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RESEARCH ARTICLE

Genetic structure of the critically endangered plant *Tricyrtis ishiiana* (Convallariaceae) in relict populations of Japan

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Abstract *Tricyrtis ishiiana* is a relic endemic plant taxon of the Convallariaceae that inhabits two nearby gorges in Kanagawa Prefecture, Japan. The distribution range and number of populations are thought to have been reduced to the present refugial populations during the Quaternary climatic oscillations. Because of its showy flowers, this plant has faced illegal removal from its natural habitats for horticultural use and has been designated a critically endangered species (class IA). In this study, we analyzed the genetic structure of the relict populations of *T. ishiiana* in order to contribute to the conservation strategies of the prefectural government. Our analyses of nine nuclear microsatellite loci detected high genetic diversity ($H_E = 0.704$ and $H_O = 0.541$) for the two populations. The two populations were slightly differentiated ($R_{ST} = 0.032$), accompanied by faint substructure across the populations ($K = 3$). In addition, each population exhibited spatial genetic structuring. The relatively low inbreeding coefficient for both populations together ($F_{IS} = 0.233$) and each population separately ($F_{IS} = 0.217$–0.246) may be attributable to crossing among descendants within a population along with occasional gene flow between the populations. These results suggested that the extant populations have not experienced a severe bottleneck. The two extant populations were genetically differentiated at a very low level, accompanied by occasional pollen flow via pollinators and/or seed dispersal by gravity in the mountainous environment. Occasional gene exchange between the populations has allowed *T. ishiiana* to harbor high genetic diversity despite being a relic plant confined to two small refugial populations.

Keywords Convallariaceae · Gene flow · Microsatellite · Refugia · *Tricyrtis ishiiana*

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Introduction

Tricyrtis ishiiana (Kitag. et T. Okuyama) Ohwi et Okuyama is a perennial herb of the Convallariaceae that is confined to two gorges in the Tanzawa Mountains in Kanagawa Prefecture, Japan (Fig. 1). This species inhabits precipices of gorges in the cool-temperate forest at elevations ranging from ~800 to 1000 m. The large (approximately 5 cm in length) showy flowers are yellow and bell-shaped, and are pollinated by bumblebees (Takahashi 1993). This species is vulnerable because of its limited population number and illegal removal from its natural habitats for horticultural use. It has been designated a critically endangered species (class IA) based on IUCN criteria (Japan Society of Plant Taxonomists 1993; Ministry of the Environment of Japan 2000).

Increased outcrossing is a result of the protandry of *T. ishiiana* flowers (Takahashi 1993); however, crossing between kin-individuals may result in lower genetic diversity of the relic population. In addition, this plant extends horizontal rhizomes and propagates clonally. Consequently, the genetic diversity of the two confined populations may decrease as a result of inbreeding, clonal propagation, and a bottleneck effect for population establishment. Evaluation of the population genetic diversity

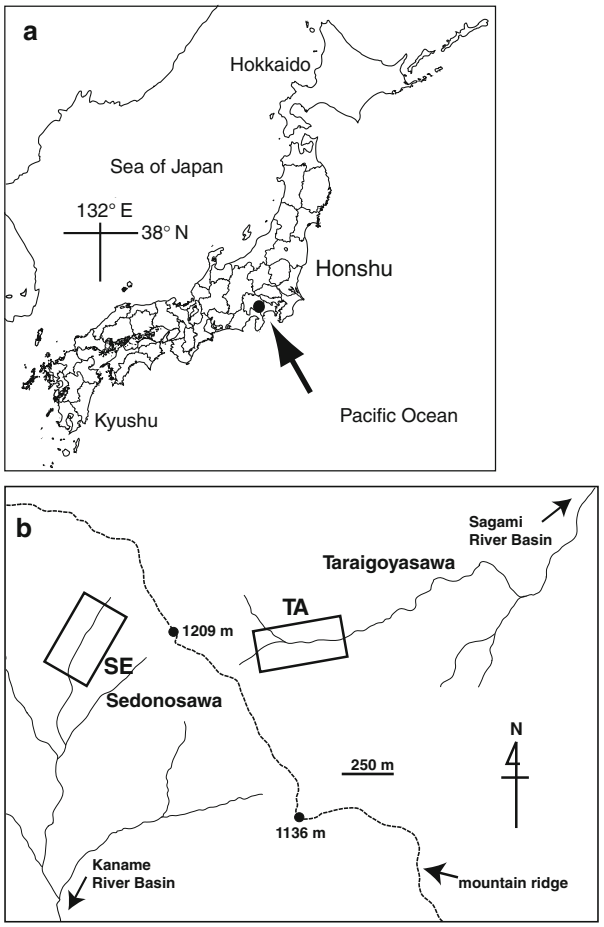

Fig. 1 Locations of the study sites. **a** Location of the study sites within the Japanese islands. *Closed circles with arrows* indicate the locations of the two *T. ishiiana* populations. **b** Map of the Tanzawa Mountains, showing river systems and the locations of the two *T. ishiiana* populations examined (*enclosed by squares*)

and population genetic structure would provide basic information for the conservation of the remaining populations.

In addition, estimation of the gene flow between the two populations would provide conservation strategies, delimiting whether the two populations should be protected individually or inclusively. The two habitat gorges are approximately 640 m apart (Fig. 1), and pollen flow between the two populations may be mediated within this distance by bumblebees. In addition, seed dispersal is believed to occur by gravity or strong wind, based on the relatively small seed size (3–4 mm in diameter) and the presence of a narrow wing. Thus, there are a few opportunities for seed dispersal between the populations. On the other hand, this plant grows on precipices of gorges, and seed and pollen flows may tend to remain confined within a gorge. In this case, genetic differentiation would be high between the populations.

Relic endemic species confined to small refugial populations may harbor either low or high genetic diversity. The extant populations were reduced and retreated to their distribution range during the Quaternary climatic oscillations. The small distribution range and low number of populations suggests that the species has a diminished genetic diversity due to a bottleneck effect. In general, narrowly restricted species tend to harbor lower levels of a genetic diversity than widespread species (e.g., Sherman-Broyles et al. 1992; Linhart and Permoli 1993; Edwards and Wyatt 1994; Purdy and Bayer 1995a, b, 1996; Godt et al. 1997). On the other hand, phylogeographic examinations of Quaternary climatic oscillations and range shifts in biota have revealed that persisting populations tend to exhibit higher genetic diversity and/or haplotype diversity than those of recolonized populations (e.g., Lumaret et al. 2002; Palmé and Vendramin 2002; Petit et al. 2002, 2003; Schönswetter et al. 2005; Ikeda et al. 2006). Although *T. ishiiana* has only two neighboring populations with small ranges, these populations (or at least, one of the two populations) may harbor high genetic diversity originating from the ancestral population. In the latter case, a serious bottleneck effect might not have occurred at the time of population establishment and/or division into the two gorges.

The Kanagawa prefectural government has recently been concerned with the genetic signature of *T. ishiiana* in terms of conservation strategy planning. In this study, we analyzed the genetic structure of the two relict populations of this plant located in nearby gorges. Knowledge of the gene flow mediated by pollen and seed dispersal could provide new insights into the population dynamics of the refugial populations and would contribute to the conservation of these relict populations. We examined the population genetic structure based on highly polymorphic nuclear microsatellite loci, which allow the determination of fine-scale genetic structure.

Materials and methods

Sampling

Tricyrtis ishiiana is a deciduous perennial herb that grows in shaded precipices on the slopes of two gorges: Taraigoyasawa Gorge (population TA) and Sedonosawa Gorge (SE) in the Tanzawa Mountains of Kanagawa Prefecture. No specimens have been found in the forest understory or in sunny locations. The two populations are located at the upper ends of the gorges, ranging about 350 and 410 m along the mountain stream in TA and SE, respectively. The two populations are separated by a mountain ridge and approach most closely at a distance of approximately

640 m (Fig. 1). The large showy flowers have a corolla approximately 5 cm in length and are yellow and bell-shaped, and visited by bumblebees (Takahashi 1993). Seed dispersal is thought to occur via gravity, given the small seed size (~2–3 mm across) and the narrowly winged appendages. Seeds are not produced by selfing under cultivated conditions, and outcrossing is assumed.

Fresh leaves of mature individuals were sampled from the two populations (Fig. 1). This plant is usually lithophytic, i.e., grows in or on rocks; thus, we could not observe its root system without tearing plants and moss from rocks. Therefore, plants located at least several meters apart were selected, to avoid sampling kin-individuals and/or clonal ramets. Details of the sampling localities and the number of individuals used for the analyses are shown in Table 1. Population TA was larger, with more than 107 available ramets growing on a cliff facing the gorge; the smaller SE population had 62 samples available for analysis. In total, 169 samples were used for DNA analysis.

Table 1 Localities and sample sizes for the two *T. ishiiana* populations

Localities	Abbreviation	Altitude (m)	Coordinates	n[a]
Taraigoyasawa	TA	800–1000	35°44–45′N, 139°19–20′E	107
Subpopulations	TA-a			6
	TA-b			3
	TA-c			3
	TA-d			14
	TA-e			10
	TA-f			11
	TA-g			25
	TA-h			25
	TA-i			2
	TA-j			3
	TA-k			5
Sedonosawa	SE	900–1000	35°43–44′N, 139°17–18′E	62
Subpopulations	SE-a			4
	SE-b			1
	SE-c			9
	SE-d			8
	SE-e			2
	SE-f			1
	SE-g			7
	SE-h			4
	SE-i			4
	SE-j			6
	SE-k			8
	SE-l			3

[a] The sample sizes (n) represents number of individuals analysed

DNA extraction, PCR amplification, and microsatellite genotyping

Fresh leaf material was frozen in liquid nitrogen and then ground into a fine powder. After the polysaccharides were removed from the powder using HEPES buffer (pH 8.0; Setoguchi and Ohba 1995), genomic DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990). The extracted DNA was dissolved in 100 µl of TE buffer and used for PCR. The genotypes of 169 individuals were determined using nine nuclear simple sequence repeat (nSSR) markers developed for this study (TI-1, TI-2, TI-3, TI-4, TI-5, TI-6, TI-7, TI-8, TI-9; Setoguchi et al. 2009). The primers used are listed in Table 2.

PCR was performed in a 6-µl reaction volume containing 40–60 ng of genomic DNA, 1× Multiplex PCR Master Mix (Qiagen), and 0.2 µM of each primer. Samples were amplified in a DNA thermal cycler (Takara Bio) programmed for denaturation at 95°C for 15 min, followed by 30 cycles at 94°C for 30 s, 58°C for 90 s, and 72°C for 60 s, and a final 30-min extension at 60°C. The amplified products were loaded onto an ABI 3100 autosequencer (Applied Biosystems) using POP6 polymer and a 50-cm capillary array, and their sizes were determined using Genemapper (Applied Biosystems). The use of the polymer and capillary array enabled us to determine relatively short fragments (<100 bp).

Data analyses

The number of alleles, allelic richness (El Mousadik and Petit 1996), observed heterozygosity (H_O), gene diversity (H_E; Nei 1987), and fixation index ($F_{IS} = 1 - H_O/H_E$) were calculated for each locus and each population (TA: 107 samples; SE: 62 samples; total number of samples: 169) using FSTAT ver. 2.9.3.2 (Goudet 1995). Departures from Hardy–Weinberg equilibrium (HWE) at each locus and the linkage disequilibrium between loci were tested using FSTAT (alleles were randomized 1,000 times over all samples). These parameters were calculated for 107 and 62 individuals in the TA and SE populations, respectively.

Genetic differentiation among populations (R_{ST}; Weir and Cockerham 1984; Slatkin 1995; Rousset 1996) were estimated using FSTAT. R_{ST} is the coefficient of genetic differentiation among populations assuming the stepwise mutation model. Significant differences among these parameters at each locus were tested by the log-likelihood (G)-based exact test (Goudet et al. 1996) using a Monte Carlo Markov chain (MCMC) method.

Population differentiation based on pairwise R_{ST} between populations was estimated using ARLEQUIN version 3.1 (Excoffier et al. 2005).

Table 2 Characteristics of the nine nSSR loci isolated from *T. ishiiana*

Locus	Primer sequences (5′–3′)	Size range (bp)	No. of alleles	H_O	H_E	*P*-value
TI_1	F: GGTTGGGCAAAACCATTAGA	145–157	7	0.707	0.672	0.055
	R: TCTCTCTCTCTCACACACACAC					
TI_2	F: GGTATCGAGTGTGGCAAGGT	124–145	12	0.727	0.821	0.066
	R: ACACACACACACAGAGAGAGAG					
TI_3	F: CCTATTTAGGGATTTACGCTAGGA	84–96	7	0.329	0.684	0.070
	R: ACACACACACACAGAGAGAGAG					
TI_4	F: TCTATCTTTTCGACAACCACCA	93–141	25	0.569	0.924	0.000
	R: ACACACACACACAGAGAGAGAG					
TI_5	F: ATTGGGTAATTTATTTGTTATTG	90–164	40	0.714	0.952	0.000
	R: ACACACACACACAGAGAGAGAG					
TI_6	F: CCATCTTCCTCCTGCATTGT	71–75	3	0.365	0.391	0.453
	R: TCTCTCTCTCTCACACACACAC					
TI_7	F: TCCTCTATTCTCTCATGAAACTATTGT	89–97	5	0.185	0.183	0.303
	R: TCTCTCTCTCTCACACACACAC					
TI_8	F: ACAAAGTAACCGGGTGTCCA	94–114	12	0.760	0.868	0.002
	R: TCTCTCTCTCTCACACACACAC					
TI_9	F: AACACTACGAAGAATAAACACAC	107–152	26	0.540	0.934	0.027
	R: ACACACACACACAGAGAGAGAG					
Average			15.2	0.544	0.714	0.098

Relatedness coefficients (*r*-values) between all possible pairs of individuals were estimated using KINSHIP 1.3.1 (Queller and Goodnight 1989). The calculation is based on the frequency of alleles in the population. This measure of relatedness ranges from −1 to +1, where a positive value indicates that two individuals share more identical alleles than expected by chance.

In addition, we used a Bayesian clustering approach implemented in STRUCTURE ver. 2.2 (Pritchard et al. 2000) to infer population structure. This approach assumes HWE and attempts to find population groupings that are not in linkage or Hardy–Weinberg disequilibrium. To quantify the variation in likelihood for each number of clusters (*K*), we performed a series of 20 independent runs for each value of *K*, ranging from 1 to 9. As recommended in the software manual, we assumed an admixture model of ancestral populations (Falush et al. 2003) with a correlated model of allele frequencies using 1×10^5 burn-in time periods and 5×10^6 MCMC iterations. Previous studies have shown that in many cases, the posterior probability for a given *K* increases slightly, even after the real *K* is reached. Therefore, we used the ad hoc statistic of Evanno et al. (2005), ΔK, to determine the true value of *K*.

The genetic distance and model-based clustering methods described above estimate recent gene flow among populations. In addition, the method described by Wilson and Rannala (2003) estimates the amount and direction of recent (i.e., over the past few generations) gene flow. We estimated recent gene flow using the program BayesAss ver. 1.3, which estimates recent migration rates between all pairs of populations, allele frequencies, and inbreeding coefficients for each population. These analyses were performed by identifying individuals as immigrants or as descendents of recent immigrants from the observed temporary disequilibrium of multi-locus genotypic frequencies. The parameters (migration rate, allele frequency, and inbreeding coefficient) were estimated numerically with a MCMC simulation by inferring the estimated posterior probability. To estimate the posterior probability distribution of the parameters, the program was run with 999,999 burn-in periods and 3×10^6 total iterations. Five independent runs were conducted, and the mean values were compared among populations. We conducted the analysis for two populations, downstream and upstream, from each of the two gorges (TA and SE), thus examining four populations in total.

The patterns of spatial genetic structure described as isolation-by-distance (IBD) model (Wright 1943) were evaluated according to Rousset (1997). A Mantel test with 10,000 random permutations was performed with the matrix of pairwise genetic differentiation between subpopulations using $R_{ST}/(1 - R_{ST})$ and a matrix of the *ln* (geographic distance) using the program GENALEX version 6.1 (Peakall and Smouse 2006). A spatial autocorrelation analysis was performed with SPAGEDI version 1.2 (Hardy and Vekemans 2002). This analysis used R_{ST} and evaluated four to seven spatial distance classes with similar sample sizes. The 95% confidence intervals were estimated for each distance class using 10,000 random permutations.

To detect recent bottlenecks due to reductions in effective population size, the observed gene diversity was compared with equilibrium gene diversity given the observed number of alleles (Watterson 1978, 1986) using BOTTLENECK 1.2.02 (Piry et al. 1999). Two models for locus evolution, the infinite allele model (IAM; Maruyama and Fuerst 1985) and stepwise mutation model (SMM; Cornuet and Luikart 1996), were used for the analyses, with a Sign test (Cornuet and Luikart 1996) and a Bayesian Wilcoxon signed-rank test (Luikart et al. 1998).

Results

Genetic diversity

Characteristics of the nine nSSR loci examined are shown in Table 2. In total, 137 alleles for the nine nSSR loci were detected. These alleles distinguished all samples as different genets, implying that no clone was found among samples. Significant deviation ($P < 0.05$) from HWE was detected in five of the nine loci, possibly due to depressed gene flow between the TA and SE populations and low genetic diversity of the loci (in particular TI-6 and TI-7). High levels of genetic diversity were consistently observed in each population, as indicated by H_E estimates of 0.706 (population TA) and 0.702 (SE), with an average of 0.704 (Table 3). H_O was relatively low compared to H_E and ranged from 0.529 (SE) to 0.553 (TA), with an average of 0.541. Consequently, F_{IS} calculated for each population ranged from 0.217 (TA) to 0.246 (SE), with an average of 0.232. No significant linkage disequilibrium between loci across the two populations was observed, and all loci were used for further analyses. The degrees of relatedness represented by the *r*-values were -0.004 ± 0.003 (mean $\pm$ standard error; population TA) and -0.002 ± 0.006 (population SE; Table 3). In addition, subpopulations g and h within the TA population (as large subpopulations comprising $n > 20$) harbored relatedness coefficients of -0.015 ± 0.014 and 0.018 ± 0.016, respectively.

Genetic differentiation and gene flow between populations

R_{ST} between the two populations was 0.032, whereas most ($\sim$93%) of the genetic variation was explained by differentiation within the populations (Table 4). Genetic differentiation was significant ($P < 0.001$) among populations at all loci (Goudet et al. 1996).

Table 3 Genetic diversity parameters estimated at nine nSSR loci in two *T. ishiiana* populations

Population	No. of samples	No. of alleles	*A*	AR	H_O	H_E	F_{IS}	*P*	r^a
TA	107	114	12.22	10.997	0.553	0.706	0.217	<0.001	−0.004 (0.003)
SE	62	110	12.11	12.057	0.529	0.702	0.246	<0.001	−0.002 (0.006)
Average	83.5	112	15.22	12.550	0.541	0.704	0.232	<0.001	−0.002 (0.002)

A number of alleles per locus, *AR* allelic richness, H_O observed heterozygosities, H_E gene diversities, F_{IS} fixation indexes; *P* probability of HWE, *r* relatedness coefficient, *TA* Taraigoyasawa, *SE* Sedonosawa

[a] Standard errors are shown in parenthesis

Table 4 Results of analysis of molecular variance (AMOVA) of nSSR data from *T. ishiiana* populations

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	*P*-value
(i) Total subpopulations (24 subpopulations)					
Among populations	23	141.351	0.22814	6.97	<0.001
Within populations	314	955.696	3.04362	93.03	<0.001
(ii) TA a–f, TA g–k, SE a–d, and SE e–m					
Among groups	3	37.843	0.07326	2.23	<0.001
Among populations within group	20	103.508	0.17085	5.2	<0.001
Within populations	314	955.696	3.04362	92.58	<0.001
(iii) TA a–k and SE a–m					
Among groups	1	18.153	0.07199	2.18	<0.001
Among populations within group	22	123.198	0.192	5.8	<0.001
Within populations	314	955.696	3.04362	92.02	<0.001

The STRUCTURE analysis detected three appropriate clusters (K). Individual assignments into the three clusters were consistent in the 20 replicate runs. Population SE belonged to one cluster, and population TA was split into two clusters (Fig. 2). In addition, the genetic composition determined by the Bayesian clustering is indicated by pie charts for each population (Fig. 3). Populations TA and SE were clearly distinguished, whereas population substructure and special distribution of the genetic composition were slightly recognizable within each population: upstream and downstream populations tended to be occupied by different clusters of genotypes (e.g., dark and light gray, respectively, in TA; Fig. 3). The spatial genetic structure as isolation by distance was detected at different spatial scales (Mantel test; $P < 0.05$): at scales of the whole distribution range (Fig. 4, W), within TA (Fig. 4, TA), and within SE (Fig. 4, SE). The geographic pattern of genetic variation was analyzed by the spatial autocorrelation analysis (Fig. 5). Within the TA population, R_{ST} value between subpopulations separated by more than about 370 m was significantly higher than the expected value (Fig. 5, TA). The SE population also harbored a similar geographic structure; a higher average genetic distance (R_{ST}) was estimated as being more than about 450 m separating the subpopulations (Fig. 5, SE). At the whole distribution range level, subpopulations between about 200 m and about 1000 m exhibited spatial autocorrelation in the average genetic distance (Fig. 5, W).

Estimates of recent migration rates between populations (up to the second generation of migrants) are shown in Table 5a. The 95% confidence interval is also presented in parentheses. Recent gene flow among populations was calculated as 0.188 from SE to TA and 0.198 from TA to SE, of which the direction of gene flow could not be evaluated (ANOVA, $P > 0.05$). Analyses for gene flow among four subpopulations are presented in Table 5b. Gene flow from upstream to downstream (mean, 0.035) was significantly higher than that from downstream to upstream (mean, 0.008) in TA (ANOVA, $P < 0.05$). In another gorge (SE), gene flow from upstream to downstream was relatively high (mean, 0.129) but this value was nonsignificant based on 95% confidence intervals and also the manual of the BayesAss program. This value was higher than that of the opposite direction (mean, 0.050), but the difference again was not significant (ANOVA, $P > 0.05$).

The results of tests for mutation-drift equilibria are shown in Table 6. The Sign test under the IAM showed no evidence of a recent bottleneck in either the whole

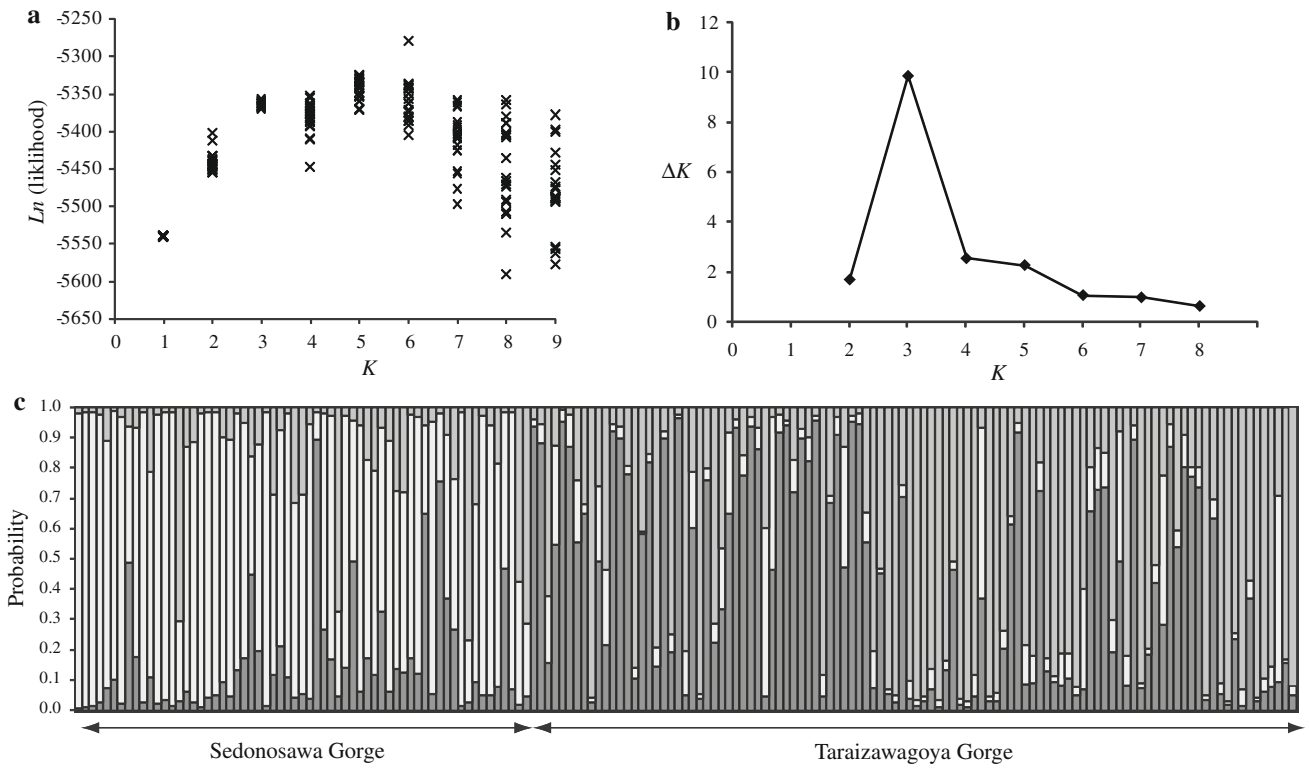

Fig. 2 Results of STRUCTURE analysis. **a** Distribution of likelihood values, $Ln(K)$. All estimated values from 20 replicates in each K were plotted in $Ln(K)$. **b** Distribution of the model parameter (ΔK). The estimated values based on the 20 replicates are shown in $\Delta(K)$. **c** Individual assignment by STRUCTURE analysis: the number of clusters (K) was three. *Bold lines* within the *squares* distinguish populations. *Abbreviations* for each population name are shown under the *bars*

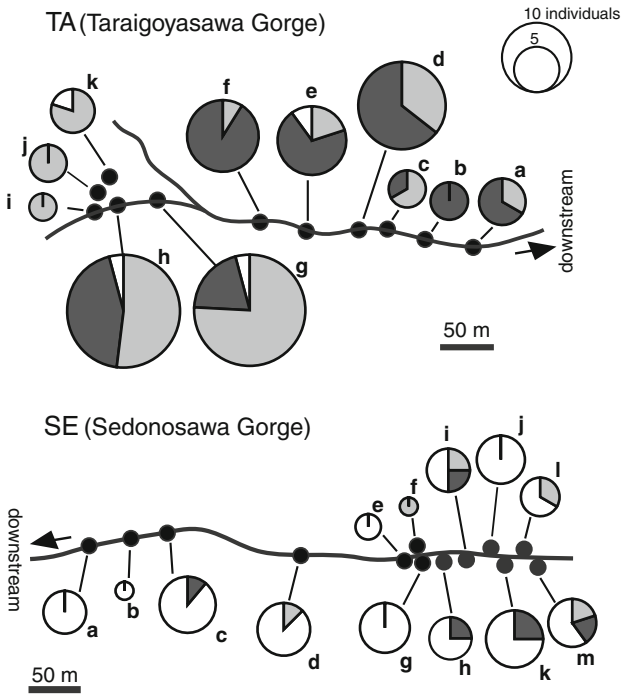

Fig. 3 Location and genetic structure of the subpopulations of *T. ishiiana*. The *pie chart* indicates the genetic composition of clusters determined by Bayesian clustering (STRUCTURE analysis). The size of the chart corresponds to the number of individuals. Codes for the subpopulations correspond to those in Table 1

population or each individual population ($P > 0.05$), whereas significant bottleneck effects were detected over all populations and for each population under the SMM. The Wilcoxon test estimated a bottleneck effect in one population (TA) and in over all populations, but not SE. Thus, three of the four analyses detected a bottleneck effect in the TA population, and a bottleneck effect in the SE population was detected by one of the four analyses.

Discussion

Genetic diversity of relict populations

These results indicated that both populations of *T. ishiiana* in the two gorges harbor high genetic diversity ($H_O = 0.541$ and $H_E = 0.704$), although both populations are very small and confined to two narrow gorges (distribution range is about 400 m in each gorge). These values are almost equal to those of other *Tricyrtis* species in Japan, which are outcrossing and narrowly restricted ones: *T. perfoliata* (distribution is confined to one mountain): $H_E = 0.693$; *T. ohsumiensis* (restricted to a few mountains in southern Kyushu): $H_E = 0.788$; and *T. flava* (restricted to southeastern Kyushu): $H_E = 0.747$ and (Takahashi et al., in press). In terms of relict populations, isolated

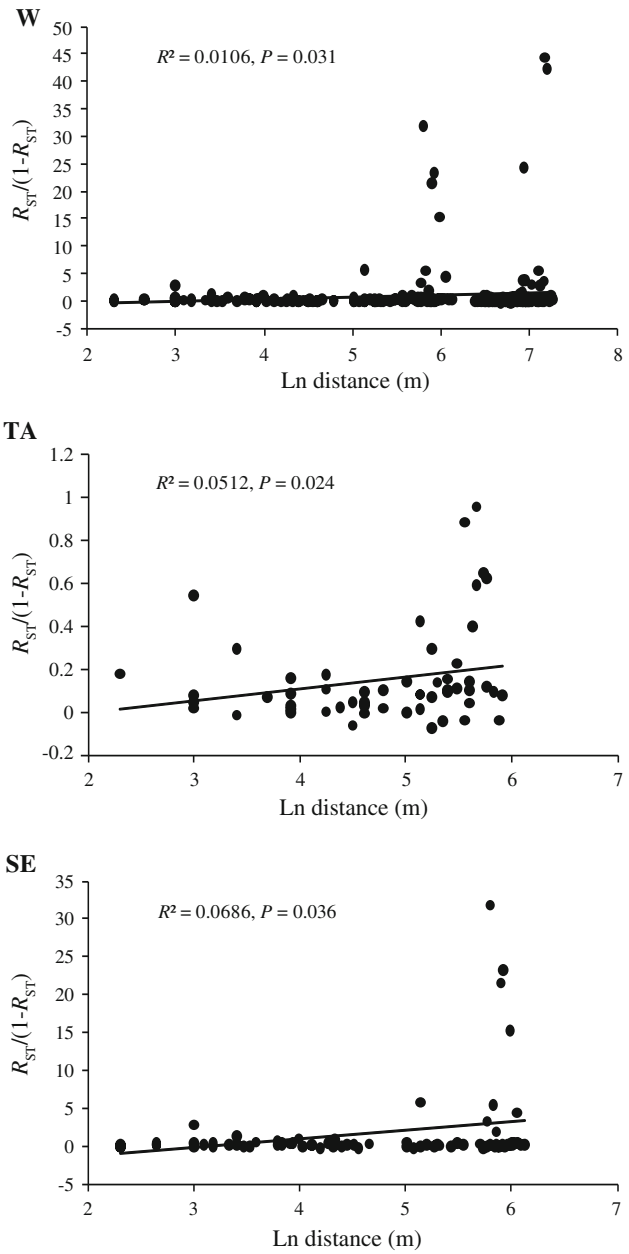

Fig. 4 Relationship among the matrix of pairwise differentiation described as $R_{ST}/(1 - R_{ST})$ and the matrix of the natural logarithm of geographical distance among individuals of *T. ishiiana* for the whole distribution range (W), Taraigoyasawa Gorge (TA), and Sedonosawa Gorge (SE)

populations of *Kirengeshoma palmata* (outcrossing perennial herb of the Hydrangeaceae) in Japan harbor genetic diversity as I (Shannon's diversity index) = 0.261 and $H_E = 0.175$ (ISSR analysis: Qiu et al. 2009). Although these data are based on different locus or molecular markers, the genetic diversity of *T. ishiiana* is high as well as congeners and other relict endemic plant, possibly due to its population history (range shifts and population size change) during the Quaternary climatic oscillations. Many

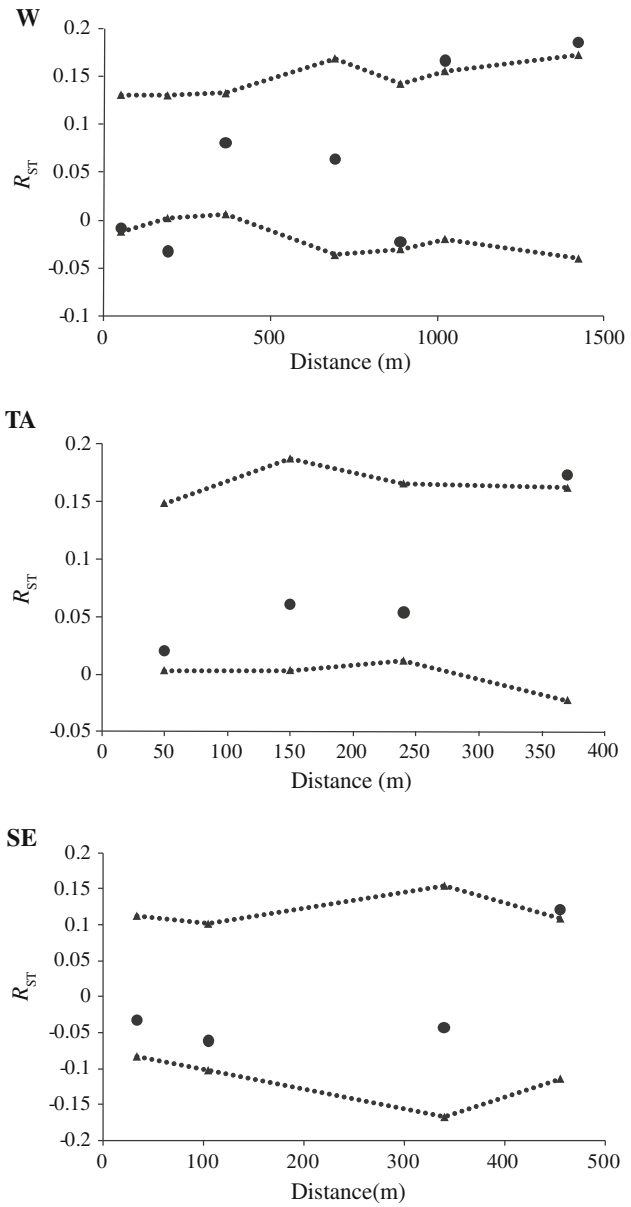

Fig. 5 Average genetic distance (R_{ST}) for special distance classes for seven classes in the whole distribution range (W), four classes in Taraigoyasawa Gorge (TA), and four classes in Sedonosawa Gorge (SE). *Broken lines* indicate the boundaries of the 95% confidence intervals estimated by permutation

phylogeographic studies have revealed that refugial populations tend to have higher genetic and/or haplotype diversity than those of recolonized populations (e.g., Lumaret et al. 2002; Palmé and Vendramin 2002; Petit et al. 2002, 2003; Schönswetter et al. 2005; Ikeda et al. 2006). Narrowly restricted populations of *T. ishiiana* harbor high genetic diversity, but this plant has only two relict populations without any recolonized ones. Therefore, we should be careful as to identifying the extant populations as having been in "refugia" during the Quaternary.

The high genetic diversity in *T. ishiiana* appears to contradict the result of the BOTTLENECK analyses, which showed evidence of reductions in effective population size. The entire *T. ishiiana* population would have certainly decreased in size following range contraction during the postglacial period and ultimately reached safety in the relict populations. Therefore, the larger ancestral populations harboring genetic diversity might have migrated to the present locality and reduced the distribution range. The retreated populations had experienced population size reduction, but they have kept the aforementioned genetic diversity. Thus, the present populations can be viewed as populations that survived during the postglacial warm periods and maintained genetic diversity.

Genetic structure of populations in the two gorges

This study also indicated that the two extant populations of *T. ishiiana* are genetically differentiated at a very low level ($R_{ST} = 0.032$), whereas a faint substructure across the populations was suggested by the STRUCTURE analysis ($K = 3$). The SE population was composed of a single cluster of genotypes in the STRUCTURE analysis, and the TA population included two clusters of genotypes. The presence of potential differentiation between the two populations implies the presence of a weak barrier to gene flow via pollen flow and seed dispersal between the two populations. The TA and SE populations are approximately 640 m apart as the minimum distance, partitioned by a mountain ridge (Fig. 1). Therefore, the topography might have restricted gene flow and sculpted the population substructure. On the other hand, the Bayesian assessment determined by the BayesAss program estimated recent migration between the two populations. These results along with a very low R_{ST} value suggested that the two extant populations were genetically differentiated at a very low level, accompanied by occasional pollen flow via pollinators and/or seed dispersal by gravity in the mountainous environment.

On the other hand, we were able to identify spatial genetic structuring within each gorge. The TA and SE populations harbored spatial genetic structuring within less distance (less than about 370 m and less than about 450 m, respectively), implying the presence of gene flow within a gorge, possibly attributable to pollen flow and/or seed flow. The significant but slight gene flow from upstream to downstream in the TA gorge may suggest the presence of occasional gene flow by water along the river, but we cannot interpret this result in terms of pollen and seed dispersal based on our data. The spatial genetic structure of *Tricyrtis* seeds is presumed to be dispersed by gravity or strong winds, without adaptations for water dispersal, and pollen is moved by pollinator insects such as bumblebees. Thus, the spatial genetic structuring of *T. ishiiana* could

Table 5 Mean value of the posterior distribution of the migration rate (m) of each population of *Tricyrtis ishiiana* estimated using the program BayesAss

a. Between populations

To	From	
	TA	SE
TA	0.812 (0.09)	0.188 (0.09)
SE	0.198 (0.09)	0.789 (0.09)

b. Among subpopulations

To	From			
	TA a–f	TA g–k	SE a–d	SE e–m
TA a–f	0.956 (0.902–0.994)	0.035 (0.002–0.086)	0.004 (0.000–0.019)	0.005 (0.000–0.022)
TA g–k	0.008 (0.000–0.044)	0.984 (0.938–0.999)	0.003 (0.000–0.014)	0.006 (0.000–0.030)
SE a–d	0.009 (0.000–0.039)	0.010 (0.000–0.050)	0.851 (0.850–0.941)	0.129* (0.049–0.125)
SE e–m	0.010 (0.000–0.046)	0.009 (0.000–0.039)	0.050 (0.002–0.157)	0.932 (0.816–0.992)

Values on the diagonal are the proportions of individuals derived from source populations. 95% confidence intervals are shown in parentheses

* indicates this value is out of 95% confidence intervals, and also evaluated to be not significant based on evaluation in BayesAss's manual

Table 6 Probability of a bottleneck estimated using the program BOTTLENECK

Population	SIGN		Wilcoxon	
	IAM	SMM	IAM	SMM
TA	0.05317	0.02346*	0.01367*	0.01953*
SE	0.05689	0.02569*	0.12891	0.12891
All	0.05697	0.00357*	0.00371*	0.00586*

IAM infinite allele model, *SMM* stepwise mutation model, *SIGN* sign test, *Wilcoxon* Wilcoxon sign-rank test

* indicate evidence for bottleneck

represent a signature of pollen and seed dispersal for this plant.

Tricyrtis ishiiana is a typical relic endemic species, confined to a small distribution range of cool riparian crags. Based on the high genetic diversity within the extant descendents, the ancestral populations would have to have been large enough to harbor high genetic diversity. During the subsequent postglacial range contraction, the retreating populations might have maintained a population size large enough to retain genetic diversity without experiencing a bottleneck effect. The high genetic diversity of *T. ishiiana* populations may imply the genetic signature of relic populations of narrowly endemic plants.

Conservation perspectives

This study provides genetic information on refugial populations of *T. ishiiana*. Maki et al. (1999) compared fixation indices for four species of *Tricyrtis* in terms of mating systems. Adichogamous and putative selfing *T. nana* showed high values of Wright's fixation index ($f = 0.534$–0.780), whereas those of protandrous and putative outcrossing species varied: $f = 0.021$–0.243 for widespread *T. flava*, $f = 0.040$ for narrowly endemic *T. perfoliata*, and $f = 0.249$ for narrowly endemic *T. ohsumiensis*. Later, Takahashi et al. (in press) reported Wright's fixation index, $F_{IS} = 0.661$, for *T. nana* (no data for other species). Thus, our result ($F_{IS} = 0.232$) can be compared to those of protandrous and outcrossing congeners, and agree with the breeding system described by Takahashi (1993). Accordingly, the fairly high heterozygosity of *T. ishiiana* ($H_O = 0.541$, $H_E = 0.704$) would be partly attributable to breeding system.

Low genetic differentiation between the populations ($R_{ST} = 0.032$) suggested that the entire habitat (including both populations) should be protected as one unit, without treating the two populations as separate. Nevertheless, transplantation and/or artificial seed movement should be avoided because the two populations harbor their inherent clusters of genotype, as detected in the Bayesian clustering analysis.

This plant should be considered for protection, as it reflects Quaternary climatic oscillation. Considering the presence of spatial genetic structure in extant populations, we recommend to protect each population (and subpopulation) without transplantation among/within populations. The genetic diversity and genetic signature of each population and subpopulations should be protected from illegal removal for horticultural use.

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