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GENETIC DIVERSITY AND POPULATION STRUCTURE OF THE NARROW ENDEMIC AND ENDANGERED SPECIES *HETEROPLEXIS MICROCEPHALA* Y. L. CHEN. IN CHINA REVEALED BY RANDOM AMPLIFIED POLYMORPHIC DNA MARKERS

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Abstract: *Heteroplexis microcephala* Y. L. Chen. is an endemic and endangered species found only in karst limestone regions in the Yangshuo County of the Guangxi Zhuang Autonomous Region in China: it is a habitat representative of species in the *Heteroplexis* genus. To provide basic genetic information for its conservation, in this study we evaluated the genetic variation and differentiation among six wild populations of *H. microcephala* by random amplified polymorphic DNA markers (RAPD). The leaves of 141 individuals were sampled. Based on 12 primers, 113 DNA fragments were generated. Genetic diversity was low at the population level (Nei's gene diversity (h)=0.0579; Shannon information index (I)=0.0924; percentage of polymorphic bands (PPB)=23.30%), but relatively high at the species level (h=0.1701; I=0.2551; PPB=46.34%). The coefficient of genetic differentiation based on Nei's genetic diversity analysis (0.6661) was high, indicating that there was significant genetic differentiation among populations, which was confirmed by AMOVA analysis exhibiting population differentiation among populations of 68.77%. Low gene flow among populations (0.2507) may result from several factors, such as a harsh pollination environment, population isolation and low seed dispersal distance. Limited gene flow and self-compatibility are the primary reasons for the high genetic differentiation observed for this species. We propose the collection of seeds from more populations with fewer individuals and core populations for *ex situ* conservation and suggest methods to increase seed germination rates.

Key words: endangered species; random amplified polymorphic DNA; genetic variability; population differentiation; conservation

INTRODUCTION

Heteroplexis Chang was first reported as a new genus belonging to the family Asteraceae. At present, the genus includes the five species: *H. vernonioides* C. C. Chang, *H. microcephala* Y. L. Chen, *H. sericophylla* Y. L. Chen, *H. impressinervia* J. Y. Liang and *H. incana* J. Y. Liang, all of which are endemic to Guangxi, China [1]. *H. microcephala* distribution is restricted to Yangshuo County in Guangxi, China, and occurs on the steep cliffs or upper slopes of karst limestone regions at an elevation of approximately 150-450 m, where the environment is very severe. Our investigations indicated that the range of this species has significantly decreased, and the number has been reduced to zero in Bilianfeng, the site where the model specimen was discovered. *H. microcephala* can provide important information leading to a better understanding of the evolution of Asteraceae. It has been designated as a class II nationally protected plant in China [2].

Genetic diversity is likely to affect the evolutionary potential of species [3]. If species lose their genetic diversity due to accelerated inbreeding, genetic drift or other reasons, the reproductive ability within populations can be reduced, leading to extinction [4]. Knowledge of genetic differentiation can provide important information to understand the evolution of

Table 1. Population location, number and sample size of six populations of *H. microcephala*.

Population code	Geographic locality	Latitude (N)	Longitude (E)	Altitude (m)	Sample size (n)
XP	Xingping Town, Yangshuo County	24°42′33″N	110°25′37″E	415	28
PY	Puyi Town, Yangshuo County	24°42′31″N	110°32′29″E	348	29
YS	Yangshuo Town, Yangshuo County	24°46′22″N	110°29′02″E	430	27
GT	Gaotian Town, Yangshuo County	24°46′41″N	110°29′06″E	160	15
BS	Baisha Town, Yangshuo County	24°48′57″N	110°24′06″E	226	27
YD	Yangdi Town, Yangshuo County	24°59′31″N	110°24′32″E	172	15

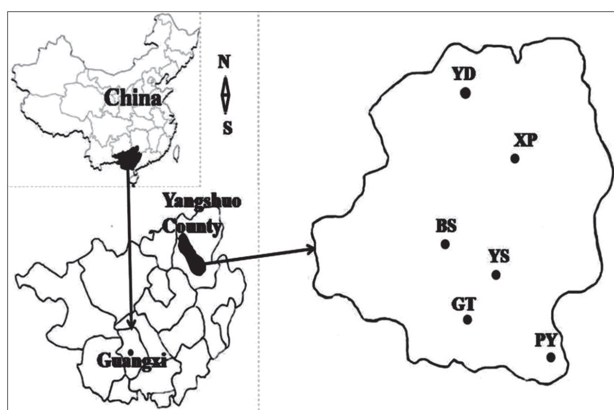

Fig. 1. The distribution of six *H. microcephala* populations from Yangshuo County in Guangxi, China surveyed in 2013. The names of six populations are abbreviated according to local town name. XP – Xingping Town; PY – Puyi Town; YS – Yangshuo Town; GT – Gaotian Town; BS – Baisha Town; YD – Yangdi Town.

species and make plans for conservation [5]. Previous studies of *H. microcephala* were primarily devoted to its chemical components [6], karyotype analysis [7] and biological characteristics [8]. However, no genetic variability has been carried out on this species, and we have no information about the genetic diversity of *H. microcephala*.

A number of PCR-based DNA markers have been used to analyze the population genetic structure of endemic and endangered plants [9], such as random amplified polymorphic DNA analysis (RAPD), inter simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) [10,11]. RAPD does not require prior genomic knowledge, and is a fast and relatively cheap method for evaluating a population's genetic differentiation, especially when molecular information is lacking on a certain species [12]. The limitation of low reproducibility and dominant nature in early RAPD can now be overcome through improved laboratory techniques and band scoring procedures, and analysis of molecular variance (AMOVA) [12-15]. Although RAPD analysis has some disadvantages, it is a useful technique for investigating population genetic diversity and has been applied to many rare plants [16,17]. The aim of this study was to use RAPD markers to characterize genetic diversity in *H. microcephala*, to investigate the genetic variation and to propose conservation strategies for *H. microcephala*.

MATERIALS AND METHODS

Plant materials

In our field investigation, six populations of *H. microcephala* from Yangshuo County in Guangxi, China, were surveyed and 141 samples representing six populations were collected in 2013. The sampling sites are shown in Table 1 and Fig. 1. Tender leaves were collected from six populations and placed in a bag container with approximately 100 g of silica gel, then stored at -80°C until DNA extraction.

DNA extraction and polymerase chain reaction (PCR) amplification

Equal amounts (0.5 g dry weight) of leaf tissue were placed in porcelain mortars with liquid nitrogen. Genomic DNA was extracted according to the CTAB method. The DNA quality was checked by gel electrophoresis [18]. The RAPD primers were synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd., according to the primers published by the University of British Columbia (UBC) [19]. For preliminary screening, 100 primers were tested on six individuals from six populations. Twelve primers (Table 2) that produced clear and reproducible bands were chosen for analysis.

PCR was performed in a 25-μL reaction volume containing 50 ng DNA template, 1.5 mM $MgCl_2$, 0.5 μM primer, 0.2 mM dNTP, 2.5 μL 10×buffer, and 1.5 units Taq polymerase. Amplifications were conducted in a Biometra TProfessional Thermocycler as follows: initial denaturation at 94°C for 5 min; 40 cycles of 94°C for 40 s, annealing temperature (Table 2) for 45 s, 72°C for 1.5 min; final extension at 72°C for 7 min [20].

Electrophoresis was performed with 5 μL of amplified products in a 1.5% agarose gel at 120 V for 1 h. The bands were detected with ethidium bromide under UV light. Molecular weights were estimated using a 2000-bp DNA ladder (Takara Biotech). To ensure the reproducibility, each amplification reaction and gel running was repeated three times and only clear and reproducible fragments were recorded. Negative controls, replacing template DNA with ddH_2O, were included in each amplification to check the possibility of contamination.

Data analysis

RAPD profiles were scored as the presence (1) or absence (0) of specific bands. Statistical analysis was performed using POPGENE 32 [21], including the percentage of polymorphic loci (PPB), Nei's genetic distance (h), Shannon's information index (I), observed allele number (Na), effective allele number (Ne), gene diversity within populations (Hs), total gene diversity (Ht), the coefficient of Nei's gene differentiation among populations (Gst), gene diversity among populations (Dst), and gene flow among populations (Nm). Analysis of molecular variance (AMOVA) was performed using WINAMOVA 1.55 [22]. An unweighted pair group method using arithmetic averages (UPGMA) cluster analysis was carried out using the NTSYS-pc version 2.02 software package [10].

Table 2. Characteristics of the 12 RAPD primers used for amplified DNA products in this research.

Primer	Sequence (5´to 3´)	TA (°C)	N_{PL}/N_L
812	CCTTGACGCA	36.9	3/8
819	ACCCCCGAAG	41	6/11
831	CAATCGCCGT	36.9	4/9
837	GACCGCTTGT	36.9	5/9
846	ACCTGAACGG	36.9	5/10
866	GAACGGACTC	36.9	3/10
867	GTCCCGACGA	41	3/8
869	CTCACCGTCC	41	5/8
871	AAAGCTGCGG	36.9	4/9
880	ACTTCGCCAC	36.9	6/10
881	CTACGGAGGA	36.9	8/12
886	GTGCCTAACC	36.9	5/9

TA – annealing temperature (°C); N_L – number of loci scored; N_{PL} – number of polymorphic loci scored

RESULTS

Genetic diversity of *H. microcephala*

One hundred RAPD primers were screened in six selected individuals. A total of 113 bands were obtained from the 12 selected primers across 141 individuals of the six populations, corresponding to an average of 9.41 bands per primer (Table 2). The percentage of polymorphic loci and average genetic diversity are summarized in Table 3. The percentages of polymorphic loci (PPB) for the populations ranged from 18.58 to 28.32% with an average of 23.32%. The average observed allele number (Na) per locus is 1.2330 and the effective number of alleles (Ne) per locus is 1.0926. Shannon indices (I) and Nei's gene diversity (h) varied

Table 3. Genetic diversity parameters of six sampled population of *H. microcephala* based on 113 RAPD loci using POPGENE 32.

Populations	Np	PPB (%)	Na	Ne	h	I
XP	32	28.32	1.2832	1.0987	0.0640	0.1049
PY	30	26.55	1.2655	1.1029	0.0657	0.1057
YS	24	21.24	1.2124	1.0782	0.0481	0.0776
GT	21	18.58	1.1858	1.0896	0.0531	0.0822
BS	26	23.01	1.2301	1.0663	0.0455	0.0769
YD	25	22.12	1.2212	1.1200	0.0707	0.1076
Mean	26	23.30	1.2330	1.0926	0.0579	0.0924
Species level	57	46.34	1.4867	1.2859	0.1701	0.2551
SD			0.5021	0.3495	0.1956	0.2838

Note: Np – number of polymorphic loci; PPB – percentage of polymorphic loci; Na – observed allele number; Ne – effective allele number; I – Shannon's information index; h – Nei's gene diversity

Table 4. Analysis of genetic variability within and among six H. microcephala populations based on RAPD data using POPGENE 32.

	Ht	Hs	Dst	Gst	Nm
Mean	0.1732	0.0579	0.1153	0.6661	0.2507
S.D	0.0391	0.0080			

Ht – total gene diversity; Hs – gene diversity within populations; Dst – gene diversity among populations; Gst – the coefficient of Nei's gene differentiation among populations; Nm – gene flow among populations

from 0.0769 to 0.1076 and 0.0455 to 0.0707, with an average of 0.0924 and 0.0579 at the population level and 0.2551 and 0.1701 at the species level. The XP population exhibited the greatest level of variability (PPB=28.32%, Na=1.2832, Ne=1.0987, h=0.0640, I=0.1049), whereas the GT exhibits the lowest level (PPB=18.58%, Na=1.1858, Ne=1.0896, h=0.0531, I=0.0822, respectively) (Table 3).

Genetic differentiation of *H. microcephala*

The genetic differentiation (Gst) among populations was 0.6661 (Table 4), which indicated that 66.61% of the genetic variability was distributed among populations, and only 33.39% of the variation existed within populations. Molecular variance was also examined using AMOVA based on RAPD banding patterns. The variance component found within populations was 31.23%, and a variance of 68.77% was found among populations (Table 5). The number of individuals exchanged between populations per generation (Nm) was 0.2507, indicating that there is a low migration rate.

Genetic relatedness among populations

Table 6 shows Nei's genetic identities and genetic distance for all population pairs. The highest genetic distance was 0.2232 between populations XP and GT, while the lowest was 0.0576 between XP and PY. Six populations clustered into two main groups (Fig. 2), one containing populations XP and PY, and the other containing YS, GT, BS, and YD. The latter was divided into three subgroups, one containing populations GT and BS, one population YS, and the last population YD.

DISCUSSION

Genetic diversity

Low genetic diversity is a common feature of endemic and endangered plant species [23,24]. Loss of genetic diversity reduces the ability of species to cope with environmental change, which is the main reasons for plant rarity. In our study, the genetic diversity of *H. microcephala* was relatively low. PPB values ranged from

Table 6. Nei's measures of genetic identity (above diagonal) and genetic distance (below diagonal) among six populations of *H. microcephala.*

Populations	XP	PY	YS	GT	BS	YD
XP	****	0.9440	0.8280	0.8000	0.8309	0.8304
PY	0.0576	****	0.8825	0.8306	0.8448	0.8267
YS	0.1888	0.1250	****	0.8900	0.8535	0.8141
GT	0.2232	0.1856	0.1165	****	0.8908	0.8585
BS	0.1852	0.1687	0.1584	0.1156	****	0.8703
YD	0.1858	0.1903	0.2056	0.1526	0.1389	****

Table 5. Analysis (AMOVA) of genetic variability within and among six *H. microcephala* populations based on RAPD data.

Source of variance	d.f.	Sum of squares	Mean squares	Variance component	Percentage of total (%)	P value
Among populations	5	855.2977	171.060	7.2351	68.77%	<0.001
Within populations	135	443.5675	3.286	3.2856	31.23%	<0.001

Note: d.f. – degrees of freedom. P-value denotes the probability of null hypothesis; significance tests after 1000 permutations

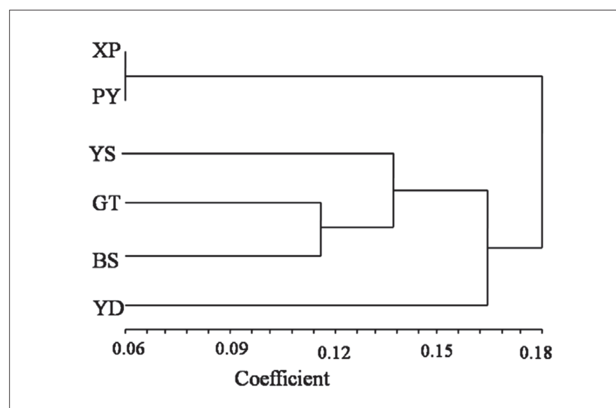

Fig. 2. Dendrogram of the six sampled locations of *H. microcephala* obtained by unweighted pair-group method with arithmetic average (UPGMA) cluster analysis and SM coefficient using NTSYS-pc version 2.02 software package.

18.58 to 28.32% with an average of 23.32%, which are smaller than seed plants in general (PPB=34.2%) and other endangered plants that have been examined using RAPD markers, such as *Dracocephalum austriacum* L. (PPB=80.14%) [25] and *Centaurea nivea* (PPB=72.90%) [26]. The genetic variation was also low compared to some other species in the Asteraceae family, such as *Centaurea nivea* (PPB=72.90%) [26], *Aster spathulifolius* Maxim (PPB=43.74%) [27] and *Nouelia insignis* (PPB=65.05%) [28]. Shannon's information index and Nei's gene diversity also revealed low genetic variation at the population (0.1328 and 0.0880) and species levels (0.2551 and 0.1701). The low genetic diversity observed in *H. microcephala* might be due to its restricted geographic distribution, breeding system, or inbreeding, as well as the decline in population size.

H. microcephala grows in harsh environments, which can easily result in a low survival ratio. It usually forms small populations at the top of hills with a discrete distribution area of only 600~1000 m^2. In its habitat, some seedlings fall to the bottom layer of vegetation with weak light during the growth process, leading to unbalanced seedling growth. The environment reduces the seedling survival ratio, and the species' natural regeneration ability is poor. However, the distribution of *H. microcephala* was expected to be narrower and more fragmented than the current one.

In general, clonal and selfing plants have high differentiation and low genetic diversity compared to out-crossing species [29]. According to our field investigation, the breeding system of *H. microcephala* prioritizes out-crossing but maintains self-compatibility. The *H. microcephala* population is fragmented, so pollen travel between populations is difficult. In general, the number of individuals in a population is between 10 to 100, which is very small and results in a decrease of population genetic diversity because of long-term self-compatibility.

All these factors may have led to a decrease in population size and increase in inbreeding, perhaps explaining the low genetic diversity observed in *H. microcephala*.

Population genetic structure

In this study, analyses of the RAPD markers using Shannon's diversity index, Nei's genetic diversity analysis and AMOVA yielded similar interpretations of the *H. microcephala* population genetic structure. The estimated Nei's genetic differentiation (Gst) calculated for all polymorphic loci suggested that a genetic differentiation has occurred (66.61%) among populations of *H. microcephala*. Furthermore, AMOVA showed that 68.77% of the total variation results from differentiation among populations. The genetic structure of plant populations reflects interactions among various factors, including habitat fragmentation and population isolation, mating system, genetic drift and gene flow [30].

One possible explanation for the lack of genetic variation within populations of *H. microcephala* may be genetic drift. The endemic plant possesses generally low genetic variation due to genetic drift. Genetic drift decreases the variation within populations and increases the differentiation among populations.

Another possible explanation for the genetic differentiation between populations is gene flow. The gene flow was only 0.2507, which is far below the baseline value of 1, indicating a genetic differentiation among populations of *H. microcephala*. As in our

study, genetic differentiation was found in several rare or endangered plant species and attributed to low or absent gene flow [31-33].

This species grows on the upper slopes or steep cliffs of karst limestone regions known for drastic climate change during the flowering season. According to our field observations, gene flow among populations of *H. microcephal*a occurs mainly through pollinators (the flowers are visited by bees such as *Eristalis cerealis* and *Vespa ducalis*). Changes in the weather lead to the quick loss of pollen vitality and affect the number of pollinators. The restrictive geographical distribution and isolated populations separated by several limestone regions can make pollen interaction between populations difficult.

Seed produce varies widely from 2000 to 5000 per seeding, and the seeds are small (weight per thousand seeds is only 0.34 g). The distance of seed dispersal of *H. microcephala* is short. Seeds usually drop to the ground during the rainy season and stay on the soil surface, although others are dispersed by wind or birds. Seeds falling around mother plants might be partly responsible for the low population genetic diversity in this species. In addition, seed germination rates are extremely low under natural conditions, even if rates are below 1% under experimental conditions. Germination and propagation are very rare events even though the seeds can disperse. Gene flow via seed and pollen dispersal may be very restricted.

Some genetic studies have confirmed a positive correlation between population size and genetic diversity [9]. Our results indicated that genetic diversity is related to population size. The genetic diversity of the smallest population, GT (approximately 15 plants), was the lowest, while that of the largest population, XP (more than 100 plants remaining), was the highest.

Conservation recommendations

Based on the observed genetic diversity, conservation strategy proposals for *H. microcephala* are as follows: first, the large populations with higher genetic variation in *H. microcephala* should be given priority for conservation: the PY and XP populations should be a priority for conservation action. Second, replacement of the lost population can be achieved by transplanting seedlings, and through other actions to artificially increase the gene flow and maintain sufficient genetic richness. Specifically, we propose a practical *ex situ* conservation strategy of collecting seeds for propagation under greenhouse conditions. This could increase the genetic diversity in combination with transplanting seedlings among extant populations. The seed germination ratio in nature is extremely low, so successful propagation and planting will require the development of methods to increase seed germination.

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Authors' contributions: Xiao Wei designed the experiments; Jianming Tang performed the experiments; Jiqing Wei and Yongtao Li analyzed the data; Yancai Shi and Shengfeng Chai wrote the paper.

Conflict of interest disclosure: This manuscript has not been published before and is not under consideration for publication anywhere else. The publication of this article was approved by all authors.

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