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DNA fingerprinting study on the intraspecific variation and the origin of *Prunus yedoensis* (Someiyoshino)

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ABSTRACT

In order to investigate the intraspecific variation of *Prunus yedoensis* (Someiyoshino) and interspecific relationship among *P. yedoensis*, *P. lannesiana* (Oshimazakura) and *P. pendula* (Edohigan), DNA fingerprinting study was conducted by using two different kinds of probes, M13 repeat sequence and $(GACA)_4$ synthetic oligonucleotide. In this study, 68 plants of *P. yedoensis* grown in 46 prefectures in Japan were investigated. All the *P. yedoensis* individuals investigated showed the completely same banding pattern, indicating their clonal origin from a single plant. On the other hand, each of *P. lannesiana* and *P. pendula* individuals investigated showed a unique banding pattern, suggesting a considerable amount of genetic variation in these two species. About 90% of bands in DNA fingerprints of *P. yedoensis* were detected in either *P. lannesiana* or *P. pendula*. This result supports the hypothesis that *P. yedoensis* is an interspecific hybrid between *P. lannesiana* and *P. pendula*. From those results, it is concluded that *P. yedoensis* was produced only once through hybridization between *P. lannesiana* and *P. pendula*, and that this particular hybrid plant has been spread vegetatively all over Japan.

1. INTRODUCTION

Repetitive DNA sequences are found at many locations throughout the genome of most of eukaryotes (Tautz and Renz, 1984; Jeffreys et al., 1985a). A repetitive DNA sequence when used as a probe produces a very complex Southern hybridization pattern that is called DNA fingerprint (Jeffreys et al., 1985a). At each locus, the difference in the number of repeat units and the change of restriction sites adjacent to the repeat cluster are two main causes for the presence of numerous allelic forms of DNA fingerprints (Jeffreys et al., 1985a). However, the former seems to be the major cause of this polymorphism (Jeffreys et al.,

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1990). The difference in the number of repeat units may be the results from mitotic or meiotic unequal crossing over, DNA slippage and gene conversion during the replication (Jeffreys et al., 1985a, b). Recently, DNA fingerprinting technique has been applied to a wide variety of organisms; human (Rogstad et al., 1988), dog and cat (Jeffreys and Morton, 1987), bird (Burke and Bruford, 1987), rice (Dallas, 1988), apple (Nybom and Schaal, 1990), Rosaceae (Nybom et al., 1990), and others (Weising et al., 1991b). It has been shown that DNA fingerprinting is a powerful means to detect variations among individuals.

In this study, in order to investigate the intraspecific variation of *Prunus yedoensis* (Someiyoshino), and interspecific relationship among *P. yedoensis*, *P. lannesiana* (Oshimazakura) and *P. pendula* (Edohigan), DNA fingerprinting was carried out. *P. yedoensis* is the most popular and widely cultivated cherry tree in Japan. *P. yedoensis* has been spread all over Japan except Okinawa prefecture, and most of meteorological observatories and stations in Japan make use of this species as the standard tree for "sakura front line" that is forecasting blooming time of cherry flower. One of the purpose of this study was to examine the genetic uniformity among the standard cherry trees. Genetic uniformity of *P. yedoensis* implies that difference in the blooming time is not caused by the genetic variation among the standard trees, and ensures that the sakura front line based on the standard trees reflects the change in climatic conditions such as temperature and day light.

In 1916, Wilson (cited by Iwasaki, 1986) suggested that *P. yedoensis* is the interspecific hybrid between *P. lannesiana* and *P. pendula*, based on the morphological comparison. Takenaka (1963) succeeded in producing artificial hybrids by reciprocal crosses between *P. lannesiana* and *P. pendula*, which are very similar to *P. yedoensis*. His results supported the Wilson's hypothesis experimentally. There are several hypotheses on the origin of *P. yedoensis*. By investigating the intra- and interspecific variation in DNA fingerprints on the above three species, it was expected to obtain some new finding on the origin of *P. yedoensis*.

2. MATERIALS AND METHODS

Plant materials

Fresh leaves of *P. yedoensis* were obtained from individual trees grown in 68 different locations of 46 prefectures in Japan, except for Okinawa. One sample was from the National Institute of Genetics, Mishima, Shizuoka prefecture, and all the rest were from the standard trees used for forecasting blooming time of the cherry flower in 67 meteorological observatories and stations in 46 prefectures. Locations of the standard trees sampled are shown in Fig. 1 and Table 1. Leaf samples of each of *P. lannesiana* and *P. pendula* were obtained from both the National Institute of Genetics and the Botanical Garden, Faculty of Science, University of Tokyo. Leaf sample of an artificial hybrid between *P. lannesiana*

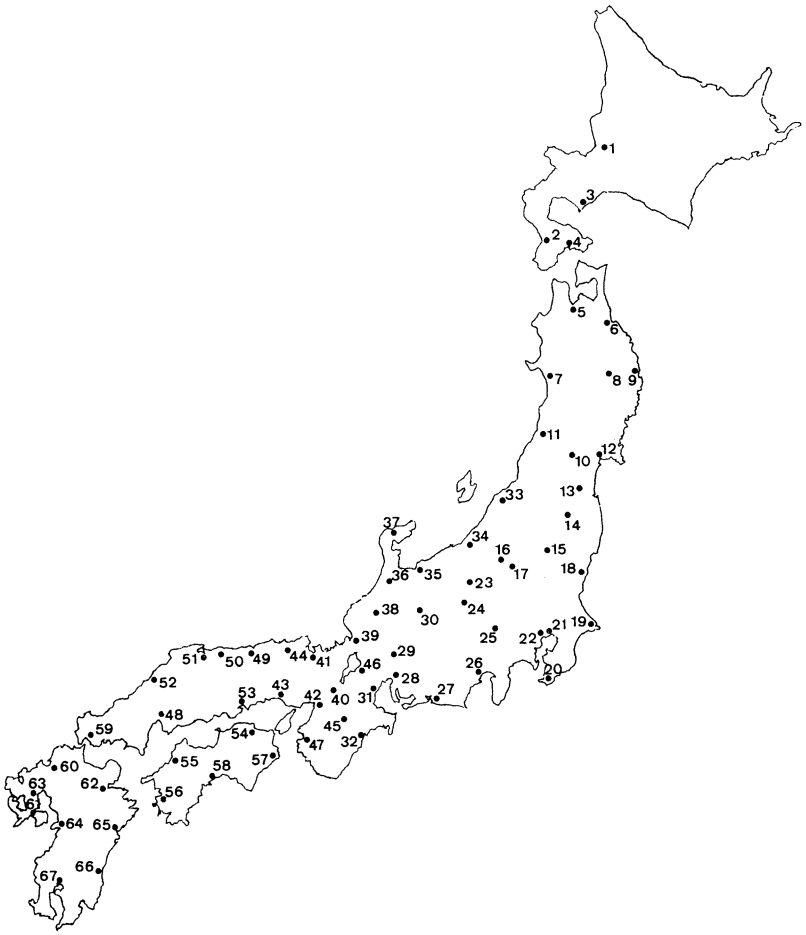

Fig. 1. Locations of the standard trees of *P. yedoensis* investigated in this study. The number corresponds to the location of meteorological observatories and stations listed in Table 1.

and *P. pendula* produced by late Dr. K. Furusato was obtained from the Hamamatsu City Flower Park, Shizuoka prefecture, leaf samples of its maternal parent, *P. lannesiana*, was from the Jungle Park in Minami-izu cho, Shizuoka prefecture and leaf sample of the paternal parent, *P. pendula*, was from the garden of Mr. T. Yamashiro in Kawazu cho, Shizuoka prefecture.

DNA extraction and membrane preparation

Total DNA was extracted by CTAB method according to Weising et al. (1991a). Collected leaves were stored at −80°C until the DNA extraction. About 15 g of leaf tissue was ground into powder with liquid nitrogen using an electric mill. The powder was placed in 60 ml of CTAB buffer (100 mM Tris-Cl, 1.4 M NaCl, 20

Table 1. Meteorological observatories and stations from which leaf samples of *P. yedoensis* were obtained

1	Sapporo DMO, Hokkaido	35	Toyama LMO, Toyama
2	Esashi MS, Hokkaido	36	Kanazawa LMO, Ishikawa
3	Muroran LMO, Hokkaido	37	Wazima MS, Ishikawa
4	Hakodate OMO, Hokkaido	38	Fukui LMO, Fukui
5	Aomori LMO, Aomori	39	Tsuruga MS, Fukui
6	Hachinohe MS, Aomori	40	Kyoto LMO, Kyoto
7	Akita LMO, Akita	41	Maizuru OMO, Kyoto
8	Morioka LMO, Iwate	42	Osaka DMO, Osaka
9	Miyako MS, Iwate	43	Kobe OMO, Hyogo
10	Yamagata LMO, Yamagata	44	Toyooka MS, Hyogo
11	Sakata MS, Yamagata	45	Nara LMO, Nara
12	Sendai DMO, Miyagi	46	Hikone LMO, Shiga
13	Fukushima LMO, Fukushima	47	Wakayama LMO, Wakayama
14	Shirakawa MS, Fukushima	48	Hiroshima LMO, Hiroshima
15	Utsunomiya LMO, Tochigi	49	Tottori LMO, Tottori
16	Maebashi LMO, Gunma	50	Yonago MS, Tottori
17	Kumagaya LMO, Gunma	51	Matsue LMO, Shimane
18	Mito LMO, Ibaraki	52	Hamada MS, Shimane
19	Choushi LMO, Chiba	53	Okayama LMO, Okayama
20	Tateyama MS, Chiba	54	Takamatsu LMO, Kagawa
21	Tokyo DMO, Tokyo	55	Matsuyama LMO, Ehime
22	Yokohama LMO, Kanagawa	56	Uwazima MS, Ehime
23	Nagano LMO, Nagano	57	Tokushima LMO, Tokushima
24	Matsumoto MS, Nagano	58	Kochi LMO, Kochi
25	Kofu LMO, Yamanashi	59	Simonoseki LMO, Yamaguchi
26	Shizuoka LMO, Shizuoka	60	Fukuoka DMO, Fukuoka
27	Hamamatsu MS, Shizuoka	61	Oita LMO, Oita
28	Nagoya LMO, Aichi	62	Nagasaki OMO, Nagasaki
29	Gifu LMO, Gifu	63	Saga LMO, Saga
30	Takayama MS, Gifu	64	Kumamoto LMO, Kumamoto
31	Tsu LMO, Mie	65	Miyazaki LMO, Miyazaki
32	Owase MS, Mie	66	Nobeoka MS, Miyazaki
33	Niigata LMO, Niigata	67	Kagoshima LMO, Kagoshima
34	Takada MS, Niigata		

DMO, OMO and LMO: Direct, Ocean and Local Meteorological Observatory, respectively. MS: Meteorological Station.

mM EDTA, 2% CTAB and 5% mercaptoethanol). After mixing 50 ml of chloroform (24:1 chloroform/isoamylalcohol), the mixture was centrifuged at $4,000 \times g$ for 20 min. To precipitate the total DNA, 40 ml of isopropanol was added to the aqueous phase and centrifuged at $4,000 \times g$ for 15 min. After washing with 76% ethanol, the pellet was dissolved in 2 ml of distilled H_2O. Total DNA was

purified in 4.4 M CsCl density gradient by ultracentrifugation at 320,000×*g* for 2.5 h. Five micrograms of the extracted DNA was digested with the restriction enzyme *Taq*I. Digested DNA was electrophoresed in 1% agarose gel at 25 V for 48 h. The DNA was Southern-blotted onto a hybridization nylon membrane (Hybond-N^+, Amersham).

DNA fingerprinting

For hybridization with the minisatellite sequence, tandem repetitive sequence in the protein III gene of M13 bacteriophage (Vassart et al., 1987) was used. This 589 bp repetitive sequence was amplified by polymerase chain reaction (PCR) for 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 3 min. Primer sequences for amplification were 5′-TCC TAT TGG GCT TGC TAT CC-3′ and 5′-TTT CGG TCA TAG CCC CCT TA-3′. After denaturation at 94°C for 10 min, the amplified M13 region was labeled with [α-^{32}P]dCTP by random priming DNA labeling method. Hybridization was conducted in 10 ml of hybridization solution (7% SDS, 1 mM EDTA, 525 mM Na_2HPO_4 and 1% bovine serum albumin) with the labeled probe at 58°C overnight. The membrane was washed once with 2×SSC containing 0.1% SDS at room temperature for 20 min. Hybridization and washing conditions followed David et al. (1988).

For the hybridization with the synthetic repetitive sequence, the oligonucleotide $(GACA)_4$ made by a DNA synthesizer was used as probe. In order to label the probe, the 5′ end of this oligonucleotide was end-labeled with [γ-^{32}P] dATP by T4 kinase. Hybridization was conducted in 10 ml of hybridization solution (5×SSPE, 5×Denhardt's solution, 10 μg/ml *E. coli* DNA and 0.1% SDS) with the labeled probe at 43°C for 1.5 h. After hybridization, the membrane was washed three times with 6×SSC at 48°C for 45 min. Hybridization and washing conditions followed Weising et al. (1989). The filter was exposed to X-ray film for 3–10 days with two intensifying screens.

3. RESULTS

Intraspecific variation of P. yedoensis

Fig. 2 shows the DNA fingerprints of 37 accessions of *P. yedoensis* probed with M13 repeat sequence. Thirty seven accessions in Fig. 2 and all the others had the completely same fingerprinting pattern. There are 22 distinct bands, ranging from 3.0 kb to 5.2 kb.

Twelve accessions randomly selected from 62 accessions of *P. yedoensis* were hybridized with probe $(GACA)_4$ synthetic oligonucleotide. The autoradiograph is shown in Fig. 3. Again, all the accessions had the completely same banding pattern. Twenty-five of scored bands are distributed in a range of 2.0 kb to 6.0 kb.

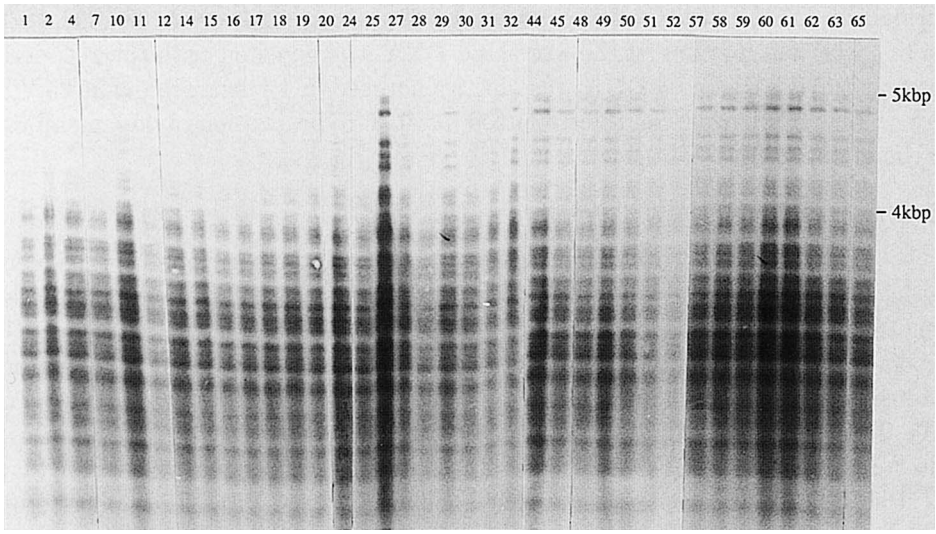

Fig. 2. The autoradiograph of the standard trees of *P. yedoensis* obtained by M13-probed DNA fingerprinting. The lane numbers correspond to the number of the meteorological observatories and stations in Table 1.

By using two different probes, it was shown that all the *P. yedoensis* investigated have the same DNA fingerprinting patterns. This result suggests that all the *P. yedoensis* accessions investigated in this study were derived from a single plant.

Interspecific relationship among P. yedoensis, P. lannesiana *and* P. pendula

Fig. 4 shows DNA fingerprints of *P. yedoensis*, *P. lannesiana*, *P. pendula* and an artificial hybrid between *P. lannesiana* as female parent and *P. pendula* as male parent probed with the oligonucleotide $(GACA)_4$. Scored bands were diagramed in Fig. 5. Each accession of *P. lannesiana* and *P. pendula* investigated showed a distinct fingerprinting pattern. This observation implies that there is a considerable intraspecific variation in both species, despite no intraspecific variation in *P. yedoensis*.

It is possible to investigate the interspecific relationship between *P. yedoensis* and other two species, *P. lannesiana* and *P. pendula* by comparing their fingerprinting patterns shown in Figs. 4 and 5. In total 26 bands were scored in *P. lannesiana*, and 36 bands in *P. pendula* (Fig. 5). It can be noted that 22 of the 25 bands in *P. yedoensis* are found either in *P. lannesiana* or *P. pendula*. The remaining three bands (No. 9, 12 and 26) in *P. yedoensis* are found in neither *P. lannesiana* nor *P. pendula*. In other words, almost all the bands present in *P. yedoensis* are found in either *P. lannesiana* or *P. pendula*. This result strongly suggests that *P. yedoensis* is a hybrid between *P. lannesiana* and *P. pendula*.

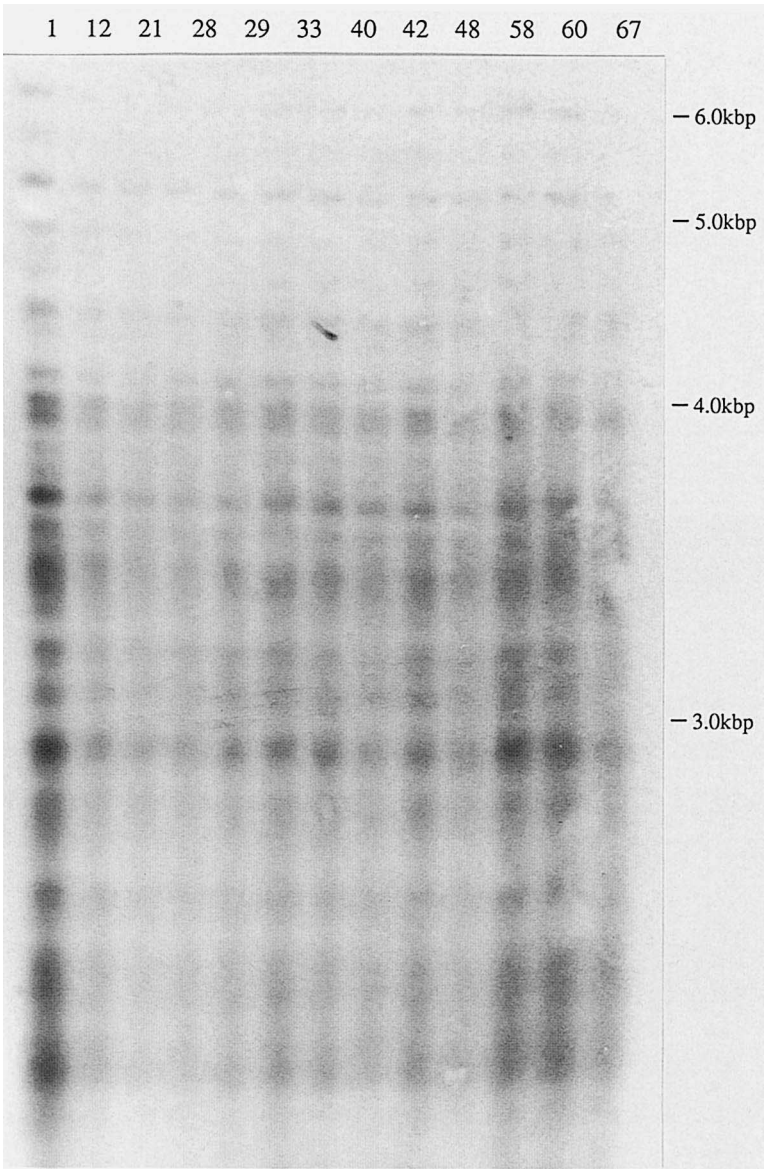

Fig. 3. The autoradiograph of the standard trees of *P. yedoensis* obtained by $(GACA)_4$-probed DNA fingerprinting. The lane numbers correspond to the number of the meteorological observatories and stations in Table 1.

Fig. 5 also shows the fingerprinting pattern of the artificial hybrid between *P. lannesiana* and *P. pendula*. In the hybrid 23 bands were scored. In spite of the similarity in morphology, the banding pattern of the hybrid is different from that of *P. yedoensis*, although they share 62% of bands in common.

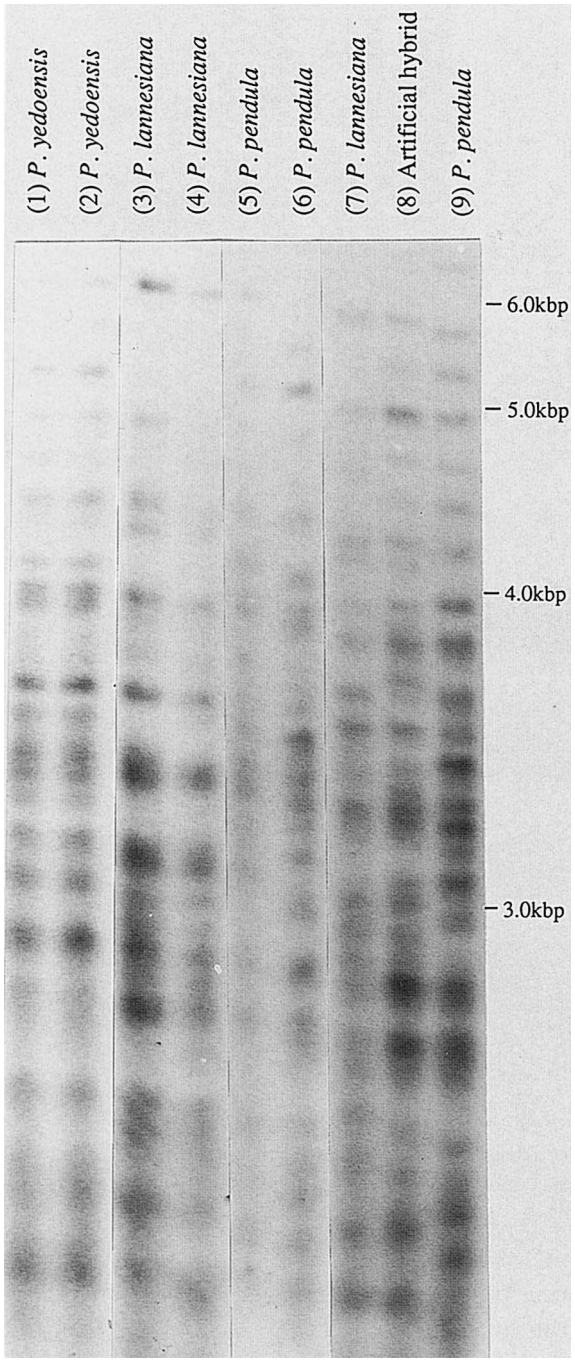

Fig. 4. The autoradiograph of three cherry species obtained by $(GACA)_4$-probed DNA fingerprinting. (1), (3) and (5) were from the National Institute of Genetics. (2) was from Sapporo Meteorological Observatory. (4) and (6) were from the Botanical Garden, Faculty of Science, University of Tokyo. Artificial hybrid (8) was produced by crossing between *P. lannesiana* (7) and *P. pendula* (9).

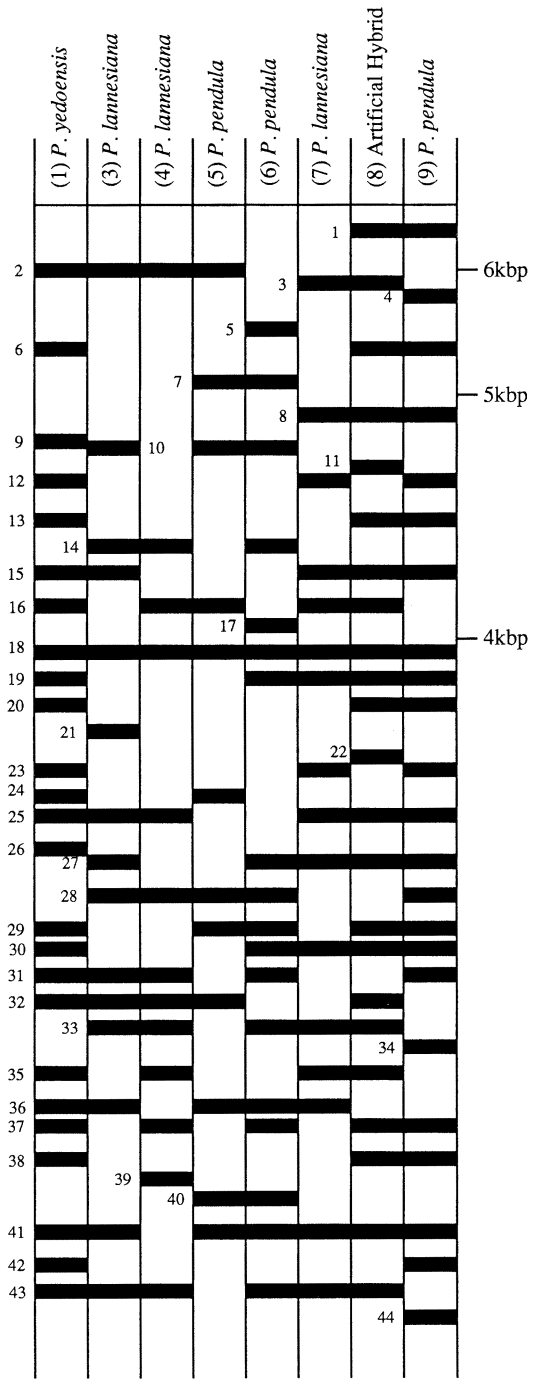

Fig. 5. Diagrams of scored bands from $(GACA)_4$ synthetic oligonucleotide-probed DNA fingerprints in Fig. 4. The band number given is shown at the left of each band.

Twenty of the 23 bands of the hybrid were found in its parents. Namely five bands (No. 3, 16, 33, 35 and 43) were found only in *P. lannesiana*, seven (No. 1, 6, 13, 20, 29, 37 and 38) were only in *P. pendula*, and eight bands (No. 8, 15, 18, 19, 25, 27, 30 and 41) were present in both *P. lannesiana* and *P. pendula*. The remaining three bands (No. 11, 22 and 32) were specific to the artificial hybrid. The occurrence of such hybrid-specific bands may be due to mutations caused by unequal crossing over, DNA slippage or gene conversion occurred during or after the hybridization.

4. DISCUSSION

Intraspecific variation of P. yedoensis *and interspecific variation of three* Prunus *species*

In this study, all the accessions of *P. yedoensis* revealed the completely same DNA fingerprinting pattern by using two different kinds of probe. This result suggests that all the accessions of *P. yedoensis* are derived from a single individual and that this species has been spread all over Japan by vegetative propagation. No genetic variation in this species assures that the sakura front line is not influenced by the genetic variation of standard trees and that the front line is reliable enough to reflect the climatic changes. On the contrary, each accession of *P. lannesiana* and *P. pendula* investigated has unique DNA fingerprint. The proportions of shared bands between individuals are 55% in *P. lannesiana* and 38% in *P. pendula*. This result suggests that there is a considerable amount of intraspecific variation in these two species. About 90% of bands found in *P. yedoensis* are detected in either *P. lannesiana* or *P. pendula*. This result supports the idea that *P. yedoensis* is a hybrid between *P. lannesiana* and *P. pendula*. The remaining about 10% of bands specific to *P. yedoensis* might have existed in the parental individuals or might have been produced by mutations after the origin of *P. yedoensis*.

From the comparison of the artificial hybrid and its parents, it can be noted that *P. lannesiana* transmitted 13 of the 16 bands to the hybrid, and that *P. pendula* did 15 of the 23 bands to the hybrid. If a parent is completely heterozygous at all the DNA fingerprint loci, 50% of its bands are transmitted to the offspring. On the other hand, if it is completely homozygous, 100% of the bands will appear in the offspring. In the case of the artificial hybrid and its parents, *P. lannesiana* and *P. pendula*, about 70% of the parental bands were detected in the hybrid. This result suggests a high heterozygosity of the investigated loci in *P. lannesiana* and *P. pendula*.

The origin of P. yedoensis

As shown in this study, *P. yedoensis* is likely to be a hybrid between *P. lannesiana* and *P. pendula*. However, as to the question that which species is

the maternal parent of *P. yedoensis*, there is a controversy. Funazu (1966) reported his grandfather's note in which he described that *P. yedoensis* was bred by crossing *P. lannesiana* as the maternal parent and *P. pendula* as the pollen parent. Iwasaki (1981) suggested that the maternal parent of *P. yedoensis* is *P. lannesiana* based on the morphological comparison of pollen and bark, and the content of sakuranetin, a kind of flavonoids. His conclusion was based on the matroclinous inheritance which is known to exist in some plants (Kakizaki, 1925; Tokumasu, 1965). The matroclinous inheritance is the phenomenon that the maternal characters are strongly inherited to the offsprings. On the other hand, from the RFLP analysis on the chloroplast DNA (ctDNA), Kaneko et al. (1986) showed that *P. yedoensis* and *P. pendula* shared the same restriction pattern of ctDNA, and that *P. lannesiana* had a different pattern. Their result suggested that the maternal parent of *P. yedoensis* is *P. pendula* if the ctDNA is assumed to be inherited maternally. Iwasaki (1986) argued against the suggestion of Kaneko et al. (1986), saying that there must be two types of *P. yedoensis* which were derived from the reciprocal crosses between *P. lannesiana* and *P. pendula*. Summarizing those previous studies, there are three hypotheses on the origin of *P. yedoensis*. The first hypothesis is that *P. lannesiana* is the maternal parent (Funazu and Iwasaki hypothesis), the second is that *P. pendula* is the maternal parent (Kaneko hypothesis), and the third is that both *P. lannesiana* and *P. pendula* are maternal parents (Iwasaki hypothesis).

Although it was not possible to specify the maternal parent of *P. yedoensis* from this study, the results obtained do not support the third hypothesis, from which we expect at least two kinds of fingerprinting patterns segregating in *P. yedoensis*, considering a relatively high heterozygosity in the parental species. However, no intraspecific variation was detected in *P. yedoensis*. In order to obtain the decisive answer on the origin of *P. yedoensis*, more detailed study on organellar DNAs by using DNA sequencing or SSCP analysis is necessary.

Summarizing the results obtained in this study, it is possible to conclude that *P. yedoensis* was produced only once by hybridization between single plants of *P. lannesiana* and *P. pendula*, and that the clone of this particular plant has been spread all over Japan.

We would like to express our sincere thanks to late Dr. K. Furusato, Past Director of the Hamamatsu City Flower Park, Shizuoka Prefecture, Dr. M. Kato, Faculty of Science, University of Tokyo, Mr. H. Iwai in the Hamamatsu City Flower Park, Mr. E. Kikuchi in the Irozaki Jungle Park, Mr. T. Yamashiro and all the persons in 67 Meteorological Observatories and Stations referred in Table 1 for their kind supply of materials used in the present study and to Dr. G. Kahl, Johann Wolfgang Goethe University, Germany for providing us the DNA fingerprinting protocols.

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