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Are rhododendron hybrids distinguishable on the basis of morphology and microsatellite polymorphism?

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ABSTRACT

Sequence Tagged Microsatellite Sites (STMSs) and morphological trait markers were used to evaluate 33 rhododendron germplasm for genetic diversity assessment and discrimination power. The average genetic diversity estimates were 0.724 (morphological traits) and 0.174 (STMSs) marker datasets. The Shannon index was higher for morphological traits (1.797) than STMS (0.302). The correlation coefficients obtained by the Mantel matrix correspondence test, which was used to compare the cophenetic matrices for the two markers, showed that estimated values of relationships given for morphological and STMS were not significantly related ($p > 0.05$). The dataset from STMS, supported by the total probability of identity (1.13×10^{-9}) and total paternity exclusion probability (0.9999), allowed all accessions to be uniquely identified. In summary, STMS marker proved to be an efficient tool in assessing the genetic variability among old broad leaf rhododendron genotypes. The pattern of variation appeared to be consistent, and it can be used for germplasm conservation and management for restoration of historical genetic resources.

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1. Introduction

Rhododendrons are one of the most popular landscape plants in the Northern Hemisphere. The genus belongs to the *Ericaceae* family and contains over 1000 species, sorted in eight subgenera: *Rhododendron*, *Hymenanthes*, *Pentanthera*, *Tsutsusi*, *Azaleastrum*, *Therorhodion*, *Mumeazalea* and *Candidastrum* (Chamberlain et al., 1996). The basic chromosome number is 13 and most of the species and hybrids are diploid (Väinölä, 2000) but natural polyploids (tetraploids, octoploids and dodecaploids) can be found (Janaki Ammal et al., 1950). Besides the immense number of species and cultivars derived by intra and inter subgeneric hybridization (Contreras et al., 2007), the diversity of this genus is also consequence of the plurality of its habitats. Most of the species are present in Asia, in North America and in Australia, while in Europe only six species (*R. hirsutum* L., *R. ferrugineum* L., *R. palustre* sbp. *palustre* L., *R. myrtifolium* K., *R. lapponicum* L. and *R. ponticum* L.) are indigenous.

During the end of the XVIII and the beginning of the XIX century, many species and new hybrids were introduced from Asia to Europe, especially in England and Germany, by means of the plant hunters. The hybridization activity reached remarkable levels and rhododendrons became the most popular flowering, broadleaf evergreens and ornamental woody plant, especially in northern locations with cold winters (Väinölä, 2000). As for most of the cultivated tree species, the identification of rhododendron hybrids is convoluted. The presence of numerous species and the wide geographical distribution, together with the high level of interspecific hybridization, make genetic relationships within the genus confusing. The same accession name could have been accidentally given to different genotypes or one genotype can have several synonyms. In addition, the parentage of most hybrids is unknown and their relationship with horticultural groups is often not available.

The study of genetic diversity of old cultivated plants, recovered in private and public gardens, as source of desirable genes is of current interest (Khlestkina et al., 2004) and the development of new hybrids adapt to different conditions, such as Alpine area (Nybom et al., 2004; Kjolner et al., 2004) is particularly intended. Morphological and molecular characterization could be of help for preserving and using these genetic resources and, through the study of pre-breeding and breeding germplasm diversity, for determining unique and distinct traits.

Among molecular markers, Sequenced Tagged Microsatellite Sites (STMSs) are considered to be neutral markers and more informative for characterizing germplasm collections thanks to several characteristics, including abundance in eukaryotic genomes, high levels of polymorphism, Mendelian inheritance, co-dominance, and locus specificity (Merdinoglu et al., 2005; Scariot et al., 2006;

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Table 1
Accessions name, parentage and locality of the 33 *Rhododendron* genotypes sampled in Burcina Park (B.P.), Villa Taranto (V.T.) and Missouri Botanical Garden (M.B.G.).

Accession name	Parentage	Locality	Accession name	Parentage	Locality
Madame Masson	*R. catawbiense* × *R. ponticum*	B.P.	Sappho	Unknown	B.P.
Lady Eleanor Cathcart	*R. maximum* × *R. arboreum*	B.P.	Michael Waterer	*R. ponticum* × *R. arboreum* ssp. *arboreum*	B.P.
Memoire de Dominique Vervaene	Unknown	B.P.	Mrs. R.S. Holford	Unknown	B.P.
John Walter	*R. catawbiense* × *R. arboreum* ssp. *arboreum*	B.P.	Lady Rolle	Unknown	B.P.
White Pearl	*R. griffithianum* × *R. maximum*	V.T.	Pink Pearl	'George Hardy' × 'Broughtonii'	B.P.
James Marshall Brooks	Unknown	B.P.	The strategist	*R, griffithianum*	B.P.
Fastosum Flore Pleno	*R. catawbiense* × *R. ponticum*	B.P.	Purity	*R. edgeworthii* × *R. formosum*	B.P.
Cunningham's White	*R. caucasicum* × *R. ponticum* var. *album*	B.P.	Prince Camille de Rohan	*R. maximum* × *R. caucasicum*	B.P.
Princesse Hortense	Unknown	B.P.	*R. arboreum* A.		V.T.
Everestianum	*R. catawbiense*	B.P.	*R. catawbiense* M.	V.T.	
Madame Patti	Unknown	B.P.	*R. fortunei* L.		V.T.
Haydeé	Unknown	B.P.	*R. ponticum* L.	V.T.	
Madame Boyer	Unknown	B.P.	*R. edgeworthii* H.	V.T.	
Perspicuum	Unknown	B.P.	*R. griffithianum* H.	V.T.	
Emperor de Maroc	Unknown	B.P.	*R. maximum* L.		M.B.G.
Onsloweanum	Unknown	B.P.	*R. caucasicum* P.	M.B.G.	
Nigrescens	Unknown	B.P.			

Marchese et al., 2007). However, the development of STMSs is laborious and at present only a few STMS primers were designed in *Rhododendron* (Dunemann et al., 1998; Kameyama et al., 2002; Dendauw et al., 2001). Numerous types of other molecular markers have been developed and used for phylogenetic studies and cultivar fingerprinting, such as *trn*K and *mat*K (Kron, 1997; Kurashige et al., 1998, 2001), nuclear ITS sequences (Gao et al., 2002; Tsai et al., 2003), Random Amplified Polymorphic DNA (RAPD; Scariot et al., 2007; Lanying et al., 2008), and EST derived markers (De Keyser et al., 2009). Only a few refer to subgenus *Hymenanthes* and *Rhododendron*. Jin et al. (2006) developed ISSR markers in *R. fortunei* L. and Contreras et al. (2007) and Wei et al. (2006) studied species diversity in *R. ponticum* L. and *R. catawbiense* M. using AFLP and EST derived markers.

This study evaluated (1) the usefulness of four STMS markers for establishing relationships in old broad leaf rhododendrons, and (2) the relatedness among cultivated hybrids (many of them never previously DNA-typed) and seven species belonging to subgenus *Hymenanthes* section *Ponticum* (*R. griffithianum* H., *R. fortunei* L., *R. ponticum* L., *R. maximum* L., *R. catawbiense* M., *R. caucasicum* P and *R. arboreum* A.) and *R. edgeworthii* H. (subgenus *Rhododendron*, section *Rhododendron*) by means of morphological and DNA markers.

2. Materials and methods

2.1. Plant material, DNA isolation and STMS analysis

Thirty-three rhododendron accessions (eight species and 25 cultivars) of the genus *Rhododendron* subgenus *Hymenanthes* section *Ponticum* and subgenus *Rhododendron* section *Rhododendron* located in the Burcina Park and Villa Taranto (Northern Italy), and in the Missouri Botanical Garden (U.S.A.) were selected for this study (Table 1). Genomic DNA was extracted as described by Thomas et al. (1993), from approximately 0.20 g leaf tissue. The DNA extraction buffer (CNB; Crude Nuclei chromatin Buffer) contained 2.5% PVP (polyvinyl pyrrolidone K40), 0.2 M Tris–HCl pH 7.6, 0.05 M Na_2EDTA pH 8, 0.25 M NaCl and 2.5% β-mercaptoethanol. This method yielded up to 240 ng/μl of genomic DNA per extraction. DNA quality was examined by electrophoresis on a 0.8% agarose gel and DNA concentration was quantified by means of a spectrophotometer. Four STMS primer sets developed by Dunemann et al. (1998), labelled with a specific fluorochrome (6-FAM or HEX), were used: GA211, RDC46, RDC45 and RDC27. Amplification reactions were carried out in a final volume of 20 μl containing 50 ng template DNA, 2 μl 10× PCR reaction buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl), 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM of each primer and 0.5 U AmpliTaq Gold® DNA polymerase (Applied Biosystems, Foster City, CA). The PCR amplifications were performed using the following temperature program: initial step of 9 min at 95 °C, followed by 28 cycles of 30 s at 95 °C, 45 s at 50 °C, 1 min 30 s at 72 °C, with a final extension step of 45 min at 72 °C. One microliter of a mix containing amplification products was added to 3 μl of a mix containing 5:2:1 parts of formamide, GeneScan-500 Liz size standard and loading dye (25 mM EDTA, 50 mg ml^{-1} blue dextran). Fluorescent samples were denatured at 95 °C for 5 min and detected on a sequencing gel (5% acrylamide, 6 M urea, 1× TBE buffer) using an ABI-PRISM®377 DNA sequencer (Applied Biosystems, Foster City, CA).

2.2. Morphological characterization

All the plants were described by means of 11 morphological traits referring to habitus, flower and leaf (Table 2) as previously assessed by Remotti et al. (2003). Three flowers in full bloom and three mature leaves were measured on each plant.

Multistate characters were treated as follows: leaf shapes = 0 (lanceolate), 1 (oval-lanceolate), 2 (spatulate-lanceolate), 3 (oval), and 4 (spatulate); leaf surface = 0 (flat), 1 (convex), 2 (concave), 3 (corrugated), and 4 (smooth); plant habit was considered = 0 (straggly shrub), 1 (thick shrub), 2 (straggly tree), and 3 (thick tree); blooming time = 0 (early; from 1st to 30th April), 1 (semi-early; from 1st to 15th May), 2 (semi-late; from 15th to 30th May), and 3 (late; from 1st to 15th June); bloom density = 0 (low; distance between truss >50 cm), 1 (medium; distance between truss ranged from 25 to 50 cm), and 2 (high; distance between truss <25 cm); truss shapes = 0 (hemispheric), 1 (conic-hemispheric), and 2 (conic).

2.3. Data analysis

The presence or absence of fragments amplified by STMS primers was coded by 1 or 0 respectively and scored as a binary data matrix. Allele frequencies (Fig. 1), number of effective alleles, Shannon's index, diversity (*h*), unbiased diversity (uh) were calculated using GenAlEx 6.3 (Peakall and Smouse, 2006). Genetic distances based on STMS data were computed according to Nei (1978). Cluster analysis was performed using Neighbor-joining method, on arithmetic means (Sneath and Sokal, 1973), by means of the TREECON software (Van de Peer and De Wachter, 1994). This software, was also used to estimate the statistical stability of the branches in the tree by bootstrap analysis with 1000 replicates.

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