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# Genetic Diversity Assessment of *Trollius* Accessions in China by RAPD Markers

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## Introduction

Over the long term, the ability of a species to respond adaptively to environmental changes depends on its genetic variability (Ayala and Kiger 1984). The amount and distribution of variation among and within populations result from dynamic processes such as gene flow, selection, inbreeding, genetic drift, and mutation (Hartl and Clark 1997). A species without enough genetic diversity is thought to be unable to survive in a changing environment or protect itself against evolving competitors and parasites. Therefore, investigations of genetic diversity and the genetic structure of populations within a species may not only illustrate the evolutionary process and mechanism, but also provide useful information for biological conservation and phylogenetic analysis (Schaal et al. 1991).

Many studies of *Trollius* have focused on the medically effective compounds it contains, such as alkaloids and flavonoids, but so far no work has concentrated on its conservation. As the medical use of *Trollius* has grown, the plant has become increasingly rare in the wild, though scientists have succeeded in breaking the dormancy of *Trollius* seeds and established cultivation technology (Hepher and Roberts 1985; Bailey et al. 1996; Ding et al. 2003). The decreasing genetic diversity of domestic *Trollius* has made it more vulnerable to diseases, insect pests, and unpredictable climate changes, reducing its survival ability and putting it at risk of extinction.

Random amplified polymorphic DNA (RAPD) (Williams et al. 1990) is an effective means of investigating genetic diversity within or among populations, used

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in many plant species (Sales et al. 2001; Jover et al. 2003). Once established, RAPD–PCR (polymerase chain reaction) has the advantage of being quick and easy and requiring little genomic DNA as a template. Also, its amplified loci are randomly distributed on genomic DNA (Bronzini de Caraffa et al. 2002). Furthermore, RAPD profiling appears to be a useful tool for population analysis as well as phylogenetic analysis (Kafkas and Perl-Treves 2001).

RAPD markers were used here to analyze the genetic polymorphism and population genetic structure of *Trollius* accessions at the molecular level. The purpose of the study was to assess the genetic diversity and divergence among accessions of this rare, endemic plant in China and to provide genetic data and a theoretical basis for its protection.

## Materials and Methods

In summer 2006 and 2007, 24 *Trollius* accessions were sampled from the Chinese provinces of Inner Mongolia, Hebei, Jilin, Sichuan, and Beijing. For each accession, 50–200 plants were sampled randomly and pooled to increase the possibility of detecting potential among-individual variation. Samples collected were dried in silica in the field.

## RAPD–PCR

Genomic DNA of *Trollius* accessions was extracted from about 30 mg silica-dried leaf tissues using the modified sodium dodecyl sulfate (SDS) method of Dellaporta et al. (1983). Samples were ground in liquid nitrogen, mixed with 700 µl extraction buffer (1.4 mol/l NaCl, 100 mmol/l Tris–HCl, pH 8.0, 20 mmol/l EDTA), and incubated at 65°C for 45 min with shaking every 15 min. Proteins in samples were extracted twice with 500 µl chloroform–isoamyl (24:1) for 10 min, followed by centrifugation at 12,000 rpm for 5 min. RNase (10 µg/ml) was added to the resulting supernatants and incubated at 37°C for 30 min, and the mixture was centrifuged at 12,000 rpm for 10 min. Pellets were washed twice in 70% ethanol, vacuum dried, and resuspended in 200 µl TE buffer (10 mmol/l Tris–HCl, 1 mmol/l EDTA, pH 8.0). The extracted genomic DNA was then purified with a gel extraction kit (Omega). The concentration of genomic DNA was estimated by comparison with a standard $\lambda$ DNA digested by *Eco*RI/*Hin*dIII through electrophoresis on 1% agarose gel in 1× TAE (89 mmol/l Tris base, 89 mmol/l HAC, 2 mmol/l EDTA, pH 8.3) and stained with GoldView I nucleic acid dye.

The PCR protocol was modified based on Williams et al. (1990). PCR reactions were performed in a 25 µl volume, containing 20 ng template DNA, 0.2 µmol/l primer, 100 µmol/l each dNTP, 1× PCR buffer (10 mmol/l Tris–HCl, pH 8.0, 50 mmol/l KCl, 1.5 mmol/l $MgCl_2$), and 1 U *Taq* DNA polymerase. Amplifications were performed in a T Gradient 96 Thermal Cycler (Biometra). The cycling program consisted of an initial denaturation of 40 s at 94°C, followed by 45 cycles of 20 s at 94°C, 1 min at 35°C, 1 min at 72°C, and a final extension at 72°C for 5 min.

Amplification products were separated on 1.5% agarose gels run in 1× TAE buffer, detected by GoldView I dye staining, and photographed under ultraviolet light.

As RAPD–PCR is sensitive to reaction parameters, 60 random 10-mer primers were initially screened against five DNA samples from 24 *Trollius* accessions. RAPD primers that generated strong amplification products were selected for further analysis. The gels were scored conservatively (i.e., only the most reliable and distinct bands were scored) as present (1) or absent (0). Staining intensity of bands was not considered as a difference.

## Statistical Analysis

Genetic diversity was assessed by the percentage of polymorphic loci. The Popgene 1.32 program (Yeh et al. 2008) was employed to calculate similarity coefficients for all accessions. These coefficients were then used to construct a dendrogram using the unweighted pair group method (UPGMA; Sneath and Sokal 1973) and the Shan (sequential, hierarchical agglomerative, and nested clustering) program in NTsys-pc 2.1 software (Rohlf 1994) to assess the relationships of accessions. The Dice coefficient was also calculated by NTsys-pc.

## Results

### RAPD Polymorphism in *Trollius* Accessions

As RAPD primers were randomly amplified in the genomic DNA, and genomic information for *Trollius* species is not available, selecting RAPD markers to study its genetic diversity was appropriate. In total, 46 out of 60 RAPD primers that amplified distinctive bands were used for further study.

The statistical results (Table 1) show that RAPD primer OPA-9 has the highest number of amplified bands (13 bands) and OPA-2 the least (2 bands). RAPD primer OPA-8 has the most polymorphic bands (10 bands). RAPD primers OPA-2, OPA-4, OPA-8, OPA-12, OPB-5, OPB-6, OPB-17, and OPH-9 have the highest percentage of polymorphism (100%), and OPH-17 has the lowest (16.67%). Of the 305 distinctive bands amplified, 189 were polymorphic. On average, one primer can amplify 6.63 bands, and 4.11 were polymorphic.

Nei's gene diversity index among 24 *Trollius* accessions was 0.2619, and Shannon's information index was 0.4025, indicating low genetic diversity among accessions. The study also found significant variation in genetic diversity of gene loci amplified by different RAPD primers. The effective gene number ranged from 1.0430 to 1.5998, and the extent of variation was 0.5568; gene diversity ranged from 0.0412 to 0.3716, and the extent of variation was 0.3304; Shannon's information index ranged from 0.0879 to 0.5535, and the extent of variation was 0.4656. These statistics indicate that though Nei's gene diversity and Shannon's information index varied substantially, on the whole, the genetic variance of *Trollius* accessions was low, and genetic diversity among them was not abundant.

**Table 1** RAPD amplified results of *Trollius* accessions

| Primer | Loci | | | Diversity indices | | |
|---|---|---|---|---|---|---|
| | Total | Polymorphic | % Polymorphic | *Ne* | *H* | *I* |
| OPA-2 | 2 | 2 | 100.00 | 1.5891 ± 0.1524 | 0.3678 ± 0.0606 | 0.5535 ± 0.0671 |
| OPA-4 | 5 | 5 | 100.00 | 1.1921 ± 0.2831 | 0.1312 ± 0.1575 | 0.2315 ± 0.2098 |
| OPA-7 | 8 | 5 | 62.50 | 1.4387 ± 0.4382 | 0.2551 ± 0.2052 | 0.3974 ± 0.2536 |
| OPA-8 | 10 | 10 | 100.00 | 1.3676 ± 0.3868 | 0.2197 ± 0.1950 | 0.3455 ± 0.2559 |
| OPA-9 | 13 | 3 | 23.08 | 1.5952 ± 0.4641 | 0.3307 ± 0.2204 | 0.4900 ± 0.2758 |
| OPA-10 | 5 | 2 | 40.00 | 1.3699 ± 0.4624 | 0.2260 ± 0.2612 | 0.3515 ± 0.3527 |
| OPA-11 | 7 | 4 | 57.14 | 1.7096 ± 0.4493 | 0.3716 ± 0.2210 | 0.5315 ± 0.2868 |
| OPA-12 | 4 | 4 | 100.00 | 1.4572 ± 0.4688 | 0.2584 ± 0.2309 | 0.3931 ± 0.2972 |
| OPA-15 | 7 | 4 | 57.14 | 1.4430 ± 0.4618 | 0.2493 ± 0.2403 | 0.3760 ± 0.3163 |
| OPA-16 | 8 | 5 | 62.50 | 1.5738 ± 0.3910 | 0.3275 ± 0.1892 | 0.4872 ± 0.2435 |
| OPA-17 | 10 | 3 | 30.00 | 1.1524 ± 0.3335 | 0.0866 ± 0.1771 | 0.1301 ± 0.2533 |
| OPA-18 | 5 | 2 | 40.00 | 1.1260 ± 0.2134 | 0.0902 ± 0.1439 | 0.1503 ± 0.2272 |
| OPA-19 | 7 | 5 | 71.43 | 1.4388 ± 0.4325 | 0.2495 ± 0.2178 | 0.3719 ± 0.3008 |
| OPA-20 | 4 | 2 | 50.00 | 1.2481 ± 0.4391 | 0.1391 ± 0.2271 | 0.2109 ± 0.3156 |
| OPB-1 | 10 | 5 | 50.00 | 1.2744 ± 0.4072 | 0.1562 ± 0.2081 | 0.2360 ± 0.2931 |
| OPB-3 | 9 | 5 | 55.56 | 1.2965 ± 0.3902 | 0.1737 ± 0.2077 | 0.2638 ± 0.2939 |
| OPB-4 | 7 | 4 | 57.14 | 1.2283 ± 0.3756 | 0.1348 ± 0.1970 | 0.2105 ± 0.2776 |
| OPB-5 | 6 | 6 | 100.00 | 1.2097 ± 0.3200 | 0.1364 ± 0.1689 | 0.2342 ± 0.2259 |

**Table 1** continued

| Primer | Loci | | | Diversity indices | | |
|---|---|---|---|---|---|---|
| | Total | Polymorphic | % Polymorphic | *Ne* | *H* | *I* |
| OPB-6 | 4 | 4 | 100.00 | 1.5998 ± 0.4017 | 0.3401 ± 0.1874 | 0.5054 ± 0.2333 |
| OPB-7 | 5 | 4 | 80.00 | 1.5105 ± 0.3986 | 0.2964 ± 0.2005 | 0.4406 ± 0.2778 |
| OPB-8 | 7 | 6 | 85.71 | 1.3572 ± 0.3189 | 0.2298 ± 0.1698 | 0.3626 ± 0.2388 |
| OPB-10 | 8 | 2 | 25.00 | 1.1289 ± 0.2956 | 0.0770 ± 0.1634 | 0.1182 ± 0.2384 |
| OPB-11 | 6 | 3 | 50.00 | 1.1752 ± 0.2396 | 0.1219 ± 0.1604 | 0.1980 ± 0.2482 |
| OPB-12 | 6 | 4 | 66.67 | 1.3264 ± 0.4581 | 0.1793 ± 0.2326 | 0.2695 ± 0.3171 |
| OPB-13 | 4 | 3 | 75.00 | 1.3043 ± 0.4609 | 0.1741 ± 0.2251 | 0.2718 ± 0.3042 |
| OPB-14 | 8 | 6 | 75.00 | 1.1378 ± 0.2859 | 0.0879 ± 0.1514 | 0.1543 ± 0.2085 |
| OPB-16 | 10 | 8 | 80.00 | 1.5148 ± 0.4481 | 0.2815 ± 0.2211 | 0.4128 ± 0.2990 |
| OPB-17 | 3 | 3 | 100.00 | 1.0897 ± 0.0472 | 0.0812 ± 0.0398 | 0.1725 ± 0.0687 |
| OPB-18 | 8 | 2 | 25.00 | 1.0925 ± 0.2447 | 0.0565 ± 0.1438 | 0.0879 ± 0.2104 |
| OPB-19 | 9 | 7 | 77.78 | 1.3825 ± 0.4188 | 0.2217 ± 0.2060 | 0.3409 ± 0.2772 |
| OPB-20 | 5 | 1 | 20.00 | 1.2088 ± 0.0000 | 0.1727 ± 0.0000 | 0.3150 ± 0.0000 |
| OPH-1 | 11 | 7 | 63.64 | 1.3607 ± 0.3931 | 0.2093 ± 0.2137 | 0.3130 ± 0.3011 |
| OPH-2 | 6 | 5 | 83.33 | 1.3285 ± 0.4620 | 0.1801 ± 0.2321 | 0.2749 ± 0.3100 |
| OPH-4 | 4 | 2 | 50.00 | 1.2086 ± 0.3319 | 0.1329 ± 0.1938 | 0.2101 ± 0.2840 |
| OPH-5 | 9 | 5 | 55.56 | 1.1743 ± 0.2978 | 0.1095 ± 0.1730 | 0.1758 ± 0.2490 |
| OPH-6 | 4 | 2 | 50.00 | 1.3345 ± 0.4156 | 0.1966 ± 0.2337 | 0.2900 ± 0.3403 |

**Table 1** continued

| Primer | Loci | | | Diversity indices | | |
|---|---|---|---|---|---|---|
| | Total | Polymorphic | % Polymorphic | *Ne* | *H* | *I* |
| OPH-7 | 8 | 7 | 87.50 | 1.4119 ± 0.4410 | 0.2335 ± 0.2185 | 0.3589 ± 0.2830 |
| OPH-8 | 6 | 3 | 50.00 | 1.3620 ± 0.5525 | 0.1941 ± 0.2649 | 0.2991 ± 0.3412 |
| OPH-9 | 5 | 5 | 100.00 | 1.5096 ± 0.4519 | 0.2862 ± 0.2167 | 0.4307 ± 0.2773 |
| OPH-10 | 7 | 5 | 71.43 | 1.5030 ± 0.4313 | 0.2912 ± 0.1906 | 0.4470 ± 0.2303 |
| OPH-11 | 12 | 8 | 66.67 | 1.4521 ± 0.4289 | 0.2580 ± 0.2080 | 0.3949 ± 0.2662 |
| OPH-13 | 3 | 1 | 33.33 | 1.0430 ± 0.0000 | 0.0412 ± 0.0000 | 0.1021 ± 0.0000 |
| OPH-14 | 8 | 6 | 75.00 | 1.4739 ± 0.4499 | 0.2660 ± 0.2193 | 0.4037 ± 0.2794 |
| OPH-17 | 6 | 1 | 16.67 | 1.0888 ± 0.0000 | 0.0815 ± 0.0000 | 0.1760 ± 0.0000 |
| OPH-18 | 3 | 2 | 66.67 | 1.4813 ± 0.0000 | 0.3249 ± 0.0000 | 0.5061 ± 0.0000 |
| OPH-19 | 3 | 1 | 33.33 | 1.4813 ± 0.0000 | 0.3249 ± 0.0000 | 0.5061 ± 0.0000 |
| Total | 305 | 189 | | | | |
| Average | 6.63 | 4.11 | 61.97 | 1.4483 ± 0.3779 | 0.2619 ± 0.1855 | 0.4025 ± 0.2387 |

*Note*: *Ne* effective number of alleles, *H* Nei's gene diversity, *I* Shannon's information index

## Genetic Distance and Cluster Analysis of *Trollius* Germplasm Resource

The Dice genetic coefficient index showed genetic distances of 0.0159–0.5768 for the 24 *Trollius* accessions. *Trollius* accessions GZ and AET had the highest genetic distance (0.5768), and DL and WC had the lowest (0.0159). Genetic identity among the 24 *Trollius* accessions ranged from 0.4232 to 0.9841.

Based on the genetic coefficient index, 24 *Trollius* accessions were clustered by the UPGMA method (Fig. 1). Under the 0.70 coefficient threshold, the dendrogram separates these accessions into four groups: *Trollius* accession GZ from Sichuan in group IV, AET from Xinjiang in group III, SPKKG from Sichuan in group II, and the other 21 accessions in group I. Under the 0.80 coefficient threshold, the 21 accessions in group I were then separated into six subgroups, with CB and DB from Inner Mongolia in subgroup i and subgroup ii, respectively; JXLC-A from Hebei in subgroup iii; CBS from Jilin in subgroup v; and GZJD-A from Hebei in subgroup vi. The other 16 accessions, from Inner Mongolia, Hebei, and Beijing, form subgroup iv.

## Genetic Structure Analysis of *Trollius* Accessions

Assuming Hardy–Weinberg equilibrium, the mean genetic differentiation coefficient estimated by Nei's index within *Trollius* groups was 0.0849, and the total genetic differentiation coefficient was 0.2619. Based on the total genetic diversity and genetic diversity within groups, the gene differentiation index was 0.7381 (i.e.,

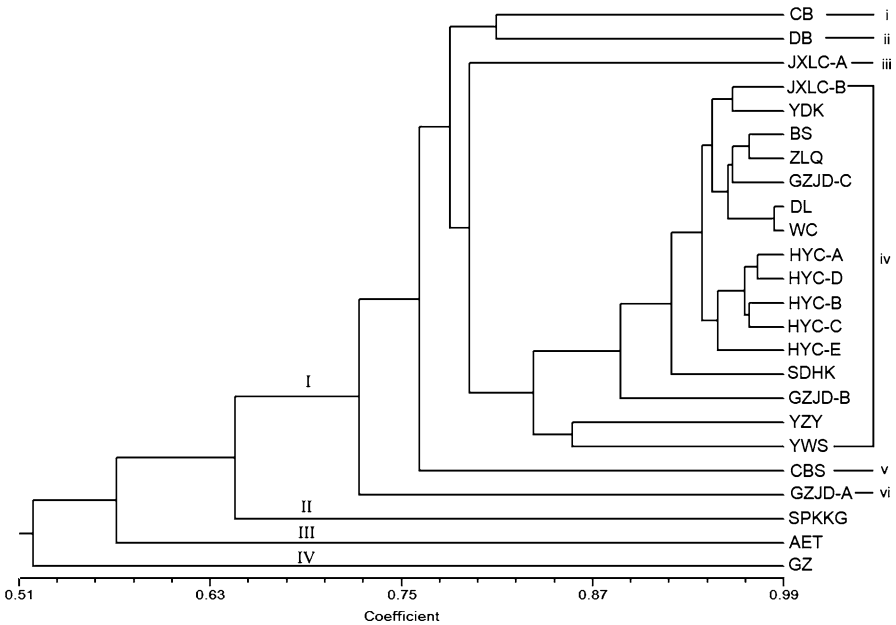


**Fig. 1** UPGMA dendrogram of 24 *Trollius* accessions based on Nei's genetic coefficient

73.81% of the variation occurred among *Trollius* groups and 26.19% within groups). The mean gene flow among *Trollius* groups was only 0.1684.

## Discussion

### Genetic Diversity and Accession Genetic Structure

RAPD markers require no genomic structure information on the material studied, and the binding sites of RAPD markers are randomly distributed on the genome DNA, so their use is appropriate to the study of genetic characteristics of a germplasm resource or the genetic diversity of closely related materials (Galván et al. 2001; Chen et al. 2005). The variance of population genetic estimates did not decrease substantially if more than 30 RAPD markers were used (Aagaard et al. 1998). In this study, 46 of 60 RAPD markers were used to analyze the genetic diversity of 24 *Trollius* accessions (including wild, semiwild, and cultivated germplasm). Those 46 primers amplified 305 distinctive bands, 189 of which were polymorphic among accessions, and the percentage of polymorphic loci was 61.97%. Genetic similarity and cluster analysis results indicated that the RAPD marker was appropriate for study of the *Trollius* germplasm resource's genetic diversity.

The endemic and endangered medicinal herb *Trollius* showed a high percentage of polymorphic loci (61.97%), which was near the percentage for *Changium smyrnidoies*'s (69%; Fu et al. 2003) but higher than that of *Dacydium pierrei* (33.3%; Su et al. 1999) and *Cathaya argyrophylla* (32%; Wang et al. 1996). Though the percentage of polymorphic loci was high, the genetic variation index was low (0.2619), and under Hardy–Weinberg equilibrium, the genetic diversity within and gene flow among *Trollius* groups was very low (0.0849 and 0.1684, respectively). Most genetic variation was among accessions that were far apart geographically. Such a pattern indicates a high rate of recombination among accessions dispersed in adjacent regions and a very low gene flow among *Trollius* accessions living at greater geographic distances.

The RAPD analysis showed high genetic diversity in *Trollius* accessions growing in different environments, and low diversity in *Trollius* accessions in the same or adjacent regions, with a few exceptions. For example, *Trollius* accessions GZJD-A, GZJD-B, and GZJD-C all grew at Weichang in Hebei province, but their genetic distance was great. The same was true of *Trollius* accessions JXLC-A and JXLC-B. In this study, the division of 24 *Trollius* accessions into four groups based on their genetic coefficient index allowed very little chance for gene flow among accessions that were geographically distant, but the probability of naturally occurring genetic cross and gene flow should be high among accessions growing near each other. So this study concluded that the high genetic diversity among accessions in adjacent regions was mostly attributable to artificial introduction, not natural genetic differentiation.

## Implications for Conservation

Maintaining or enhancing the genetic diversity of a given species will promote its ability to adapt to the environment and thus decrease its risk of extinction (Luan et al. 2008). Resources available for conservation are limited, and it can be asked whether small populations of plants are worth preserving (Lesica and Allendorf 1992). An important aim of any conservation program, however, 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 variation of the species. If a species has no more than 20% variation among populations, the samples taken from two populations are enough to get the same results (Pei et al. 1995; Yun et al. 1998).

In the case of *Trollius* germplasm, it was not possible to analyze all *Trollius* accessions in China, but the 24 *Trollius* accessions from different growing environments that were analyzed provided useful information about the genetic diversity level of the *Trollius* germplasm resource. This study found that Nei's gene diversity index and Shannon's information index were low. The genetic differentiation of the 18 accessions in subgroup iv of group I was low, but this was not true for subgroups i, ii, iii, v, and vi. The observed high levels of genetic diversity were mostly among accessions at significant geographic distance. This implies that management should aim to conserve more of the rare accessions and accessions with high genetic variation, even if their populations are very small.

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