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Evolutionary analysis of *Pinus densata* (Masters), a putative Tertiary hybrid.

2. A study using species-specific chloroplast DNA markers

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Summary. Restriction fragment analysis and heterologous hybridization of chloroplast (cp) DNA was used to develop species-specific markers for *P. tabulaeformis*, *P. yunnanensis* and *P. massoniana*. Fragment patterns created by the *Bcl*I and *Dra*I restriction enzymes and hybridization patterns to the *psb*C and *psb*D probes were distinctive among the three species. No intraspecific variation was detected with respect to any of the cpDNA markers developed in this study. The cpDNA markers obtained were subsequently used to examine the parentage of *P. densata*, a putative Tertiary hybrid between *P. tabulaeformis* and *P. yunnanensis*. The analysis demonstrated for the first time that *P. densata* populations accommodate chloroplast genomes of *P. tabulaeformis* and *P. yunnanensis*, which strongly supports earlier suggestions of the hybrid origin of this species. It appears that *P. densata* represents a stabilized natural hybrid that has become adapted to high mountain environments where neither of the parental species can normally grow.

Key words: *Pinus* – Species hybridization – Chloroplast DNA – Molecular markers – Evolution

Introduction

Species' hybridization is often regarded as an important factor in plant evolution (Anderson and Stebbins 1954; Stebbins 1959; Grant 1981; Barton and Hewitt 1989). The occurrence of several crossable, sympatric species from subsection *Sylvestres* in Asia has led to suggestions that hybridization has played a significant role in the evolution of the genus *Pinus* on this continent (Wu 1956; Mirov 1967).

Pinus densata (Masters) is a well-known case of a putative ancient *Pinus* hybrid from Asia. Paleobotanical and morphological evidence suggests that *P. densata* arose from a hybridization between *P. tabulaeformis* (Carrière) and *P. yunnanensis* (Franchet) (Cheng 1930; Wu 1956; Mirov 1967). *P. tabulaeformis* is a widespread species in the northern and central parts of China, whereas *P. yunnanensis* is distributed in the southwestern part (Wu 1956). Some authors believe that the two species intercrossed in the Yunnan and Sichuan region as early as the Tertiary (Wu 1956; Mirov 1967). It is also possible that a third species, *P. massoniana*, which overlaps with *P. tabulaeformis* and *P. yunnanensis*, was involved in the evolution of *P. densata* through hybridization. Today, *P. densata* occurs in the high mountains at elevations between 2,700–3,900 m where neither of the putative parental species can normally grow (Cheng 1930; Li and Liu 1984). The advanced evolutionary age of *P. densata* as compared with other known *Pinus* hybrids (Zobel 1951; Critchfield 1975; Christensen 1987) makes it a particularly interesting object for a genetic analysis of species' hybridization. However, the hybrid origin of this taxon has not been demonstrated with any genetic method prior to this study.

In a previous study utilizing allozyme markers we provided the first genetic documentation of *P. densata* and its putative parents (Wang et al. in press). However, this analysis did not permit us to unambiguously determine the hybrid nature of this taxon and to identify its parents. Precise DNA markers may provide the best means for resolving ambiguous cases of hybridization in plants (Rieseberg et al. 1988, 1990; Szmidt 1990). The recent development of chloroplast (cp) DNA markers has furnished a qualitative means for the genetic analysis of hybrids in conifers (Szmidt et al. 1988a, b; Szmidt 1990; El-Kassaby et al. 1988; Sigurgeirsson et al. 1990;

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Wang et al. 1990). In this paper we present the first results of our ongoing evolutionary analysis of *P. densata* and other pines from Asia using cpDNA markers. Our objectives in the present study were: (1) to find species-specific cpDNA markers for *P. tabulaeformis*, *P. yunnanensis* and *P. massoniana* and (2) to assess the parentage of *P. densata*.

Material and methods

Plant material

The origins and designations of the investigated plant material are given in Table 1. Seeds used in this study were taken from bulk natural stand collections made by the Department of Forest Sciences of the Nanjing Forestry University. Seedlings from two allopatric populations of *P. tabulaeformis* and *P. yunnanensis* were grown for 1.5 years in a greenhouse. These two populations originate from outside the region of sympatry with *P. densata* and can thus be regarded as pure species. A composite needle sample consisting of 20 individual seedlings was collected from each of the two populations and used for cpDNA extraction. Seedlings from one natural population of *P. densata* were grown in a greenhouse for 8 months. Three composite needle samples consisting of 10, 20 and 30 seedlings, respectively, were used for analysis of cpDNA variation. In addition to the composite samples, needles were collected from several individual trees of *P. tabulaeformis*, *P. yunnanensis*, *P. massoniana* and *P. densata* in China, in the Hørsholm Arboretum, Denmark and in the Arboretum of the Hokkaido Forest Tree Breeding Institute, Japan, and used for analysis of cpDNA variation (Table 1).

Table 1. List of *Pinus* samples used in this study and their geographic origin

Species	Sample designation	Number of plants	Latitude (°N)	Longitude (°E)
P. tabulaeformis	Pti# 1	1	35	113
	Pti# 2	1	35	113
	Pti# 3	1	35	113
	Pti# 4	1	40	116
	Pti# 5	1	40	116
	Pti# 6	1	40	116
	Pti# 7	1	36	117
	Pti# 8	1	36	117
	Ptc#20	20	40	111
P. yunnanensis	Pyi# 1	1		unknown
	Pyi# 2	1		unknown
	Pyi# 3	1		unknown
	Pyi# 4	1		unknown
	Pyi# 5	1	25	103
	Pyi# 6	1	25	103
	Pyi# 7	1	25	103
	Pyi# 8	1	25	103
	Pyc#20	20	23	105
P. massoniana	Pmi# 1	1	32	117
	Pmi# 2	1		unknown
	Pmi# 3	1		unknown
	Pmi# 4	1		unknown
P. densata	Pdi# 1	1	31	102
	Pdi# 2	1	31	102
	Pdi# 3	1	31	102
	Pdi# 4	1	31	102
	Pdc#10	10	32	103
	Pdc#20	20	32	103
	Pdc#30	30	32	103

DNA isolation, digestion and separation

Fresh needles were used for cpDNA isolation following Szmidt et al. (1986). cpDNA from *P. tabulaeformis* and *P. yunnanensis* was digested to completion separately with the following 13 restriction enzymes, all of which recognize six-base nucleotide sequences: *Acc*I, *Ava*I, *Bam*HI, *Bcl*I, *Cla*I, *Dra*I, *Hind*III, *Hpa*I, *Kpn*I, *Pst*I, *Sal*I, *Sma*I and *Xba*I (Boehringer®, Mannheim). cpDNA from *P. massoniana* and *P. densata* was digested only with *Bcl*I and *Dra*I. Digestion was made according to the manufacturer's instructions. Digested cpDNA samples were separated by electrophoresis on 0.8% agarose gels for 14–16 h, at 3V/cm in TAE buffer, pH 8.0, in the presence of ethidium bromide (Maniatis et al. 1982). The gels were photographed under UV light using a Polaroid® camera.

DNA transfer and hybridization

After denaturation and neutralization, the separated cpDNA fragments were transferred from agarose gels to nylon membranes (Southern 1975). The DNA size marker used was the BRL® 1 kilobase (kb) ladder. Five heterologous gene-specific probes for *psb*A, *psb*C, *psb*D, *rbc*L and 23S rDNA were used in this study (Table 2). Radioactive probes were prepared using the random priming procedure (Feinberg and Vogelstein 1984) and hybridized to filters containing digested cpDNA from the investigated *Pinus* species following the scheme presented in Table 3. Hybridization was carried out at 60 °C overnight in the solution of 6 × SSC, 5 × Denhardt, 2.0 m*M* EDTA, pH 8.0, 0.2% SDS and 0.01% denatured calf thymus DNA. The filters were washed in 0.1% SDS and 0.1 × SSC, 3 × 15 min at room temperature and 2 × 15 min at 50 °C and exposed to X-ray films. To remove the probe prior to rehybridization, the filters were washed in 0.1 × SSC, 0.1% SDS at 95 °C for 15 min. The size of the cpDNA fragments was determined using a digitizer and DIGDNA computer program (Szmidt in preparation) employing an algorithm developed by Schaffer and Sederoff (1981).

Table 2. List of heterologous gene-specific cpDNA probes used in this study

Organism	Clone	Probe	Reference
Anacystis nidulans	AN23	23SrDNA	Tomioka et al. (1981)
Spinacia oleracea	SA	*psb*A	Bohnert and Löffelhardt (1982)
Spinacia oleracea	pPSII44	*psb*C	Alt et al. (1984)
Spinacia oleracea	pPSII32/2	*psb*D	Alt et al. (1984)
Chlamydomonas reinhardtii	CRRBCL	*rbc*L	Dron et al. (1982)

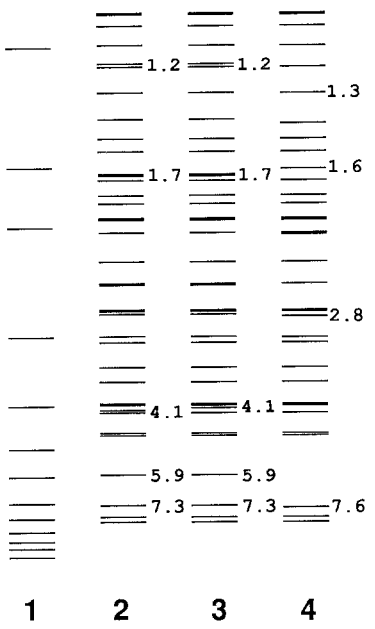

Fig. 1. Schematic drawing of cpDNA restriction fragment patterns created by *Bcl*I. *Lane 1* 1 kb ladder, *lane 2 P. tabulaeformis, lane 3 P. yunnanensis, lane 4 P. massoniana. Numbers* denote size of fragments (in kb) showing interspecific variation

Table 3. Hybridization scheme to five heterologous cpDNA probes used in this study and the size (in kb) of hybridizing cpDNA fragments in *P. tabulaeformis* (*Pt*), *P. yunnanensis* (*Py*), *P. massoniana* (*Pm*) and *P. densata* (*Pd*)

Enzyme	Species	Probe					
		*psb*A		*psb*C	*psb*D	*rbc*L	23S
*Bcl*I	*Pt*	2.9	2.1	1.7	1.2	4.1	8.1
	Py	2.9	2.1	1.7	1.2	4.1	8.1
	Pm	2.9	2.1	1.7	1.2	4.1	8.1
	Pd	–[a]		1.7	1.2	–	–
*Dra*I	*Pt*	2.4		14.0	14.0	–	–
	Py	2.4		3.1	3.1	–	–
	Pm	2.4		2.3	2.3	–	–
	Pd	2.4		14.0/ 3.1	14.0/ 3.1	–	–

[a] Not tested

Results

I. Restriction fragment analysis

To find out whether *P. tabulaeformis* can be distinguished from *P. yunnanensis* on the basis of its cpDNA restriction fragment pattern, we applied 13 different restriction enzymes. Of these 13 enzymes, *Bcl*I and *Dra*I showed variation in restriction fragment pattern between the two species. As demonstrated earlier, *P. massoniana* can be distinguished from these two species on the basis of the cpDNA restriction fragment pattern created by the same

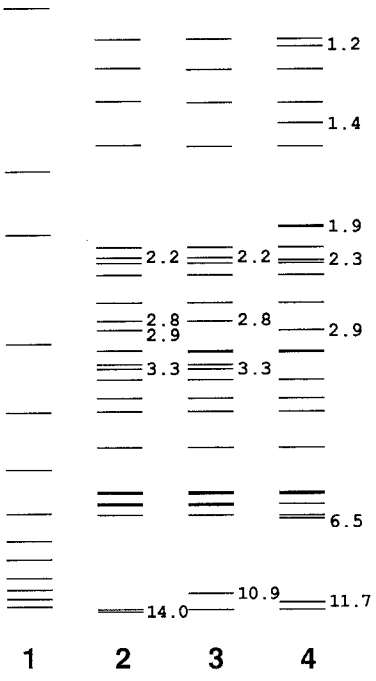

Fig. 2. Schematic drawing of cpDNA restriction fragment patterns created by *Dra*I. *Lane 1* 1 kb ladder, *lane 2 P. tabulaeformis, lane 3 P. yunnanensis, lane 4 P. massoniana. Numbers* denote size of fragments (in kb) showing interspecific variation

two enzymes (Wang et al. 1990; Wang unpublished results). For all three species the restriction fragment patterns created by *Bcl*I and *Dra*I are presented in Figs. 1 and 2, respectively. No variation was found within and among individuals and composite samples from either of the three species with respect to any of the restriction enzymes used.

cpDNA from *P. densata* individual trees and composite samples was digested with *Bcl*I and *Dra*I, which are the enzymes that can distinguish among *P. tabulaeformis, P. yunnanensis* and *P. massoniana*. Two types of *Bcl*I and *Dra*I restriction fragment patterns were found among individual trees of *P. densata*. Trees Pdi#1 and Pdi#2 showed *Bcl*I and *Dra*I restriction patterns that are unique to *P. tabulaeformis*. On the other hand, trees Pdi#3 and Pdi#4 had *Bcl*I and *Dra*I restriction patterns unique to *P. yunnanensis*. In composite samples of *P. densata* all of the cpDNA fragments that are unique to *P. tabulaeformis* as well as all fragments that are unique to *P. yunnanensis* were found (data not shown). All other *Bcl*I and *Dra*I fragments found in *P. densata* also occurred in *P. tabulaeformis* and *P. yunnanensis*. Neither *P. densata* individuals nor composite samples showed the *Bcl*I or *Dra*I fragments that are unique to *P. massoniana*.

II. Heterologous DNA hybridization

In addition to restriction analysis, heterologous cpDNA hybridization was also used to distinguish among *P. tabu-*

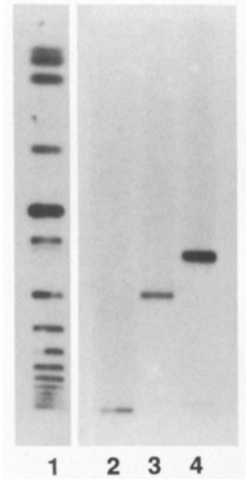

Fig. 3. Hybridization pattern of *Dra*I cpDNA fragments to *psb*C probe: *lane 1* 1 kb ladder, *lane 2 P. tabulaeformis*, *lane 3 P. yunnanensis*, *lane 4 P. massoniana*

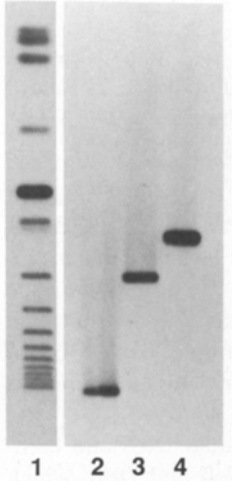

Fig. 4. Hybridization pattern of *Dra*I cpDNA fragments to *psb*D probe: *lane 1* 1 kb ladder, *lane 2 P. tabulaeformis*, *lane 3 P. yunnanensis*, *lane 4 P. massoniana*

laeformis, *P. yunnanensis* and *P. massoniana*. The five probes used in this study were hybridized individually to the cpDNA digests following, the scheme presented in Table 3. We found that both the *psb*C and *psb*D probes hybridized to three different *Dra*I fragments (14.0, 3.1 and 2.3 kb, respectively) in these three species (Table 3, Figs. 3 and 4). On the other hand, other hybridizations did not detect differences among the three species (Table 3). Furthermore, no intraspecific variation was detected in either species with respect to any of the probe and enzyme combinations tested.

The results of *psb*C and *psb*D hybridization to *P. densata* cpDNA digested with *Dra*I are presented in Fig. 5. An examination of *P. densata* individuals gave results similar to those obtained employing restriction

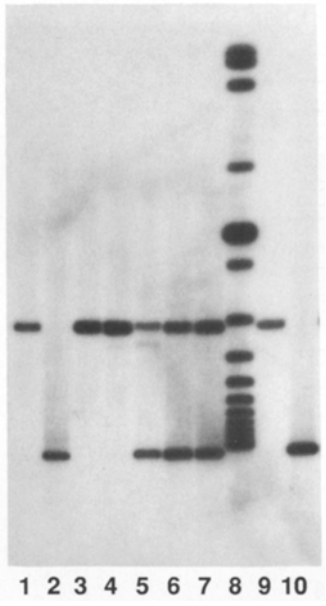

Fig. 5. Hybridization pattern of *Dra*I cpDNA fragments to *psb*C probe: *lane 1 P. densata* (Pdi#3), *lane 2 P. densata* (Pdi#2), *lane 3 P. densata* (Pdi#4), *lane 4 P. yunnanensis* (Pyi#5), *lane 5 P. densata* (Pdc#10), *lane 6 P. densata* (Pdc#20), *lane 7 P. densata* (Pdc#30), *lane 8* 1 kb ladder, *lane 9 P. yunnanensis* (Pyc#20), *lane 10 P. tabulaeformis* (Ptc#20)

fragment analysis. We found that trees Pdi#1 (not shown in Fig. 5) and Pdi#2 had the hybridization pattern unique to *P. tabulaeformis*, while trees Pdi#3 and Pdi#4 had the hybridization pattern unique to *P. yunnanensis*. In composite cpDNA samples from *P. densata* digested with *Dra*I, the *psb*C and *psb*D probes hybridized to two different (14.0 and 3.1 kb) fragments that are specific to *P. tabulaeformis* and *P. yunnanensis*, respectively. On the other hand, the *psb*C and *psb*D hybridization patterns unique to *P. massoniana* were absent in the *P. densata* individuals and composite samples. The hybridization of *psb*C and *psb*D probes to *Bcl*I digests of *P. densata* cpDNA showed no variation among the samples analyzed. As in *P. tabulaeformis*, *P. yunnanensis* and *P. massoniana* the two probes hybridized to the 1.7 and 1.2 kb fragments, respectively (Table 3). Similar results were obtained after hybridizing the *psb*A probe to the *Dra*I cpDNA digest from *P. densata*. In all samples this probe hybridized to one (2.4 kb) fragment, which was also found in three other species analyzed in this study (Table 3).

Discussion

I. Species-specific cpDNA markers

For the genetic analysis of hybrid populations it is necessary to distinguish between individual parental species. A

species-specific marker must show stable qualitative differences between the species under investigation, while showing little or no variation within populations of individual parental species (Rieseberg et al. 1988, 1990; Szmidt 1990). The present study well illustrates the considerable usefulness of cpDNA markers for studies of species' hybridization in conifers. Our results clearly show that all three *Pinus* species analyzed can be distinguished easily by using restriction fragment analysis and heterologous hybridization of cpDNA.

Although the conservative nature of cpDNA is usually reflected in the paucity of intraspecific variation, some differences within species and individuals have been found in several conifers (Govindaraju et al. 1988, 1989; White 1990; Wang unpublished results). However, no intraspecific variation was detected in any sample and with any cpDNA marker obtained in the present study. In addition, except for *psb*C and *psb*D all of the other probes used did not detect differences among the investigated species. It thus appears that the analyzed cpDNA regions are relatively conservative in the investigated *Pinus* species. Another potential problem that may arise while using heterologous hybridization in the analysis of cpDNA variation is cross-homology between the chloroplast probes used and mitochondrial and/or nuclear DNA (Timmis and Scott 1983; Sederoff et al. 1986; Cheung and Scott 1989). From our results it appears however that also this phenomenon was not important in the material analyzed. Purified cpDNA samples were used for hybridization, and all five probes hybridized to distinct cpDNA fragments. We therefore conclude that the polymorphism of cpDNA found in this study can be used as a stable species-specific marker to distinguish among *P. tabulaeformis, P. yunnanensis* and *P. massoniana.*

II. Parentage of P. densata

The presence of cpDNA fragments specific for *P. tabulaeformis* and *P. yunnanensis* in composite samples from *P. densata* clearly demonstrates the heterogeneous nature of this taxon with respect to the chloroplast genome. Further support for this conclusion comes from the observed pattern of cpDNA variation among individual trees of *P. densata* possessing cpDNA fragments unique to either *P. tabulaeformis* or *P. yunnanensis*. The latter observation is in agreement with other evidence indicating that cpDNA is uniparentally inherited in conifers, and thus only one parental cpDNA type is normally expected in an individual (Neale et al. 1986, 1989; Szmidt et al. 1987, 1988 b; Wagner et al. 1987; Stine et al. 1989; Wang unpublished results). This result also reduces the possibility that the cpDNA hybridization pattern to the *psb*C and *psb*D probes observed in composite samples may result from the occurrence of two different *Dra*I fragments homologous to these two probes in one individual, which would suggest biparental cpDNA transmission or gene duplication.

Wu (1956) has proposed two possible explanations for the evolution of *P. densata*. His first hypothesis asserts that *P. densata* may represent an ancient ancestor to *P. tabulaeformis* and *P. yunnanensis*. The alternative hypothesis maintains that *P. densata* arose as a result of hybridization between *P. tabulaeformis* and *P. yunnanensis*. While the latter hypothesis has been viewed more favorably in later descriptions of this taxon (Mirov 1967; Farjon 1984), genetic evidence supporting either suggestion was lacking. Our present results strongly support the hypothesis implying the evolution of *P. densata* through hybridization between *P. tabulaeformis* and *P. yunnanensis*. Populations of the two latter species analyzed in this study are located outside the range of *P. densata*. Hence, our material can be viewed as representing pure reference species. Each of the two pure species could be characterized by several different cpDNA markers showing no intraspecific variation. On the other hand, cpDNA markers specific for *P. tabulaeformis* and *P. yunnanensis* were detected in the two different populations of *P. densata* analyzed in this study. We believe that this situation results from hybridization during earlier contact between *P. tabulaeformis* and *P. yunnanensis*.

Previous suggestions on the hybrid origin of *P. densata* implied that *P. tabulaeformis* and *P. yunnanensis* are parents of this taxon (Wu 1956; Mirov 1967; Farjon 1984). However, from the distribution of *Pinus* species in Asia it is apparent that also *P. massoniana,* which occurs sympatrically with the other two species (Mirov 1967; Farjon 1984), could have been involved in the hybridization. Our results show, however, that only *P. tabulaeformis* and *P. yunnanensis* cpDNA markers are present in *P. densata*. Therefore, *P. massoniana* can probably be excluded as a possible parent of this taxon.

A characteristic geographic feature of *P. tabulaeformis, P. densata* and *P. yunnanensis* distribution in China is the gradual replacement of one species by another from north to south (Wu 1956; Mirov 1967; Guan 1981). The vertical distribution of pure *P. densata* stands is always above that of *P. tabulaeformis* and *P. yunnanensis* (2,700–3,900 m). In fact, the present-day vertical range of this taxon is higher than that of any other *Pinus* species occurring in China (Cheng 1930; Wu 1956; Cheng and Fu 1978). Earlier attempts at artificial crossing *P. tabulaeformis* with *P. yunnanensis* have ended in failure (Duffield 1952; Wright and Gabriel 1958; Wang Zhang-Rong and Shen Xi-Huan personal communications). Even if at present the two species are to some extent reproductively isolated, it may not have been the case when they originally came into contact. The fertility of interspecific *Pinus* hybrids has been noticed by many authors (Duffield 1952; Mirov 1967; Critchfield 1975).

At present, *P. densata* appears to be fully fertile (Cheng 1930; Wu 1956; Li and Liu 1984), hence it cannot be regarded as an evolutionary relict with an impaired reproductive system that would prevent its proliferation. The present-day normal fertility and firm entrenchment of *P. densata* at higher elevations that remain inaccessible to either parent indicate the possible adaptive advantage of this hybrid.

Due to the uniparental inheritance of cpDNA in conifers this study does not provide definite proof for the occurrence of hybridization between *P. tabulaeformis* and *P. yunnanensis*. Taking into account all of the evidence currently available it nevertheless appears justifiable to assume that hybridization actually occurred and has led to an establishment of a new taxon, *P. densata*. Using cpDNA markers developed in this study we are currently analyzing the relative contribution of each of the two parental species in populations of *P. densata*. This information will be helpful in elucidating genetic processes that were responsible for the creation and maintenance of this hybrid.

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