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

1. Allozyme variation

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Summary. Allozyme differentiation at 13 loci was studied in populations of *Pinus tabulaeformis, P. densata*, and *P. yunnanensis* from China. It was previously suggested that *P. densata* represents a Tertiary hybrid between *P. tabulaeformis* and *P. yunnanensis*. The observed levels of allozyme variation within and among the investigated species were comparable to those of other conifers. *P. tabulaeformis* differed markedly from *P. yunnanensis* with respect to allozyme frequencies, while *P. densata* was intermediate between the two putative parents. There was evidence of homozygote excess in embryos from all investigated species, as compared to Hardy-Weinberg expectations. The observed allozyme composition of *P. densata* conformed to earlier morphological and molecular evidence indicating hybrid origin of this taxon. It was proposed that fusion of gene pools from *P. tabulaeformis* and *P. yunnanensis* has led to adaptive evolution of a new species, *P. densata*.

Key words: *Pinus* – Species hybridization – Allozymes – Evolution

Introduction

In the past few years, several studies have addressed the genetic composition of putative hybrids in conifers using biochemical and molecular methods (Copes and Beckwith 1977; Prus-Glowacki and Szweykowski 1979; Florence and Hicks 1980; Millar 1983; Yeh and Arnott 1986; Wagner et al. 1987; Millar et al. 1988; Sigurgeirsson et al. 1990). However, all this earlier work focused on species occurring in Europe and North America, while taxa native to Asia have not been investigated to date. The occurrence of several crossable, sympatric species from subsection Sylvestres of the genus *Pinus* in this region has led to suggestions that some species arose as a result of hybridization (Wu 1956; Mirov 1967). Nothing is known about the genetic composition of such putative natural hybrids in Asia.

P. tabulaeformis is a widespread species occurring in the northern and central parts of China. *P. yunnanensis* has its distribution in the southwestern part of this country (Wu 1956; Mirov 1967; Farjon 1984). According to some authors, during the Tertiary Period the two species met in northern Yunnan and formed a natural hybrid species, *P. densata* Masters (Cheng 1930; Wu 1956; Mirov 1967; Farjon 1984). Today, *P. densata* occurs at high mountain elevations between 2,700 and 3,900 m, where neither of the two putative parental species can normally grow (Cheng 1930; Li and Liu 1984). Despite the potential value of *P. densata* for the evolutionary analysis of species hybridization, the genetic documentation of this taxon and its potential parents is lacking. The hybrid origin of *P. densata* has not been demonstrated with any genetic method, and all earlier suggestions are based on limited morphological evidence.

In our recent study of chloroplast DNA variation in *P. densata*, we demonstrated that it harbors chloroplast genomes from *P. tabulaeformis* and *P. yunnanensis*, which strongly supports earlier suggestions on the hybrid origin of this species and the identity of its parents (X.-R. Wang and A.E. Szmidt, in preparation). The present study aims at quantifying allozyme variation in these taxa. In addition, the following questions were addressed. (i) Does the pattern of allozyme variation conform to chloroplast DNA evidence indicating hybrid origin of *P. densata*? (ii) Are *P. tabulaeformis* and *P. yunnanensis* likely parents of *P. densata*?

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Materials and methods

Seed material

Bulked seed samples were obtained from three individual natural populations of *P. tabulaeformis, P. densata,* and *P. yunnanensis* from China (Table 1). The populations of *P. tabulaeformis* and *P. yunnanensis* were located outside the range of *P. densata.* The exact number of trees included in these collections is unknown, but was apparently greater than 50. Seeds were kept at 0°C until analysis. Samples comprising 106, 195, and 110 macrogametophytes and their corresponding embryos were analyzed from each population of *P. tabulaeformis, P. densata,* and *P. yunnanensis,* respectively.

Electrophoresis

Before analysis, seeds were germinated for approximately 10 days until a 3-mm radicle emerged from the seed coat. Enzyme extraction was performed as described by Szmidt (1984). Isoenzyme separation was carried out in 12% starch gels, and seven enzyme systems comprising 13 loci were scored. Information concerning the enzyme systems analyzed and electrophoresis is given in Table 2. Staining procedures and inheritance of individual isoenzymes were described elsewhere (Szmidt 1984; Wang et al. 1990; X.-R. Wang, unpublished results). All 13 loci were assessed simultaneously in each macrogametophyte and its corresponding embryo.

Statistical methods

Allozyme frequencies, expected (Nei 1978) and observed heterozygosities, gene diversity statistics (Nei 1973), and unbiased genetic distance measures (Nei 1978) were calculated using release 1.7 of the BIOSYS-1 program (Swofford and Selander 1981). A locus was considered polymorphic if the frequency of the most common allele did not exceed 0.95. Fixation indices at polymorphic loci were computed based on total observed and total expected heterozygosities in embryos (Curie-Cohen 1982). Departures of the observed genotype frequencies from those expected under panmixia were evaluated by χ^2 tests, using observed genotype frequencies and those expected under Hardy-Weinberg equilibrium (Sokal and Rohlf 1969).

Table 1. Geographic origin of seed samples used in allozyme analyses

Population	Latitude	Longitude
1. *P. tabulaeformis*	40°00′N	111°00′E
2. *P. densata*	31°40′N	102°30′E
3. *P. yunnanensis*	22°30′N	105°00′E

Table 2. Enzyme systems and their Enzyme Commission (EC) numbers, number of loci scored, and buffer systems used

Enzyme	Abbreviation	EC no.	No. of loci	Buffer system
Phosphoglucomutase	*Pgm*	2.7.05.01	2	A
Malate dehydrogenase	*Mdh*	1.1.01.37	2	A
Fluorescent esterase	*Fes*	3.1.01.01	1	A
Leucine amino peptidase	*Lap*	3.4.11.01	2	B
Glutamate oxaloacetate transaminase	*Got*	2.6.01.01	3	B
Glutamate dehydrogenase	*Gdh*	1.4.01.02	1	B
Shikimate dehydrogenase	*Sdh*	1.1.01.25	2	B

Buffer system A: Conkle et al. (1982); buffer system B: Clayton and Tretiak (1972)

Results

Thirteen loci were studied in each of the three investigated *Pinus* populations. Of these 13 loci, 9 were polymorphic (0.95 criterion) in at least one population and 6 were polymorphic in all three populations. Allozyme frequencies found in the investigated populations are given in Table 3. Among the most polymorphic loci were *Fes, Lap-2, Got-3,* and *Sdh-1.* Little polymorphism was found at *Mdh-1, Mdh-2, Pgm-2,* and *Sdh-2* loci. Allozyme frequencies at some loci varied markedly among the three *Pinus* populations analyzed. Particularly distinct differences were found at the *Lap-1* locus, which was highly variable in *P. densata* but nearly monomorphic in *P. tabulaeformis* and *P. yunnanensis. Lap-2* and *Sdh-1* loci were nearly monomorphic in *P. yunnanensis,* but highly variable in *P. tabulaeformis* and *P. densata* (Table 3). Differences among the investigated populations were further accentuated by the occurrence of unique allozymes. Despite smaller sample sizes, the most unique allozymes were found in *P. tabulaeformis* and *P. yunnanensis* (Table 3).

A summary of measures of genetic variability at 13 loci in the investigated populations is given in Table 4. The mean number of allozymes per polymorphic locus was high and ranged from 2.2 in *P. yunnanensis* to 2.8 in *P. tabulaeformis.* The proportion of polymorphic loci ranged from 46.2 in *P. yunnanensis* to 61.5 in *P. densata* (Table 4). Observed heterozygosity and unbiased estimate (Nei 1978) of expected heterozygosity in embryos ranged from 0.158 to 0.179 and 0.169 to 0.210, respectively, and were highest in *P. densata* (Table 4).

In all three investigated populations there was an excess of homozygotes in embryos, indicating deviation from Hardy-Weinberg expectations. At some loci, these deviations were statistically significant as revealed by χ^2 tests (data not shown). This was reflected in positive mean values of fixation indices in all three populations (Table 5). The highest mean fixation index (0.094) was found in embryos from the *P. densata* population.

The results of partitioning gene diversity using Nei's (1973) statistics of population subdivision are presented in Table 6. Some loci, e.g., *Got-3* and *Sdh-1,* contributed a particularly great amount to the observed levels of differentiation among the three populations. Of the total diversity over all three populations ($H_T = 0.239$), 13.4% resided among populations ($D_{ST} = 0.032$).

Table 3. Allele frequencies in populations of *P. tabulaeformis* (1), *P. densata* (2), and *P. yunnanensis* (3)

Locus	Population		
	1	2	3
Pgm-1			
(N)	110	194	106
1	0.132	0.049	0.000
2	0.814	0.884	0.788
3	0.045	0.067	0.212
4	0.009	0.000	0.000
Pgm-2			
(N)	45	164	96
1	0.000	0.000	0.005
2	1.000	1.000	0.979
3	0.000	0.000	0.016
Mdh-1			
(N)	110	195	106
1	0.018	0.033	0.033
2	0.973	0.964	0.967
3	0.009	0.003	0.000
Mdh-2			
(N)	110	195	106
1	0.986	0.962	1.000
2	0.014	0.038	0.000
Fes			
(N)	110	192	106
1	0.341	0.443	0.245
2	0.436	0.461	0.410
3	0.155	0.096	0.042
4	0.055	0.000	0.000
5	0.014	0.000	0.000
6	0.000	0.000	0.302
Lap-1			
(N)	109	195	106
1	0.982	0.885	0.995
2	0.000	0.000	0.005
3	0.018	0.115	0.000

Table 3. (continued)

Locus	Population		
	1	2	3
Lap-2			
(N)	110	194	103
1	0.014	0.003	0.000
2	0.932	0.863	0.767
3	0.027	0.131	0.233
4	0.027	0.003	0.000
Got-1			
(N)	110	195	106
1	0.100	0.079	0.099
2	0.891	0.897	0.901
3	0.009	0.023	0.000
Got-2			
(N)	110	195	106
1	0.005	0.000	0.028
2	0.923	1.000	0.873
3	0.073	0.000	0.000
4	0.000	0.000	0.099
Got-3			
(N)	107	190	104
1	0.224	0.605	0.837
2	0.734	0.379	0.139
3	0.042	0.011	0.005
4	0.000	0.005	0.000
5	0.000	0.000	0.019
Gdh			
(N)	110	195	106
1	1.000	1.000	1.000
Sdh-1			
(N)	106	194	106
1	0.618	0.209	0.000
2	0.217	0.152	0.000
3	0.146	0.637	0.995
4	0.014	0.003	0.005
5	0.005	0.000	0.000
Sdh-2			
(N)	110	163	106
1	0.000	0.077	0.000
2	1.000	0.923	1.000

Table 4. Genetic variability at 13 loci in populations of *P. tabulaeformis*, *P. densata*, and *P. yunnanensis* (standard errors in parentheses)

Population	Mean sample size per locus	Mean no. of alleles per locus	Percentage of loci polymorphic[a]	Mean heterozygosity	
				Observed	Expected[b]
1. *P tabulaeformis*	104.4 (5.0)	2.8 (0.4)	53.8	0.165 (0.050)	0.195 (0.062)
2. *P. densata*	189.3 (3.2)	2.5 (0.3)	61.5	0.179 (0.047)	0.210 (0.056)
3. *P. yunnanensis*	104.8 (0.8)	2.2 (0.3)	46.2	0.158 (0.050)	0.169 (0.057)

[a] A locus was considered polymorphic if the frequency of the most common allele did not exceed 0.95
[b] Unbiased estimate (Nei 1978)

Table 5. Fixation indices (F) in embryos of *P. tabulaeformis* (1), *P. densata* (2), and *P. yunnanensis* (3)

Locus	Population		
	1	2	3
Pgm-1	0.071	0.070	−0.044
Fes	0.187	0.319	0.240
Lap-1	–[a]	0.221	–
Lap-2	0.070	0.104	0.022
Got-1	−0.029	0.053	−0.004
Got-2	0.044	–	−0.146
Got-3	0.067	0.031	0.016
Sdh-1	0.540	0.098	–
Sdh-2	–	0.264	–
Mean	0.073	0.095	0.014

[a] A dash indicates that F values were not calculated if the frequency of the most common allele was higher than 0.95

Table 6. Gene diversity estimates (Nei 1973) within and among populations of *P. tabulaeformis, P. yunnanensis,* and *P. densata*

Locus	H_T	H_S	D_{ST}
Pgm-1	0.298	0.288	0.010
Pgm-2	0.014	0.014	0.000
Mdh-1	0.062	0.062	0.000
Mdh-2	0.034	0.034	0.000
Fes	0.673	0.642	0.030
Lap-1	0.088	0.083	0.005
Lap-2	0.254	0.242	0.012
Got-1	0.188	0.188	0.000
Got-2	0.130	0.123	0.006
Got-3	0.517	0.393	0.124
Sdh-1	0.558	0.362	0.195
Sdh-2	0.050	0.047	0.003
Mean	0.239	0.207	0.032

H_T=total gene diversity; H_S=gene diversity within populations; D_{ST}=gene diversity between populations

The values of unbiased estimates of genetic distance (Nei 1978) ranged from 0.026 to 0.104. The largest genetic distance (0.104) was found between *P. tabulaeformis* and *P. yunnanensis*. The distance between *P. densata* and the two putative parents was much lower (0.037 between *P. tabulaeformis* and *P. densata*; 0.026 between *P. densata* and *P. yunnanensis*).

Discussion

Despite extensive research on allozyme variation in conifers, there are few reports concerning *Pinus* species from Asia (Szmidt 1982; Huang et al. 1988; Krutovskii et al. 1988; Shiraishi 1988; Wang et al. 1990). Levels of allozyme variation and polymorphism found in single populations of *P. tabulaeformis*, *P. densata*, and *P. yunnanensis* analyzed in this study were similar to those reported for other conifers (see Loveless and Hamrick 1984; Govindaraju 1988, for summaries). Since only one population of each species was analyzed, the observed levels of allozyme variation may not be representative of the species. Nevertheless, from our data it appears that *Pinus* species from Asia are at least as differentiated as those from Europe or North America.

The pattern of allozyme variation observed in this study was also similar to that reported for most other conifers (Loveless and Hamrick 1984). Millar et al. (1988) analyzed the apportionment of gene diversity at 32 loci among *Pinus* species from subsection Oocarpae, and found that 24% of the total variation was distributed among species. The level of differentiation among the investigated species from subsection Sylvestres (13.4%) was smaller than that reported by these authors, but usually larger than estimates from other studies where different populations of the same species were compared (Wheeler and Guries 1982; Wheeler et al. 1983; Cheliak et al. 1988; Huang et al. 1988).

Genetic distance measures found in this study were similar to measures reported among other conifers (Szmidt 1982; Millar et al. 1988; Wheeler et al. 1983; Yeh and Arnott 1986), but were lower than those reported among *Pinus* species from different sections (Karalamangala and Nickrent 1989). From our results, it appears that the levels of genetic divergence among the investigated species are similar to those observed among species of other conifers.

The fertility of interspecific hybrids of *Pinus* species suggests that hybridization may play an important role in the evolution of this genus (Mirov 1967; Christensen 1987). There is mounting biochemical and molecular evidence that evolution of at least some conifer species has been affected by this process (Copes and Beckwith 1977; Prus-Glowacki and Szweykowski 1979; Wheeler and Guries 1987; Wagner et al. 1987; Szmidt et al. 1988; Sigurgeirsson et al. 1990). The usual occurrence in speciation is ecological expansion involving adaptation to new environments (Anderson and Stebbins 1954; Lewontin and Birch 1966). The ecological range of any species is the result of an evolutionary process involving more or less profound genetic changes. It appears that the history of *P. densata* conforms well to such an evolutionary scenario.

We demonstrated recently that *P. tabulaeformis* and *P. yunnanensis* populations analyzed in the current study can easily be distinguished based on restriction fragment and hybridization patterns of chloroplast DNA (X.-R. Wang and A. E. Szmidt, in preparation). In addition, we found that the investigated population of *P. densata* harbors both *P. tabulaeformis* and *P. yunnanensis* chloroplast DNA. This finding has led us to conclude that

earlier suggestions as to the hybrid origin of *P. densata* were correct. The results obtained in this study agree well with this conclusion. Assuming that *P. densata* is a product of natural hybridization, many of its allozymes should still be present in the two putative parental species. In fact, except for two rare allozymes, all other allozymes occurred in one or both putative parents, although often with different frequencies. On the other hand, despite smaller sample size, as many as five unique allozymes were found in each of the two putative parents. Thus, the observed allozyme composition of *P. densata* could easily be extracted from allozyme polymorphism already present in *P. tabulaeformis* and *P. yunnanensis*. The observed higher diversity in *P. densata* as compared to *P. tabulaeformis* and *P. yunnanensis* may be the result of hybridization between two genetically distinct parental populations.

Further evidence suggesting the hybrid origin of *P. densata* comes from the nearly equidistant position of this taxon from *P. tabulaeformis* and *P. yunnanensis*. The smaller distance from *P. yunnanensis* than from *P. tabulaeformis* conforms to the patterns of geographic distribution of the former species. To date, *P. yunnanensis* occurs sympatrically with *P. densata* at higher elevations in the Yunnan Plateau, where the two species can possibly still exchange genes to some extent.

A slight homozygote excess at the seed stage is usually expected for a mixed-mating system (selfing and outcrossing), which is typical for conifers (Shaw and Allard 1982; Muona and Szmidt 1985; Szmidt and Muona 1985; Plessas and Strauss 1986; Cheliak et al. 1988). The excess of homozygosity found in the investigated embryos may result from the family structure causing mating among relatives, positive assortative mating, and selection for homozygotes. In the case of *P. densata*, it can also result from the variance in allozyme frequencies among the sub populations from which the embryo genotypes were drawn (Wahlund 1928). Unfortunately, our data were derived from bulked embryo samples and thus do not allow discrimination between these factors. A more detailed analysis of the genetic structure and mating system in the investigated species is thus required.

A characteristic geographic feature of *P. tabulaeformis, P. densata,* and *P. yunnanensis* is gradual replacement of one species by another from north to south (Wu 1956; Mirov 1967; Guan 1981). *P. tabulaeformis* is widely distributed in colder temperate regions from approximately 32 to 43° N and 102 to 125°E. In some of the most southern parts of its distribution it overlaps horizontally, but not vertically, with *P. densata* (Cheng 1930; Wu 1956; Cheng und Fu 1978). *P. yunnanensis* distribution is limited to the Yunnan Plateau, with the range of 22 to 29°N and 98°30' to 105°E (Li and Liu 1984). It meets *P. densata* in the most northwestern part of its range (Wu 1956; Li and Liu 1984). The natural stands of *P. yunnanensis* occur mainly in warm and humid subtropical regions at 1,500–2,800 m. Vertical distribution of pure *P. densata* stands is always higher than that of *P. tabulaeformis* and *P. yunnanensis* (2,700–3,900 m). In fact, the altitudinal range of this taxon is higher than for any other *Pinus* species occurring in China (Cheng 1930; Wu 1956; Cheng and Fu 1978). In the lower part of its vertical distribution, *P. densata* can overlap with *P. yunnanensis* where the two species form distinct replacement. However, poor growth of *P. yunnanensis* at higher elevations and the absence of *P. densata* at lower elevations suggest discrete adaptation to climate (Guan 1981; Li und Liu 1984). Unlike *P. yunnanensis*, a subtropical forest species, *P. densata* is regarded as a mountain species well adapted to lower temperatures, hence its Chinese name: Gaoshan-song, which means high mountain pine.

From these ecological characteristics and from the results of the present and earlier studies, it appears that the fusion of gene pools from *P. tabulaeformis* and *P. yunnanensis* provided genetic variability for the selection of genotypes better adapted to a relatively harsh environment at high mountain elevations. This fusion of gene pools consequently gave rise to the formation of a new species, *P. densata*. Such a conclusion is consistent with the hypothesis put forward by some authors, which stated that introgression of genes, although not adaptive per se, can lead to rapid adaptive evolution (Anderson and Stebbins 1954; Lewontin and Birch 1966).

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