

# Effect of gene flow on spatial genetic structure in the riparian canopy tree *Cercidiphyllum japonicum* revealed by microsatellite analysis

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Few studies have analyzed pollen and seed movements at local scale, and genetic differentiation among populations covering the geographic distribution range of a species. We carried out such a study on *Cercidiphyllum japonicum*; a dioecious broad-leaved tree of cool-temperate riparian forest in Japan. We made direct measurement of pollen and seed movements in a site, genetic structure at the local scale, and genetic differentiation between populations covering the Japanese Archipelago. Parentage analysis of seedlings within a 20-ha study site indicated that at least 28.8% of seedlings were fertilized by pollen from trees outside the study site. The average pollination distance within the study site was 129 m, with a maximum of 666 m. The genotypes of 30% of seedlings were incompatible with those of the nearest female tree, and the maximum seed dispersal distance within

the study site was over 300 m. Thus, long-distance gene dispersal is common in this species. The correlation between genetic relatedness and spatial distance among adult trees within the population was not significant, indicating an absence of fine-scale genetic structure perhaps caused by high levels of pollen flow and overlapping seed shadows. Six populations sampled throughout the distribution of *C. japonicum* in Japan showed significant isolation-by-distance but low levels of genetic differentiation ( $F_{ST}=0.043$ ), also indicating long-distance gene flow in *C. japonicum*. Long-distance gene flow had a strong influence on the genetic structure at different spatial scales, and contributes to the maintenance of genetic diversity in *C. japonicum*.

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## Introduction

Spatial genetic structure in natural plant populations is defined as the nonrandom distribution of genetic variation among individuals. It is determined by the interaction of many factors, such as gene flow, random genetic drift, natural selection, colonization history, and the spatial distribution of individuals or populations (Epperson, 1993; Hamrick and Nason, 1996). Patterns and levels of gene flow via pollen and seed dispersal are one of the most critical determinants in the establishment of genetic structure (Hamrick and Nason, 1996). When gene flow is restricted to shorter distances, levels of genetic differentiation between pairs of individuals and/or populations will increase as a function of the spatial distance between them, as expressed by the isolation-by-distance model of Wright (1943). Species with restricted gene movement should exhibit greater genetic differentiation than species with widely dispersed pollen and/or seed. Species that have large and heavy seeds, such as oak or beech, exhibit genetic structure at small spatial

scales (Berg and Hamrick, 1995; Streiff *et al.*, 1998; Ueno *et al.*, 2000). In contrast, some studies report that species with extensive gene dispersal via pollen and/or seed have a random genetic structure within localized populations (Xie and Knowles, 1991; Doligez and Joly, 1997), and genetic structuring in such species may occur at spatial scales larger than those investigated.

Revealing patterns and levels of gene flow thus contributes substantially to understanding the genetic structure of a plant population both on smaller and larger scales. Although a variety of genetic markers are available for such kinds of studies for different spatial scales, few studies have analyzed pollen and seed movements at local scale, and genetic differentiation among populations covering the geographic distribution range of a species simultaneously.

In this study, gene flow and spatial genetic structure in *Cercidiphyllum japonicum*, a dioecious broad-leaved tree of cool-temperate riparian forest in Japan, were investigated at distinct spatial scales: direct measurement of pollen and seed movements in a site, genetic structure at the local scale, and genetic differentiation between populations covering the Japanese Archipelago.

Recently, highly polymorphic microsatellite markers have become a popular genetic tool in ecological studies, and have been utilized for various analyses, such as

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parentage analysis to identify realized contemporary gene flow (Dow and Ashley, 1996; Isagi *et al*, 2000; Kameyama *et al*, 2001; Jones and Ardren, 2003), detection of fine-scale genetic structure (Streiff *et al*, 1998; Ueno *et al*, 2000; Kameyama *et al*, 2002), and the analysis of among-population genetic structure (Kikuchi and Isagi, 2002; Heuertz *et al*, 2004). In this study, we used five nuclear microsatellites for genetic analyses at different spatial scales.

## Materials and methods

### Study species

*C. japonicum* Sieb. et Zucc is a deciduous, broad-leaved tree of cool-temperate riparian forests in Japan. At maturity, it reaches 30 m in height and 2 m in diameter, and it may live for several hundred to 1000 years. The standing density of adult trees is relatively low at a few trees per hectare. Most adults have numerous sprouts, which may contribute to its persistence at a given location. The species is dioecious and wind-pollinated, flowering in early spring before the leaves shoot. Its seeds are winged, wind-dispersed and approximately 6 mm in length and 2 mm in width (Kubo *et al*, 2000), with an average weight of 0.667 mg (Seiwa and Kikuzawa, 1996). Current-year seedlings reach only approximately 1.5 cm in height, emerging from bare soil on steep slopes, rocky ground and fallen logs, but most die within a year (Kubo *et al*, 2000).

### Study site and sample collection

Gene flow and fine-scale genetic structure within a population were investigated in a 20-ha study site in the Kanumazawa Riparian Research Forest located on the southern foothills of Mt. Yakeishi in Iwate prefecture, northern Japan (Figure 1). The study site comprises a cool-temperate deciduous forest along a stream, and is dominated by *C. japonicum*, *Aesculus turbinata*, *Pterocarya rhoifolia*, and *Fagus crenata*. All mature trees of *C. japonicum* in the study site were mapped, and the sex of individuals determined by observation of the flowers. There were 20 mature female and 29 mature male trees,

and leaves were sampled from all of these for DNA extraction. We established circular seedling plots of 5 m radius around the main stem of seven female trees. From 24 to 37 current-year seedlings were sampled from each plot, 212 in total.

To study the among-populations genetic structure, six populations (Nakagawa, Iwanazawa, Kanumazawa, Oyamasawa, Yoshiwa, and Kujyu) throughout the distribution of *C. japonicum* in the Japanese Archipelago (Figure 1) were sampled. For each population, leaves were sampled from 36 to 117 trees, 333 samples in total (Table 1). All samples were stored at  $-30^{\circ}\text{C}$  pending DNA extraction.

### DNA extraction and microsatellite genotyping

Genomic DNA was extracted from leaves using a modified CTAB method (Milligan, 1992). Genotypes of the 545 samples were determined by five microsatellite markers developed for *C. japonicum* (MSCJ35, MSCJ86, MSCJ92, MSCJ93, and MSCJ95; Isagi *et al*, 2005). There was no evidence of linkage disequilibrium between these loci (Isagi *et al*, 2005). PCR amplifications were performed under the following conditions: initial denaturation at  $95^{\circ}\text{C}$  for 9 min; then 30 cycles of denaturation at  $95^{\circ}\text{C}$  for 1 min, annealing at primer-specific temperature for 1 min and extension at  $72^{\circ}\text{C}$  for 1 min; and final extension at  $72^{\circ}\text{C}$  for 10 min. The size of PCR products was measured using a 3100 Genetic Analyzer with GENESCAN analysis software (Applied Biosystems).

### Gene flow within a population: parentage analysis of established seedlings

Initially, parentage assignment of seedlings was performed by simple exclusion, based on multilocus genotypes for all candidate parents within the study site (Dow and Ashley, 1996; Jones and Ardren, 2003). This technique is most powerful when there are few candidate parents and highly polymorphic markers are available (Jones and Ardren, 2003). Exclusion probabilities and null allele frequency of each locus in the Kanumazawa Riparian Research Forest was calculated with CERVUS 2.0 (Marshall *et al*, 1998). To prevent the exclusion of true candidate parents, individuals that appeared to be homozygous at a locus with a putative null allele were assumed heterozygous with null allele. This method may increase the number of matches within the study site but would



**Figure 1** Distribution of six sampled populations of *Cercidiphyllum japonicum* in the Japanese Archipelago.

**Table 1** Number of samples (N), average number of alleles per locus (A), average observed heterozygosities ( $H_O$ ), average expected heterozygosities ( $H_E$ ), and fixation indices ( $F_{IS}$ ) in six populations of *Cercidiphyllum japonicum*

Population	N	A	$H_O$	$H_E$	$F_{IS}$
Nakagawa	36	12.6	0.823	0.854	0.036
Iwanazawa	117	18.0	0.821	0.849	0.036*
Kanumazawa	49	14.8	0.842	0.843	-0.028
Oyamasawa	47	15.8	0.836	0.857	0.024
Yoshiwa	45	14.2	0.805	0.819	0.006
Kujyu	39	11.6	0.755	0.774	0.027
Average	55.5	13.8	0.814	0.833	

Levels of significance for  $F_{IS}$  were determined after 1000 permutations: \* $P < 0.05$ .

not exclude any true parents (Dow and Ashley, 1996; Kameyama *et al.*, 2001). Where there were more than two nonexcluded parents, the categorical allocation method (Marshall *et al.*, 1998) was used to select the most likely parent from the pool of nonexcluded parents. This method involves calculating a logarithm of the likelihood ratio (LOD score), the latter being the likelihood of an individual (or pair of individuals) being the parent (or parents) of a given offspring divided by the likelihood of these individuals being unrelated (Jones and Ardren, 2003). The process of categorical allocation was carried out with CERVUS 2.0 (Marshall *et al.*, 1998).

#### Fine-scale genetic structure

The fine-scale genetic structure of mature trees in the Kanumazawa Riparian Research Forest study site was examined by calculating correlations between their genetic relatedness and spatial distance. Genetic relatedness values based on Queller and Goodnight (1989) were calculated for all pairs of reproductive trees using RELATEDNESS version 5.0.8 (<http://www.gsoftnet.us/Gsoft.html>). Correlations were assessed with the Mantel test (Mantel, 1967) with 5000 permutations, calculated using R PACKAGE version 4.0 (Casgrain and Legendre, 2000).

#### Genetic diversity and among-populations genetic structure

Standard measures of genetic variation, including average number of alleles per locus ( $A$ ), observed heterozygosity ( $H_O$ ), and expected heterozygosity ( $H_E$ ), were obtained for each population. Weir and Cockerham's  $F_{IS}$  (Weir and Cockerham, 1984) was calculated for each population and its significance ( $F_{IS} \neq 0$ ) tested after 1000 permutations. Overall and pairwise  $F_{ST}$  were calculated with GENEPOP web version 3.4 (Raymond and Rousset, 1995; <http://wbiomed.curtin.edu.au/genepop>). The significance of pairwise  $F_{ST}$  values was tested by permuting the individuals between the populations. To evaluate spatial genetic structure among populations, the isolation-by-distance model was tested by regressing the population pairwise  $F_{ST}$  values against the geographical distances between populations. Analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992) was performed to examine the hierarchical genetic structure, using the program ARLEQUIN version 2.000 (Schneider *et al.*, 2000). We used two populations that adjoined as regional groups in AMOVA. Genetic variation was partitioned into three levels: within populations, among populations within regional groups, and among regional groups. The significance of  $F$  statistics was tested with 10000 permutations.

## Results

#### Microsatellite polymorphism

At the species level, all five microsatellite markers were highly variable, having 21–30 alleles per locus with an average of 26.2, and 131 alleles in total. At the intrapopulation level, the number of alleles per locus ranged from 11.6 to 18.0, with an average of 14.5. The observed and expected heterozygosities ( $H_O$  and  $H_E$ )

ranged from 0.755 to 0.842 and from 0.774 to 0.857, with averages of 0.814 and 0.833, respectively (Table 1). Positive null allele frequency was observed at one locus; MSCJ35. Fixation indices ( $F_{IS}$ ) that measure deviation from Hardy–Weinberg equilibrium were calculated by using four loci except MSCJ35, because the presence of null allele has an impact on the estimation of  $F_{IS}$ .  $F_{IS}$  ranged from  $-0.028$  to  $0.036$ , with only the Iwanazawa population differing significantly from zero (Table 1  $P < 0.05$ ).

#### Gene flow within a population: parentage analysis of current-year seedlings

At the Kanumazawa Riparian Research Forest, the total exclusion probabilities over the five loci for first and second parents were 0.989307 and 0.998929, respectively. In spite of the relatively small number of loci ( $n = 5$ ), the small number of candidate parents due to the low standing density and the dioecious nature of *C. japonicum* made the results of the assignment reliable.

We could assign parentage to 179 out of 212 seedlings (84.4%) by the simple exclusion method, and a further 30 seedlings (14.2%) by a combination of simple exclusion and categorical allocation, 209 in total. Three seedlings (1.4%) were not assigned because more than two candidate parents (or pair of parents) could explain their genotype.

Of the 209 seedlings analyzed, 149 (70.8%) were assigned to a male parent within the study site. For the other 61 seedlings (29.2%), no male parent was assigned, indicating that the male parent was outside the study site. The average distance of definite pollination events was 129 m ( $\pm 134$  SD), and the longest was 666 m (Figure 2a), the latter almost the diagonal length of the study site.

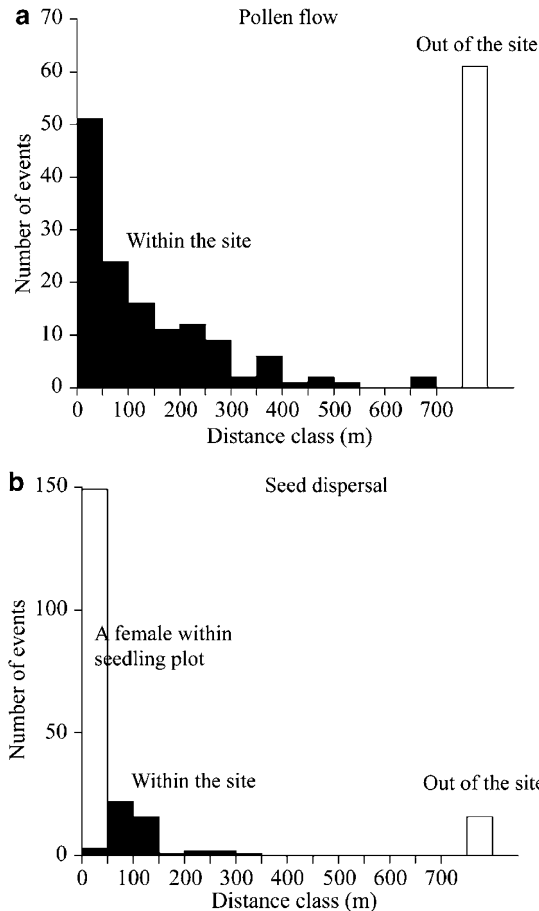
For 146 seedlings (69.9%), the female parent was the mature female within the plot in which the seedling was found, 48 seedlings (23.0%) had genotypes compatible with a female tree within the study site but outside the seedling's plot, and 15 seedlings (7.2%) had genotypes that were incompatible with those of all female trees in the study site (Figure 2b). Some documented seed dispersal events were more than several hundred meters, the longest being 302 m.

#### Fine-scale genetic structure

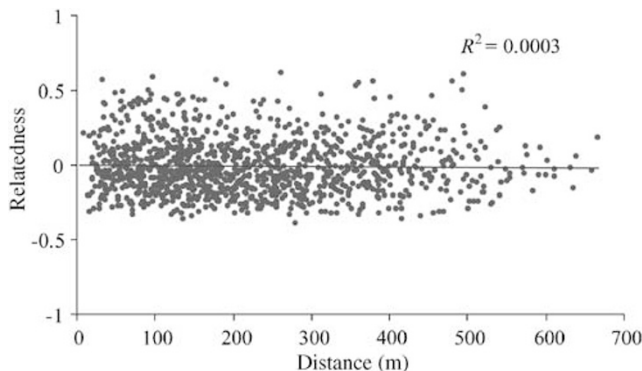
The correlation between spatial distance and genetic relatedness among mature trees in the Kanumazawa Riparian Research Forest was not significant (Mantel test,  $r = -0.0355$ ,  $P > 0.05$ , Figure 3), indicating that there is no spatial genetic structure within the population of established trees.

#### Among-populations spatial genetic structure

The overall  $F_{ST}$  estimate was 0.043. The population pairwise  $F_{ST}$  values (six populations, 15 pairs) ranged from 0.0186 to 0.0710, and all of the 15 pairwise  $F_{ST}$  were significantly larger than zero ( $P < 0.05$ ). The averaged values of pairwise  $F_{ST}$  were 0.046, and the test for isolation-by-distance were significant (Figure 4). Percentages of variation within populations, among populations within regional groups, and among regional groups were 95.39, 2.8, and 1.81%, respectively.



**Figure 2** Distance class distributions of (a) pollen flow, and (b) seed dispersal inferred from parentage analysis of current-year *Cercidiphyllum japonicum* seedlings within the Kanumazawa Riparian Research Forest.

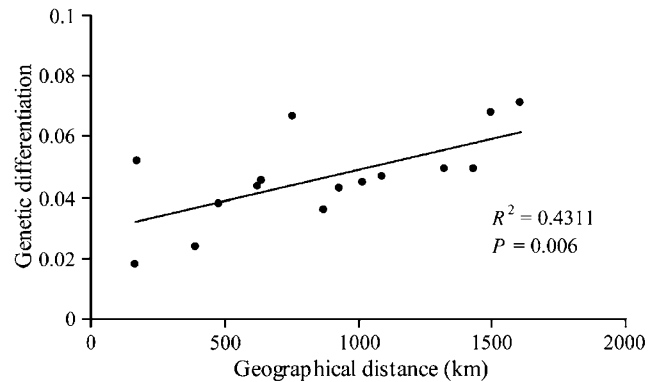


**Figure 3** Genetic relatedness (y-axis) plotted against spatial distance (x-axis) for adult *Cercidiphyllum japonicum* trees in the Kanumazawa Riparian Research Forest. The correlation is not significant (Mantel  $r = -0.0355$ ,  $P > 0.05$ , 5000 permutations). The solid line is the slope of a least-squares fit.

## Discussion

### Levels of gene flow inferred from parentage analysis

High levels of pollen dispersed at distances exceeding several hundred meters were observed within the study site of 20 ha, and 28.8% of the seedlings were sired by pollen from outside the study site. This suggests that the



**Figure 4** Relationship between pairwise genetic differentiation and geographical distance for six populations of *Cercidiphyllum japonicum*.

average pollination distance of 129 m found within the study site may be an underestimate of the real average. These distances are similar to those reported for other wind-pollinated species such as pine and oak (Dow and Ashley, 1996; Streiff *et al*, 1999; Schuster and Mitton, 2000). For example, Schuster and Mitton (2000) investigated pollen dispersal in an isolated population of limber pine, *Pinus flexilis*, and reported movements of more than 2 km. Pollen dispersal distances in wind-pollinated plants have a leptokurtic distribution (Richards, 1997), and parentage or paternity analyses have often shown high proportions of offspring with no potential father within the study population (Streiff *et al*, 1999; Lian *et al*, 2001). Relatively long distance pollination may therefore be a common phenomenon in wind-pollinated trees. Long-distance pollen flow presently found for *C. japonicum* might allow reproduction in populations with low densities.

We detected seed dispersal in *C. japonicum* of over several hundred meters, beyond the ridge and from outside the study site, although 70–80% of seedlings were under the crown of the maternal tree. Thus, seed shadows of maternal trees overlapped. A simulation study has shown that seeds lifted above the canopy by wind may disperse at least several hundred meters, and perhaps tens of kilometers (Nathan *et al*, 2002). This study also demonstrated the possibility of seed dispersal of more than 10 km in yellow poplar (*Liriodendron tulipifera*), a species that produces larger and heavier winged seeds than *C. japonicum*. Lighter seeds are more likely to be lifted and dispersed further by wind (Nathan *et al*, 2002). In addition, the seeds of *C. japonicum* are released in autumn and winter when the absence of leaves in the deciduous riparian forest may increase the number of seeds lifted by wind and thus lead to dispersal over longer distances.

### The effect of gene flow on fine-scale genetic structure

Near-neighbor mating and/or localized seed dispersal can produce significant levels of genetic structure within plant populations (reviewed in Hamrick and Nason, 1996). Wind-dispersed seeds are not expected to move as far as wind-dispersed pollen, because of their much larger volume and weight. Thus, limited seed dispersal that results in half-sib patches is likely to have a stronger influence on spatial genetic structure at local scales than pollen dispersal. Several studies of fine-scale genetic structure in plants have detected significant negative relationships between genetic relatedness and spatial

distance, which were often explained by limited seed dispersal (Berg and Hamrick, 1995; Ueno *et al.*, 2000). In this study, parentage analysis revealed potential for long-distance dispersal in both pollen and seed that may weaken fine-scale genetic structure, and also revealed that seed dispersal distances were shorter than those of pollen. We could not detect genetic structure within the study site.

Processes influencing germination and seedling survival also influence spatial genetic structure. Seeds that disperse longer distances may have higher survival probabilities than those near maternal trees because they escape the mortality caused by density-dependent processes such as competition, predation, and disease (Janzen, 1970; Clark and Clark, 1984). In the case of *C. japonicum*, seedlings that germinate under the crown of a mature tree cannot survive because the light conditions are not suitable for establishment (Seiwa and Kikuzawa, 1996; Kubo *et al.*, 2000). The preferred site for *C. japonicum* seedlings is bare ground formed by infrequent, large disturbances such as floods with debris avalanches (Kubo *et al.*, 2000), and seed shadows may overlap at such disturbed sites. Therefore, seedlings of *C. japonicum* do not play an important role as successors in the absence of disturbance. On the other hand, *C. japonicum* is long lived and has many sprouts, and so may persist through periods with a low probability of seedling establishment (Kubo *et al.*, 2001). In summary, the absence of fine-scale genetic structure in *C. japonicum* might be the result of extensive gene flow via pollen and seed, combined with a regeneration process that favors seeds that have dispersed further.

#### Genetic structure and gene flow among populations

One of the objectives of the present study was to investigate whether isolation-by-distance could be observed in a species featuring longer-distance dispersal. We could not detect isolation-by-distance within the Kanumazawa population, but did detect weak genetic structure at the larger spatial scale in the form of significant isolation-by-distance and a low level of genetic differentiation among populations. Our pollen dispersal data appear to fit a leptokurtic distribution and, from these data, it is inferred that gene flow among populations is closely approximated by the isolation-by-distance model (Hamrick and Nason, 1996; Richards, 1997). Under this model, gene flow rates are a function of spatial distance, being higher between neighboring populations and lower among populations that are spatially more remote (Wright, 1943). The result of AMOVA is also consistent with isolation-by-distance among populations, indicating that gene flow among populations within regional groups was higher than among groups. *C. japonicum* has a hierarchical genetic structure, with no fine-scale genetic structure within the Kanumazawa population but significant genetic structure among regional groups and throughout Japan.

We found most genetic diversity (95.4%) within populations of *C. japonicum*. Wind-pollinated, outcrossing trees with wide and continuous distributions tend to have high levels of genetic diversity within populations (usually more than 90% of the total genetic variation) and low levels of genetic differentiation of populations, a pattern contrasting with that found in species with alternate breeding systems and/or restricted distribu-

tions (Hamrick *et al.*, 1992; Hamrick and Nason, 1996). In Japan, population genetic studies have been intense in coniferous species utilized for forestry. Widely distributed coniferous species with well-dispersed pollen, such as *Chamaecyparis obtusa*, *Larix kaempferi* and *Cryptomeria japonica*, showed low levels of genetic differentiation at allozyme loci, with  $G_{ST}$  values of less than 0.050 (reviewed in Tsumura, 2001). In contrast, trees with well-dispersed pollen but restricted distributions showed moderate level of genetic differentiation as indicated by  $G_{ST}$  values of 0.144 in *Abies mariesii* and 0.170 in *Pinus pumila* (reviewed in Tsumura, 2001). Furthermore, Kikuchi and Isagi (2002) reported remarkable levels of genetic differentiation in *Magnolia sieboldii* spp. *japonica*, a deciduous tree that is insect-pollinated and restricted to high elevation areas of discontinuous occurrence, as indicated by an  $F_{ST}$  value of 0.488 and  $R_{ST}$  value of 0.538. It has been suggested that species with more seed and pollen movement should have less genetic differentiation than species with restricted gene flow (Govindaraju, 1988; Hamrick and Nason, 1996), and weak genetic structure (smaller  $F_{ST}$ ) is used as an indication of massive gene flow among populations. We have revealed by parentage analysis that *C. japonicum* has potential for extensive gene flow through both pollen and seed dispersal, consistent with low levels of genetic differentiation among populations, and such long-distance gene flow may homogenize allele frequencies between populations.

#### Conclusion and remarks

In this study, the effect of gene flow on spatial genetic structure in *C. japonicum* was explored through genetic analyses at different spatial scales. The simultaneous study of gene flow within a population and among populations provided more information than either analysis alone. Our result, indicating extensive gene flow in *C. japonicum*, was supported by both within- and among-population genetic structure. Such high levels of gene flow may enable reproduction at low population densities, increase the local effective size of the population ( $N_e$ ) and maintain the genetic diversity of this species.

Riparian forests have important ecological functions in the maintenance of biodiversity (Sakio and Yamamoto, 2002), but large areas of riparian forest in Japan have been destroyed and fragmented by human activities such as the construction of dams or channelization of rivers. In the present study, we found high levels of genetic diversity in *C. japonicum* even within a population. However, we should treat this result with caution. The effect of forest fragmentation on genetic diversity may not be detectable (Collevatti *et al.*, 2001) because long-lived tree species may initially retain the genetic diversity that they had before fragmentation. Since adult trees of *C. japonicum* last a very long time, adult trees retain genetic traits for several hundred years before when their habitats were more abundant, and populations existed more closely with each other before the industrialization of Japan. This might be one of the factors that caused the low genetic differentiation between populations located far apart. A possible scenario is that the genetic diversity will decrease in future generations. Conservation and re-establishment of

riparian forest are urgent issues, and the studies of spatial genetic structure and gene flow provide essential data for the selection of gene sources for revegetation, planting at appropriate densities, and development of management programs.

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