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Spatial Distribution of CpDNA and MtDNA Haplotypes in a Hybrid Zone between *Pinus pumila* and *P. parviflora* var. *pentaphylla* (Pinaceae)

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Pinus **species exhibit paternal chloroplast inheritance and maternal mitochondrial inheritance. This independent inheritance of two cytoplasmic genomes provides an exceptional environment for discriminating female (seeds) and male (pollen) components of gene flow across hybridizing species. We obtained mitochondrial genetic markers diagnostic to *P. parviflora* var. *pentaphylla* and *P. pumila* by PCR amplification of the intron of *nad*1 on mtDNA, and examined the spatial-distribution pattern of the mtDNA haplotypes in a hybrid zone between *P. parviflora* var. *pentaphylla* and *P. pumila* in the Tanigawa Mountains of Japan. These data, in conjunction with previous information on cpDNA haplotypes and needle morphology, revealed contrastive patterns of introgression of two cytoplasmic genomes. CpDNA introgression has occurred uni-directionally from *P. parviflora* var. *pentaphylla* to *P. pumila*. Conversely, mtDNA introgression has occurred in the opposite direction, from *P. pumila* to *P. parviflora* var. *pentaphylla*. Levels of introgression are roughly equivalent for cpDNA and mtDNA. The contrastive spatial distribution pattern of cpDNA and mtDNA haplotypes could be caused by differential movement of seeds and pollen for interspecific genetic exchange.**

Key words: Choroplast DNA — Introgressive hybridization — Mitochondrial DNA — *Pinus*

Most angiosperms studied to date transmit their cytoplasmic genomes, chloroplast DNA (cpDNA) and mitochondrial DNA (mtDNA), predominantly from female parent to sexual progenies (Mogensen 1996). In contrast to this, several conifers, such as *Pinus* (Neale and Sederoff 1989), *Pseudotsuga* (Neale *et al.* 1986, Marshall and Neale 1991), and *Taxus* (Pennell and Bell 1988), exhibit paternal chloroplast inheritance and maternal mitochondrial inheritance. This contrasting uni-parental transmission of two cytoplasmic genomes provides an exceptional environment for studying maternal and paternal genetic lineages within a single species (Palmer 1992), and for discriminating female (seeds) and male (pollen) components of gene flow across hybridizing species.

In a previous study on the introgressive hybridization in *Pinus* (Watano *et al.* 1995), we documented the spatial distribution of cpDNA haplotypes in a hybrid zone between *Pinus pumila* (Pallas) Regel and *P. parviflora* Sieb. et Zucc. var. *pentaphylla* (Mayr) Henry in the Tanigawa Mountains of Japan. CpDNA haplotypes were determined by PCR-SSCP of the intergenic spacer between the *trn*L (UAA) and the *trn*F (GAA) of cpDNA. We found that all morphological intermediates sampled in the Tanigawa Mountains had the same cpDNA haplotype of *P. parviflora* var. *pentaphylla*. Furthermore, some individuals classified on the basis of needle morphology as belonging to *P. pumila* showed the same cpDNA haplotypes of *P. parviflora* var. *pentaphylla*. We therefore concluded that uni-directional cpDNA introgression from *P. parviflora* var. *pentaphylla* to *P. pumila* occurs in this mountain region.

In addition to cpDNA haplotypes, the present study examined the spatial distribution of mtDNA haplotypes in the hybrid zone examined by Watano *et al.* (1995). Two distinct means of genetic movement, pollen and seed, are employed in seed plants. Genetic transfers by pollen and seed across species could not be coupled, necessarily, but could be differential. Arnold *et al.* (1991) found that introgression of nuclear DNA markers had occurred in the absence of cpDNA introgression in a hybrid zone between *Iris fulva* and *I. hexagona*, indicating pollen-mediated gene flow across species. By contrast, Rieseberg and Soltis (1991) compiled studies on cpDNA introgression, and found that cpDNA introgression was frequently observed without evidence of nuclear introgression. This may imply a higher contribution by seed than by pollen dispersal in genetic transfer across species.

In the present paper, we address the following questions. 1) What is the extent of mitochondrial introgression between the two pine species ? 2) Is mitochondrial introgression uni-directional or bi-directional ? 3) Is spatial pattern of mitochondrial introgression congruent with that of chloroplast introgression ?

Materials and Methods

Pinus pumila is distributed from the middle Honshu, Japan, northward to eastern Siberia, while *P. parviflora* var. *pentaphylla* is distributed from the middle Honshu northward to

Hokkaido, Japan. The Japanese *P. pumila* is a creeping alpine shrub, and the *P. parviflora* var. *pentaphylla* is an erect tree in the montane to subalpine zones. Although the two species are usually isolated due to their different vertical distributions, plants that are morphologically intermediate to them are often found in the alpine to subalpine zones of some mountains (Isii 1941).

The study site of the present paper was the Tanigawa Mountains, located on the borders of the Niigata and Gunma Prefectures, central Honshu, Japan. We collected 35 individuals of five-needle pines from the Tanigawa Mountains, which comprised morphologically of typical *P. pumila*, typical *P. parviflora* var. *pentaphylla*, and various types of morphological intermediates (Watano *et al.* 1995). In order to obtain species-specific markers of mtDNA, we also examined representative individuals of the parental species from other mountains: fourteen samples of *P. pumila* and twelve of *P. parviflora* var. *pentaphylla*. The examined samples from the Tanigawa Mountains and *P. pumila* from other mountains were the same as those examined in the previous paper (Watano *et al.* 1995). Samples of *P. parviflora* var. *pentaphylla* from other mountains were newly collected in the present paper, because we could not amplify a mtDNA fragment by polymerase chain reaction (PCR) successfully using samples used in Watano *et al.* (1995). Sources of the materials of *P. parviflora* var. *pentaphylla* are as follows. D5, Jyoudodaira, Mt. Azumayama, Fukushima, Japan; D6-D14, Shiramine-mura, Ishikawa, Japan; D15, Nagawa-mura, Minamiazumi-gun, Nagano, Japan; D16, Uematsu-machi, Kiso-gun, Nagano, Japan. Vouchers of D5-D14 are deposited in the Herbarium of Kanazawa University (KANA). Samples of D15 and D16 were provided by the seed bank of Forestry and Forest Products Research Institute (FFPRI), Kukizaki, Ibaragi, Japan.

Genomic DNA was extracted from leaf or seed materials by the CTAB isolation method (Doyle and Doyle 1987) and then used as a template in the PCR. We amplified an intron between exons B and C of the mitochondrial gene encoding subunit 1 of NADH dehydrogenase (*nad*1) by PCR, using primers designed by Demesure *et al.* (1995). The reaction mixture (50 μl) contained 1× reaction buffer, 100 μM dNTPs, 0.2 μM of each primer, 50 ng of genomic DNA, and 1 unit of Taq polymerase (Ex Taq, TAKARA). The amplification was carried out using one cycle of 3 min at 94C, 30 cycles of 1 min at 92C, 1 min at 55C, 3 min at 72C and one cycle of 10 min at 72C. After amplification, 5 μl of the PCR products and 1 μg of λ *Hin*dIII digest were run on 1.5% agarose gel and visualized with ethidium bromide staining to check yield and overall length polymorphisms of the amplified DNAs. The remaining PCR products were extracted with chloroform, and concentrated up to 20 μl by ammonium acetate/ethanol precipitation. Digestions with two restriction endonucleases (*Sau*3AI, *Rsa*I) were also conducted for some PCR products. Two aliquot (10 μl) of the concentrated samples were digested and then separated in 3% NuSieve agarose (FMC) with 1 μg of ΦX174 *Hae*III digest and stained by ethidium bromide.

Results

Species-specific mtDNA markers of *P. pumila* and *P. parviflora* var. *pentaphylla* were obtained by amplifying the *nad*1 intron of mtDNA. The PCR products of *P. pumila* were about 2200 base pairs (bp) long, and those of *P. parviflora* var. *pentaphylla* were about 2600 bp long. The length variation between the two *Pinus* species was apparent by 1.5% agarose electrophoresis of the uncut PCR products (Fig. 1). Although the PCR products obtained from the two pine species are rather different in length, their homology is

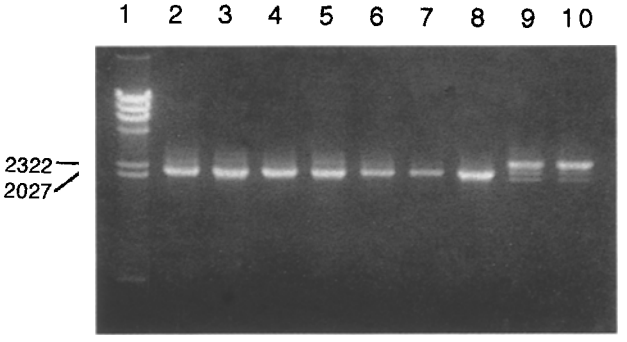

Fig. 1. Amplification of a mtDNA intron from exon B to exon C of the *nad* 1 gene in *Pinus parviflora* var. *pentaphylla* and *P. pumila*. Lane 1, molecular size marker (λ *Hin*d III digest); lanes 2-8, *P. pumila* (A1, A2, A9, B5, B7, B9, and C1, see Watano *et al.* (1995) for sources of the samples); Lanes 9 and 10, *P. parviflora* var. *pentaphylla* (D5 and D6). Numbers at left indicate sizes in bases.

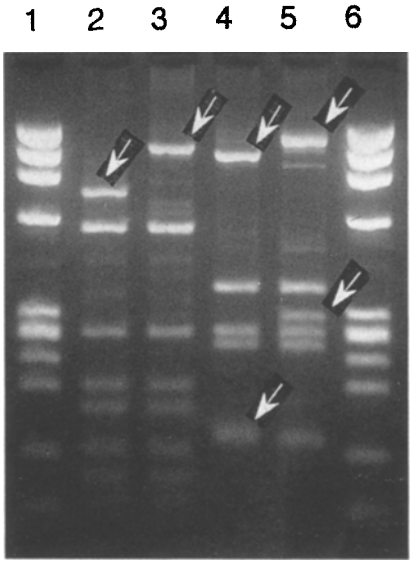

Fig. 2. Restriction endonuclease digestions of mtDNA amplification products from *P. parviflora* var. *pentaphylla* (Lanes 3 and 5) and *P. pumila* (Lanes 2 and 4). Lanes 1 and 6, molecular size marker (ΦX174 *Hae* III digest); Lanes 2 and 3, *Sau* 3AI digest; Lanes 4 and 5, *Rsa* I digest. Arrows indicate the digested fragments showing size variation between the two pine species.

apparent from the band patterns of the samples digested with restriction enzymes (Fig. 2). In the case of *Sau*3AI digest (Lanes 2 and 3), eight fragments were resolved for both species, and length variation was observed for one fragment pair. As for *Rsa*I digest (Lanes 4 and 5), six fragments were detected for both species, and two fragment pairs differed in length. Therefore, at least two insertion/deletion events cause the length difference between the PCR products of *P. pumila* and *P. parviflora* var. *pentaphylla*. Intraspecific variation of the *nad*1 intron was not observed.

Figure 3 shows sampling sites for the 35 individuals examined in the Tanigawa Mountains, as well as their mtDNA haplotypes defined by the length variation of the *nad*1 intron. CpDNA haplotypes (Watano *et al.* 1995), estimated from the same DNA samples that we used for the mtDNA analyses, are also shown in Fig. 3. Plants of W30 to W35 below the altitude of 1,500 m had an erect tree habit typical of *P. parviflora* var. *pentaphylla*. Their mtDNA and cpDNA haplotypes were the same as those of *P. parviflora* var. *pentaphylla*. The other 29 plants were all creeping shrubs like the typical plants of *P. pumila*. However, most plants (23/29) were chimera of cytoplasmic genomes: mtDNA of *P. pumila* type and cpDNA of *P. parviflora* type. Four plants (W11, W14, W42, and W43) growing near the peaks of Mt. Asahidake and Mt. Tanigawadake had the same mtDNA and cpDNA haplotypes as *P. pumila*. The last two (W21, W25) on the southward ridge of Mt. Kasagatake showed the same mtDNA and cpDNA haplotypes as *P. parviflora* var. *pentaphylla*.

Three different shoots were collected for each individual of W1 to W5 and W7 to W25 to test for mtDNA variation within individuals. However, no heteroplasmic individuals

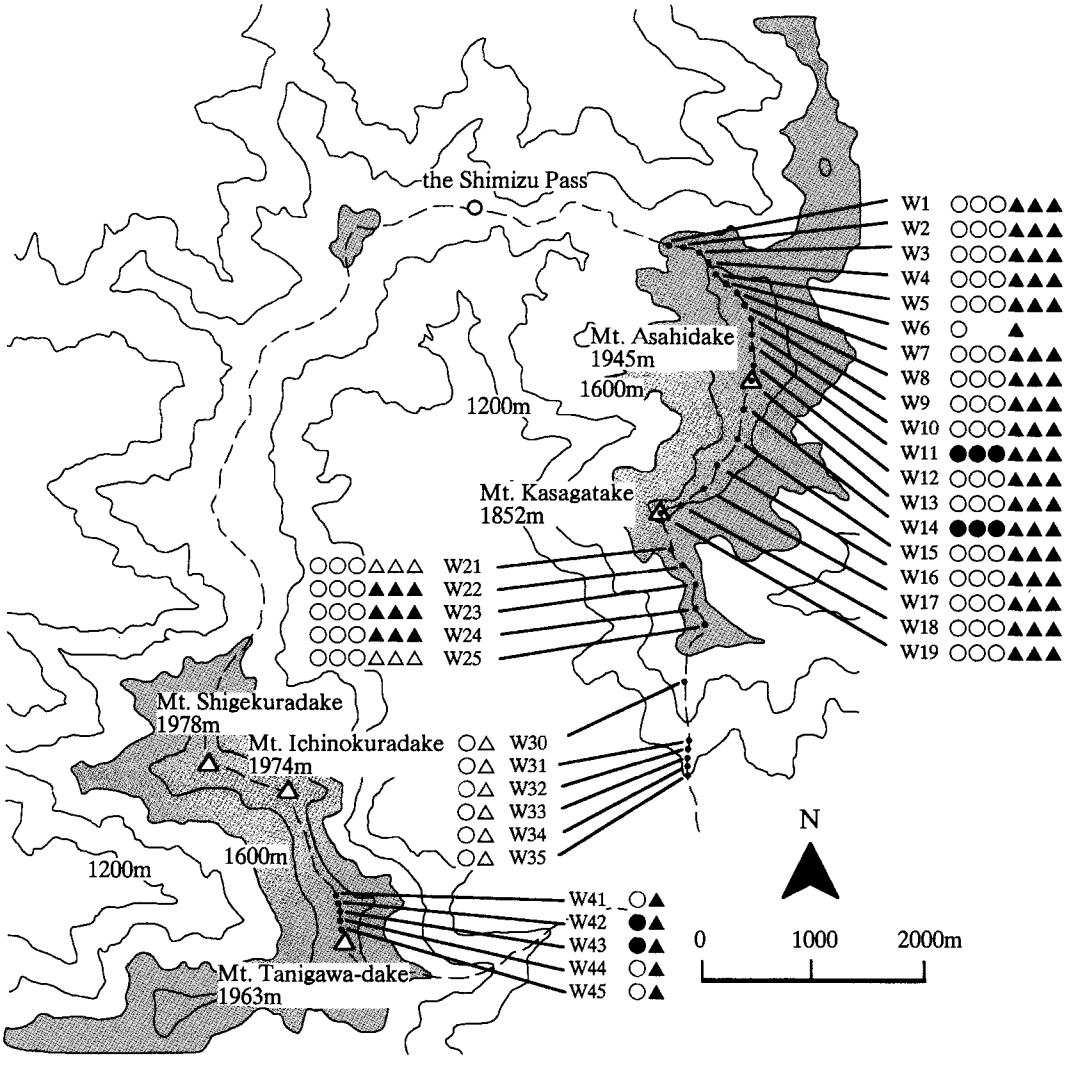

Fig. 3. Sampling sites of five-needle pines, and their cpDNA and mtDNA haplotypes in the Tanigawa Mountains. Open circles and triangles show the cpDNA and mtDNA haplotypes of *Pinus parviflora* var. *pentaphylla*, respectively. Closed circles and triangles indicate the cpDNA and mtDNA haplotypes of *P. pumila*, respectively. Number of circles or triangles indicates number of shoots sampled per individual.

were detected.

Voucher specimens were made for parts (18/35) of the samples of the Tanigawa Mountains. The anatomical character of their needles has been examined in order to classify the plants into three categories: *P. pumila*, *P. parviflora* var. *pentaphylla*, and morphological intermediates (Watano *et al.* 1995). Needle types, mtDNA and cpDNA haplotypes of the plants were listed in Table 1 in the order of the altitude of each collection site. *Pinus parviflora* var. *pentaphylla* were distributed at a lower altitudinal range below 1700 m, *P. pumila* at a higher altitudinal range above 1730 m, and the morphological intermediate plants at a middle range having overlaps with those of *P. parviflora* var. *pentaphylla* and *P. pumila*. The spatial distribution patterns of cpDNA and mtDNA haplotypes were clearly different. In the case of the cpDNA haplotype, that of *P. parviflora* var. *pentaphylla* predominated, while that of *P. pumila* were found only near mountain peaks. Contrastingly, mtDNA haplotype of *P. parviflora* var. *pentaphylla* were detected only at the lowest range, and that of *P. pumila* predominated.

Discussion

Plant mtDNAs are more rarely used for systematic studies

Table 1. Morphological characters, cpDNA and mtDNA haplotypes of samples collected from the Tanigawa Mountains

Sample No.	Altitude (m)	Habit	Needle type	CpDNA haplotype	MtDNA haplotype
W30	1500	tree	par.	par.	par.
W1	1610	shrub	par.	par.	**pum.**
W2	1665	shrub	*int.*	par.	**pum.**
W22	1675	shrub	par.	par.	**pum.**
W21	1700	shrub	par.	par.	par.
W3	1730	shrub	**pum.**	par.	**pum.**
W4	1790	shrub	*int.*	par.	**pum.**
W5	1850	shrub	**pum.**	par.	**pum.**
W19	1850	shrub	**pum.**	par.	**pum.**
W6	1890	shrub	**pum.**	par.	**pum.**
W15	1900	shrub	*int.*	par.	**pum.**
W7	1920	shrub	*int.*	par.	**pum.**
W16	1935	shrub	*int.*	par.	**pum.**
W10	1935	shrub	**pum.**	par.	**pum.**
W11	1940	shrub	**pum.**	**pum.**	**pum.**
W14	1945	shrub	**pum.**	**pum.**	**pum.**
W43	1960	shrub	**pum.**	**pum.**	**pum.**
W45	1960	shrub	**pum.**	par.	**pum.**

Samples were listed in the order of alltitude. Abbreviations: par., *p. parviflora* type; *int.*, intermediate type; **pum.** *p. pumila* type.

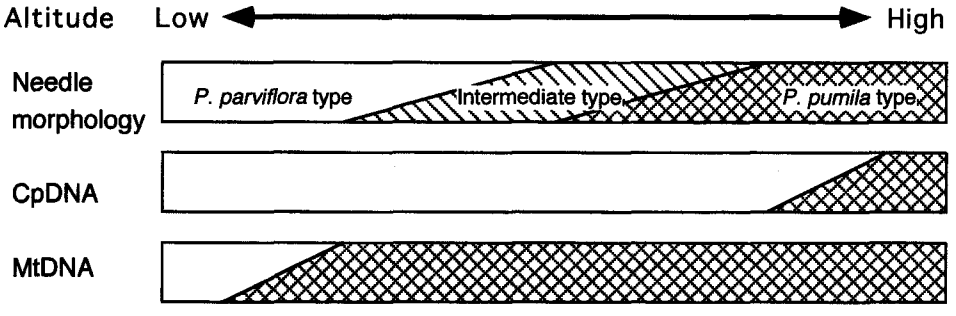

Fig. 4. Schematic representation of the relationship among altitude, needle morphology, cpDNA and mtDNA haplotypes in the Tanigawa mountains. The cpDNA haplotype of *P. parviflora* var. *pentaphylla* permeates into *P. pumila*, while the mtDNA haplotype of *P. pumila* permeates into *P. parviflora* var. *pentaphylla*.

than cpDNAs. Unlike chloroplasts, plant mtDNAs are characterized by an abundance of short dispersed repeats and high rates of structural rearrangement (Palmer 1992). These features, combined with low rates of point mutations, limit the applicability for the restriction site-based reconstructions of phylogeny, for which cpDNA is so well-studied. Although the complex nature of mtDNA variation has limited power for phylogenetic reconstruction, it may nonetheless be a useful tool for rapid surveys and classifications of genome types at a lower taxonomic level (Palmer 1992, Strauss *et al.* 1993). In the present study, PCR amplifications of a non-coding region of mtDNA were undertaken in order to obtain species-specific genetic maker of mitochondria. This may be the most rapid method for obtaining mtDNA genetic markers. In certain instances, however, we should pay attention to this method, because the movements of mtDNA segments to the nucleus have been reported in certain legumes (Palmer 1992) and an ecotype of *Arabidopsis* (Sun and Callis 1993). In the present study, however, PCRs using universal primers for an intron of mitochondrial gene *nad*1 (Demesure *et al.* 1995) generated a single band of 2200 bp or a single band of 2600 bp in all samples including representative individuals of *P. pumila* and *P. parviflora* var. *pentaphylla*, and morphologically intermediate individuals. Heterozygous band pattern, as would be expected for nuclear DNA, was never observed. This argues that our PCR products are mtDNA fragments, and not of nuclear DNA origin.

CpDNA and mtDNA molecular markers specific to parental species clarified that plants derived from hybridization occupied wide altitudinal ranges from 1610 (W1) to 1960 m (W45) in the Tanigawa Mountains (Fig. 3, Table 1). Although the number of plants classified as morphological intermediates was relatively small, alien cytoplasmic genomes were found, both in plants classified as *P. pumila* and as *P. parviflora* var. *pentaphylla*. The most striking point is that hybridization is supposed to have occurred strictly directionally; only one type of cytoplasmic chimera (cpDNA of *P. parviflora* type and mtDNA of *P. pumila* type) occurred in the Tanigawa Mountains. This suggests that *P. parviflora* var. *pentaphylla* always behaved as male, and *P. pumila* as female in the formation of this hybrid zone.

The relationship among altitude, needle morphology, cpDNA and mtDNA haplotypes are schematically shown in Fig. 4. The spatial-distribution pattern of cpDNA haplotypes suggested that cpDNA introgression had occurred uni-directionally from *P. parviflora* var. *pentaphylla* to *P. pumila*. Conversely, mtDNA introgression seems to have occurred in the opposite direction, from *P. pumila* to *P. parviflora* var. *pentaphylla*. Levels of introgression are roughly equivalent for cpDNA and mtDNA; alien cytoplasmic genomes were detected in six of nine plants classified as *P. pumila*, and in two of four plants of *P. parviflora* var. *pentaphylla*.

In *Pinus*, cpDNA is paternally inherited, and hence migrates through pollen, while maternally inherited mtDNA migrates through seeds, but not pollen (Neale and Sederoff 1989). The differential contribution of seeds and pollen to interspecific genetic exchange, therefore, could lead to the distinct pattern of introgression of cpDNA and mtDNA. As for the hybrid zone in the Tanigawa Mountains, it remains a difficult question whether seeds or pollen contributed more to interspecific gene flow. This is because the present distribution can be explained by predominant uni-directional pollen flow and also by predominant uni-directional seeds flow.

The present study revealed contrastive spatial-distribution patterns of cpDNA and mtDNA haplotypes and suggested differential movement of seeds and pollen for the formation of the hybrid zone between the two pine species. However, the mechanistic aspect of the observed phenomenon remains unresolved. Which pecies is the more likely maternal parent in crosses ? Is there pollen competition between the two species? Are hybrids with different maternal parents equally fertile? Artificial-crossing experiments designed to answer the above questions will be essential for further understanding of the hybrid zone between the two pine species.

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