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Full Length Research Paper

Genetic diversity analysis among and within populations of Vatica mangachapoi from China with RAPD and AFLP makers

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Genetic diversity and relationships of five populations of Vatica mangachapoi, which was overexploited for rubber, banana, pineapple and lichi planting by the civilian in Hainan Island, China, were investigated by Random amplified polymorphic DNA (RAPDs) and Amplified fragment length polymorphism (AFLP) method. A total of 354 highly reproducible and discernible loci were generated from 100 individuals of 5 natural populations with 20 primers by RAPD, of which polymorphic loci were 73.01%. Nei`s gene diversity was 0.2187 and Shannon information index was 0.3270. With 5 primer combinations by AFLP, 337 highly reproducible and discernible loci (frequency >5.00%) were generated and populations polymorphic loci were 48.83%. Nei`s gene diversity was 0.1956 and Shannon information index was 0.2437. Based on Nei`s Gst value, a large proportion of genetic variation (71.31% by RAPD; 61.73% by AFLP) was resided among individuals within populations, however, only (28.69% by RAPD; 38.27% by AFLP) genetic varience was resoded among populations. UPGMA cluster analysis based on Nei`s genetic distance, the populations in Shimeiwan (SMW) do not form one group by RAPD and AFLP makers. It proved V. mangachapoi Blanco ssp. Hainanensis was not the new variety of V. mangachapoi specie. Further works involving suitable human interferences and afforestation approaches were suggested for the conservation and development of Vatica mangachapoi in Hainan Island, China.

Key words: Vatica mangachapoi, RAPD, AFLP, genetic diversity, conservation biology.

INTRODUCTION

The genus Vatica Linn. is a member of the Dipterocarpaceae family, which consists of 17 genera and approximately 500 species of mainly tropical lowland rainforest trees. Their distribution is pantropical, from northern South America to Africa and Asia, the Seychelles, India, Indochina, Indonesia, Malaysia and China(Ashton P.S,1982; Xiang et al,2008). The greatest diversity of Dipterocarpaceae occurs in Borneo(Ashton P.S,1982, 2004). Some species are now endangered as a result of overcutting, extensive illegal logging and habitat conversion. They provide valuable woods, aromatic essential oils, balsam, resins and are a source for plywood. In China, there are three species belonging to Vatica Linn., V. xishuangbannaensis, V. guangxiensis and V. mangachapoi (Chong et al, 2008). V. mangachapoi can only be found in Hainan Island. Furthermore, it is the only one species and has limited number of individuals (Flora of china,1990; Flora of Guangdong, 2005). Other research also reported one new variety: V. mangachapoi Blanco ssp. hainanensis which was found in Shimeiwan (SMW) coastal V. mangachapoi forest (Fu et al, 2008). But it has no molecular proof rather than morphological evidence. In China, V. mangachapoi played an essential role in the ecosystem of Hainan tropical rain forests. With the high

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Table 1: Population location and sample size

Population location and Code	Latitude(N0	Longitude(E)	Altitude/m	Forest type	Sample size
Shimeiwan(SMW)	18°39′	110°16′	100~175	Coastal rainforest	20
Bawangling(BWL)	19°32′	109°07′	110~530	Seasonal rainforest	20
Sanya(SY)	18°15′	109°15′	25~200	Coastal rainforest	20
Jianfengling(JFL)	18°40′	108°57′	141~685	Coastal rainforest	20
Wengchang(WC)	19°44′	110°41′	23~185	Lowland rainforest	20

adaptability, the separated or concentrated distribution of V. mangachapoi is from the tropical and subtropical coastline to the field of country and hill under 1000m altitude. On the seashore it has functions of withstanding winds and waves and consolidating the sea dikes (Wei et al,2007). But, the population of V. mangachapoi forest has been damaged by human activity, especially by deforestation and cultivation of tropical crop, such as the reclamation for rubber, banana, pineapple and lichi planting. From 1999, V. mangachapoi has been listed in 'National protected key wild plants (the first listing)' in category II in China (Chun et al,2003). The overexploitation of the key wild plants has led to losses of valuable plant resources, which in turn may lead to losses of genetic diversity as well. In order to have a successful V. mangachapoi conservation, breeding program or afforestation scheme, attention needs to be paid to the genetic diversity and structure within and among populations.

Genetic diversity is of special long-term survival and development potential of the physical infrastructure. For the protection of endangered plants, both the ecological features and the genetic diversity, hereditary constitution should be considered, whatever in situ conservation or off-site conservation (Clarke et al, 2000). Extensive fieldwork was used as the basis for studying the characteristics and species diversity of the V. mangachapoi community (Zhang et al, 2007; Li et al , 2001,2006,2008). Based on the morphological and anatomical evidence, chloroplast genes, RFLP（Tsumura et al,1996）, AFLP(Cao et al,2006), microsatellite markers (chadapom et al, 2011) were used to analysis the genetic diversity, phylogeny and biosystematics of Dipterocarpaceae.

Polymerase chain reaction (PCR) technology has promoted the development of a range of molecular assay systems that detected polymorphism at the DNA level. Molecular markers successfully developed during the last two decades have largely overcome the problems that are associated with phenotype-based classification. RAPD, ISSR (Inter simple sequence repeats), AFLP and SSR (Simple sequence repeat), provide DNA markers that are dispersed throughout plant genomes and are easier to reproduce and analyze (Awasthi et al,2004; Kafkas et al, 2006). Among them, RAPD has been the most commonly used method for the easy operation and cost effective way to detect nucleotide sequence polymorphisms using a single primer of arbitrary nucleotide sequence requiring no prior sequence information (Williams et al, 1990). Furthermore, due to the high multiplex ratio (Rafalski et al, 1996) and reproducibility (Jones et al,1997), AFLP is an efficient marker technique for fingerprinting and assessing genetic polymorphisms(Garcia et al, 2004; Xiao et al, 2006; Bouajila et al, 2007; Masum Akond et al, 2008). This marker technique has often been applied to study of genetic variation in forest tree species (Gailing et al, 2004; Castillo-Càrdenas et al, 2005; Tang et al,2008; Huang et al,2008).

The objectives of the present study are: (1) to test whether V. mangachapoi Blanco ssp. Hainanensis is the new variety of V. mangachapoi using molecular makers. (2) to evaluate genetic variation of this endangered plant using Random amplified polymorphic DNA (RAPDs) and Amplified fragment length polymorphism (AFLP) makers in order to provide the baseline information for the development of a conservation program for the specie.

METHODS

Plant materials

The wild species obtained from Shimeiwan, Bawangling, Sanya, Jianfengling, Wenchang in Hainan Island, China. The population locations and sample sizes are presented in Table 1. The fresh leaves were dried quickly by using silica gels in field, and stored at room temperature for further use.

DNA extraction

Genomic DNA was extracted from leaf tissue by the CTAB method of Doyle and Doyle(1987) with minor modifications (Kafkas and Perl-Treves, 2002). Concentration DNA was dissolved in 0.1xTE(1mmol/L Tris-HCL(pH8.0), 0.1mmol/L EDTA(pH8.0)) and subjected to PCR amplification after adjusting concentration.

Table2: Primers sequences used in RAPD amplification

Primer	Sequence
G09	5-GGGTAACGCC-3
G10	5-GTGATCGCAG-3
G18	5-AGGTGACCGT-3
G21	5-GTTTCGCTCC-3
H04	5-GTTGCCAGCC-3
H06	5-GGACCCAACC-3
H07	5-GTCGCCGTCA-3
H10	5-TTGGCACGGG-3
H11	5-GTGTGCCCCA-3
H14	5-AGCGCCATTG-3
H29	5-AGATGCAGCC-3
S82	5-GGCACTGAGG-3
S93	5-CTCTCCGCCA-3
S169	5-TGGAGAGCAG-3
S172	5-AGAGGGCACA-3
S350	5-AAGCCCGAGG-3
S371	5-AATGCCCCAG-3
S387	5-AGGCGGGAAC-3
S393	5-ACCGCCTGCT-3
S399	5-GCGTGGTGAC-3

RAPD amplification

Twenty arbitrarily primers that could amplify reproducible and clear DNA bands were selected from 250 primers (Sheng gong, China) for further amplification (Table 2). PCR reactions were performed according to the protocol of Williams et al, (1990). Briefly, PCR amplifications were carried out in an MJ Research Thermal Cycler (Takara, Japan), in a reaction volume of 20μL containing 20mM Tris-HCl (pH8.4), 50mM KCl, 50mM MgCl2 ,0.2 um primer, 0.1 mM each of dATP, dTTP, dCTP, dGTP, 0.5 U of Taq DNA polymerase (Takara, Dalian). DNA amplification was performed according to the following cycle profile: Initial denaturation at 94℃ for 4 min followed by 40 cycles at 94℃ for 1 min,72℃ for 2 min and a final 10 min extension at 72℃. PCR products were electrophoresed on a 1.5% agarose gel according to Sambrook et al (1989). in 1X TBE buffer (89mM Tris-borate, 2mM EDTA, pH8.0) and stained with golden View (Takara, Dalian).The gel image was recorded using a Gel Documentation System(LED, Sweden).

AFLP amplification

AFLP analysis was performed as originally proposed (Reineke A et al, 2000) with minor modifications. 250ng of DNA were used for each reaction. DNA was digested with 10U EcoRI and 3U of TruI (both enzymes from Takara,Dalian) in buffer recommended by the manufacturer in a total volume of 15ul at 37℃ for 120 min, followed by 120 min at 65℃.10μL of a solution with a final concentration of 5pmol of EcoRI dapter,50 pmol of Tru1I adapter, 1xT4 DNA ligase buffer and 1U T4 DNA ligase (Takara, DaLian) were added to the digested DNA. The solution was incubated at 20℃ for 2h, T4 ligase was inactivated by heating to 65℃ fir 10 min and the mixture was diluted 10-fold with TE buffer. Following ligation, a first amplification was carried out with primers containing one selective nucleotide (cytosine and adenine for MseI and EcoRI primers, repectively), dNTPs (0.125 mM Takara, DaLian), 1xPCR buffer (MBI Fermentas, Germany), 1.5 mM MgCl2 and 1UTaq polymerase(MBI Fermentas, Germany) were added in a total volume of 10ul.PCR was performed for 20 cycles, which consisted of 30s at 94℃,1min at 56℃ and 1min at 72℃ in a Tpersonal thermocycler (Takara, Japan). The PCR products were diluted 10-fold with TE buffer. The second amplification was carried out with five pair of primers listed in Table 2. The PCR mixture consisted of 2ul of diluted preamplified DNA, 4.2 ng of EcoRI primer,11.4 ng of MseI primer 0.25mM, dNTPs (Takara, Dalian), 1xR buffer (Takara,Dalian), 1.5mM MgCl2 and 1U Taq polymerase (Takara, Dalian) in a total volume of 10μL .The thermocycler program consisted to two segments. The first segment comprised 12 cycles with the annealing temperature decreased from 65℃ by 0.7℃ in each cycle: 30S at 94℃,30S at 65℃to 57.3℃ and 1min at 72℃. The second segment sonsisted of 23 cycles of 30S at 94℃,1 min at 56℃ and 1 min at 72℃. The PCR products were mixed with 10μL of loading buffer (98% formamide, 10mM EDTA and 0.025% bromophenolblue), denatured for 4 minutes at 90℃ and 5μL of the mixture were loaded onto a 6% denaturing polyacrylamide gel in a sequencing gel system (LKE, Sweden). The gels were stained with silver nitrate using silver staining kit (Sigma USA). Five pairs of primers were listed in table 3.

Table3: Primers sequences used in amplification

Primer Sequence	Primer Sequence
M3 5′-gat gag tcc tga gta a-CAG-3	E6 5′-gac tgc gta cca att c -ACG
M4 5′-gat gag tcc tga gta a-CAT-3	E2 5′-gac tgc gta cca att c -AAG
M7 5′-gat gag tcc tga gta a-CTG-3	E1 5′-gac tgc gta cca att c –AAC-3
M7 5′-gat gag tcc tga gta a-CTG-3	E2 5′-gac tgc gta cca att c -AAG
M6 5′-gat gag tcc tga gta a-CTC-3	E8 5′-gac tgc gta cca att c -AGG

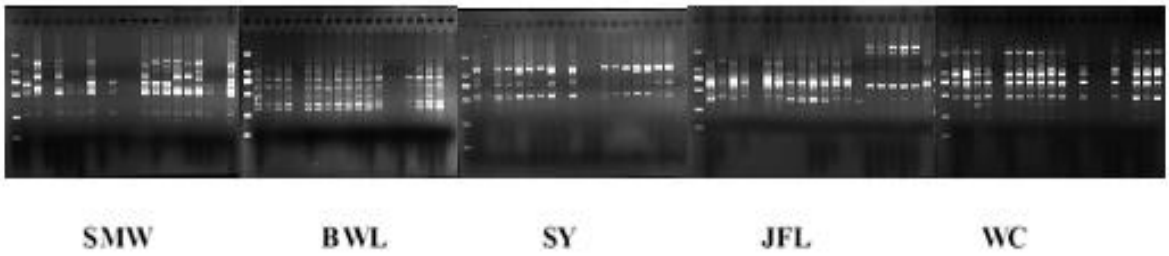

Figure 1. Amplification products of V. mangachapoi using primer S350 on the populations located from Shimeiwan(SMW), Bawangling(BWL), Sanya(SY), Jianfengling(JFL), Wenchang(WC) in Hainan Island, China.

Date analysis

The bands were marked by Quantity One software, and the bands were watched at the same position (molecular weights). Amplified fragments were scored for the presence (1) absence (0) of homologus bands. Binary character matrices were compiled for further analysis. The matrix of the RAPD and AFLP phenotypes were assembled for the following analysis: Percentage of polymorphic loci (P) %, Shannon diversity index (I), Nei's gene diversity(H), Observed number of alleles(Na) by using POPGENE Version 3.01(Yeh et al.,1994). At the species level, genetic diversity measures total gene diversity (Ht), gene diversity within population (Hs), coefficient of gene differentiation (Gst), average gene idrntity (I,) and gene flow (Nm) were also analyzed. An unweighted pairgroup method arithmetic averages (UPGMA) dendrogram was construsted using the program NTSYS2.1 (Rohlf 2000).

RESULTS

RAPD and AFLP profile

For the 100 individuals of V. mangachapoi from the five populations were analyzed. Twenty RAPD primers have produced totally 354 makers out of which 325 makers were found to be polymorphic. Markers obtained for each primer varied from 5 to 23. Figure 1 shows the RAPD PCR fingerprints of 100 samples come from five locations using primer S350. Combinations which gave good fingerprints were selected for molecular analysis. In total 337 AFLP markers out of which 311 makers were found to be polymorphic and used to study molecular diversity analysis. The bands amplified by primer S35 are shown in Figure 2.

Genetic diversity and differentiation

The percentage of polymorphic bands (P) within populations ranged from 65.34% (JFL) to 79.90 (SY), with an average of 73.01% by RAPD, from 40.27% (SMW) to 58.74% (BWL), with an average of 48.83 by AFLP. Nei's gene diversity (H) of JFL is 0.1914 (by RAPD) and 0.1609 (by AFLP), which are lower than that of other populations. The Shannon`s information index of diversity (I) also shows an identical trend (Table 4). In addition, total gene diversity (Ht), gene diversity within population (Hs), and coefficient of gene differentiation (Gst) are shown in Table 5 respectively. The distribution of gene diversity revealed a large proportion of gene differentiations (71.31% by RAPD; by 61.73% AFLP) based on the difference of individuals within populations, whereas only 28.69% by RAPD; 38.27%by AFLP among populations based on their location sites.

Cluster analysis

On the basis of the genetic distances, a dendrogram of five populations was generated by using UPGMA cluster analysis. The five populations were divided two groups by RAPD (Figure 3): SMW, BWL and WC were in one group, SY and JFL were in the other group. Relative to

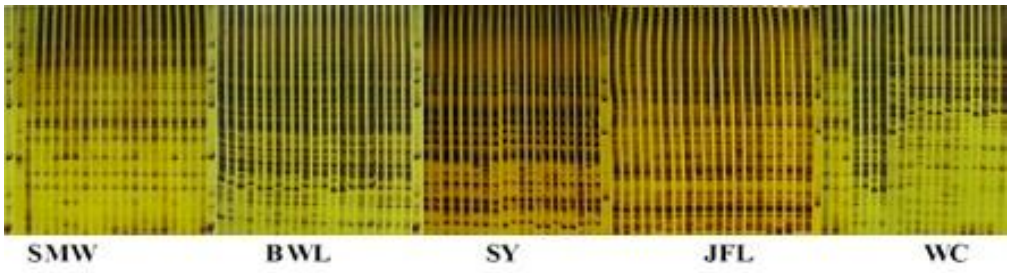

Figure 2. Amplification products of *V. mangachapoi* using primer combination of M3E6 on the populations located from Shimeiwan(SMW), Bawangling(BWL), Sanya(SY), Jianfengling(JFL), Wenchang(WC) in Hainan Island, China.

Table 4: The genetic variation statistics among populations of *V. mangachapoi*

Popultaion		P %	Na	Ne	H	I
SMW	RAPD	75.05	1.7505	1.5062	0.2343	0.3488
	AFLP	40.27	1.2027	1.1210	0.1696	0.2033
BWL	RAPD	76.99	1.7699	1.5045	0.2343	0.3496
	AFLP	58.74	1.3874	1.2114	0.2248	0.2879
SY	RAPD	79.90	1.7990	1.4805	0.2256	0.3421
	AFLP	49.73	1.2973	1.1813	0.2063	0.2581
JFL	RAPD	65.34	1.6534	1.4287	0.1914	0.2865
	AFLP	41.17	1.2117	1.0979	0.1609	0.1946
WC	RAPD	67.77	1.4640	1.4640	0.2080	0.3083
	AFLP	54.23	1.3423	1.1977	0.2162	0.2744
mean	RAPD	73.01	1.6874	1.4768	0.2187	0.3270
	AFLP	48.83	1.2883	1.1619	0.1956	0.2437
Species level	RAPD	99.41	2.0000	1.5262	0.3095	0.4696
	AFLP	80.81	1.6081	1.2405	0.2512	0.4388

Table 5: Genetic diversity estimated by RAPD and AFLP makers

	Total genetic diversity Ht	Gene diversity with population Hs	The coefficient of differentiation Gst	Nm^+ gene flow
RAPD	0.3067	0.2187	0.2869	1.2430
AFLP	0.1447	0.0893	0.3827	0.8065

AFLP, two groups were also found in Figure 4. SMW and BWL were in one group, WC, SY and JFL were in the other group.

DISCUSSION

Whether V. mangachapoi Blanco ssp. Hainanensis is the new variety?

The question about the new variety of V. mangachapoi in Hainan Island has been researched as early as 80`s last century. Wan (1982) reported there had one genus and two new varieties: V. hainanensis H. T. Chang et L. C. Wang, V. mangachapoi H. T. Changet L. C. Wang var. parvifoli Chang and V. mangachapoi H. T. Chang et L. C. Wang var. glandipetala L. C. Chang. In 2008, Fu reported a new variety V. mangachapoi Blanco ssp. Hainanensis belonging to V. Linn. But the proof is only about the differences of the seed numbers, nervation numbers, and the size of vegetative organ. For the instability of the leaf

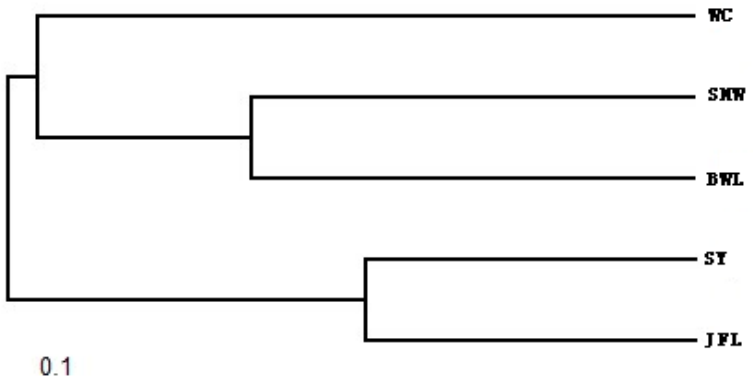

Figure 3: UPGMA dendrogram based on Nei`s genetic distance by RAPD analysis of *V. mangachapoi* on Hainan Island, China

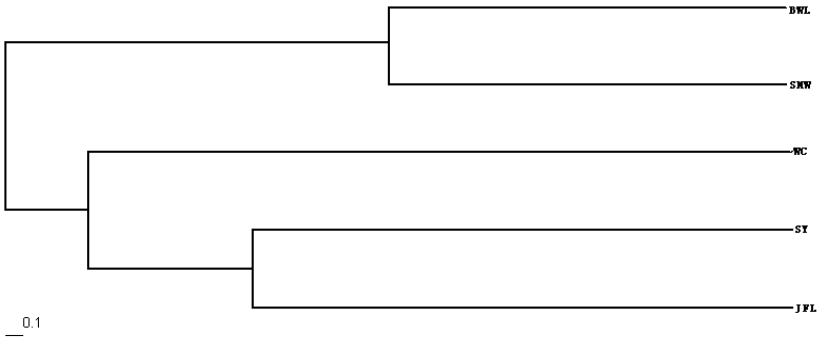

Figure 4: UPGMA dendrogram based on Nei`s genetic distance by AFLP analysis of *V. mangachapoi* on Hainan Island, China

sub-veins and the size winged perianth, Zhu (1992) believed V. xishuangbannaensis G. D. Tao et J.H. Zhang and V. guangxiensis X. L. Mo were one genus. In the flora of China (1990) and the flora of Guangdong (2005), V. mangachapoi was believed only one genus in Hanan Island. In this research, RAPD and AFLP makers were used to identify whether V. mangachapoi Blanco ssp. Hainanensis was the new variety. According to the dendrogram of UPGMA cluster analysis, the populations in SMW do not form the separated group with others (Figures 3, 4). The same results can also be found in the research of Huang (2008) by AFLP analysis. So our point is V. mangachapoi Blanco ssp. Hainanensis is not the new variety of V. mangachapoi.

Genetic diversity and Population genetic structure

Both RAPDs and AFLPs polymorphism have revealed a little high level of genetic diversity in V. mangachapoi compared with other Dipterocarpaceae species. An average of 73.01% RAPD bands being polymorphic is higher than 32.46% in V. guangxiensis (Li et al., 2002) and 20.8% in parashorea chinensis (Li et al., 2005). At the same time, An average of 48.83% AFLP bands being polymorphic is also a little high level in despite of a little lower than 53.32% in Shorea leprosula (Cao et al., 2006) and 51.79% in shore paroifolia (Cao et al., 2006). But in Huang`s research, 79.45% is higher than 48.83% in this study. It may conducted by the different sample collection.

Nei`s gene diversity (He) and Shannon`s information index (that does not rely on the estimation of allele frequencies) showed identical tendencies (Table3). Compared to other tree species studied using AFLPs (Gomez et al, 2005; Luu 2005; Tang et al, 2008; Cao et al, (2009), V. mangach apoi investigated here showed a little higher levels of He (0.2153). The Shannon

information index (I) using RAPDs (0.3270), AFLPs (0.2437) in V. mangachapoi investigated here showed moderate levels compared with other tree species: V. guangxiensis (0.1552) (Li et al, 2002), Pharashore chinensis (0.787) (Li et al, 2005) using RAPDs, and S. leprosula (0.245), S paroifolia(0.215) (Cao et al, 2006) using AFLPs.

The little high genetic diversity of V. mangachapoi was consistent with the characteristics of it`s natural distribution and ecological features. V. mangachapoi `s distribution range was wide in the global and also in Hainnan Island. It still keeps high genetic diversity even this species is vulnerable to extirpation in China due to habitat loss for the logging and destroy during the recent fifty years (Yang et al, 2005). During field research, there happened the differences in the size of leaves and winged fruits, numbers of seeds even in the same biotype. In Jiangfengling wildness V. mangachapoi population, big and small leaves type can live in the same population (Xu 2007).

The population genetic structure of a species is affected by a number of evolutionary factors including its mating system, gene flow, seed dispersal and its mode of reproduction as well as its natural selection (Hamrick and Godt 1990). The mating system plays a critical role for the population genetic structure. RAPD-based Gst values are available for 35 plants species, with an average of 15.5% for 27 out-crossing species, and 59.6% for eight in breeding species (Bussell 1999). Therefore, compared with the proportion of genetic diversity found among populations of outcrossing species as presented above, V. mangachapoi is probably an outcrossing species (Gst=28.69% by RAPD; Gst=0.3827 by AFLP). During field investigation, it has tape authotaxy. There are many flowers on each inflorescence. The stigma is higher than the anther. This is the apt configuration to avoid self pollen. The florescence of V. mangachapoi begins from June and ends in September. In Hainan island, there is often typoon weather during form June to September. The heavy wind can promote the separate of pollen and seed. Furthermore, V. mangachapoi can be pollinated by insects such as ants. The mature seed has the winged persistent calyxes can spread in virtue of the wind. Those may also to some extent show that V. mangachapoi has an outcrossing mating system. For V. mangachapoi, the indirect estimation of the level of gene flow based on Gst was moderated (Nm=1.2430), which means that the numbers of migrants per generation are greater than one successful migrant, and the level of genetic diversity maintained within a population is less susceptible to genetic drift. A migration rate of 0.5 was considered sufficient to overcome the diversifying effects of random drift (Ellsrand and Elam 1993). The Nm value of 1.2430 is near to the average value reported for outcrossed animal-pollinated species (Nm=1.154) and higher than that of mixed—mating species (Nm=0.727) (Hamrick and Godt 1990).

Conservation consideration

Genetic diversity is very important for the conservation and management of rare and endangered species, and is also important for the long-term survival and evolutionary process of these tropical area plants. V. mangachapoi is the native tree species of Hainan Island in China. It can normally live in the leanness coast sand soil. Though V. mangachapoi in Hainan Island is widely distributed, it is endangered at present, it faces great pressures subsequent to people`s economic activities, especially the logging for medicine extracts (C10H18O) and tropical cash crop planting. Their overall number is steadily decreasing. The tendency will heavy introduce the inbreeding and genetic drift; consequently lead the loss of genetic diversity and high genetic differentiation (Naito et al, 2005). So some conservation measures must be adopted.

In situ conservation: In field study, it was found that the seeds number of V. mangachapoi was great. Many seedlings were found under the mature trees, but the young tree was few. This may be coursed by the loss of enough sunshine. In the V. mangachapoi nature reserve, more measures should be done to help the seedlings growth into the young tree.

Out situ conservation can also be thought viable and get primary success (Meng et al, 2005). Much attention should be paid on the collection of seeds of different populations. If the seeds come from one population, it should be gathered from different circumstances in order to protect the abundant genetic genus.

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