Research Article

Title

Comparative analysis of spatial genetic structures in sympatric populations of two riparian plants, *Saxifraga acerifolia* and *Saxifraga fortunei*

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Running Head

Impact of habitat differentiation on genetic structure in two *Saxifraga*

Abstract

Premise of the study

The genetic structure between plant populations is facilitated by the spatial population arrangement and limited dispersal of seed and pollen. *Saxifraga acerifolia*, a local endemic species in Japan, is a habitat specialist that is confined to waterfalls in riparian environments. Its sister species, *Saxifraga fortunei*, is a generalist that is widely distributed along riverbanks. Here, we examined sympatric populations of the two *Saxifraga* species to test whether the differences in habitat preference and colonization process influenced regional and local genetic structures.

Methods

To reveal genetic structures, we examined chloroplast microsatellite variations and genome-wide nucleotide polymorphisms obtained by genotyping by sequencing. We also estimated the gene flow among and within populations and performed landscape genetic analyses to evaluate seed and pollen movement and the extent of genetic isolation related to geographic distance and/or habitat differences.

Key results

We found a strong genetic structure in the specialist *S. acerifolia*, even on a small spatial scale (< 1 km part); each population on a different waterfall in one river system had a completely different predominant haplotype. By contrast, the generalist *S. fortunei* showed no clear genetic differentiation.

Conclusions

Our findings suggest that the level of genetic isolation was increased by spatially fragmented habitat and limited seed and pollen dispersal over waterfalls in *S. acerifolia*. Habitat differentiation between the sister taxa could have contributed to the different patterns of gene flow and then shaped the contrasting genetic structures.

Keywords

Fragmented population; Habitat differentiation; Japan; Landscape genetics; Local endemics; *Saxifraga*; Saxifragaceae; Sister species; Specialist species; Waterfall

INTRODUCTION

A species' habitat, the physical place where it lives, is determined by its physiological and ecological requirements. The actual and potential presence of sessile autotrophic plants is strongly influenced by abiotic elements such as climate conditions, terrain features, and nutrient resources (Grinnell, 1917; Whittaker et al., 1973; Kearney, 2006; Soberón, 2007). A habitat generalist species with a larger habitat can grow in a wide gradient of environments; by contrast, a specialist is restricted to specific environmental conditions, which are often too harsh for the successful growth of common species (Futuyma and Moreno, 1988; Kassen, 2002). Such special habitats (e.g., alpine, special soil, and riparian environments) are often spatially fragmented, so specialist species tend to have discontinuous distributions (Bazzaz, 1991; Brouat et al., 2003; Komonen et al., 2004). In addition, habitat specialists with patchy distributions have small populations. Population genetic studies have suggested that specialist species show low genetic diversity and high genetic differentiation between fragmented populations (e.g., Hughes et al., 1999; Medrano and Herrera, 2008).

The pattern of genetic structure within a plant species is influenced by several factors that contribute to restricted geneflow, such as reproductive mode, breeding system, life history traits, geographic distribution and population history (Brown, 1979; Hamrick et al., 1979; Hamrick, 1983; Carson and Templeton, 1984; Loveless and Hamrick, 1984). Especially on a fine scale, a species' spatial arrangement based on its habitat can affect the patterning of genetic variation (Loveless and Hamrick, 1984). In a fragmented population, reduced seed and pollen dispersal between patches would promote genetic differentiation. Another factor is modes of seed and pollen dispersal, as the two means of independent gene exchange (Hamrick et al., 1993). In general, shorter dispersal distances of seeds and/or pollen lead to greater levels of genetic isolation between populations (Loiselle et al., 1995; Brouat et al., 2003; Harata et al., 2012).

Mountainous landscapes with complex arrangements of ridges and gorges within a narrow range can be strong geographic barriers to gene flow among populations, resulting in divergent genetic structures (Ohsawa and Ide, 2008). At the bottom of a gorge, water currents develop to form a riparian environment inhabited by specific plant species, riparian plants, that have adapted morphologically or ecologically to strong water pressure (van Steenis, 1987; Imaichi and Kato, 1997; Nomura et al., 2010; Mitsui et al.,

2011; Ekar et al., 2019). Population genetic studies of riparian plants have found strong genetic differentiation between different river systems because their suitable habitats are confined to riverbanks and mountain ridges prevent their movement across the gorges (Mitsui et al., 2010; Werth et al., 2014). Within a river system, landscape heterogeneity can influence gene flow via seeds and pollen (Mitsui et al., 2010; Wang et al., 2012). Landscape geneticists have proposed several hypotheses to account for genetic structure in a riparian environment. In addition to the classical isolation-by-distance (IBD) concept (Wright, 1943), a linear correlation between pairwise genetic distance and Euclidean distance, isolation-by-river distance (isolation-by-landscape: IBL), is an important pattern (Richards-Zawacki, 2009; Zalewski et al., 2009, Murray et al., 2019). In a riparian landscape, plant habitats develop along a river and gene exchange by seeds and pollen flow occurs within a linear habitat; the river connectivity between individuals affects genetic structure.

Japan consists of mountainous islands on the eastern edge of the Eurasian continent, where numerous rivers run along gorges between mountain ridges. At the most upstream areas in such undulating topography, rivers are often composed of stepwise arrangements of rapid and/or gentle streams and waterfalls with vertical or acutely angled rocky walls, providing harsh environments for plant growth. However, in the very narrow range of two river systems in Japan (Fig. 1A, B, and C), a local endemic species, *Saxifraga acerifolia* Wakab. et Satomi (Saxifragaceae; section *Irregulares*), adheres to the rock surface of waterfalls, exposed to splashing water spray, but never lives on the riverbanks between waterfalls (Wakabayashi, 1973) (Fig. 2A and B). Thus, each population is spatially fragmented within a river system. Contrasting the disjunct distribution of *S. acerifolia*, with its narrow distribution because of its special habitat, its sister species *Saxifraga fortunei* Hook. f. (Magota et al., unpublished) grows sympatrically as a larger population. *S. fortunei* grows in a wider environment, inhabiting contiguous areas along riverbanks and wet precipices (but not on the rock surfaces of waterfalls) over the habitat range of *S. acerifolia*, as well as along other branch streams that lack *S. acerifolia*. These sister taxa are clearly distinguished by their leaf morphology: *S. acerifolia* has deeply dissected and bright-colored leaves, while *S. fortunei* has broadly ovate, deep green leaves (Fig. 2C and D). They share propagation characteristics, such as reproductive systems and seed morphologies (Wakabayashi, 1973). Therefore, the

differentiation of fine-scale genetic structures between the two species is likely influenced by their spatial population arrangements based on their habitats: spatially fragmented or isolated waterfall habitats for *S. acerifolia* and continuous distribution along riverbanks for *S. fortunei*.

Here, we reveal how the spatial population arrangement based on the habitat differentiation of the sister species *S. acerifolia* and *S. fortunei* affects their regional and local genetic patterns. We addressed three questions: (1) Between different river systems geographically divided by mountain ridges, is each population of *S. acerifolia* and *S. fortunei* genetically isolated? (2) Within a river system, are the fragmented waterfall populations of *S. acerifolia* more genetically isolated than the populations of *S. fortunei* that show continuous distribution along the river? (3) Does the degree of habitat continuity along a river influence the relationship between genetic isolation and geographic distance in both species? We used chloroplast microsatellite variation and genome-wide nucleotide polymorphism patterns for analyses at multiple scales. Comparing maternally and biparentally inherited markers, we could infer gene flow via seeds and pollen among populations independently.

MATERIALS AND METHODS

Study species

Saxifraga acerifolia is a spring-flowering perennial herb with white-petal flowers, pollinated by small bees and flies. The mating system is estimated as predominantly outcrossing based on the observation that, in the protandrous flower, the anthers are caducous soon after anthesis and the flowers become functionally female (Fig. 2E), which would lead to a low self-pollination rate [e.g. *Saxifraga cernua* (Molau, 1992; Molau and Prentice, 1992)].This species is confined to the two most upstream areas in the transitional area between warm-temperate and cool-temperate forests at elevations ranging from 480 m to 650 m of the Kuzuryu (KUZ) and Daishoji (DAI) Rivers systems in Fukui and Ishikawa Prefectures, Japan, respectively (Fig. 1A and C). In KUZ, this species is distributed within 1 km on 12 waterfalls of the main stream and three branch streams, whereas its distribution in DAI is confined to a single waterfall. The two habitat gorges are approximately 10 km apart separated by undulating topography (Fig. 1B and C). This species has been designated as an endangered species [Category II (VU): Ministry of the Environment, Japan, 2019] due to its narrow distribution and small number of individuals: approximately 300 and 100 mature individuals in KUZ and DAI, respectively (Magota, personal observation). In contrast, *S. fortunei* is an autumn-flowering perennial herb and grows on the riverbank ranging from the upstream to downstream of KUZ and DAI with larger populations.

The samples of *S. acerifolia* used in this study covered the entire distribution in KUZ (36°08′ N, 136°22′ E) and DAI (36°11′ N, 136°27′ E). In KUZ, we partitioned the entire population into five subpopulations according to the geographical arrangement of the waterfalls (Fig. 1C). Three subpopulations (A1, A2, and A3) were located on the three branch streams, A4 was located at the catchment of the A2 and A3 branch streams, and A5 was located in the most downstream area of the study site. The branch stream of A1 was connected just upstream of A5 through underground flow. In addition, we sampled the sympatric population of *S. fortunei*, and divided it into five subpopulations (F1, F2, F3, F4, and F5) according to the same partitions. *S. fortunei* inhabits continuous areas along the streams; we collected samples from plants located at least several meters apart to avoid sampling kin individuals. In DAI, we sampled individuals from a large population at a single waterfall; *S. acerifolia* adheres to the vertical rocky wall and *S.*

fortunei grows on the upstream riverbank. The numbers of individuals from each population used for the analyses are shown in Table 1.

DNA extraction and chloroplast DNA analysis

Total genomic DNA was extracted from dried leaf samples of 183 individuals of *S. acerifolia* and 191 individuals of *S. fortunei* using the cetyltrimethylammonium bromide method (Doyle and Doyle, 1987) after washing the leaf powder twice with HEPES buffer (pH = 8.0; Setoguchi and Ohba, 1995). Chloroplast DNA (cpDNA) haplotypes were determined for 161 and 177 individuals of *S. acerifolia* and *S. fortunei*, respectively. Five chloroplast microsatellite (simple sequence repeat; SSR) markers were used to determine the cpDNA haplotypes of the two species, including the four previously reported markers Sacer_cp4155, Sacer_cp5080, Sacer_cp11875, and Sacer_cp30071 (Magota et al., 2018), as well as the marker Sacer_cp10072, which was designed in the present study (Appendix S1; see the Supplementary Data with this article). PCR amplifications were performed in 5-μL reaction volumes containing ~0.5 ng DNA, 2.5 μL 2× Multiplex PCR Master Mix (Qiagen, Hilden, Germany), 0.01 μM forward primer, 0.2 μM reverse primer, and 0.1 μM fluorescence-labelled M13 primer. The following PCR thermal profile was used: initial denaturation at 95°C for 30 min; followed by 35 cycles of 95°C for 30 s, 58°C for 3 min, and 68°C for 1 min; and a final extension at 68°C for 20 min. The amplified PCR products were loaded onto an ABI 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA) with GeneScan 600 LIZ Dye Size Standard v2.0 (Thermo Fisher Scientific, Carlsbad, California, USA), and the fragment length was determined using GeneMapper software (Applied Biosystems).

To evaluate the haplotype diversity and geographic structure, we calculated unbiased diversity (*uh*) and conducted analysis of molecular variance (AMOVA) for the KUZ samples using GenAlEx ver. 6.503 (Peakall and Smouse, 2006). We also calculated haplotype richness (*HR*) using the R package 'hierfstat' (Evanno et al., 2005) in R ver. 3.5.2 (http://www.R-project.org). To reveal genetic differentiation and phylogeographical structure within species, we used two indices of genetic differentiation: G_{ST} and R_{ST} (Pons and Petit, 1996). G_{ST} is calculated based on allele frequency among populations, while R_{ST} accounts for relatedness of haplotypes estimated by allele size variation of microsatellite markers under a stepwise mutation model. Calculations were performed

using PermutCpSSR ver. 1.2.1 (https://www6.bordeaux-aquitaine.inra.fr/biogeco/Production-scientifique/Logiciels/Contrib-Permut/Permut) and the significance of the differentiation between two indices was estimated from 1000 permutations. This analysis verifies whether distinct haplotypes are more closely related within populations or between populations. In addition, a median joining network was generated using Network ver. 5.0.0.3 (Bandelt et al., 1999) to resolve the relationships among haplotypes.

MIG-seq library preparation and SNP calling

We used multiplexed inter-simple sequence repeat (ISSR) genotyping by sequencing (MIG-seq) to detect genome-wide single nucleotide polymorphisms (SNPs); in this method, loci between two ISSR regions were amplified by PCR and sequenced using a next-generation sequencer (Suyama and Matsuki, 2015). Three MIG-seq libraries were prepared under a protocol slightly modified from that of Suyama and Matsuki (2015), with one library each for *S. acerifolia* and *S. fortunei* and one library that included both *S. acerifolia* and *S. fortunei*. The first PCR was conducted using MIG-seq primer set 1 with an annealing temperature of 38°C. The first PCR products were purified using AMPure XP (Beckman Coulter, Brea, California, USA), and then used for a second PCR. The second PCR was conducted using primer pairs containing tail sequences, adapter sequences for Illumina sequencing, and the index sequences of forward and reverse primers to identify each individual sample. After purification, fragments in the size range of 350–800 bp were isolated. The libraries were then sequenced on the Illumina MiSeq platform (Illumina, San Diego, California, USA) using a MiSeq Reagent Kit v3 (150 cycles, Illumina).

Sequence data for MIG-seq primer regions and low-quality reads were removed using FASTX-Toolkit ver. 0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit) and TagDust ver. 1.13 (Lassmann et al., 2009). Both read1 and read2 were used for *de-novo* assembly. After combining the data for the respective species, SNPs were called using Stacks ver. 2.1 (Catchen et al., 2013) with the following parameter settings: minimum number of identical reads required to create a stack, 6; nucleotide mismatches between loci within a single individual, 2; and mismatches between loci when building the catalogue, 2. After exporting genotype data for individual-based analysis, we filtered the SNP data using

PLINK ver. 1.07 (Purcell et al., 2007) with the following settings to obtain the same number of individuals with high overall genotyping rates: markers with a minor allele frequency (maf) < 0.05, markers within Hardy–Weinberg equilibrium (hwe) < 0.01, loci with a missing individual rate (geno) > 0.5, and individuals with a missing locus rate (mind) > 0.86 (for *S. acerifolia*) or > 0.89 (for *S. fortunei*).

Population genetic analysis and estimations of gene flow among populations

For genome-wide SNPs, the calculation of genetic diversity parameters for each population, including observed heterozygosity (H_O), expected heterozygosity (H_E), and fixation index (F_{IS}), as well as the AMOVA analysis, were performed using GenAlEx ver. 6.503. Allelic richness (*AR*) was calculated using the R package 'hierfstat', and deviation from Hardy–Weinberg equilibrium was assessed using 'diveRsity' (Keenan et al., 2013) in R ver. 3.5.2. We also calculated G_{ST} as a parameter of genetic differentiation using SPAGeDi ver. 1-5a (Hardy and Vekemans, 2002). To infer population genetic structure, Bayesian clustering analysis was performed using STRUCTURE ver. 2.3.4 (Pritchard et al., 2000). The population model was set to allow admixture, correlation of allele frequencies between clusters, and use sampling location as a prior (Falush et al., 2003, Hubisz et al., 2009). We simulated 20 independent runs for each K (K = 1–10) with 100,000 Markov chain Monte Carlo (MCMC) iterations after a burn-in of 100,000 iterations. The most meaningful number of genetic clusters (K) was detected based on the Ln probability of the data [LnP(K)] (Pritchard et al., 2000) and the second-order rate of change in the Ln probability of the data (ΔK) (Evanno et al., 2005). The ΔK values were calculated and visualized using STRUCTURE HARVESTER (Earl, 2012). We used CLUMPP (Jakobsson and Rosenberg, 2007) and Distruct (Rosenberg, 2004) to generate STRUCTURE plots from the admixture values for the best K.

The amount and direction of recent migration over the last few generations were estimated based on the Bayesian assignment method between two regional populations or subpopulations of KUZ were calculated using BayesAss ver. 1.3 (Wilson and Rannala, 2003). To calculate the posterior probability distribution of parameters, we ran a burn-in of 30,000,000 steps followed by 50,000,000 steps for populations from KUZ and DAI and subpopulations from KUZ. In addition, we calculated the pollen-to-seed migration ratio ($r = m_p / m_s$) to estimate the contribution ratio of seed and pollen flow using an

equation of Ennos (1994): $r = m_p / m_s = \{2 (1 / G_{STc} - 1) - (1 / G_{STn})\} / \{1 - (1 / G_{STc})\}$. G_{STc} and G_{STn} are differentiation indices that correspond to subdivisions of maternally inherited and biparentally inherited markers, respectively. In plant populations, there are two methods of gene flow: seed and pollen migration. For biparentally inherited nuclear markers, gene flow occurs by both seed and pollen, whereas chloroplast markers involve only maternally inherited genes. We used cpDNA polymorphisms and genome-wide SNPs from MIG-seq for maternal (via seed dispersal) and biparental (via seed and pollen dispersal) components, respectively.

Landscape genetic analysis

Landscape features limit gene flow among populations, subsequently shaping the fine-scale genetic structure. Two hypotheses were addressed here: IBD (isolation-by-distance) and IBL (isolation-by-landscape). The IBD model proposes a correlation between the pairwise genetic distance and shortest straight-line distance. But if a riparian landscape including rivers and steep slopes has promoted gene exchange via seeds and pollen along gorges, the path distance along the river should better correlate with the genetic distance, supporting the IBL model. In particular, in this study, because the species' distribution is confined to a river, which includes some branch streams, and the levels of population continuity in *S. acerifolia* and *S. fortunei* differ greatly, we could uncover the effect of river connectivity on genetic structure by comparing the IBL of the two species. Therefore, we tested whether the pairwise genetic distance between individuals within KUZ could be ascribed to the two geographic distances. Note that the river distance is significantly correlated with the straight-line distance, but it is always greater (Appendix S2).

We calculated the pairwise genetic distances between individuals in KUZ based on cpDNA polymorphisms and genome-wide SNPs from MIG-seq. The procedures were performed following Kosman et al. (2005) using the 'PopGenReport' package (Adamack and Gruber, 2014) in R ver. 3.6.0. We also calculated the two geographic distances among individuals across a landscape mosaic for each hypothesis: (1) The Euclidean distance for IBD, the straight-line geographical distance between pairs of individuals, was calculated using the Universal Transverse Mercator coordinates of the original sites of individuals with GenAlEx ver. 6.503. (2) The path distance along the river (river distance) for IBL was calculated using the Network Analysis toolbox in ArcMap ver. 10.5 (ESRI, Redlands,

California, USA). The river distance did not include the elevation difference and consisted only of the horizontal distance along the curves of the river.

We tested both the IBD and IBL hypotheses using Mantel and partial Mantel correlations between pairwise genetic distances and the two geographic distances. Typically, Mantel tests tend to show spurious correlations if two geographic distances are highly correlated (Cushman and Landguth, 2010). Then, we used the simple Mantel's test for IBD and sIBL and partial Mantel test for pIBL, partialling out the effects of IBD because the river distances for IBL were correlated with the Euclidean distances. Each (partial) Mantel test was performed with 999 permutations using the 'ecodist' package (Goslee and Urban, 2007) in R ver. 3.6.0.

RESULTS

Haplotype diversity and population differentiation based on cpDNA analysis

We identified ten and eight haplotypes in *Saxifraga acerifolia* and *S. fortunei*, respectively, based on five cpDNA SSR loci (Table 1). The phylogenetic relationships among the haplotypes are shown in the median joining network diagram presented in Figure 3A. Two well-separated haplotype groups were detected in the network: one containing ten haplotypes (H1–H10) from *S. acerifolia*, and the other containing eight haplotypes (H11–H18) from *S. fortunei*. The two species-specific haplotype groups are separated by 15 steps (between H10 of *S. acerifolia* and H11 of *S. fortunei*), clarifying the absence of shared haplotypes between the sympatric sister taxa.

In *S. acerifolia*, the haplotype compositions of the two populations from KUZ and DAI were completely differentiated: KUZ and DAI harbored six (H5–H10) and four (H1–H4) closely related population-specific haplotypes, respectively. These population-specific haplotypes are demarcated by three steps between H4 of KUZ and H6 of DAI. By contrast, two populations of *S. fortunei* shared two (H15 and H16) of the eight haplotypes. In *S. acerifolia*, the haplotype differentiation between KUZ and DAI was estimated as G_{ST} = 0.326 and R_{ST} = 0.801 (Table 2), indicating high relatedness between haplotypes within populations and definite separation of retained haplotypes between populations. By contrast, much lower values, 0.169 (G_{ST}) and 0.156 (R_{ST}), were estimated for *S. fortunei*, suggesting low genetic differentiation with haplotype admixture between the populations. The total haplotype richness (*HR*) and unbiased diversity (*uh*) were higher for *S. acerifolia* (2.60 and 0.352, respectively) than for *S. fortunei* (2.00 and 0.206, respectively) (Table 1). The results of AMOVA are shown in Table 3. The proportions of genetic variability among and within populations of *S. acerifolia* were 31.7% and 57.2%, respectively, whereas for *S. fortunei*, most of the variation was explained by differentiation among individuals (85.0%) and subpopulations (14.6%).

In the river system of KUZ, the three subpopulations of *S. acerifolia* (A1–A3) in three different branch streams were dominated by different haplotypes (Fig. 4A). The five *S. acerifolia* subpopulations were highly differentiated (G_{ST} = 0.727, R_{ST} = 0.783), whereas *S. fortunei* exhibited lower differentiation among subpopulations (0.121 and 0.087, respectively) (Table 2). The *HR* of *S. acerifolia* subpopulations ranged from 1.00 (A2) to 1.38 (A3), and that of *S. fortunei* subpopulations ranged from 1.20 (F2 and F5) to

1.42 (F1) (Table 1). The results of AMOVA showed that most of the total genetic variation within KUZ was found among subpopulations in *S. acerifolia* (86.6%), whereas *S. fortunei* exhibited the most genetic variability among individuals (86.3%), not among populations (13.7%) (Table 3).

Genetic diversity and genetic differentiation among populations based on SNP data using MIG-seq

In the MIG-seq analysis, 58,598,110 raw reads (320,208 reads/sample) were obtained for *S. acerifolia* and 68,239,670 (357,276) were obtained for *S. fortunei*. After SNP filtering, 264 SNPs were genotyped from 117 *S. acerifolia* individuals, and 349 SNPs were genotyped from 102 *S. fortunei* individuals. The overall genotyping rates were 0.629 and 0.620 for *S. acerifolia* and *S. fortunei*, respectively. MIG-seq data were deposited in databases of the NCBI Sequence Read Archive under the following accession numbers: BioProject, PRJDB7742; BioSample, SAMD00153271–SAMD00153644; Experiment, DRX155130–DRX155503; and Run, DRR164511–DRR164884.

The genetic diversity parameters are summarized in Table 1. The total H_E was 0.243 in *S. acerifolia*, and 0.216 in *S. fortunei*. The *AR* of both *S. acerifolia* and *S. fortunei* showed higher values in KUZ (1.24 and 1.61, respectively) than in DAI (1.22 and 1.56). The F_{IS} of all populations of *S. acerifolia* and *S. fortunei* were approximately zero, except for the *S. fortunei* subpopulation F5 (−0.287) (Table 1). Hardy–Weinberg equilibrium exact tests indicated that the populations from KUZ as well as the total populations of both *S. acerifolia* and *S. fortunei* were in deviation. The G_{ST} values among all individuals were 0.080 and 0.029 for *S. acerifolia* and *S. fortunei*, respectively (Table 2).

The AMOVA results are shown in Table 3. Most of the variation was explained by differentiation among individuals within subpopulations (58.1% and 70.7% for *S. acerifolia* and *S. fortunei*, respectively) and within individuals (30.5% and 26.2%), whereas the amount of genetic variability among populations was 6.3% and 0.8%, respectively. In the STRUCTURE analysis, intraspecific genetic structure was assessed; the Ln probability of the data [LnP(*K*)] increased as *K* increased for both *S. acerifolia* and *S. fortunei*, although ΔK indicated a single peak ($K = 2$, $\Delta K = 182.652$) in *S. acerifolia*, but no significant peak in *S. fortunei* (Appendix S3). Clear genetic differentiation between KUZ and DAI was revealed in *S. acerifolia*, whereas analysis of *S. fortunei* resulted in an

ambiguous structure between the two regions (Fig. 3C).

Within KUZ, each subpopulation harbored higher *AR* in *S. acerifolia* (1.23–1.26) than in *S. fortunei* (1.21–1.22) (Table 1). The AMOVA among subpopulations of KUZ revealed that most genetic differentiation existed among individuals in both species (Table 3). The result of STRUCTURE analysis indicated that A1 is the most differentiated of the five subpopulations of *S. acerifolia* (Fig. 4C), whereas F1 of *S. fortunei* harbored an admixed structure (Fig. 4D). LnP(*K*) and Δ*K* values are shown in Appendix S4.

Gene flow among populations

In the Bayesian assignment test based on genome-wide SNPs, *S. acerifolia* and *S. fortunei* exhibited different gene flow patterns between the KUZ and DAI populations in different river systems. In *S. acerifolia*, the high self-recruitment rates suggested that most individuals were derived from their source populations, whereas significant unidirectional gene flow from KUZ to DAI was detected between populations of *S. fortunei* (migration rate [m] = 0.318) (Table 4A and B). Among *S. acerifolia* in KUZ, every subpopulation had a high self-recruitment rate ([m] > 0.683), and gene flow among subpopulations was also detected (Table 4C). Migration from upstream to downstream subpopulations was detected from A1 to A4 ([m] = 0.153), from A3 to A4 ([m] = 0.109), and from A3 to A5 ([m] = 0.212). A2 and A3 were assumed to have bidirectional gene flow ([m] = 0.263 and 0.214, respectively). In *S. fortunei*, the migration rates could not be calculated because the MCMC calculation did not converge with all possible combinations of variables.

The estimated ratios of pollen flow to seed flow (*r*) in KUZ were 93.5 and 1.43 for *S. acerifolia* and *S. fortunei*, respectively.

Landscape genetic analysis

To investigate whether riparian habitat connectivity along a river in KUZ influenced the spatial genetic structure, we tested the correlations between genetic distance and the two geographic distances (IBD and IBL). For the IBL model, we performed two calculations: the simple Mantel test of genetic distance and river distance for sIBL and the partial Mantel test for pIBL, partialling out the effect of the straight-line distance. In *S. acerifolia*, the IBD and sIBL based on cpDNA variation were significant ($P < 0.005$), and the correlation coefficient (Mantel *r*) of IBD (0.535) was slightly lower than that of sIBL

(0.540). After partialling out the effects of geographic distance, the correlation coefficient of the pIBL model approached zero (0.096), but was still significant ($P < 0.005$). For genome-wide SNPs, we determined the IBD (0.166) and sIBL (0.518) ($P < 0.005$), but pIBL was not significant ($P > 0.05$). In *S. fortunei*, no correlation model was significant ($P > 0.05$).

DISCUSSION

Genetic structures in different river systems

Overall, this study revealed contrasting genetic structures between the riparian sister taxa *Saxifraga acerifolia* and *S. fortunei* in the KUZ and DAI populations, attributed to their geographic distributions. For riparian plants, undulating topographical features act as strong geographical barriers because the movements of seed and pollen over mountain ridges are restricted, creating linear habitat connectivity along gorges (Liao and Hsiao, 1998; Kitamoto et al., 2005; Pollux et al., 2007).

In *S. acerifolia*, the two populations in different river systems of KUZ and DAI harbored heterogeneous structures in both cpDNA and genome-wide SNPs. The median joining network showed that the cpDNA haplotype composition was clearly differentiated, demarcated by steps in the network (Fig. 3A). This was corroborated by the results of AMOVA, in which genetic variability among populations accounted for 31.7% of the total variation (Table 3). STRUCTURE analysis using genome-wide SNPs also revealed a clear genetic differentiation between KUZ and DAI (Fig. 3C), corroborated by a lack of inter-population gene flow based on the Bayesian assignment test (Table 4). Reduced gene flow strongly influences the genetic distinction between populations (Slatkin, 1985, 1987). The disjunct distribution in the mountainous landscape strongly limited genetic exchange via seed and pollen, resulting in genetic isolation between the KUZ and DAI populations. In addition, the low mobility of *S. acerifolia*, with its specialized habitat in limited climatic conditions, would likely restrict the expansion of its distribution (e.g., Yoshimura et al., 2019). The lack of suitable waterfall habitats and the discontinuity of the populations in the undulating topology likely encouraged the formation of the two small segregated regional populations, even though they are only 10 km apart.

Contrasting the clear geographical structure in *S. acerifolia*, we found no clear genetic differentiation between the two populations of *S. fortunei*. They shared predominant cpDNA haplotypes, and Bayesian clustering implied an ambiguous structure between KUZ and DAI (Fig. 3C). These results suggest two possibilities: gene flow between the two populations, or shared ancestral polymorphisms between the two populations. The presence of gene flow in a few recent generations was detected by the Bayesian assignment test (Table 4). In this study, we sampled from limited populations of *S. fortunei* in KUZ and DAI as sympatric populations to *S. acerifolia*, but there are

435 many *S. fortunei* individuals in the mountain areas around and between these two
436 populations. Thus, the presence of unsampled populations around the two gorges would
437 probably contribute to gene flow between KUZ and DAI. We could also consider the
438 possibility of ancestral polymorphisms, because the two regional populations share
439 dominant haplotypes, contrasting the complete differentiation in *S. acerifolia*. It is
440 difficult to distinguish shared ancestral variation from gene flow between populations
441 (Muir and Schloetterer, 2005; Lexer et al., 2006). This comparison between *S. acerifolia*
442 and *S. fortunei* shows that different spatial population arrangements and levels of gene
443 flow due to a special habitat can shape genetic structures even at a fine geographic scale.
444
445 ***The unique genetic structure of* S. acerifolia *growing on a waterfall***
446 Strong fine-scale spatial genetic structures are often sculpted by limited seed dispersal
447 and settlement processes (Nason et al., 1997; Vekemans and Hardy, 2004; Harata et al.,
448 2012). Especially for plants living in harsh habitats, the frequency of opportunities for
449 recruiting newly arrived seeds to the population may affect the pattern of genetic diversity
450 (Honnay et al., 2010) because the habitats will depress seed-settlement (Caujapé-Castells
451 et al., 1999). Waterfalls, as specialized, harsh habitats, might prevent *S. acerifolia* seeds
452 from settling. In KUZ, each subpopulation had a characteristic cpDNA haplotype on a
453 fine geographic scale of less than 1 km (Fig. 4A), and the results of the AMOVA (Table
454 3B) showed that most of the differentiation (86.6%) was among subpopulations, unlike
455 the genetic differentiation based on genome-wide variation in SNPs (Fig. 4C). The low
456 contribution of seed dispersal (pollen-to-seed migration ratio: r = 93.5) was likely
457 influenced by the limited seed movement, creating a predominant haplotype. We could
458 also consider the effect of demographic processes (i.e., founder and bottleneck effects)
459 for such a haplotype distribution. By contrast, for *S. fortunei*, seed settlement is easier
460 within its widespread suitable habitat along rivers and occurs more frequently, similar to
461 the case for other riparian plants (Schneider and Sharitz, 1988; Merritt and Wohl, 2002;
462 Opgenoorth et al., 2010), and all of the subpopulations shared haplotypes (Fig. 4B). The
463 lower pollen-to-seed migration ratio (r = 1.43) also suggested a strong contribution of
464 seed dispersal to the colonization of new habitats.
465 Landscape genetic analysis revealed the contrasting consequence of habitat
466 preference between *S. acerifolia* and *S. fortunei*. We detected significant correlations

between genetic distances and the two geographic distances in *S. acerifolia*, but there was no significant correlation in *S. fortunei* for either marker (Table 5). The observed genetic structure in *S. acerifolia* was related to the geographic distance between individuals, suggesting that landscape features restricted the gene flow via seeds and pollen. The higher correlations for the cpDNA data than the genome-wide SNP data indicated that the levels of seed and pollen flow were biased and seed dispersal was strongly restricted by landscape (McCauley, 1994; Latouche-Hallé et al., 2003). Moreover, correlation values of IBD and sIBL were similar in both genetic markers (Table 5), suggesting simple geographic isolation itself was the important factor to promote genetic differentiation, although population connectivity along a river could also have a slight influence on it. The fragmented habitat confined to waterfalls prevents pollen dispersal depending on reduced pollinator movement, as is the case in other plant species with fragmented populations (Segelbacher et al., 2003; Epperson, 2007; Reding et al., 2013). Therefore, frequent short-distance pollination by small bees and flies with low mobility would have induced substantial genetic structure, even within a river system spanning 1 km. We need, however, more observations of the effective pollinator insects for the species and their flight distances. By contrast, *S. fortunei* showed no IBD-like pattern in common with other riparian plants (Leck and Graveline, 1979; Mitsui et al., 2010), probably because of long seed dispersal by water currents. If hydrochory was effective, biased genetic diversity could be generated in which downstream populations have higher genetic diversity than upstream populations (Russell et al., 1999; Mitsui et al., 2010). *Saxifraga fortunei* did not show such a pattern (Table 1), suggesting that hydrochory is not the dominant seed dispersal mode. However, the continuous habitat along the river would have promoted frequent seed and pollen dispersal for *S. fortunei*.

Genetic diversity in S. acerifolia*, a local endemic species*

The genetic diversity of a plant species with a small population size tends to be lower than that in larger populations due to genetic drift and increased inbreeding depression (Ellstrand and Elam, 1993; Frankham, 1996). *S. acerifolia* is a typical local endemic species that is distributed within a very small range with small population sizes and a discrete waterfall-confined population arrangement. It was found to harbor slightly higher genetic diversity (*HR* and H_E: 2.60 and 0.243 in all individuals, respectively; Table 1)

than the more widespread *S. fortunei* (2.00 and 0.216) in the same study area. The F_{IS} values were approximately zero in each population and subpopulation (e.g., −0.016 and −0.033 in KUZ and DAI, respectively), suggesting that most regeneration occurs by outcrossing. This tendency indicates that the two *S. acerifolia* populations might not have experienced a severe bottleneck effect due to low population size. Their historical population dynamics should be evaluated in future studies.

The mating system characteristics of *S. acerifolia* also support the retention of high genetic diversity, promoting outcrossing. Individuals being crowded into narrow waterfall habitats may also increase the level of pollinator visitation (Kunin, 1997), which can result in frequent local gene exchange via pollen. Moreover, a small geographic scale of ~800 m containing each whole population within a river system likely induced gene flow between spatially separated subpopulations. The presence of these factors is likely to maintain a high level of genetic diversity, even in a rare or endangered species with a small population size (Wu et al., 2015).

CONCLUSIONS

The results of our study highlight how the strong differentiation of habitat sculpted a contrasting pattern of genetic diversity between the sister taxa *Saxifraga acerifolia* and *S. fortunei*. The fragmented habitat by waterfalls strongly limited seed dispersal and colonization, even on a small spatial scale (< 1 km). The unique ecological traits of *S. acerifolia*, a specialist that adheres to the vertical rocky surfaces of waterfalls, are important for its coexistence with other riparian plants, including *S. fortunei*. Future investigations of the adaptation mechanisms of *S. acerifolia* will provide new insights into plant species diversity.

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Author Contributions

K.M., S.S., K.A., and H.S. conceived and designed the experiments. K.M., K.A., and H.S. contributed to sample collection. K.M., S.H., and Y.T. performed the molecular experiments and K.M. conducted the molecular analysis and drafted the manuscript. All authors participated in manuscript modifications and approved the final version for publication.

Data Availability Statement

Raw sequence reads of the MIG-seq data were deposited in the NCBI Sequence Read Archive as follows. BioProject: PRJDB7742 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJDB7742), BioSample: SAMD00153271–SAMD00153644, Experiment: DRX155130–DRX155503, Run: DRR164511–DRR164884.

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773 **Tables**

Table 1 Genetic diversity estimated from cpDNA and genome-wide SNPs in local populations of KUZ and DAI and subpopulations of KUZ for *Saxifraga acerifolia* and *S. fortunei*.

	S. acerifolia											*S. fortunei*										
Population	Subpop.	cpDNA				genome-wide SNPs						Subpop.	cpDNA				genome-wide SNPs					
		N	*A*	*HR*	*uh*	*N*	*AR*	H_O	H_E	F_{IS}			*N*	*A*	*HR*	*uh*	*N*	*AR*	H_O	H_E	F_{IS}	
KUZ		137	6	1.80	0.284	94	1.24	0.250	0.240	-0.016	*		145	6	1.74	0.230	82	1.61	0.195	0.217	0.121	*
	A1	54	4	1.19	0.035	46	1.23	0.243	0.226	-0.043		F1	36	4	1.42	0.278	19	1.22	0.202	0.211	0.037	
	A2	26	1	1.00	0.000	14	1.23	0.257	0.216	-0.147		F2	40	5	1.20	0.132	19	1.21	0.189	0.204	0.070	
	A3	27	3	1.38	0.104	15	1.24	0.268	0.228	-0.129		F3	30	3	1.30	0.199	17	1.21	0.190	0.205	0.074	
	A4	16	3	1.35	0.048	9	1.25	0.243	0.225	-0.091		F4	36	5	1.34	0.224	24	1.21	0.197	0.207	0.052	
	A5	14	2	1.20	0.088	10	1.26	0.267	0.235	-0.113		F5	3	2	1.20	0.133	3	1.21	0.181	0.139	-0.287	
DAI		22	4	1.60	0.134	23	1.22	0.221	0.209	-0.033			32	4	1.25	0.059	20	1.56	0.187	0.195	0.036	
Total		159	10	2.60	0.352	117	2.00	0.243	0.243	0.016	*		177	8	2.00	0.206	102	2.00	0.193	0.216	0.126	*

N, number of individuals; *A*, number of alleles; *HR*, haplotype richness; *uh*, unbiased diversity; *AR*, allelic richness; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, fixation index. *Significant deviation from Hardy–Weinberg equilibrium.

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Table 2 Estimation of population differential indices between local populations and among subpopulations in KUZ of *Saxifraga acerifolia* and *S. fortunei*.

		S. acerifolia		*S. fortunei*	
		G_{ST}	R_{ST}	G_{ST}	R_{ST}
cpDNA	Among populations	0.326	0.801	0.169	0.156
	Among subpopulations in KUZ	0.727	0.783	0.121	0.087
genome-wide SNP	Among populations	0.080	-	0.029	-
	Among subpopulations in KUZ	0.028	-	0.040	-

No significant differentiation between G_{ST} and R_{ST} was detected among subpopulations in FUK for cpDNA and genome-wide SNP analyses.

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Table 3 Results of AMOVA using cpDNA and genome-wide SNP analysis (A) in KUZ and DAI and (B) in subpopulations of *S. acerifolia* and *S. fortunei* in KUZ.

	S. acerifolia								*S. fortunei*							
	cpDNA					genome-wide SNPs			cpDNA					genome-wide SNPs		
	d.f.	Sum of squares	Variance components	Percentage of variation	d.f.	Sum of squares	Variance components	Percentage of variation	d.f.	Sum of squares	Variance components	Percentage of variation	d.f.	Sum of squares	Variance components	Percentage of variation
(A) KUZ and DAI																
Among populations	1	70.5	0.434 *	31.7%	1	619.0	4.565 *	6.3%	1	6.2	0.002	0.4%	1	272.5	0.675	0.8%
Among subpopulations within populations	4	161.2	0.783 *	57.2%	4	897.1	3.633 *	5.0%	4	20.7	0.078 *	14.6%	4	846.5	2.045 *	2.3%
Among individuals within subpopulations	153	46.1	0.151 *	11.0%	111	11771.4	41.996 *	58.1%	171	154.4	0.452 *	85.0%	96	14176.1	62.283 *	70.7%
Within individuals	-	-	-	-	117	2580.5	22.056 *	30.5%	-	-	-	-	102	2356.5	23.103 *	26.2%
(B) subpopulations in KUZ																
Among subpopulations	4	161.2	0.784 *	86.6%	4	897.1	3.577 *	5.2%	4	22.0	0.081 *	13.7%	4	846.5	2.064 *	2.4%
Among individuals within subpopulations	132	32.0	0.121 *	13.4%	89	9599.9	42.748 *	62.2%	140	143.9	0.514 *	86.3%	77	11324.2	61.851 *	70.9%
Within individuals	-	-	-	-	94	2102.5	22.367 *	32.6%	-	-	-	-	82	1916.0	23.366 *	26.8%

*$P < 0.01$

Table 4 Mean values of the posterior distributions of the migration rates [m] by BayesAss analysis. (A) Between KUZ and DAI in *Saxifraga acerifolia*; (B) between KUZ and DAI in *S. fortunei*; (C) among subpopulations of *S. acerifolia* in KUZ.

(A)

	From	
To	KUZ	DAI
KUZ	**0.989**	0.012
DAI	0.038	**0.962**

(B)

	From	
To	KUZ	DAI
KUZ	**0.992**	0.008
DAI	**0.318**	**0.682**

(C)

	From				
To	A1	A2	A3	A4	A5
A1	**0.959**	0.007	0.015	0.007	0.013
A2	0.018	**0.684**	**0.263**	0.018	0.018
A3	0.051	**0.214**	**0.683**	0.033	0.018
A4	**0.153**	0.024	**0.109**	**0.690**	0.024
A5	0.055	0.022	**0.212**	0.022	**0.689**

Values on the diagonal indicate the proportion of individuals derived from the source populations. Migration rates > 0.10 are in bold.

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Table 5. Results of Mantel test for the correaltion between genetic distance and geographic distances

	S. acerifolia						*S. fortunei*					
	IBD		sIBL		pIBL		IBD		sIBL		pIBL	
	Genetic dist. ~Euclidean dist.		Genetic dist. ~River dist.		Genetic dist. ~River dist.+Euclidean dist.		Genetic dist. ~Euclidean dist.		Genetic dist. ~River dist.		Genetic dist. ~River dist.+Euclidean dist.	
cp DNA	0.535	**	0.540	**	0.096	**	0.029		0.023		-0.015	
genome-wide SNPs	0.166	**	0.158	**	-0.038		0.035		0.018		-0.055	

Simple Mantel test for IBD and sIBL model and partial Mantel test for pIBL model were performed. Correlation coefficients (Mantel *r*) were shown with *P*-value (*$P < 0.005$).

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Figures

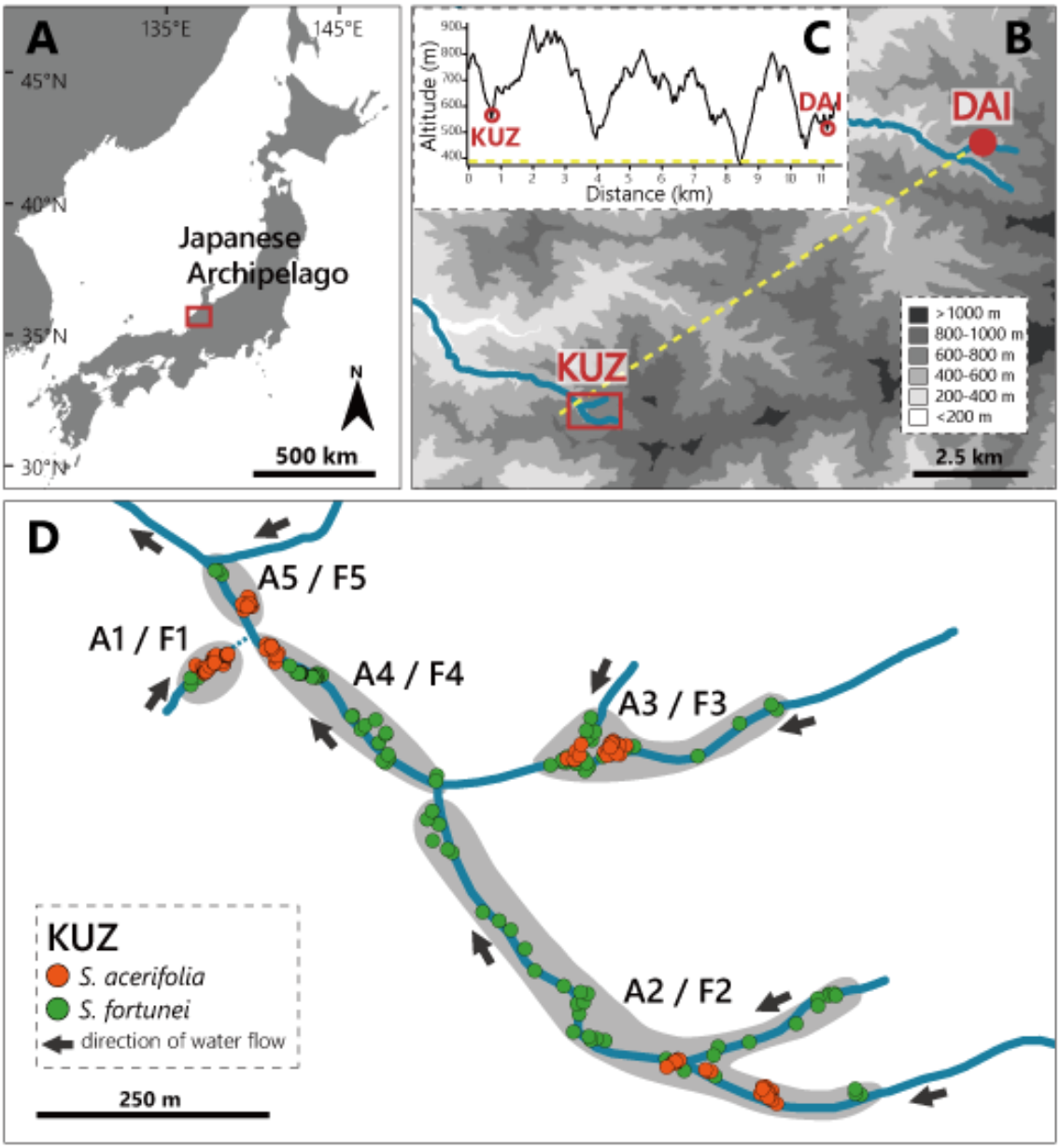


Figure 1

Geographical information. (A) Map of the Japanese archipelago and studied sites (indicated by red squares). (C) Enlarged map of the studied site. The red square and circle indicate the KUZ and DAI populations, respectively. Vertical section view of geographical undulations along the yellow line is shown in (B). (D) Individuals of *Saxifraga acerifolia* (orange dots) and *S. fortunei* (green dots), and partitions into five subpopulations depending on branch currents and spatial arrangements (A1–A5 for *S. acerifolia*; F1–F5 for *S. fortunei*).

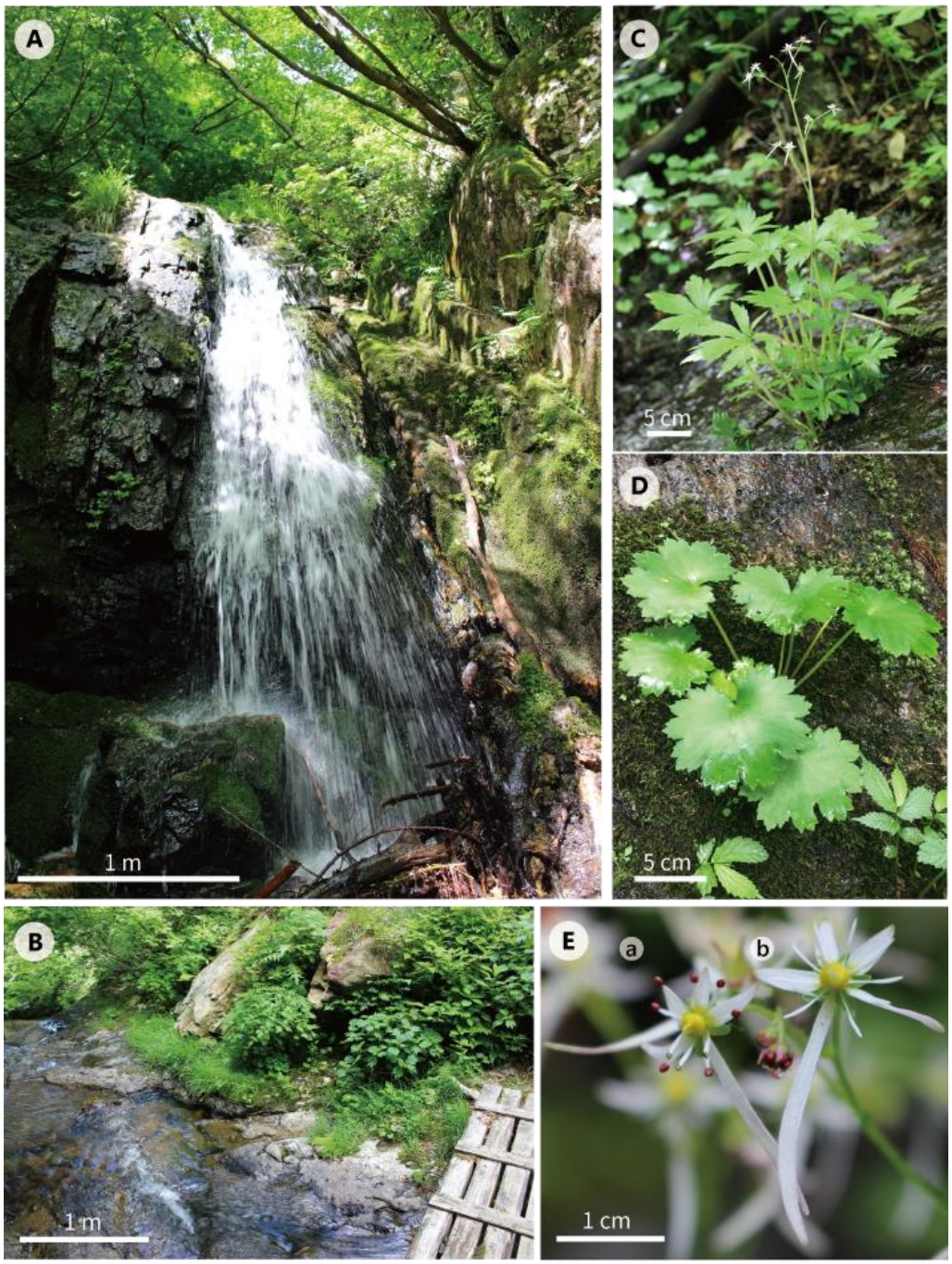


Figure 2

Photographs of habitat environments of *Saxifraga acerifolia* and *S. fortunei*. (A) Rocky surface of the waterfall where (C) *S. acerifolia* adheres. (B) Riverbank with gentle water flow where (D) *S. fortunei* grows. (E) The protandrous flowers of *S. acerifolia*. (a) A male flower with red anthers in one florescence and (b) a functionally female flower with elongated stigmas. Anthers are caducous soon after anthesis, and the flower becomes functionally female.

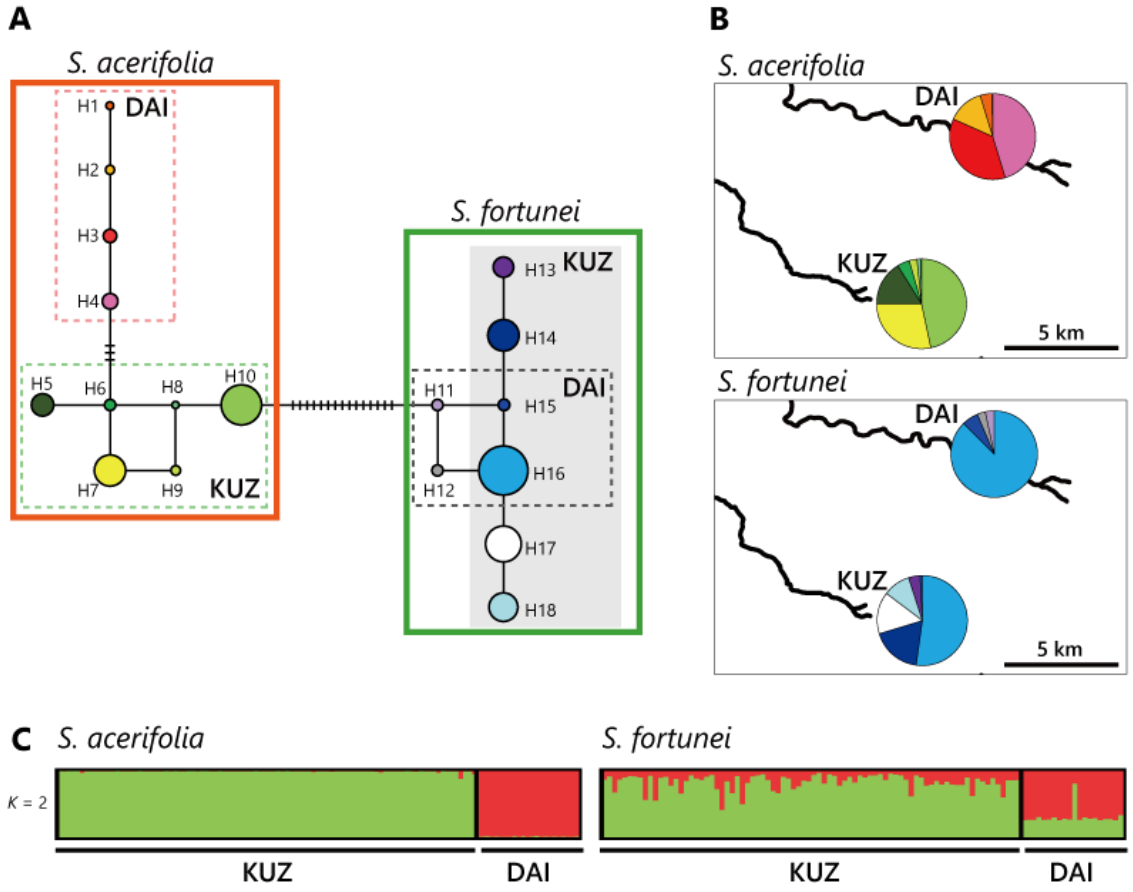


Figure 3

Haplotype and genetic structure. (A) Haplotype networks of *Saxifraga acerifolia* and *S. fortunei* based on five chloroplast microsatellite markers. The ten haplotypes (H1–H10; within the orange line) of *S. acerifolia* and eight haplotypes (H11–H18; within the green line) of *S. fortunei* are separated by 15 steps. There are two *S. acerifolia* clades: KUZ (H5–H10; within the light green dotted line) and DAI (H1–H4; within the red dotted line). For *S. fortunei*, KUZ harbored H13–H18 (shaded area), whereas DAI contained H11–H16 (within the black dotted line). (B) Haplotype distribution between KUZ and DAI. Pie charts indicate the composition ratio of the haplotypes. (C) Results of STRUCTURE analysis with genome-wide SNPs ($K = 2$) between KUZ and DAI for each species.

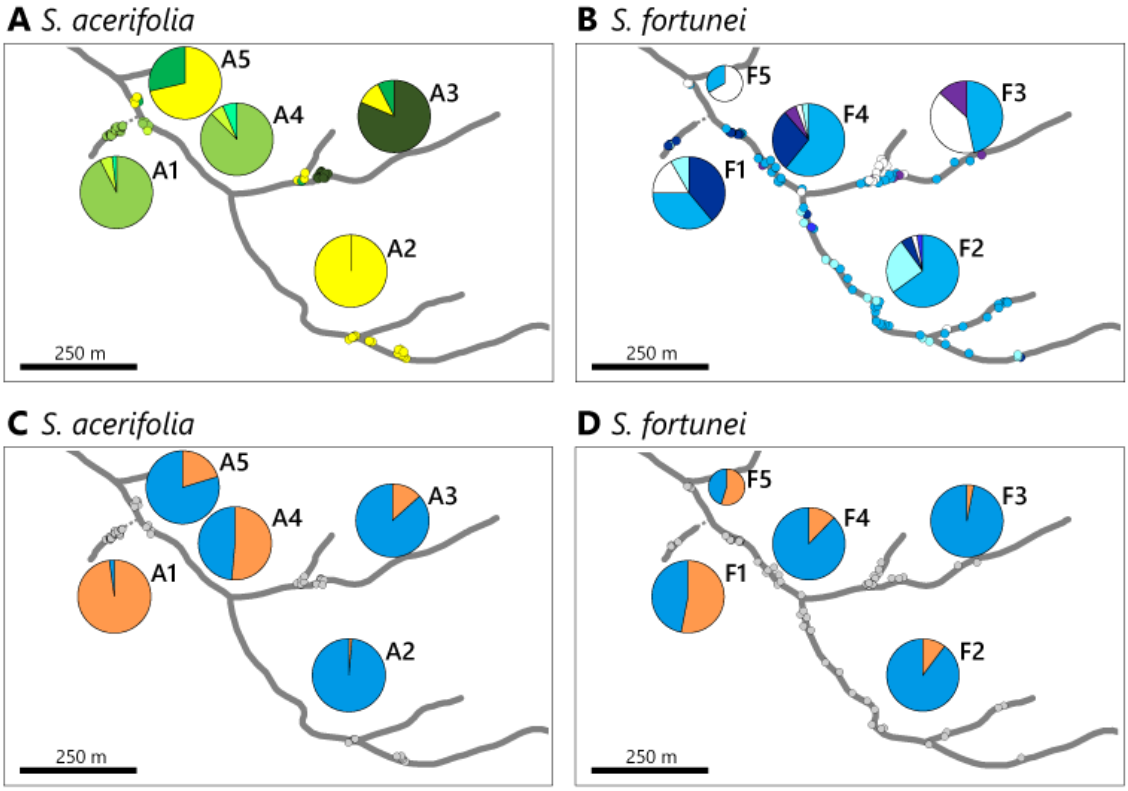


Figure 4

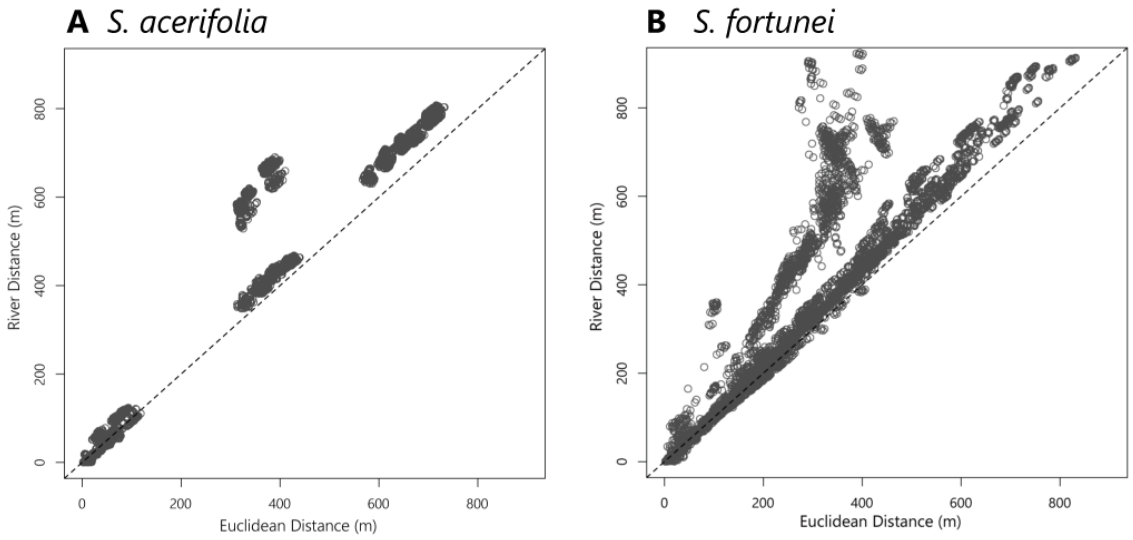
Results of haplotype and STRUCTURE analyses. Pie charts indicate the haplotype distribution ratios of *Saxifraga acerifolia* (A) and *S. fortunei* (B) in KUZ. Each dot indicates the geographic position of an individual, with colors corresponding to their haplotypes. Pie charts in (C) and (D) show the results of STRUCTURE analysis ($K = 2$) of *S. acerifolia* and *S. fortunei*, respectively. F5 was drawn with a small circle because it contains only three individuals, the smallest population size for which population structure could be estimated.

817 **Supporting Information**

818

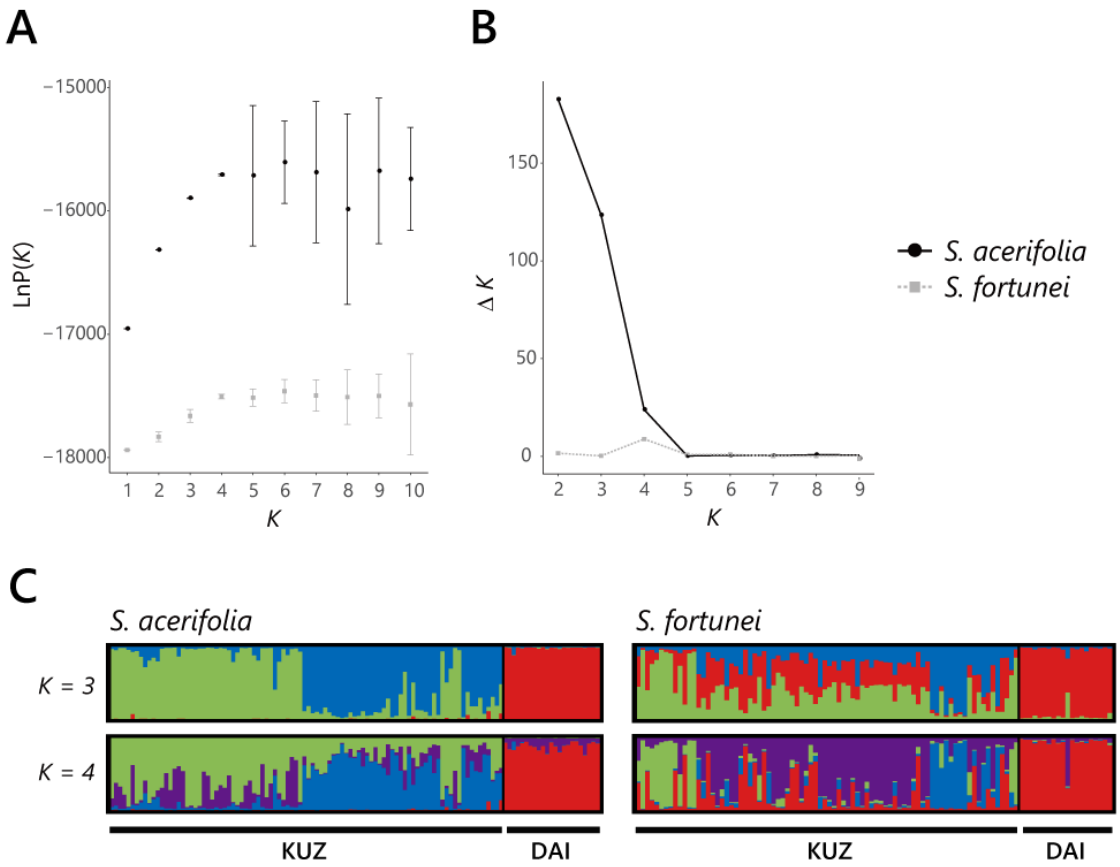
819 Appendix S1 The five chloroplast microsatellite markers used in this study.

Name	Primer sequence (5'-3')	Repeat motif	Source
Sacer_cp4155	F: TGTGGAATTGTGAGCGGTGCATGACCCAATCAAAACA	$(A)_{22}$	Magota *et al.*, 2018
	R: GTTTCTTAGCTGACGGGTTCGTTGA		
Sacer_cp5080	F: CGGAGAGCCGAGAGGTGCGGTAGACCGCTCATTGG	$(C)_{10}$	Magota *et al.*, 2018
	R: GTTTCTTCTCGAGCCGTACGAGGAG		
Sacer_cp10072	F: CACGACGTTGTAAAACGACTAACCCCTAGCCTTCCAAGC	$(T)_9$	Designed in this study. GeneBank accession no. LC432088
	R: GTTTCTTGACAATGGACGCCTTTCATTCC		
Sacer_cp11875	F: CGGAGAGCCGAGAGGTGAGCAATGCCATCGCCTAC	$(A)_{10}$	Magota *et al.*, 2018
	R: GTTTCTTTTGGGGCGATGAAAGAAA		
Sacer_cp30071	F: TGTGGAATTGTGAGCGGTCAAATCGATTCATCGTCCA	$(T)_{11}$	Magota *et al.*, 2018
	R: GTTTCTTTACCCCGAAGGCGGTAGT		



Appendix S2

Correlation of river distance with Euclidean distance between individuals of (A) *Saxifraga acerifolia* and (B) *S. fortunei* analyzed using cpDNA markers. The black dotted line represents equal values of both geographic distances.

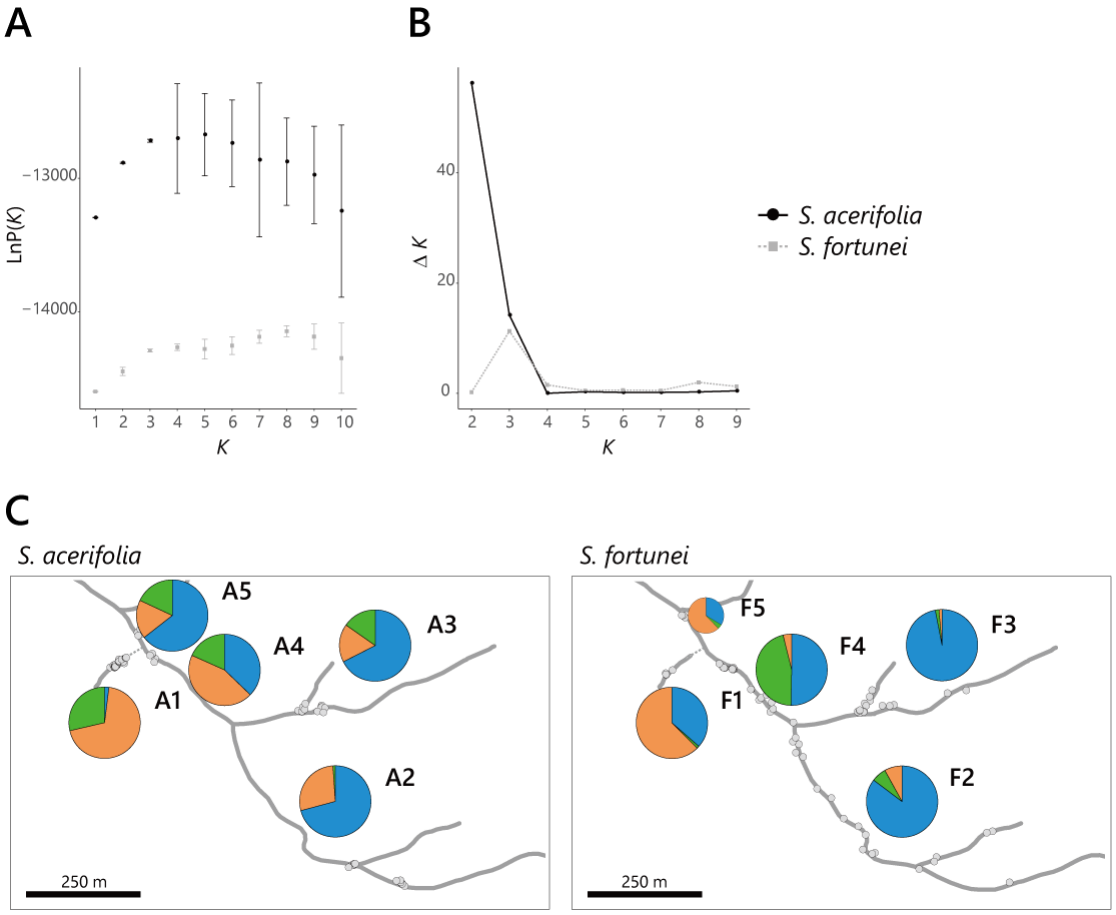


826

827 Appendix S3

828 Clustering results of KUZ and DAI for *Saxifraga acerifolia* and *S. fortunei* using
829 STRUCTURE. Plots indicate mean posterior probability [LnP(*K*)] values with error bars
830 of standard deviation per cluster (*K*) (A), and Δ*K* values (B). The results (*K* = 3 and 4)
831 are shown in C, where each bar represents an individual.

832



Appendix S4

Results of STRUCTURE analysis for *Saxifraga acerifolia* and *S. fortunei* in KUZ. Mean LnP(*K*) is shown in A, where error bars represent standard deviation; Δ*K* values for each *K* are shown in B. The ratio of clusters (*K* = 3) for each subpopulation is shown with pie charts (C).