Development and Characterization of Microsatellite Markers for Rhododendron Purdomii Using Next-Generation Sequencing

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Short Report

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Abstract

Rhododendron purdomii, an endangered ornamental species endemic to the Qinling Mountains, is an important component of montane ecosystem in central China. Due to the impact of climate change and human disturbance, management and conservation of this species are in urgent needs. In this study, we developed 13 novel microsatellite markers for R. purdomii based on next-generation sequencing data, and tested these markers’ utility in congeneric species R. concinnum. For the 13 microsatellite markers in three R. purdomii populations, number of alleles ranged from two to 12, number of effective alleles was from 1.000 to 8.892, Shannon’s information index was from 0.000 to 2.320, and the observed and expected heterozygosity were from 0.000 to 1.000 and from 0.000 to 0.888, respectively. Cross-species amplification for R. concinnum indicated eight microsatellite loci were successfully amplified and polymorphic. The microsatellite markers developed in this study will provide opportunities for examining the genetic diversity and population structure of R. purdomii and contribute to the effective conservation of this species.

Introduction

Rhododendron is the largest genus of the family Ericaceae, containing more than 1000 species worldwide [1]. This genus has many important ornamental plants. There are approximately 571 Rhododendron species in China, of which 409 species are endemic [2]. Rhododendron has been suggested as a good model system for evolutionary, ecological, and horticultural studies [1], and there are many related studies on species located in the hotspot areas (e.g. Hengduan Mountains) in South China [3, 4]. However, little is known about the Rhododendron species restricted to the central region of China [5].

Rhododendron purdomii Rehder & E. H. Wilson is an evergreen shrub or small tree endemic to the Qinling Mountains of China, occurred in Henan, Shaanxi and Gansu provinces (Fig. 1). R. purdomii was originally collected on Taibai Mountain of Shaanxi province by William Purdom in 1910, so it is named in honour of the collector [2]. It is an important component of the montane ecosystem at 1,800 - 3,500 m altitude, and plays a vital role in erosion control and climate regulation [4]. In addition, this species is a horticulturally significant plant with colorful flowers (Fig. 2). Based on our recent field survey in Henan, we found that local people collected and transplanted wild individuals of R. purdomii together, in order to build Rhododendron garden to attract tourists. Moreover, this species has been subject to some level of habitat disturbance due to human destruction and climate change [1, 4]. Based on recent evaluation, R. purdomii is listed as vulnerable to extinction in the Red List of China Higher Plants [6]. Therefore, understanding the genetic diversity and structural patterns of R. purdomii is urgently required to effectively monitor and conserve this species.

Previous studies on R. purdomii are mainly about ornamental horticulture and resource development, such as evaluation on morphological traits [7], and investigation of germplasm resources [8]. Despite its great value for horticulture research, genetic diversity and population structure of R. purdomii are not clear, with the exception that only some populations in Shaanxi were assessed based on amplified fragment length polymorphism (AFLP) [5]. Due to the characteristics of codominant inheritance, high polymorphism and wide distribution in genome, microsatellites are proven to be useful in population genetic studies [9, 10]. In this study, we developed microsatellite loci for R. purdomii based on restriction-site associated DNA sequencing (RAD-seq), and cross-species amplification was conducted for these newly developed markers in R. concinnum.

Materials And Methods

Sample collection and DNA extraction

To develop the microsatellite markers and evaluate the polymorphism of the markers, three natural populations of R. purdomii were collected from Laojun Mountain (LJS, n = 11), Longyuwan (LYW, n = 15) and Laojiejing (LJJ, n = 17) in Henan province, China, respectively. Furthermore, to validate the selected microsatellite primers in other Rhododendron species, we sampled two populations of R. concinnum from Yao Mountain (CYS, n = 10) and Laojun Mountain (CLJ, n = 10) in Henan, respectively. The locality information of the sampled populations was detailed in Table S1. Fresh and healthy leaves of the investigated individuals were collected and sampled individuals were at least 10 m apart within one site. Permissions were obtained from the local nature reserve for collecting plant materials. Leaves were dried by silica gel and stored in plastic bags until DNA extraction, and voucher specimens were deposited in the Herbarium of Zhengzhou University (ZZU).

RAD sequencing and microsatellite mining

Genomic DNA of all the investigated individuals was extracted from dried leaf tissues using a modified CTAB method [11]. A restriction-site associated DNA (RAD) library of one R. purdomii individual was constructed using the EcoRI (5’-GAATTTC-3’) enzyme following Miller et al. [12] and Baird et al. [13]. The library was sequenced at Novogene (Beijing, China) using the Illumina HiSeq 2000 platform with 150 bp paired-end reads. After filtering low-quality reads, de novo assembling of the clean reads was performed by Velvet [14] with default parameters. Microsatellite motifs with a repeat unit of 2-6 bp and a minimum number of four repeats were detected using MISA [15] with default settings. The program Primer3 [16] was used to design microsatellite primers, the length of primers ranged from 20 to 28bp, the annealing temperature was 60-65°C.

Validation of microsatellite loci and cross-species amplification

The validation of microsatellite loci was performed through three steps as follows. Firstly, we randomly selected three individuals from three different populations of R. purdomii to test the success of amplification for the designed primer pairs. Then, the forward primers of microsatellites successfully amplified in three individuals were labeled with fluorescent dye, and fluorescent PCR products of the selected six individuals from three different populations were analyzed for polymorphism. Thirdly, microsatellites showing expected size range on agarose gels, clear peaks and polymorphism during capillary electrophoresis in six individuals were further amplified in all the investigated individuals of R. purdomii. In the above processes, PCR reaction mixture used was 50 μL in volume, consisting of distilled water (22 μL), 2 × PCR Mixture (25 μL, Beibei Biotech, Henan, China), 10 μM forward and reverse primers (1 μL for
each primer), genomic DNA (1 μL). The PCR reaction conditions were: an initial denaturation at 94°C for 4 min; 35 cycles at 94°C for 30 s, annealing temperature for 30 s, 72°C for 30 s; a final extension at 72°C for 7 min. The success of amplification was determined by electrophoresis on a 1% agarose gel. The fluorescent PCR products were analyzed through capillary electrophoresis on an ABI 3730XL DNA Sequencer (Applied Biosystems, Foster City, CA, USA) at the Sangon Biotech Corporation (Shanghai, China), and the genotypes were obtained using GeneMarker (Soft Genetics).

Samples of R. concinnum were used to evaluate the transferability of the developed microsatellite markers in congeneric species. The screening of primer pairs suitable for R. concinnum was consistent with three steps described above, excepting that two individuals from two different populations were used for amplification validation and four from two populations respectively were for polymorphism test.

Population statistics

For population genetics analyses, number of alleles ($N_A$), number of effective alleles ($N_E$), Shannon's information index ($H$), observed heterozygosity ($H_O$) and expected heterozygosity ($H_E$) were estimated using GenAlEx [17]. Polymorphism information content (PIC) was calculated by CERVUS [18]. Test for Hardy-Weinberg equilibrium and linkage disequilibrium between microsatellite loci was conducted using GENEPOP [19]. Frequencies of null alleles were estimated by Micro-Checker [20].

Results And Discussion

For the RAD-seq data of R. purdomii, a total of 16,152,280 paired-end reads were generated after quality filtering. After de novo assembling of the clean reads, 301,199 contigs with the mean size of 353 bp and the mean GC content of 40.17% were obtained. The software MISA detected 6,853 microsatellite loci in the assembled contigs, and 6,714 of which were suitable for primer design. Of the 60 primer pairs selected for the initial tests, 31 of them can be amplified with clear bands. In these 31 microsatellite loci, 13 loci presenting reproducible amplification, clear peaks and rich polymorphism were selected for R. purdomii finally. In the amplification for R. concinnum, nine of the 13 loci were amplified successfully, of which one locus showed monomorphism and eight polymorphic loci were detected.

Of the 13 polymorphic microsatellite loci, five were dinucleotide repeats, three were trinucleotides, three were tetranucleotides and two were hexanucleotides (Table 1). All the 13 sequences were submitted to GenBank (MW736532-MW736544). The number of alleles per locus ranged from four (P24, P60) to 18 (P23) over all investigated R. purdomii individuals, while polymorphism information content values were from 0.240 (P58) to 0.915 (P23). In three investigated populations of R. purdomii, the number of alleles ranged from two to 12, number of effective alleles ranged from 1.000 to 8.892, Shannon's information index ranged from 0.000 to 2.320, observed heterozygosity ranged from 0.000 to 1.000, and expected heterozygosity ranged from 0.000 to 0.888 (Table 2). Significant deviations from Hardy-Weinberg equilibrium in terms of heterozygosity deficiency were found in three loci of LJS population, four of LYW, and four of LJL population, respectively. Null alleles were found in the locus P23 in three populations, P28 in LJS population, P33 in LYW population, P57 and P58 in LJL population, and P59 in LJS and LYW population. Deviations from Hardy-Weinberg equilibrium of some loci might be related to the presence of null alleles. Linkage disequilibrium was detected in one pair loci (P16 & P60) in LJL population. The linkage disequilibrium might indicate physical proximity of the loci on the chromosome or some evolutionary processes, such as selection, introgression and genetic drift, and those loci should be used with caution in different analyses [21, 22].

In two populations of R. concinnum, the number of alleles ranged from three to 10, number of effective alleles ranged from 2.198 to 7.692, Shannon's information index ranged from 0.856 to 2.155, observed heterozygosity ranged from 0.500 to 1.000, and expected heterozygosity ranged from 0.545 to 0.870 (Table 3). Two loci in CYS population and one locus in CLJ population deviated from Hardy-Weinberg equilibrium. No evidence of null alleles was identified. Linkage disequilibrium was found in loci P28 and P57 in CYS population.

Declarations

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**Tables**

| Table 1 | Characterization of the microsatellite markers for *Rhododendron purdomii* |
| Locus | Primer sequences (5′–3′) | Repeat motif | Size range (bp) | \( N_A \) | PIC | Ta (°C) | GenBank accession no. |
|-------|--------------------------|--------------|----------------|--------|------|--------|---------------------|
| P3    | F:AAGAATGCTGAAAATGTCTTCCA R:ACCATCTGGCTTTTATTTTCC | (TC)_{16}    | 133-167        | 13     | 0.813 | 52     | MW736532            |
| P16   | F:GTTTCTAGATCCAGCCTCTTG | (TC)_{15}    | 97-133         | 15     | 0.910 | 53     | MW736533            |
| P23   | F:TTGGGTGTCAAAAATAAAAACGAAG R:GAAGCCCGAAGGACATAAAT | (AG)_{16}    | 135-207        | 18     | 0.915 | 52     | MW736534            |
| P24   | F:GTCTTAGAGAAAAAGTGCTCTACA R:CGCTACGAGTGATTCAGCTA | (TTT)_{7}   | 118-127        | 4      | 0.460 | 54     | MW736535            |
| P28   | F:GCTATTTACCTTTCTTGGCAGC R:TCTGGGACAGAGTGATACCA | (CT)_{15}    | 113-157        | 13     | 0.802 | 53     | MW736536            |
| P33   | F:GTATACGATGCTATCGCTCCAC R:TCAATTGAATTTCGCTTACCA | (CT)_{15}    | 121-157        | 15     | 0.898 | 52     | MW736537            |
| P41   | F:ATGAAATTGGAGAACGAGAATGGA | (AGA)_{8} | 100-127        | 7      | 0.687 | 51     | MW736538            |
| P42   | F:GCACGCAAAACTATAAATCAACATT R:ATAGATTGAAAAACCACATCGCA | (TAA)_{8}   | 101-188        | 13     | 0.789 | 50     | MW736539            |
| P46   | F:GGGCTTCTCAAGATTGATTTTAAAGGT | (TTTA)_{5} | 136-156        | 6      | 0.625 | 54     | MW736540            |
| P57   | F:TGGGTCTCTACTTTACCCCCCAATTTT R:AACGTAACGACCGACGAAAGATTTT | (TGTA)_{5} | 121-141        | 5      | 0.647 | 53     | MW736541            |
| P58   | F:GGTACACATCGAAAGCTCTCTCTGTACGACAAGGCAAGATTTT R:TCTTACGCTGCTTTTATT | (CGGGAG)_{4} | 131-179        | 6      | 0.240 | 54     | MW736542            |
| P59   | F:GTGGGCGACAAGAAAGATTTGGTTGCC | (TTTTAT)_{6} | 125-149        | 5      | 0.572 | 52     | MW736543            |
| P60   | F:GTAATAGGGTTGTAGTGGGAGAAG R:ATAATCGAATGACGTACGCGCA | (TTCT)_{5} | 145-157        | 4      | 0.596 | 52     | MW736544            |

\( N_A \) number of alleles, PIC polymorphism information content, Ta annealing temperature.

**Table 2** Genetic diversity parameters of 13 polymorphic microsatellite loci in three populations of *Rhododendron purdomii*
Geographic distribution of Rhododendron purdomii in China. The locality information was based on specimen records. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.
Figure 2
Photographic images of Rhododendron purdomii. a A typical individual of R. purdomii. b Leaves and leaf buds. c R. purdomii inflorescence. d Capsule of R. purdomii

Supplementary Files
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- TableS1.docx