Assessment of genetic relationship among *Rhododendron* cultivars using amplified fragment length polymorphism and inter-simple sequence repeat markers

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ABSTRACT. Genetic relationships of 17 *Rhododendron* cultivars, China, were assessed using inter-simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) markers. A total of 133 bands were obtained using nine selected ISSR primers, 129 (96.99%) of which were polymorphic; 267 bands were amplified by four AFLP primer pairs, 251 (94.01%) of which exhibited polymorphism. Based on these polymorphic products, a cluster analysis revealed similarities between the results of the ISSR and AFLP. All of the cultivars were clustered into two major branches; one branch contained the same four cultivars, and the other cultivars were separated into different groups in the other branch. The cluster results showed that the genetic relationships of the 17 cultivars were partly related to their morphological characteristics, particularly the flowering

phase. Therefore, the results of this study support the classification of *Rhododendron* cultivars according to flowering phase. In addition, the cluster results can be used to select suitable parents for breeding.

Key words: *Rhododendron*; Genetic relationship; AFLP; ISSR; Morphological characteristic; Cluster analysis

INTRODUCTION

Many species of *Rhododendron* are native to Asia, and were introduced to Europe to create new cultivars in the 18th century (Zhou et al., 2013). In China, they are used as indoor ornamental plants because of their flower types, brilliant colors, and other ornamental features, and are classified into three different groups according to their flowering phase (Chen, 2001): spring azaleas, summer azaleas, and spring and summer azaleas. However, other classification systems exist, including classification into four groups based on their phenotypes and origins (Huang and Qiang, 1984).

Molecular markers are powerful tools for estimating genetic diversity and distinguishing between different individuals that have different origins or morphologies (Peng et al., 2014). Several polymerase chain reaction (PCR)-based markers have been used to investigate genetic diversity and relationships, such as random amplified polymorphic DNA (Wang et al., 2012), sequence-related amplified polymorphism (Mokhtari et al., 2013), simple sequence repeats (Souza et al., 2009; Liu et al., 2012), inter-simple sequence repeats (ISSRs) (Al-Turki and Basahi, 2015; Zhan et al., 2015), and amplified fragment length polymorphism (AFLP) (Elameen et al., 2008; Larsen et al., 2015). The ISSR technique amplifies DNA fragments that are located between two identical microsatellites, and is a simple and efficient technique to estimate genetic relationships; AFLP is the best choice for analyzing population genetic and genetic relationships when there is little genetic information available (Zhao et al., 2012).

The assessment of genetic relationships is important for breeding and cultivar classification. There are a large number of *Rhododendron* resources, including wild resources and cultivars. The genetic relationships and diversity of wild *Rhododendron* plants have been estimated in previous studies (Liu et al., 2012; Zhao et al., 2015), but few studies have investigated genetic relationships among *Rhododendron* cultivars. In the present study, we investigated the genetic relationships among 17 *Rhododendron* cultivars using ISSR and AFLP markers, and compared their morphological characteristics. Characterizing cultivars using molecular tools can accurately elucidate genetic relationships, in order to identify the correct taxonomic status of varieties and contribute to possible breeding programs (Valadez-Moctezuma et al., 2015).

MATERIAL AND METHODS

Plant materials and morphological characterization

The 17 *Rhododendron* cultivars that were used belonged to three groups: 14 were spring azaleas, two were summer azaleas, and one was a spring and summer azalea (Table 1). Ten individuals of each cultivar were used. All of the samples (fresh leaves taken from annual shoots) were collected from Jiashan, China, and planted in a greenhouse at Yangling, China.

Table 1. Samples and their morphological characteristics.

No.	Name	Group	Flowering phase	Floret number	Flower color	Leaf length (cm)	Leaf width (cm)	Stipe length (cm)
1	R. 'Xiao Qing Lian'	spring azalea	End of March	2-5	violet	2.7	1.4	0.259
2	R. 'Bi Zhi'	spring azalea	Mid-March	3-4	violet	1.8	1	0.3
3	R. 'WaiGuo Hong'	spring azalea	End of March	2-3	pink	2.5	1.3	0.27
4	R. 'Tao Ban Zhu Sha'	spring azalea	End of March	3	cinnabar	1.7	0.9	0.29
5	R. 'Lv Se GuangHui'	summer azalea	End of April	2-5	green	3	1.8	0.27
6	R. 'Yu Ling Long'	spring azalea	End of April	3-4	pink white	2.7	1.5	1.22
7	R. 'Song Jiang Da Tao Hong'	spring azalea	End of April	2-3	pink	2.6	1.1	0.25
8	R. 'Ning Bo Hong'	spring azalea	End of April	3-4	red	2.1	1	0.23
9	R. 'Xiao Tao Hong'	spring azalea	Early April	2-5	pink	2	1	0.3
10	R. 'Hong Yue'	spring azalea	Early April	2-4	pink	3.3	1.5	0.24
11	R. 'HuoFeng Huang'	spring azalea	End of March	2	red	3.3	2	0.4
12	R. 'Ai Ding Bao'	spring azalea	End of April	3-4	violet	3.2	1.3	0.33
13	R. 'Zi Chen Dian'	summer azalea	May to June	2-3	violet	2.2	1.2	0.33
14	R. 'Hong Shan Hong'	spring and summer azalea	End of April	2-3	red	1.8	1	0.23
15	R. 'Zhuang Yuan Hong'	spring azalea	End of March	2-4	pink	2.1	1	0.19
16	R. 'Lan Ying'	spring azalea	Early April	2-3	violet	2.8	1.7	1.17
17	R. 'TuRui Mei Gui'	spring azalea	End of March	2-5	cinnabar	2.2	1.7	0.17

A morphological characterization of the flowers was conducted in the greenhouse during the full-bloom stage, and mature leaves were used to record leaf characteristics. Six characteristics were investigated: flowering phase, flower color, floret number, leaf length, leaf width, and the ratio of petiole length and leaf length. A detailed description of the samples and the morphological characterization is provided in Table 1.

DNA extraction

Total genomic DNA was extracted from approximately 100 mg leaf tissue suspended in double distilled H_2O (ddH_2O) using a Plant Genomic DNA Kit (Tiangen, Beijing, China). The quantity and quality of the extracted DNA was evaluated by ultraviolet spectrophotometry (Thermo Fisher NanoDrop, USA) and determined on 1% agarose gels and 1X TAE buffer [40 mM Tris-acetate and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0]. The DNA was stored at -20°C until use.

ISSR analysis

Forty primers were evaluated for DNA amplification, which were synthesized according to the sequence obtained from the University of British Columbia, Canada; nine were selected to amplify informative fingerprints (Table 2). The PCR mixture contained 2.5 µL DNA (50 ng/µL), 10 µL ddH_2O, and 12.5 µL primer mix (TaKaRa *Taq*™ Version 2.0). Amplifications were conducted as follows: 10 min of denaturation at 94°C; 35 cycles with three steps: 45 s of denaturation at 94°C, 45 s of annealing at 55°C, and 90 s of elongation at 72°C; and a final elongation at 72°C for 8 min. The amplification products were determined on 2% agarose gels using a 2000-bp DNA Ladder, with 1X TAE buffer for 40 min at 120 V. The gels were visualized by ethidium bromide staining and were then photographed. The ISSR was conducted twice.

AFLP analysis

AFLP analysis was performed according to Vos et al. (1995), with minor modifications. Firstly, 5 µL genomic DNA from each sample was digested with 5 U *Mse*I and 5 U *Eco*RI, and then the restricted DNA fragments were ligated to *Eco*RI and *Mse*I adapters. The digestion and connection was conducted at 37°C for 4 h, and the products were diluted 10-fold with ddH$_2$O for pre-amplification.

The pre-amplified reaction mixture contained 4.4 µL ddH$_2$O, 0.6 µL primers based on the *Eco*RI and *Mse*I adapter sequences, 10 µL premix *Taq* (TaKaRa), and 5 µL DNA fragments. The PCR was performed with the following profile: 2 min at 94°C; 30 s at 94°C, 30 s at 56°C, 1 min at 72°C for 24 cycles; and 10 min at 72°C. The products were diluted 10-fold with ddH$_2$O for selective amplification.

Initially, 64 primer pairs were tested for amplification of the pre-amplified DNA; four were used to amplify the samples (Table 2) with the following profile: 4 min at 94°C, 12 cycles of 30 s at 94°C, 30 s at 65°C (decreasing the temperature by 0.7°C over each cycle), 1 min at 72°C, 26 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, an extension at 72°C for 1 min, and a final extension at 72°C for 8 min.

For the analysis, the selective amplification products were mixed with 7 µL denaturation buffer, denatured at 98°C for 8 min, and cooled in ice. The products were separated by 6% polyacrylamide gel electrophores in 1X TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.0) for 90 min at 65 W, and stained with silver-staining solution. In order to prevent experimental error and ensure the reliability of the results, pre-amplification, selective amplification, and gel electrophoresis were conducted twice.

Data analysis

The amplified fragments were manually scored as 1 for present and 0 for absent for all of the ISSR unambiguous fragments (Figure 1) and distinct bands of the AFLP fragments (60-600 bp) (Figure 2). Two matrices were then created, which were used in further analysis. Dendrograms were constructed in order to determine relationships among the 17 cultivars by the unweighted pair group method with arithmetic mean based on Nei's unbiased genetic distance matrix, using NTSYSpc ver. 2.10.
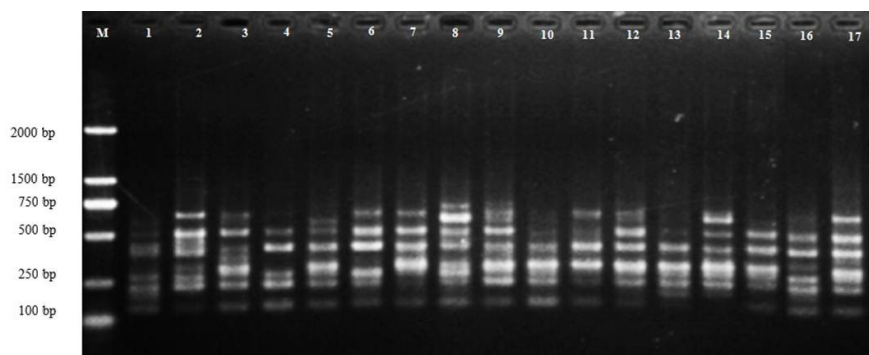

Figure 1. Inter-simple sequence repeat fingerprinting patterns of samples using the primer UBC841. *Lane M = molecular marker; lanes 1-17 = Rhododendron* cultivars.
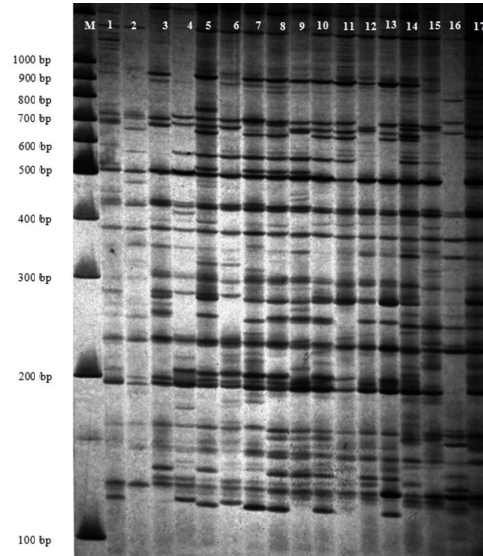
Figure 2. Amplified fragment length polymorphism fingerprinting patterns of samples using the primer combination M-CAT/E-ACC. *Lane M* = molecular marker; *lanes 1-17 = Rhododendron* cultivars.

RESULTS

Primer selection and polymorphism of amplified bands

In the ISSR analysis, the 17 *Rhododendron* cultivars were assessed using nine ISSR primers (Table 2), which generated different numbers and sizes of polymorphic fragments (Figure 1). A total of 133 bands were amplified that ranged between 100 and 1700 bp long; 129 were polymorphic with a minimum of 12 bands (UBC811), a maximum of 17 bands (UBC816, UBC817, and UBC841), and an average of 14.78 bands per primer. The percentage of polymorphic bands ranged from 92.86 (UBC819) to 100% (UBC811, UBC818, UBC823, UBC830, and UBC842).

Table 2. Polymorphisms obtained by inter-simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) markers.

ISSR				AFLP			
Primer	Bands generated	N	PPB (%)	Primer combination	Bands generated	N	PPB (%)
UBC811	12	12	100.00	M-CTG/E-ACT	49	48	97.96
UBC816	17	16	94.12	M-CTG/E-ACC	58	52	89.66
UBC817	17	16	94.12	M-CAT/E-ACC	88	84	95.45
UBC818	15	15	100.00	M-CAT/E-ACG	72	67	93.06
UBC819	14	13	92.86	Total	267	251	
UBC823	14	14	100.00	Average	66.75	62.75	94.01
UBC830	13	13	100.00				
UBC841	17	16	94.12				
UBC842	14	14	100.00				
Total	133	129					
Average	14.78	14.33	96.99				

PPB, percentage of polymorphic bands.

In the AFLP analysis, the samples were evaluated using four primer pairs (Table 2), which amplified 267 bands; 251 of them were polymorphic, with a maximum of 88 bands (M-CAT/E-ACC). The bands ranged between 60 and 600 bp long, and the percentage of polymorphic bands per primer pair varied from 89.66 (M-CTG/E-ACC) to 97.96% (M-CTG/E-ACT).

Genetic relationship analysis

Genetic relationship parameters of the 17 cultivars were compared using the molecular marker and cluster analysis results. Using ISSR markers, the genetic coefficient was between 0.56 and 0.82, the maximum was between 'Ning Bo Hong' and 'Yu Ling Long', and the minimum was between 'Lv Se Guang Hui', 'Zhuang Yuan Hong', and 'Tu Rui Mei Gui'. The cultivars were clustered into two groups (Figure 3) when setting the threshold value at 0.63; four were clustered in Group II ('Hong Shan Hong', 'Zhuang Yuan Hong', 'Tu Rui Mei Gui', and 'Lan Ying') and the rest were clustered in Group I. The flowering phases of the cultivars in Group II were earlier than those of the others, and their leaves were wider than those in Group I.
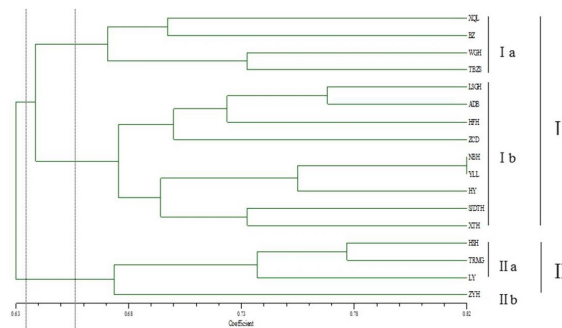

Figure 3. Dendrogram of polymorphic DNA of 17 *Rhododendron* genotypes by inter-simple sequence repeat cluster analysis.

Using AFLP markers, the genetic coefficient varied from 0.57 to 0.85, the maximum was between 'Ning Bo Hong' and 'Yu Ling Long', and the minimum was between 'Ai Ding Bao' and 'Tu Rui Mei Gui'. The AFLP-based dendrogram had two major branches (Figure 4) with the threshold value set at 0.66. One branch contained the same four cultivars as Group II by ISSR, and the other cultivars shared the other branch.
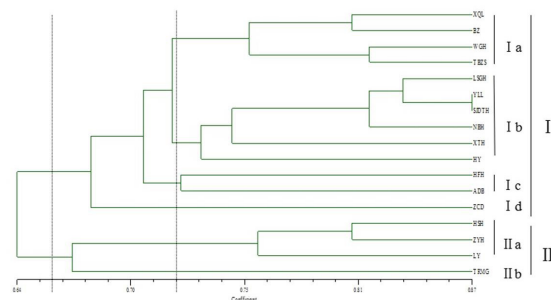

Figure 4. Dendrogram of polymorphic DNA of 17 *Rhododendron* genotypes by amplified fragment length polymorphism cluster analysis.

DISCUSSION

Recently, ISSR and AFLP techniques have been used in studies of genetic diversity (Kumar et al., 2015; Zhang et al., 2015), plant classification, and genetic relationships (Miyashita et al., 2015), including in the *Rhododendron*. Scariot et al. (2007a,b) classified evergreen azalea cultivars using AFLP markers, and compared the capacity and effectiveness of AFLP, sequence-tagged microsatellite site, and expressed sequence tag markers in assessing genetic relationships among evergreen azaleas. The authors found that AFLP could be used in assessing genetic relationships among *Rhododendron* varieties. Morphological characterization is the first step in discovering new resources, and is the most direct method of classifying plants and determining their genetic relationships, e.g., *Chrysanthemum* cultivars (Guan et al., 2013) and pea (Jha et al., 2013). In the present study, six morphological characteristics were selected to cluster samples in order to compare AFLP and ISSR.

The average percentage of polymorphic bands detected by ISSR primers was 96.99%, and by AFLP, it was 94.01%. Liu et al. (2012) reported 87.43% polymorphism in *R. aureum* at four different altitudes using ISSR, and Zhao et al. (2012) found 92.04% polymorphism in five species of *Rhododendron* using AFLP.

The clustering patterns that were obtained by ISSR and AFLP had a certain similarity. The cultivars were clustered into two groups by both ISSR and AFLP, with four in Group II and the remainder in Group I. The difference between the two results was that four cultivars were clustered into two different groups in Group II. The ISSR clustered 'Tu Rui Mei Gui' into Group IIb; however, the AFLP clustered it into Group IIa, and 'Zhuang Yuan Hong' into Group IIb. The flowering phases of the cultivars in Group II were earlier than those of the other cultivars, and their leaves were wider than those in Group I. There were two groups (Ia and Ib) in Group I according to the ISSR (with a coefficient value of 0.64), and four groups (Ia, Ib, Ic, and Id) according to the AFLP (with a coefficient value of 0.72).

The cultivars in ISSR Group Ia were the same as those in AFLP Group Ia, and the flowering phases of the species in Group Ia were earlier than those of the others. These four species were clearly separated into two branches, consistent with their flower colors: one branch contained 'Xiao Qing Lian' and 'Bi Zhi', and the other contained 'Wai Guo Hong' and 'Tao Ban Zhu Sha'. In ISSR Group Ib, one branch contained the cultivars 'Lv Se Guang Hui', 'Ai Ding Bao', 'Huo Feng Huang', and 'Zi Chen Dian', and the flowering phases of these cultivars were later and their leaves longer than those of the others; the other branch contained five cultivars. In the AFLP analysis, nine cultivars were separated into four branches, and six of them shared the same branch: 'Lv Se Guang Hui', 'Yu Ling Long', 'Song Jiang Da Tao Hong', 'Ning Bo Hong', 'Xiao Tao Hong', and 'Hong Yue'. 'Huo Feng Huang' and 'Ai Ding Bao' were in Group Ic and 'Zi Chen Dian' was alone in Group Id. The classification of the nine cultivars corresponded to their flowering phases.

Several previous studies that used morphological characteristics and molecular markers have reported a lack of consistency between them (Pham et al., 2011; Peng et al., 2014). This study demonstrated that the clustering results obtained by AFLP and ISSR are generally related to morphological characterization, particularly of the flowering phase. Therefore, our results support the classification of *Rhododendron* cultivars based on their flowering phases. In addition, the cluster results revealed the genetic relationships among the 17 *Rhododendron* cultivars studied, which can be used to select suitable parents for breeding.

ACKNOWLEDGMENTS

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