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GENETIC STRUCTURE OF HYBRID ZONES BETWEEN *PINUS PUMILA* AND *P. PARVIFLORA* VAR. *PENTAPHYLLA* (PINACEAE) REVEALED BY MOLECULAR HYBRID INDEX ANALYSIS[1]

YASUYUKI WATANO,[2,4] AKIKO KANAI,[2] AND NAOKI TANI[3]

[2]Department of Biology, Faculty of Science, Chiba University, Yayoi, Inage, Chiba 263-8522, Japan; and [3]Department of Forest Genetics, Forestry and Forest Products Research Institute, Kukizaki, Ibaraki 305-8687, Japan

Pinus species have three differently inherited genomes: paternal chloroplast, maternal mitochondrial, and biparental nuclear. Our previous study on the hybrid zones between alpine *Pinus pumila* and montane to subalpine *P. parviflora* var. *pentaphylla* demonstrated contrasting patterns of introgression of two cytoplasmic genomes, i.e., the paternal cpDNA flowed from *P. parviflora* var. *pentaphylla* to *P. pumila*, and the maternal mtDNA flowed in the reverse direction. In the present study, we developed codominant nuclear DNA markers diagnostic or mostly diagnostic for each parental species by single-strand conformation polymorphism (SSCP) of polymerase chain reaction (PCR) products, using expressed sequence tag (EST) primers of *Pinus taeda*. To describe the introgressive patterns of the nuclear genes, the molecular hybrid index (MHI) showing the overall proportion of alleles inferred to be derived from *P. pumila* was determined for each plant collected in hybrid zones on Mt. Asahidake and Mt. Higashiazuma, Japan. At Mt. Asahidake, the MHI values changed clinally according to the altitudes at which the plants were collected. However, at Mt. Higashiazuma, there was a gap in the MHI values between the plants above and below the *Abies* and *Tsuga* forest zone (alt. 1800–1900 m). This suggested that the zone plays a role in creating an effective barrier to gene flow in the hybrid zone.

Key words: EST primers; hybrid index; hybrid zone; introgression; Japan; Pinaceae; *Pinus*; SSCP.

Biological diversification over time has been portrayed as a series of dichotomous branching events. However, natural hybridization among species is a widespread phenomenon in plants, and many plant species seem to be occasionally interconnected by a limited gene exchange through space and time (Rattenbury, 1962; Grant, 1981). This biological perspective on plant ''species'' has been emphasized by recent investigations using molecular markers, particularly abundant examples of chloroplast DNA capture through introgressive hybridization (Rieseberg and Wendel, 1993; Rieseberg, 1995; Arnold, 1997).

Hybrid zones between *Pinus pumila* (Pallas) Regel and *P. parviflora* Siebold et Zucc. var. *pentaphylla* (Mayr) Henry have several interesting characteristics suitable for the study of interspecific genetic exchange and its evolutionary significance. First, *Pinus* species exhibit a paternal chloroplast inheritance and a maternal mitochondrial inheritance (Neale and Sederoff, 1989; Mogensen, 1996). Therefore, two cytoplasmic genomes can transgress independently across species boundaries. In fact, previous papers on the hybrid zones between *P. pumila* and *P. parviflora* var. *pentaphylla* indicated that the paternal chloroplast DNA (cpDNA) flowed from *P. parviflora* var. *pentaphylla* to *P. pumila*, and, in contrast, the maternal mitochondrial DNA (mtDNA) flowed from *P. pumila* to *P. parviflora* var. *pentaphylla* (Watano et al., 1995, 1996; Senjo et al., 1999). The independent introgression of the cpDNA and mtDNA provides a unique opportunity to study the relationship among modes of inheritance and the levels and patterns of interspecific gene flow. Two distinct means of genetic movement, pollen and seed, are employed in seed plants. The paternal cpDNA is transmitted by both pollen and seed, and in contrast, the maternal mtDNA is carried only by seed. Therefore, the patterns of introgression of the cpDNA and mtDNA can be also viewed as a window to assess pollen- and seed-mediated gene flow across species boundary. Second, several hybrid zones in Japan seem to have formed independently. *Pinus pumila* is a creeping shrub, which dominates the vegetation zone above the forest limit of the high mountains in Japan, while *P. parviflora* var. *pentaphylla* is a tall tree in subalpine to montane zones. Hybrid zones are observed in the ecotonal zone of the two species in several mountains. Because the populations of *P. pumila* have an island-like geographic distribution due to the alpine habitat of the species, hybrid zones in different mountain regions seems to have been formed independently. A comparison of independent hybrid zones will allow us to elucidate the necessary genetic consequence of hybridization. Finally, the genomics project for *Pinus taeda* L. is ongoing (Brown et al., 2001; Temesgen et al., 2001), and the database of expressed sequence tags (ESTs) is available to the public (http://dendrome.ucdavis.edu/). The ESTs developed in *P. taeda* are also available in other pine species (Brown et al., 2001).

In the previous studies on the introgressive patterns of cpDNA and mtDNA (Watano et al., 1995, 1996; Senjo et al., 1999), we examined cpDNA and mtDNA haplotypes and morphology in each individual sampled and then assessed the direction and level of cpDNA and mtDNA introgressions based on morphological criteria. Although *P. pumila* and *P. parviflora* var. *pentaphylla* can be discriminated by many morphological characters such as cones, seeds, and needle anatomy (Isii, 1941), the previous studies used only needle anatomical

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[4] E-mail: watano@faculty.chiba-u.jp.

characters. This is because plants that have cones with seeds are relatively rare in nature. In the present study, we developed codominant nuclear markers, then elucidated the genetic structure of the hybrid zones by estimating the molecular hybrid index (MHI) for each individual. The MHI measures are much more unbiased criteria for the genetic makeup of hybrids than the morphological one with limited characters (Carney et al., 2000). The combination of the MHI and cytoplasmic genome composition allowed us to compare the introgressive patterns of three independently inherited genomes: the biparental nuclear, paternal chloroplast, and maternal mitochondrial DNAs.

To develop nuclear DNA markers in our materials, we used polymerase chain reaction (PCR) primers developed from *P. taeda* ESTs (Temesgen et al., 2001). First, we tested the cross-amplification of *P. taeda* PCR primers in *P. pumila* and *P. parviflora* var. *pentaphylla*, which belong to a different subgenus from *P. taeda* (Price et al., 1998). Next, the allelic variation of the amplified DNA fragments was analyzed using the single-strand conformation polymorphism (SSCP) method. The PCR-SSCP of nuclear DNAs generates codominant markers (Bodenes et al., 1996; Ishikawa et al., 2002). Diagnostic or mostly diagnostic markers of *P. pumila* and *P. parviflora* var. *pentaphylla* were selected, and the multilocus genotype of each sample were determined. Finally, the genetic structure of the hybrid zones was described by comparing the MHI of each sample to previous data from the cytoplasmic genomes.

MATERIALS AND METHODS

Plant materials—Two hybrid zones, Mt. Asahidake (in the Tanigawa Mountains) and Mt. Higashiazuma (in the Ohu Mountains), were examined in this study. Mt. Asahidake is located at the border of Niigata and Gunma Prefectures, central Honshu, Japan. A total of 45 individuals collected at altitudes from 874 m to 1945 m were examined. Samples W1–W25 were the same as those used by Watano et al. (1995, 1996). Samples TA1–TA49 were newly collected in 1998. Mt. Higashiazuma is located in the southern part of the Ohu Mountains, which form the backbone of northern Honshu. A total of 69 individuals collected from 1220 m to 1974 m in elevation were examined. The samples from Mt. Higashiazuma were the same as those used by Senjo et al. (1999). Control samples of pure parental species were collected from the following four locations: (1) Mt. Hakusan, at the border between Ishikawa and Gifu Prefectures, Japan (*P. pumila*), (2) Mt. Norikuradake, at the border between Nagano and Gifu Prefectures, Japan (*P. pumila*), (3) Shiramine, Ishikawa Prefecture, Japan (*P. parviflora* var. *pentaphylla*), and (4) Moriyoshiyama, Akita Prefecture, Japan (*P. parviflora* var. *pentaphylla*). Vouchers were deposited at the Herbarium of Kanazawa University (KANA) for samples from Mt. Asahidake, Mt. Higashiazuma, Shiramine, and Moriyoshiyama. No vouchers were collected for the samples from Mt. Hakusan and Mt. Norikuradake.

Development of diagnostic nuclear markers by PCR-SSCP—DNA from each plant was extracted from fresh leaves by the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). To test cross-amplification of the *P. taeda* primers developed by Temesgen et al. (2001) in our materials, we carried out PCR amplifications using 93 primer sets with 12 DNA templates (six *P. pumila* and six *P. parviflora* var. *pentaphylla*). The PCR reaction was conducted in a total volume of 25 μL containing 25 ng of template DNA, 0.2 μmol/L of each primer, 0.2 mmol/L of each dNTP, 1× PCR buffer, 1.5 mmol/L $MgCl_2$, and 0.625 unit of TaKaRa ExTaq DNA polymerase (TaKaRa Bio., Tokyo, Japan). The thermal profile for PCR was as follows: initial 3 min denaturation at 95°C, three cycles at 95°C for 1 min, 58°C for 1 min, 72°C for 2 min, followed by three cycles at 95°C for 1 min, 55°C for 1 min, 72°C for 2 min, followed by 34 cycles at 95°C for 45 s, 52°C for 45 s, 72°C for 1 min 30 s, and a final extension at 72°C for 10 min. The samples obtained from the PCR reaction were resolved by 2% agarose gel, and the primer sets that generated the expected size of DNA fragments with no artificial bands were screened. The sequence variation of PCR products amplified in *P. pumila* and *P. parviflora* var. *pentaphylla* were then analyzed by SSCP. A portion of PCR sample (1 μL) was mixed with 19 μL of formamide-dye solution (90% formamide, 0.005% bromophenol blue, 8% glycerol), and then denatured for 3 min at 95°C. The denatured samples were cooled on ice, and 4 μL of the sample was loaded on a nondenatured 50% MDE gel (TaKaRa Bio., Shiga, Japan) with dimensions of 135 mm wide, 130 mm long, and 0.75 mm thick. Electrophoresis was carried out at two electrophoretic conditions (gels with 2% and 5% glycerol) at 20°C in 50% TBE (50 mmol/L Tris, 41.5 mmol/L boric acid, and 1 mmol/L EDTA-2Na) using an electrophoretic apparatus with a thermostat-controlled cooled water circulator (AE-6290, ATTO, Tokyo, Japan). The DNA bands were visualized using a DNA Silver Staining Kit (Amersham Biosciences, Piscataway, New Jersey, USA). Finally, we selected primer sets for which the PCR products had diagnostic SSCP band patterns for *P. pumila* and *P. parviflora* var. *pentaphylla*.

Molecular hybrid index—Some of the marker loci used in this study were not perfectly diagnostic. To calculate the hybrid index of each plant, therefore, we employed the maximum-likelihood method of Rieseberg et al. (1998). Here, we hypothesize that a hybrid individual has a hybrid index h, which represents the overall proportion of alleles in the hybrid individual inferred to be derived from *P. pumila*. Under this hypothesis, the probability that the hybrid individual has an allele x is

$$\Pr(x|h) = hu_x + (1 - h)a_x$$

where u_x and a_x are the frequencies of that allele in the *P. pumila* and *P. parviflora* var. *pentaphylla* populations, respectively. Therefore, the likelihood function for h ($h = 0$ to 1) of a hybrid individual is calculated as the sum of the log probability over each of the individual's alleles as,

$$\mathrm{L}(h) = \sum_{i=1}^{2n} \log(hu_{xi} + (1 - h)a_{xi})$$

where n is the number of loci examined. We can find the maximum likelihood estimate (MLE) for h, which gives the maximum value of L(h). The significance of hybridity for each individual was tested by the two-unit support level: $\mathrm{L}(0) < \mathrm{L}(\mathrm{MLE}) - 2 > \mathrm{L}(1)$. Calculations were made using Mathematica 4.1 (Wolfram Research, Champaign, Illinois, USA). Some alleles were found only in hybrid zones and not in the control populations of parental species. These orphan alleles were assigned minimum frequencies (1/[1 + 2× number of samples examined]) in the parental populations for the purposes of calculating the MLEs of the hybrid index. This hypothesized that the allele in question would have been observed in the control population if one more allele had been sampled (Banks and Eichert, 2000).

RESULTS

Codominant nuclear markers—Among the 93 EST primer sets examined (Temesgen et al., 2001), 36 primer sets (39%) generated clear single-band patterns in 2% agarose gel from template DNAs of *P. pumila* and *P. parviflora* var. *pentaphylla*. In subsequent SSCP analyses of these PCR products, the PCR products of 25 primer sets generated interpretable SSCP band patterns, but those of the other 11 primer sets produced complex band patterns, which could be from amplification of multiple genomic regions. Finally, the PCR products of 10 primer sets showed diagnostic SSCP band patterns for each parental species. These were PtIFG_0464, 0893, 1454, 2009, 2615, 8728, 9008, 9076, 9098, and 9157 (Temesgen et al., 2001). The eight markers except PtIFG_8728 and 9098 were also selected as anchored reference loci, which allowed the synthesis of genetic maps from different *Pinus* species by Brown et al. (2001). Among the 10 markers, PtIFG_2009 and 9157 were not used for further analyses in the present study because too many alleles were detected. The electrophoretic

TABLE 1. Primer sequences, locations marked on the *Pinus taeda* linkage map, and electrophoretic conditions of eight nuclear markers used for the hybrid index estimates in the hybrid zones between *P. pumila* and *P. parviflora* var. *pentaphylla*.

Locus	Primer sequences (5′–3′)	Linkage group/cM	Electrophoretic condition in SSCP[a] analysis
PtIFG_0464	TGT CAC TGC CCA GAG CTA TTC ATC ACA GCC GCT CCA AAA C	2/58	Digestion by *Hin*dIII[b], 2% glycerol, 20°C, 300 V, 7 h
PtIFG_0893	GGA CTG AAG GGA TCT AGC TGG CAG CCC AAA TTC CAT CGT C	5/41.3	2% glycerol, 20°C, 350 V, 8 h
PtIFG_1454	ACA TCA ATC AAG TTG GCC TTG ACG ACC ATC TTC AAC CAC TC	5/75.9	2% glycerol, 20°C, 300 V, 7 h
PtIFG_2615	CAC TCT TTA TTC TTG CCC TTC G TCG GTT AGG TAA CGA CTG GAC	11/49.5	10% glycerol, 20°C, 300 V, 10 h
PtIFG_8728	CCA AAG CCC AAA TCC ATG CCA ATT TGC ACT TTG CCC	no info.[c]	2% glycerol, 20°C, 300 V, 6 h
PtIFG_9008	GGT AAA CTG GGA TGG ATT GC[d] TCT CGG ATA GGG CAA TAT GC	5/71.5	2% glycerol, 20°C, 300 V, 8 h
PtIFG_9076	AGA ATT TAC TGG CCG CTC G CTC TAT TGC AAA AAT GTG CCA C	11/39.1	10% glycerol, 20°C, 300 V, 7 h
PtIFG_9098	GTG GGC TTG CTA TAA ATG C[d] AGT GCA TCG TTC ACA ATT CTC	no info.[c]	2% glycerol, 20°C, 300 V, 5 h

[a] SSCP = Single-strand conformation polymorphism.
[b] PCR products of PtIFG_0464 were digested by restriction enzyme *Hin*dIII, and genotyping was carried out based on the band pattern of a larger DNA fragment.
[c] Map locations were not examined because of the complex DGGE (denatured gradient gel electrophoresis) band pattern (8728) and lack of polymorphism (9098) in *P. taeda* (Temesgen et al., 2001).
[d] Original primer sequence of Temesgen et al. (2001) has a GC-clump for DGGE analysis. The GC-clump was omitted in the present study.

conditions for the other eight markers were improved using some control samples (Table 1). The representative SSCP band patterns of the markers under the optimal electrophoretic conditions (Table 1) are shown with their assumed genotypes in Fig. 1.

Table 2 shows the estimated allele frequencies for pooled samples from two *P. pumila* populations (Mt. Hakusan and Mt. Norikuradake) and two *P. parviflora* var. *pentaphylla* populations (Shiramine and Moriyoshiyama). Among the alleles observed in hybrid zones, only three (*a2* at PtIFG_8724, *a3* at PtIFG_1454, and *a3* at PtIFG_0464) were found to be not represented in the pure parental populations. Frequencies of these ''orphan'' alleles were also assigned in both species, for the purpose of calculating a maximum likelihood estimate of hybrid index.

A larger scale examination than the initial screening with six individuals in each parental taxa revealed that the marker alleles of either *P. pumila* or *P. parviflora* var. *pentaphylla* were shared at low frequencies by the other parental species, except PtIFG_2615 and 8728 (Table 2). Because of the sharing of some marker alleles, simple additive estimates of the hybrid index in the pure parental populations varied from 0.857 to 1 in *P. pumila* and from 0 to 0.071 in *P. parviflora* var. *pentaphylla*. However, the maximum likelihood method effectively narrowed the ranges of the hybrid index: from 0.949 to 1 in *P. pumila* and from 0 to 0.026 in *P. parviflora* var. *pentaphylla*.

Genetic makeup of hybrid zones—Nuclear genotypes at eight marker loci were determined for 46 individuals from Mt. Asahidake and 69 from Mt. Higashiazuma. The multilocus genotypes are shown in Appendix 1 (see Supplemental Data accomanying the online version of this article) together with data on the altitude, horizontal distance from the first sample collected, cytoplasmic haplotypes, and needle morphology. No apparent difference of introgressive patterns was observed among eight marker loci (Appendix 1). The changes in the MLEs of the hybrid index and in the haplotypes of the cpDNA and mtDNA along the sampling routes are illustrated in Fig. 2. Because the MHI represents the overall proportion of alleles in the hybrid individual inferred to be derived from *P. pumila*, a high MHI value corresponds to a *P. pumila*-like plant and a low MHI to a *P. parviflora*-like plant.

At Mt. Asahidake (Fig. 2a), the distribution of five-needle pines was separated by a gap between sample numbers TA45 to TA47: 385 m in elevation and 1015 m in distance. The individuals below the gap were nearly pure ''*P. parviflora* var. *pentaphylla*.'' Immediately above the gap, however, the values of the hybrid index started to increase according to the change in elevation. On a plateau (above 1900 m), the values of hybrid index varied from 0.585 to 0.938 with a mean value of 0.753. Only in individuals W11 and W18 were the values of hybrid index 1 or not significantly deviated from 1. Although W11 had *P. pumila*-type cpDNA and mtDNA, W18 had *P. parviflora*-type cpDNA. These results suggest that it would be difficult to find a pure ''*P. pumila*'' individual on this mountain; even the population at the top contained a considerable portion of *P. parviflora* var. *pentaphylla* genes (approximately 25%).

It is clear that the values of the hybrid index at Mt. Higashiazuma (Fig. 2b) rapidly changed between sample numbers AZ43 (hybrid index = 0.016) and AZ44 (0.875). In this mountain, a gap in the distribution of five-needle pines existed between AZ44 and AZ45: 125 m in altitude and 350 m in distance. This gap was covered with dense forest of *Abies mariesii* Masters and *Tsuga diversifolia* Masters. The values of the hybrid index below the *Abies* and *Tsuga* forest zone varied from 0 to 0.203 with a mean of 0.075 except for AZ44, while the hybrid index above the zone varied from 0.173 to 1 with a mean of 0.945. Only one individual (AZ58) at the top of mountain had a low value of the hybrid index (0.173). That individual was specifically collected because it was the only individual that had an erect stem, which is a key character of *P. parviflora* var. *pentaphylla*.

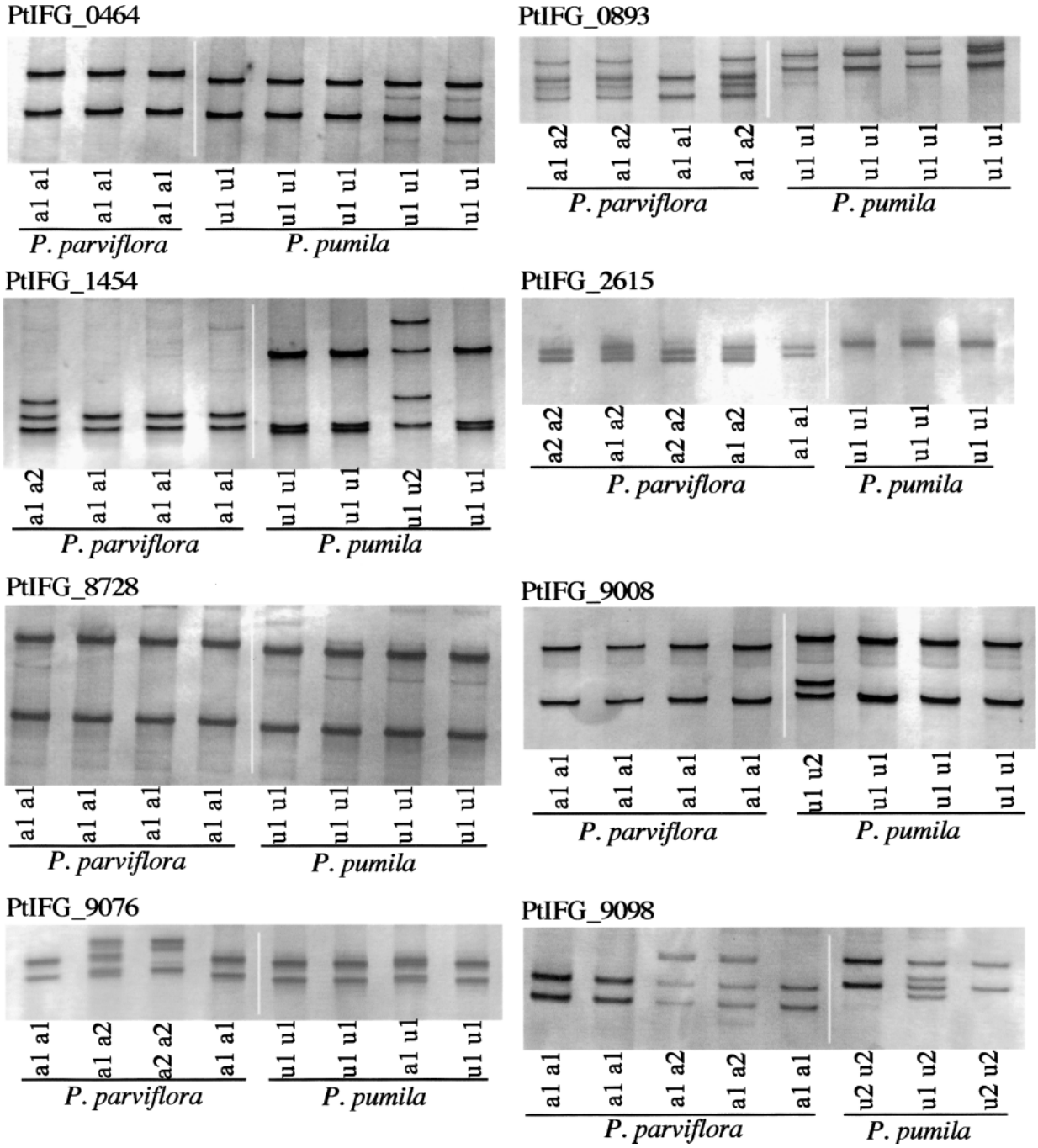

Fig. 1. Species-specific PCR-SSCP band patterns at the eight marker loci used to calculate the hybrid index measures. Samples of *Pinus parviflora* var. *pentaphylla* are from Shiramine and Moriyoshiyama and those of *P. pumila* from Mts. Hakusan and Norikuradake. The interpreted genotype is shown under each lane, and the marker loci are in top left corner.

Comparison of the levels of nuclear introgression with cpDNA and mtDNA introgression—To compare nuclear introgression with cpDNA and mtDNA, we divided samples into several zones mainly based on altitudinal discontinuity and calculated the mean value of the hybrid index and the frequencies of the cpDNA and mtDNA haplotypes in each zone (Table 3). As mentioned in the introduction, the introgression of cytoplasmic genomes are unidirectional in hybrid zones of *P. pumila* and *P. parviflora* var. *pentaphylla*: cpDNA flowed from *P. parviflora* var. *pentaphylla* to *P. pumila* (from the bottom to the top), and, in contrast, mtDNA flowed from *P. pumila* to *P. parviflora* var. *pentaphylla* (from the top to the bottom) (Watano et al., 1996; Senjo et al., 1999).

On Mt. Asahidake, the mean value of the hybrid index in the highest zone (Top) was 0.753, indicating that 25% of the genes were from *P. parviflora* var. *pentaphylla*. The frequency of cpDNA from *P. parviflora* var. *pentaphylla* was 0.8 in this zone. An excess of cpDNA introgression over nuclear introgression was also observed in the Middle 1 and 2 zones. It is clear that the level of cpDNA introgression is higher than the

TABLE 2. Allele frequencies for pooled samples from two *Pinus pumila* populations and from two *P. parviflora* var. *pentaphylla* populations. Alleles diagnostic or mostly diagnostic for *P. parviflora* var. *pentaphylla* and *P. pumila* are denoted by *a* and *u*, respectively.

Locus	N[a]	Allele					
		*a*1	*a*2	*a*3	*a*4	*u*1	*u*2
P. pumila							
PtIFG_0893	20[a]	0.025				0.925	0.050
PtIFG_1454	20	0.024		0.024[b]		0.902	0.049
PtIFG_2615	20					1.000	
PtIFG_9008	20	0.075				0.900	0.025
PtIFG_0464	20			0.024[b]		0.952	0.024
PtIFG_9076	20	0.175				0.825	
PtIFG_8728	20		0.024[b]			0.927	0.049
PtIFG_9098	18					0.222	0.778
P. parviflora var. *pentaphylla*							
PtIFG_0893	31	0.742	0.258				
PtIFG_1454	31	0.873	0.111	0.016[b]			
PtIFG_2615	31	0.774	0.193	0.032			
PtIFG_9008	30	0.950	0.050				
PtIFG_0464	31	0.905	0.016	0.016[b]	0.016	0.047	
PtIFG_9076	31	0.694	0.306				
PtIFG_8728	30	0.967	0.016[b]	0.017			
PtIFG_9098	24	0.729	0.250			0.021	

[a] N = number of samples examined.
[b] Frequencies of the alleles found only in hybrid zones (''orphan'' alleles).

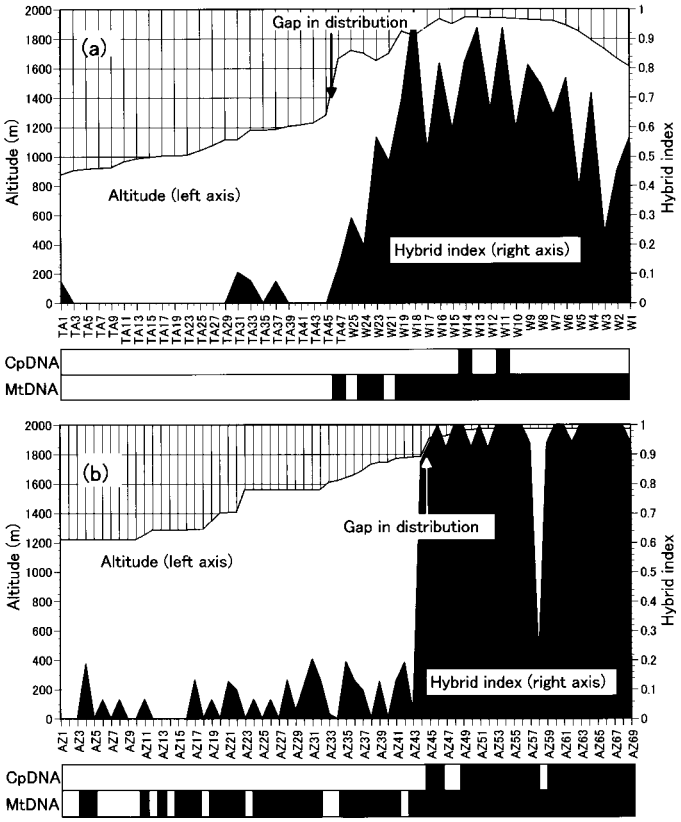

Fig. 2. Genetic structure in hybrid zones of *Pinus parviflora* var. *pentaphylla* and *P. pumila* on (a) Mt. Asahidake and (b) Mt. Higashiazuma. Samples are arranged from left to right in the order in which they were collected along the sampling route. The altitude of each sample is shown by the white line graph (left axis) and the value of the hybrid index by the black line graph (right axis). The chloroplast and mitochondrial DNA haplotypes of each sample are indicated under the name of the sample by the white box (*P. parviflora* var. *pentaphylla* type) or black box (*P. pumila* type).

nuclear one on Mt. Asahidake. In contrast to the cpDNA, the introgression of *P. pumila* mtDNA stopped at the gap between TA45 and 47 (between Bottom 2 and Middle 1) and did not penetrate into the lower zones (Bottom 1 and 2).

On Mt. Higashiazuma, in contrast to Mt. Asahidake, the introgression of *P. pumila* mtDNA to the lower zones was prominent; the frequencies of the *P. pumila* mtDNA haplotype was much higher than the values of the hybrid index in Bottom 1 and 2. The level of introgression of *P. parviflora* var. *pentaphylla* cpDNA to the highest zone (Top) was low (0.120) and was nearly identical to the level of the nuclear portion of *P. parviflora* var. *pentaphylla* ($0.055 = 1 - 0.945$).

Molecular hybrid index and needle morphology—Watano et al. (1995) and Senjo et al. (1999) classified each sample into three categories, the *P. pumila* type, the *P. parviflora* type, and the intermediate type, based on the anatomical characters of needles. The characters used were (1) the number and position of resin canals, (2) the presence or absence of sclerenchymatous cells above and below a fibrovascular bundle, and (3) the presence or absence of idioblasts. Table 4 summarizes the relationship among morphological categories and the hybrid index.

The available morphological data for samples from Mt. Asahidake was limited to the part of the samples named W** (Watano et al., 1995). Surprisingly, the mean values for the hybrid index of the three morphological categories for Mt. Asahidake did not differ significantly ($P = 0.72$).

As for Mt. Higashiazuma, morphological data were available for all samples (Senjo et al., 1999). Sixteen intermediate types were classified into two different types. The type with an intermediate resin canal arrangement had low values of hybrid index with the mean of 0.084, which did not significantly deviate from the mean value (0.051) of the *P. parviflora* type ($P = 0.17$). All samples of this type, except AZ58, were below the gap of distribution between AZ44 and AZ45 (Fig. 1b). The other intermediate type had high values of the hybrid

TABLE 3. The mean values of molecular hybrid indexes and frequencies of the cpDNA and mtDNA haplotypes in the altitudinal zones of Mt. Asahidake and Mt. Higashiazuma.

Zone	Sample	Sample size	Mean altitude (m)	Hybrid index	Frequency of cpDNA haplotype of *Pinus parviflora* var. *pentaphylla*	Frequency of mtDNA haplotype of *Pinus pumila*
Mt. Asahidake						
Bottom 1	TA1–15	8	935	0.009	1.000	0.000
Bottom 2	TA17–45	14	1130	0.032	1.000	0.000
Middle 1	TA47–W17	8	1748	0.482	1.000	0.750
Top	W16–W7	10	1932	0.753	0.800	1.000
Middle 2	W6–W1	6	1765	0.517	1.000	1.000
Mt. Higashiazuma						
Bottom 1	AZ1–11	11	1223	0.035	1.000	0.273
Bottom 2	AZ12–22	11	1324	0.038	1.000	0.727
Middle 1	AZ23–32	10	1560	0.073	1.000	0.800
Middle 2	AZ33–44	12	1713	0.148	1.000	0.833
Top	AZ45–69	25	1965	0.945	0.120	1.000

index, with the mean of 0.950, and all samples except AZ44 grew above the gap.

DISCUSSION

Distribution gap and interspecific gene flow—The hybrid index analysis of the hybrid zone of Mt. Higashiazuma (Fig. 2b) clearly showed that this hybrid zone can be divided into a *P. parviflora*-like (low hybrid index) zone and a *P. pumila*-like (high hybrid index) zone below and above the gap of distribution located in the altitudinal range from 1800 m to 1900 m, respectively. The introgression of cpDNA from *P. parviflora* var. *pentaphylla* was also stopped by this gap. However, the introgression of mtDNA from *P. pumila* transgressed the gap and reached the lowermost region. The gap in the pines was covered with a dense forest of *Abies mariesii* and *Tsuga diversifolia*, which are dominant constituents of the subalpine forest in the northern and middle parts of Honshu, Japan. *Pinus parviflora* var. *pentaphylla* grows mainly on the sharp ridge or cliff sites in subalpine to montane zones and does not become a dominant constituent of the subalpine coniferous forest. Pines generally have lower tolerance to shade than other conifers such as *Abies*, *Picea*, and *Tsuga* (Rundel and Yoder, 1998), thus possibly, ecological interactions between pines and the dominant species of the subalpine coniferous forest result in the formation of the gap in the pine range. Although the gap on Mt. Higashiazuma is only 125 m in elevation and 350 m in distance, it seems to be effective in preventing nuclear and cpDNA gene flow between the pine populations below and above the gap. Unlike the developed subalpine coniferous forest zone on Mt. Higashiazuma, Mt. Asahidake lacks the vegetation zone of the subalpine coniferous forest and broad-leaved deciduous shrubs such as *Quercus crispula* Blume var. *horikawae* H. Ohba and *Sorbus commixta* Hedl. are distributed in place of it (Kaji, 1982). The values of the hybrid index at Mt. Asahidake (Fig. 2a) showed a clinal change that is representative of hybrid zones (Barton and Gale, 1993). In the top zone on Mt. Asahidake, about 25% of the nuclear genes and 80% of the cpDNA was from *P.*

TABLE 4. Categories of needle morphology (Watano et al., 1995; Senjo et al., 1999) and hybrid index.

Category of needle morphology	Sample size	Scl.	Resin	Idi.	Hybrid index
Mt. Asahidake					
Pinus parviflora type	2	+	PAR	−	0.522
Intermediate type					
W2	1	+	INT	+	0.450
W4	1	+	PUM	−	0.715
W15	1	−	PAR	+	0.586
W16	1	−	INT	−	0.817
W7	1	−	PUM	+	0.637
Total	5				0.641
P. pumila type	7	−	PUM	−	0.630
Mt. Higashiazuma					
P. parviflora type	33	+	PAR	−	0.051
Intermediate type					
AZ16, 23, 29, 32, 35, 36, 38, 39, 41, 43, 58	11	+	INT	−	0.084
AZ44, 45, 59, 64, 65	5	+	PUM	−	0.950
Total	16				0.355
P. pumila type	20	−	PUM	−	0.978

Abbreviations: PAR, *Pinus parviflora* type; INT, intermediate type; PUM, *Pinus pumila* type; Scl., presence (+) or absence (−) of sclerenchymatous cells above and below the fibrovascular bandle; Resin, type of arrangement of resin canals; Idi., presence (+) or absence (−) of idioblasts in mesophyll.

parviflora var. *pentaphylla* (Table 3). The contrasting genetic structure of the hybrid zone on Mt. Asahidake to that on Mt. Higashiazuma indirectly indicates the importance of subalpine coniferous forests as a barrier to gene flow in hybrid zones.

Although Mt. Asahidake lacks a subalpine coniferous forest, a gap in the pine distribution exists at the lower region, from 1300 to 1600 m in elevation. Nuclear and mtDNA introgression from *P. pumila* were stopped by the gap, while nuclear and cpDNA introgression from *P. parviflora* var. *pentaphylla* transgressed the gap. It is interesting to note that the effects of the distribution gap on gene flow are different in the two hybrid zones. One possible explanation is the difference in the altitudinal locations of the distribution gaps on these two mountains. The gap at the high elevation (Mt. Higashiazuma) may be more effective in stopping the upward gene flow from *P. parviflora* var. *pentaphylla*, and the gap at the low elevation (Mt. Asahidake) may be a more effective barrier for the downward gene flow from *P. pumila*.

The seeds of *P. pumila* have no wings and are dispersed mainly by the Eurasian Nutcracker (*Nucifraga caryocatactes*) (Kajimoto, 2002). The dispersal of pine seeds by nutcrackers and the range of seed dispersal may be critical to understanding detailed gene flow patterns in hybrid zones. In this respect, it should be noted that only AZ58 had a low hybrid index (0.173) in the top zone of Mt. Higashiazuma. All plants surrounding AZ58 had high hybrid indices over 0.9. Because the hybrid index of the seeds produced by the plants in the top zone should be higher than 0.45, AZ45 cannot be the direct offspring of the plants in the top zone. AZ58 probably originated from seed dispersed from the zones below the gap. This indicates that the transport of pine seeds by nutcrackers may occasionally become a medium for gene flow between the zones above and below the gap. As a whole, however, the contrasting hybrid index values above and below the gap in Mt. Higashiazuma suggest that such occasional seed flow has been ineffective in mixing the two zones.

Nuclear vs. cytoplasmic introgression—The paternal cpDNA had a higher level of introgression than the nuclear DNAs at Mt. Asahidake, while the maternal mtDNA introgression exceeded nuclear introgression at Mt. Higashiazuma (Fig. 2, Table 3). These results indicated that the levels of introgression of the two differently inherited cytoplasmic genomes were largely affected by unique situations on each mountain. As discussed earlier, the nature and the location of the distribution gap in the hybrid zone could be one of the factors determining the introgressive pattern of each genome. In addition to the present situation on each mountain, historical factors should also be considered. An excess of cpDNA introgression over nuclear introgression has been reported in many angiosperm species (Rieseberg and Wendel, 1993). Because cpDNA is maternally inherited in most angiosperms, the examples of extensive cpDNA introgression in angiosperms may correspond to extensive mtDNA introgression, which is the case with Mt. Higashiazuma in our study. Senjo et al. (1999) examined mtDNA haplotypes in many populations throughout the distribution range of *P. parviflora* var. *pentaphylla* and found that the populations that had high frequencies of the mtDNA haplotype of *P. pumila* were clustered within the southern and middle parts of the Ohu Mountains in Tohoku. Mt. Higashiazuma is located in the southernmost position of an extensive mtDNA introgression area. Several authors (Dong and Wagner, 1993; Martinsen et al., 2001) discussed that such extensive introgression of cytoplasmic genomes may reflect ancient introgression, namely, a footprint of the ancient location of the hybrid zone. It is not certain that the ancient introgression hypothesis holds true in our study. However, it may be a good working hypothesis for further studies.

Molecular hybrid index and morphology—Based on the comparison of the hybrid index for categories of needle morphology, needle morphology was a poor indicator for assessing the nuclear genetic constitution of individuals in hybrid zones. For example, on Mt. Asahidake, there were no significant differences in mean values of hybrid index among the three morphological categories for samples above 1600 m in elevation (Table 4). Carney et al. (2000) examined both the morphological and molecular hybrid index in a hybrid population between *Helianthus annuus* and *H. bolanderi*. The hybrid population was divided into western and eastern halves by a central patch of grass. In the eastern half, there was no significant correlation between the morphological and molecular hybrid index measures, and morphologically pure *H. annuus*-like plants contained a considerable portion of *H. bolanderi* genes (approximately 20%). The authors suggested that this might be the result of the ecological selection on hybrids for *H. annuus* morphological traits. Although our morphological data are limited, the situation observed in the hybrid population of *Helianthus* may be very similar to that in the pine hybrid zone on Mt. Asahidake. *Pinus pumila* is adapted to severe environments in the alpine zone. Although the pines of the highest region on Mt. Asahidake had a highly introgressed nuclear genome (25%), they still survive in a ''pure'' *P. pumila* habitat. It is possible that a strong conflict between gene flow and natural selection occurred in the past and may still be ongoing. A detailed description of the morphological and physiological characters of this mountain population is needed.

Summary and conclusions—In this study, we developed codominant nuclear DNA markers diagnostic or mostly diagnostic for *P. pumila* and *P. parviflora* var. *pentaphylla*. The molecular hybrid index measures calculated by these nuclear markers clarified the genetic structure of the hybrid zones on two mountains, Mt. Higashiazuma and Mt. Asahidake. On Mt. Higashiazuma, a *P. parviflora*-like (low hybrid index) zone and a *P. pumila*-like (high hybrid index) zone were clearly recognized below and above the subalpine coniferous forest zone located in the altitudinal range from 1800 to 1900 m. Mt. Asahidake lacks the subalpine coniferous forest, and the hybrid zone at this mountain showed a gradual increase in the hybrid index from the bottom to the top. The contrasting genetic structure of the two mountains suggested that even a small distribution gap (approximately 100 m in elevation) could play a role in creating an effective barrier to nuclear gene flow in the hybrid zone. As for the relationship among inheritance modes of genomes and the levels of interspecific gene flow, the two hybrid zones had different trends. The paternal cpDNA introgression was more extensive than the nuclear introgression on Mt. Asahidake, but not on Mt. Higashiazuma. In contrast, the maternal mtDNA introgression exceeded the nuclear introgression on Mt. Higashiazuma, but not on Mt. Asahidake. It is possible that the level and pattern of introgression of each genome could be affected by unique circumstances on each mountain, such as the size and position of the distribution gap.

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