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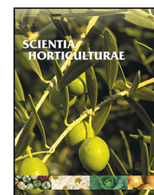

Genetic diversity of endangered primrose (*Primula heterochroma* Stapf.) accessions from Iran revealed by ISSR and IRAP markers

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ABSTRACT

Primula heterochroma Stapf. is an endemic plant to the north of Iran. In this study, 24 individuals and combinations of ISSR and IRAP markers were used for evaluation of genetic diversity among 50 *P. heterochroma* accessions. Analysis of polymorphic bands using simple matching coefficient indicated that genetic similarity ranged from 0.39 to 0.95. Among the markers and marker combinations tested, IRAP had the higher mean values of genetic diversity parameters. The results indicated that the UBC873, UBC817, TOS-2 and UBC813 + UBC815 primers were the most informative which could be used to assess the diversity of wild primrose accessions. The cluster analysis with UPGMA method separated the accessions into six main groups. Accessions from the same regions generally were clustered into the same group or subgroups. The results supported that ISSR and IRAP analyses could be used for the characterization and grouping of *P. heterochroma* accessions.

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1. Introduction

The *Primula* L. is one of the most popular horticultural plants (Zhang and Kadereit, 2004) that belongs to the family Primulaceae. Considering the latest evaluations, it includes 500 species, mainly located in the temperate and cold regions of the northern hemisphere and in the tropical mountains (Hao et al., 2001). *Primula heterochroma* is an endemic threatened species of *Primula* genus in northern Iran (Alinezhad et al., 2011) and covers low slopes of the forests of the Caspian coasts. However, irregular and delayed germination together with pasturage and severe winter cause failure of its maturity and consequently, resulted in mortality and extinction. Biotechnological methods are now an essential component of plant genetic resources management (Benson et al., 2000; Morozowska and Wesołowska, 2004) and exponentially becoming important for the conservation of rare and endangered plants. A number of these valuable techniques include cryopreservation, micropropagation and molecular fingerprinting (Glover and Abbott, 1995; Sudha et al., 1998).

Previously, *in vitro* seed germination and micropropagation of *P. heterochroma* using shoot tip explants have been reported (Noroozisharaf et al., 2011). However, preserving the genetic diversity of endangered species is one of the primary aims in conservation strategies, because survival of the species depends on the maintenance of sufficient genetic variability (Vicente et al., 2011). Many molecular techniques have been used to identify crop cultivars and accessions (Pivoriene and Pasakinskiene, 2008) and overcome problems largely associated with phenotype-based classification (Awasthi et al., 2004). In recent years, a number of molecular techniques such as AFLPs (Reisch and Kellermeier, 2007), ISSRs (Crema et al., 2009; Shao et al., 2009), RAPDs (Reisch et al., 2005), SSRs (Van Geert et al., 2008) and cpDNA sequencing (Honjo et al., 2004; Kitamoto et al., 2005) have been widely used to detect genetic diversity or phylogenetic relationships in primroses. Inter-simple sequence repeat (ISSR) is one of the most efficient molecular marker methods in terms of ability to produce abundant polymorphic markers within a short time and limited budget (Pivoriene and Pasakinskiene, 2008). Compared to many other techniques, ISSR can discriminate between closely related genotypes (Hodkinson et al., 2002) and detect polymorphisms without any previous knowledge of the crop's DNA sequence (Kumar et al., 2006).

Retrotransposons are highly abundant and dispersed components of most plant genomes, showing activity at transcription and integration levels, providing an excellent basis for the development of molecular marker systems (Kalendar et al., 2004). Inter-retrotransposon amplified polymorphism (IRAP), a PCR-based technique, detects high levels of polymorphism which have

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Table 1
List of 50 germplasm accessions of *Primula heterochroma* collected from Guilan Province of Iran.

Accession name	Collected site	Altitude (m)	Accession name	Collected site	Altitude (m)
G1	Saravan	81	G26	Jokleh Bandan	132
G2	Saravan	41	G27	Jokleh Bandan	157
G3	Saravan	47	G28	Imamzadeh Hashem	174
G4	Saravan	60	G29	Imamzadeh Hashem	108
G5	Saravan	61	G30	Imamzadeh Hashem	111
G6	Kacha	230	G31	Imamzadeh Hashem	111
G7	Kacha	225	G32	Jokleh Bandan	111
G8	Kacha	222	G33	Jokleh Bandan	158
G9	Kacha	228	G34	Jokleh Bandan	167
G10	Qazian	230	G35	Jokleh Bandan	188
G11	Qazian	137	G36	Mushangah	186
G12	Qazian	136	G37	Mushangah	184
G13	Qazian	186	G38	Mushangah	198
G14	Kacha	184	G39	Siyahkal	256
G15	Kacha	200	G40	Tutaki	346
G16	Kacha	170	G41	Lunab	542
G17	Kacha	161	G42	Lunab	545
G18	Oskoolak	181	G43	Qaleh Rudkhan	103
G19	Oskoolak	202	G44	Khararud	555
G20	Siahrud	137	G45	Khararud	574
G21	Jokleh Bandan	55	G46	Khararud	549
G22	Jokleh Bandan	59	G47	Pirkooh	590
G23	Jokleh Bandan	72	G48	Pirkooh	613
G24	Jokleh Bandan	112	G49	Deylaman	612
G25	Jokleh Bandan	103	G50	Deylaman	499

no DNA digestion, ligations or probe hybridization to generate marker data, thus increasing the reliability and robustness of the assay (Kalendar et al., 2004). IRAP technique has recently been exploited to study genetic diversity and phylogeny in the plant genus such as *Musa* (Teo et al., 2005), *Citrus* (Bretó et al., 2001) and *Oryza* (Hirochika et al., 1992).

The high genetic diversity maintained in *Primula* L. is probably due to distylous (pin and thrum) floral morphs (Kery et al., 2000). The pin and thrum flowers are generally self-incompatible and reciprocally pollinated by insects. Floral dimorphism facilitates pollen exchange among different individuals. This breeding system will tend to maintain the genetic diversity, and promote gene flow within populations, increasing the effective population size and reducing the effects of drift (Van Rossum and Triest, 2006).

To our knowledge, no literature is available about analysis of genetic diversity and relationships of the exclusive species of *P. heterochroma* in Iran. The objectives of the present study were: (i) to assess the genetic relationships among 50 germplasm accessions of *P. heterochroma* using ISSR and IRAP markers and (ii) to provide elementary information for future conservation strategies.

2. Materials and methods

2.1. Plant materials

Fifty primrose accessions (*P. heterochroma* Stapf.) were collected from different regions of Guilan province, Iran (Table 1). Fresh foliage samples were harvested from two or three individuals of each accession and kept at −80 °C for genomic DNA extraction.

2.2. DNA extraction

DNA was extracted from frozen plant material of the individual plants using a cetyltrimethyl ammonium bromide (CTAB) method (Rogers and Bendich, 1994) modified as follows: approximately 80–100 mg leaf material was ground in liquid nitrogen in a 2 ml Eppendorf tube followed by addition of 1000 μl extraction buffer (250 mM Tris HCl, pH 8.5; 20 mM EDTA, pH 8.3; 1.4 M NaCl; 3% CTAB; 0.1% PVP; 2 μ1/m1 β-mercapto ethanol). Samples were incubated at 65 °C for 20 min and shaken every 3–5 min during this time. Subsequently the mixture was extracted twice with an equal volume of cold chloroform/isoamylalcohol (24:1), and centrifuged at 10,000 × *g* at 4 °C for 10 min. By addition of an equal volume of isopropanol (4 °C), the DNA was precipitated and pelleted by centrifugation at 10,000 × *g* at 4 °C for 15 min. The DNA was washed with 70% cold ethanol (4 °C) for 5 min and air-dried for 20–30 min. It was re-suspended in 200 ml TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA, pH 8.3). Extracted DNA was qualified using 1% (w/v) agarose gel electrophoresis. Afterwards, the DNA concentration was estimated spectrophotometrically (PG Instrument +80, Leicester, UK) at 260 nm, and the purity measured by the ratio of the absorbance at 260 nm and 280 nm. For PCR, only template DNA was used with a purity of 2 in a dilution of 10 ng/ml.

2.3. PCR amplification

Total volume of the PCR reaction mixture was 10 μl, containing 30–40 ng of template DNA, 10 mM dNTP, 0.3 mM primer, 1× PCR buffer and 1 U DNA Taq polymerase. DNA amplification was carried out using a BioRad thermocycler (BioRad Laboratories Inc., Hercules, CA, USA) as follows: initial denaturation at 94 °C for 4 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 41 °C/51 °C (depending on the primers used) (Table 2) for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. The PCR products were separated on 1.5% (w/v) agarose gel in 1× TAE buffer at a constant voltage of 90 for 60 min. The resulting gel images were captured using the Biometra gel documentation system (Whatman Biometra, Gottingen, Germany). The size of produced fragments was defined by comparing to size marker (GeneRuler 1 kb DNA ladder, SM0241, Fermentase, Ontario, Canada).

2.4. Data analysis

In all, 24 individual ISSR and IRAP primers with their combinations were used to amplify regions of genomic DNA (Table 2). Only reproducible and well defined bands in the replications were considered as potential polymorphic markers. The polymorphic bands

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