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RAPD analysis for genetic diversity in *Changium smyrnioides* (Apiaceae), an endangered plant[1]

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Abstract. Random Amplified Polymorphic DNA (RAPD) markers were used to measure genetic diversity of *Changium smyrnioides* Wolff (Apiaceae), a genus endemic to eastern China and an endangered medicinal plant, collected from five populations along the Yangzi River. A total of 92 amplified bands were scored from the 13 RAPD primers, and a mean of 7.1 amplified bands per primer and 69% (64 bands) percentages of polymorphic bands (*PPB*) was found. The Shannon's index was used to partition genetic diversity. Genetic diversity estimates indicated that 51.2% of total diversity was among populations and 48.8% within populations. The species shows higher genetic diversity between populations than other endangered plants. In this study, the RAPD results of genetic diversity are similar to those of previous studies employing allozymes (58% among populations and 42% within populations). The RAPDs provide a useful tool for assessing genetic diversity of rare, endemic species and for resolving relationships among populations. The results show that the genetic diversity of this species is high, possibly allowing it to more easily adapt to environmental variations. A pattern of greater variation among rather than within populations was observed with both markers. This distributive pattern of genetic variation of *C. smyrnioides* populations provides important baseline data for conservation and collection strategies for this species. It is suggested that most of the populations should be sampled in ex-situation protection so as to retain as much genetic diversity as possible.

Keywords: Allozymes; *Changium smyrnioides*; Genetic diversity; RAPD.

Introduction

Changium smyrnioides Wolff, a species of monotypic genus in the family Apiaceae, is an endangered herbaceous perennial plant in China. It is endemic to eastern China, and is distributed only in fragments in Zhejiang, Anhui, Jiangsu, and Hubei Provinces (see Figure 1). The species is not only of important medicinal value; it is also of interest to plant systematists. The drugs made from this species promote the production of body fluid to quench thirst, nourish "yin" to moisten the lung, soothe the throat, and clear away toxic material that may cause skin infection. As there might be some internal reasons, such as questions of reproductive biology and population ecology, this species has been assigned an endangered status and is listed in the Red Data Book as an endangered plant by the government of China (Zhang, 1991).

The species was named *C. smyrnioides* by Wolff (1924) based on a specimen from Wuxin, Zhejiang. Various aspects of the genus have been discussed in earlier papers (Qiu et al., 2000; Qiu and Fu, 2001), but it should be emphasized that natural populations of *C. smyrnioides* are very small and rare. Because allozymes have sometimes proven insufficiently variable for assessing genetic diversity within and among populations of rare endemic plants, attention has turned to more variable regions of the genome. A more recently employed approach in plant systematics and population biology is random amplified polymorphic DNA (RAPD) markers, a PCR-based (polymerase chain reaction) technique.

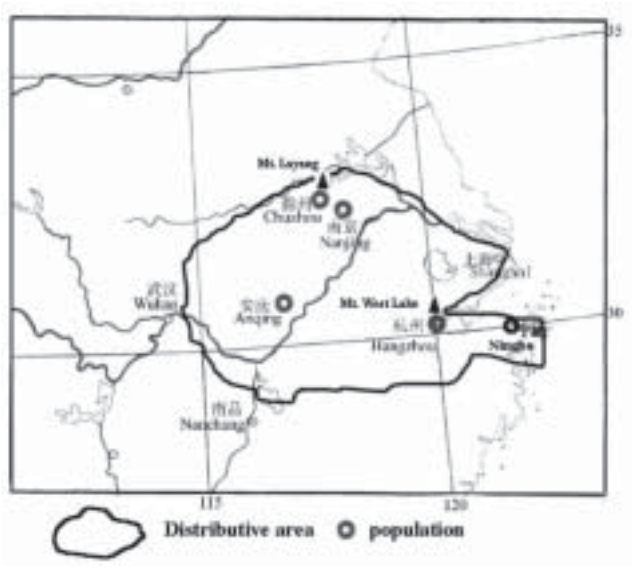

Figure 1. The distribution area and sampling locations of *Changium smyrnioides*.

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Although earlier studies (Pan et al., 1983, 1995; Zhang et al., 1983; Li et al., 1987; Sheh and Su, 1987; Su and Sheh, 1990; Cheng et al., 1993) have reported some results of research on chemical, cytological, pollen morphological, ecological, geographical, and karyotypic characters of the species; neither the genetic diversity nor the divergence were clear. The purpose of the present study is to assess genetic diversity and divergence within and among populations of the species using RAPD markers and to compare the results with those obtained from allozymes. Another important aim is to provide genetic data and a theoretical basis for protection of the species.

Materials and Methods

Population Sampling

Total DNA was extracted from plants collected in the field. Leaves were dried in plastic bags with silica gel until extracted in the laboratory at Zhejiang University, Hangzhou, Zhejiang, China. A total of 35 individuals from five populations (Ningbo, Langya, Nanjing, Anqing, Hangzhou) were included in the study. The distance between plants collected was at least 10 m to increase the possibility of detecting the variation potential within each population. Details on material and vouchers are given in Table 1.

DNA Extraction and Amplification

The CTAB (hexadecyltri-methylammonium bromide) method of Dellaporta et al. (1983) was used to extract total DNA. Leaf material was powdered in liquid nitrogen, mixed with 2 ml extraction buffer [1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 2% CTAB, and 1% 2-mercaptoethanol] at 65°C, and incubated at 65°C for 45 min with slow shaking every 10 min. Proteins were extracted twice with 2 ml chloroform: iso-amylalcohol (24:1), then centrifuged at 13,000 rpm for 2 min. RNase (10 µg/ml) was added to the supernatants for 30 min at 37°C, and the mixture was centrifuged at 13,000 rpm for 2 min again. The sediment was washed twice in 70% ethanol, vacuum-dried, and resuspended in 100 µl Tris-EDTA buffer (1 mM Tris-HCl and 0.1 mM EDTA, pH 8.0).

Amplification of genomic DNA was made on a Perkin Elmer DNA Cycler (BIOMETRA, Germany), using the arbitrary decamers. The 13 primers were selected from the "S" RAPD primer kit purchased from Sangon (Shanghai, China) (Table 2); these primers included S14, S15, S16, S21, S23, S24, S31, S34, S35, S38, S39, S44 and S45. Amplifications of genomic DNA were performed in 25-µl reaction volumes containing 1.2 units of *Taq* polymerase (Sangon, Shanghai, China), 10 mM Tris-HCl (pH 9.0), 25 mM KCl, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 24 ng each of random primer and 40 ng of template DNA. The cycle program included an initial 75 sec denaturation at 94°C, followed by 45 cycles of 15 sec at 94°C, 30 sec at 42°C and 75 sec at 72°C, with a final extension at 72°C for 7 min. RAPD fragments were separated electrophoretically on 1.5% agarose gels in 1X TBE buffer, stained with ethidium bromide, and photographed on a UV transilluminator using a digital camera. DNA from each plant was amplified with the same primer more than once, and the banding patterns were compared.

Optimization of RAPD Protocol

Because the RAPD-PCR technology is sensitive to changes in experimental parameters, a total of 50 primers were initially screened against ten plants selected from all populations. The effects of magnesium, template DNA concentrations, pH values, and length of the denaturation stage of the amplification were all examined. When trying to optimize annealing temperatures, we ran the test reactions at 37°C, 39°C and 42°C. The decamer primers can be clearly amplified at 42°C. A subset of 13 primers for further analysis were based on the following criteria: (i) consistent, strong amplification products, and (ii) production of uniform, reproducible fragments between replicate PCRs.

Table 1. Populations of *Changium smyrnioides* used as sources of DNA.

Population code	Sample No	Localities	Vouchers
HZ	H1—H8	Mt.Nangao, Hangzhou, Zhejiang	Y.X. Qiu et al., 9901-9908
NB	N1—N7	Mt. Tiantong, Ningbo, Zhejiang	Y.X. Qiu et al., 9911-9917
LY	L1—L6	Mt. Longya, Chuzhou, Anhui	Y.X. Qiu et al., 9921-9926
AQ	A1—A7	Mt. Dalong, Anqing, Anhui	Y.X. Qiu et al., 9931-9937
NJ	NJ1—NJ8	Mt. Laoshan, Nanjing, Jiangsu	Y.X. Qiu et al., 9941-9948

Table 2. Sequences of the random nucleotide primers.

Primers	Sequence	Primers	Sequence
S14	5′-TCCGCTCTGG-3′	S34	5′-TCTGTGCTGG-3′
S15	5′-GGAGGGTGTT-3′	S35	5′-TTCCGAACCC-3′
S16	5′-TTTGCCCGGA-3′	S38	5′-AGGTGACCGT-3′
S21	5′-CAGGCCCTTC-3′	S39	5′-CAAACGTCGG-3′
S23	5′-AGTCAGCCAC-3′	S44	5′-TCTGGTGAGG-3′
S24	5′-AATCGGGCTG-3′	S45	5′-TGAGCGGACA-3′
S31	5′-CAATCGCCGT-3′		

Table 3. Polymorphic amplified bands detected with thirteen primers for five populations of *Changium smyrnioides* (percentages of polymorphic band, PPB).

Primer	Number of amplified bands	Number of polymorphic bands (*PPB*)					Total number of polymorphic bands (*PPB*)
		NB	HZ	NJ	AQ	LY	
S14	6	2 (0.33)	2 (0.33)	1 (0.17)	2 (0.33)	2 (0.33)	3 (0.50)
S15	8	3 (0.38)	4 (0.50)	2 (0.25)	3 (0.38)	4 (0.50)	7 (0.88)
S16	4	2 (0.50)	2 (0.50)	1 (0.25)	1 (0.25)	1 (0.25)	3 (0.75)
S21	6	2 (0.33)	3 (0.50)	2 (0.33)	2 (0.33)	3 (0.50)	4 (0.69)
S23	10	6 (0.60)	2 (0.20)	1 (0.10)	3 (0.30)	2 (0.20)	7 (0.70)
S24	4	1 (0.25)	2 (0.50)	2 (0.50)	2 (0.50)	2 (0.50)	3 (0.57)
S31	9	1 (0.11)	5 (0.56)	1 (0.11)	3 (0.33)	6 (0.67)	6 (0.67)
S34	5	1 (0.20)	2 (0.40)	3 (0. 60)	3 (0.60)	1 (0.20)	3 (0.60)
S35	6	3 (0.50)	3 (0.50)	3 (0.50)	3 (0.50)	3 (0.50)	5 (0.83)
S38	7	2 (0.29)	3 (0.43)	1 (0.14)	1 (0.14)	3 (0.43)	3 (0.43)
S39	9	2 (0.22)	3 (0.33)	1 (0.11)	3 (0.33)	2 (0.22)	5 (0.56)
S44	9	7 (0.78)	2 (0.22)	4 (0.44)	2 (0.22)	7 (0.78)	8 (0.89)
S45	9	4 (0.44)	5 (0.56)	3 (0.33)	2 (0.22)	5 (0.56)	7 (0.78)
Average	7.1	2.8 (0.38)	2.9 (0.43)	1.9 (0.28)	2.3 (0.32)	2.4 (0.43)	4.9 (0.69)

Data Analysis

Fragment sizes were designated as amplified bands, and bands were shared as diallelic characters (present = 1, absent = 0). Those bands amplifying in each instance were scored and included in the analyses. The number of multilocus genotypes (unique arrays of amplified bands) was calculated for each population. RAPD locus diversity was calculated with the Shannon-Weaver information statistic, employing the Brilliouin formula for eliminating the bias of finite sample size (Yun et al., 1998; Peet, 1974; Whitkus et al., 1998). The formula is Ho=-$\Sigma Pi\log_2(Pi)$ (Pi for frequency of the i band, Ho for diversity), which estimates the genetic diversity within and among populations of the species.

Results

A total of 92 bands were scored for the 13 RAPD primers for a range from 4 to 10, corresponding to an average of 7.1 bands per primer, and 69% (64 bands) of these were polymorphic. Percentages of polymorphic bands (*PPB*) for each primer ranged from 43% to 89%. *PPB* within populations for each primer ranged from 0.10 of S23 in NJ to 0.78 of S44 in NB and LY, which can be seen in Table 3. Mean RAPD similarities for individuals of different populations ranged from 0.22 to 0.89, with an average of 0.31 for all pairwise comparisons. Populations HZ and LY exhibit the highest level of variability (*PPB*=0.43), and population NJ exhibits the lowest (*PPB*=0.28).

Shannon's index of phenotypic diversity was used to class diversity into components within and among populations. Table 4 shows that the diversity within populations related to different primers and different populations. Primer S44 detected the highest genetic diversity within these populations while primer S34 detected the lowest. As for different populations, the populations HZ and LY showed greater average variation (1.246 and 1.215) than the other populations while the population NJ on average showed less variation (0.465) than the others.

Table 4. Estimates of gene diversity within population for *Changium smyrnioides* from five locations with Shannon's index.

Primers	NB	HZ	NJ	AQ	LY
S14	0.530	0.722	0.530	0.500	1.040
S15	0.516	1.782	0.722	1.500	1.855
S16	0.698	0.970	0.000	0.000	0.516
S21	0.000	1.368	0.440	1.000	1.053
S23	2.222	1.434	0.000	1.500	0.000
S24	1.370	0.460	0.930	1.000	0.980
S31	0.530	2.054	0.000	1.500	2.636
S34	0.000	0.258	0.258	0.810	0.460
S35	1.162	1.060	0.258	0.500	0.800
S38	0.994	1.524	0.000	0.500	1.260
S39	0.440	1.252	0.000	1.500	0.800
S44	2.902	0.928	1.922	1.000	2.490
S45	1.988	2.386	0.980	1.000	1.906
Average	1.027	1.246	0.465	0.947	1.215

Table 5. Partitioning of the genetic diversity within and among populations of *Changium smyrnioides* for RAPD.

Primer	Genetic diversity within population	Total genetic diversity	Percentage of diversity within population	Genetic differentiation coefficient among populations (*Gst*)
Average	0.980	2.012	0.488	0.512

Usually, Shannon's index of mean phenotypic diversity can be divided into two types: within populations and among populations (Table 5). In brief, a little more than half of the total variation (51.2%) is presented among the populations, and 48.8% of the variation is presented within the populations. Of course different primers had different percentages. For example, the variation among populations detected by primer S24 was only 18.8% whereas primer S34 detected 73.5%.

Discussion

Allozymes' value is sometimes limited for rare endangered plants because of their limited genetic variation although they have been widely and effectively used to analyze genetic diversity within and among plant populations (Crawford et al., 1994; Hamrick and Godt, 1996). RAPD markers have been employed as an alternative (Brauner et al., 1992; Dawson et al., 1993; Pei et al., 1995; Su et al., 1999; Wolfe and Liston, 1998; Yun et al., 1998; Esselman et al., 2000). Data from RAPD analysis has indicated that their diversity is usually similar to or greater than diversity from allozymes in plant species (Esselman et al., 1999, 2000). Comparing allozyme and RAPD diversity in rare endangered plants, the latter often prove more variable than the former. For example, no allozyme variation was found in some species of *Dendroseris* although RAPD diversity was detected (Esselman et al., 2000).

Both the Shannon-Weaver index (King and Schaal, 1989) and the Nei index (Nei, 1972) can be used to estimate the genetic diversity of populations, but the data analyzed by the Nei index need strict dominant and recessive allelic frequency. The data from RAPDs do not depend on these criteria. Estimating *Gst* using RAPDs has been problematical due to their dominance, and analytical methods usually rely on knowledge of the selfing rate or assume Hardy-Weinberg equilibrium. This assumption does not hold when populations exhibit fixed heterozygosity, so an alternative method, the Shannon-Weaver index, was used to partition the genetic diversity. However, this index is, to some extent, in accord with the analysis of RAPD data. Wei et al. (1999) used the two indices to analyze RAPD data from *Caragana* and found that both were consistent in showing the variation of populations. Do allozymes and RAPD markers provide similar estimates of the genetic diversity for rare endangered plants? As discussed above, we know that RAPD markers are inherited as dominants although the banding patterns cannot be used to analyze gene diversity. This is in contrast to the analysis of allozyme data, in which band frequencies can be directly interpreted as allelic frequencies. Esselman et al. (2000) found that the two species of *Dendroseris* were with the highest allozyme diversity and also exhibited the highest RAPD diversity, and one species showed low diversity with both markers.

In the studies, the level and distribution of genetic diversity detected by RAPDs are in agreement with previous results by allozymes (Qiu et al., 2000). The allozyme study found significantly lower gene diversity within populations of NJ and AQ compared with the other three populations. Likewise, the results from this RAPD analysis show that the NJ and AQ populations are of lower genetic polymorphism at the population levels. The RAPD genetic diversity within and among populations was also similar to the results from allozyme analysis, which showed about 51.2% among and 48.8% within populations in the former (Table 5) and 57.8% among and 42.2% within population in the latter. This is only slightly lower than the diversity seen with allozymes. Considering the data from both markers, we can conclude that genetic diversity among populations is higher than within populations of *Changium smyrnioides*. It was found from field investigations that the distributive pattern of the species is becoming island-like as a result of human activity and forest destruction. Results from RAPD and allozymes indicate that genetic drift might have occurred among the studied populations, thereby producing population differentiation. Genetic distance among populations of *Changium smyrnioides* at allozyme loci ranged from 0.386 for HZ and AQ to 0.006 for NJ and LY. It was found from field observations that the NJ population was a small, artificial group that was planted there from the Zijing Mountain near the LY population decades ago. This might explain why the genetic identity between them is high (0.994).

The percentage of polymorphic bands (69%) of RAPD in the species was higher than in other endangered plants, e.g. *Lactoris fernandeziana* (Lactoridaceae) 24.5% (Brauner et al., 1992), *Cathaya argyrophylla* 32% (Wang et al., 1996), *Paeonia suffruticosa* 22.5% and *P. rockii* 27.6% (Pei et al., 1995), and *Dacydium pierrei* 33.3% (Su et al., 1999). This shows that the species' genetic diversity is not low, and it should be able to fit the environmental variation. Accordingly, it is a main reason that the human's activities to damage their habitats and dig them excessively for medicine make the species' population decrease in size and their habitat be island-like in distribution. The populations HZ and LY, with higher genetic diversity, are located in the West Lake Mountains and in the Forest Park of Langya Mountain, respectively, and both sites enjoy good environmental protection. With a larger area of population, the probability of crossing among the individuals increases, which results in the retention of genetic

variation. Though many individuals of the species have been recorded in the Lao Mountains of Jiangsu Province, they have disappeared gradually along with environmental changes in their habitat.

Only with a firm grasp of the genetic structure and the diversity grade of populations in rare and endangered species can we make an efficacious measurement and strategy of protecting them. For a species with limited gene flow and over 50% variation among populations, it is necessary to collect samples from at least six populations in order to conserve 95% of the genetic diversity of the species. For a species with only 20% variation among populations, the samples taken from two populations are enough to get the same results above (Hamrick et al., 1991; Pei et al., 1995; Yun et al., 1998). The 51.2% genetic differentiation coefficient of *Changium smyrnioides* from RAPD (Table 5) suggests that the species is of a higher genetic diversity among populations than other endangered plants. The damage to their habitats is a main reason they are so rare. It is, therefore, a good strategy to protect more of their habitats. The results of RAPD and isozyme also show that we need to take individuals from more different populations if we are to construct an artificial conservation area so as to preserve their diversity for the future.

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瀕危植物明黨參（傘形科）遺傳多樣性的 RAPD 分析

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利用隨機擴增多形性 DNA (RAPD) 標記方法，對特產於長江流域的特有藥用植物明黨參（*Changium smyrnioides* Wolff ）的 5 個族群進行了遺傳多樣性分析。從 13 個引子共發現了 92 個條帶，平均每個引子 7.1 個條帶，其中 64 個條帶是多態的，多態性條帶百分率（*PPB*）為 69%。使用 Shannon 指數對該種的遺傳多樣性作了評價，結果表明總遺傳多樣性的 51.2% 發生在族群之間，48.8% 發生在族群之內。該種比其他瀕危植物具有更高的族群間遺傳多樣性。在本研究中，RAPDs所檢測的遺傳多樣性與前已獲得的異構酶比較（57.8% 的變異在族群間，42.2% 的變異在族群內，PPB=33.35%）具有相似的結果，因此 RAPDs對評價珍稀特有植物的遺傳多樣性和揭示族群間關係提供了一個很有用的工具。結果表明該種的遺傳多樣性並不低，具備適應環境變化的能力。在兩個標記中，均體現族群間的變異要大於族群內。該種遺傳變異特徵的確立為該植物的保護和取樣策略提供了一個重要的基礎。本研究建議要保護明黨參的棲息地，並在遷地保護中，為保存盡可能多的遺傳多樣性，必須在大多數族群中取樣。

關鍵詞：明黨參；遺傳多樣性；隨機擴增多形性DNA；異構酶。