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## Genetic diversity analysis of *Hepatacodium miconioides* at different altitude in Tiantai Mountain, Zhejiang Province, China and its relationship with environmental factors

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**Abstract** The genetic diversity within and among populations of *Hepatacodium miconioides* collected at three different altitudes in Tiantai Mountain, Zhejiang Province and its relationships to environmental factors were analyzed by random amplified polymorphic DNA (RAPD) technique. Amplification using 12 random primers of 60 plants and 122 repetitive loci were produced. The percentage of polymorphic loci of three populations ranged from 18.85% to 23.77% with an average of 21.86%, indicating the relatively low genetic diversity of *H. miconioides*. The average Shannon index of phenotypic diversity (0.1329) and Nei index (0.0925) within populations were relatively low. A distinct genetic differentiation existed among populations of *H. miconioides* in spite of the relatively small geographical distribution. The average genetic diversity within populations of *H. miconioides* accounted for 33.58% of the total genetic diversity while the genetic diversity among populations accounted for 66.42% as estimated by the Shannon index of phenotypic diversity. The genetic differentiation among populations of *H. miconioides* was 0.6546, as estimated by Nei index. The gene flow estimated from  $G_{ST}$  was only 0.2656 and it indicated that gene flow among populations of *H. miconioides* was relatively low. The mean value of the genetic identity among populations of *H. miconioides* was 0.7126 and the average of genetic distance of *H. miconioides* was 0.3412. The genetic identity between populations at the elevation of 990 m and at the elevation of 780 m was the highest. The genetic identity between population at the elevation 500 m and other two populations was relatively low. The correlation analysis showed that the

genetic diversity within populations was significantly related with the soil total nitrogen.

**Keywords** *Hepatacodium miconioides*, random amplified polymorphic DNA (RAPD), genetic diversity, altitude, environmental factors

### 1 Introduction

Random Amplified Polymorphic DNA (RAPD) technique is a simple, rapid and straightforward molecular marker widely applied in genetic diversity and structure studies of woody plants (Wachira 1995; Palacios and Gonzalez 1997; Li and Peng 2001; Xia et al., 2001). Genetic diversity studies not only impart the history, adaptive potential and mechanisms involved in the dying out of a certain plant species, but also provide scientific and efficient conservation strategies for the endangered plant (Ge and Hong 1999). These studies also provide important information for the analysis of the evolutionary potential of the endangered species, factors equally significant for species conservation.

*Heptacodium miconioides* is a small endemic deciduous arbor belonging to the family Caprifoliaceae. It is listed as a Class II endangered species and can be found only in a few areas such as Zhejiang Province (Yicheng City), Anhui Province (Jingxie City) and Hubei Province (Xingshan City), with Zhejiang Province having the highest concentration of these endangered plant. Habitat breakage and long-term deforestation resulted in the decline of the species, reducing population levels to almost zero even in Xianshan City, Hubei Province (Yu et al., 2003), the site where the model specimen was first collected. Populations of *H. miconioides* intermittently distribute in a few island-like regions in the northwestern part of Zhejiang Province, such as Xiaokou Town and Longgang Town in

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Linan City, and the east of Zhejiang Province, such as Tiantai Mountain and Kuocang Mountain. Current studies show relatively high numbers of *H. miconioides* individuals distributed over a larger area in Tiantai Mountain. Previous research on population structure and dynamics, community traits and ecophysiological characteristics of the *H. miconioides* found in Tiantai Mountain gave the researchers insights on incomplete age stage, declining age structure, impaired natural regeneration, and the day-to-day danger severity of *H. miconioides* (Jin 1997, 1998; Jin and Ke 2002). Despite these informations, no genetic diversity study has been conducted on *H. miconioides*.

In this study, the genetic diversity level and dynamics of *H. miconioides* within populations, its relationship to the environmental factors and the genetic differentiation among populations at three different altitudes in Tiantai Mountain in Zhejiang Province were investigated using random amplified polymorphic DNA (RAPD) technique. This study aims to provide genetic differentiation and evolution potential data, as well as academic foundation for the species' conservation strategy.

## 2 Materials and methods

### 2.1 Materials

Materials were collected in Shiziyankeng, Huangdingshan (the highest peak in Tiantai mountain) of Zhejiang Province. Three plots containing *H. miconioides* were set according to the altitude. The environmental conditions of 3 plots were listed in Table 1. Twenty adult trees with heights longer than 30 m in each population were randomly selected. Young tender leaves were collected and preserved in the 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.

**Table 1** The environmental conditions of three *H. miconioides miconioides* populations

Environmental conditions	Populations		
	P1	P2	P3
Altitude / m	990	780	500
Aspect	NW30°	NE15°	NW20°
Slope	30°	25°	35°
pH value	5.02	5.18	5.13
Water content / %	26	28	24
Total nitrogen / (mg·kg <sup>-1</sup> )	5.38	5.66	4.88
Total phosphorus / (mg·kg <sup>-1</sup> )	0.519	0.256	0.398
Organic matter / (mg·kg <sup>-1</sup> )	46.57	48.34	41.23

### 2.2 Methods

#### 2.2.1 DNA Extraction and quantification

The improved SDS (Sodium dodecyl sulfate) 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 diluted to 10 ng / μL and was stored at -20°C for RAPD amplification.

#### 2.2.2 RAPD Amplification and determination of the products

The random primers were bought from Shanghai Sangon Inc., China. Amplification of genomic DNA was made on a P × 2 Thermo DNA Cycler (Li et al., 2002a). Products amplified by PCR were resolved using 1.4% agarose gel (Sangon Inc., Shanghai, China) 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 (200bp DNA ladder, Huamei Inc., Shanghai, China) was used to assign molecular weights for RAPD bands.

### 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, or "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 variation 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 RAPD amplification results of *H. miconioides*

Total DNA was extracted from the leaves of *H. miconioides*. Twelve out of the 60 random primers (Table 2) were chosen for formal amplification based on two criteria: (i) strong and clear amplification products, (ii) production of uniform and reproducible fragments between replicate PCRs.

**Table 2** Sequences of 12 arbitrary primers

Primer	Sequence	Primer	Sequence
S125	CCGAATTCCC	S305	CCTTCCCTC
S71	AAAGCTGCGG	S122	GAGGATCCCT
S344	CCGAACACGG	S31	CAATCGCCGT
S315	CAGACAAGCC	S24	AATCGGGCTG
S160	AACGGTGACC	S25	AGGGGTCTTG
S124	GGTGATCAGG	S335	CAGGGCTTTC

### 3.2 Percentage of polymorphic loci

A total of 122 bands were produced from 12 RAPD primers after the analysis on the 60 DNA samples from the 3 populations of *H. miconioides*. The highest percentage of polymorphic loci came from the P2 population with an average of 23.77%. The P1 population has an average of 22.95%, while the P3 population had the lowest percentage with an average of 18.85%. The mean of the percentage of polymorphic loci was 21.86%. The difference between the P1 and P2 populations was near and a single locus difference was observed. The difference between the P3 population and P1 or P2 population was relatively larger.

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

Population	Number of samples	Total of loci	No. of polymorphic loci	Percentage of polymorphic loci /%
P1	20	122	28	22.95
P2	20	122	29	23.77
P3	20	122	23	18.85
Average	200	122	26.67	21.86

### 3.3 Genetic diversity within populations

The genetic diversity within 3 populations of *H. miconioides* estimated by Shannon informative index is shown in Table 4. The results indicated that there were definite differences in the genetic diversity within the populations with an average of 0.1329. The genetic diversity of the P2 population was the highest; the P1 population was slightly lower than the P2 population and the P3 population was the lowest. The genetic diversity detected by the different primers ranged from 0 (detected by primer S24) to 0.2423 (detected by primer S335).

**Table 4** Genetic diversity within 3 populations of *H. miconioides* estimated by Shannon index of phenotypic diversity

Primer	P1	P2	P3	Average
S125	0.096 8	0.034 3	0.104 0	0.078 4
S71	0.082 9	0.237 5	0.097 8	0.139 4
S344	0.062 5	0.093 9	0.089 6	0.082 0
S315	0.142 5	0	0.034 2	0.058 9
S160	0.097 3	0.286 1	0.046 7	0.143 4
S124	0.097 1	0.076 7	0	0.057 9
S335	0.358 4	0.056 6	0.089 2	0.168 1
S305	0.091 2	0.081 4	0.193 1	0.121 9
S122	0	0.088 3	0.156 7	0.081 7
S31	0.033 0	0.140 7	0.110 2	0.094 6
S24	0	0	0	0.000 0
S25	0.085 1	0.115 9	0.047 5	0.082 8
Average	0.095 6	0.101 0	0.080 8	0.092 5

The gene diversity estimated by Nei's index is shown in Table 5. The mean gene diversity of 3 populations of

*H. miconioides* was 0.0925, lower than that estimated by Shannon informative index, but the rank sequence of the genetic diversity suggested by two indices was coincident, i.e. that of P2 population was the highest, followed by P1 population and P3 population. The gene diversity estimated by the different primers was different. Gene diversity estimated by primer S335 was the highest with an average of 0.1681 while that of primer S24 was the lowest with an average of 0. It was consistent with that estimated by Shannon informative index.

**Table 5** Genetic diversity within 3 populations of *H. miconioides miconioides* estimated by Nei's index

Primer	P1	P2	P3	Average
S125	0.134 9	0.052 5	0.158 1	0.115 2
S71	0.120 0	0.332 4	0.135 8	0.196 1
S344	0.105 6	0.131 8	0.127 5	0.121 6
S315	0.199 5	0	0.052 3	0.083 9
S160	0.135 3	0.400 0	0.065 7	0.200 3
S124	0.135 1	0.113 6	0	0.082 9
S335	0.509 2	0.090 6	0.127 0	0.242 3
S305	0.129 1	0.118 7	0.269 1	0.172 3
S122	0	0.132 5	0.226 4	0.119 6
S31	0.060 6	0.197 6	0.164 4	0.140 9
S24	0	0	0	0.000 0
S25	0.122 8	0.168 5	0.066 5	0.119 3
Average	0.137 7	0.144 8	0.116 1	0.132 9

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

The genetic diversity estimated by Shannon informative index could be divided into two parts, within populations and among populations (Table 6). The genetic diversity within *H. miconioides* populations averaged 0.1329 and the total genetic diversity was 0.3958. In the whole genetic variation, the most part existed among populations (66.42%) and that within populations was only 33.58%. It suggested that the genetic diversity of 3 *H. miconioides* populations was low, but the genetic differentiation was high and most of the variation existed among populations.

The mean gene diversity of 3 populations estimated by Nei's index was 0.2678 and that within populations was 0.0925. The genetic differentiation coefficient among population was 0.6546 (Table 7). It indicated that most of the genetic variation of 3 *H. miconioides* populations existed among populations, and then within populations. It was similar to the results estimated by Shannon informative index. Gene flow estimated by *Gst* was 0.2656 which indicated that the gene flow among population was small. The greater the differentiation, the less the gene flow among population. Gene flow of 3 *H. miconioides* populations was less than 1, which suggested that the differentiation among populations was high.

**Table 6** Genetic diversity among and within 3 populations of *H. miconioides* estimated by Shannon index of phenotypic diversity

Primer	Within-population genetic diversity $H_{pop}$	Total genetic diversity $H_{sp}$	Proportion of genetic diversity within-population $H_{pop}/H_{sp}$	Proportion of genetic diversity among population $(H_{sp}-H_{pop})/H_{sp}$
S125	0.115 2	0.574 7	0.200 5	0.799 5
S71	0.196 1	0.474 5	0.413 3	0.586 7
S344	0.121 6	0.369 1	0.329 5	0.670 5
S315	0.083 9	0.364 0	0.230 5	0.769 5
S160	0.200 3	0.472 9	0.423 6	0.576 4
S124	0.082 9	0.363 9	0.227 8	0.772 2
S335	0.242 3	0.540 8	0.448 0	0.552 0
S305	0.172 3	0.294 9	0.584 3	0.415 7
S122	0.119 6	0.464 7	0.257 4	0.742 6
S31	0.140 9	0.409 8	0.343 8	0.656 2
S24	0.000 0	0.251 2	0.000 0	1.000 0
S25	0.119 3	0.168 7	0.707 2	0.292 8
Average	0.132 9	0.395 8	0.335 8	0.664 2

**Table 7** Genetic differentiation within and among 3 populations of *H. miconioides* estimated by Nei index

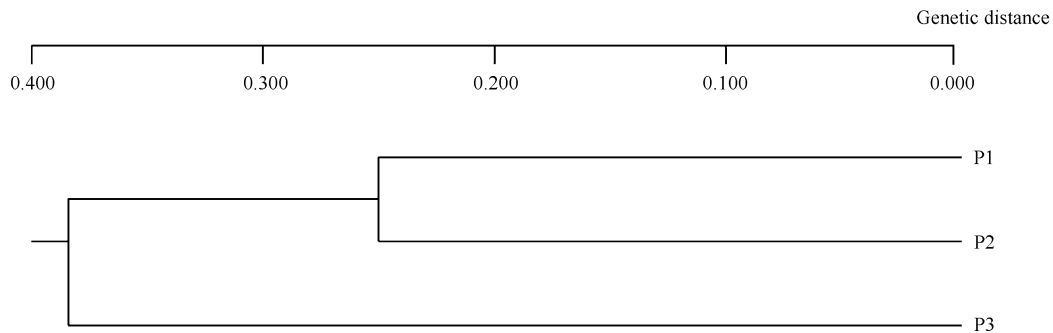
Primer	Total gene diversity, $H_T$	Within population gene diversity, $H_S$	Genetic differentiation among populations, $G_{ST}$
S125	0.404 6	0.078 4	0.806 2
S71	0.313 1	0.139 4	0.554 8
S344	0.256 2	0.082 0	0.679 9
S315	0.243 1	0.058 9	0.757 7
S160	0.326 2	0.143 4	0.560 4
S124	0.243 4	0.057 9	0.762 1
S335	0.354 3	0.168 1	0.525 5
S305	0.189 6	0.121 9	0.357 1
S122	0.317 5	0.081 7	0.742 7
S31	0.276 8	0.094 6	0.658 2
S24	0.175 6	0.000 0	1.000 0
S25	0.113 9	0.082 8	0.273 0
Average	0.267 8	0.092 5	0.654 6

The genetic similarity among *H. miconioides* populations as calculated by the Popgen 32 software (Nei 1972) is shown in Table 8. The genetic similarity between the P1 and P2 populations was the highest (0.7736), that of between the P1 and P3 populations took the second place (0.7124), and that of between the P2 and P3 populations was the lowest (0.6519). The mean genetic similarity was 0.7126, which indicated that the genetic similarity of 3 populations was not too high and there was relatively high genetic differentiation among populations. The genetic distance ranged from 0.2567 to 0.4378, and the mean was 0.3412. The genetic distance between the P1 and P2 populations was the lowest, while that between the P2 and P3 populations was the highest. Based on the genetic distances, the cluster analysis was conducted using UPGMA. The P1 and P2 populations were clustered into one group, then they clustered with P3 population.

**Table 8** Genetic identity and genetic distance among 3 populations of *H. miconioides miconioides*

Populations	P1	P2	P3
P1	-	0.7736	0.7124
P2	0.2567	-	0.6519
P3	0.3392	0.4278	-

Note: Nei's genetic identity (above diagonal) and genetic distance (below diagonal)



**Fig.1** The cluster figure produced by POPGENE soft for 3 populations of *H. miconioides*

### 3.5 Correlation between the genetic structure of *H. miconioides* populations and ecological factors

The correlation between the genetic structure of 3 *H. miconioides miconioides* populations and ecological factors is shown in Table 9. The genetic diversity within populations estimated by the Shannon informative index was significantly and positively correlated with the soil total nitrogen ( $r = 0.9925$ ,  $P < 0.01$ ). It was also positively correlated with altitude, soil pH value and soil water content with no significant difference ( $P > 0.05$ ). It was negatively correlated with soil organic matter and total phosphorus with no significant difference ( $P > 0.05$ ). The correlation between the genetic diversity estimated by Nei's index and ecological factors were identical to the above.

**Table 9** Correlation of the genetic diversities within 3 populations of *H. miconioides miconioides* and the ecological factors

Ecological variables	Genetic diversity estimated by Shannon index of phenotypic diversity	Genetic diversity estimated by Nei index
Altitude /m	0.7769	0.6157
pH Value	0.0266	0.0479
Water content / %	-0.9600	0.9658
Total nitrogen / (mg·kg <sup>-1</sup> )	0.9925**	0.9949**
Total phosphorus / (mg·kg <sup>-1</sup> )	-0.2820	-0.3024
Organic matter / (mg·kg <sup>-1</sup> )	-0.0424	-0.0210

\*\*  $P < 0.01$ , high notable correlativity

## 4 Discussion

The genetic diversity of 3 wild type populations of *H. miconioides miconioides* found at different altitude in Tiantai Mountain, Zhejiang Province was analyzed using the RAPD technique, and the correlation between genetic diversity and ecological factors was further discussed using mathematical and statistical methods. The genetic diversity of 3 populations was low. The percentage of polymorphic loci average 21.86%, while the mean genetic diversity estimated by the Shannon informative index and the Nei's index was 0.1329 and 0.0925, respectively. Genetic

differentiation among populations was relatively high, and most of the variation existed among populations. Shannon informative index indicated that 66.42% existed among populations and only 33.58% existed within populations.  $G_{st}$  estimated by Nei's index was 0.6546.

Genetic differentiation of plants comes about at the geographical distribution and at the microenvironment level. In the large geographical scope, genetic differentiation rests with the gene diffusion ability of the species. But at microenvironment level, genetic difference means the spatial heterogeneity of genotype or gene within a single population. Even if there is no evident difference of microenvironment, the limited gene flow can result in genetic differentiation (Chen and Song 1998). In this paper, the samples were collected at different altitudes but only in a small area. But there was relatively high genetic differentiation among the 3 populations of *H. miconioides* and it seems that the limited gene flow play an important role. Wright indicated that if the values of gene flow among population are larger than 1, the species would become genetically homogeneous and if the values are less than 1, gene flow might be the main cause of genetic differentiation (Wright 1931). There is a possibility that the limited gene flow of *H. miconioides* may be the result of lack of immigration of outer pollen (genes) in entomophilous *H. miconioides* caused by slow movement for the pollen, high self-pollination (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. The species distributes in cliff and a valley with extremely poor growth conditions and its declining age structure is no seedling in the forest of *H. miconioides* with declined age structure. The limited gene flow causes an increase in genetic differentiation among populations. Gene flow estimated by  $G_{st}$  was only 0.2656 – far below the baseline value 1 and the extremely low gene flow can facilitate the genetic differentiation among populations. on the other hand, the heterogeneity of the microenvironment is one of the factors that determine the genetic differentiation among populations. Genetic differentiation comes into play when the different adaptation of different genotypes on different habitats accumulate the individuals with

same genotype in a relatively adaptive microenvironment (Chen and Song 1998). It is sure that the adaptation to different ecological microenvironment can form diverse genetic structure in different populations (Su et al., 1997). Although the 3 populations of *H. miconioides* distribute in the limited geographical area, their microenvironment is different and genetic differentiation comes into play under the selection pressure imposed by the habitat. The low genetic diversity within 3 populations may be a result of simple and monotonous genetic structure induced by the loss of the genetic variance for the unclear sexual reproduction and low gene flow.

Among the 3 populations, the percentage of polymorphic loci of the P2 population was the highest, the P1 population took the second place, with a value close to the P2 population and the P3 population was the lowest. The same order was observed in the genetic diversity of the 3 *H. miconioides* populations as estimated by Shannon informative index and Nei's index. The mean genetic similarity was 0.7126 with the highest similarity between the P1 and P2 populations and the lowest similarity between the P2 and P3 populations, which indicated that there exist high genetic differentiation between the populations. Similar results were obtained from the genetic distance analysis among populations. The clustering result based on the Nei's genetic distance showed that the P1 and P2 populations clustered first, and then the P3 population came into together. Although there was significant genetic differentiation among 3 populations and the relative high genetic distance between every 2 populations, the P3 population sampled from the lowest altitude was different to the P1 and P2 populations, because the sampling site is near the village and has human interference, such as severe chopping of firewood. Genetic diversity is lessened for the close relative propagation at a great degree and the increase of the genetic homologous is (Li et al., 1998) caused by human interference. Once the genetic diversity is lost, the resume rate is extremely slow and the low genetic diversity pattern form. The P2 and P1 population with low human disturbance listed in Huading National Forest Park maintain relative high genetic diversity.

Based on the statistic analysis of the correlation between the genetic diversity and the environment factors, there is a significantly positive correlation between the genetic diversity of 3 *H. miconioides* populations and total soil nitrogen content. Greater levels of total nitrogen content correlates to greater population genetic diversity. This suggests that nitrogen might be important in the maintenance of genetic diversity of *H. miconioides* populations because the soil nitrogen absorbed by plants might be important in its growth and development. This nitrogen is used as components of protein, nucleic acid, enzyme, chlorophyll, some vitamins and hormones. Genetic diversity of *H. miconioides* in the soil correlates to the levels of total nitrogen; high nitrogen levels result in high genetic diversity. It was observed that there is no significant correlation between the genetic diversity and the altitude.

Aside from the geographical factors, human interference and the biological trait of the species also influence genetic diversity.

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