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杜鹃红山茶遗传多样性的ISSR分析

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摘要:运用ISSR分子标记技术,利用筛选的10条ISSR引物,对珍稀濒危植物杜鹃红山茶(*Camellia changii* Ye)2个亚种群60个单株的遗传多样性进行了研究。结果表明:物种水平上的多态位点百分率(PPB)为55.29%,Nei's基因多样性指数(H)及Shannon多态性信息指数(I)分别为0.2191和0.3215。种群间的遗传分化系数(G_{ST})仅为0.0922。研究结果揭示了杜鹃红山茶的遗传多样性较低,亚种群间遗传分化较小。小种群和人为活动干扰是杜鹃红山茶现存种群的主要限制因素。影响杜鹃红山茶种群发展的其它因素亟需进一步研究。

关键词:杜鹃红山茶;特有种;遗传多样性;ISSR
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Genetic Diversity of *Camellia changii* Ye (Theaceae) Using ISSR Markers

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Abstract: *Camellia changii* Ye is a rare and endangered species endemic to China. It is confined to the E'huangzhang Nature Reserve at Yangchun City, southwestern Guangdong Province, South China. The genetic diversity was studied using Inter-Simple Sequence Repeat (ISSR) markers. A total of 60 individuals from two subpopulations were sampled. Ten primers with discernible DNA bands were applied. The percentage of polymorphic bands (PPB), Nei's Index and Shannon's Index were 55.29%, 0.2191 and 0.3215, respectively. The coefficient of gene differentiation (G_{ST}) was 0.0922. These results indicate that *C. changii* has low genetic diversity and low differentiation between the two subpopulations. Small population size and human disturbances are the main limiting factors for the extant population. Further studies on the factors limiting population development are urgently required.

Key words: *Camellia changii*; Endemic species; Genetic diversity; ISSR

Camellia changii Ye is a critically endangered and endemic species. It is found only in the E'huangzhang Nature Reserve (111°21' 29" – 111°36' 03" E, 21°50' 36" – 21°58' 40" N) [1], ca. 60 km away from Yangchun City, southwestern Guangdong Province, China (Fig. 1). Recent field studies have showed that only one population of *C. changii* survives, with a total known range of less than 100 km^2. It is distributed in degraded riparian woodlands along a stream. The population has been greatly reduced as a result of cutting between the 1950s and 1970s because it has showy, bright red flowers. The construction of hydropower stations in

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the surrounding areas is the major cause of disturbance in the recent decade. A survey of the population found fewer than 1 000 individuals (Luo et al., unpublished data). As a species of limited extent and numbers, *C. changii* has been listed as critically endangered (CR) in the China Species Red List [2].

Endemic species with restricted geographic distributions have become a central concern of biologists. Understanding the level and structure of genetic variation is of critical importance in formulating conservation and management plans for *C. changii*'s long-term survival, *ex situ* collection and conservation. A preliminary study using Random Amplified Polymorphic DNA (RAPD) markers showed that the genetic diversity of *C. changii* was low [3], but other biological information about this species is lacking.

Several methods are currently employed to measure genetic variation within and among populations of a species, the most commonly used recently being the Inter-Simple Sequence Repeat (ISSR) technique. It is a highly sensitive, reproducible and effective technique for the genetic studies of plants [4-5]. It has several advantages over the RAPD markers: (1) the target sequence is not required beforehand [6]; (2) the reproducibility of amplified bands is higher than with RAPDs [7-9]; (3) the polymorphic bands produced by ISSRs are higher than those produced by RAPDs [10-12]. The ISSR technique has been successfully applied in studies of many cultivated species [11,13], and has recently been shown to be useful in population genetic studies, especially in detecting genetic diversity in rare and endangered species [12,14].

The main purpose of the present study was to reveal the level of ISSR variation and genetic differentiation between the two subpopulations of *C. changii*. It will provide the basic information for effective conservation.

1 Materials and methods

1.1 Field collections

A total of 60 individuals of *C. changii* were sampled at intervals of 100 m along two stream banks in March 2005. Thirty-four samples (Subpop-1) were from the east bank while the other 26 samples (Subpop-2) were from the west bank (Table 1). Young leaves were collected and dried quickly with silica gel in sealed plastic bags in the field and then stored at -80℃ for further use.

1.2 DNA extraction and quantification

Total DNA was extracted using the modified SDS protocol of Luo et al.[15]. 0.1 g dried leaf tissue was ground to a fine powder with liquid nitrogen and 4% w/v PVP in a mortar, followed by incubation in 800 μl of preheated 1% w/v SDS extraction buffer containing 0.2% v/v mercaptoethanol for 1 hour at

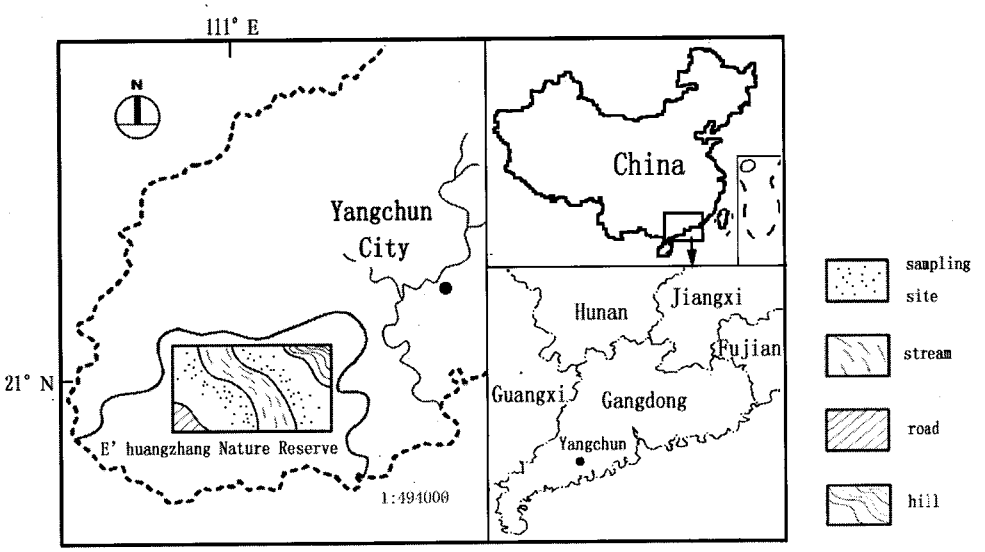

Fig. 1 Map showing the locations of the population of *C. changii* sampled

Table 1 Population of *C. changii* examined for ISSR variation

Collection no.	Population	Samples	Location	Altitude (m)
1–34	Subpop-1	34	East stream bank	40–175
35–60	Subpop-2	26	West stream bank	50–130

65℃. The homogenate was extracted twice with 800 μl chloroform: isoamyl alcohol (24:1, v/v) and samples were shaken gently for 10 min and centrifuged at 5 668 × g for 10 min at room temperature. The supernatant was reserved and precipitated in 2 volumes of precooling ethanol and 1/10 volumes of 3 mol/L NaAc and collected by centrifugation at 3 354 × g for 1 min at room temperature. The pellet was washed 3 times with 70% ethanol, air dried, and dissolved in 150 μl 0.1×TE buffer. DNA quality and quantity were determined in 0.8% agarose gels.

1.3 Selection of primers and PCR amplification

A total number of 96 ISSR primers (Sangon Inc., Shanghai, China) were screened using two DNA samples to select the appropriate primers. Primers with clarity, scorability, and reproducibility of banding patterns were used for ISSR analysis. All the reactions were performed in a volume of 20 μl containing 5 ng template DNA, 2.0 μl 10 ×Buffer, 1.5 mmol/L $MgCl_2$, 0.15 mmol/L 10 × dNTPs, 0.15 μmol/L primer, 1 U Taq polymerase. Amplifications were carried out in PTC-100™ Peltier Thermal Cycler, programmed for a 5 min initial denaturation at 94℃, followed by 40 cycles of 30 s denaturation at 94℃, 45 s annealing at 54℃, and an extension of 80 s at 72℃, and ended up with 5 min extension at 72℃. Amplified products were electrophoresed in 1.5% agrose (containing EB 0.5 μg ml^{-1}) — 1 ×TBE gel at 4 V cm^{-1} for 3 h and photographed under ultraviolet by the Bio-Rad imaging devices (Gel Doc 2000 Gel Documentation System) supported by Quantity One (version 4.2) software. Fragment sizes were estimated based on 100 bp ladder size standards (Sangon).

1.4 Data analysis

Each unique band size was designated as a locus for each primer and scored as diallelic (present = 1, absent = 0). Population genetic parameters, the percentage of polymorphic bands (PPB), Nei's gene diversity (H), Shannon diversity index (I), the coefficient of gene differentiation (G_{ST}) and Nei's genetic identity were calculated using POPGENE (version 1.32). Gene flow was estimated according to the formula: $Nm = (1 - G_{ST})/4G_{ST}$ [16]. Based on Nei's gene diversity, UPGMA dendrogram was drawn by NTSYSpc (version 2.10).

2 Results

2.1 Genetic diversity of *C. changii*

A total of 85 bands were scored from 10 screened primers. Among the 85 loci, 47 of which were polymorphic (PPB = 55.29%), and PPB varied from 42.9% to 61.5% per primer (Table 2). An example of PCR amplification of DNA samples from the population with primer 810 is given in Fig. 2.

Table 2 Primers used for ISSR amplification

Primer code	Sequence of primer	Total bands	Polymorphic bands	%
810	$(GA)_8T$	7	4	57.1
811	$(GA)_8C$	7	3	42.9
815	$(CT)_8G$	11	6	54.5
816	$(CA)_8T$	6	4	66.7
817	$(CA)_8A$	10	6	60.0
818	$(CA)_8G$	10	6	60.0
825	$(AC)_8T$	8	4	50.0
827	$(AC)_8G$	7	3	42.9
855	$(AC)_8YT$	6	3	50.0
857	$(AC)_8YG$	13	8	61.5
Average	-	8.5	4.7	55.29

Nei's Index (H) and Shannon's Index (I) were 0.2191 and 0.3215 at species level, respectively. At

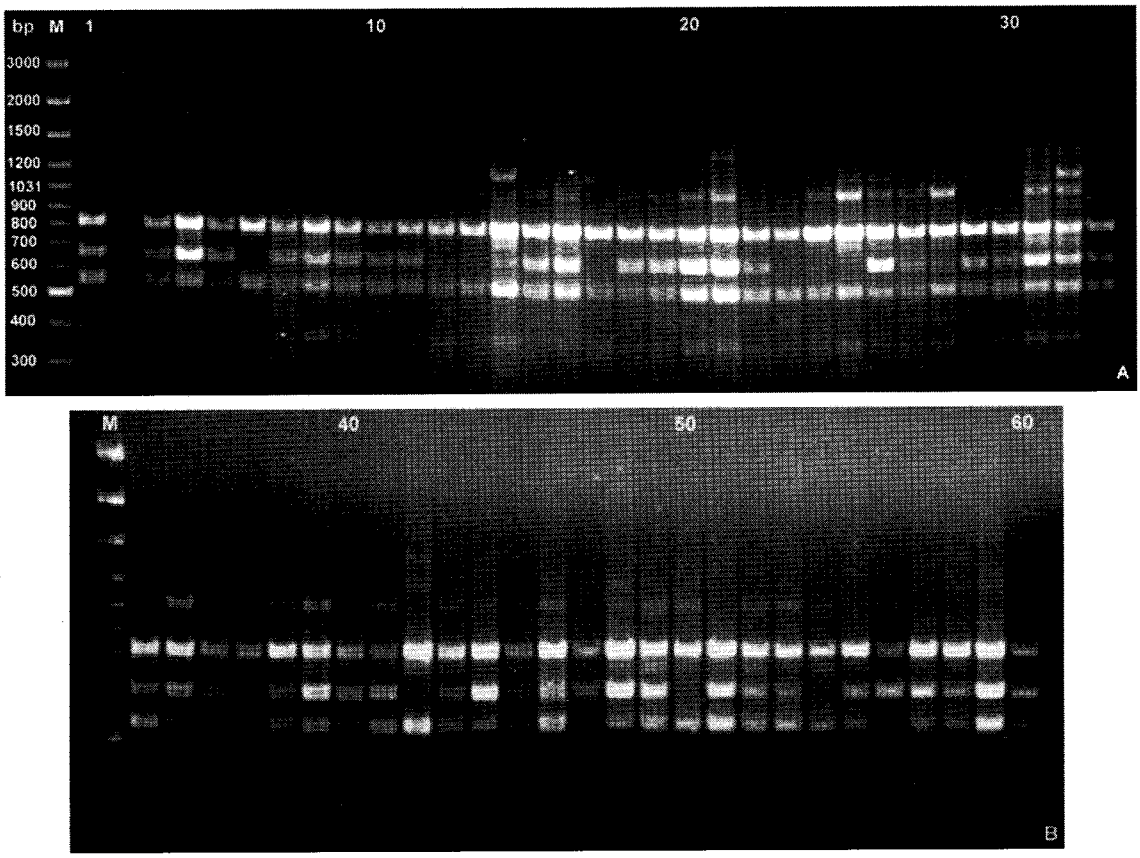

Fig.2 ISSR bands of *C. changii* from the samples amplified with primer 810
M: Molecular marker. 1– 60: Sample numbers.

subpopulation level, PPB value of Subpop-1 was higher than that of Subpop-2, and the Shannon index (I) and Nei's gene diversity index (H) showed the same trends (Table 3).

Table 3 Genetic diversity of *C. changii*

Subpopulation	Samples	Polymorphic loci	PPB	H	I
Subpop-1	34	45	52.94	0.2200	0.3191
Subpop-2	26	38	44.71	0.1726	0.2535
Species level	60	47	55.29	0.2191	0.3215

PPB=Percentage of polymorphic bands; H = Nei's gene diversity; I = Shannon's information index

2.2 Genetic differentiation of *C. changii*

The coefficient of gene differentiation (G_{ST}) between the two subpopulations of *C. changii* was 0.0922, which indicated only 9.22% variation presented among subpopulations and great majority of genetic variation (90.78%) resided among individuals. Nei's genetic identity value was 0.9508. The level of gene flow (N_m) was 2.4619. The results showed that the two subpopulations were genetically similar.

2.3 Cluster analysis

The population was divided into four groups (Fig.3). Group I is the largest, with all the individuals on the west bank and 17 individuals on the east bank. All the individuals of Groups II to IV are from the east bank. The subpopulation on the east bank (Subpop-1) had higher genetic diversity than that on the west bank (Subpop-2).

3 Discussion

3.1 Genetic diversity of *C. changii*

ISSR has been applied in genetic studies of many rare and endangered species in recent years. The results showed that the genetic diversities of some species were low, but some were high. For example,

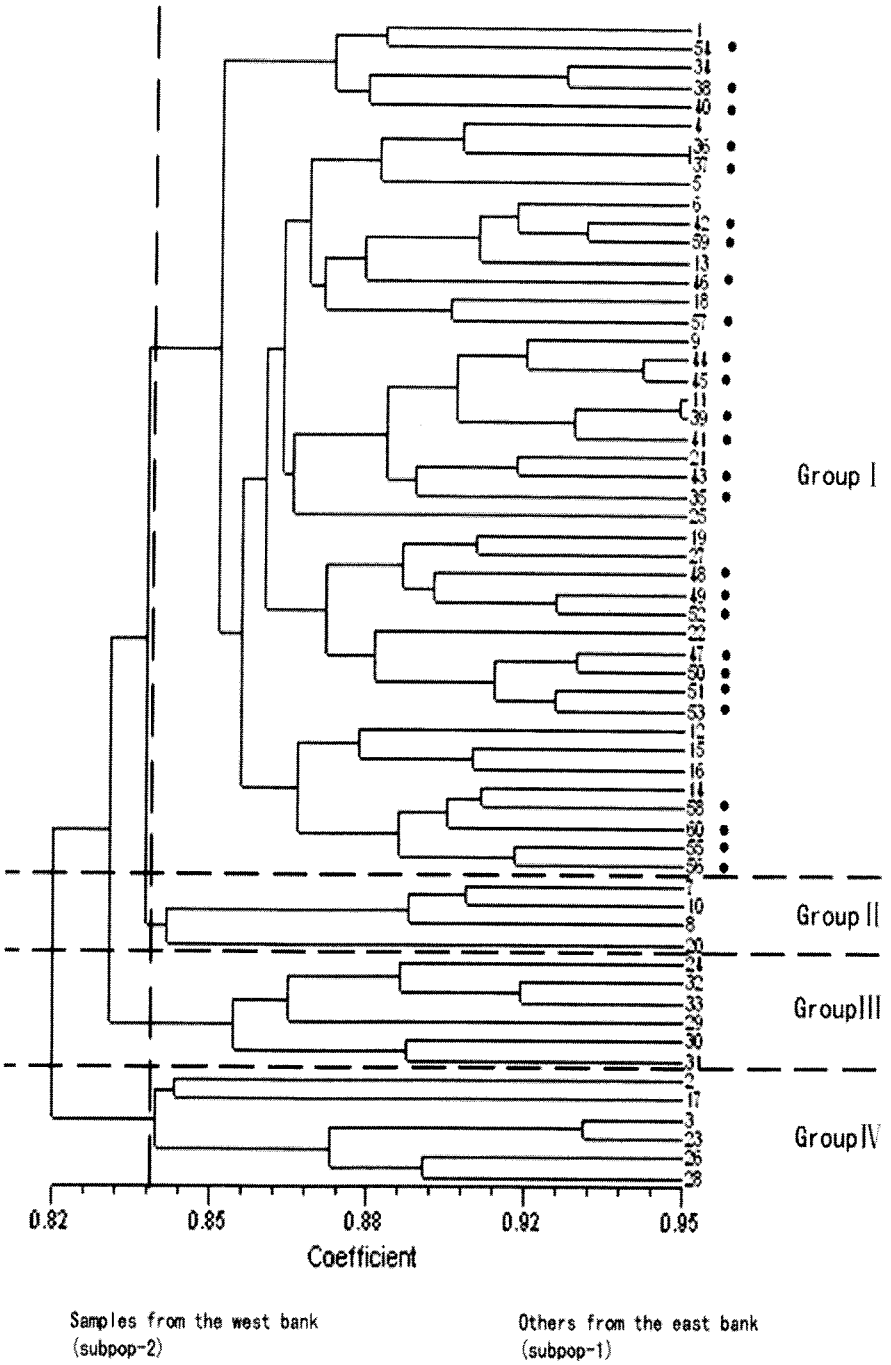

图 3 UPGMA dendrogram of *C. changii* based on Nei's genetic identity

the PPB values of the rare species, *Cycas guizhouensis* and *Glycine soja*, were lower than 50%[17-18], while those of *Changium smyrnioides* and *Psathyrostachys huashanica* were greater than 80% [14,19]. The genetic diversities of both subpopulations of *C. changii* were low, but that of Subpop-1 (H=0.22) was slightly higher than that of the Subpop-2 (H=0.17). Hamrick and Godt found that the species endemic to narrow distributions usually had low genetic diversity (0.21–0.23)[20] and *C. changii* was consistent with this.

As stated by Karron, it would be more meaningful to compare restricted plants to more widespread congeners because they have a similar phylogenetic history and share certain ecological attributes[21]. A few

studies have investigated *Camellia* species by ISSR. The PPB values of *C. sinensis* varied between 77.6% and 84%[22-24] and that of *C. nitidissima* was 75.24% [25]. By comparison, the genetic diversity of *C. changii* was the lowest (55.29%).

3.2 Factors affecting the genetic diversity of *C. changii*

The geographical range is the main factor affecting the genetic variation of a species. Hamrick and Godt analyzed data from 449 species to elucidate general trends and concluded that there was a positive correlation between geographical range and genetic diversity, and that rare, endemic, or narrowly distributed plants tended to contain less variation than widespread species[20]. Karron compared congeners of 11 groups and found that the diversities of endemic or rare species were lower than those of widespread congeners [26]. The low diversity of *C. changii* is consistent with its extremely narrow range.

Human disturbance may possibly have had an impact on the genetic diversity. Although the physical environments of the two banks are quite similar as the stream is narrow, they differ in human disturbance. The west bank is close to the access road and has more frequent and stronger human disturbances than the east bank at the bottom of hills. Therefore, the pollinators of *C. changii* may be more easily affected. However, the impact of human disturbances on the insect richness and diversity between two subpopulations are not clear yet. It needs further study.

3.3 Factors affecting the genetic structure of *C. changii*

ISSR markers revealed that *C. changii* held more genetic variation within subpopulations than among subpopulations (G_{ST} = 0.0922), which was consistent with our previous study by RAPD markers (G_{ST} = 0.1242)[3]. Hamrick and Godt stated that the reproductive biology was the most important factor in determining the genetic structure of plant populations. They showed that outcrossing plant species tend to exhibit between 10% and 20% genetic variation among populations while selfing species exhibit, on average, 50% variation between populations[20]. Our field observations confirmed *C. changii* is self-incompatible, the lower G_{ST} value was attributable to its obligate outcrossing breeding system.

Besides the reproductive biology, the population genetic structure of a species is affected by a number of factors including long term evolutionary history, mutation, gene flow and seed dispersal, as well as natural selection[20]. For *C. changii*, the effective gene flow per generation (N_m) was 2.4619, which indicated that there was a considerable gene flow between the two subpopulations. Insect activities may greatly promote gene flow. The result of UPGMA cluster analysis showed 50% of the individuals sampled on the east bank clustered with those on the west bank, showing that the separation between the two subpopulations was well within the foraging range of the pollinators, so there was high gene flow between subpopulations. Therefore, there was no distinct genetic differentiation between the two subpopulations.

Pollen transfer and seed dispersal determine a plant's reproductive success, range expansion, and population genetic structures. *C. japonica* in Japan[27] and *C. hongkongensis* in HK (personal communication with R. T. Corlett), are bird-pollinated-mostly by the Japanese white-eye (*Zosterops japonica*). While Diptera are considered to be the most numerous and effective pollinators of *Camellia sinensis*[28]. There is very little information on seed dispersal of camellias. *C. japonica* is dispersed by scatter-hoarding rodent (*Apodemus speciosus*) in Japan, and that is probably true for all the large and nutritious-seed Camellia species [29]. Therefore, pollination by birds (long-distance) and seed dispersal by rodents (short-distance) would result in most gene flow. Pollen would be dispersed across water but not seeds.

3.4 Comparison of the efficiency of RAPD and ISSR markers

With a few exceptions[14], most studies have found that the ISSR technique could detect more polymorphism than RAPD[4-5,12,30]. In an earlier study, the genetic

diversity (PPB) of *C. changii* detected by RAPD was 38.83% [3], while the present study by ISSR was 55.29%. Both methods showed similar results that *C. changii* had low genetic diversity, but the polymorphism detected by ISSR was higher.

3.5 Implications for conservation

Studies of rare and/or endemic species are becoming increasingly important with the growing awareness of the need for data on which to base conservation decision. It is obvious that the most important thing is to protect *C. changii*'s natural habitat if we want to protect its diversity. Since the existing population has dramatically declined in the past few decades, *C. changii* and its habitat are protected by law in the E'huangzhang Nature Reserve. Although *in situ* protection has alleviated the decline of *C. changii*, protecting the habitat by itself is not enough to recover and spread its population. So *ex situ* protection must be carried out. Although G_{ST} for *C. changii* is low, the number of existing individuals is small. This indicates a need to protect as much of this species as possible. *Ex situ* strategies such as establishing a conservation centre and developing fast propagation techniques should be taken into consideration.

Only a few seedlings of *C. changii* were seen in natural habitats, suggesting that there may be reproduction and regeneration problems. Therefore, further study on the reproductive biology is urgently required in order to identify the barriers to population recovery.

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