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RAPD Profiling in Detecting Genetic Variation in Endemic *Coelonema* (Brassicaceae) of Qinghai-Tibet Plateau of China

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Random amplified polymorphic DNA (RAPD) markers were used to measure genetic diversity of Coelonema draboides *(Brassicaceae), a genus endemic to the Qilian Mountains of the Qinghai-Tibet Plateau. We sampled 90 individuals in 30 populations of* Coelonema draboides *from Datong and Huzhu counties of Qinghai Province in P.R. China. A total of 186 amplified bands were scored from the 14 RAPD primers, with a mean of 13.3 amplified bands per primer, and 87% (161 bands) polymorphic bands (PPB) was found. Analysis of molecular variance (AMOVA) shows that a large proportion of genetic variation (84.2%) resides among individuals within populations, while only 15.8% resides among populations. The species shows higher genetic diversity between individuals than other endemic and endangered plants. 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 adapt more easily to environmental variations. The main factor responsible for the high level of differentiation within populations and the low level of diversity among populations is probably the outcrossing and long-lived nature of this species. Some long-distance dispersal, even among far separated populations, is also a crucial determinant for the pattern of genetic variation in the species. This distributive pattern of genetic variation of* C. draboides *populations provides important baseline data for conservation and collection strategies for*

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the species. It is suggested that only populations in different habitats should be studied and protected, not all populations, so as to retain as much genetic diversity as possible.

KEY WORDS: *Coelonema*; genetic variation; RAPD; Qinghai-Tibet Plateau.

INTRODUCTION

Over a long term, the ability of a species to respond adaptively to environmental changes depends on the level of genetic variability it contains (Ayala and Kiger, 1984). The amount and partitioning of genetic variation among and within populations result from the dynamic processes of gene flow, selection, inbreeding, genetic drift, and mutation (Hartl and Clark, 1994). A species without an appropriate amount of genetic diversity is thought to be unable to cope with changing environments or evolving competitors and parasites. Therefore, investigations of population genetic diversity and the structure of populations within a species may not only illustrate the evolutionary process and mechanism but also provide information useful for biological conservation and phylogenetic analysis (Schaal *et al*., 1991).

Increasing human activity and overgrazing have decreased the number of populations of the endemic plant *Coelonema draboides* of the Qilian Mountains in the Qinghai-Tibet Plateau, where it is now considered endangered (Xue *et al*., 2002). It is distributed only in Huzhu and Datong counties of Qinghai Province and Tianzhu County of Gansu Province in the Qilian Mountains of the Qinghai-Tibet Plateau. According to our field survey from 1999 to 2000, about 10% of natural populations of the species have become extinct, and about 70% are on the verge of extinction. We did not find any populations in Tianzhu County because it has become more arid during the last two decades.

So far, many investigations have applied molecular tools to population studies of alpine plants, and all studies have focused on arctic plants (Steinger *et al*., 1996; Bauert *et al*., 1998; Abbott and Comes, 2003; Kjølner *et al*., 2004), but no work centers on endemic plants of the Qinghai-Tibet Plateau. The Qinghai-Tibet Plateau is the highest plateau in the world. Its short period of formation, since the Pliocene, has considerable influence on the structure and evolution of its component flora (Shi *et al*., 1998). The plateau has an exceptionally diverse flora, with about 4385 species of 1174 genera in 189 families (Wu, 1980). It is estimated that more than 25% of those species are endemic to the plateau (Wu, 1987); however, only about 20 genera are considered endemic (Wu, 1980, 1987; Wu *et al*., 1995). The genera endemic to the plateau were hypothesized to be closely related to and to have originated from local or adjacently distributed genera (Wu, 1980, 1987; Wu *et al*., 1995).

Coelonema is a monotypic genus of the Brassicaceae. The genus *Coelonema* was named by Maximowicz (1880). It is endemic to the Qilian Mountains of the Qinghai-Tibet Plateau. *Coelonema draboides* Maxim. is a perennial and stoloniferous herb. Plants germinate in spring, form a rosette during the year, flower in the autumn, and die above ground in winter. Flowering plants reach a height of 4–8 cm. The typical habitats of the species are alpine meadow, rocky crevices, and shrubland.

Random amplified polymorphic DNA (RAPD) technology (Williams *et al*., 1990) is a means of investigating genetic diversity within and between populations and has been applied to many plants (Nebauer *et al*., 1999, 2000; Ester *et al*., 2001; Jover *et al*., 2003). Once established, RAPD-PCR (polymerase chain reaction) has the advantage of being quick and easy, requiring little plant material, and having a high resolution (Gugerli *et al*., 1999; Bronzini *et al*., 2002). Furthermore, RAPD profiling appears to be useful for population as well as phylogenetic analysis (Hadrys *et al*., 1992; Lynch and Milligan, 1994; Kafkas and Perl-Treves, 2001). Because of the efficiency and the convenience of this technique, we decided to analyze our collection with RAPD markers.

In this study, the genetic polymorphism and population genetic structure of this rare and endemic plant of *Coelonema* populations in the Qinghai-Tibet Plateau of China at molecular level were analyzed. The purpose of the study is to assess genetic diversity and divergence within and among populations of this species. Another important aim is to provide genetic data and theoretical basis for protection of the species.

MATERIALS AND METHODS

Plant Material

In the autumns of 1999 and 2000 we sampled plants from 38 populations of *Coelonema draboides* in Datong and Huzhu counties of Qinghai Province along a 200-km stretch of the Qilian Mountains in the Qinghai-Tibet Plateau of China. Huzhu County is more than 200 km east of Datong County (Fig. 1). The distance between populations is about 2 km. In each population we sampled 3–6 plants at distances of 10 m from each other to increase the possibility of detecting potential among-individual variation. The fresh leaves were dried in silica in the field.

RAPD-PCR

Ninety individuals from 30 populations were included in the study (Fig. 1; Table I). Total DNA was extracted from ∼30 mg silica-dried leaf tissue using a modification of the cetyltrimethylammonium bromide (CTAB) protocol of Saghai-Maroof *et al*. (1984), differing primarily in the use of a 2× extraction buffer and RNase

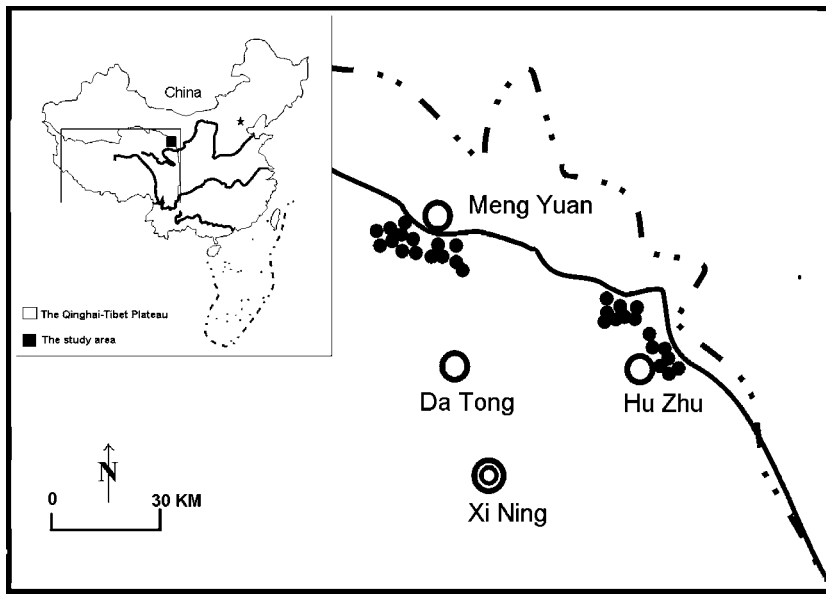

Fig. 1. Geographic location of the 30 populations of *Coelonema drabaoides* in Qilian Mountains of the Qinghai-Tibet Plateau. (•) Population symbols.

treatment. Leaf material was ground in liquid nitrogen, mixed with 700 μL extraction buffer at 65°C (1.4 mol/L NaCl, 100 mmol/L Tris–HCl (pH 8.0), 20 mmol/L EDTA, 2% CTAB, and 1% 2-mercaptoethanol), and incubated at 65°C for 45 min with shaking every 10 min. Proteins were extracted twice with 500 μL chloroform-isoamyl alcohol (24:1) for 10 min and then centrifuged at 12,000 rpm for 5 min. RNase (10 μg/mL) was added to the resulting supernatants and incubated for 30 min at 37°C, and the mixture was centrifuged at 12,000 rpm for 10 min. The pellet was washed twice in 70% ethanol, vacuum dried, and resuspended in 150 μL TE buffer (10 mmol/L Tris–HCl, 1 mmol/L EDTA, pH 8.0). Approximate DNA concentrations were estimated by visual inspection of

Table I. Study Populations of *Coelonema draboides*

Location	No. of sampled plants	Longitude (east)	Latitude (north)	Altitude (m)	Habitat
Huzhu 1–8	30	102°10′70″	37°00′50″	3500	Alpine meadow, rocky crevices
Huzhu 9–15	30	102°20′34″	37°08′48″	3500	Shrubland
Datong 16–30	30	101°47′09″	37°13′08″	3700	Alpine meadow, rocky crevices

0.7% agarose gels run in TBE buffer (89 mmol/L Tris base, 89 mmol/L boric acid, 2 mmol/L EDTA, pH 8.3) and stained with ethidium bromide, by comparing staining intensity of the samples to a λ *Eco*RI/*Hin*dIII marker.

PCRs were modified after Williams *et al*. (1990). Reaction volumes were 25 μL with 2.5 ng genomic DNA, 0.2 μmol/L primer (Sangon Biotechnology, Shanghai), 100 μmol/L of each dNTP (Promega, Madison, WI), 1× PCR buffer [10 mmol/L Tris–HCl (pH 9.0), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.01% gelatin, and 0.1% Triton X-100; Promega, Madison, Wisconsin, USA], and 0.5 U *Taq* polymerase (Promega). Amplifications were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA) using microtiter plates. An initial denaturing step for 2 cycles of 2 min at 94°C, 20 s at 37°C, 2 min at 72°C, was followed by 36 cycles of 40 s at 94°C, 45 s at 37°C, and 3 min at 72°C. A final extension step at 72°C for 8 min was included for extra elongation. A negative control was run for each primer. Amplification products were separated on 1.5% agarose gels run in 1× TBE buffer, detected by ethidium bromide staining, and photographed under UV light. GeneRuler 100 bp DNA Ladder (Fermentas) was used as a marker.

Because RAPD-PCR is sensitive to reaction parameters, 50 random 10-mer primers (Sangon kit S) were initially screened against 30 plants selected from 30 populations of *Coelonema*. The effects of Mg^{2+} and template DNA concentrations, Taq polymerase concentrations, and different times and temperatures during the annealing stage of amplification were examined. Twenty out of 50 primers generated strong amplification products. Then a subset of 14 primers that were insensitive to DNA template concentrations and produced reproducible bands was selected for further analysis (Table II). Each DNA sample was replicated running two separate amplifications. In the few cases where the two replicates were not identical, we repeated amplification to confirm the banding pattern. In a preliminary primer screening, we tested the 10-mer primers that had produced the best results in the study on primer rating by Fritsch *et al*. (1993). The gels were scored conservatively (i.e., only the most reliable and distinct bands were scored), as 1, present, or 0, absent. Staining intensity of bands was not considered as a difference.

Statistical Analysis

Genetic diversity was measured by the percentage of polymorphic bands (PPB), which was calculated by dividing the number of polymorphic bands at population, region, and species levels by the total number of bands surveyed. An RAPDistance program was employed to calculate Jaccard similarity coefficients for all pairs of individuals (J. Armstrong, A. Gibbs, R. Peakall, G. Weiller, Australian National University, Canberra). These coefficients were used as operational taxonomic units (OTUs) to construct a dendrogram using the unweighted pair group method (UPGMA) (Sneath and Sokal, 1973) and the SHAN (sequential, hierarchical,

Table II. Sequences of RAPD Primers Used in RAPD Analyses of *Coelonema draboides*

RAPD primer	Nucleotide sequence	Size of polymorphic markers (bp)
S1441	CAAGGGGCGG	500, 700, 900, 1100, 1200, 1300, 1600, 1800, 2000, 2200, 2500
S1446	AAGTGCACGG	400, 500, 600, 700, 750, 1000, 1031, 1100, 1200, 1500, 1600, 1800, 2000, 2100, 2600
S1449	TCGCTTCTCC	500, 600, 650, 800, 900, 950, 1100, 1400
S1450	AAGAGGCCAG	400, 600, 620, 720, 780, 800, 820, 840, 900, 960, 1031, 1100, 1200, 1400, 1600, 1800, 2200, 2800
S1454	GAACGAGGGT	800, 900, 1000, 1031, 1200, 1500, 2200, 2500, 3000
S1455	TTTGCCCCGT	300, 500, 640, 800, 1400
S1457	GGCAAACCCT	500, 820, 900, 1000, 1200, 1800, 2200, 2600
S1458	ACGAGAGGCA	300, 400, 550, 600, 650, 700, 800, 900, 920, 1031, 1100, 1200, 1300, 1500, 1600, 1800, 2200, 3000
S1507	CACAGACCTG	300, 400, 500, 600, 700, 910, 1031, 1200, 1220, 1300, 1600, 1800, 2000
S1509	CCGAGGGGTT	510, 550, 600, 620, 640, 700, 800, 850, 900, 1100, 1600, 1800, 2200
S1510	ACTGCCCGAC	350, 400, 650, 700, 800, 1200, 1300, 1500
S1513	GGCTTGGCGA	600, 700, 750, 850, 900, 1100, 1200, 1500, 1600, 1800, 2000, 2800
S1514	CTCTCGGCGA	300, 480, 500, 600, 750, 800, 900, 950, 1200, 1300, 1600, 2000
S1515	CCCACACGCA	500, 650, 700, 840, 950, 900, 1031, 1200, 1500, 2100, 3000

agglomerative, and nested clustering) routine in NTSYS software (Rohlf, 1994) to assess the association between genetic relationships of individuals and their habitats.

To describe genetic structure and variability among populations, the nonparametric analysis of molecular variance (AMOVA) program, version 1.55, was used (Excoffier *et al*., 1992). AMOVA analyses were based on the pairwise squared Euclidian distances, among molecular phenotypes, which are equal to the number of different band states. Because a sum of squares in a conventional analysis of variance (ANOVA) can be written as the sum of all squared pairwise differences, AMOVA is closely related to ANOVA. It allowed us to calculate variance components and their significance levels for variation among populations and within populations. Because significance tests in AMOVA are based on permutation procedures, they are essentially assumption free.

RESULTS

RAPD Polymorphism

Genetic data for 30 populations of *Coelonema* are summarized in Table III. Very high levels of genetic variation of the species of *Coelonema* were detected using RAPDs: a total of 186 bands ranging from 300 to 3000 bp were scored,

Table III. Polymorphic RAPD Bands in *Coelonema draboides*

Location	No. of RAPD polymorphic bands[a]	% of polymorphic bands of RAPDs
Huzhu 1–8	66	35.48
Huzhu 9–15	76	40.86
Datong 16–30	128	68.82
Total	186	86.56

[a] A total of 186 RAPD bands were scored in the present study.

corresponding to an average of 13.3 bands per primer; of these, 87% (161 in total) were polymorphic among plants. The percentage of polymorphic bands (PPB) for each location is shown in Table III. Datong 16–30 exhibited the highest level of variability (68.8), whereas Huzhu 1–8 was the lowest (35.5).

Genetic Structure of Populations

The AMOVA program was used to partition the genetic variation by hierarchical analysis from the distance matrix. In a nested analysis of two groups, the variance component between the two counties (Huzhu and Datong) was negative (−6.21%) but insignificant. AMOVA showed a highly significant ($p < 0.001$) genetic difference within populations as well as within regions (Table IV). Examination of the proportion of diversity between locations, among populations within locations, and within populations indicated that, on average, most of the

Table IV. Analysis of Molecular Variance (AMOVAs) for 90 Individuals of *Coelonema draboides* Using RAPDs

Source of variation	df	SSD	MSD	Variance components	% of total variance
Huzhu vs Datong	1	3.1358	3.136	−2.149	−6.21
Among populations within regions	1	3.6868	3.687	7.204	20.82
Within populations	87	50.8229	0.295	29.54	85.39
Among populations	29	6.8225	3.411	5.544	15.80
Within populations	60	50.8229	0.295	29.55	84.20
Among locations	2	3.1358	3.136	3.247	9.34
Within locations	87	54.5096	0.315	3.151	90.66

Note. All p values obtained were <0.001, except for one negative variance component. Levels of significance were based on 3000 iteration steps. Abbreviation: df, degrees of freedom; SSD, sum of squared deviations; MSD, mean squared deviations.

variance (85.4%) occurred within populations. Only 20.8 and 9.3% of variance was distinguished among populations within locations and among locations, respectively. For the among-population analysis, the majority of genetic variation (84.2%) resided within populations, and a small amount of variation (15.8%) represented differences between populations.

A UPGMA dendrogram based on Jaccard similarity coefficients between populations of *Coelonema* is shown in Fig. 2. A cluster analysis indicated that the populations of *Coelonema* grown in the same habitat were separated into two major clusters (Huzhu 1–8 and Datong 16–22, 24–27, 30 in alpine meadow and Huzhu 9–15 in shrubland), while populations from the same geographical region (Huzhu or Datong County) did not cluster as a clade. Both the AMOVA analysis and the cluster dendrogram demonstrated that there was no distinct genetic differentiation between the two counties.

DISCUSSION

Genetic Diversity and Population Genetic Structure

The variance of population genetic estimates does not decrease substantially if more than 30 RAPD markers are used (Aagard *et al*., 1998). Therefore, 50 markers used in our study should be appropriate. The endemic and endangered

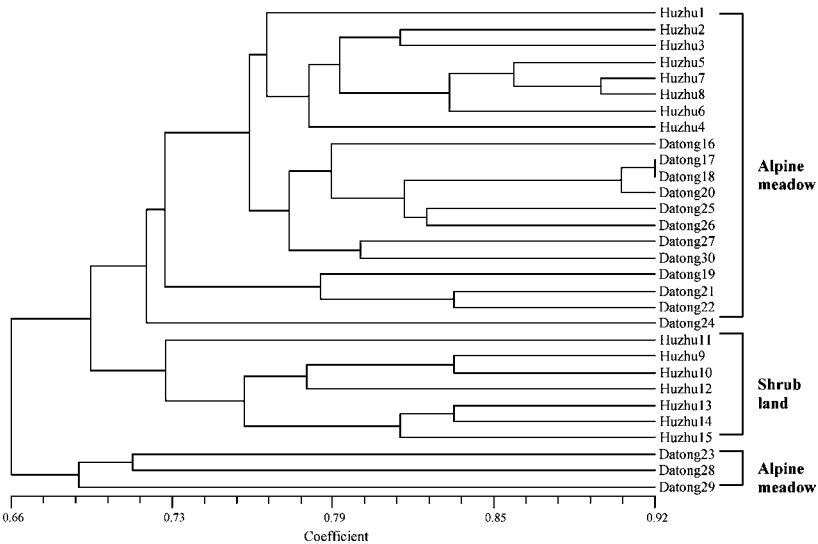

Fig. 2. Dendrogram illustrating genetic relationships among 30 populations of *Coelonema draboides*, generated by the UPGMA cluster analysis (NTSYS) calculated from 186 RAPD markers by 14 primers.

herb *Coelonema draboides* showed a high degree of genetic variation, with 86.6% of bands being polymorphic among plants from 30 populations in the Qilian Mountains of Qinghai-Tibet Plateau. The percentage of polymorphic bands (PPB = 86.6%) of RAPD in the species was higher than in other endemic plants, such as *Changium smyrnioides* 69% (Fu *et al*., 2003), *Dacydium pierrei* 33.3% (Su *et al*., 1999), and *Cathaya argyrophylla* 32% (Wang *et al*., 1996). This shows that the species genetic diversity is not low, and it would be able to fit the habitat variation.

The major proportion (84.2%) of the total genetic variation of *Coelonema* was found within populations. Only 15.8% of the total genetic variation resided among populations (Table IV). These results are similar to those found by Gugerli *et al*. (1999) in populations of the widespread species *Saxifraga oppositifolia* from the Swiss Alps. They found approximately 95% of the total genetic variation within populations and less than 5% among populations. According to overviews on genetic variation within and among plant populations, outcrossing, nonclonal, long-lived, and widespread species exhibit high intrapopulational variation, whereas interpopulational variation is low (Loveless and Hamrick, 1984; Hamrick and Godt, 1996). Such a pattern is explained mainly by a high rate of recombination within populations and a relatively high degree of gene flow between them, which inhibits their genetic differentiation. This suggests that the breeding system of *Coelonema* should be outcrossing. The long-lived nature of the species also promotes the exchange of alleles among individuals of different generations and favors high genetic variation within populations, and this is exactly what we detected. However, the species of *Coelonema* is endemic in the Qilian Mountains, suggesting that this species may be the remnant of a more widespread species in the Qinghai-Tibet Plateau before the latest uplift at about 1.6 million years ago, which was a turning point for climate change and vegetation replacement by alpine shrub, alpine meadow, and the expanded coniferous forests (Shi *et al*., 1998).

Populations from the two geographical regions were to a large extent intermingled in the UPGMA dendrogram, in agreement with the AMOVA analyses, which suggest that the populations from different geographical regions are not more differentiated than populations within the same geographical region, although the two counties are 200 km apart. The UPGMA dendrogram of genetic distance of populations grown in the same habitat gave one tight cluster from different geographical regions (Fig. 1, Table IV). This result may be due to gene flow by seed dispersal because seed can be blown over long distances across the snow surface during the winter storms or washed away in creeks. As the two regions appeared to be far enough from each other, gene flow by pollen should be low. Once a new individual becomes established within a new population, its alleles may spread across that population by extensive outcrossing. This lack of a geographical genetic pattern was also found in alpine plants in the Alps (Gugerli *et al*., 1999; Zhang *et al*., 2001; Holderegger *et al*., 2002). Historical events,

however, also have a profound influence on determining and partitioning of the genetic variation in plant species (Stehlik *et al*., 2001). A very short time has been available for genetic divergence, and the present geographic distribution of the species *Coelonema draboides* results from speciation after the uplift of the Qinghai-Tibet Plateau. The genus *Coelonema* is closely related to *Draba* (Maximowicz, 1880) and is considered to originate from *Draba* by taxonomists (He *et al*., 1997). *Draba* is the largest genus of the Brassicaceae, distributed primarily in the Northern Hemisphere, especially in the subarctic to arctic regions and alpine or mountainous portion of the temperate regions (Al-Shehbaz, 1987). *Draba* shows strong affinities to high alpine regions (e.g., in the Alps, Scandinavia, the Himalayas, the Andes), and therefore, its evolution appears to have been influenced by glaciation and deglaciation periods throughout the Pleistocene (1.8 million years ago, Koch *et al*., 2003). This suggests that *Coelonema* may have occurred in the Pleistocene, which corresponds well with the hypothesis of the latest uplift in the Qinghai-Tibet Plateau. The lack of genetic divergence among different geographical regions of *Coelonema draboides* is clearly most consistent with the general view on the historical origin of the Qinghai-Tibet Plateau flora that the flora had receded from or had been extinguished by the Quaternary ice sheet (Wulff, 1944) and all endemics in the plateau originated in situ or from adjacently distributed genera with the uplift of the plateau (Wu, 1987).

Conservation Implications

Resources available for conservation are limited, and it can be asked whether small populations of plants are worth preserving (Lesica and Allendorf, 1992). However, an important aim of any conservation program must be the preservation of genetic variability. For a species with limited gene flow and over 50% of 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 (Hamrick *et al*., 1991; Pei *et al*., 1995; Yun *et al*., 1998). In the case of *C. draboides*, the observed high degree of genetic diversity within populations indicates that management should aim to conserve more of their habitat, not all populations. The decline in their habitat is the main reason that they are so rare.

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