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Genetic Diversity of *Pinus massoniana* Revealed by RAPD Markers

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Abstract

Random amplified polymorphic DNA (RAPD) markers were used to measure genetic diversity within and among *Pinus massoniana* populations, a multipurpose economic tree species in China. Concordant with previous studies based on allozymes, we also detected high genetic diversity within and low genetic differentiation among populations. Unexpected UPMGA clustering results indicated genetic uniformity throughout South China caused probably by large-scale artificial afforestation. The significant positive correlations between population genetic diversity and its elevation suggested the populations at lower elevation harbor less genetic diversity than those at higher elevation.

Key words: AMOVA; Genetic differentiation; Genetic diversity; *Pinus massoniana*; RAPD.

Introduction

Knowledge on genetic variation of forest tree populations is fundamental for sustainable forest management. However, information on genetic diversity of tree species in China is limited only to few studies.

Pinus massoniana, a conifer native to China, is widely distributed from 21°41' to 33°56' degree latitude and from 102°10' to 123°14' degree longitude, including 17 provinces, growing at elevation up to 1500-1650m. Being one of the most economically important forest trees (e.g., for timber and pulp production), it has been widely used for plantations and afforestation (MEN and LUO, 1987). In order to improve its economic value, many studies have been carried out on quantitative genetic variation of the rate of growth, height, biomass and other characters (LAI and WANG, 1997; WANG, 1993), and high diversity within geographic provenances has been found. The studies on the genetic diversity of *P. massoniana* using allozyme markers also demonstrated high genetic diversity within and low genetic differentiation among populations (GE et al., 1988; HUANG and ZHANG, 2000), however indicating greater differentiation along latitude than altitude gradients (RONG and ZHOU, 1989).

However, allozymes can detect only a limited number of coding regions of genome and its expression might be affected by environmental conditions and different stages of plant development. In contrast, DNA-based markers, such as RAPD (random amplified polymorphic DNA), overcome these disadvantages and are proved to be more powerful than allozyme markers when used to reveal genetic structure and diversity of populations (SZMIDT et al., 1996; WU et al., 1999). In addition, once established, RAPD has the advantage of being quick and easy,

Table 1. – Detailed description of 10 populations of *Pinus massoniana* used for sample collection.

Population locations	Subpopulations	Latitude	Longitude	Altitude (m)	Community type where *P. massoniana* samples collected	Density (/100m^2)
Dinghu Mountain (DH)	DH1	23°08'	112°35'	20	Pure *P. massoniana* forest	14
	DH2	23°08'	112°35'	180	Mixed *P. massoniana* and broad-leaved forest	6
	DH3	23°08'	112°35'	290	Pure *P. massoniana* forest	10
	DH4	23°08'	112°35'	490	Mixed *P. massoniana* and broad-leaved forest	4.5
Conghua (CH)		23°38'	113°40'	150	Pure *P. massoniana* forest	15
Luoke Mountain (LK)	LK1	23°57'	112°32'	200	Pure *P. massoniana* forest	12
	LK2	23°57'	112°32'	520	Mixed *P. massoniana* and broad-leaved forest	4.8
	LK3	23°57'	112°32'	830	Evergreen broad-leaved forest	1.5
Xiaojiang (XJ)		24°37'	112°36'	320	Pure *P. massoniana* forest	22
Hengshan (HS)		26°56'	112°35'	630	Pure *P. massoniana* forest	14

allowing resolution of complex patterns of genetic variation while the DNA sequence information is not available (Lynch and Milligan, 1994).

In *P. massoniana*, RAPD has been successfully used to construct the molecular linkage maps (Yi et al., 1997) and to detect the genetic diversity of three populations in Dinghu Mountain, Guangdong Province, China (Li and Peng, 2001). However, the large-scale genetic diversity of *P. massoniana* populations with DNA-based marker is still unknown. Therefore, in this paper, we have used the RAPD technique to investigate the genetic diversity of *P. massoniana* and provide baseline information for forest management.

Materials and Methods

Sample collection

Ten *P. massoniana* populations were sampled from five locations of Guangdong and Hunan Province in China (*Figure 1; Table 1*). In Dinghu Mountain and Luoke Mountain locations, four and three populations were chosen, respectively, along elevation in order to analyze possible correlation between a population genetic diversity and its altitude. All sampled populations are known as natural and unmanaged by man. For each population, ten individuals with height ≥ 5 m, dbh (diameter at breast height) ≥ 10 cm were chosen randomly.

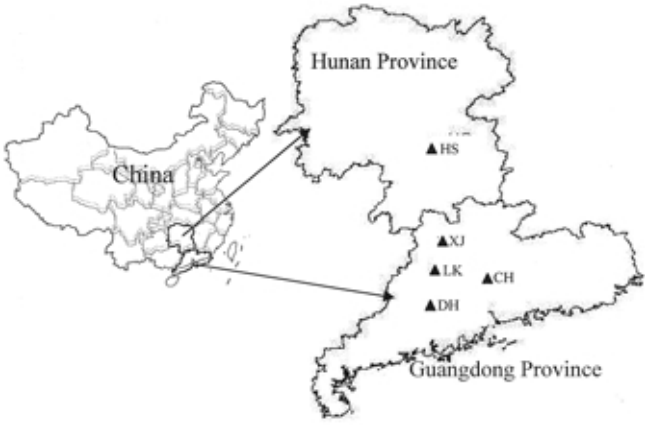

Figure 1. – Locations of studied *P. massoniana* populations. Four sample locations in Guangdong Province China: Dinghu Mountain (DH), Conghua (CH), Luoke Mountain (LK), Xiaojiang (XJ). One sample location in Hunan Province China: Hengshan Mountain (HS).

RAPD analysis

Genomic DNA was extracted from fresh leaf material using a CTAB method modified by Li and Lin (2000). Eighty-four decanuclotides of arbitrary sequence obtained from Sangon Biological Engineering Company (China) were tested for PCR amplification. Nine of them were chosen to assess the genetic variability of the samples: S191 (AGTCGGGTGG), S237 (ACCGGCTTGT), S447 (CAGCACTGAC), S2198 (CTGGCGAACT), S238 (TGGTGGCCTT), S461 (GTAGCACTCC), S226 (AGGCCCAGGT), S446 (CCACGGGAAG), S464 (GTGTCTCAGG).

PCR reactions were performed in volumes of 25 µl containing 1 x PCR buffer, 20 ng DNA, 0.2 µM primers , 0.25 mM dNTP, 4 mM $MgCl_2$, 1 U *Tag* polymerase on Biometra UNW-Thermoblock (Germany). Amplification was performed as followed: two cycles at 94°C for 1 min, 40°C for 30s and 72°C for 1 min.; 43 cycles at 94°C for 30s, 40°C for 20s, 72°C for 1 min and 72°C for 10 min. The amplification products were examined on 1.5% agarose gel in 0.5 x TBE containing 0.5 µg/ml of ethidium bromide, and photographed using Polaroid T667 film.

Data analysis

Nei's unbiased genetic diversity (expect heterozygosity, H_e) was calculated for each population using Popgene software (Version 1.31, Yeh et al., 1997). The same package was used to estimate genetic distance between populations (after Nei, 1978). The distance matrices were then used to produce dendrograms using the UPGMA cluster analysis as implemented in Neighbor from PHYLIP 3.57c software package (Felsenstein, 1993). A hierarchical analysis of molecular variance (AMOVA, Excoffier et al., 1992) was performed to calculate genetic divergence among groups (locations), among populations within groups and within populations, and the significance was tested by 1000 permutations.

Results and Discussion

RAPD analysis indicated the overall genetic diversity of *P. massoniana* was 0.2451. For five locations, the highest genetic diversity was found in Henshan (HS) (0.2620), the lowest, whereas, was in Dinghu Mountain (DH) (0.2116) (*Table 2*). For four subpopulations in DH, the highest genetic diversity was found in DH4 (0.2269), the lowest in DH1 (0.1895). For Luoke Mountain (LK) the highest was 0.2245 in Lk3 (*Table 2*).

Table 2. – Genetic diversity of *P. massoniana* populations.

Population	H_e	Subpopulation	H_e
DH	0.2116	DH1	0.1895
		DH2	0.2116
		DH3	0.2184
		DH4	0.2269
CH	0.2306		
LK	0.2180	LK1	0.2118
		LK2	0.2176
		LK3	0.2245
XJ	0.2417		
HS	0.2620		

The variance components assessed by AMOVA showed that 87.57% genetic variance was within populations, 3.76% among populations within groups, and 8.67% ($P < 0.01$) among groups. This means that the majority of genetic variation is maintained within rather than among populations of *P. massoniana.*

In agreement with previous allozyme results (H_e = 0.2172, GE et al., 1988; H_e = 0.2730, HUANG and ZHANG, 2000), the present study also revealed high genetic diversity as compared to other gymnosperm species (e.g. mean heterozygosity based on allozymes is 0.151, HAMRICK et al., 1992). Considering other pine species studied with the same kind of markers, our results are moderate, higher than for California closed-cone pines (*P. attenuata, P. muricata, P. radiata*) (WU et al., 1999) and *P. sylvestris* (SZMIDT et al., 1996), but lower than for *P. halepensis* (GOMEZ et al., 2001).

The level of genetic differentiation between populations revealed in this paper and earlier allozyme studies tend to be fairly low, in general, consistent with wind dispersed and wind pollinated woody plant species, especially gymnosperms (HAMRICK et al., 1992). However, some pine species with disjunct populations showed more genetic differentiation among populations, for example, 32–34% (RAPD and AMOVA results) for California closed-cone pines (WU et al., 1999).

RAPD and allozymes revealed similar level of genetic diversity reported in other pine species (AAGAARD et al., 1998a). However due to dominant character of RAPDs sampling diploid tissue may lead to slight bias in genetic parameter estimates (SZMIDT et al., 1996; ISABEL et al., 1999). Different sampling size and number of loci detected may also result in different genetic diversity even with the same marker type (SZMIDT et al., 1996; AAGAARD et al., 1998b). For example, in Douglas-fir (*Pseudotsuga menziesii*), RAPD markers of mitochondrial DNA exhibited lower population diversity and higher differentiation than RAPDs of nuclear DNA (AAGAARD et al., 1998b).

UPMGA clustering of populations resolved them into three main groups. Not surprising, four populations, DH1, DH2, DH3 and DH4, in Dinghu Mountain and three populations, LK1, LK2 and LK3, in Luoke Mountain clustered together, indicating limited differentiation within a relative small distribution areas.

Although statistically significant difference was found among groups (locations, see above), a surprising result was that three geographically distant populations, Conghua (CH), Xiaojiang (XJ) and Hengshan (HS) clustered together (*Figure 2*). We suspect that such genetic uniformity could be largely caused by mixing artificial and natural regeneration. *P. massoniana* is a tree species suitable for airplane planting (usually with seeds) and has been widely used for afforestation in southern China effectively (MEN and LUO, 1987). The seeds of *P. massoniana* are light and can be dispersed by wind. Therefore, the artificial seeds might fly a long distance and mix with natural populations.

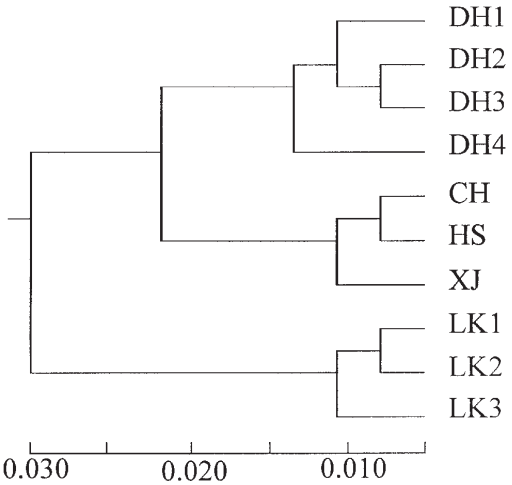

Figure 2. – UPGMA dendrogram based on NEI's unbiased genetic distance.

Populations in Dinghu Mountain and Luoke Mountain, located along elevational gradient showed significant positive correlations between populations genetic diversity and its elevation (correlation coefficient was 0.998 and 0.952, respectively, $P < 0.05$), indicating that populations at lower elevation have less genetic diversity than those at higher elevation.

Such correlations have previously been observed between elevation and cold-resistance ability (WANG, 1993) and chromosome pattern (FANG and LU, 1990) of *P. massoniana*, and all these studies suggested temperature gradients was the main reason.

Further studies are needed to better understand how human-mediated activities influence distribution of genetic variation of *P. massoniana*, which is important for the species genetic conservation and sustainable forest management.

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Nuclear Microsatellite Markers for the Identification of *Quercus ilex* L. and *Q. suber* L. hybrids

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Summary

We report the transfer of a set of nuclear microsatellite markers from *Quercus macrocarpa* Michx. and *Q. petraea* (Matts) Liebl. to *Q. ilex* L. and *Q. suber* L. as a useful tool for further genetic studies on these species. Their specific applicability for the praecox and doubtless identification of *Q. ilex* x *Q. suber* hybrids is also shown. Hybrids were obtained by controlled pollinations on *Quercus ilex* L. with pollen from *Quercus suber* L. trees. This is the first work in which nSSR have been used in *Q. ilex.*

Key words: Q. ilex, Q. suber, microsatellites, hybridisation.

Introduction

Cork oak (*Quercus suber* L.) and especially holm oak (*Quercus ilex* L.) are widely distributed along the Western Mediterranean region, where they share part of their distribution areas and often occur in mixed stands. From ecological point of view, *Q. ilex* L. is considered one of the most important tree species in this region. Its eurioic temperament enables successful settlement in a broad range of different habitats and climates. *Q. ilex* L. is reckoned as a climactic species in extended regions of the Iberian Peninsula, where its acorns are very esteemed for pork feeding. In contrast, cork oak is a more demanding species. It is less resistant to extreme temperatures and lives only in non-calcareous or decarbonated substrates. Cork production makes *Q. suber* L. one of the most important non-timber forest trees in Western Mediterranean region.

Hybridisation between these two species has been proposed as a feasible mechanism in their evolution, as suggested by cytoplasmic DNA studies (BELAHBIB *et al.,* 2001). Since several interspecific barriers have been described (BOAVIDA *et al.,* 2001), hybridisation is not likely a frequent event; however, it has negative effects from an economical point of view, as it implies a decrease in the quality of both cork and acorns, the most important products obtained from these species. Therefore, identification of hybrids in mixed stands could be of great interest, with both scientific and practical implications. At present, this identification is based on morphological criteria, not always reliable on young plants.

Despite ecological and economical interests, few studies have been carried out on the variability and population genetics of these species. Some recent works have focused on the chloroplast genome variation (LUMARET *et al.,* 2002; BELAHBIB *et al.,* 2001, and only few studies utilised nuclear molecular markers, mostly isozymes (JIMÉNEZ *et al.,* 1999; TOUMI and LUMARET, 1998; ELENA-ROSELLÓ and CABRERA, 1996). Isozymes are inexpensive, fast, technically not demanding and reproducible, with high transferability among species, so they are very often used as a first approach in the study of population genetics of a species. However, they are often difficult to score and interpret, and they usually reveal relatively low levels of polymorphism, so they may not be suitable for more detailed studies.

Microsatellites frequently show much higher levels of polymorphism than isozymes; they are codominant and usually considered as neutral markers, so they are suitable for a number of different research problems, including paternity analysis, studies on pollen dispersal, etc. In this paper we report the transfer of six nuclear microsatellites (nSSR) developed in other *Quercus* species to holm and cork oaks. We also present the specific applicability of the set of microsatellites for the praecox and doubtless identification of *Q. ilex* L. and *Q. suber* L. hybrids.

Material and Methods

Eleven holm oak and nine cork oak adult trees were used to test the transferability of nSSR from *Quercus macrocarpa* Michx. and *Q. petraea* (Matts.) Liebl.

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