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Genetic Diversity and Relationship of *Rhododendron* Species Based on RAPD Analysis

Zhou Lanying, Wan Yongqing and Zhang Li

College of Forestry and Horticulture, Sichuan Agricultural University, China

Abstract: A total of 43 samples belonging to *Rhododendron* species were studied among 49 using RAPD analysis, they including 25 samples of 23 species in subgenus *Hymenanthes*, 18 samples of 18 species belong to subgenus *Rhododendron* and six samples of two species belong to subgenus *Pseudorhodorastrum*. An obtained 407 DNA fragments were amplified using 24 primers, among which 399 were polymorphic with 98.03%. The genetic Similarity Coefficient (GS) was ranging from 0.2623 to 0.9059. The dendrogram obtained by unweighted pair group method using an arithmetic average (UPGMA) showed that RAPD analysis could divided the samples into three subgenus based on morphological characters. Subgenus *Hymenanthes* had a closer relationship with subgenus *Rhododendron* than with subgenus *Pseudorhodorastru*, however subgenus *Pseudorhodorastru* can not be merged into subgenus *Rhododendron.* The obtained RAPD analysis results suggested that subgenus *Hymenanthes* was more primitive in phylogeny and subgenus *Pseudorhodorastru* was the evaluative group in morphological characters, while subgenus *Rhododendron* was a transitional group.

Key words: *Rhododendron* % RAPD % analysis % UPGMA % genetic diversity

INTRODUCTION

Rhododendron is the largest genus in Ericaceae family and 1000 species have been found up to now [1]. *Rhododendron* distributes mountainous region widely in southeastern China and formed many large populations, which plays a great role in conserving water and soil and maintaining ecological environment. Many species of *Rhododendron* are famous ornamental flowers in the world and traditional flowers in China. Studies on its relationship and classification system are good for the reasonable utilization and resource conservation. After the genus of *Rhododendron* was raised in 1753, a large number of studies have been done on its classification and many forms of classification systems were proposed, including eight subgenus reported by Sleumer [2, 3], Chamberlain [5, 6], Philipson and Philipson [7], five subgenus by Cullen [4] and nine subgenus by Fang [1]. The classification of *Rhododendron* is still under arguing and the one of focus is that whether lepidote *Pseudorhodorastrum* whose anthotaxy is axillary is belong to subgenus *Pseudorhodorastrum* or be as a subgroup of lepidote subgenus *Rhododendron.* Molecular markers could reflect the difference between species directly not affected by environment and phase of development, which is used to identify the cultivars, clones, species and homologous genes [8]. Up to now, there had been few reports on *Rhododendron* in molecule level. Kron [9] and Kurashige *et al.* [10, 11] analyzed two genes trnK and matK of *Rhododendron.* Gao *et al.* [12, 13] studied the relationship between subgenus Tsutsusi and the phylogeny and development of section Azaleastrum. RAPD analysis only was reported on 11 *Rhododendron* [14]. Southwestern of China is the largest distribution center of *Rhododendron*, Sichuan plays an important role in its distribution, where has a larger distribution of numerous and valuable subgenus Hymenanthes subgenus *Rhododendron*. However studies on this region were few, due to its wide remote. In this paper, genetic diversity, relationship and phylogeny of 43 species distributed in southeast of China were analyzed by RAPD, which can provide some proof for the classification of *Rhododendron* and know more about its phylogeny and its adaptation so as to protect and utilize *Rhododendron* resources.

MATERIALS AND METHODS

Plant materials: Forty three samples represented 29 species were collected from southwestern mountainous region of Sichuan in China, where the altitude is 2020~3435m, the other 14 species were collected from West China Subalpine Botanical Garden, Institute of Botany, the Chinese Academy of Sciences, Dujiangyan in

Corresponding Author: Dr. Wan Yongqing, College of Forestry and Horticulture, Sichuan Agricultural University, China

Table 1: Plant materials used in the study and their locality regions

No.	Species	Locality
Subgenus *Hymenanthes*		
Section *Ponticum*		
	Subsection *Fortunea Sleumer*	
1	*R. davidii*	Yingjing
2	*R. calophytum*	Yingjing
3	*R. decorum.*	Mianning
4	*R. vernicosum*	Maerkang
5	*R. gonggashanense*	Maerkang
6	*R. gonggashanense*	Maerkang
7	*R. fortunei*	Maerkang
8	*R. fortunei*	Maerkang
9	*R. orbiculare*	Longchi
10	*R. oreodoxa*	Longchi
11	*R. asterochnoum*	Baoxin
	Subsection *Argyrophylla*	
12	*R. argyrophyllum*	Yingjing
13	*R. hunnewellianum*	Longchi
14	*R. floribundum*	Longchi
	Subsection *Taliensia*	
15	*R. bureavii*	Huili
16	*R. balangense*	Longchi
17	*R. wiltonii*	Longchi
18	*R. phaeochrysum*	Longchi
	Subsection *Falconera*	
19	*R. galactinum*	Baoxin
20	*R. rex*	Huili
	Subsection *Maculifera*	
21	*R£®strigillosum*	Baoxin
22	*R. pachytrichum*	Longchi
	Subsection *Irrorata*	
23	*R. sikangense*	Yingjing
	Subsection *Neriiflora Sleumer*	
24	*R. sperabile*	Longchi
	Subsection *Lanata Chamb.*	
25	*R. circinnatum*	Huili

Table 1: Continued

No.	Species	Locality
Subgenus *Rhododendron*		
Section *Rhododendron*		
	Subsection *Triflora*	
26	*R. oreotrephes*	Longchi
27	*R. polylepis*	Yingjing
28	*R.davidsonianum*	Mianning
29	*R. rigidum*	Mianning
30	*R. tatsienense*	Zhaojue
31	*R. trichanthum*	Xichang
32	*R. yunnanense*	Xichang
33	*R. concinnum*	Huili
34	*R. augustinii*	Huili
35	*R. amesiae*	Baoxin
36	*R. siderophyllum*	Huili
37	*R. triflorum.*	Longchi
38	*R. ambiguum*	Longchi
39	*R. lutescens*	Longchi
	Subsection *Heliolepids*	
40	*R. rubiginosum*	Huili
	Subsection *Lapponica*	
41	*R. intricatum*	Zhaojue
42	*R. thymifolium*	Maerkang
43	*R. nitidulum*	Longchi
Subgenus *Pseudorhodorastrum*		
	Section *Rhodobotrys*	
44	*R. racemosum*	Zhaojue
45	*R. racemosum*	Zhaojue
46	*R. racemosum*	Zhaojue
	Section *Trachyrhodion*	
47	*R. hemitrichotum*	Xichang
48	*R. hemitrichotum*	Xichang
49	*R. hemitrichotum*	Xichang

Sichuan. Each sample was collected from four individuals and mixed together, then placed into silica gel temporarily and conserved in -80° C for a long time. Eighty nine specimens collected from wild were housed in the collection of Sichuan Agricultural University.

All plant materials used in the study including 43 species belonging to three subgenus's; *Hymenanthes*, *Rhododendron* and *Pseudorhodorastrum* were presented in Table 1. Many of them just has one specie as a sample, but for those that have obvious differences in one specie were as 2~3 samples.

DNA extraction and RAPD amplification reaction conditions: Genomic DNA extraction method was followed by Zou *et al.* [15]. The concentration and quality of DNA samples were calculated from the Optical Density (OD) values at 260 and 280 nm and they were prepared of standardized DNA (20ng/ìl). The RAPD-PCR reaction was carried out in 20 ìl and was prepared as follows: 40 ng of genomic DNA, 1.5 U *Taq E*, 1.5 Fl $MgCl_2$, 25 mM, 2.0 ìl 10×reaction buffer, 5 mM dNTPs and 0.36 ìM primer. Each reaction solution was overlaid with one drop of mineral oil to prevent evaporation. Amplification was performed in a Thermal Cycler PCR (Eppendorf) programmed as follows: an initial denaturizing at 94° for 3 min, followed by 40 cycles of 1 min at 94°, 39s at 36°, 1 min at 72° and finally extended at 72° for 10 min. The amplified products were analyzed for band presence and absence after electrophoretic separation on 1.5% agarose ge1 and staining with ethidium bromide. Each amplified reaction

Table 2: Nucleotide sequences of 24 primers and the number of amplified fragments of *Rhododendron*

Primer name	Nucleotide sequence (5′-3′)	Total bands	Size of fragment	Polymorphic bands	Polymorphic (%)
G14	GGATGAGACC	24	150-2000	23	95.83
I5	TGTTCCACGG	18	150-2000	17	94.44
I6	AAGGCGGCAG	12	300-1800	12	100.00
I9	TGGAGAGCAG	19	250-2200	18	94.74
J4	CCGAACACGG	14	400-2100	14	100.00
J7	CCTCTCGACA	22	400-2200	21	95.45
J8	CATACCGTGG	12	300-2000	12	100.00
J9	TGAGCCTCAC	18	300-2000	18	100.00
J11	ACTCCTGCGA	19	250-2000	18	94.74
J15	TGTAGCAGGG	15	250-2000	15	100.00
K11	AATGCCCCAG	16	200-2000	16	100.00
K12	TGGCCCTCAC	19	200-2000	18	94.74
M5	GGGAACGTGT	16	200-1800	16	100.00
R4	CCCGTAGCAC	16	300-1900	16	100.00
R6	GTCTACGGCA	17	250-1900	17	100.00
R10	CCATTCCCCA	19	200-2000	19	100.00
X5	CCTTTCCCTC	14	250-2000	13	92.86
X7	GAGCGAGGCT	15	350-1900	15	100.00
X10	CCCTAGACTG	24	180-2100	24	100.00
Y1	GTGGCATCTC	17	250-2200	17	100.00
Y16	GGGCCAATGT	16	250-1800	16	100.00
Z9	CACCCCAGTC	17	200-1800	17	100.00
AA7	CTACGCTCAC	17	250-1500	17	100.00
AB2	GGAAACCCCT	11	250-1900	10	90.91
Total		407	150-2200	399	98.03

was carried out three times to ensure result consistency. Twenty four primers have been screened out based on the standard of bands with clarity and more quantity and even distribution (Table 2).

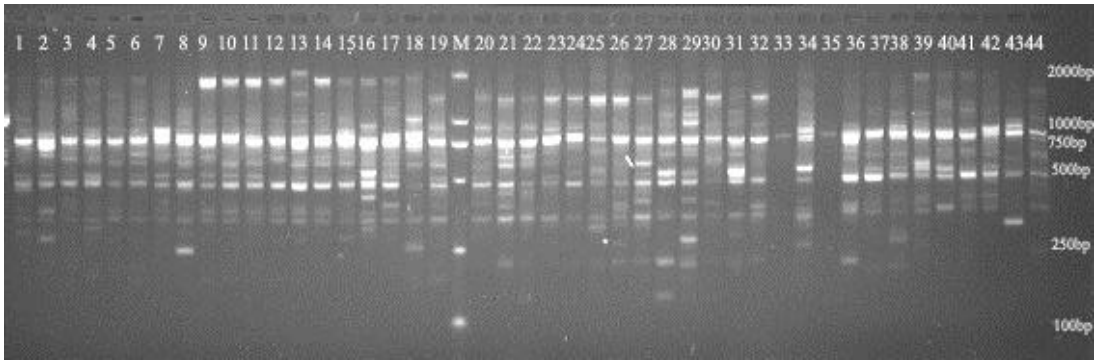
Genetic analysis: RAPD bands were scored as present (1) or absent (0). The data was used for similarity-based analysis using the software program NTSYSpc. RAPD analyses were analyzed using the Nei genetic similarity index (Nei and Li, 1979) on the basis of the equation, GS=2Nij/(Ni+Nj). Nij represents the number of bands sample i and j amplified, Ni only stands the bands amplified by sample i and Nj only stands the bands amplified by sample j. A dendrogram was constructed on the basis of the similarity matrix data by unweighted pair group method with average (UPGMA) cluster analysis.

RESULTS AND DISCUSSION

A total of 407 bands were amplified among 49 samples using 24 primers and the number of polymorphic bands was 399. There were 11~24 amplified bands by each primer and the averaged bands amplified by a primer were 16.96. The mean percentage of polymorphic bands was 98% with molecular sizes ranged from 0.15 to 2.2 kb. The results in Table 2 showed that different primers can amplify different bands on the same sample and the different samples can amplify different bands by the same primer, which reflect the complexity of their genetic background and genetic diversity. Only eight bands of the 407 bands were commonly detected in all samples, which reflected certain homology of the samples. Among the three subgenus's, there were some bands shared by one subgenus, six bands in subgenus *Hymenanthes*, five bands among subgenus *Rhododendron* and four bands in subgenus *Pseudorhodorastrum*, which showed the characteristics of the subgenus. Figure 1 showed the amplified results by primer J_9 and X_3.

The average of genetic similarity coefficient (GS) of 49 samples was 0.4631, which reflected a small difference between the samples. The genetic similarity, between *R. rubiginosum* and *R. hemitrichotum* was the least with 0.2623, while it was the largest between two of *R. gonggashanenses* with 0.9059 and followed by 0.8736

Primer SBS-J9



Primer SBS-X3

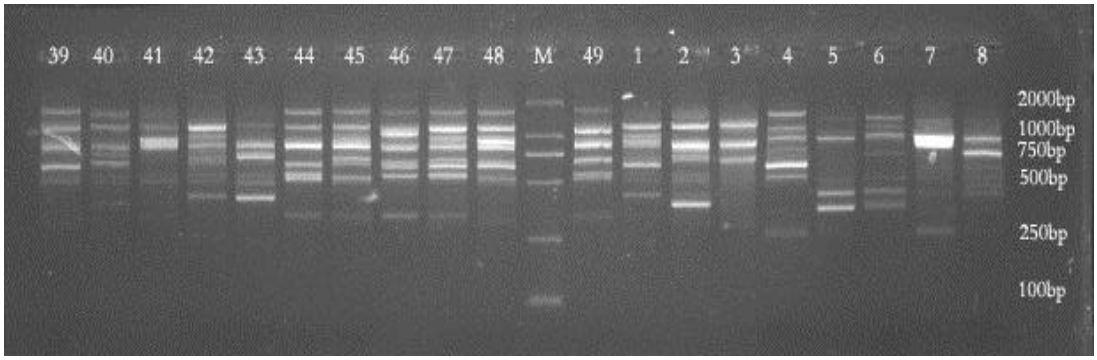


Fig. 1: RAPD amplified bands using primers SBS-J9 and SBS-X3. M is DNA ladder; 1~49 and sample numbers as in Table 1

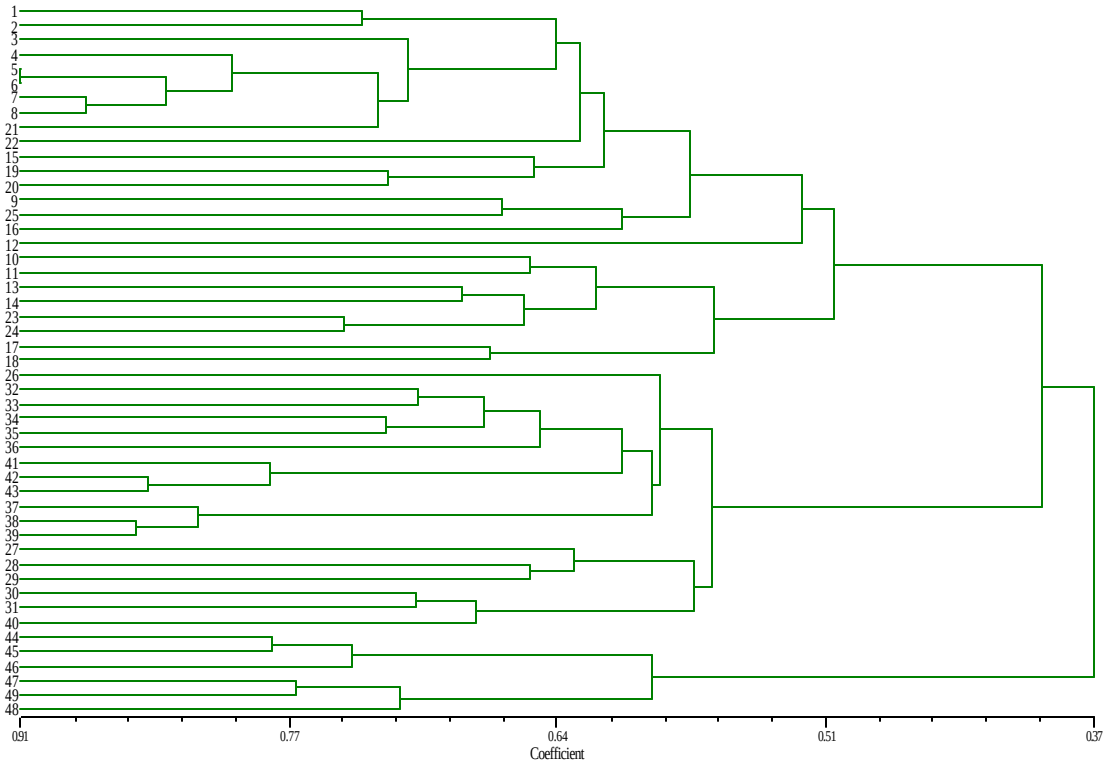


Fig. 2: Dendrogram obtained from RAPD data of 43 species (49 specimens) of *Rhododendron* by UPGMA

between two of *R. fortunei*. Three of *R. racemosum* and three of *R. hemitrichotum* had a higher GS in their species, which was 0.7547 and 0.7342, respectively. Although some *Rhododendron* belonged to the same spices, their GS was higher, which reflected their high homology in genetic background. The GS of 49 samples between three subgenuses were different; GS between subgenus *Hymenanthes* and subgenus *Rhododendron* was 0.3982 which was higher than the GS 0.3713 between subgenus *Rhododendron* and subgenus *Pseudorhodorastrum*. GS 0.3519 between subgenus *Hymenanthes* and subgenus *Pseudorhodorastrum* was the least, which reflected that subgenus *Rhododendron* and subgenus *Hymenanthes* had a closer relativeship than with subgen *Pseudorhodorastrum*. The relative ship between subgenus *Pseudorhodorastrum* and subgenus *Rhododendron* was farther, while subgenus *Pseudorhodorastrum* had a much farther relative ship with subgenus *Hymenanthes*. The low GS between 3 subgenuses showed their great differences of background. GS in one subgenus was high, GS of 25 samples in subgenus *Hymenanthes* was 0.4457~0.8714, 18 samples in subgenus *Rhododendron* was 0.4457~0.8488, 2 samples in subgenus *Pseudorhodorastrum* was 0.5291~0.7059, their averages were 0.5660, 0.5983 and 0.6527, respectively. The results showed that the difference in subgenus less than between them.

Forty nine samples were divided into three groups by UPGMA based on the GS (Fig. 2). The first group contained 25 samples from 1 to 25 belonged to subgenus *Hymenanthes*; the second group contained 18 samples from 26 to 43 belonged to subgenus *Rhododendron* and 44 to 49 of subgenus *Pseudorhodorastrum* were divided into the third group. The results were according with the classification divided by morphology. The first two groups clustered in GS 0.40 then gathered with the third group in GS 0.37.

Only one section was divided in the first group that was section *Ponticum*, but the 25 samples can be divided into eight subsections. GS between species was 0.4508 to 0.8714, which is different obviously. The 25 samples gathered two groups in GS 0.54, 17 samples belonged to 15 species were divided into the first group contained subsection *Fortunea*, subsection *Falconera* and s*ubsection Maculifera*. The other eight species were divided into the second group. Eighteen samples in the second group were 18 species, which could be divided into 3 subsections by morphology. There was a large difference between the species in each subsection; their GS was from 0.4457 to 0.8488. The 18 samples were divided into two groups in GS 0.59. Twelve species were in the first group containing subsection *Heliolepids* and most of subsection *Triflora*. There were six species in the second group containing 5 species of subsection *Triflora* and *R. rubiginosum* of subsection *Heliolepids*. The results showed that in section *Ponticum, R. triflorum, R. ambiguum* and *R. lutescens* had a closest relativeship and they were gathered together in GS 0.83. Followed were *R. intricatum*, *R. thymifolium* and *R. nitidulum* in subsection *Lapponica*, they were clustered together in GS 0.80. In the third group, six samples of subgenus *Pseudorhodorastrum* were divided into two small group, three samples of *R. racemosum* were in the first small group. All the other three samples in the second small group were *R. hemitrichotum*. The result was consisted with the classification of species.

A total of 407 bands were amplified by RAPD among 49 samples, the number of polymorphic bands was 399 with 98% (Table 2 and Fig. 1), which reflected high genetic diversity among 49 Rhododendron samples. The morphology diversity resulted from genetic diversity and their complex environments produced different Rhododendron with high value, which can be cultivated in different region. The high genetic diversity was good to the breeding to select excellent cultivars from the wild populations and can select new ones with high value and strong adaptation by crossing with different advantages and different habitation. High diversity of Rhododendron is the reflection of adaptation to environment, which is beneficial to its propagation, resources conservation, the domestication of wild species and the screen of aim genes. The clustering result is according to the classification divided by morphology, which showed that morphological traits can reflect the genetic characteristic. Figure 2 showed that subgenus *Hymenanthes* and subgenus *Rhododendron* gathered together first and then gathered with subgenus *Pseudorhodorastrum*, which indicated that subgenus *Hymenanthes* and subgenus *Rhododendron* had a closer relativeship than with subgenus *Pseudorhodorastrum*. Subgenus *Hymenanthes* always were divided into one subgenus, it was not improper to divide subgenus *Pseudorhodorastrum* as a subsection in subgenus *Rhododendron*. However, the obtained results did not support Cullen and Chaimberlain's view, but agreed with Sleumer's that considered *Pseudorhodorastrum* as a subgenus. It could show the difference between subgenus *Pseudorhodorastrum* and subgenus *Rhododendron*.

The genetic differentiation was obvious between species in a subgenus. *R. gonggashanense*, *R. davidii*

and *R. fortunei* in subgenus *Hymenanthes*, their GS can up to 0.814, which was approached to the division by morphology. *R. davidii* and *R. wiltonii* had a close relationship, their GS was 0.4508. *R. triflorum*, *R. ambiguum* and *R. lutescens* in section *Ponticum* were sole semi-deciduous species and with yellow flowers, the RAPD marker showed these characteristic, their GS was the largest (0.8280). Although *R. intricatum*, *R. thymifolium* and *R. nitidulum* in subsection *Lapponica* were collected far away, they clustered together in GS 0.80 reflecting their high homology in genetic. GS between *R. rubiginosum* in subsection *Heliolepids* and 14 species in subsection *Triflora* was 0.5000~0.6867, the average was 0.6039, while the GS in subsection *Triflora* was 0.4700~0.8488, the mean was 0.5981, which showed that subsection *Heliolepids* had a closer relationship with subsection *Triflora*. It can be reflected on the morphology. So we could consider merging subsection *Heliolepids* and subsection *Triflora* as one subsection. *R. racemosum* and *R. hemitrichotum* in subgenus *Pseudorhodorastrum* were divided into section *Rhodobotrys* and section *Trachyrhodion*, respectively based on morphology and their GS was 0.5915 approached to the GS 0.5983 in section *Rhododendron* and was higher than the GS 0.5660 in section *Ponticum*. It was obviously that section *Rhodobotrys* and section *Trachyrhodion* had a close relationship; it should reconsider that whether they could be divided into different sections.

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