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RESEARCH ARTICLE

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Genetic diversity of natural *Hepatacodium miconioides* populations in Zhejiang Province

Abstract *Hepatacodium miconioides* is the Class II protected plant species in China. This paper studies the genetic diversity and differentiation of its nine natural populations in Zhejiang Province by using random amplified polymorphic DNA (RAPD) technique. Twelve random primers were selected in the amplification, and 164 repetitive loci were produced. The percentage of polymorphic loci in each *H. miconioides* population ranged from 14.60% to 27.44%, with an average of 20.73%. Among the test populations, Kuochangshan had the highest percentage of polymorphic loci, Simingshan took the second place, and Guanyinping had the lowest percentage. As estimated by Shannon index, the genetic diversity within *H. miconioides* populations accounted for 27.28% of the total genetic diversity, while that among *H. miconioides* populations accounted for 72.72%. The genetic differentiation among *H. miconioides* populations as estimated by Nei index was 0.715,7. This figure was generally consistent with that estimated by Shannon index, i.e., the genetic differentiation among populations was relatively high, but that within populations was relatively low. The gene flow among *H. miconioides* populations was relatively low (0.198,7), and the genetic similarity ranged from 0.655,7 to 0.811,9, with an average of 0.730,6. The highest genetic distance among populations was 0.422,9, while the lowest was 0.208,3. All the results showed that there was a distinct genetic differentiation among *H. miconioides* populations. The genetic distance matrix of nine test populations was calculated using this method, and the clustering analysis was made using the unweighted pair group method with arithmetic mean (UPGMA). The cluster analysis suggested that the nine populations of *H. miconioides* in Zhejiang Province could be divided into two groups, the eastern Zhejiang group and the western Zhejiang group.

Keywords genetic differentiation, genetic diversity, *Hepatacodium miconioides*, natural population, random amplified polymorphic DNA (RAPD)

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1 Introduction

Genetic diversity is an important constituent of biodiversity, and a fundamental aspect of species and ecosystem diversity (Zeng et al., 2003). Studies of the subject not only describe the history, adaptive potential, and mechanisms involved in the dying out of certain plant species, but also provide scientific and efficient conservation strategies for the endangered plants (Falk and Holsinger, 1991; Ge and Hong, 1999).

Heptacodium miconioides is a small endemic deciduous arbor belonging to the monotypic genus *Heptacodium* in the family Caprifoliaceae. It is scattered in the cliff, gouge, and under shrubs on the hillside. It can be found only in a few areas such as the provinces of Zhejiang (Yicheng City), Anhui (Jingxie City), Hubei (Xingshan City), and Zhejiang has the highest concentration of this endangered plant. The population level reduces to almost zero even in Xingshan (Yu et al., 2003), from where the model specimen was first collected. Due to human destruction, long-term deforestation and habitat breakage, the species has declined in recent decades with population levels reducing and is listed as national secondary class protection plants (Yu et al., 2003). To our knowledge, previous researches focused on community structures, population dynamics and ecophysiological characteristics of *H. miconioides* (Jin, 1997, 1998; Jin and Ke, 2002), and no studies on genetic diversity and genetic differentiation have been conducted on *H. miconioides*.

Random amplified polymorphic DNA (RAPD) technique is a simple, rapid, and straightforward molecular marker widely applied in genetic diversity and structure studies of

plants (Dawson et al., 1993; Wachira, 1995; Palacios and Gonzalez 1997; Wang et al., 2000; Zhang et al., 2000; 2001; Xia et al., 2001; Guo et al., 2002; Liu et al., 2004). In this study, we assess the genetic diversity and genetic differentiation within and among populations using RAPD markers in order to reveal the genetic variation level and genetic differentiation degree of *H. miconioides*. Then, we can illustrate the genetic variation of *H. miconioides* populations, predicate the evolution potential, and formulate recommendations for conservation strategies of this endangered species.

2 Materials and methods

2.1 Materials

Nine wild populations of *H. miconioides* were sampled throughout its distribution range (Table 1). Twenty adult trees with distances longer than 30 m in each population were randomly selected. Young tender leaves were collected and preserved in sealed plastic bags. Samples were transported back to the laboratory using 4°C containers kept in ice bags and later transferred to a −70°C freezer until DNA extraction.

2.2 Methods

2.2.1 DNA extraction and quantification

The improved sodium dodecyl sulfate (SDS) method was used to extract total DNA (Li et al., 2002b). DNA was then run on a 0.8% agarose gel and photographed on a GIS-2008 gel imaging and analysis system. The DNA concentration was determined and stored at −20°C for RAPD amplification.

2.2.2 RAPD amplification and determination of the products

The random primers were bought from Sangon Inc., Shanghai, China. The amplification reaction conditions were according to Li et al. (2002a). The optimal amplification procedure included an initial 5 min denaturation at 94°C, followed by 35 cycles each of 30 s at 94°C, 45 s at 52.4°C, and 1.5 min at 72°C, and 5 min final extension at 72°C. Amplification of genomic DNA was made on a P×2 Thermol DNA Cycler (Thermo Hybaid, Inc.). Products amplified by polymerase chain reaction (PCR) were resolved using 1.4% agarose gel containing 0.5 μg/mL ethidium bromide electrophoresed in 0.5×TBE buffer. Images of each gel were photographed on a GIS-2008 gel imaging and analysis system. A molecular size marker (200 bp DNA ladder, Huamei Inc., Shanghai, China) was used to assign molecular weights for RAPD bands (Fig. 1).

2.3 Data statistics and analysis

2.3.1 Statistics of the RAPD polymorphic bands

Using the 200 bp DNA ladder as a molecular size marker, a data matrix of RAPD profiles for fragments of a similar molecular weight was scored as "1" when present, and "0" when absent.

2.3.2 Data analysis

The percentage of polymorphic loci (*P*%), Nei's gene diversity (*h*), Shannon's information index (I), and genetic differentiation coefficient were calculated with the software Popgen32 (Yeh and Boye, 1997). The unweighted pair group method arithmetic average (UPGMA) clustering was conducted.

3 Results and analysis

3.1 Primer selection

Nine DNA sampled selected from every population of *H. miconioides* were used for RAPD amplification and the negative control was conducted by replacing template DNA with ddH_2O. Amplification products were selected according to the agarose gel patterns. A subset of 12 random primers (Table 2) from 150 primers for further analysis was chosen based on the following criteria: i) consistent, strong amplification products; ii) product of uniform and reproducible fragments between replicate PCRs; and iii) no amplification in negative control.

Table 1 Basic conditions of each spot of *H. miconioides* populations

Population	Locality	Geographical location	Elevation /m
Xiaojiang (XJ)	Xiaojiang Forestry Center, Xinchang City	29°23′N, 121°06′E	880
Simingshan (SMS)	Siming Mountain Forestry Center, Ningbo City	29°39′N, 120°59′E	560
Dapanshan (DPS)	Dapan Mountain, Panan City	28°59′N, 120°32′E	720
Kuochangshan (KCS)	Kuochang Mountain, Linhai City	28°49′N, 120°55′E	950
Tiantaishan (TTS)	Tiantai Mountain, Tiantai City	29°15′N, 121°06′E	780
Beishan (BS)	Bei Mountain, Jinhua City	29°13′N, 119°38′E	600
Dongbaishan (DBS)	Dongbai Mountain, Dongyang City	29°30′N, 120°26′E	930
Gankeng (GK)	Gankeng, Xiakou Town, Linan City	30°08′N, 119°01′E	980
Guanyinping (GYP)	Guangyinping, Longgang Town, Linan City	30°10′N, 119°06′E	1,140

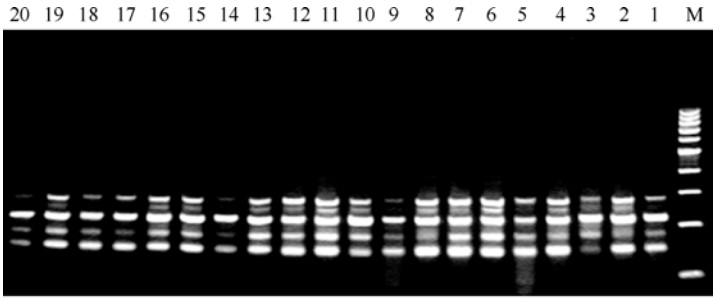

Fig. 1 RAPD amplification of *H. miconioides* in the Dongbaishan population produced with primer S125
1–20: individual 1–20; M: 200 bp DNA ladder molecular weight marker.

Table 2 Sequences of 12 random primers used in RAPD analysis

Primers	Sequences
S125	CCGAATTCCC
S313	ACGGGAGCAA
S328	GGGTGGGTAA
S160	AACGGTGACC
S169	TGGAGAGCAG
S71	AAAGCTGCGG
S323	CAGCACCGCA
S23	AGTCAGCCAC
S24	AATCGGGCTG
S315	CAGACAAGCC
S65	GATGACCGCC
S89	CTGACGTCAC

3.2 Percentage of polymorphic loci

A total of 164 bands were produced from 12 RAPD primers after the analysis of the 180 DNA samples from the nine populations of *H. miconioides*. Percentage of polymorphic loci of every population was different (Table 3). The highest percentage of polymorphic loci came from the KCS population with an average of 27.44%, that of the SMS population took the second place (23.17%), and that of the GYP population was the least (14.63%). The percentage of polymorphic loci at population level ranked in a descendant order: KCS > SMS > BS > TTS > XJ > DBS > DPS > GK > GYP. The average percentage of polymorphic loci of *H. miconioides* population was 20.73%. The number and percentage of unique loci were relatively low. There was no unique locus detected in SMS, BS, and GYP populations.

3.3 Genetic diversity within populations

The genetic diversity within nine populations of *H. miconioides* estimated by Shannon informative index was 0.125,7 (Table 4); that of the KCS population was the highest, that of the SMS population took the second place, and that of the GYP population was the lowest. The genetic diversity within nine populations of *H. miconioides* estimated by Nei's gene index was 0.086,9, which is less than that estimated by Shannon informative index. Some genetic diversity estimated by Nei's gene index was different from that estimated by Shannon informative index, but even then that of the KCS population was the highest, that of the SMS population took second place, and that of the GYP population was the lowest.

Table 3 Percentage of polymorphic loci within populations of *H. miconioides*

Population	Number of samples	Total of loci	Number of polymorphic loci	Percentage of polymorphic loci, *P* /%	Number of unique loci	Percentage of unique loci, *P* /%
XJ	20	164	34	20.73	2	1.22
SMS	20	164	38	23.17	0	0.00
DPS	20	164	31	18.90	2	1.22
KCS	20	164	45	27.44	1	0.61
TTS	20	164	35	21.34	1	0.61
BS	20	164	37	22.56	0	0.00
DBS	20	164	33	20.12	1	0.61
GK	20	164	29	17.68	2	1.22
GYP	20	164	24	14.63	0	0.00
Average	20	164	34	20.73	1	0.61

Table 4 Genetic diversity within 9 populations of *H. miconioides*

Index	XJ	SMS	DPS	KCS	TTS	BS	DBS	GK	GYP	Average
Shannon information index	0.107,9	0.140,7	0.116,3	0.170,4	0.134,6	0.135,2	0.124,3	0.110,9	0.090,9	0.125,7
Nei index	0.071,7	0.097,4	0.080,9	0.118,4	0.093,9	0.093,1	0.086,1	0.077,5	0.063,4	0.086,9

Table 5 Genetic diversity index analysis of nine populations of *H. miconioides*

Shannon information index		Nei index	
Within-population genetic diversity, H_{pop}	0.125,7	Within-population gene diversity, H_S	0.086,9
Total genetic diversity, H_{sp}	0.460,8	Total gene diversity, H_T	0.305,7
Ratio of genetic diversity within population, H_{pop}/H_{sp}	0.272,8	Ratio of gene diversity within population, Hs/H_T	0.284,3
Ratio of genetic diversity among populations, $(H_{sp}\text{-}H_{pop})/H_{sp}$	0.727,2	Genetic differentiation among populations, G_{ST}	0.715,7
		Gene flow, N_m	0.198,7

Table 6 Genetic similarity and genetic distances among nine populations of *H. miconioides*

Populations	XJ	SMS	DPS	KCS	TTS	BS	DBS	GK	GYP
XJ	—	0.756,2	0.764,6	0.752,4	0.730,0	0.709,5	0.718,5	0.722,7	0.670,8
SMS	0.279,5	—	0.730,3	0.743,3	0.734,9	0.708,5	0.720,7	0.682,5	0.655,7
DPS	0.268,4	0.314,3	—	0.753,1	0.778,1	0.756,2	0.734,8	0.686,2	0.669,6
KCS	0.284,5	0.296,6	0.283,6	—	0.769,6	0.757,9	0.740,2	0.727,8	0.707,2
TTS	0.314,7	0.308,0	0.250,9	0.261,9	—	0.760,4	0.748,1	0.758,1	0.687,5
BS	0.343,1	0.344,6	0.279,4	0.277,2	0.273,9	—	0.779,4	0.752,5	0.714,2
DBS	0.330,6	0.327,6	0.308,1	0.300,9	0.290,2	0.249,2	—	0.724,5	0.684,4
GK	0.324,8	0.382,0	0.376,5	0.317,8	0.277,0	0.284,4	0.322,2	—	0.811,9
GYP	0.399,3	0.422,0	0.401,0	0.346,4	0.374,7	0.336,6	0.379,3	0.208,3	—

Numbers above the diagonal indicate Nei's genetic similarity, and those below the diagonal indicates genetic distances.

3.4 Genetic differentiation within and among *H. miconioides* populations

The genetic diversity within *H. miconioides* populations estimated by Shannon informative index averaged 0.125,7 and the total genetic diversity was 0.460,8 (Table 5). In the whole genetic variation, the most part existed among populations (72.72%) and that within populations was only 27.28%. The genetic diversity within *H. miconioides* populations estimated by Nei's gene diversity averaged 0.086,9 and the total genetic diversity was 0.305,7 (Table 5). The genetic differentiation coefficient was 0.715,7. It was similar to the results estimated by Shannon informative index. This indicated that the genetic diversity within population was relatively low and most of the variation existed among populations. Gene flow among *H. miconioides* populations was 0.198,7, which was very low.

Genetic similarity and genetic distance estimated by Nei (1973) is shown in Table 6. The results show that the genetic similarity ranged from 0.655,7 to 0.811,9 with an average of 0.730,6. The genetic similarity between the GYP population and GK population with nearest geographical distance was highest. In addition, genetic similarity between the GYP population and other populations was relatively low and it indicated that the genetic differentiation among them was high. The pairwise genetic distance ranged from 0.208,3 to 0.422,9, which suggested that significant differentiation existed among populations. The genetic distance between the GYP population and GK population with very near geographical distance was 0.208,3, which indicated that there was definite differentiation between these two populations.

On the basis of the genetic distances, nine populations of *H. miconioides* were clustered into two groups, the eastern Zhejiang group and the western Zhejiang group, using UPGMA (Fig. 2). The eastern population included seven populations: XJ, SMS, DPS, TTS, KCS, BS, and DBS. Among them, populations with near geographical distance such as XJ and SMS, DPS, TTS and KCS, BS and DBS were grouped together. The GYP population and GK population with near geographical and genetic distances in the eastern group were far from other populations. It was in accordance with the geographical distribution pattern, i.e., genetic distance between populations with relatively near geographical distance is relatively low.

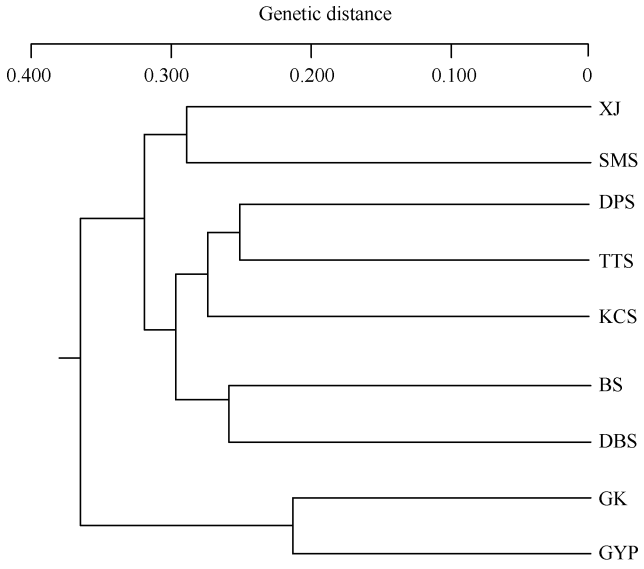

Fig. 2 Dendrogram produced by POPGENE software for nine populations of *H. miconioides*

4 Discussion

RAPD analysis showed that the percentage of polymorphic loci of nine *H. miconioides* populations ranged from 14.60% to 27.44% with an average of 20.70%. The genetic diversity estimated by Shannon informative index and Nei's gene index was relatively low with an average of 0.125,7 and 0.086,9, respectively. There was significant genetic differentiation among populations of *H. miconioides*. Shannon informative index estimated that within-population genetic diversity occupied 27.28%, while among- populations genetic diversity occupied 72.72%. Nei's gene index showed that genetic variation within *H. miconioides* populations was relatively low and that among populations was relatively high. It indicated that genetic differentiation existed among populations and individuals within populations, and genetic differentiation among populations was significantly higher than that within populations. Genetic differentiation coefficient among populations was 0.715,7.

Many factors, such as selection, reduction of population effective size, genetic drift and inbreeding, can cause genetic depression within populations (Zou et al., 2001). The natural habitat of *H. miconioides* has already been completely destroyed and the population size has been reduced to a very small range. It can cause inbreeding reproduction in a great degree, increase the genetic homology within populations and then increase the genetic homogeneity within populations (Li et al., 1998). *H. miconioides* is a light-loving, shade-intolerant and heavily light-intolerant species with narrow ecological amplitude of light acclimation (Jin, 1998). It is at a disadvantage in the existing competition and return to extremely bad habitats such as cliff and valley with barren soil and bare rock, giving place to other evergreen broad-leaved trees on the hillside with relatively good site conditions (Jin, 1999). *H. miconioides* is gradually limited to small isolated areas, and eventually fragmented into island-like small populations. Two genetic consequences of small population sizes are increased drift and inbreeding. Inbreeding depression and reduction of heterozygosity can influent the fitness of individuals and further result in the loss of genetic diversity. Moreover, low genetic diversity within populations was correlated with the biological traits such as reproductive mechanisms. *H. miconioides* is entomophilious, but has a high self-pollination rate (Bian et al., 2002), long dormancy period, extremely low seed germination (Wang et al., 1995), unclear sexual reproduction, and difficulties during the settling of seeds. All of these factors can limit gene flow, cause the loss of genetic diversity, and result in a simplified and monotonous genetic structure. The decreasing of the seed fitness and the change of reproductive system retard the renewal of *H. miconioides* populations, decrease the genetic diversity, reduce the adaptability to the environment, and then accelerate the endangered status of *H. miconioides* populations.

Gene flow is one of the main factors that can influence the genetic differentiation among populations, to homogenize the genetic structure of populations. Genetic differentiation of species with limited gene flow is higher than that of species with broad gene flow. Wright (1931) indicated that when $N_m > 1$ is typical in high gene flow species, the species will become genetically homogeneous, whereas when $N_m<1$ is typical, differential selection may be very strong, and population differentiation may be maintained. Gene flow among *H. miconioides* populations was only 0.198,7 — far below 1 — and the extremely low gene flow is beneficial for genetic differentiation. On the other hand, the heterogeneity of the microenvironment is one of the factors that determines the genetic differentiation among populations. Genetic differentiation comes into play when the different adaptations of different genotypes on different habitats gather the individuals with the same genotype in a relatively adaptive microenvironment (Chen and Song, 1998). It is sure that the adaptation to different ecological microenvironments can form diverse genetic structures in different populations (Gehring and Linhart, 1992; Su et al., 1997). The geographical locations of nine *H. miconioides* populations were different with different altitudes. For example, the altitude of the GYP population is at 1,140 m, while the SMS population is at 560 m. The difference of habitat and changes in long periods of adaptation produce the genetic differences among *H. miconioides* populations.

The genetic similarity ranged from 0.655,7 to 0.811,9 with an average of 0.730,6. Genetic similarity between the GYP population and GK population in Linan City with the smallest geographical distance was the highest. Genetic similarity between the GYP population and SMS population was the lowest. There was significant genetic differentiation among *H. miconioides* populations. The pairwise genetic distance ranged from 0.208,3 to 0.422,9. The genetic distance between the GYP population and GK population with very small geographical distance was 0.208,3, which indicated that there was a definite differentiation between these two populations. On the basis of the genetic distances, nine populations were clustered into two groups, the eastern group and the western group, using UPGMA (Fig. 2). The eastern group had seven populations: XJ, SMS, DPS, TTS, KCS, BS, and DBS. The western group comprised the GYP population and GK population. It was in accordance with the geographical distribution pattern, i.e., the genetic distance between populations with relatively small geographical distance is relatively low.

Recently, there have been a lot of arguments on the roles of genetic diversity in the conservation of species. Some scholars think that genetic diversity plays a crucial role in the existence and development of species, while others believe that ecological factors such as habitat destruction and environment transition are the direct causes for the endangerment and extinction of species, and these should be the problems primarily considered in species conservation (Ge and Hong, 1999). In fact, genetic diversity, life history traits, and ecological factors can all influence the existence and development of species. Hence, comprehensive conservation measures instead of single steps are encouraged to be considered to prevent population depression. First, to pre-

serve all the existing individuals and populations, slashing and denudation should be banned. Second, given that most of the genetic differentiation exists among populations, the preservation of all the populations should be considered as soon as possible. On-the-spot protection should be enforced to preserve the suitable habitat. Transplantation from populations with genetic variation can artificially increase gene flow, enhance genetic diversity and improve the adaptation to the environment. Third, given the high gene differentiation among populations, the sample size of populations should be as large as possible to ensure the representation of the diversity of populations when the ex-site conservation is being carried out. Fourth, in order to preserve the genetic diversity of *H. miconioides*, further studies on seed germination should be conducted to break the dormancy period, increase seed germination, compensate plants in a relatively large space, and artificially increase the seedlings in populations to create the conditions of gene flow and recombination.

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