IDENTIFICATION OF 33 CHINESE *RHODODENDRON* SPECIES USING *matK* SEQUENCES AND AFLP DATA

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Abstract

The current assortment of pot azalea (*Rhododendron simsii* hybrids) has been created from a relatively narrow basis of collectors material brought from the far east. *R. simsii*, the main ancestor, originates from hilly areas in China, Thailand, Laos and Burma. However several other species from the *Tsutsusi* subgenus, from South-Asia and Japan might have contributed e.g. *R. indicum*, *R. mucronatum*, and *R. scabrum*. From the Kunming Institute of Botany (China) 33 seed lots from natural populations in mountain area's with an altitude ranging from 250 to 3500 m were obtained. The majority of these *Rhododendron* species belong to the *Tsutsusi* subgenus; 8 are *R. simsii*. Per population 10 plants were analysed by AFLP using 3 primer combinations. They were compared to the breeders pot azalea genepool (70 plants, some with common pedigree and bud sports). AFLP was performed using the commercially available kit for fluorescent fragment detection on an ABI Prism 377 DNA Sequencer. The genetic diversity of the different gene pools was analysed by comparison of the marker frequencies, by calculating similarity indices, by multivariate analysis and AMOVA. Small differences within populations were observed. Large variation was observed within the *R. simsii* species and between the different species from the *Tsutsusi* subgenus. The pot azalea genepool was clearly distinguishable from the Chinese accessions. On dendrograms it was more closely clustered to *R. simsii* and *R. mucronatum* than to less related species. For *matK* sequencing a subset of plants from the Chinese accessions was selected and compared to examples from the breeders pot azalea genepool. For amplification and sequencing 4 primer couples were applied that covered in total a DNA sequence of 2529 bp. Multiple alignment from all sequences was used as template for a phylogenetic analysis. One outgroup was defined for rooted tree construction. The dataset was analysed in three ways: Neighbour Joining based clustering, parsimony and maximum likelihood analysis. The topology of the trees achieved by AFLP and *matK* analysis were compared. Several corresponding clusters could be identified, showing the complementarities of both approaches.

Abbreviations

matK maturase K, **AFLP** Amplified Fragment Length Polymorphism, ***rbcL*** rubisco large subunit

1. Introduction

The genus *Rhododendron* is well known for the beauty and diversity of floral and vegetative form of its more than 1000 species. Because of the many species within this genus, during history taxonomists have made several classifications mainly based on morphological data (i.e. flowers, leafs, hairs, etc.). Morphological data are relatively easy to obtain, but only a small number of characters can be evaluated. An alternative for this

reason classification may be based on molecular data. Molecular data provide the possibility to study a tremendous number of genetic loci in three distinct genomes (nuclear, mitochondrial, and chloroplast DNA). In plant systematics the chloroplast genome has proven to be very informative. Two of the genes that have been useful in constructing evolutionary trees in the *Ericaceae* are *rbcL* (rubisco large subunit) and *matK* (maturase K). The *matK* is known to evolve approximately three times faster than *rbcL*, and is therefore a powerful tool for phylogenetic reconstruction within angiosperm families and genera (Hilu and Liang, 1997). On the other hand, the use of molecular markers can be very effective to study within species diversity or the relationships between closely related species.

2. Material and methods

2.1. Plant material

From the Kunming Institute in China we received seed lots from several locations (Table 1) within the provinces Yunnan, Hunan, Fujian and Jiangxi, from a range of altitudes. After sowing and growing the plants in the greenhouse, young leafs were harvested, lyophilised and stored in -80 °C until use. For the other plants (mainly *R. simsii* hybrids from the DvP-genepool) young leaf material was directly available.

2.2. DNA isolation

From the young leafs total DNA was extracted using a two step protocol, a modification of the method described by Greenwood *et al.* (1989).

2.3. AFLP reactions and PAGE

AFLP reactions were run on an ABI Prism 377 DNA Sequencer using the commercially available kit for fluorescent fragment detection (AFLP Amplification Core Mix Module (P/N 402005 from Applied Biosystems). Selective amplification was done using 3 fluorescent-labelled *EcoRI/MseI* primer combinations with 6 selective bases : *EcoRI*-ACT / *MseI*-CTA, *EcoRI*-ACT/*MseI*-CAT and *EcoRI*-AAG/*MseI*-CTA (De Riek *et al.*, 1999).

2.4. matK amplification and sequencing

A specific PCR to amplify the *matK* gene was performed on the 33 Chinese *Rhododendron* species. For this purpose 4 primer couples were used (Kurashige *et al.*, 1998) (Table 2 and 3; Fig. 1). The amplified PCR fragments were directly used for sequencing, using the PCR primers as sequencing primers (forward and reverse). Dideoxy sequencing was done using the Big Dyes Chemistry on a ABI Prism 377 DNA sequencer (Applied Biosystems). The obtained sequences from the several contigs were assembled using the Auto Assembler software (Applied Biosystems).

2.5. Data analysis

For the scoring of the AFLP fingerprints, the settings for semi-automated scoring of fluorescent reactions defined by De Riek *et al.* (1999) were applied. Statistical analysis was based on 179 scored AFLP-markers. Calculations of similarity coefficients, construction of dendrograms (UPGMA) and principal co-ordinates analysis were performed by the modules SIMIL, CLUSTER and PCOORD of the “R package” (Legendre and Vaudor, 1991).

Alignment of the *matK* sequences prior to parsimony analysis was performed by ClustalW 1.81 and a alignment control was done with GeneDoc 2.3. Parsimony analysis

was done with PAUP (Phylogenetic Analysis Using Parsimony), version 4B.8 (Swofford, 1993). Trees were obtained using 100 X bootstrapping, stepwise addition and tree bisection recognition as branch swapping algorithm.

3. Results

3.1. AFLP fingerprinting

To evaluate the genetic conformity between and within populations and the gene pool collection used in the breeding program, two approaches were applied : ordination analysis (clustering and principal co-ordinate analysis) and analysis of molecular variance (not reported here). Both type of analysis take a similarity or distance matrix as an input.

A pair wise similarity matrix was calculated on the basis of the frequencies of the markers in each population (Euclidean distance). In Fig.2, a cladogram is showing the genetic similarity between assayed populations. The Belgian pot azaleas, Hirado and Kurume azaleas were considered to be separate and well defined populations to be able to calculate marker frequencies. Bootstrap analysis was used to evaluate the reproducibility of the obtained groupings. Being supported by high bootstrap values, three major clusters and some outlying species can be distinguished. The first group contains *R. florulentum*, *R. rufulum*, *R. seniavinii* and two populations of *R. rivulare*. All these species belong to the *Tsutsusi* section of the *Tsutsusi* subgenus. AFLP fingerprints for *R. florulentum* and *R. rufulum* appeared to be very alike. These species are also phenotypically very similar; taxonomic difference is based on the size of the flowers. The second group solely holds *R. simsii* populations clustered with its subspecies *mesembrinum*. The third group consists of the in China free proliferating cultivated species *R. x pulchrum* and *R. mucronatum* and the different groups of cultivated azalea hybrids. The remaining species (*R. racemosum*, *R. yunnanense* and *R. mariesii*) are clearly separated based on AFLP data.

3.2. Phylogenetic analysis based on matK sequencing

Phylogenetic analysis based on the *matK* sequences was used to confirm the taxonomic position of the different species of the *Tsutsusi* subgenus. For that purpose, some extra species belonging to the subgenera *Hymenanthus* and *Rhododendron* were also analysed.

After sequencing and alignment of the different contigs a fragment of 2529 bp long was obtained covering *matK* and its flanking *trnK* sequences. For the analysis using PAUP, from the 2529 characters, 2438 characters were constant, 30 characters were variable and parsimony uninformative, 61 characters were variable and parsimony informative. Trees were generated using 100 resampled bootstrap datasets, stepwise addition and tree bisection recognition as branch swapping algorithm. The nodes that separated the different subgenera and sections all received high bootstrap values. In Fig.3, a parsimony cladogram is showing the genetic similarity between the assayed Chinese *Rhododendron* species and some *R. simsii* hybrids.

Five major clusters can be distinguished. The first group contains *R. mariesii* and *R. farerrae*, which was used as an outgroup species to root the dendrogram. Both species belong to the section *Brachycalyx* from the subgenus *Tsutsusi*. The second cluster contains *R. yunnanense*, *R. spiciferum*, *R. x duclouxii*, and two populations of *R. racemosum*. All these species belong to the subgenus *Rhododendron*. A third clusters contains *R. fortunei*, *R. sinofalconeri*, *R. argyrophyllum*, *R. irroratum* and two populations of *R. decorum*. All these species belong to the subgenus *Hymenanthus*. The fourth cluster contains *R. florulentum*, *R. rufulum* and two populations of *R. rivulare*. All these species belong to the subgenus *Tsutsusi*.

The last and largest cluster contains species all belonging to the section *Tsutsusi*, it can be divided in two subclusters. A first contains four *R. simsii* populations, two *R. simsii* var. *mesembrinum* populations, *R. scabrum*, *R. kiusianum* and the Kurume hybrid

Cupido. A second subcluster contains five *R. simsii* hybrids, *R. pulchrum*, *R. mucronatum*, and *R. scabrum*.

4. Discussion

Computation of similarity or distance measures and ordination analysis allowed assessing genetic conformity and relatedness of the studied species and cultivated hybrids. The use of marker frequency data (Fig. 2) assures that all available genetic information is represented by the ordination. The *matK* data confirm the previously obtained AFLP data. In general four distinct groups and some outlying species can be distinguished. The first group contains *R. florulentum*, *R. rufulum*, *R. seniavinii* (no *matK* data) and two populations of *R. rivulare*. All these species belong to the *Tsutsusi* section of the *Tsutsusi* subgenus. The marker data for *R. florulentum* and *R. rufulum* appeared to be very alike. These species are also phenotypically very similar; taxonomic difference is based on the size of the flowers. A second group contains the *R. simsii* populations and cultivated *R. simsii* hybrids. *R. simsii*, generally considered to be the most important ancestor for pot azaleas due to type of flowers and the only ancestor exclusively distributed from China, seemed to be more apart. However, because a predominant number of *R. simsii* populations is included in this study, this might somewhat distort the AFLP ordination. The *R. simsii* populations are apart from the cultivated *R. simsii* hybrids; the latter are clustering with *R. x pulchrum*, *R. scabrum*, and *R. mucronatum*. This was confirmed by the *matK* sequencing data. A third group consists of species belonging to subgenus *Rhododendron*, namely, *R. yunnanense*, *R. racemosum*, *R. spiciferum* (no AFLP data) and *R. x duclouxii* (no AFLP data). A last group consists of *R. mariesii* and *R. farerrae* (outgroup for *matK* analysis) both belonging to the Section *Brachycalyx* of the Subgenus *Tsutsusi*.

Regarding the parentage of the cultivated hybrids, a closer clustering of *R. x pulchrum* and *R. mucronatum* with Hirado (AFLP data) and *R. simsii* hybrids or pot azalea (AFLP data and *matK* data) was observed (Fig 2 & 3). Kurume azaleas (AFLP and *matK* data) were more distant (Fig. 2). *R. x pulchrum* is regarded to be a hybrid between *R. indicum* and *R. mucronatum*. Both species are also related to pot azaleas. According to Sweet, who described the species *R. x pulchrum* first, this taxon may be loosely grouped with the *Azalea indica* hybrids (a former erroneous name for *R. simsii* hybrids but also a term to describe the Satsuki azaleas) although it is not certain that both type of hybrids have the same parentage (Chamberlain and Rae, 1990). *R. mucronatum* has been cultivated at least for 300 years in Japan and China. The original wild form, native to Japan, is var. *ripense* with rose-pink flowers but widely cultivated together with var. *mucronatum*, an albino form. Hirado and pot azaleas share at least one important common ancestor, *R. scabrum*, originating from some small southern Japanese islands. 'Phoenixium', one of the early crossing parents with supposed *R. simsii* plants from Sanghai that lead to the current *R. simsii* hybrids, is accepted to be a cross between *R. mucronatum* and *R. scabrum* (Galle, 1987).

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Table 1. List of used species with there classification and origin.

Species	Classification	Population
<i>R. mariesii</i>	Sect. <i>Brachycalyx</i>	Manshan Hunan
<i>R. farerrae</i>	Sect. <i>Brachycalyx</i>	
<i>R. argyrophyllum</i>	Subg. <i>Hymenanthus</i>	Yulong Caojian Yunnan
<i>R. decorum</i> (a)	Subg. <i>Hymenanthus</i>	
<i>R. decorum</i> (b)	Subg. <i>Hymenanthus</i>	
<i>R. fortunei</i>	Subg. <i>Hymenanthus</i>	
<i>R. irroratum</i>	Subg. <i>Hymenanthus</i>	
<i>R. sinofalconeri</i>	Subg. <i>Hymenanthus</i>	
<i>R. x duclouxii</i>	Subg. <i>Rhododendron</i>	Yulong Caojian Yunnan
<i>R. racemosum</i> (a)	Subg. <i>Rhododendron</i>	
<i>R. racemosum</i> (b)	Subg. <i>Rhododendron</i>	Huaping Yunnan
<i>R. spiciferum</i>	Subg. <i>Rhododendron</i>	
<i>R. yunnanense</i>	Subg. <i>Rhododendron</i>	
<i>R. florulentum</i>	Sect. <i>Tsutsusi</i>	Yanzhong Fujian
<i>R. kiusianum</i>	Sect. <i>Tsutsusi</i>	
<i>R. mucronatum</i>	Sect. <i>Tsutsusi</i>	Lushan Jiangxi
<i>R. x pulchrum</i>	Sect. <i>Tsutsusi</i>	Lushan Jiangxi
<i>R. rivulare</i> (a)	Sect. <i>Tsutsusi</i>	Daoxian Xiaojiangjiao Hunan
<i>R. rivulare</i> (b)	Sect. <i>Tsutsusi</i>	Manshan Hunan
<i>R. rufulum</i>	Sect. <i>Tsutsusi</i>	Dongxiaobaitu Fujian
<i>R. scabrum</i>	Sect. <i>Tsutsusi</i>	Yiliang Yunnan
<i>R. simsii</i> (a)	Sect. <i>Tsutsusi</i>	Jianshui Yunnan
<i>R. simsii</i> (b)	Sect. <i>Tsutsusi</i>	Daoxian Hunan
<i>R. simsii</i> (c)	Sect. <i>Tsutsusi</i>	Xishan Yunnan
<i>R. simsii</i> (d)	Sect. <i>Tsutsusi</i>	Kutiankang Fujian
<i>R. simsii</i> (e)	Sect. <i>Tsutsusi</i>	Jiuyidianzhan Hunan
<i>R. simsii</i> (f)	Sect. <i>Tsutsusi</i>	Daoxian Xiaojiangjiao Hunan
<i>R. simsii</i> var. <i>mesembrinum</i> (a)	Sect. <i>Tsutsusi</i>	Lushan Jiangxi
<i>R. simsii</i> var. <i>mesembrinum</i> (b)	Sect. <i>Tsutsusi</i>	Manshan Hunan
<i>R. semavinii</i>	Sect. <i>Tsutsusi</i>	DvP genepool
<i>R. simsii</i> hybrids (pot azalea)	Sect. <i>Tsutsusi</i>	DvP genepool
Kurume (winterhardy)		DvP genepool
Hirado's (<i>R. scabrum</i>)		DvP genepool
Kurume (not winterhardy)		

Table 2. Primer codes, primer sequence (IUPAC codes) and T_m used for amplification of the *matK* locus.

Primer	5' sequence 3'	T_m (°C)
<i>trnK</i> 3914F	GGG GTT GCT AAC TCA ACG G	56.4
<i>trnK</i> 1 MF	GAT AAG TTT ACC GAG GTA GC	47.6
<i>matK</i> 462F	AAT ACC CTA YCC CRT YCA TC*	48.9
<i>matK</i> 3MF	GTG GTC TCA ACC AAG AAG G	50.5
<i>matK</i> 174R	CGA KTA ATT AAM CGT TTC AC*	46.8
<i>matK</i> 1MR	GTA GAA AAA ATC GTA ATA GC	47.6
<i>matK</i> 1848R	TAT CGA ACT TCT TAA TAG C	40.9
<i>trnK</i> 2R	AAC TAG TCG GAT GGA GTA G	44.5

* Degenerated primers for PCR

Table 3. The 4 primer pairs used (length of the expected amplicon).

Primer pair	Forward primer	Reverse primer	Length Amplicon
I	<i>trnK</i> 3914 F	<i>matK</i> 174R	± 920 bp
II	<i>trnK</i> 1MF	<i>matK</i> 1MR	± 720 bp
III	<i>matK</i> 462 F	<i>matK</i> 1848R	± 740 bp
IV	<i>matK</i> 3MF	<i>trnK</i> 2R	± 860 bp

Figure 1. The *matK* locus showing the position of the primers used for amplification of the partial sequences (Kurashige *et al.*, 1998).

Figure 2. UPGMA cladogram based on Euclidean distance between different populations or groups for AFLP marker frequencies.

Figure 3. Consensus parsimony tree based on *matK* data for a selection of Chinese *Rhododendron* species and cultivated *R. simsii* hybrids, length = 98; consistency index (excluding uninformative characters)= 0.9403; retention index = 0.9987.