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Fine-scale initiation of non-native *Robinia pseudoacacia* riparian forests along the Chikumagawa River in central Japan

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Abstract

Robinia pseudoacacia has become invasively naturalized in Japan. We investigated the role of sexual reproduction in the development of *R. pseudoacacia* riparian forests along the Chikumagawa River in Japan, by using five chloroplast (cpSSR) and seven nuclear (nSSR) markers. We identified eight chloroplast haplotypes and 147 nuclear genotypes from 619 *R. pseudoacacia* trees sampled in three plots (Plots A, B, and C) and along two line transects (Lines D and E). CpSSR analyses showed that multiple maternal lines were distributed along the river, and that some haplotypes from different populations overlapped. In addition, while Plots A and B were separated by a short distance, only these two plots exhibited genetic differentiation in the haplotypes. In the nSSR analysis, all pairwise F_{ST} values among the three plots were significantly different from zero. Kinship analysis based on nSSR markers revealed that kinship connected many individuals to another individual from the same plot. These results indicate that seed dispersal near to mother trees contributes to the fine-scale genetic structure of *R. pseudoacacia* riparian forests. Our results indicate that sexual reproduction, in addition to asexual reproduction, is a major contributor to the fine-scale formation of *R. pseudoacacia* forests.

Key words: exotic tree species, invasion, riparian forest, *Robinia pseudoacacia*, sexual reproduction

INTRODUCTION

Robinia pseudoacacia L. was introduced to Japan more than 100 years ago for several purposes, including use as ornamental plants, serving as sources of nectar, and to prevent erosion. However, recently, this species has become invasively naturalized, and is recognized as an invasive tree species in Japan by the Ministry of the Environment of Japan (http://www.env.go.jp/nature/intro/1outline/caution/list_sho.html). This invasion has led to concerns about whether this species is contributing to possible declines in plant biodiversity or having adverse effects on native species (Maekawa and Nakagoshi 1997). Thus, it is necessary to assimilate baseline datasets about *R. pseudoacacia* for future environmental management planning.

The establishment of an invasive woody species in a habitat that would normally be inhabited by native species might alter ecosystem functioning, consequently having a major impact on native ecosystems (Vitousek 1990, Parker et al. 1999). To address this issue, it is essential to understand how the propagules of such species disperse from the parent plants. While an accurate history of the expansion of a naturalized species is difficult to obtain, the radiation of some invasive plants has been demonstrated through historical records of naturalization assimilated via landscape-scale analyses (Landenberger et al. 2009) and population-genetic analysis (Bossdorf et al. 2005, Rosenthal et al. 2008, Sakio 2009, Pairon et al. 2010). For example, landscape-scale information, such as aerial

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photographs and GIS, indicate that the *R. pseudoacacia* riparian forests have gradually become more widely distributed in Japan (Society for History of Chikumagawa and Saigawa River 2003). In addition, population-genetic analysis of *R. pseudoacacia* populations along the Arakawa River in Japan has indicated no significant correlation between geographical distance and genetic similarity at the watershed level (Sakio 2009). However, while these studies might provide a general explanation for the process of *R. pseudoacacia* habitat expansion at a landscape level, fine-scale information about the process of forest establishment by *R. pseudoacacia* remains unresolved.

After the establishment of *R. pseudoacacia* forests, trees appear to reproduce asexually via adventitious buds sprouting on tree stumps and horizontal roots (Boring and Swank 1984, Jung et al. 2009, Kurokochi et al. 2010). However, evidence of sexual reproduction within forests has also been reported. For example, *R. pseudoacacia* trees have been observed to generate large seed banks (Takahashi et al. 2008, Masaka et al. 2010), from which some seeds germinate without the necessity for dormancy breaking treatments, such as heat shock (Masaka and Yamada 2009). In addition, direct field observations of seedlings have been reported (Fukuda et al. 2005). Therefore, *R. pseudoacacia* populations may expand through a combination of fine-scale sexual (i.e., seed dispersal) and asexual (i.e., adventitious roots) reproduction.

Our research group has previously used dendroecological and microsatellite polymorphism analyses to investigate the regeneration process of *R. pseudoacacia* riparian forests after the clear-cutting of mature stands along the Chikumagawa River in Japan. We demonstrated that, within several years of clear-cutting activity, most *R. pseudoacacia* trees became established through asexual reproduction. In addition, we observed that some *R. pseudoacacia* trees regenerated asexually around a fallen *R. pseudoacacia* tree (Kurokochi et al. 2010). Our results indicate that an established *R. pseudoacacia* tree of a certain nuclear genotype could maintain its genotype in the forest. Hence, the exploration of relationships among *R. pseudoacacia* trees in existing forests might indicate the fine-scale process of *R. pseudoacacia* riparian forest establishment.

In our previous study, various sizes of *R. pseudoacacia* genets were found to have four nuclear SSR markers (Kurokochi et al. 2010). This result supported the fine-scale role of asexual reproduction in the formation of *R. pseudoacacia* riparian forests; however, the extent to which sexual reproduction contributes has yet to be determined. Microsatellite markers for polymorphism analysis are known to be effective for analyzing the reproductive mechanisms of many organisms, ranging from fungi to higher plants and animals. Nuclear microsatellite (nSSR) markers with high polymorphism might also contribute to the analysis of parenthood, kinship, and population genetics (Streiff et al. 1998, Sato et al. 2006). In plants, chloroplast microsatellite (cpSSR) markers also contribute toward distinguishing maternal lines (Lian et al. 2003). Furthermore, the precise reproductive characteristics of some woody plants have been successfully elucidated through the simultaneous use of cpSSR and nSSR markers (Lian et al. 2003, Geng et al. 2008). Therefore, the simultaneous application of cpSSR and nSSR polymorphism analyses might provide more accurate information about the actual fine-scale contributions of sexual and asexual reproduction in the forest initiation process of *R. pseudoacacia*.

To improve our understanding about the radiation of *R. pseudoacacia* in Japan, in this study, we used simultaneous polymorphism analyses of cpSSR and nSSR markers to investigate the fine-scale genetic structure of *R. pseudoacacia* at both the tree and population level. On the basis of our findings, we discuss the fine-scale process of *R. pseudoacacia* riparian forest initiation and expansion along the banks of the Chikumagawa River in Japan.

MATERIALS AND METHODS

Study site and sample collection

The current study was conducted in *R. pseudoacacia* riparian forests established along the banks of the Chikumagawa River in Japan. The Chikumagawa River is one of the major rivers in Japan where *R. pseudoacacia* trees have become naturalized, forming large monospecific forests. According to the administrators of this river, in 2005, *R. pseudoacacia* represented more than 500 ha (1 ha = 10,000 m^2) of the total 2,000 ha of natural vegetation.

We established three plots (Plots A, B, and C) and two transect lines (Lines D and E) along the river banks of the middle section of the Chikumagawa River, where large *R. pseudoacacia* riparian forests with tall trees (approx. 10 m in height) were intermittently distributed (Fig. 1). Moreover, stands of almost pure *R. pseudoacacia* forests have been establishing at this study site and the surrounding areas for more than 40 years. As *R. pseudoacacia* was not planted or seeded along the banks of the middle section of the river, these trees are believed to have originated from mother trees that were previously planted along the

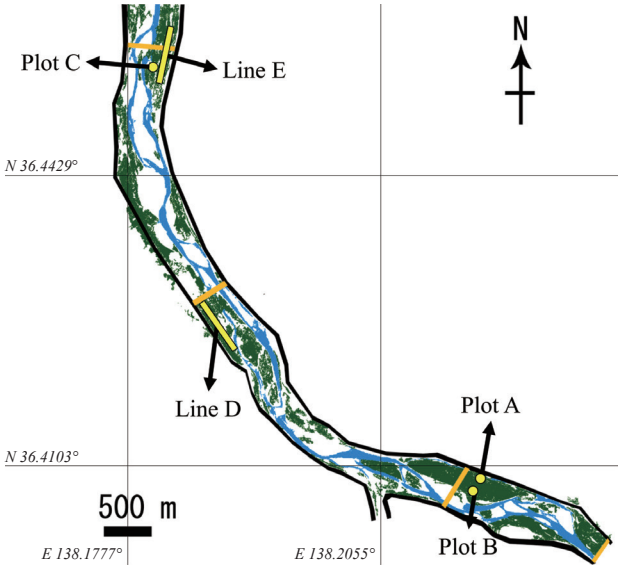

Fig. 1. The study site location (showing the positioning of plots and transect lines) along the Chikumagawa River, Japan**.** The river flows from the lower right to the upper left. Green areas on the riverside represent *Robinia pseudoacacia* riparian forests. Orange bars across the river indicate bridges. Fine lines denote levees. Blue lines of irregular thickness between levees indicate the river channel.

upper reaches of the river for deliberate afforestation. In the present study, we primarily focused on the fine-scale investigation of three specific plots. However, in case we did not detect genetic differentiation among plots and/or stands within plots, two transect lines were established to determine whether the absence of genetic differentiation was due to the narrow range of plots.

Plots A, B, and C were 60 × 30 m, 30 × 30 m, and 30 × 30 m in size, respectively. The average tree diameter at breast height (DBH) in all plots ranged from 9 to 14 cm (Kurokochi et al. 2010). Lines D and E were both approximately 600 m in length, and were established parallel to the river bank. Line E passed Plot C at a distance of 10 m (Fig. 1).

Because most *R. pseudoacacia* tree were very tall and had leaves only at their canopy, it was very difficult to collect leaf samples from many tree. In this study, therefore, a fragment of cambial tissue was collected from the trunk of all *R. pseudoacacia* trees in the three plots. Along each of the two line transects, 25 *R. pseudoacacia* trees of >10 cm DBH were selected at 20–25 m intervals, from which cambial fragments were also collected. Each cambial tissue fragment was dried separately in a plastic bag containing silica gel, and stored at room temperature until use.

PCR amplification and length determination of cpSSR and nSSR markers

DNA was extracted from each fragment using a modified cetyltrimethylammonium bromide (CTAB) method (Kurokochi et al. 2013). Five cpSSR markers (Ropscp03, Ropscp04, Ropscp06, Ropscp07, and Ropscp08) developed by Kimura et al. (in press), three nSSR markers (Rops05, Rops06, and Rops08) developed by Lian and Hogetsu (2002), and four nSSR markers (RP109, Rp200, RP206, and RP01B) developed by Mishima et al. (2009) were used in this study. All types of markers were amplified by PCR in a manner similar to that reported by Kurokochi et al. (2010). PCR products were electrophoresed using a Hitachi SQ5500E analyzer (Hitachi, Tokyo, Japan), and their lengths were determined using FRAGLYS ver. 3 (Hitachi). To avoid misreading the band sizes, the PCR products that were preliminarily judged as being the same length in the first electrophoregrams were re-electrophoresed side by side to confirm this.

Data analyses

Regarding the five cpSSR markers, the number of alleles (Na) that were detected at each marker was visually counted. For the seven nSSR markers, the number of alleles (Na), observed heterozygosity (Ho), expected heterozygosity (He), polymorphic information content (PIC) of each marker, and combined non-exclusion probabilities of all nSSR markers were evaluated using CERVUS 3.0.3 (Marshall et al. 1998). In the analysis, trees of the same nSSR genotype and the same cpSSR haplotype with no mismatching on each locus were regarded as belonging to the same genet and the same maternal line, respectively.

Genetic differentiation among plots was evaluated using a dendrogram constructed based on pairwise genetic distances for all samples using the neighbor-joining (NJ) method of the Populations 1.2.30 (Langella 2007) from datasets of nSSR markers. Pairwise F_{ST} values were calculated using Arlequin ver. 2.0 (Schneider et al. 2000) from datasets of cpSSR and nSSR markers. The NJ tree was visualized using the Tree View (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

The presence of potential kinship between nuclear genotypes was tested at a significance level of 0.001, using KINGROUP ver. 2 (Konovalov et al. 2004) from the nSSR markers by 10,000,000 random permutations. Genotype pairs, in which the relationships of full-siblings, half-siblings, or parent-offspring were detected, were regarded to have kinship.

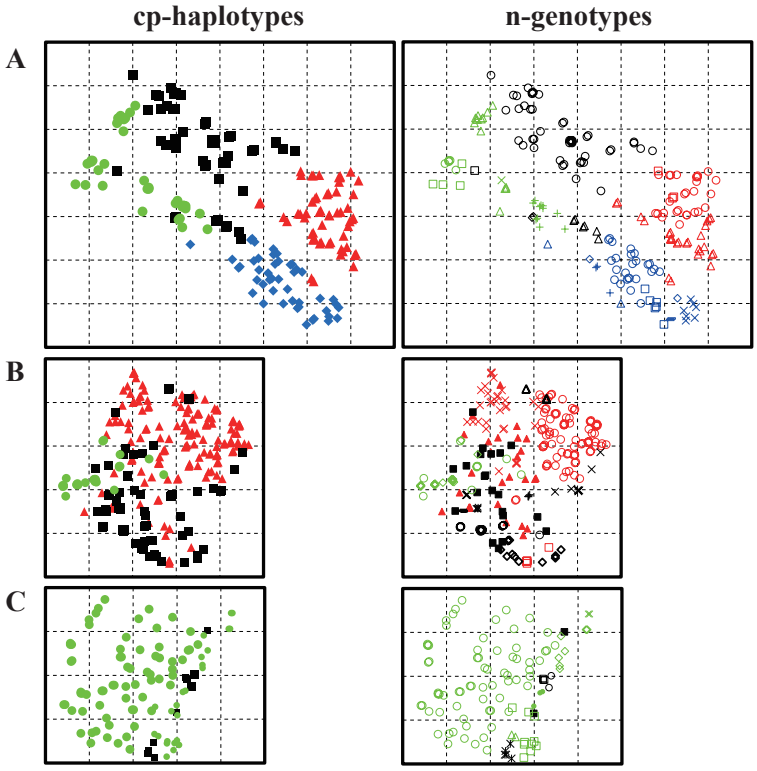

Fig. 2. Distribution of cp-haplotypes and n-genotypes of *Robinia pseudoacacia* in Plots A, B, and C. In cp-haplotypes (graphs in left side), symbols of the same color and shape represent trees of the same cp-haplotype. Cp-haplotypes H1, squares; H2, circles; H3, triangles; and H5, diamonds. In n-genotypes (graphs in right side), symbols of the same color and shape represent trees of the same n-genotype. Painted squares and triangles in Plots B and C represent a tree for which the n-genotype was unique in the plot. Grid width is 10 m.

RESULTS

Chloroplast haplotypes (cp-haplotypes) and nuclear genotypes (n-genotypes)

We successfully completed PCR amplification of cpSSR and nSSR markers in 192 samples from Plot A, 273 samples from Plot B, 104 samples from Plot C, 25 samples from Line D, and 25 samples from Line E (Table 1).

The five cpSSR markers contained between two and six alleles, with an average of four (Table 2). In Plots A, B, and C, and Lines D and E, four, three, two, five, and five cp-haplotypes were found, respectively, with a total of eight unique cp-haplotypes being present (Table 1). Cp-haplotypes H1 and H2 were found in all Plots and Lines.

The seven nSSR markers were highly variable, containing between nine and 16 alleles, with an average of 10.9 alleles. The averages of Ho, He, and PIC were 0.72, 0.75, and 0.72, respectively (Table 2). The combined non-exclusion probability of the first parent was 0.025, and genotypic

Table 1. Baseline data at each study site using chloroplast and nuclear microsatellite markers

Study site	Latitude [a]	Longitude [a]	Area or length	Analyzed trees	No. of analyzed samples	No. of detected genotypes at each chloroplast haplotype								
						H1	H2	H3	H4	H5	H6	H7	H8	total
Plot A	36°24′33.6″	138°13′08.2″	1800 (m^2)	All trees in the plot	192	4	6	4	-	8	-	-	-	22
Plot B	36°24′32.8″	138°13′07.3″	900 (m^2)		273	33	30	9	-	-	-	-	-	72
Plot C	36°26′52.4″	138°10′55.5″	900 (m^2)		104	5	8	-	-	-	-	-	-	13
Line D	36°25′29.8″	138°11′13.9″	600 (m)	Trees at 20–25 m intervals along the river	25	14	2	2	3	-	1	-	-	22
Line E	36°26′52.3″	138°10′58.3″	600 (m)		25	8	7	-	1	-	-	1	1	18

[a] Latidude and longitude were recorded in the middle of each Plot and Line.

consistency probabilities between two unrelated individuals and between two unrelated siblings were <0.001 and 0.002, respectively (Table 2). Since the probability of genet discrimination ability by the present analysis using seven nSSR markers was high (Table 2), ramets with the same n-genotype were assumed to belong to the same clonal genet. In Plots A, B, and C, and Lines D and E, we identified 22, 72, 13, 22, and 18 n-genotypes, respectively, with a total of 147 n-genotypes being present (Table 1). Each n-genotype was found at only one study site.

Trees with the same cp-haplotype (i.e., maternal line) were aggregated (Fig. 2), with some being included in different n-genotypes (i.e., genets) (Table 1). Trees in the same genet were continuously established in clusters, and always belonged to the same maternal line (Fig. 2). In cases where a certain n-genotype was detected in more than one tree within each population, we selected one tree as a representative of the n-genotype, and used its n-genotype and cp-haplotype for the analyses in this study.

Genetic differentiation among Plots

The dendrogram and NJ tree revealed that the plot populations were genetically differentiated, and the populations in Plots A and B were marginally close (Fig. 3). For pairwise F_{ST} values between the plot populations, one out of three pairwise F_{ST} values, based on cp-haplotypes, and all pairwise F_{ST} values, based on n-genotypes, were significantly different from zero (Table 3; $P < 0.01$).

Kinship analysis

As shown in Table 4, a total of 84 n-genotype pairs were detected to have kinship. Kinship was primarily recorded between two n-genotypes within the same plots or lines. Only a few pairs were between n-genotypes of spatially different plots and/or lines (Table 4).

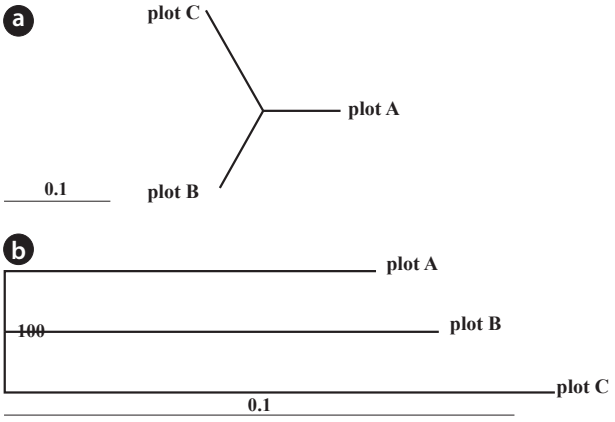

Fig. 3. (a) Neighbor-joining tree and (b) dendrogram of *Robinia pseudoacacia* in Plots A, B, and C. Bar 0.1 indicates genetic distance between Plots. 100 means 100 % of bootstrap probability (No. of bootstrap test is 1,000).

DISCUSSION

In this study, cpSSR and nSSR polymorphism analyses provided information about the reproduction process leading to the initiation of *R. pseudoacacia* riparian forests situated along the banks of the Chikumagawa River.

Fine-scale genetic structure of *R. pseudoacacia*

CpSSR analyses showed that multiple maternal lines were distributed along the river, and that some haplotypes from different populations overlapped (Table 1).

Table 2. Traits of five chloroplast and seven nuclear microsatellite markers of *Robinia pseudoacacia* based on 147 individual nuclear genotypes

Chloroplast SSR markers								
Locus name	Ropscp03	Ropscp04	Ropscp06	Ropscp07	Ropscp08			Average
Na	6	3	5	4	2			4.0
Nuclear SSR markers								
Locus name	Rops05	Rops06	Rops08	RP109	RP200	RP206	RP01B	Average
Na	13	7	9	9	11	16	11	10.9
Ho	0.81	0.57	0.79	0.76	0.61	0.76	0.76	0.72
He	0.86	0.56	0.77	0.76	0.71	0.88	0.70	0.75
PIC	0.84	0.53	0.73	0.73	0.69	0.86	0.68	0.72
Combined non-exclusion probability of first parent						0.025		
Combined non-exclusion probability of individual identity						<0.001		
Combined non-exclusion probability of sib identity						0.002		

Na, number of detected alleles; Ho, observed heterozygosity; He, expected heterozygosity; PIC, polymorphic information content.

These results were similar to the distribution patterns recorded for *R. pseudoacacia* chloroplast haplotypes along the Arakawa River in Japan (Sakio 2009). The distribution patterns of different admixed haplotypes in a population have been reported for several invasive plant species (Rosenthal et al. 2008, Pairon et al. 2010, Kurokochi et al. 2013), with *R. pseudoacacia* populations at our study site exhibiting similar characteristics. In addition, though Plots A and B were spatially and collectively closer (Figs. 1 and 3), genetic differentiation in the cp-haplotypes was only detected between these two plots (Table 3). This result indicates that seed dispersal was spatially limited, and might proceed independently at each plot.

As for nuclear DNA analyses, with respect to several invasive woody plants, an absence of genetic structure, which is related to geographical distance, has been previously reported at a broad (country-wide and/or landscape) scale (Dunphy and Hamrick 2005, Aldrich et al. 2010, Le Roux et al. 2011). At the same scale, studies using nuclear DNA polymorphism analysis have also reported this trend for *R. pseudoacacia* in Japan (Sakio 2009), with genetic differentiation among *R. pseudoacacia* populations being relatively small (Sun et al. 2009). However, in the present study, all pairwise F_{ST} values were significantly different from zero in the nSSR analysis (Table 3). This result indicated that genetic differentiation was large at a fine-scale, and that the forests might be derived from a large number of offspring from pioneer trees in each population.

Table 3. Pairwise F_{ST} values by nuclear and chloroplast microsatellite markers

	Plot A	Plot B	Plot C
Plot A		0.039*	0.032*
Plot B	0.131*		0.041*
Plot C	0.152	0.005	

The upper right and left lower three values represent F_{ST} values using nSSR and cpSSR markers, respectively. F_{ST} values with "*" are significantly larger than zero ($P < 0.01$; 1,000 per mutations).

Table 4. Number of pairs connected by kinship site-by-site (Full-sibs/Half-sibs/Parent-offspring)

Study site	Plot A (22)	Plot B (72)	Plot C (13)	Line D (22)	Line E (18)
Plot A (22)	15/13/10 16	0/1/0 1	0/1/1 1	0/1/1 1	1/1/0 1
Plot B (72)		29/22/32 44	0/0/0 0	0/0/1 1	0/0/0 0
Plot C (13)			5/4/5 7	1/1/1 1	3/3/3 3
Line D (22)				2/3/5 6	0/1/0 1
Line E (18)					1/1/1 1

Integer numbers shown in parentheses represent the number of detected n-genotypes at each study site. Integer numbers shown with underlines represent the total sum of kinship pairs for which overlapping pairs were deleted.

Relationship among *R. pseudoacacia* trees

Kinship analysis provided insights into the seed propagation (sexual reproduction) of *R. pseudoacacia*. This analysis showed that kinship connected many individuals to another individual in the same plot (Table 4). In addition, the tendency for genets belonging to the same maternal line being aggregated supported local seed dispersal and establishment (Fig. 2). These data indicate that new genets were established from seeds dispersed from mother trees in the immediate vicinity. This observation might explain the presence of genetic differentiation among plots (Table 3). Although the genetic differentiations were detected at a plot level, we found only a few pairs exhibiting kinship from two individuals belonging to spatially different study sites at an individual level (Table 4). This observation indicates that only a few seeds may disperse relatively long distances, or that there is limited gene flow among trees of spatially different populations. Indeed, some *R. pseudoacacia* seeds have been directly observed being transported by the river (Sakio 2009).

In most cases, *R. pseudoacacia* trees appear to reproduce asexually in the field (Boring and Swank 1984, Jung et al. 2009). However, our results suggest that sexual reproduction, in combination with asexual reproduction, is a major contributor in the fine-scale formation of *R. pseudoacacia*.

Fine-scale initiation and maintenance of *R. pseudoacacia* riparian forests

Only a small number of *R. pseudoacacia* trees appeared to be present on the riverside at the forest initiation stage (Society for History of Chikumagawa and Saigawa River 2003), with only a few *R. pseudoacacia* trees being initially introduced in the form of seeds. Therefore, we propose two possible mechanisms for the initiation and development of *R. pseudoacacia* riparian forests from small numbers of *R. pseudoacacia* trees at a fine-scale. The forests in each plot may have been derived (1) from the offspring of small numbers of *R. pseudoacacia* trees that were initially established in each plot through the intra-plot

dispersal of seeds, or (2) from the offspring of mature *R. pseudoacacia* populations inhabiting the upper reaches of the river. In general, most plants including both trees and herbs disperse seeds nearby mother plants. Previous research has indicated that many seeds of woody species are often released in the immediate vicinity of the mother tree (Cousens et al. 2008). Similarly, *R. pseudoacacia* seeds might disperse close to mother trees, since seed banks of *R. pseudoacacia* often form where *R. pseudoacacia* trees are distributed (Takahashi et al. 2008). On the basis of existing knowledge combined with the results of the current kinship analysis, we suggest that the initiation and development of *R. pseudoacacia* riparian forests partly depends on short-distance seed dispersal.

In our previous study (Kurokochi et al. 2010), we provided information on the regeneration and distribution characteristics of developed *R. pseudoacacia* riparian forests, using dendroecological and microsatellite polymorphism analyses. The regeneration of developed *R. pseudoacacia* riparian forests requires certain stimuli, such as clear-cutting and fallen trees, which appear to trigger the formation of new shoots, as observed for other species (Negreros-Castillo and Hall 2000, Keim et al. 2006, O'Hara et al. 2007). In addition, most shoots reproduce asexually, when underground disturbance is absent (Kurokochi et al. 2010). Moreover, we previously found that ramet trees belonging to the same genet were distributed in clusters in riparian forests, indicating that trees established from seeds that were distributed close to mother ramets, as a result of asexual reproduction through residual horizontal roots (Kurokochi et al. 2010).

The combined results of our current research (sexual reproduction) and previous research (asexual reproduction and forest regeneration; Kurokochi et al. 2010) allowed us to elucidate the process of *R. pseudoacacia* forest formation during initiation and development. First, only a small number of naturalized *R. pseudoacacia* trees became established on the river banks of the middle reach of the river, grew, and produced seeds. In parallel, trees expanded their genet areas by asexually extending horizontal roots and producing ramets. Second, seeds produced from the initially established trees dispersed in the local vicinity, and initiated new seedlings. The initially established trees and seedlings grew and, at the same time, developed horizontal roots, from which new shoots sprouted. Hence, *R. pseudoacacia* forests have developed on a meshwork of horizontal roots. Finally, when *R. pseudoacacia* trees in forests fall naturally, or the aboveground parts are removed by clear-cutting, which is a typical management strategy in Japan, this action creates gaps, in which new saplings sprout from the residual horizontal roots and cut stumps, within several years after gap formation. Therefore, the forest is restored asexually, without seed reproduction.

Our result was one of the reminders that the asexual and sexual reproductive ability of *R. pseudoacacia* trees is very high. In protective zones where establishment of *R. pseudoacacia* is undesirable, regular monitoring is required and *R. pseudoacacia* should be actively removed by falling trees and digging away roots, even if number of its establishment is small.

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