Analysis of genetic relationships in *Rosa rugosa* using conserved DNA-derived polymorphism markers

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**ABSTRACT**

The genetic diversity and relationships among populations of Chinese-endemic rugged rose (*Rosa rugosa*) were studied at the molecular level, to provide theoretical guidance and technical support for the scientific evaluation, preservation and development of the germplasm resources of this endangered species. DNA samples of 120 specimens of *R. rugosa*, originating from six different Chinese source populations were amplified with CDDP (conserved DNA-derived polymorphism) primers. A total of 121 polymorphic loci out of the 128 total bands (94.53%) were recovered using 13 informative and reliable primers. The average number of amplified bands per primer was about 9.84. The number of specific bands was 8, accounting for 6.25%. Nei’s gene diversity (*H*), Shannon’s information index (*I*) and the effective number of alleles (*N*<sub>e</sub>) were 0.3057, 0.4646 and 1.5131, respectively. A UPGMA (unweighted pair group method with arithmetic mean) cluster analysis suggested that these populations of rugged rose could be divided into six major groups, with a similarity coefficient of 0.81. Here we show that the CDDP marker technique can effectively reveal the genetic diversity among Chinese-endemic populations of the rugged rose. Based on the results from the cluster analysis, we suggest that although the population genetic structure mirrors the geographic structure to some extent, it is clear that other factors besides geographic distance contribute to the generation of genetic variation.

**Introduction**

The rugged rose (*Rosa rugosa*; family Rosaceae) is a deciduous shrub native to northeastern China and adjacent areas of North Korea, Japan and eastern Russia [1,2]. In China, the shrub is endemic to the Jilin Tumen River Estuary, the south coast of Liaoning and the coastal area of Shandong (Figure 1) [1]. The rugged rose possesses many desirable traits, including strong fragrance and resistance to cold, drought and other challenging environmental conditions [3]. As *R. rugosa* is hardier than most cultivated roses, its germplasm is an important resource for rose cultivation. This species is also important ecologically, as it can grow on sandy substrates and thus protect delicate coastal areas from erosion. However, in recent years, many of the coastal areas that comprise the endemic habitat of the rugged rose have been subject to increasing industrial development, tourism, aquaculture and other detrimental human activities, resulting in a decrease in suitable habitats [1]. For this reason, the rugged rose was deemed one of the two nationally protected plants of China in 1992 and was incorporated into the China Plant Red Data Book [1]. In order to better protect endemic Chinese populations of this ecologically important plant, it is necessary to have an in-depth understanding of genetic variation among various rugged rose populations.

Several studies of genetic diversity among populations and varietals within the Rosaceae have already been conducted. It has been shown with RAPD (random amplified polymorphic DNA) that cultivars of the Pinying rose display slightly more genetic diversity among varieties than among populations [4]. RAPD has also been used to analyze rose (*R. rugosa*) of Korean origin [5]. It was also shown via AFLP (amplified fragment length polymorphism) that the diversity of Chinese *R. rugosa* germplasm resources was low, and there was little genetic distance among cultivars [6]. However, the same study showed that wild *R. rugosa* populations from different locations were genetically distinct, and that there were many differences between wild and cultivated germplasm [6]. In a study of three Chinese-endemic rose species, it was shown with SRAP molecular markers that *R. rugosa* and *R. multiflora* were closely related, while *R. xanthina* was genetically more distant [7]. Unfortunately, studies of genetic diversity in *R. rugosa* cultivars have been limited by the high costs of AFLP, SRAP and RAPD as well the drawbacks of the these methods [8].
Recently, a lower-cost molecular marker technique, CDDP (conserved DNA-derived polymorphism), has been developed [9]. CDDP is based on a single primer amplification reaction, and primers are designed specifically to target conserved sequences of plant functional genes. As such, CDDP can be used in many different plant species. CDDP molecular markers tend to generate candidate markers, where the gene markers may be part of or closely linked to known genes, such as dominant markers or high polymorphic genes. The PCR products of CDDP can be separated with agarose gel electrophoresis [10–13]. Studies of rice (Oryza sativa) have shown that CDDP molecular markers have many advantages, including convenience, lower cost and rich polymorphism, which can effectively produce markers related to the target traits [9]. CDDP technology is an effective supplement to labeling methods (such as RAPD, ISSR, TRAP, CoRAP and SCoT) and is prospectively useful in many applications [9]. CDDP molecular markers have already proved useful in the analysis of several plant species such as Chrysanthemum and Paeonia suffruticosa [14,15], but these markers have not yet been used in the study of R. rugosa. In order to evaluate the application of CDDP technology to genetic analysis of Chinese R. rugosa germplasm resources and to provide recommendations for the development of these resources, we aimed to use polymorphic CDDP markers to assess the genetic diversity of the current R. rugosa germplasm and to investigate the relationship between CDDP polymorphisms and biological traits.

**Materials and methods**

**Plant materials**

We collected fresh leaves from 120 different R. rugosa plants in various locations in northeastern China (Table 1). All specimens were preserved in silica gel at −20 °C.

**Genomic DNA extraction**

We extracted the total genomic DNA of each plant from the leaves (n = 120) using the cetyl trimethyl ammonium bromide (CTAB) method with minor modifications. We quantified the genomic DNA with a spectrophotometer. All DNA samples were stored at −20 °C before use.

**Table 1. Geographical location of the 120 samples of R. rugosa.**

| ID code | Sample site             | Latitude   | Longitude  | Altitude (m) | Annual mean air temperature (°C) | Frost-free season (number of days) | Annual precipitation (mm) |
|---------|-------------------------|------------|------------|--------------|---------------------------------|-----------------------------------|---------------------------|
| 1–45    | Hunchun, Jilin Province (A) | 42.36°     | 130.31°    | 5.5          | 5.6                             | 152                               | 606.8                     |
| 46–60   | Mingyang Town, Liaoning (B) | 39.33°     | 122.40°    | 3            | 8                               | 165                               | 890.6                     |
| 61–75   | Muping District, Yantai (C) | 37.46°     | 121.69°    | 4.5          | 11.6                            | 180                               | 737                       |
| 76–90   | Jianggezhuang Town, Yantai City (D) | 37.45° | 121.29°    | 5            | 11.6                            | 180                               | 737                       |
| 91–105  | Shichang Road, Weihai (E) | 37.50°     | 122.00°    | 3            | 12                              | 190                               | 810                       |
| 106–120 | Chengshan town, Weihai (F) | 37.40°     | 122.54°    | 3            | 12                              | 190                               | 810                       |
Polymerase chain reaction (PCR)

We tested 21 CDDP primers (synthesized by Shanghai Biotechnology Engineering Co., Ltd.) [9]. Of these, preliminary results indicated that 13 resulted in clear, stable and polymorphic bands. These 13 primers (Table 2) were selected for PCR amplification. Each 20 μL PCR mixture contained 10 μL 2XTaq Master Mix (CWBio, CW06905, China), 1 μL 40 ng/μL DNA template, 1.0 μL 10 pmol/μL primer (Sangon Biotech, China) and 8 μL ddH₂O. A standard PCR cycle was an initial denaturation step at 94 °C for 3 min; 35 annealing cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min; and a final extension of 5 min at 72 °C. PCR products were stored at 4 °C. PCR products were separated by electrophoresis in 2% polyacrylamide gels using a vertical-gel apparatus in 1× TAE (Tris–acetate–Ethylenediaminetetraacetic acid) buffer at 120 V constant current for 1.5–2 h, along with a DL5000 marker as a size marker. Amplified fragments were stained by the silver-staining method and photographed with a digital camera. All amplifications were repeated at least twice.

Data scoring and statistical analysis

Based on the electropherogram, we selected only clear bands for statistical analysis. Only bands that were clearly polymorphic in all replicates were scored as present (1) in a data matrix. Bands without clear polymorphisms, or that were only polymorphic in one replicate were scored as absent (0). At the same location, the only present and only missing amplification sites of a sample were taken as specific bands. We calculated the total number of bands, the number of polymorphic and specific bands and the percentage of the total bands that were polymorphic or specific, using Microsoft Excel 2007. We used POPGENE 1.32 to compute: the number of effective loci, the percentage of polymorphic loci, Shannon’s information index (I), the observed number of alleles (Na), expected heterozygosity (He), effective number of alleles (Ne), Nei’s gene diversity (H), total genetic identity (Ht), gene diversity within populations (Hs) and the coefficient of genetic differentiation (Gst). Ne, I and Gst were calculated with the following formulas: Ne = 1/ ΣPi2 where Pi is the frequency of the first allele variation; I = −Σ (Pi × InPi) where Pi is the frequency of the allele variation; and Gst = (Ht−Hs)/Ht. We used the similarity function in NTSYS-pc (v2.1) to generate a genetic distance matrix. We constructed a UPGMA (unweighted pair group method with arithmetic mean) dendrogram using SHAN. We used Tree Plot to draw a cluster analysis tree [16].

Results and discussion

Polymorphisms amplified with CDDP primers

CDDP molecular technology is a simple and efficient method that can be used effectively to study polymorphism within plant species. It has been shown that polymorphism appears in up to 95.58% of the bands amplified in 299 samples of peony by 18 CDDP primers, while 90% polymorphism was found using CDDP and ITM (intro-targeting marker) in 143 Solanum dulcamara samples [12,15]. In 53 chrysanthemum cultivars, the percentage of polymorphic loci found using 19 CDDP primers was 92.53% [14]. Here, of the 128 fragments generated with the 13 selected CDDP primers, 121 were polymorphic (94.53%), an average of 9.85 polymorphic bands per primer (Table 3). The number of polymorphic fragments detected per primer ranged from 6 to 14 fragments; these bands were 77.78%–100.00% polymorphic (Table 3). This high degree of polymorphism suggests abundant genetic variation in R. rugosa germplasm. The number of bands amplified by each primer ranged from 5 (Myb1) to 14 (WRKY-R3). The 13 CDDP primers amplified a total of eight specific bands (6.25%). Figure 2 shows the amplification products obtained with the CDDP primer ERF1 in samples of R. rugosa. In previous studies of the germplasm of 19 R. rugosa samples, which used sequence related amplified polymorphism (SRAP), the percentage of polymorphic loci found using 13 SRAP primers was 68.89% [17]. In the Chinese rose, 53.2% polymorphism was found with 13 primers [3]. Therefore, we have shown here that CDDP markers produce more polymorphism than other markers. These markers can also be applied to the identification of R. rugosa varieties,
The obtained average effective values of Ne, H and I were 1.5131, 0.3057 and 0.4646, respectively (Table 4). The genetic structure analysis showed that the total genetic diversity (Ht) was as high as 0.2770. The intraspecific genetic diversity was 0.1522 (Hs), while the interspecific genetic diversity (Dst) was 0.1248. Among 16 endemic R. rugosa populations from Japan, Russia and South Korea, Ht was shown to be 0.61, while the Ht of seven invasive populations from Sweden, Denmark, Norway, Germany and other European countries was shown to be 0.63 using SSR (simple sequence repeat) with seven pairs of primers [18]. Ht of four Chinese-endemic R. rugosa populations was shown to be 0.23 with RAPD [19]. Here, we found that, among six Chinese-endemic R. rugosa populations, Ht is 0.2770. The genetic diversity of the Chinese R. rugosa population is generally low, about 1/3 to 1/2 of that of invasive European and Asian populations [12]. Although the results of different molecular markers are not fully comparable, they still provide some useful information.

In this study, the coefficient of genetic differentiation (Gst) was 0.4505, which implies genetic variation of 45.05% among populations and 54.95% variation within populations. These results are consistent with previous work in R. rugosa, where genetic variation within populations has been found to be greater than genetic variation among populations [17,19]. The rugged rose is a typical plant with insect cross-pollination; this type of pollination can promote gene exchange among populations. As R. rugosa populations are relatively concentrated, pollen and seed dispersal by bees and birds can easily facilitate the frequent exchange of genes within populations. This high potential gene flow may have led to the large genetic divergences observed within populations [3].

Table 3. Amplification results obtained using 13 CDDP primers.

| Code | Primer name | TNB | NPB | PPB (%) | NSB | PSB |
|------|-------------|-----|-----|---------|-----|-----|
| 1    | MADS-2      | 7   | 7   | 100     | 0   | 0   |
| 2    | ABP1-1      | 11  | 11  | 100     | 0   | 0   |
| 3    | ERF1        | 9   | 7   | 77.78   | 0   | 0   |
| 4    | MADS-1      | 12  | 10  | 83.33   | 1   | 8.33|
| 5    | Myb1        | 6   | 5   | 83.33   | 1   | 16.67|
| 6    | ERF2        | 7   | 7   | 100     | 0   | 0   |
| 7    | WRKY-R3B    | 13  | 11  | 84.62   | 1   | 6.99|
| 8    | WRKY-R1     | 8   | 8   | 100     | 0   | 0   |
| 9    | WRKY-R3     | 14  | 14  | 100     | 2   | 14.29|
| 10   | KNOX-2      | 9   | 9   | 100     | 1   | 11.11|
| 11   | WRKY-F1     | 9   | 9   | 100     | 2   | 22.22|
| 12   | ABP1-2      | 11  | 11  | 100     | 0   | 0   |
| 13   | Myb2        | 12  | 12  | 100     | 0   | 0   |

| Total | 128 | 121 | 8 |
|-------|-----|-----|--|
| Average | 9.85 | 9.31 | 94.54 | 0.62 | 6.18 |

TNB, total number of bands; NPB, number of polymorphic bands; PPB, percentage of polymorphic bands; NSB, number of specific bands; PSB, specific band ratio.

Analysis of the genetic diversity of R. rugosa

The obtained average effective values of Ne, H and I were 1.5131, 0.3057 and 0.4646, respectively (Table 4). The genetic structure analysis showed that the total genetic diversity (Ht) was as high as 0.2770. The intraspecific genetic diversity was 0.1522 (Hs), while the interspecific genetic diversity (Dst) was 0.1248. Among 16 endemic R. rugosa populations from Japan, Russia and South Korea, Ht was shown to be 0.61, while the Ht of seven invasive populations from Sweden, Denmark, Norway, Germany and other European countries was shown to be 0.63 using SSR (simple sequence repeat) with seven pairs of primers [18]. Ht of four Chinese-endemic R. rugosa populations was shown to be 0.23 with RAPD [19]. Here, we found that, among six Chinese-endemic R. rugosa populations, Ht is 0.2770. The genetic diversity of

to the classification of germplasm resources and to the construction of a genetic map.

Analysis of the genetic distance between populations

We found the highest degree of genetic distance between samples from Hunchun and Mingyang town (0.2988), and the lowest distance (0.0636) between samples from Muping and Shichang Road (Table 4). Nei’s genetic identity ranged from 0.7417 to 0.9383 (Table 5). These results indicate that the genetic diversity among the studied populations is abundant at the molecular level.

The genetic distance between populations D and C was the smallest, with the highest degree of similarity (0.9383), indicative of a close relationship. The genetic distance among populations D, E and F was also low. In contrast, the genetic distance between populations A

Figure 2. Amplification results obtained using CDDP primers.
and B was the largest (0.2988), with the lowest degree of similarity (0.7417), indicating a more distant genetic relationship. These genetic relationships are not correlated with geographical distance, which may indicate that the genetic similarity is independent of geographic location in *R. rugosa* germplasm.

### Cluster analysis

The genetic similarity coefficient (GS) for all 120 samples ranged from 0.512 to 1.000, suggesting a close genetic relationship among all populations. Of these, two populations from northeastern Hunchun (No. 4 and No. 12) and two populations from Chengshan (No. 112 and No. 118) have a GS of 1.000, indicating an extremely close relationship. However, because *R. rugosa* has a well-developed rhizome system, it is highly likely that these four samples are part of a single plant. Similarity, a GS of 0.994 was observed between samples No. 110 and No. 112. We also noted a GS of 0.512 was observed between samples No. 7 and No. 85.

The UPGMA dendrogram showed that the analyzed populations of *R. rugosa* could be divided into six major groups when the genetic coefficient is 0.81 (Figure 3).

The first group contained some germplasm samples from northeastern Hunchun; the second group contained some germplasm samples from Hunchun and three germplasm samples from Jianggezhuang (No. 88, No. 89 and No. 90); the third group contained the germplasm samples from Liaoning Ming Yang, Shichang Road, Chengshan and Jianggezhuang; the fourth group contained only the germplasm sample No. 44; the fifth group contained germplasm samples from Muping and Jianggezhuang; and the sixth group contained only germplasm samples No. 85 and No. 86 (Figure 3). In general, samples from the same geographic area tend to cluster together, but this is not strictly true. Our results suggest that population genetic diversity within *R. rugosa* is not solely due to geographical distance, but may be the result of multiple factors including gene exchange among populations. For example, Mingyang town in the Liaoning province and Weihai city in the Shandong province are widely separated, but these populations show a high genetic similarity coefficient (0.8172, Table 5). This is consistent with results obtained from a cluster analysis of phenotypic traits in populations of *R. rugosa* [20]. According to geological history, Shandong and the Liaoning peninsula were once connected to the ancient continent Jiao Liao. Beginning in the Mesozoic Era, this ancient continent slowly began to sink. Due to the movement of tectonic plates, the Strait of Bohai and the Miaodao islands were formed between Shandong and the Liaodong Peninsula; thus, any plant present in both areas has great relevance [21]. In the early Quaternary–Holocene, sea water entered the Bohai Strait to create a semi-enclosed inland sea. This flooding led to the death of large numbers of plants. This area is now part of the Liaoning Changhai County Changshan island and has endemic populations of the rugged rose [22]. Our clustering results are consistent with the ancient continuous range.

### Table 4. Genetic diversity parameters based on amplification using 13 CDDP primers.

| Code | Primer name | Percentage of polymorphic base pairs | Observed number of alleles (No.) | Effective number of alleles (Ne) | Nei’s gene diversity (H) | Shannon’s information index (I) |
|------|-------------|-------------------------------------|----------------------------------|---------------------------------|--------------------------|-------------------------------|
| 1    | MADS-2      | 100                                 | 2.0000                           | 1.5619                          | 0.3276                   | 0.4969                        |
| 2    | ABP1-1      | 100                                 | 2.0000                           | 1.3665                          | 0.2469                   | 0.4062                        |
| 3    | ERF1        | 77.78                               | 1.7778                           | 1.3380                          | 0.2114                   | 0.3353                        |
| 4    | MADS-1      | 83.33                               | 1.8333                           | 1.4226                          | 0.2598                   | 0.3999                        |
| 5    | Myb1        | 83.33                               | 1.8333                           | 1.3484                          | 0.2141                   | 0.3370                        |
| 6    | ERF2        | 100                                 | 2.0000                           | 1.6864                          | 0.3920                   | 0.5752                        |
| 7    | WRKY-R8B    | 84.62                               | 1.8462                           | 1.4063                          | 0.2386                   | 0.3642                        |
| 8    | WRKY-R1     | 100                                 | 2.0000                           | 1.6856                          | 0.3995                   | 0.5866                        |
| 9    | WRKY-R3     | 100                                 | 2.0000                           | 1.5549                          | 0.3439                   | 0.5151                        |
| 10   | KNOX-2      | 100                                 | 2.0000                           | 1.5188                          | 0.3172                   | 0.4850                        |
| 11   | WRKY-F1     | 100                                 | 2.0000                           | 1.6445                          | 0.3664                   | 0.5416                        |
| 12   | ABP1-2      | 100                                 | 2.0000                           | 1.6300                          | 0.3566                   | 0.5295                        |
| 13   | Myb2        | 100                                 | 2.0000                           | 1.4662                          | 0.2983                   | 0.4668                        |
| Average |            | 94.54                               | 1.9454                           | 1.5131                          | 0.3057                   | 0.4646                        |

### Table 5. Nei’s genetic identity and genetic distance among different populations of *R. rugosa*.

| Population | A | B | C | D | E | F |
|------------|---|---|---|---|---|---|
| A          | **** | 0.7417 | 0.8109 | 0.7805 | 0.8418 | 0.7430 |
| B          | 0.2988 | **** | 0.7728 | 0.8320 | 0.7988 | 0.8172 |
| C          | 0.2097 | 0.2577 | **** | 0.8218 | 0.9383 | 0.8148 |
| D          | 0.2478 | 0.1839 | 0.1962 | **** | 0.8528 | 0.9210 |
| E          | 0.1722 | 0.2247 | 0.0636 | 0.1592 | **** | 0.8735 |
| F          | 0.2970 | 0.2019 | 0.2049 | 0.0823 | 0.1352 | **** |

Note: Data above the diagonal indicate the genetic similarity between populations, and data below the diagonal indicate the genetic distance. Population codes are consistent with Table 1. **** is because the two refers to the same community, so there is no difference and numerical value.
Figure 3. UPGMA dendrogram of the genetic similarity of 120 samples of rugged rose.
Conclusions

In this study, the genetic diversity of *R. rugosa* was analyzed by CDDP molecular markers. The results showed that the genetic diversity among wild roses was more abundant. Using cluster analysis to assess the relationship between *R. rugosa*, we showed that the geographical origin is not the only factor determining genetic variation. This study demonstrated the effectiveness of CDDP markers in assessing the genetic relationship of *R. rugosa* germplasm. As a target molecular marker, it can be further applied to the study of genetic diversity and relationships in *R. rugosa* or other plants.

Disclosure statement

No potential conflict of interest was reported by the authors.

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