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Assessment of genetic diversity among six populations of *Rhododendron triflorum* in Tibet using ISSR and AFLP markers

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ABSTRACT

In order to assess genetic variations among *Rhodendron trifolium* populations sampled from Tibet and determine the correlation among genetic variations, the geographic location of a population, and factors that influence high-level genetic diversity, in total of 107 *R. triflorum* samples using inter simple sequence repeats (ISSR) and amplified fragment length polymorphisms (AFLP). All genotypes were collected from six different areas. Eleven ISSR primers and five AFLP primer pairs were screened, and 118 and 169 amplification products were produced, of which, 96.61% in ISSR and 95.27% in AFLP were polymorphic. High genetic diversity was observed at the species level: Nei's genetic diversity (H) was 0.3382 and 0.306, and Shannon's information index (I) was 0.5085 and 0.4642 in ISSR and AFLP, respectively. Both the coefficient of gene differentiation (G_{ST} 0.3752 in ISSR, 0.31 in AFLP) and AMOVA analysis (75% in ISSR, 71% in AFLP) indicated that most genetic diversity was distributed within populations. Gene flow (Nm) was 0.8326 in ISSR and 1.1127 in AFLP. The analyze of unbiased genetic distances determined by an unweighted pair group method using arithmetic mean (UPGMA) phonograms indicated that there was a certain degree correlation between the genetic distance and the geographic distance, which was confirmed by a principal coordinate analysis. The results maybe indicated that difference of geographical environment and variation of habitat types caused the genetic differentiation of *R. triflorum.* At last, some conservation strategies for *R. triflorum* germplasm were put forward according to these results.

1. Introduction

The Tibetan and adjacent regions of China was one of the most diverse alpine plants region in the world (Yu and Zhang, 2013). Tibetan provides the best habitat for growing azaleas and, together with Yunnan and Sichuan (Geng, 2010), forms the azalea distribution center of the world. It is important to understand the resources of *Rhododendron* in Tibetan. *Rhodendron trifolium*, the species in this study, is a perennial evergreen dwarf shrub inhabiting alpine regions, and has a high ornamental value. As *R. triflorum* is a hardy ornamental plant that grows in the wild and has the potential to enrich the alpine plant community and increase plant material for use in constructing high altitude landscapes, both Xiao et al. (2010) and Xing et al. (2011) suggested that this plant should be preferentially developed. Guidelines of reasonable exploitation and utilization of the germplasm resources should have been established to protect the germplasm resources of *R. triflorum*.

Determination of genetic diversity is essential for conservation and increased exploitation of genetic resources (Dvorakova et al., 2015). Genetic diversity differs widely among species; therefore, it is necessary to use population genetics of individual species to establish strategies for reasonable utilization and protection (Zhao et al., 2012). In addition, determining genetic diversity can help identify the genotypes and horticultural characteristics of the species, and can help to establish methods for gene transfer, which can shorten breeding programs (Okcu et al., 2015). Presently, research on genetic diversity is an important means for protecting and utilizing germplasm, and it provides an important basis for breeding. Liu et al. (2012) demonstrated that life forms, taxonomic status, and geographic locations of plants strongly impact genetic variation, and that these factors partition the genetic diversity within and among plant populations. In studies in genetic diversity of a wild species of sorghum, Basahi (2015) demonstrated that genetic diversity is influenced by human and natural factors, including altitude, soil, climate, and gene flow. Different altitudes and regions may restrict gene flow between the populations in different geographical locations, and then may lead to genetic differentiation (Arnaud-Haond et al., 2006). Recently, many studies of genetic diversity have focused on geographic variation, such as elevation gradients (Thiel-Egenter et al., 2009; Liu et al., 2012). Assessment of the genetic diversity in populations that belong to different geographic regions could reveal gene flow and indicate strategies for utilization and conservation.

A number of molecular markers have been used successfully to evaluate genetic relationship (Abraha et al., 2016) and genetic diversity

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of natural plants (Mokhtari et al., 2013; Brandao et al., 2015; Larsen et al., 2015; Moreira et al., 2015; Okcu et al., 2015; Basu et al., 2016; Ngailo et al., 2016). Additionally, many molecular markers had been used to study the genetic diversity of wild *Rhododendron* resources, including horizontal starch gel electrophoresis, RAPD, ISSR (Liu et al., 2012), SSR (Li et al., 2015), and AFLP (Tikhonova et al., 2012). Therefore, it is necessary to choose the most suitable technique to assess the genetic diversity of *R. triflorum*, based on cost and qualities of the techniques.

ISSR is a molecular markers technique based on inter-tandem repeats of short DNA sequences (Al-Turki and Basahi, 2015), and is widely used to determine genetic distances between organisms. ISSR has many advantages, including simplicity, reproducibility, and low cost. It does not require information about the DNA sequences (Morshedloo et al., 2015), and it can produce a large number of fragments per primer. ISSR uses the SSR motifs anchored with 2–4 random nucleotides as primers, which are anchored to genomic sequences to generate either side of the targeted simple sequence repeats (Al-Turki and Basahi, 2015). Compared to RAPD, ISSR can detect a greater level of genetic diversity (Dvorakova et al., 2015). Wolfe and Liston (1998) demonstrated that ISSR can provide more reliable and reproducible results than RAPD when using longer primers and higher annealing temperatures.

Powell et al. (1996) demonstrated that AFLP is a suitable technology for assessing genome-wide marker distributions and estimating genetic diversity. Vos et al. (1995) also demonstrated that AFLP is a powerful method for detecting DNA polymorphisms and marker analysis, because the primer combinations allow analysis of a large number of loci and have high polymorphism as the same as reproducibility. The primary step AFLP is the digestion step, in which the DNA is digested into fragments by restriction enzymes without requiring sequence information. Then, the fragments are selected and amplified. Zhang et al. (2014) proposed that AFLP markers are currently the better choice, if sequence information is not known.

To our knowledge, research reports of the genetic diversity of *R. triflorum* have never been published. Moreover, although ISSR and AFLP markers have never been used to study *R. triflorum*, they have been used to study other species, including *Rhododendron aureum* Georgi (Liu et al., 2012), *Rhododendron* species from Far East of Russia (Tikhonova et al., 2012), and *Rhododendron ferrugineum* (Escaravage et al., 1998). ISSR and AFLP are promising technologies for the estimation of genetic diversity with high degrees of reliability (Zhao et al., 2012; Jena et al., 2015; Kumar et al., 2015). Furthermore, there have been assessments of genetic diversity in other plants, based on ISSR and AFLP markers (Das et al., 2015).

Keeping into perspective view about the above discussed facts, the present study was to clarify the genetic diversity of *R. triflorum* using ISSR and AFLP markers, as there is not any information of the genome of this species. Moreover, the efficiency and usefulness of these two techniques in estimating genetic variation were compared. Ultimately, the results of these two techniques were compared and complemented mutually, in order to evaluate the genetic diversity of *R. triflorum* more comprehensively. This analysis is expected to provide the information needed to improve future methods for germplasm collection and to help establish reasonable strategies for the utilization, management, and conservation of *R. triflorum*.

2. Materials and methods

2.1. Plant materials

Plant materials of *R. triflorum* used in the present study totaled 107 and were collected from 6 locations of Tibet which were coded as Milin1 (M), Milin2 (ML), Bomi (B), Sejila Mountain (S), Linzhi (LH), and Galongla (G). The detailed information of 6 populations is enumerated in Table 1. Cluster sampling method has been used to sample the materials; samples were always at least 3 m away from each other to avoid sampling ramets from the same vegetative clone (Liu et al., 2012). Young leaves were sampled from plants of *R. triflorum*, and dried directly in silica gel for transportation, then been frozen at −20 °C for storage till DNA extraction.

Table 1
Overview of *Rhododendron triflorum* populations, located in different regions of Tibetan China.

No.	Population	Sample size	Coordinates	Altitude(m)
1	Milin1 (M)	19	E94°00′48″ N29°11′22″	2984
2	Milin2 (ML)	20	E94°16′33″ N29°14′56″	2965
3	Bomi (B)	17	E95°21′47″ N29°57′52″	2610
4	Galongla (G)	20	E95°34′39″ N29°35′06″	3543
5	Sejila (S)	15	E94°43′36″ N29°49′27″	3526
6	Linzhi (LH)	16	E94°19′36″ N29°40′17″	2980

2.2. DNA extraction

Total genomic DNA was extracted from 0.03 g powdered leaf materials using a plant genomic DNA Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The purity and the quality of the extracted DNA were detected by 1% (*w*/*v*) agarose gel electrophoresis and a UV spectrophotometer (Liuyi, Beijing, China). The gels were stained with ethidium bromide (EtBr and EB) and photographed using a Bio-rad gel documentation system (Bio-Rad Laboratories, Shanghai, China). Finally, each DNA was diluted to 50 ng μL^{-1} and stored at −20 °C for further fingerprinting analysis.

2.3. ISSR amplification

The ISSR analyses were performed according to Liu et al. (2012) with minor modifications. A total of 40 primers were designed by the University of British Columbia (UBC) and synthesized by AuGCT (Beijing, China). 11 primers, which could produce strong, clear and reproducible bands, were screened from 40 primers using 6 individuals from 6 populations and used for the amplification reactions for all samples in this study (Table 2). Briefly, ISSR-PCR amplification reactions were performed following Xu et al. (2016), This experiment had been repeated three times.

2.4. AFLP amplification

The AFLP analyses in this study were carried out following Vos et al. (1995) and Xu et al. (2016) with some adjustment as. Selective amplifications were performed in 20 μL volume by using 5 selected primer combines (Table 2) and conducted in a gradient type PCR machine with the following profile: 4 min at 94 °C; using 12 cycles of 30 s at 94 °C, 30 s at 65 °C (lowering the annealing temperature by 0.7 °C over each cycle) and 1 min at 72 °C; then 24 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 1 min; extension at 72 °C for 8 min at last.

For estimation, the products of selective amplification, which were mixed with 7 μL denaturation buffer, were denatured for 8 min at 98 °C and cooled on ice immediately. Each 4 μL of the denatured products was separated by 6% (*w*/*v*) polyacrylamide gel electrophoresis (PAGE) in 1× TBE buffer using 100 bp Ladder (Cwbiotech, Beijing, China), stained by silver staining solution ultimately.

2.5. Genetic analysis and construction of dendrogram

For the genetic diversity analysis, ISSR and AFLP amplified fragments which are distinct, reproducible and unambiguous were assembled into binary matrix by manually scoring present band as (1) or absent band as (0). The resulting binary matrix was subjected to estimate genetic parameters using the genetic software program POPGENE version 1.31 (Yeh et al., 1997), including observed number of alleles (Na), effective