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Effect of regeneration method on RAPD-based genetic variation of *Cyclobalanopsis glauca* (Fagaceae)

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Abstract *Cyclobalanopsis glauca* is a dominant species of evergreen broad-leaved forests in mainland China. This study compares the genetic variation of an artificially regenerated population with its donor population and two other wild populations, by using RAPD markers. A total of 74 clear, reproducible bands were scored for 12 RAPD primers; 72 were polymorphic (P = 97.3%). AMOVA revealed that most genetic variation was within populations and only 10.35% was among populations. Various measures indicated that there is no difference in genetic diversity between the planted and the original populations. Φ_{ST} between the planted offspring population and the donor population was larger than those between the planted and other two natural populations, indicating that artificial regeneration might lead to biased genetic composition, given that temporal differentiation is usually lower than spatial differentiation. This divergence may be due to unequal seed production among the maternal individuals and viability differences among seeds.

Keywords Regeneration · Genetic diversity · RAPDs · *Cyclobalanopsis glauca*

Introduction

Genetic diversity is the raw material for adaptation, evolution and survival of populations. Since dominant tree species usually play important roles in forest ecosystems, their genetic

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diversity has a specific significance in forest sustainability and ecosystem stability (Rajora and Pluhar 2003). It has been recognized that the long-term consequences of sustainable forest management should not lead to the decline in the genetic diversity of forest species (Montreal Process Working Group 1999). Despite the potential for regeneration – reduced changes in population genetics, little empirical information has been generated concerning the influence of restoration on genetic structure and diversity of forests. Studies examining the impact of forest regeneration on genetic diversity have provided conflicting indications of the impact of regeneration on genetic composition. For example, Knowles (1985) reported that artificial regeneration had no impact on the genetic structure of jack pine and black spruce. Thomas et al. (1999) also found no significant effects between planted and natural stands. In contrast, Gomory (1992) found that planted stands of Norway spruce had significantly less genetic diversity than unharvested or naturally regenerated stands. Even with wide seed collections, inadvertent loss of genetic diversity or shift in allele frequencies may result from selection during seed collection, processing and seedling production (Gomory 1992). Considering the number of tree species that are used in restoration, the impact of regeneration method has been studied only in a limited number of trees and all of these species were commercially important (Gomory 1992; Knowles 1985; Stoehr and El-Kassaby 1997).

Evergreen broad-leaved forests (EBLFs) are the climax vegetation of subtropical areas of eastern Asia (Wu 1980). They were thought to have low commercial value and most of them in China have been eliminated to provide residential areas, paddy fields, tea gardens and plantations of commercially important species, such as *Cunninghamia lanceolata* and bamboos. It is estimated that EBLFs occur only in 4.4% of the land area in east China based on remote sensing data (NOAA-AVHRR NDVI data) (Dr Li, JX, personal communication). In recent years, the central government emphasized that, besides commercially important plantations, ecological plantations should be established. Late-successional species thus are critical for reforestation.

Based on the potential vegetation theory, Miyawaki (1993) proposed a rapid method to restore forests of potential natural vegetation. The potential natural vegetation is the predicted natural vegetation based on the climate and soil conditions without human disturbance (Song 2001), and can be identified by the remaining natural vegetation judged by remaining native trees in the field (Miyawaki 1993). Provided with appropriate conditions by artificial measurements, seedlings of dominant species of potential vegetation are densely planted to accelerate the growth though high mortality rate might be expected. It is reported that forests established by this method can grow into 5–7 m within 4 years (Miyawaki 1998). Since late 1990s, Miyawaki's method was introduced to China to restore ''approx-natural forests'' (Wang and Chen 1999). In 2000, a forest about 3000 m^2 was set up in Shanghai. The forest is mainly constituted by indigenous species, such as *Cyclobalanopsis glauca*, *C. myrsinaefolia*, *Castanopsis sclerophylla* and *Machilus thunbergii*.

Cyclobalanopsis glauca is a widely distributed species of East Asia. It is a medium-sized evergreen tree, capable of attaining heights of 25 m and a DBH of 0.8 m. The species exhibits wide ecological amplitude and is a dominant species in evergreen broad-leaved forests or mixed evergreen and deciduous broad-leaved forests in mainland China (Wu 1980). Outcrossing rate was estimated to be 0.332 based on allozymes (Chen and Song 1997). Allozymic analysis indicated that *C. glauca* had moderate genetic diversity (H_e = 0.2252, P = 50%) and small differentiation among populations (G_{ST} = 5.6%) (Chen et al. 1997).

Miyawaki's method aims to restore forests as close as possible to the natural ones. Besides the species composition of a planted forest, genetic variation of the dominant species is an important aspect to evaluate the similarity. The objective of the present paper is to study the effects of artificial regeneration on genetic diversity of a planted *C. glauca* population relative to the donor and other wild populations.

Materials and methods

Study sites and sample collection

A forest built according to Miyawaki's method is located in Pudong New District, Shanghai Municipality. *Cyclobalanopsis glauca* is one of the dominant species of the native EBLFs in the studied region (Wu 1980). Seeds of *C. glauca* were collected from more than 400 trees of a natural population located in Western Huangshan of Anhui Province in October of 1999, and were germinated in greenhouses. When seedlings reached about 10 cm in height, they were transferred into small pots with a diameter of about 10 cm and each pot contained one seedling. In June of 2000, about 12,350 seedlings of 11 plant species, among them 2500 being *C. glauca*, were densely planted to accelerate the height growth, in a 3000 m^2 afforestation site, Pudong of Shanghai. In 2004, the forest reached the height of about 4 m, there were about 400 *C. glauca* individuals remained (unpublished data), with the mortality rate of about 84%.

In 2004, 35 samples were collected randomly in the restored population. The donor population, Western Huangshan of Anhui Province, and two other wild populations (Hangzhou and Tiantong populations) (Fig. 1) were also sampled to reflect the genetic variation of naturally regenerated populations. In Western Huangshan and Hangzhou, *C. glauca* is the dominant species, whereas in Tiantong *C. glauca* scatters in communities dominated by *Castanopsis fargesii*, *Ca. carlesii*, and *Lithocarpus glaber*, etc. In donor and wild populations, 23–35 samples (Table 3) were collected randomly with a distance between each other at least of 20 m. Two to four fully expanded healthy leaves were sampled. Before silica gel-dried, fresh leaves were cleaned using wet gauze to decrease contamination of fungi and bacteria.

DNA extraction and PCR condition

We extracted DNA for PCR according to a protocol modified from Doyle and Doyle (1987). Leaves (0.05 g) were ground to powder using cold homogenate buffer (100 mmol/l Tris–HCl (pH 8.0), 1.4 mol/l NaCl, 20 mmol/l EDTA (pH 8.0), 0.3% β-mercaptoethanol (V/V) and 2% PVP (W/V)). The sample was then incubated for 5 min on ice and centrifugated for 15 min at 10,000 rpm. The sediment was suspended with 750 µl 65°C 2× CTAB buffer (2% CTAB (W/V) in homogenate buffer) and incubated for 60 min. After two chloroform–isoamyl alcohol extractions, the aqueous phase was collected and digested with RNase A for 30 min. We added equal-volume of chloroform–isoamyl alcohol to the solution followed by centrifugation at 14,000 rpm for 10 min. The nucleic acid was precipitated with cold 70% ethanol and resuspended in TE buffer.

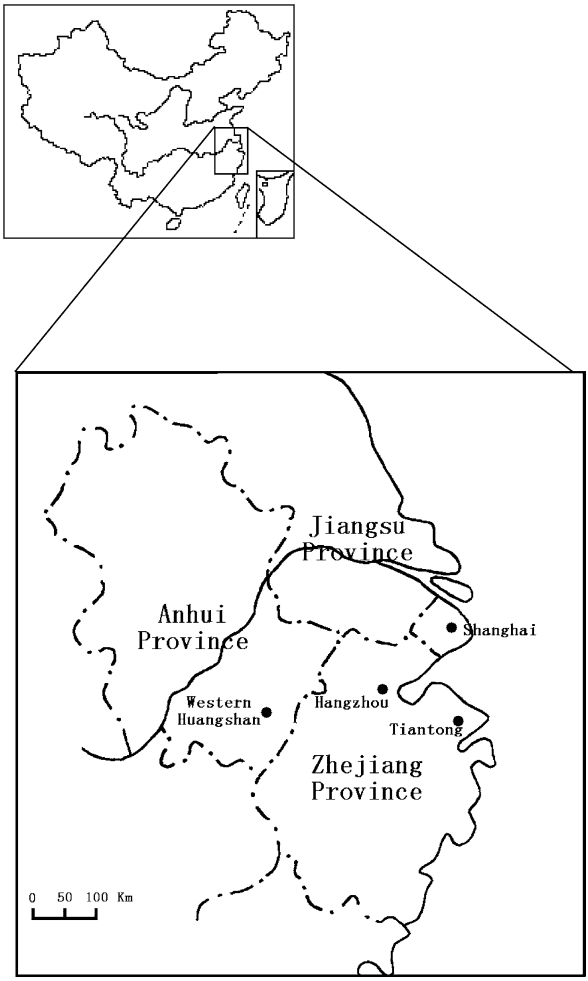

Fig. 1 Localities of sampling sites of *Cyclobalanopsis glauca* populations

A set of random 10-mer primers was purchased from a commercial source (Sagon Inc., Shanghai). After initial tests for more than 150 primers, 12 primers that amplified clear and reproducible banding patterns were used for further studies (Table 1). We performed RAPD assays in 25 μl volume of 10 mM Tris–HCl (pH 9.0), 8 mM $(NH_4)_2SO_4$, 3.0 mM $MgCl_2$, 200 μM dNTPs, 1 U of *Taq* DNA polymerase, 0.8 μM primer and 40 ng template DNA (Fan et al. 2004), using a PTC-220 thermal cycler (MJ Research). Each sample was amplified twice. The amplification product (5 μl) was separated on 1.6% agarose gel in 0.5× TBE buffer and visualized by staining with ethidium bromide and photographed over UV light with Bio-RAD Gel Doc2000™.

Table 1 Sequences of the RAPD primers used in the present study

Primers	Sequences	Primers	Sequences
A07	5′-GAAACGGGTG-3′	B12	5′-CCTTGACGCA-3′
AD13	5′-GGTTCCTCTG-3′	S2090	5′-AAGCGGCCCT-3′
AF05	5′-CCCGATCAGA-3′	S286	5′-AAGGCTCACC-3′
AG01	5′-CTACGGCTTC-3′	S307	5′-GAGCGAGGCT-3′
AN01	5′-ACTCCACGTC-3′	S399	5′-GAGTGGTGAC-3′
B02	5′-TGATCCCTGG-3′	S90	5′-AGGGCCGTCT-3′

Data analysis

The RAPD bands separated on agarose gels were scored and transformed into a binary matrix. Expected heterozygosity (H_e) at population and species level were calculated based on Lynch and Milligan's Taylor expansion estimate (Lynch and Milligan 1994) using TFPGA (Tools For Population Genetic Analyses) v1.3 (Miller 1997). Shannon index (SI) (Lewontin 1972) was calculated to provide a relative estimate on the degree of variation within each population by using the formula: $SI=-\Sigma\, p_i \log_2 p_i$, where p_i is the frequency of presence or absence of each RAPD band. SIs were estimated by Popgene 1.31 (Yeh et al. 1999).

Genetic variation within and among populations was determined by analysis of molecular variance (AMOVA) (Excoffier 1993). AMOVA-PREP version 1.01 (Miller 1998) was used to construct a data matrix for AMOVA. Φ_{ST} statistics, analogous to Wright's F_{ST}, were generated. The significance level for Φ_{ST} was determined using 1000 bootstrap replicates.

Results

A total of 74 clear and reproducible bands were scored for the 12 RAPD primers. Among these, 72 were polymorphic (overall polymorphism=97.3%). Each individual of the four populations has a unique multilocus genotype. No population-specific band was observed in the data set. Compared with the donor population, there were 11 polymorphic bands absent in the planted population. Nine polymorphic bands in the planted population were not observed in the donor population.

The polymorphism for individual populations ranged from 73.0 to 78.4% (Table 2). Polymorphism in the Shanghai planted population was not significantly lower than in the donor population (Western Huangshan) and the other two wild populations (Hangzhou and Tiantong populations). Expected heterozygosity (H_e) based on Lynch & Milligan's Taylor expansion estimate and Shannon's index averaged 0.3145 and 0.4445, respectively. Means of H_e and Shannon's index were 0.3211 and 0.4526, respectively. Due to large variance of those estimators based on RAPD data, the two measurements of genetic variation of the planted population were not significantly lower than those of the naturally regenerated populations (Table 3).

AMOVA indicated that most variation was apportioned within populations. About 10% of variation was partitioned among populations (Table 4, $P<0.01$). Pair-wise comparisons of Φ_{ST} values were small, but significant ($P<0.001$), ranging from 0.0557 to 0.1456 with a mean of 0.1032 (Table 5). All the pair-wise Φ_{ST} values were statistically significant. Φ_{ST}

Table 2 Number and percentage (in brackets, %) of polymorphic bands amplified with 12 primers for the *Cyclobalanopsis glauca* populations

Primers	Number of amplified bands	Shanghai[a]	Huangshan	Hangzhou	Tiantong	Total
A07	5	5 (100.0)	4 (80.0)	4 (80.0)	4 (80.0)	5 (100.0)
AD13	6	4 (66.7)	5 (83.3)	4 (66.7)	5 (83.3)	6 (100.0)
AF05	6	4 (66.7)	3 (50.0)	5 (83.3)	3 (50.0)	6 (100.0)
AG01	8	7 (87.5)	7 (87.5)	7 (87.5)	7 (87.5)	8 (100.0)
AN01	8	5 (62.5)	7 (87.5)	6 (75.0)	7 (87.5)	8 (100.0)
B02	4	3 (75.0)	2 (50.0)	3 (75.0)	2 (50.0)	4 (100.0)
B12	5	5 (100.0)	4 (80.0)	5 (100.0)	5 (100.0)	5 (100.0)
S2090	7	4 (57.1)	5 (71.4)	6 (85.7)	6 (85.7)	7 (100.0)
S286	8	5 (62.5)	7 (87.5)	5 (62.5)	6 (75.0)	8 (100.0)
S307	7	7 (100)	5 (71.4)	6 (85.7)	6 (85.7)	7 (100.0)
S399	5	2 (40.0)	4 (80.0)	3 (60.0)	4 (80.0)	5 (100.0)
S90	5	3 (60.0)	3 (60.0)	3 (60.0)	3 (60.0)	3 (60.0)
Average	6.17	4.50 (73.2)	4.67 (74.1)	4.75 (76.8)	4.83 (77.1)	6.00 (96.7)
Total	74	54 (73.0)	56 (75.7)	57 (77.0)	58 (78.4)	72 (97.3)

[a] Population Shanghai is planted in 2000 and population West Huangshan is the donor population

Table 3 Expected heterozygosity (H_e) and Shannon's index (SI) of the four populations of *Cyclobalanopsis glauca*

[a] Population Shanghai is planted in 2000 and population West Huangshan is the donor population

Location	Latitude	Longitude	Sample size	H_e	SI
Shanghai[a]	N31°13.143′	E121°32.302′	35	0.2946	0.4204
Western Huangshan	N30°11.314′	E118°03.584′	35	0.3122	0.4432
Hangzhou	N30°14.790′	E120°05.822′	35	0.3276	0.4636
Tiantong	N29°48.522′	E121°47.806′	23	0.3234	0.4509
Average			32	0.3145	0.4445
Total			128	0.3680	0.5007

Table 4 Analysis of molecular variance (AMOVA) for populations of *Cyclobalanopsis glauca*

	d.f	Sum of squares	Mean squares	Variance	% of total variance	*P*
Among populations	3	113.8208	37.940	0.9395	10.35	<0.01
Within populations	124	1009.5230	8.141	8.1413	89.65	
Total	127	1123.3437				

between the planted and donor population was larger than those between planted and other wild populations (Table 5).

Discussion

In comparison with allozyme and microsatellite markers, RAPDs have some limitations, such as dominant allelic expression, occasionally low reproducibility, and relatively limited literature compared to that of allozyme markers (Lee et al. 2002). However, due to its advantages, such as randomly detecting variation in the whole genome, higher levels of polymorphism than allozyme analysis, and faster and easier analysis than microsatellites, RAPDs have been widely used in studies of plant populations (Nybom 2004; Nybom and Bartish 2000).

Table 5 Pair-wise Φ_{ST} values calculated by AMOVA

	Shanghai[a]	Western Huangshan	Hangzhou	Tiantong
Shanghai[a]	–			
Western Huangshan	0.1368	–		
Hangzhou	0.0557	0.1099	–	
Tiantong	0.0996	0.1456	0.0717	–

All the Φ_{ST} values computed were significantly larger than a random Φ_{ST} value ($P<0.001$)

[a] Population Shanghai is planted in 2000 and population West Huangshan is the donor population

RAPD analysis reveals that *Cyclobalanopsis glauca* has relatively high within-population genetic variation compared to other species. The percentage of polymorphic loci of *C. glauca* ranges from 73.0 to 78.4%. Our values are slightly higher than reported in other common tree species, such as *Populus tremuloides* (Stevens et al. 1999) and *Plathymenia reticulata* (Lacerda et al. 2001). The H_e of *C. glauca* was higher than the means for dicotyledons (0.191), long-lived perennials (0.242), and widespread (0.208), mixed (0.219), gravity-dispersal (0.212) or late-successional species (0.287) (Nybom and Bartish 2000). RAPD-based genetic diversity of *C. glauca* was higher than allozyme-based diversity (Chen et al. 1997), as also observed in other species (Nybom and Bartish 2000). Among-population genetic variation was small, as indicated by the Φ_{ST}s and AMOVA, but slightly larger than the G_{ST} (5.6%) based on allozyme markers (Chen et al. 1997).

The planted population is an offspring population of the donor. Many studies have revealed that the temporal differentiation was much lower than spatial differentiation (Chen et al. 2003; Linhart et al. 1981; Ueno et al. 2002). However, Φ_{ST} showed that the planted *C. glauca* population was more similar to other wild populations than to the donor population. These results indicate that biased genetic composition might occur during the ecological restoration. Biased genetic composition in newly founded populations had been observed in many systems (Frankham et al. 2002; Gugerli et al. 2001; Ledig 2000). In the present study, the difference between plantation and its donor population might be the result of unrepresentative seed collection. Although the seeds were collected as widely as possible from the donor population, unequal seed production among the maternal individuals and viability differences among seeds might lead to changes in gene frequencies in the offspring population.

It is interesting that, compared to the donor population, 11 polymorphic bands were absent and nine new bands were observed in the planted population. There are two sources contributing to the difference. Firstly, sampled individuals of the donor population in our study are not exactly the same as those for collecting seeds. Secondly, even if all the individuals for seed collecting were sampled to analyze the genetic composition, seed may be more diverse than the maternal trees because pollen from other populations also contribute to the gene pool of the seed (Morris et al. 2002).

Compared with the donor and other natural populations of *C. glauca*, no significantly lowered genetic variation was found in the restored population. A reduction of genetic diversity in artificial regeneration relative to unharvested control or natural regeneration has been observed in some other species, including *Picea glauca* (Gomory 1992; Rajora 1999), *Scaphium macropodum* (Ratnam et al. 2000), and *Eucalyptus sieberi* (Glaubitz et al. 2003). *Metasequoia glyptostroboides* has been widely planted though its natural distribution is very narrow. Lower genetic diversity was found in planted than in naturally regenerated populations (Li et al. 2005). However, no reduction of genetic diversity in

planted populations were found in many studies, such as jack pine and black spruce (Knowles 1985), lodgepole pine (Thomas et al. 1999), *Abies amabilis* and *Tsuga heterophylla* (El-Kassaby 2000). Diverse results of artificial regeneration on genetic composition of plant populations are related with the founder effects and regeneration methods (Montalvo et al. 1997). For example, vegetative regeneration, such as cutting, may lead to low diversity in the artificial populations (Chen 1999).

Consequence of decreased genetic variation in restored populations varies depending on the life history as well as isolation from other populations. For species of inbreeding or of short pollen dispersal distance, a reduction in genetic variation led by founder effect and isolation from other populations might decrease the fitness in the restored population. For example, low genetic variation might explain the failure in restoring seagrass beds (Williams and Orth 1998). In *C. glauca*, it might be another situation. No apparent consequence might be observed in the restored population because *C. glauca* is a wind-pollinated species with the mixed mating system (t=0.332) (Chen and Song 1997), usually having high gene flow. Furthermore, the distance of the restored population to the nearest natural population is about 50 km and it is not a gene flow barrier for *C. glauca*. However, nevertheless, the populations near the restored site are preferable donor than other populations due to potential local adaptation (Hufford and Mazer 2003; Lesica and Allendorf 1999).

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