for multiplex sets 2 and 3; and a final extension step of 30 min at 60°C. The PCR
products were loaded on an ABI 3130xl Genetic Analyzer with GS 500 LIZ size standard (Applied Biosystems, Foster City, CA, USA). Genotypes were determined using Gene Mapper version 4.0 software (Applied Biosystems). The genotypic data were provided in **SUPPORTING INFORMATION** as a supplementary material.

**Data analysis**

We initially described the basic statistics for the population genetics of each sampling site and locus. We used GenoDive software version 2.0 (Meirmans and van Tienderen 2004) to calculate observed heterozygosity \( H_o \), within-population genetic diversity \( H_s \), identical to the expected heterozygosity \( H_e \), calculated using GenoDive), total genetic diversity \( H_t \), and the inbreeding coefficient \( G_{is} \); and the Fstat version 2.9.3.2 (Goudet 2002) to calculate allelic richness (AR; El Mousadik and Petit 1996). Deviations of \( G_{is} \) from the Hardy-Weinberg equilibrium were analysed with 9,999 one-sided permutation tests using GenoDive. We also analysed the number of alleles, genetic diversities, and genetic differentiation among populations \( (H_o, H_s, H_t, \text{ and } G_{st}) \) for each locus by using GenoDive. Significances of the genetic differentiation, \( G_{st} \), were analysed for each locus based on sum-of-square of the test statistic with 9,999 permutations. Linkage disequilibrium among the 19 loci was also tested with 1,000 Markov Chain Monte Carlo (MCMC) iterations with 100 batches, using GENEPOP software version 4.2 (Raymond and Rousset 1995). Because of the possibility of clonality, the number of identical genotypes was checked by the clone assignment implemented in GenoDive.

Secondly, we used a Bayesian clustering and population phylogeny to infer patterns of genetic differentiation. We estimated population gene pools and ancestry using Structure software version 2.3.4 (Pritchard et al. 2000) for all samples. The estimated number of ancestral populations \( K \) was set from 1 to 25 with a 100,000-step burn-in and 300,000 MCMC iterations. Allele frequencies were assumed to be correlated among populations and an admixture model was chosen. This process was repeated 20 times for each \( K \). The probable \( K \) value was determined using the \( \Delta K \) statistic (Evanno et al. 2005). The mean log-likelihood and the \( \Delta K \)
statistics were calculated using Structure Harvester software version 0.6.93 (Earl 2012). We repeated this Structure analysis using the 30 populations from the Kinki area to further determine the genetic structure at the local scale. Additionally, we estimated the population structure using a phylogenetic network depicted by the SplitsTree4 version 4.13.1 (Huson and Bryant 2006). The network was constructed on the basis of Nei et al.’s (1983) genetic distance ($D_a$; calculated using GenoDive).

Thirdly, we analysed relationships of genetic differentiation and environmental dissimilarity or geographic distance. We selected $G''_{st}$ as a metric of genetic differentiation to adjust $H_s$-dependent variation in $G_{st}$ values (Meirmans and Hedrick 2011). The data from the entire study area and the Kinki area were separately analysed. According to the method of Rousset (1997), we converted pairwise $G''_{st}$ values into $G''_{st}/(1 - G''_{st})$ for each pair of sampling sites. The Mesh Climate 2000 database (Japan Meteorological Agency 2002) was used to compile the environmental variables. This database provides the information on air temperature (°C; daily mean, maximum and minimum), annual precipitation (mm), radiation (h), sunshine (MJ/m$^2$), and maximum snow cover (cm) at a 1-km$^2$ scale, and is thus suitable for our sampling scale. We added the altitude of each sampling site to these variables. These variables were summarized by a principal component analysis (PCA) to deal with multi-collinearity of the environmental variables. Sum of the first and second principal components (PC1 and PC2) explained 78% and 79% (respectively for the entire area and the Kinki area) of the total variation in habitat environments represented by the eight variables. PC1 explained approximately 54% of the variation, whereas PC2 explained roughly 25% of the variation for both the entire area and the Kinki area. PC1 was related to the three temperature variables (daily mean, maximum, and minimum air temperature) as well as the altitude, whereas PC2 was related to the light conditions (radiation and sunshine) and water availability (precipitation and snow cover). This tendency was consistent between the entire study area and the Kinki area (Fig. 1C, D). On the basis of the PCA results, we used PC1 and PC2 as representative indices to analyse genetic differentiation along environmental gradients. We used Mantel tests to analyse Pearson’s correlation between genetic and geographic distance;
or partial Mantel tests for the correlation between genetic distance and difference of principal component values by incorporating the geographic distance as a covariate. $P$-values were determined by 9,999 permutations. We utilized the prcomp function for the PCA analysis and the vegan package (Okanen et al. 2013) implemented in the R software version 3.0.1 (R Core Team 2013) for the Mantel tests. Additionally, to deal with the exaggerated sampling on the Kinki area, we repeated the Mantel tests (with 999 permutations) for the entire study area by randomly selecting 10 populations from the Kinki area. This subsampling analysis was run 9,999 times, where the significance of test statistics ($r$ and $P$) was determined by mean and 95% CI (i.e. $1.96 \times$ SD) of the resampled distribution.

Finally, we estimated demographic changes in each studied population using the Msvar programme version 1.3 (Beaumont 1999). On the basis of a coalescent simulation by hierarchical Bayesian models with a step-wise mutation assumption, this programme provides multi-locus posterior distribution estimates for current and ancient effective population size ($\log_{10}[N_0]$) and $\log_{10}[N_1]$), respectively), mutation rate ($\log_{10}[\mu]$) and the time since populations started to expand/decline ($\log_{10}[T_a]$). These parameters were allowed to vary among all 19 loci, while their initial values were equal among these loci. The generation time ($g_a$) was set as 2 years because *A. halleri* subsp. *gemmifera* is a perennial herb able to bloom during the first year of its life cycle under cultivated conditions (Y. Sato, pers. obs.). We used uninformative lognormal priors as follows: means of the prior distributions were set to 4, 4, −4 and 5; and SD equal to 4, 4, 1 and 4 for $N_0$, $N_1$, $\mu$ and $T_a$, respectively. Hyper-prior lognormal distributions were set as: mean equal to 6, 6, −4 and 8; SD equal to 2, 2, 0.25 and 2; hyper-prior of mean equal to 0, 0, 0 and 0; hyper-prior of SD equal to 0.5, 0.5, 0.5 and 0.5 for $N_0$, $N_1$, $\mu$ and $T_a$, respectively. Three independent chains of MCMC were run with 40,000 thinned updates and a thinning interval of 20,000 steps (i.e., the total number of update steps $= 8 \times 10^8$). The coda package (Plummer et al. 2006) implemented in R was used to estimate the posterior distribution and to assess convergence among three chains, where the first half of the iterations was discarded as burn-in periods. The convergence was assessed using potential scale reduction factors (Gelman and Rubin 1992). For the coalescent
estimation, we did not analyse populations with less than seven individuals due to their small sample size (the total number of populations analysed = 30).

RESULTS

Basic statistics of population genetics

The allelic richness ranged from 1.00 to 2.02 in all populations (Table 1). Within-population genetic diversities were <0.4 for all populations. $G_{is}$ values significantly deviated from zero in 23 out of 41 populations (Table 1). A few populations were found to have private alleles (Table 1). Percentages of null alleles were less than 5%, except for 7.5% in site 19. Most markers showed significant genetic differentiation among the studied populations (global $G_{st} = 0.42$; Table 2). Sixteen markers showed a significant $G_{is}$, and six of them had $G_{is}$ values >0.2 (Table 2). No marker pairs exhibited a significant linkage disequilibrium, except in three cases (ICE12-MHJ24, $\chi^2 = 14.4$, $df = 6$, $P = 0.006$; ICE10-AthZEPG, $\chi^2 = 6.05$, $df = 2$, $P = 0.048$; ATH-AthCTR1A, $\chi^2 = 91.9$, $df = 34$, $P < 0.0001$). Within the entire dataset, there were only five individuals with missing values at more than four loci. Although site 40 consisted of identical genotypes across the 19 loci, identical genotypes were less than 10% in the entire dataset.

Patterns of population genetic structure

We observed the largest value of $\Delta K$ at $K = 2$ for all the 41 populations; and at $K = 4$ for the 30 populations from the Kinki area [see SUPPORTING INFORMATION, Fig. S2]. At $K = 2$, most populations near and outside the Kinki area were assigned to one of two clusters, but admixture patterns of these two clusters were observed near the Gifu and Fukui Prefectures of the Kinki area (Fig. 2A). Within the Kinki area, the Structure results at $K = 4$ showed some distinct clusters and admixed patterns. Four populations located in the Kyoto (sites 24–27) and Hyogo Prefectures (sites 34–37) were clearly assigned to different groups (cluster 1 and 2, respectively; an inset of Fig. 2B). While admixed patterns were observed in other sites, a single cluster (cluster
was predominant near the boundary between the Kyoto and Shiga Prefectures (Fig. 2B). Another cluster (cluster 3) was predominantly observed in the eastern Shiga and Osaka areas (Fig. 2B). We also found two sites that were assigned to genetic groups different from their neighbouring sites within the Kinki area (sites 8 and 12; Fig. 2B). The expected heterozygosity of individuals within a same cluster ranged from 0.19 to 0.31 within the Kinki area (cluster 1, 2, 3 and 4 = 0.20, 0.25, 0.28, and 0.30, respectively). Genetic differentiation from an estimated ancestry ranged from 0.06 to 0.42 ($F_{st} = 0.42, 0.42, 0.06,$ and 0.17 for cluster 1, 2, 3, and 4, respectively).

In the phylogenetic network (Fig. 3), we found multiple but not clear clusters within the Kinki area (sites 7–9 and 11–37). The populations outside the Kinki area (sites 1–5, 10 and 38–41) were located on margins of the network. The north-eastern populations (sites 1 and 3–6) tended to cluster in the upper right of the network, while the south-western populations (sites 39–41) were grouped in the upper left. Within the Kinki area, there were two populations that were genetically distant from the others (sites 8 and 12; Fig. 3), corroborating the Structure result that these two sites were assigned to genetic groups different from the neighbouring ones ($K = 4$ for the Kinki area; Fig. 2B).

**Isolation-by-distance or isolation-by-environment**

The degree of genetic differentiation showed a significantly positive correlation with the geographic distance (Mantel tests, $r = 0.34$, $P < 0.001$ with 9,999 permutations; Fig. 4A) but was not correlated significantly with the environmental components among the entire study area (partial Mantel tests, $r = -0.13$, $P = 0.88$; $r = 0.04$, $P = 0.30$ for PC1 and PC2, respectively; Fig. 4B, C). These results were also supported when we subsampled the Kinki populations (Geographic distance, $r = 0.25 \pm 0.06$, $P = 0.03 \pm 0.04$; PC1, $r = -0.13 \pm 0.07$, $P = 0.77 \pm 0.16$; PC2, $r = -0.08 \pm 0.14$, $P = 0.72 \pm 0.37$: Mean ± 95% CI by 9,999-times subsamplings). On the other hand, within the Kinki area, the genetic differentiation was marginally correlated with the geographic distance (Mantel tests, $r = 0.13$, $P = 0.099$; Fig. 4D). The genetic differentiation within the Kinki area was not correlated with the difference of the environmental PC1 value (partial Mantel tests, $r = -0.11$, $P$
= 0.78; Fig. 4E) but positively correlated with the PC2 values at a marginally significant level ($r = 0.17, P = 0.054$; Fig. 4F). Although the high altitude populations (site 5, 6, and 7) were outliers in the environmental PC1 (Fig. 1C, D), significant correlations were not detected in the PC1 difference even when the altitude variable was excluded from the PCA analysis ($r = –0.16, P = 0.93$; $r = –0.10, P = 0.74$, for the entire area and Kinki samples, respectively).

**Demographic changes of local populations**

The coalescence-based analysis by the Msvar programme indicated that most populations experienced $10^2–10^3$ times reduction of the effective population size compared to the ancestral (Table 1). The time since the demographic changes was estimated at $10^4–10^5$ years except for a few populations (>10$^8$ years for sites 34 and 41; Table 1). Because estimations for four populations did not converge (multivariate potential scale reduction factors > 1.1 for sites 2, 12, 31, and 31), further MCMCs were run with 120,000 thinned updates for these populations. After this additional run, the multivariate potential scale reduction factors reduced to ≤1.1 for all populations, indicating successful convergence among the MCMC chains.

**DISCUSSION**

We did not detect more than two genetic groups across the Japanese mainland, but we identified a fine-scale differentiation and admixed pattern within the central area, as indicated by the Structure analysis. The magnitude of genetic differentiation depended on geographical distance, but not on the environmental differences across the entire study scale. However, at least within the central area, we found suggestive evidence for environment-related differentiation, although it was marginally significant. Given that the second principal component was related to the light condition (radiation and sunshine) and water availability (precipitation and snow cover), we hypothesize that microhabitat conditions can be a factor in local adaptation. Moreover, *A. halleri* subsp. *gemmafera* often occurs in transient habitats such as vegetation gaps (Ihara 1976), and we indeed observed them often growing in gravel along valleys or shady habitats nearby.
forest margins. Hence, it seems plausible that microhabitat environments rather than meteorological conditions (such as temperature or altitude) influenced the distribution of our studied species and accordingly resulted in the isolation-by-environments.

In plants, isolation-by-adaptation has been suggested for micro-environmental regimes, such as salt tolerance in *Mimulus guttatus* (Lowry *et al.* 2008) or water availability in *Boechera stricta* (Lee and Mitchell-Olds 2011, 2013). These examples may agree with our evidence in which microhabitat-related differentiation was observed at a local scale. Furthermore, in the relative *A. thaliana*, the influence of breeding systems on genetic structure has been suggested as relevant even within a scale smaller than 50 km (Bomblies *et al.* 2010). In our studied species, it is possible that the life-history traits can facilitate the formation of the fine-scale differentiation. Specifically, *A. halleri* subsp. *gemmifera* has a potential for clonal growth (Aikawa *et al.* 2010) and thereby can generate identical genotypes within a population. Despite the fact that such identical genotypes were rare in the entire dataset, similar genotypes easily occurred in our studied clonal species. In addition, seeds of *A. halleri* subsp. *gemmifera* are not specialized for long-distance dispersal. Thus, the asexual breeding system may have allowed populations from a small number of founders with low genetic diversity to become established. In total, combinations of microhabitat isolations and negative distance-dispersal relationships may be specific factors facilitating the local-scale differentiation in *A. halleri* subsp. *gemmifera*.

The coalescence-based estimation inferred a decline in the effective population size for most sites. Accompanied with the species’ life-history discussed above (clonality and lack of long-distance dispersal), such a decline of population size could account for significantly positive $G_{IS}$ and moderate heterozygosity as it might have caused genetic drifts. In Japan, bottlenecks that are associated with positive inbreeding were also reported for a clonal self-incompatible herb *Veratrum album* subsp. *oxysepalum* (Kikuchi *et al.* 2013). The bottleneck effects exerted by the reduction of effective population size may therefore explain the local-scale genetic differentiation and low levels of within-population genetic diversity in our studied clonal self-incompatible species, *A. halleri* subsp. *gemmifera*. However, other factors may also be responsible for the within- and
among-population patterns of genetic diversity. For example, in a self-incompatible relative *A. lyrata*, selfing has recently evolved in some populations (Mable and Adam 2007; Foxe et al. 2010) and consequently influenced their genetic structure (Willi and Määttänen 2011). Although the evidence for the breakdown of self-incompatibility has not been reported in our studied species yet, this factor remains possible for some populations exhibiting the extremely positive deviation of $G_{is}$ (e.g. site 20 and 21).

The pattern of genetic structure of *A. halleri* subsp. *gemmafera* seems comparable to those of the temperate deciduous broadleaved trees in Japan, i.e. admixed pattern in central Japan (Hiraoka and Tomaru 2009b; Iwasaki et al. 2010). A comparative phylogeographic study on four species of deciduous broadleaf trees has identified genetic groups specific to the coastal area of the Kinki area; this postulates the presence of multiple refugia within the Kinki area during glacial periods (Iwasaki et al. 2012). The phylogeographical scenario for the temperate deciduous broadleaved trees may be responsible for the presence of multiple genetic groups that we identified within the Kinki area. Despite the unclear genetic structure across the Japanese mainland, significant isolation-by-distance was detected across the entire study area. This may suggest that the genetic differentiation have been formed gradually along the geographic scale rather than clustered into distinct genetic groups.

The present results should be interpreted with caution. Firstly, the sample size per site in several populations was too small to accurately quantify their genetic variation or perform quantitative analysis. Secondly, our Structure analysis detected admixture of multiple genetic groups even within a population (e.g. site 7, 23, 29, and 31). The underlying genetic structure made it difficult to accurately estimate the demographic processes of highly admixed populations. Thirdly, we should note that our demographic inference has some limitations due to a simplified assumption. In particular, the Msvar estimates assumed the absence of gene flow among local populations (Beaumont 1999). Thus, this assumption prevented us from excluding a possibility that the decline of population size and the time since the demographic change were overestimated for proximate populations in the Kinki area, while the estimates of regional samples did not differ
considerably from those of local ones. These caveats should be taken into account when interpreting the patterns of genetic differentiation reported here.

**CONCLUSIONS**

In summary, this study provides suggestive evidence for microhabitat-related differentiations at a local scale despite the lack of isolation-by-environment across the Japanese mainland in A. halleri subsp. gemmifera. We also suggest the possible effects of population decline on the patterns of genetic variation. Our findings therefore highlight a potential impact of ecological and demographic factors on the genetic differentiation at a fine spatial scale. Further investigations (such as common garden experiments) are required for rigorous testing of isolation-by-adaptation in order to understand how local adaptation and geographic isolation interactively affect the pattern of genetic differentiation within a plant species.

**SUPPORTING INFORMATION**

The following [SUPPORTING INFORMATION] is available in the online version of this article:

**Figure S1.** Pictures of the study sites and plants.

**Figure S2.** Log-likelihood and $\Delta K$ values showing the likely number of ancestral populations inferred by the Structure analysis (Pritchard et al. 2000).

**Supplementary Material.** Genepop format of raw genotypic data [SatoKudoh_AhSSR_GENEPOP.txt].

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CONTRIBUTIONS BY AUTHORS

Y. Sato performed the experiment and analysed the data. Y. Sato and H. Kudoh designed the study and wrote the manuscript.

CONFLICTS OF INTEREST

No conflicts of interest.

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FIGURE LEGENDS

Figure 1. Map of sampling sites (A and B) and two principal components (PCs) summarizing eight environmental variables (C and D) for 41 populations of *Arabidopsis halleri* subsp. *gemmifera*. The site IDs (coloured blue) are mapped on the sampling location. Panel (A) focuses on the Kinki area whereas panel (B) presents the entire study area. The eight environmental variables include air temperature (°C, daily mean, maximum, and minimum), annual precipitation (mm), radiation (h), sunshine (MJ/m²), maximum snow cover (cm), and altitude (m). PC1 and PC2 are also shown for the entire study area (C) and the Kinki area (D). Red arrows represent the contributions of each environmental variable to the principal components. Numbers correspond to the site IDs. Note that high altitude populations (shaded IDs) show outlier values beyond the plot area.

Figure 2. Genetic groups inferred from the Structure analysis (Pritchard *et al.* 2000). Panel (A) and (B) show results at the number of ancestral populations (*K*) = 2 and 4 for the entire study area and within the Kinki area, respectively. The inset of the panel (B) presents a neighbour-joining tree based on allele-frequency divergence among clusters (a scale bar indicates a 0.01 unit). The site IDs correspond to those in Table 1 and Figure 1. Note that colours of clusters do not correspond between different values of *K*.

Figure 3. A split phylogenetic network between the studied *Arabidopsis halleri* subsp. *gemmifera* populations based on Nei *et al.*’s (1983) $D_a$ distances. The site IDs correspond to Table 1 and Figure 1. The IDs of sites outside the Kinki area are within parentheses. A scale bar indicates a 0.1 unit of the $D_a$ distance.

Figure 4. Isolation-by-distance or -environmental gradients across the Japanese mainland (A, B, and C) and within the Kinki area (D, E, and F). Pairwise genetic distances ($G_{st}/[1 – G_{st}]$) are plotted against geographic distances (km) or the differential values of the first or second principal
components (PC1 and 2; see also Fig. 1C, D). Solid and dashed lines indicate significant ($P < 0.05$) and marginally significant trends ($P < 0.1$), respectively. Pearson's correlation coefficient ($r$) is also shown within each panel.
(A) Entire area, $K = 2$

(B) Kinki area, $K = 4$
(A) Entire area  
$r = 0.34$

(B) Entire area  
$r = -0.13$

(C) Entire area  
$r = 0.04$

(D) Kinki area  
$r = 0.13$

(E) Kinki area  
$r = -0.11$

(F) Kinki area  
$r = 0.17$
Table 1. List of 41 populations of *Arabidopsis halleri* subsp. *gemmifera* and their genetic characteristics. Site ID, locality name, latitude, longitude, and altitude are shown for each sampling site. The number of plants sampled per site (*n*), the number of polymorphic loci (#Poly. loci), the number of private alleles per site (#PA), allelic richness (AR), observed heterozygosity, within-population genetic diversity (H₀, and Hₛ), and inbreeding coefficient (Gₛ) are shown based on the results from 19 microsatellite loci. Bold values of Gₛ indicate ≥5% significant deviation from Hardy-Weinberg equilibrium values, in which positive and negative values indicate deficit and excess of heterozygotes, respectively. Demographic estimates by the Msvar programme (current and ancient effective population size, N₀ and N₁, and time since the change of the population size, Tₐ) are also given. Mean and 95% highest posterior density intervals (within square brackets) are presented for the Msvar estimates. Bars (---) indicate a study site not analysed due to its limited sample size, and NA means no information available.

| SiteID | Name            | Latitude | Longitude | Alt. (m) | n   | #Poly. loci | #PA | AR   | H₀   | Hₛ   | Gₛ   | log₁₀(N₀) | log₁₀(N₁) | log₁₀(Tₐ) |
|--------|-----------------|----------|-----------|----------|-----|-------------|-----|------|------|------|------|-----------|-----------|----------|
| 1      | Hakodate, Hokkaido | 41°47'N  | 140°49'E  | 10       | 10  | 4           | 0   | 1.23 | 0.10 | 0.08 | -0.25 | 1.7 [0.2 - 2.7] | 5.1 [3.5 - 7.0] | 4.5 [2.6 - 6.7] |
| 2      | Mazawa, Yamagata   | 38°26'N  | 140°08'E  | 210      | 10  | 10          | 0   | 1.45 | 0.18 | 0.19 | 0.07  | 2.1 [0.8 - 3.1] | 5.0 [3.7 - 6.2] | 3.9 [2.4 - 5.4] |
| 3      | Okuniikawa, Miyagi | 38°20'N  | 140°36'E  | 320      | 4   | 10          | 2   | 1.63 | 0.25 | 0.26 | 0.05  | ---       | ---       | ---      |
| 4      | Fukuroda, Ibaraki  | 36°46'N  | 140°24'E  | 120      | 6   | 8           | 0   | 1.34 | 0.16 | 0.14 | -0.10 | ---       | ---       | ---      |
| 5      | Kaida, Nagano      | 35°59'N  | 137°36'E  | 1270     | 10  | 11          | 1   | 1.66 | 0.28 | 0.28 | 0.01  | 2.2 [1.1 - 3.1] | 5.1 [3.8 - 6.3] | 4.0 [2.6 - 5.2] |
| 6      | Kamikochi, Nagano  | 36°15'N  | 137°39'E  | 1510     | 6   | 12          | 0   | 1.53 | 0.15 | 0.20 | 0.23  | ---       | ---       | ---      |
| 7      | Hirayu, Gifu       | 36°11'N  | 137°33'E  | 1260     | 8   | 9           | 0   | 1.42 | 0.14 | 0.16 | 0.12  | 2.2 [1.2 - 3.1] | 4.6 [3.5 - 5.7] | 4.0 [2.7 - 5.3] |
| 8      | Sufudani, Gifu     | 35°19'N  | 136°27'E  | 190      | 8   | 11          | 0   | 1.68 | 0.21 | 0.27 | 0.24  | 2.8 [1.8 - 3.6] | 5.1 [2.8 - 7.9] | 5.6 [2.3 - 9.9] |
| 9      | Midoridani, Gifu   | 35°37'N  | 136°36'E  | 260      | 12  | 9           | 0   | 1.47 | 0.12 | 0.17 | 0.26  | 2.6 [1.9 - 3.3] | 5.7 [3.9 - 7.5] | 5.5 [3.9 - 7.3] |
| 10     | Funato, Toyama     | 36°28'N  | 137°14'E  | 225      | 6   | 9           | 1   | 1.58 | 0.14 | 0.21 | 0.34  | ---       | ---       | ---      |
| 11     | Takefu, Fukui      | 35°52'N  | 136°15'E  | 120      | 10  | 10          | 0   | 1.58 | 0.14 | 0.21 | 0.33  | 2.4 [1.5 - 3.3] | 5.3 [4.2 - 6.4] | 4.6 [3.2 - 6.0] |
| 12     | Katsuyama, Fukui   | 36°03'N  | 136°29'E  | 150      | 10  | 8           | 0   | 1.38 | 0.12 | 0.16 | 0.26  | 2.2 [1.4 - 3.1] | 5.2 [3.8 - 6.5] | 4.6 [3.0 - 6.1] |
| 13     | Fujiwara-Mikuni, Mie | 35°13'N | 136°27'E  | 240      | 9   | 12          | 1   | 1.95 | 0.33 | 0.34 | 0.04  | 2.6 [1.6 - 3.5] | 4.6 [3.4 - 5.8] | 4.1 [2.5 - 5.7] |
| 14     | Fujiwara-Sakamoto, Mie | 35°11'N | 136°28'E  | 250      | 6   | 9           | 1   | 1.67 | 0.17 | 0.23 | 0.28  | ---       | ---       | ---      |
| 15     | Kiwada, Shiga      | 35°06'N  | 136°22'E  | 310      | 10  | 11          | 0   | 1.50 | 0.13 | 0.19 | 0.33  | 2.6 [1.9 - 3.3] | 5.2 [4.0 - 6.5] | 4.5 [3.4 - 5.6] |
| 16     | Ryozen-Niu, Shiga  | 35°18'N  | 136°22'E  | 210      | 6   | 9           | 3   | 1.65 | 0.18 | 0.23 | 0.21  | ---       | ---       | ---      |
|   | Location                        | Latitude | Longitude | Population | Temperature | Wind Speed | Pressure | Humidity | 2018-07-29 |
|---|--------------------------------|----------|-----------|------------|-------------|------------|----------|----------|-------------|
| 17| Ojigahata, Shiga              | 35°13'N  | 136°23'E | 310        | 10          | 11         | 0        | 1.66     | 0.22        | 0.12        |
| 18| Ibuki, Shiga                 | 35°24'N  | 136°23'E | 350        | 7           | 10         | 0        | 1.56     | 0.20        | 0.15        |
| 19| Gongendani, Shiga           | 35°15'N  | 136°22'E | 380        | 6           | 11         | 0        | 2.02     | 0.31        | 0.14        |
| 20| Asibidani, Shiga            | 35°13'N  | 135°51'E | 410        | 9           | 10         | 0        | 1.59     | 0.10        | 0.22        |
| 21| Umenoki, Shiga              | 35°16'N  | 135°52'E | 430        | 10          | 6          | 0        | 1.33     | 0.01        | 0.92        |
| 22| Katsuragawa-Sakashita, Shiga | 35°11'N  | 135°51'E | 480        | 6           | 10         | 0        | 1.42     | 0.14        | 0.22        |
| 23| Kutsuki, Shiga              | 35°22'N  | 135°55'E | 180        | 14          | 10         | 0        | 1.47     | 0.18        | 0.20        |
| 24| Hanase-Yamasu, Kyoto        | 35°13'N  | 135°47'E | 410        | 10          | 6          | 0        | 1.18     | 0.09        | 0.10        |
| 25| Hanase-Bessho, Kyoto        | 35°11'N  | 135°47'E | 500        | 10          | 9          | 0        | 1.41     | 0.15        | 0.16        |
| 26| Miyama, Kyoto               | 35°18'N  | 135°42'E | 350        | 10          | 9          | 0        | 1.40     | 0.16        | 0.19        |
| 27| Kurama, Kyoto               | 35°08'N  | 135°47' | 420        | 10          | 4          | 0        | 1.12     | 0.06        | 0.15        |
| 28| Ohara, Kyoto                | 35°10'N  | 135°51'E | 330        | 10          | 10         | 0        | 1.62     | 0.13        | 0.23        |
| 29| Shizuhara, Kyoto            | 35°07'N  | 135°48' | 230        | 10          | 7          | 0        | 1.45     | 0.10        | 0.38        |
| 30| Minoo, Osaka                | 34°51'N  | 135°28' | 160        | 10          | 10         | 0        | 1.57     | 0.16        | 0.22        |
| 31| Myoken, Osaka               | 34°55'N  | 135°27' | 230        | 10          | 10         | 0        | 1.67     | 0.24        | 0.10        |
| 32| Tada, Hyogo                 | 34°54'N  | 135°21' | 140        | 10          | 10         | 0        | 1.57     | 0.19        | 0.23        |
| 33| Mikohata, Hyogo             | 35°15'N  | 134°43' | 320        | 10          | 9          | 1        | 1.47     | 0.16        | 0.08        |
| 34| Takacho-Tada, Hyogo         | 35°06'N  | 134°53' | 190        | 10          | 8          | 0        | 1.35     | 0.16        | 0.15        |
| 35| Ikuno, Hyogo                | 35°10'N  | 134°49' | 360        | 10          | 10         | 0        | 1.55     | 0.22        | 0.23        |
| 36| Omoide-gawa, Hyogo          | 35°06'N  | 134°56' | 200        | 9           | 9          | 0        | 1.35     | 0.15        | 0.16        |
| 37| Monzen, Hyogo               | 35°05'N  | 134°54' | 160        | 10          | 10         | 0        | 1.40     | 0.16        | 0.18        |
| 38| Fukiya, Okayama             | 34°52'N  | 133°27' | 280        | 10          | 5          | 0        | 1.22     | 0.05        | 0.35        |
| 39| Uga, Hiroshima              | 34°33'N  | 132°23' | 80         | 6           | 4          | 0        | 1.16     | 0.12        | 0.59        |
| 40| Enmeikyo, Hiroshima         | 34°42'N  | 133°22' | 280        | 7           | 0          | 0        | 1.00     | 0.00        | NA          |
| 41| Tokusa, Yamaguchi           | 34°26'N  | 131°41' | 550        | 10          | 5          | 0        | 1.23     | 0.10        | 0.09        |
Table 2. Primer information and basic population genetic statistics for microsatellite loci used in this study. Multiplex combinations (comb. 1–3), four types of inflorescent dye, annealing temperature (°C), size range of fragments detected in this study, and the number of alleles are shown for each locus. Observed heterozygosity, within-population and total genetic diversity (\(H_o\), \(H_s\), and \(H_t\), respectively), inbreeding coefficients (\(G_is\): Bold values indicate <5% significant deviations from Hardy-Weinberg equilibrium), and among-population genetic differentiation (\(G_st\): Bold values indicate <5% significant deviations from no differentiation. See also references for sequence information.

| Primer | Dye | Tm | Comb | Range (bp) | #Allele | Reference                              | \(H_o\) | \(H_s\) | \(H_t\) | \(G_is\) | \(G_st\) |
|--------|-----|----|------|------------|---------|----------------------------------------|--------|--------|--------|---------|---------|
| ATH    | FAM | 53 | 1    | 152-158    | 4       | Llaurens et al. (2008)                 | 0.10   | 0.13   | 0.30   | 0.26    | 0.55    |
| ELF3   | FAM | 53 | 1    | 267-300    | 10      | Llaurens et al. (2008)                 | 0.44   | 0.53   | 0.79   | 0.18    | 0.33    |
| LYR133 | VIC | 53 | 1    | 130-150    | 6       | Llaurens et al. (2008)                 | 0.09   | 0.12   | 0.21   | 0.24    | 0.42    |
| ICE12  | VIC | 53 | 1    | 226-236    | 5       | Clauss et al. (2002)                  | 0.19   | 0.22   | 0.30   | 0.16    | 0.25    |
| ICE5   | PET | 53 | 1    | 173-177    | 2       | Clauss et al. (2002)                  | 0.02   | 0.02   | 0.03   | 0.02    | 0.48    |
| nga112 | NED | 53 | 1    | 173-205    | 12      | Clauss et al. (2002)                  | 0.39   | 0.48   | 0.82   | 0.19    | 0.42    |
| ICE8   | VIC | 53 | 1    | 50-60      | 2       | Clauss et al. (2002)                  | 0.00   | 0.01   | 0.01   | 1.00    | -0.02   |
| ICE14  | FAM | 53 | 1    | 219-237    | 6       | Clauss et al. (2002)                  | 0.16   | 0.16   | 0.31   | 0.06    | 0.46    |
| nga361 | NED | 58 | 2    | 124-134    | 6       | Llaurens et al. (2008)                 | 0.37   | 0.42   | 0.58   | 0.12    | 0.29    |
| nga1145| VIC | 58 | 2    | 225-237    | 6       | Symonds & Lloyd (2003)                | 0.25   | 0.31   | 0.58   | 0.20    | 0.47    |
| AthCTR1A | VIC | 58 | 2    | 150-156    | 4       | Bell & Ecker (1994)                   | 0.12   | 0.18   | 0.36   | 0.33    | 0.51    |
| MHI24  | FAM | 58 | 2    | 129-135    | 3       | Clauss et al. (2002)                  | 0.02   | 0.04   | 0.04   | 0.42    | 0.11    |
| F21M12 | NED | 58 | 3    | 155-159    | 2       | Clauss et al. (2002)                  | 0.07   | 0.08   | 0.10   | 0.09    | 0.25    |
| ICE10  | VIC | 58 | 3    | 119-121    | 2       | Clauss et al. (2002)                  | 0.01   | 0.01   | 0.03   | 0.31    | 0.63    |
| ICE13  | FAM | 58 | 3    | 217-253    | 9       | Clauss et al. (2002)                  | 0.32   | 0.36   | 0.64   | 0.10    | 0.44    |
| F19G10 | VIC | 58 | 3    | 179-181    | 2       | Clauss et al. (2002)                  | 0.01   | 0.01   | 0.01   | -0.01   | 0.01    |
| AthZEPEG | PET | 58 | 3    | 126-165    | 15      | Clauss et al. (2002)                  | 0.36   | 0.41   | 0.73   | 0.11    | 0.44    |
|     | FAM |   |     |   | Clauss et al. (2002) |    |    |    |     |
|-----|-----|---|-----|---|----------------------|----|----|----|-----|
| nga151 |     | 58 |   |   | 92-104               |   | 5  |    |     |
| nga129 |     | 58 | 3  |   | 140-160              |   | 3  |    |     |
| Overall | --- | --- | --- | --- | 104                  | --- | --- | --- |     |

Clauss et al. (2002) | 0.02 | 0.03 | 0.03 | 0.33 | 0.02 |
|---------------------|------|------|------|------|------|
| ng129               | 0.02 | 0.19 | -0.14 | 0.93 |  
| Overall             | 0.15 | 0.19 | 0.32 | 0.16 | 0.42 |