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Genetic Diversity and Relationship of 43 *Rhododendron* sp. Based on RAPD Analysis

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Abstract: In this study, 49 samples of 43 *Rhododendron* sp. were studied by using RAPD to analyze their genetic diversity, relative ship and phylogeny. Four hundred and seven DNA fragments were amplified by 24 primers, the polymorphic rate was up to 98.03%. Their coefficient of Genetic Similarity (GS) was ranging from 0.2623~0.9059, which indicated rich genetic diversity in *Rhododendron*. The results showed that: 43 *Rhododendron* sp. were divided into 3 groups by RAPD, which was consistent with the division based on morphological characters, subgenus *Hymenanthes* had a closer relationship with subgenus *Rhododendron* than subgenus *Pseudorhodorastrum* and subgenus *Hymenanthes* was more primitive in phylogeny, subgenus *Pseudorhodorastrum* was the evolutive group in morphological characters, while subgenus *Rhododendron* was the transition one.

Key words: *Rhododendron*, RAPD, classification, genetic diversity, system of classification, China

INTRODUCTION

Rhododendron distributes mountain region widely in Southeastern China and formed many large populations. Studies on its relationship and classification system are good for resource conservation. *Rhododendron* is the largest genus in Ericaceae family, 1000 species have been found up to now (Yang *et al*., 1999). After genus of *Rhododendron* was posted in 1753, a large number of studies have been done on its classification and many kinds of classification system were proposed, including 8 subgenuses by Sleumer (1949, 1980), 5 subgenuses by Cullen (1980), 8 subgenuses by Chamberlain and Rae (1990) and Chamberlain *et al*. (1996), 8 subgenuses by Philipson and Philipson (1982) and 9 subgenuses. Which, one is more authoritative is still under arguing. Whether, lepidote *Pseudorhodorastrum* whose anthotaxy is axillary, belonged to subgenus *Pseudorhodorastrum* or should be regarded as a subgroup of lepidote subgenus *Rhododendro* is the controversial focus.

Random Amplified Polymorphic DNA (RAPD) technique is convenient to operate, with good polymorphism can be used in analyzing genetic diversity and the relation between species has been used in analyzing the relationships between strains belonging to the same genera and genetic diversity on many plants, such as Nothopanax Miq., Caragana Fabr., Prunus Persica and Lespedeza. Up to now, there had been few reports on *Rhododendron* in molecule level. In abroad, Kron (1997) and Kurashige *et al*. (1998, 2001) analyzed two genes trnK and matK of *Rhododendron*. At home, Gao *et al*. (2002, 2003) studied the relationship between subg. *Tsutsusi* and Phylogeny and development of section *Azaleastrum*. RAPD analysis only was reported on 11 *Rhododendron* (Zhao *et al*., 1996) in home.

Southwest of China is the largest distribution center of *Rhododendron*, where has a large distribution of numerous and valuable subgenus *Hymenanthes* and subgenus *Rhododendron*. In this study, genetic diversity, relationship and phylogeny of 43 species distributed in Southeast in China were analyzed by RAPD, which provided some proofs for the classification of *Rhododendron*.

MATERIALS AND METHODS

Forty nine samples belong to 43 species were studied. Twenty nine species were collected from southwestern mountain region of Sichuan in China, where, the altitude is 2020~3435 m; while, the other 14 species were collected from West China Subalpine Botanical Garden, Institute of Botany, the Chinese Academy of Sciences, Dujiangyan in Sichuan. Selected four individuals of each sample and mixed, then put into silica gel to dry and conserved at -80°C.

Eight subgenuses system proposed by Sleumer (1949, 1980) was adopted. Forty three species (Table 1) belong to 3 subgenuses, including 23 subgenus *Hymenanthes*, 18 subgenus *Rhododendron* and 2 subgenus *Pseudorhodorastrum*.

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Table 1: Materials of experiment

Species	Locality
Subgenus *Hymenanthes*	
Section *Ponticum*	
Subsection *Fortunea sleumer*	
R. davidii	Yingjing
R. calophytum	Yingjing
R. decorum	Mianning
R. vernicosum	Maerkang
R. gonggashanense	Maerkang
R. gonggashanense	Maerkang
R. fortunei	Maerkang
R. fortunei	Maerkang
R. orbiculare	Longchi
R. oreodoxa	Longchi
R. asterochnoum	Baoxin
Subsection *Argyrophylla*	
R. argyrophyllum	Yingjing
R. hunnewellianum	Longchi
R. floribundum	Longchi
Subsection *Taliensia*	
R. bureavii	Huili
R. balangense	Longchi
R. wiltonii	Longchi
R. phaeochrysum	Longchi
Subsection *Falconera*	
R. galactinum	Baoxin
R. rex	Huili
Subsection *Maculifera*	
R.strigillosum	Baoxin
R. pachytrichum	Longchi
Subsection Irrorata	
R. sikangense	Yingjing
Subsection *Neriiflora Sleumer*	
R. sperabile	Longchi
Subsection *Lanata Chamb.*	
R. circinnatum	Huili
Subgenus *Rhododendron*	
Section *Rhododendron*	
Subsection *Triflora*	
R. oreotrephes	Longchi
R. polylepis	Yingjing
R. davidsonianum	Mianning
R. rigidum	Mianning
R. tatsienense	Zhaojue
R. trichanthum	Xichang
R. yunnanense	Xichang
R. concinnum	Huili
R. augustinii	Huili
R. amesiae	Baoxin
R. siderophyllum	Huili
R. triflorum	Longchi
R. ambiguum	Longchi
R. lutescens	Longchi
Subsection *Heliolepids*	
R. rubiginosum	Huili
Subsection *Lapponica*	
R. intricatum	Zhaojue
R. thymifolium	Maerkang
R. nitidulum	Longchi
Subgenus *Pseudorhodorastrum*	
Section *Rhodobotrys*	
R. racemosum	Zhaojue
R. racemosum	Zhaojue
R. racemosum	Zhaojue
Section *Trachyrhodion*	
R. hemitrichotum	Xichang
R. hemitrichotum	Xichang
R. hemitrichotum	Xichang

DNA extraction: DNA extraction method suitable for RAPD analysis has been established by means of the nucleus deposition method (Zou *et al*., 2001). The concentration and quality of each sample of DNA were calculated from the Optical Density (OD) values at 230, 260 and 280 nm and DNA was regarded as being of good quality when, the ratio of $OD_{260/280}$ to $OD_{260/230}$ was near 1.8. DNA samples were diluted to standardized DNA (20 ng μL^{-1}).

PCR amplification: The reaction was carried out in a volume of 20 μL and was prepared as follows: 40 ng of genomic DNA, 1.5 U TaqE, 1.5 μL $MgCl_2$ (25 mmol L^{-1}), 2.0 μL 10× reaction buffer, 5 mmol L^{-1} dNTPs and 0.36 μmol L^{-1} primer. Each reaction solution was overlaid with one drop of mineral oil to prevent evaporation. Amplification reactions was performed in a 96-well thermocycler (Eppendorf Authorized Thermal Cycler PCR) programmed as follows: an initial denaturizing at 94°C for 3 min followed by 40 cycles of 1 min at 94°C, 39 sec at 36°C, 1 min at 72°C and finally extended at 72°C for 10 min. The amplified products were analyzed for band presence and absence after electrophoretic separation on 1.5% agarose gels and staining with ethidium bromide. Each amplified reaction was carried out 3 times to ensure results were consistent.

Statistics and analysis of data: Four hundred and twenty RAPD primers screened, 24 primers produced distinct, reproducible, polymorphic profiles among the samples tested (Table 2). Evaluated 1 for band and 0 for bandless to form a binary matrix. Analyzed the (0, 1) binary matrix by using NTSYSpc software and calculated GS by Nei and Li (1979) method with equation:

$$GS = 2 N_{ij}/(N_i + N_j)$$

Where,

N_{ij} = The number of bands sample i and j amplified
N_i = The number of bands amplified by sample i and
N_j = Amplified by sample j. The samples tested were classified by UPGMA based on GS.

RESULTS AND DISCUSSION

RAPD amplification results: Four hundred and seven bands were amplified by 24 primers among 49 samples, 399 were polymorphic bands, up to 98.03%. Average bands amplified by a primer were 16.96. The results (Table 2) showed that different primers could amplify different bands on the same sample and different samples could

Table 2: Sequences of 24 primers and the number of amplified strips of *Rhododendron*

Primer code	Nucleotide sequence	Total bands	Size of strip	Poly bands	Poly rate (%)
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

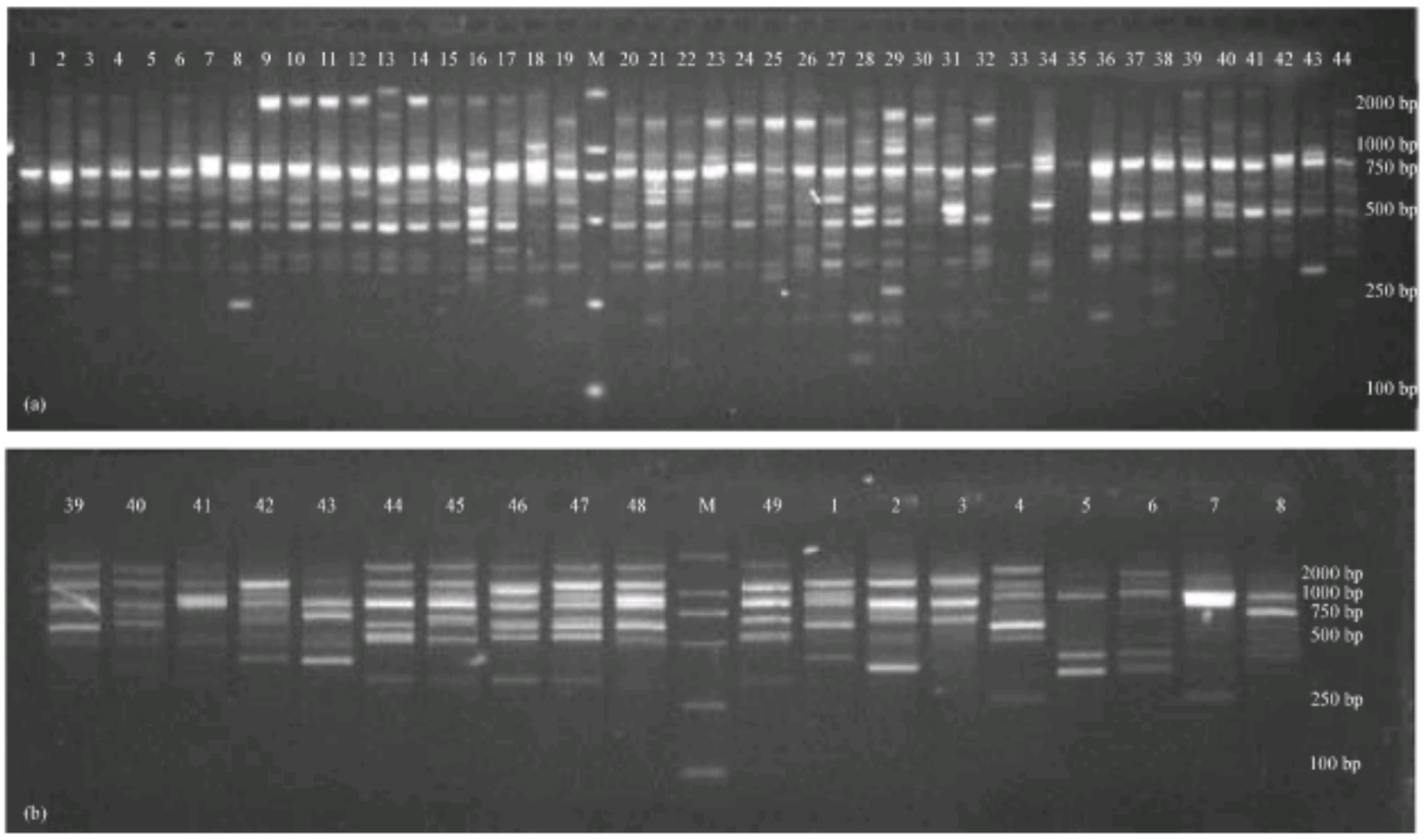

Fig. 1: RAPD figure amplified by primer SBS-J9 and SBS-X3. M is DNA Marker; 1~49: Sample number

amplify different bands by the same primer, which reflects complexity in genetic background and genetic diversity. Only 8 bands were shared by all samples, which indicated their homology to a certain extent. Among the three subgenuses, some bands shared by one subgenus, 6 in subgenus *Hymenanthes*; 5 in subgenus *Rhododendron*; and 4 in subgenus *Pseudorhodorastrum*, which showed the characteristics of subgenus. Figure 1 was the result amplified by primer J_9 and X_3.

The similarity coefficient was calculated by software. Average GS of 49 samples was 0.4631, which reflected the difference between samples was small. GS 0.2623 between *R. rubiginosum* and *R. hemitrichotum* was the least, while GS 0.9059 between 2 *R. gonggashanenses* was the largest and followed the GS 0.8736 between 2 *R. fortunei* 3 *R. racemosum* and 3 *R. hemitrichotum* had a higher GS within their species, which were 0.7547 and 0.7342, respectively. Although, some *Rhododendron* belongs to

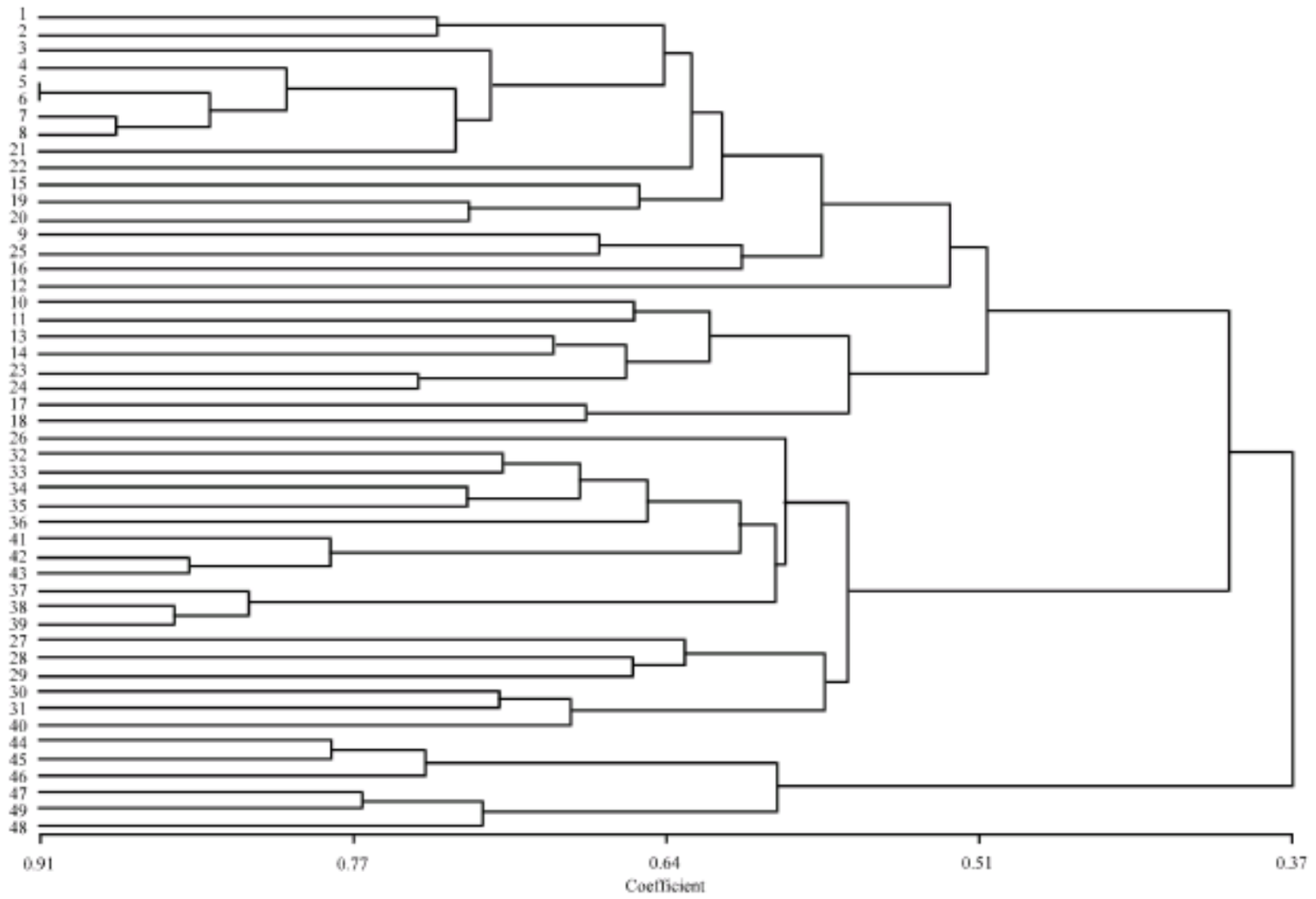

Fig. 2: Dendrogram obtained from RAPD data of 43 species (49 specimens) of *Rhododendron* by UPGMA

the same species, their GS was higher, which indicated their high homology in genetic background. The GS of 49 samples between 3 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 relationship than subgen *Pseudorhodorastrum*. The relationship between subgenus *Pseudorhodorastrum* and subgenus *Rhododendron* was far, while subgenus *Pseudorhodorastrum* had a much farther relationship with subgenus *Hymenanthes*. The low GS between 3 subgenuses showed their great differences in background. 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 3 groups by UPGMA based on GS (Fig. 2). The first group included 25 samples belonged to subgenus *Hymenanthes*; the second group contained 18 samples belonged to subgenus *Rhododendron* and the other 6 samples of subgenus *Pseudorhodorastrum* were divided into the third group. The result was accorded with the classification based on morphology. The first two groups clustered at GS 0.40 then gathered with the third group at GS 0.37.

Only section *Ponticum* was gathered into the first group, 25 samples of 23 species were divided into two subgroups at GS 0.54, 17 samples of 15 species were divided into the first subgroup including subsection *Fortunea*, subsection *Falconera* and *subsection Maculifera*, the other 8 species were divided into the second subgroup. Eighteen samples in the second group were 18 species, which could be divided into 3 subsections by morphology. While, they were divided into two subgroups in GS 0.59, 12 species of, which were in the first subgroup containing subsection *Heliolepids* and most of subsection *Triflora*. The other 6 species in the subsecond group contained 5 species of subsection *Triflora* and a species *R. rubiginosum* of subsection *Heliolepids*. The results showed that in subgenus *Rhododendron*, there was a closest relativeship among *R. triflorum*, *R. ambiguum* and *R. lutescens* and they were gathered together at GS 0.83. Followed by *R. intricatum*, *R. thymifolium* and *R. nitidulum* in subsection *Lapponica*, they were clustered together at GS 0.80. In the third group, 6 samples of subgenus *Pseudorhodorastrum* were divided into two subgroups, 3 samples of *R. racemosum* were in the first small subgroup, the other 3 samples in the second subgroup were *R. hemitrichotum*.

RAPD genetic diversity: Four hundred and seven bands were amplified by 24 primers among 49 samples, 399 of, which were polymorphic bands, up to 98.03% (Table 2 and Fig. 1), which reflected a high genetic diversity among 49 *Rhododendron* samples. The morphology diversity was caused by genetic diversity and the complex environment. High genetic diversity was good for the breeding of excellent cultivars. High diversity of *Rhododendron* reflected its strong adaptability to environment, which is benefit to its propagation, resource conservation, domestication and screen.

Clustering result is consistent with the classification based on morphology, which showed that morphological traits can reflect the genetic characters. Figure 2, 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 relationship than with subgenus *Pseudorhodorastrum*. In many classification system, *Hymenanthes* were divided as one subgenus, so it was improper to divide subgenus *Pseudorhodorastrum* as a subsection in subgenus *Rhododendron*. In this study, the results did not support Cullen and Chaimberlain's view, but we can agree Sleumer's standpoint to divide subgenus *Pseudorhodorastrum* as a subgenus, which could show the difference between subgenus *Pseudorhodorastrum* and subgenus *Rhododendron*.

The genetic difference was obvious between species in a subgenus. *R. gonggashanense*, *R. davidii* and *R. fortunei* in subgenus *Hymenanthes*, their GS can be up to 0.814, which was approached to the value calculated 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 flower, RAPD markers showed these characters, their GS was the largest (0.8280), we can show from the clustering dendrograph that their genetic difference was the least. Although, *R. intricatum*, *R. thymifolium* and *R. nitidulum* in subsection *Lapponica* were distributed far away, they clustered together in GS 0.80, which reflected their high genetic homology. 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, their average was 0.5981, which showed that subsection *Heliolepids* had a closer relationship with subsection *Triflora*. It can be reflected by morphology. So, we could consider to merge 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, their GS was 0.5915 close to the GS 0.5983 within section *Rhododendron* and higher than the GS 0.5660 within section *Ponticum*. It was obviously that section *Rhodobotrys* and section *Trachyrhodion* had a closer relationship; we should reconsider that whether they could be divided into different sections.

CONCLUSION

Forty three *Rhododendron* sp. tested can be divided into 3 groups by RAPD, which was consistent with the division based on morphological characters. The 407 bands were amplified by 24 primers among 49 samples, the number of polymorphic bands was 399 up to 98.03%, which reflected high genetic diversity in *Rhododendron*.

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