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GENETIC RELATIONSHIP AMONG NINE *RHODODENDRON* SPECIES IN QINLING MOUNTAINS, CHINA USING AMPLIFIED FRAGMENT LENGTH POLYMORPHISM MARKERS

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Abstract

Genetic relationships of nine species of *Rhododendron* in the Qinling Mountains were evaluated using amplified fragment length polymorphism (AFLP) markers. A total of 440 amplification products were obtained using nine selected AFLP markers, of which 421 (95.40%) showed polymorphism. With these polymorphic products, a dendrogram was constructed using the unweighted pair-group method with arithmetic mean (UPGMA). *R. calophytum*, *R. hypoglaucum* and *R. clementinae*, belonging to Subgen *Hymenanthes*, gathered together, and the species derived from Subgen *Rhododendron* and Subgen *Tsutsusi* formed another two groups. *R. tsinlingense*, *R. purdomii*, *R. Taibaiense* and *R. capitatum* (Subsect. *Lapponica*), and *R. concinnum* (Subsect. *Triflora*) were clustered as one group, but they belong to difference subsect. and *R. purdomii* and *R. Taibaiense* showed the closest genetic distance, but both species differed greatly in morphological characteristics.These results showed that the genetic relationships among nine *Rhododendron* species, determined by AFLP markers, were partially related to their taxonomic position, geography distribution and morphological classification. The present study will benefit the identification and conservation of *Rhododendron*, and the development of new *Rhododendron* cultivar.

Key words: *Rhododendron,* Genetic relationship, AFLP, Axonomic position, Morphological classification.

Introduction

Rhododendron L. species are constructive and accompanying species of Qinling Mountains ecosystem, *Rhododendron* in Qinling mountains usually clustered into impenetrable bushes because of low height, dense branches and developed root system, which have played a big part in keeping the alpine soil, preventing erosion and gravel sliding. So *Rhododendron* was the most excellent plant for soil and water conservation in Qinling mountains. Furthermore, some *Rhododendron* bushwood has developed moss layer, which can form dense carpeting. And this primary vegetation type was very rarely seen in other types of shrubs. Therefore they have important ecological significance and beneficial to the stability of the ecosystems. In addition, native *Rhododendron* L. plant germplasm can be important parental sources of crossing-breeding because of high cold-resistance and ornamental value (Gen, 2008). Nine *Rhododendron* species in this study have special ornamental value due to their flower shape and color, Therefore, these species should be exploited for breeding.

It is important for breeding to understand the genetic relationships of the species. At present, AFLP markers are commonly used to access the genetic structure of natural plant populations (Manel *et al.,* 2007; Gentili *et al.*, 2010; Pamidimarri *et al*., 2010; Na *et al.*, 2010) and to indentify genetic relationships in some plants (Karimi *et al.*, 2009; Deng *et al.*, 2010; Kaya *et al.*, 2011; Zhu *et al.*, 2011). Furthermore, AFLP has also been successfully applied to population analysis of congeners in different environments and genetic differentiation analysis of different species in the *Rhododendron* L. (De Riek *et al*., 1999; Dendauw *et al.*, 2002; Handa *et al.*, 2002; Scariot *et al*., 2007; Chappell *et al.*, 2008). There are many reports on the genetic relationship analysis using AFLP for cultivated accessions (Gowri *et al.*, 2010; Kaoru *et al.*, 2010), but there are very few reports regarding the wild species. Chappell *et al.* studied genetic relationships among seven deciduous Azalea species using AFLP, but the samples were all from America. Thus far, research on the genetic relationship of *Rhododendron* species in China using AFLP has not been seen (NG *et al.*, 2000). It is well known there are abundant wild *Rhododendron* resources in China, Some have more higher ornamental value, some have excellent resistence gene. And they are the most important breeding genetic resources, the analysis of genetic relationship of wild species will provide a useful reference for the selecting of new cultivars. Therefore the research for the first time assesses the genetic relationships of *Rhododendron* wild resources in China through AFLP markers.

The presence of numerous species and the vast geographical distribution, along with the high level of interspecific hybridization, make genetic relationships within the genus confusing (Caser *et al.*, 2010). Therefore detailed knowledge regarding genetic relationship among parent materials is important for the breeding of new cultivars and molecular markers are useful tools for this purpose (Nafees *et al*., 2015). Genetic relationships among nine *Rhododendron* species of high ornamental value in the Qinling Mountains were analyzed with AFLP molecular marking technique, tending to probe into the genetic classification on the molecular level, thus providing theoretical basis for the parent selection in crossbreeding and the cultivation of new *Rhododendron* cultivars with high ornamental value and stress resistance.

Materials and Methods

Plant material: The leaves of nine *Rhododendron* species in the test were picked from six counties in Shaanxi province (Figs. 1, 2 and Table 1). Fresh tender leaves were picked and sealed in ziplock bags and quickly taken back to the lab, and conserved at -20℃until DNA extraction.

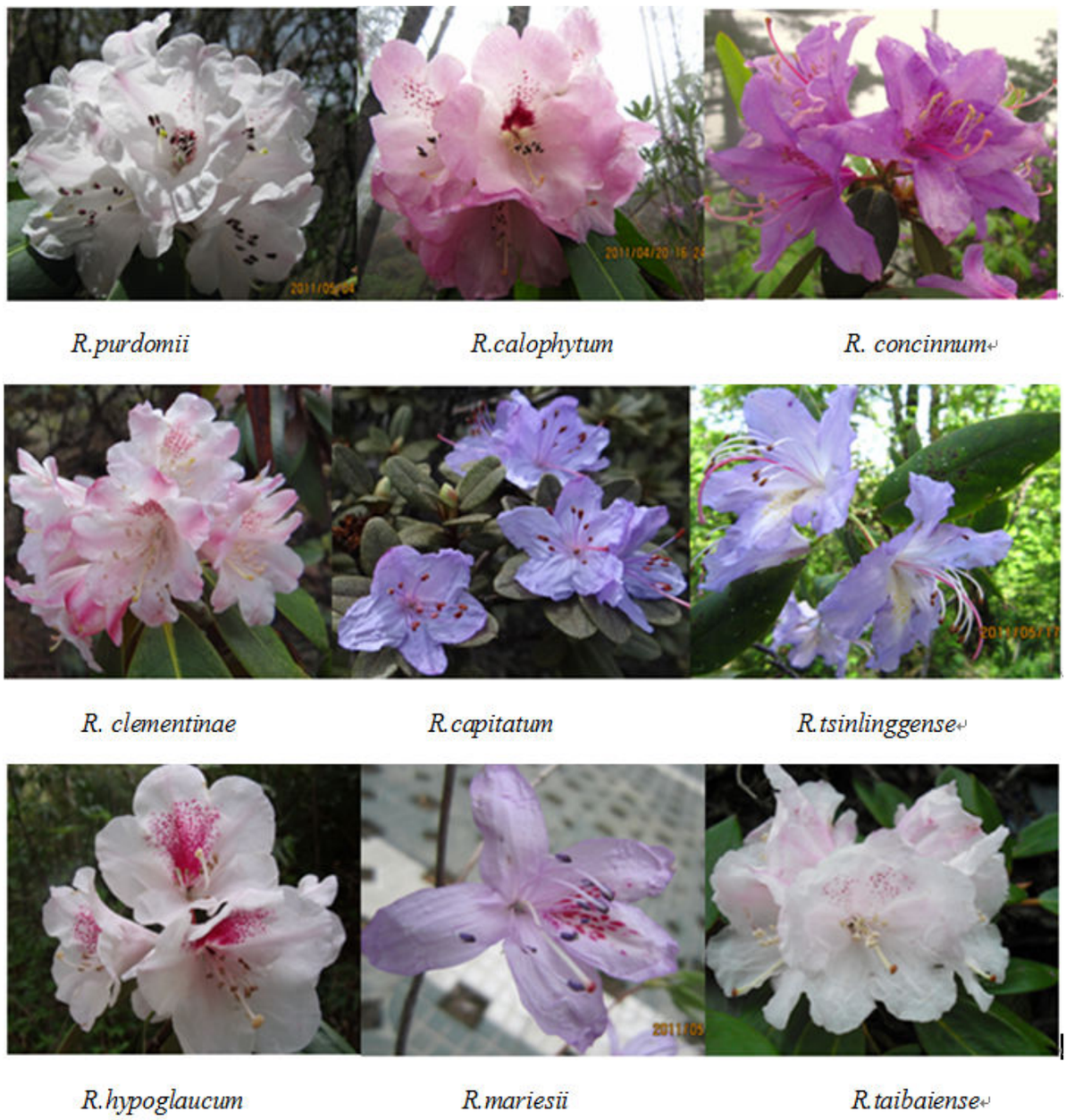

Fig. 1. Flower pictures of nine *Rhododendron* species in Qinling Mountains.

DNA extraction: Genomic DNA was extracted from leaf tissues using Plant Genomic DNA Kit (Tiangen, Beijing, China), according to the manufactory's instructions. The concentration of DNA was determined by ultraviolet spectrophotometer. The sample was diluted to 50 ng μl^{-1} in Tris-EDTA (TE) buffer and stored at -20^{o}C until usage. Fresh leaf samples were collected from individuals of each species for DNA extraction.

AFLP analysis: AFLP assays were carried out according to Vos *et al.* (1995) with minor modifications. Briefly, genomic DNA was digested and connected for 3 h at 37°C to generate template DNA for amplification. Pre-amplification (30 s at 94°C, 30 s at 56°C, and 1 min at 72°C. 24cycles) was performed using template DNA and a pair of primers based on the sequences of the EcoRI and MseI adapters. The pre-amplified PCR products were 10-fold diluted and used as the template for selective amplifications, with a total of nine primer combinations, which can generate clearer and more abundant bands. The first phase of the selective amplification was performed with 12 cycles of 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C, with the annealing temperature decreasing stepwise from 65.0°C to 56.6°C. The latter phase was performed with 24 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C. The amplified products were detected with denaturing polyacrylamide gel electrophoresis (PAGE) in 1×TBE running buffer and silver staining, using Puc19 DNA/MspI (HpaII) Marker (Fermenta-MBI, China) as molecular weight marker.

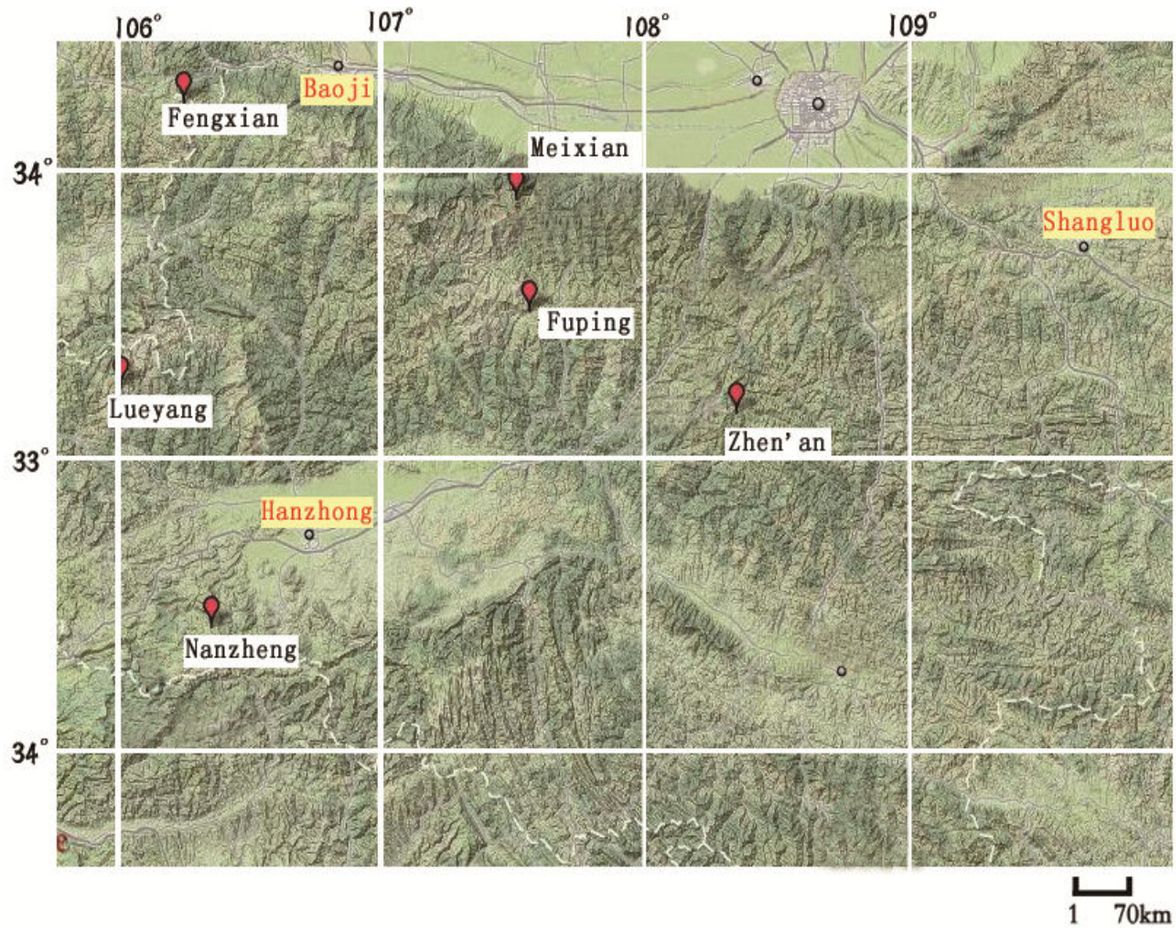

Fig. 2. Locations of nine *Rhododendron* populations in Qinling.

Table 1. Provenance of *Rhododendron* samples collected for this study and site characteristics.

Species	Taxonomic position (*)	Population	Altitude (m)	Longitude	Latitude
R. capitatum	Subgen *Rhododendron* Subsect. *Lapponica*	Baoji, Meixian	3000--3767	107 °80′	33°95′
R. concinnum	Subgen *Rhododendron* Subsect. *Triflora*	Baoji, Meixian	2250--3200	107°78′	33°99′
R. taibaiense	Subgen *Rhododendron* Subsect. *Lapponica*	Baoji, Meixian	3200--3680	107°81′	33°93′
R. purdomii	Subgen *Rhododendron* Subsect. *Lapponica*	Baoji, Meixian	2115---2172	107°79′	34°06′
R. hypoglaucum	Subgen *Hymenanthes* Subsect. *Fortunea*	Hanzhong, Nanzhen	1453-1472	106°39′	32°48′
R. mariesii	Subgen *Tsutsusi* subsect. *Tsut*	Hanzhong, Fuping	1358-1700	107°58′	33°40′
R. calophytum	Subgen *Hymenanthes* Subsect. *Fortunea*	Shangluo, Zhen'an	1448--1890	108°37′	33°24′
R. tsinlingense	Subgen *Rhododendron* Subsect. *Lapponica*	Baoji, Fengxian	1992--2019	106°33′	34°15′
R. clementinae	Subgen *Hymenanthes* Subsect. *Taliensus*	Hanzhong, Lueyang	1284--1635	106°18′	33°28′

*According to the taxonomic system of H. Sleumer

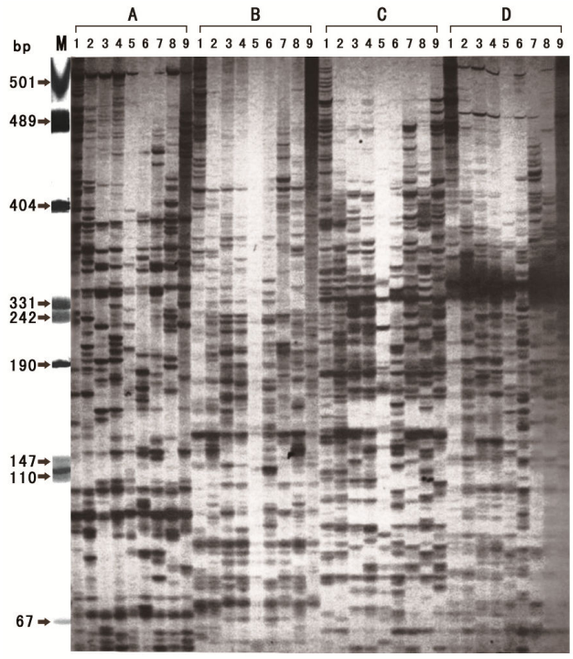

Fig. 3. AFLP fingerprinting patterns of samples using primer combination (1-9：nine species of *Rhododendron*; A-D: primer combinations M48E35, M49E41, M52E35, M50E36)

Data analysis: To confirm the reliability, the pre-amplification, selective amplification and gel electrophoresis were conducted twice for the DNA extracted from species listed in Table 1. AFLP fingerprints were manually scored for the presence (1) and the absence (0) of a band. Electrophoretic DNA bands of low visual intensity that could not be readily distinguished as present or absent were considered ambiguous markers and were not scored. A dendrogram was constructed to determine the relationships among the nine surveyed species with UPGMA based on Nei's unbiased genetic distance matrix, using NTSYSpc ver. 2.10 (Rohlf, 2000).

Results

Primer selection and polymorphism of amplification bands: One of the key steps in AFLP technique is the selection of the primers, suitable primers are the precondition of amplifying molecular marking with high resolution and high polymorphism. Out of 100, nine pairs of primers that can amplify well-distributed band type with high polymorphism and good discernibility were selected. Which includes M62E46, M64E94, M48E35, M49E41, M52E35, M19E94, M50E36, M64E46 and M50E37 (Fig. 3).

The nine AFLP primer combinations amplified a total of 440 bands ranging from 67 bp to 501 bp, 421 of which were polymorphic. The percentage of polymorphic bands was 95.40%. The average DNA bands amplified by each primer combination ranged from 34 to 62, averaging 48.89, the number of amplified DNA fragments by each primer combinations was different. The primer pair which generated the most bands was M48/ E35 (M-CAC/E-ACA) with the production of 62 DNA bands, the fewest was M64/ E94 (M-GAC/E-TTT) with only 34 DNA bands amplified (Table 3).

Genetic relationship analysis: To further analyze the genetic differentiation and relationship among species, the molecular marking results of nine species with the cluster analysis method were analyzed and the cluster tree diagram of genetic relationship was built (Fig. 4). The genetic distance between two *Rhododendron* species is larger than zero, but they can exist together, which means that nine species share the same genetic background, while differ from each other to a certain degree. The likeness coefficient range of different genotypes is between 0.68 and 1.45. The nine different *Rhododendron* species were significantly divided into three groups at the threshold value 1.28. The first group belongs to Subgen *Hymenanthes*, which includes *R. calophytum, R. hypoglaucum* and *R. clementinae*； *R. concinnum, R. Taibaiense, R. purdomii, R. capitatum* and *R. tsinlingense* were clustered into the second group, which belongs to Subgen *Rhododendron*； *R. mariesii* of Subgen *Tsutsusi* were clustered into the third group, which has distinct genetic differences from the other eight populations, therefore share a single branch.

Discussion

AFLP is an efficient molecular marking method (Vos *et a1*., 1995). In recent years, AFLP technique has been widely used in the study on plants classification and genetic relationship. This molecular marking technique is especially suitable for the molecular marking of those species with genetic relationship and with genetic differences that are not so obvious (Powell *et al.*, 1997). Therefore, AFLP molecular marking can be used in the study of genetic relationship of *Rhododendron* species and it can provide important basis for germplasm conservation and parent selection in crossbreeding (Barbara, 2011; Kunal *et al.*, 2012).

Morphological characteristics among *Rhododendron* species differ greatly because of the genetic differences, which caused different species have different ornamental values and suitable for different cultivation environments. Ng *et al.* (2000) pointed out that six *Rhododendron* species in Hong Kong, in comparison with woody plants with similar life history, have higher genetic diversity and allelic genes abundance. because high genetic diversity revealed good acclimatization ability of *Rhododendron* populations and enabled species to adapt to changing environments and provided plant breeders with the raw materials necessary for artificial selection This is also good for the reproduction of the *Rhododendron* population, the sifting of resistance genes of *Rhododendron*, the conservation of germplasm resources, the cultivation and domestication of wild species. Therefore, by selecting excellent wild species with obvious target traits from different environments, people can hopefully cultivate new species with high ornamental value and adaptability.

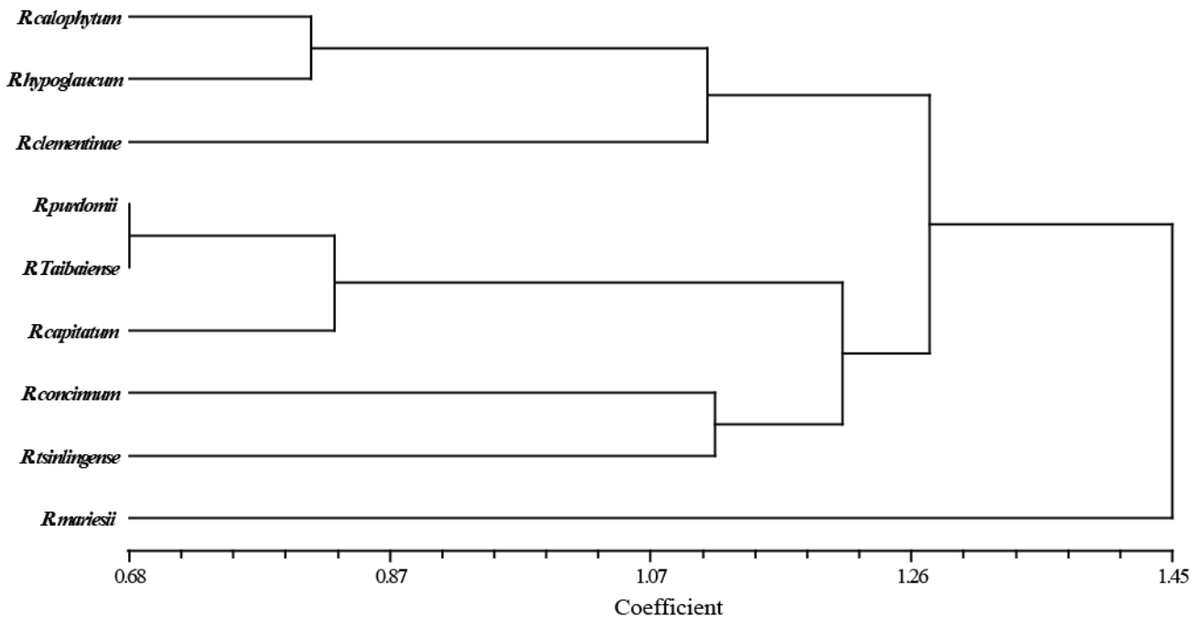

Fig. 4. Dendrogram of polymorphic DNAs of nine different genotypes of *Rhododendron* by cluster analysis.

Table 2. Morphological data of nine species.

Species	scales	Life form	Flower color	Floret number	Stamen number	The length of the leaf (cm)	The width of the leaf (cm)
R. capitatum	With scales	Evergreen shrub	purple	4-5	10	1-3	0.8-1
R. concinnum	With scales	Evergreen shrub	purple	3-5	10	3-8	1-3
R. taibaiense	No scales	Evergreen shrub	White with red spot	8-10	10	2-4.5	1-1.5
R. purdomii	No scales	Evergreen shrub	white	6-16	10	5-9.5	2.5-4.5
R. hypoglaucum	No scales	Evergreen shrub	White with red spots	4-7	10-12	5-13.5	2-5
R. mariesii	No scales	Deciduous shrub	Purple and red	1-3(5)	10	3-7	1.8-3.5
R. calophytum	No scales	Evergreen shrub	pink with red spots	15-20	10	18-30	5-7
R. tsinlingense	With scales	Evergreen shrub	Light-purple	3-5	10	7-9	2.5-3.5
R. clementinae	No scales	Evergreen shrub	White with red spots	7-15	14-15	6-15	2.5-8

Table 3. Polymorphism of AFLP bands obtained by selective amplification based on nine primer pairs.

Primer code	Selective Nucl.	Total bands	Polymorphic bands	Percentage polymorphic bands
62-46	M-CTT/E-ATT	36	33	91.67
64-94	M-GAC/E-TTT	34	33	97.06
48-35	M-CAC/E-ACA	62	58	93.55
49-41	M-CAG/E-AGG	60	60	100
52-35	M-CCC/E-ACA	41	39	95.12
19-94	M-GA/E-TTT	56	52	92.86
50-36	M-CAT/E-ACC	51	50	98.04
64-46	M-GAC/E-ATT	47	45	95.74
50-37	M-CAT/E-ACG	53	51	96.23
Total		440	421	
Average		48.89	46.78	95.40

In the present work, According to the cluster analysis graph, we can clearly see the genetic relationship of nine *Rhododendron* species and their system locations. The shorter the distance on the graph, the closer the genetic relationship, and vice versa. For example, *R. calophytum*, *R. hypoglaucum* and *R. clementinae* all belong to Subgen *Hymenanthes*, and they have some common morphological characteristics such as the leaves are evergreen, the number of stamens is 10-20 (Table 2). They gathered together on the graph, meaning they also have the most closest genetic relationship. Those belong to Subgen *Rhododendron* and Subgen *Tsutsusi* respectively formed another two groups,basically agreeing with traditional morphological classification. *R. tsinlingense, R. purdomii*, *R. Taibaiense* and *R. capitatum* belong to Subsect. *Lapponica* in terms of traditional morphological classification, but on the cluster graph of genetic distance, they are in the same group with *R. concinnum*, which belongs to Subsect. *Triflora*. *R. purdomii* and *R. Taibaiense* differ greatly in morphological characteristics, but are closest on the cluster graph. As for the geographical locations, *R. concinnum*, *R. tsinlingense, R. purdomii*, *R. Taibaiense* and *R. capitatum* were both from

Mei County, Baoji City. Especially, *R. purdomii* and *R. Taibaiense* were both from Mount Taibai in Mei County, the geographical distance is very short. This means, in the long process of evolution, the influence of geographical distance and natural selection on species formation is obvious. The shorter the geographical distance is, the smaller the genetic differences between species. The reason might be that natural crossbreeding happens more often among species that are geographically close, leading to variations in phenotypes. As a result, those species with short genetic distance are morphologically different. The result of this study is in line with the results of the researches of Falk *et al.* (2001). Which also showed that the genetic relationship among 9 *Rhododendron* species were related to their taxonomic position, geography distribution and morphological classification, to some extent .Based on the results of this study, further parent crossbreeding is possible, and hopefully new *Rhododendron* species with high ornamental value and stress resistance can be cultivated.

In conclusion, this study basically demonstrated the genetic relationships of nine *Rhododendron* species in the Qinling mountains using AFLP markers. This research not only provided an effective mean of the classification and identification of *Rhododendron* species, but also establish an genetic database, which could provide molecular evidence for selection and breeding of the *Rhododendron* germplasm.

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