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ISSR-BASED GENETIC DIVERSITY OF WILD AND *EX SITU* CONSERVED POPULATION OF *HOPEA CHINENSIS* (MERR.) HAND.-MAZZ

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Abstract

The genetic diversity and structure of 4 wild population and 2 *ex situ* conserved population of *Hopea chinensis* (Merr.) Hand.-Mazz were analyzed and compared by ISSR marker method. Fifty eight bands were amplified from total genomic DNA with 10 primers in which there were 51 polymorphic, the percentage of polymorphic bands of populations ranged from 53.45 to 79.31, with an average value of 69.82, indicating the presence of high genetic diversity. However, the percentage of polymorphic band, Nei's diversity index (He) and Shannon information index (I) of wild populations (86.21, 0.3636 and 0.5232, respectively) were higher than those of *ex situ* conservated populations (77.59, 0.3153 and 0.4597, respectively), which meant that *ex situ* conservations of *Hopea chinensis* had a narrow genetic diversity. Population structure analysis revealed that genetic variation of *Hopea chinensis* was 0.0373, so the genetic differentiation was 3.73% among populations, and 96.27% in populations. Furthermore, the strong gene flow (Nm = 12.8896) would be the main reason of limited population differentiation. It concluded that *ex situ* population had not yet fully covered the entire genetic diversity of *Hopea chinensis*.

Introduction

Hopea chinensis (Merr.) Hand.-Mazz, a tertiary relict plant unique to China, is distributed only in Daqingshan Mountain and Shiwandashan Mountain (both located in China's southern province Guangxi) (Xu and Yu 1982). It commonly grows on low mountain and in valley, regarded as one of the typical tree species in evergreen monsoon forest of southern Guangxi (Wang *et al.* 1994). It is also one of the most valuable timber tree species in Guangxi for its wood has a great economic value in architecture, shipbuilding and furniture manufacturing. However, *Hopea chinensis* is being destroyed at an alarming rate due to serious ecological environmental destruction, and has been listed as the first degree of national protection plant (Yu 1999).

In recent years, global biodiversity declined drastically, lots of plant species are estimated to be threatened with extinction (IUCN 2006). *Ex situ* conservation, as an efficient approach with the main goal of maintaining optimum genetic diversity for rare and endangered plant species has been adopted worldwide (Frankham *et al*. 2002). From 1990s, *ex situ* conservation program began to carry out for *Hopea chinensis* (Zhang *et al.* 2001), and two *ex situ* methods of conservation have been established in Guilin city, Guangxi Province and in Xisuangbanna County, Yunnan Province. Some individuals have been successfully blossomed and fruited, due to newly adopted methods conservation. Research on *ex situ* conservation of cultivated *Hopea chinensis* is progressing. Most of these studies however, are only focused on observing characteristics of growth and fruitage of *ex situ* conserved populations, not involving conservation genetics (Tang *et al*. 2009, Huang *et al.* 2008, Zhou *et al*. 2013). Studying the genetic characteristics is essential for the establishment of effective and efficient conservation practices for rare plants (Yang *et al.* 2010). Molecular markers such as inter-simple sequence repeats (ISSR) have been proposed an

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economical and reliable DNA marker system (Zietkiewicz *et al*. 1994), and widely used for analyses on genetic diversity and phylogenetic relationship in multiple species (Zhou *et al*. 1999, Culley *et al*. 2007, Zhang *et al*. 2008).

In this study, the main objectives were to discriminate and evaluate the genetic diversity and structure of *Hopea chinensis* from 4 wild populations and 2 *ex situ* conserved populations by using ISSR molecular marker technology. Our study may have important implications for further work on *ex situ* conservation.

Materials and Methods

Leaf samples of *Hopea chinensis* were originally collected from 4 wild populations (Naqin, Fulong, Nasuo and Gongyuan) in Shiwandashan mountain, Fangchenggang city, Guangxi Zhuang Autonomous Region, China, and 2 *ex situ* conserved populations (Guilin botanic garden and Xishuangbanna botanic garden), China (Table 1). Each wild population was represented by 20 individuals. For *ex situ* conserved populations, only 20 samples were obtained, due to their tiny habitat.

Table 1. Profiles of sampling population.

Population		Latitude	Altitude /m	Sample number
Wild population	Naqin (NQ)	N21°52′ E108°01′	180	20
	Fulong (FL)	N21°50′ E107°55′	193	20
	Nasuo (NS)	N21°45′ E108°06′	60	20
	Gongyuan (GY)	N21°54′ E107°54′	284	20
Ex situ conservated populations	Guilin (GL)	N25°01′ E110°17′	170	10
	Banna (BN)	N21°41′ E101°25′	570	10

Leaves were desiccated in silica gel before DNA extraction. Total genomic DNA was extracted following the modified CTAB method described by Dai *et al*. (2011). The concentration of DNA was estimated by spectrophotometer and the quality was checked using 0.8% agarose gel electrophoresis.

ISSR primers published by the University of British Columbia (UBC, Zuo *et al*. 2001) were used. One hundred ISSR primers were initially screened, of which 10 yielded bright and discernible bands were selected to further analyze genetic diversity of our samples. ISSR amplifications were performed in a 25 μl reaction volume containing 1 × PCR buffer, 2.0 mM Mg^{2+}, 0.25 mM dNTP, 0.4 U Taq polymerase, 0.2 μM primer, and 4 ng template DNA. The PCR reaction was programmed as follows: initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 45 s, primer annealing at 53°C for 45 s, extension at 72°C for 1.5 min, and a final extension at 72°C for 7 min (Dai *et al*. 2011). Amplification products were separated in 1.5% agarose gels buffered with 1 × TAE and stained with ethidium bromide.

ISSR profiles were scored for each individual as the presence (1) or absence (0) of specific bands and entered into a binomial matrix. A measure of intra- and inter-population genetic statistics were then calculated by "POPGENE32" (Yeh *et al*. 1999), including the percentage of polymorphic bands (PPB), number of alleles (Na), effective allele number (Ne), total genetic diversity (Ht), intra-population genetic diversity (Hs), Nei's gene diversity (He) and Shannon's information index (I) and the relative magnitude of genetic differentiation among population (Gst = Ht-Hs/Ht) (Nei and Li 1979). Gene flow was estimated using the formula: Nm = (1-Gst)/4Gst (Slatkin and Barton 1989). A dendrogram was constructed to evaluate the genetic relationship for

these populations by the unweighted pair group method with arithmetic average (UPGMA), based on the average genetic distances, with the SAHN module of NTSYS-pc 2.10 (Rohlf 2000).

Results and Discussion

Ten ISSR primers chosen for analysis produced a total of 58 bands, of which 51 were polymorphic, the percentage of polymorphism of the amplified products was 87.93 (Table 2). The number of bands per primer ranged from 4 to 7 with the average number of bands per primer being 5.8. The size of DNA fragments ranged from 400 to 2000 bp. The genetic diversity of the 6 populations showed that the percentage of polymorphic bands ranged from 53.45 to 79.31 with a average of 69.82 between populations. The ISSR patterns of PCR amplified by primer UBC825 is presented in Fig. 1.

Table 2. The sequences and amplifying results of 10 ISSR primers.

Primer code	Primers sequence	No. of amplified bands	No. of polymorphic bands
UBC811	$G(Ag)_7AC$	5	5
UBC813	$C(TC)_7TT$	5	5
UBC815	$C(TC)_7Tg$	6	3
UBC818	$C(AC)_7Ag$	6	6
UBC825	$A(CA)_7CT$	7	7
UBC827	$A(CA)_7Cg$	7	7
UBC841	$G(Ag)_7AYC$	4	0
UBC857	$A(CA)_7CYg$	5	5
UBC886	$VDVC(TC)_6T$	6	6
UBC890	$VHVg(Tg)_6T$	7	7
Total	/	58	51
Average	/	5.8	5.1

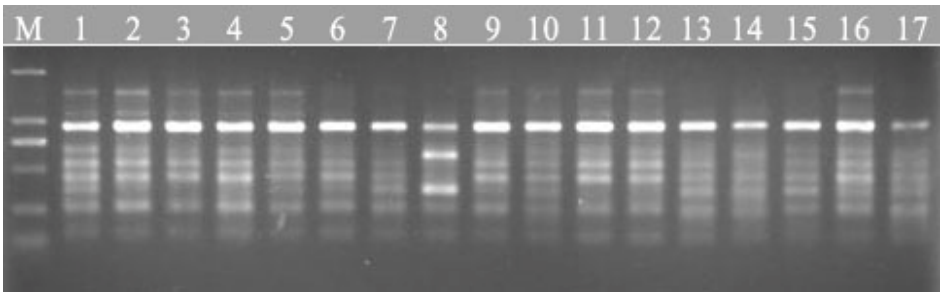

Fig. 1. Electrophoresis picture of PCR products from primer UBC825. Lane 1 - 10 and 11 - 17, were the individuals of *Hopea chinensis* in Guilin and Xishuanbanna, respectively, M indicates DNA marker.

The number of alleles (Na) per population ranged from 1.5345 to 1.7931, with an average of 1.6983. The effective number of alleles (Ne) ranged from 1.4487 to 1.6737, with an average of 1.6671. At the species level, the percentage of polymorphic bands (PPB), Nei's diversity index (He) and Shannon's information index (I) were 87.93, 0.3637 and 0.5242, respectively. Among six populations, the Shannon diversity index (I) averaged 0.4516 with a range from 0.3431 to 0.5126.

He and I together showed that the corresponding variation order of the populations was Fulong (FL) > Gongyuan (GY) > Nasuo (NS) > Xishuangbanna (BN) > Naqin (NQ) > Guilin (GL). The percentage of polymorphic bands (PPB), Nei's gene diversity (He) and the Shannon diversity index (I) of these populations had the same tendency, except Naqin (NQ) and Xishuangbanna (BN). These indexes indicated that wild populations had a larger genetic diversity compared with to *ex situ* conservated populations (Table 3).

Table 3. Genetic diversity among six populations of *Hopea chinensis*.

Population		Na	Ne	He	I	Polymorphic bands	poly-morphism/%
Wild population	NQ	1.7069	1.5729	0.3120	0.4466	41	70.69
	FL	1.7931	1.6737	0.3608	0.5126	46	79.31
	NS	1.7241	1.6251	0.3319	0.4704	42	72.41
	GY	1.7414	1.6459	0.3428	0.4851	43	74.14
	Total	1.8621	1.6688	0.3636	0.5232	50	86.21
Ex situ conservated populations	GL	1.5345	1.4487	0.2411	0.3431	31	53.45
	BN	1.6897	1.5983	0.3193	0.4519	40	68.97
	Total	1.7759	1.5597	0.3153	0.4597	45	77.59
Average		1.6983	1.5941	0.3180	0.4516	40.5	69.82
Species level		1.8793	1.6671	0.3637	0.5242	51	87.93

The total gene diversity (Ht), genetic diversity within populations (Hs) and the genetic differentiation coefficient (Gst) among 6 populations indicated that the genetic diversification intra-population was far greater than that of inter-populations, and gene flow (Nm = 12.8896) was high among populations of *Hopea chinensis* (Table 4).

Table 4. Genetic diversity and differentiation parameters between wild populations and *ex situ* conserved populations.

Population	Ht	Hs	Gst	Nm
Group of wild populations	0.3636	0.3369	0.0735	6.3020
Group of *ex situ* conservated populations	0.3153	0.2802	0.1112	3.9974
Total	0.3610	0.3475	0.0373	12.8896

The Nei's genetic distance (D) and genetic similarity coefficient among six populations were shown in Table 5. The genetic similarity coefficient ranged from 0.8957 to 0.9554, and the Nei's genetic distance (D) ranged from 0.0456 to 0.1101, with the farthest (0.1101) being between Guilin (GL) and Nasuo (NS), and the nearest (0.0456) being between Gongyuan (GY) and Nasuo (NS). An inverse relationship was found for genetic similarity coefficient. The average genetic distance among *ex situ* conservated populations (D = 0.0922) was higher than that among wild populations (D = 0.0550), which indicated a certain variation seems to be happening in *ex situ* conservated populations.

Table 5. Genetic similarity coefficient (above diagonal) and genetic distance (below diagonal) of *Hopea chinensis.*

Population	NQ	FL	NS	GY	GL	BN
NQ	****	0.9494	0.9483	0.9426	0.9016	0.9086
FL	0.0519	****	0.9402	0.9427	0.9221	0.9305
NS	0.0530	0.0616	****	0.9554	0.8957	0.9261
GY	0.0591	0.0590	0.0456	****	0.9105	0.9078
GL	0.1036	0.0811	0.1101	0.0937	****	0.9039
BN	0.0959	0.0720	0.0768	0.0967	0.1010	****

NQ, FL, NS and GY corresponding to 4 wild populations, BN and GL corresponding to 2 *Ex situ* conservated populations listed in Table 1.

The cluster results showed that populations with lower genetic distance (GY and NS) were grouped together, and then grouped together with NQ and FL. In addition, BN and GL formed independently group (Fig. 2). Indicated the genetic variation inter-population was consistent with its geographic distribution.

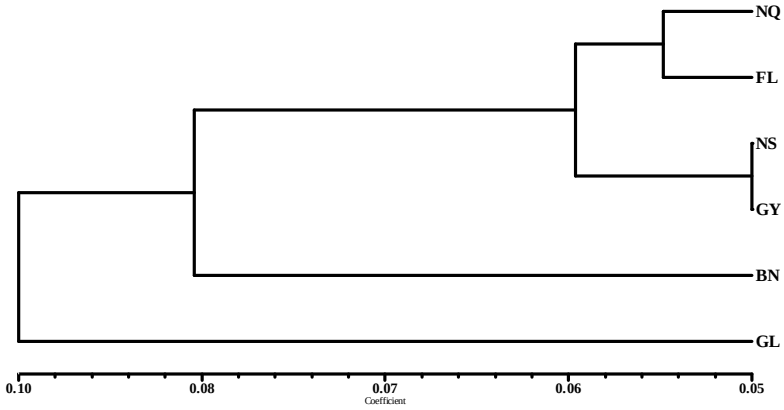

Fig. 2. UPGMA dendrogram of six pupulations of *Hopea chinensis*. NQ, FL, NS and GY corresponding to 4 wild populations, BN and GL corresponding to 2 *ex situ* conservated populations listed in Table 1.

In conclusion, knowing the levels of genetic diversity is significant for planning conservation strategies for rare and endangered species. Based on ISSR in this study, the wild populations was found to have a high genetic diversity (PPB = 77.59%, H = 0.3153, I = 0.4597). However, relatively lower genetic diversity was found among *ex situ* conservated populations (PPB = 86.21%, H = 0.3636, I = 0.5232) compared with the wild populations. This might be resulted from the small population size of *ex situ* conservation, in which the individuals only covered a limited set of wild population resources. It meant though the introduced *Hopea chinensis* had been proved to grow and fruit normally in these two *ex situ* methods conservation, but it is still not sufficient, especially on genetic diversity. The mean Gst value (0.0373) across all populations indicated that only 3.73% of the total genetic variation resides among populations of this species, it suggested that develop a conservation plan could focuses on representative populations with the greatest genetic diversity. As an example from this study, the population from Fulong (FL) has a genetic

diversity greater than that of the other populations, and it has diverse genotypes within population, thus, it should be a priority to consider its germplasm resources. Therefore, in order to conserve most genetic diversity of *Hopea chinensis*, further efforts should be made to strengthen on introduction for these two *ex situ* conservation. Our results indicated that seedlings introduction from Fulong (FL) population should be considered as a first option. It is also advisable to mix seeds or live plants collected from different populations for cross-breeding. These combinations might have potential for enhancing the genetic diversity of *Hopea chinensis*.

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