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Genetic relationships of *Rhododendron (Ericaceae)* in Taiwan based on the sequence of the internal transcribed spacer of ribosomal DNA

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SUMMARY
The genetic relationships of 20 *Rhododendron* species in Taiwan was determined based on the sequence of the internal transcribed spacer (ITS) region of ribosomal DNA. Sequences of the complete ITS region including ITS1, 5.8S rDNA, and ITS2, were obtained by direct sequencing of polymerase chain reaction (PCR)-amplified fragments. *Gaultheria itoana* was used as an outgroup. Aligned sequences of ITS1 and ITS2 from the 21 taxa resulted in 493 characters. According to the dendrogram, six main clusters were classified among the 20 species of the genus *Rhododendron* in Taiwan. *Rhododendron oldhamii*, *R. nakaharai*, *R. taiwanalpinum*, *R. simsii*, *R. lasiostylum*, *R. rubropilosum*, *R. breviperulatum*, *R. kanehirai*, and *R. noriakianum* were grouped with *R. longiperulatum* in cluster I. *Rhododendron lamprophyllum* was grouped with *R. ovatum* in cluster II. *Rhododendron pseudochrysanthum*, *R. morri*, *R. hyperythrum*, and *R. rubropunctatum* were grouped with *R. formosanum* in cluster III. In addition, *R. mariesii*, *R. ellipticum* and *R. kawakamii* formed three independent clusters. In this study, the findings based on ITS sequences are in agreement with the systematics of *Rhododendron*.

Azaleas (*Rhododendron* spp.) are horticulturally important ornamental plants and have been extensively hybridized (Kron *et al*., 1993). Approximately, 850 native azaleas species have been found in the world (Sleumer, 1966). Over 1000 horticultural hybrids had been bred by artificial hybridization to 1976 (Bean, 1976). Variability within species and an absence of distinguishing morphological characteristics between species have caused difficulties in defining taxa of azalea plants (Rayburn *et al*., 1993). Therefore, only 500 to 600 species have been identified within the genus *Rhododendron* based on different standpoints of classification (Willis, 1985).

The genus *Rhododendron* has been subdivided into eight subgenera (Kron and Judd, 1990). Four subgenera, including 20 species, were identified within the genus *Rhododendron* in Taiwan (Li, 1978; He *et al*., 1999; Yang *et al*., 1999). A taxonomic revision of this genus was conducted based on morphology, habitat, and flowering season in Taiwan. Others have suggested that only 14 species and one doubtful species occur within the genus *Rhododendron* in Taiwan (Lu and Yang, 1989).

Plant taxa of the *Ericaceae* show a great deal of morphological variability and have been thoroughly studied based on characteristics of morphology, embryology, anatomy, and chemistry (Kron *et al*., 1999). Morphological characters have traditionally been used to distinguish species. However, most morphological characters are easily influenced by environmental factors (Iqbal *et al*., 1995). Recently, molecular data have been introduced to assess several higher-level phylogenetic analyses within the *Ericaceae* or Ericales. For example, *rbc*L gene sequences of chloroplast DNA (cpDNA) were useful for elucidating the systematics of the *Ericaceae*, *Empetraceae*, and *Epacridaceae* (Kron and Chase, 1993); 18S rRNA gene sequences were useful for determining the phylogenetic relationships of the *Empetraceae*, *Epacridaceae*, *Ericaceae*, *Monotropaceae*, and *Pyrolaceae* (Kron, 1996); and *mat*K gene sequences were used for determining the phylogenetic relationships of the *Lyonia* group of the *Andromedeae* (*Ericaceae*) (Kron and Judd, 1997), as well as within the Andromedeae (*Ericaceae*) (Kron *et al*., 1999).

Because ITS regions of rDNA show great divergence, it has been suggested that ITS regions would be quite useful for comparing among closely related organisms or for the study of microevolutionary processes among or even within populations (Baldwin, 1993; Ritland and Straus, 1993). Sequence comparison of the ITS region of rDNA has been used to reconstruct the phylogeny of numerous plant taxa, such as *Calycadenia* (*Asteraceae*) (Baldwin, 1993), *Paeonia* (*Ranunculaceae*) (Sang *et al*., 1995), and *Sorghum* (*Poaceae*) (Sun *et al*., 1994). The ITS sequence also has been used to determine the genetic relationship of the section *Pentanthera (Ericaceae*) (Scheiber *et al*., 2000).

In this study, PCR-amplified products of direct DNA sequencing were used to determine the ITS sequences of rDNA of the 20 *Rhododendron* species in Taiwan. The genetic relationships among these 20 *Rhododendron* species was thus established based on their ITS sequences.

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TABLE I
Twenty taxa of Rhododendron *spp. used in this study and their systematic classification, ITS length, and accession number*

Species and classification	Source[a]	ITS length	Accession no. in GenBank
Subgenus *Hymenanthes*			
Section *Vireya*			
R. formosanum	A	642	AF285854
R. hyperythrum	A	642	AF432426
R. morri	A	642	AF432422
R. pseudochrysanthum	A	642	AF432424
R. rubropunctatum	B	642	AF285842
Subgenus *Tsutsusi*			
Section *Tsutsusi*			
R. breviperulatum	A	643	AF432425
R. kanehirai	B	643	AF172290
R. lasiostylum	B	643	AF285845
R. longiperulatum	B	643	AF285847
R. nakaharai	B	643	AF285846
R. noriakianum	A	643	AF285856
R. oldhamii	B	643	AF285843
R. rubropilsoum	B	643	AF285849
R. simsii	B	643	AF285848
R. taiwanalpinum	A	643	AF432479
Section *Brachycalxy*			
R. mariesii	B	643	AF285844
Subgenus *Azaleastrum*			
Section *Azaleastrum*			
R. lamprophyllum	A	643	AF285855
R. ovatium	A	643	AF432421
Section *Choniastrum*			
R. ellipticum	B	643	AF285841
Subgenus *Rhododendron*			
Section *Vireya*			
R. kawakamii	A	648	AF432450

[a] A = from the Taiwan Endemic Spcies Research Institute, B = from a transplant nursery.

MATERIALS AND METHODS

Total DNA extraction

Twenty species of *Rhododendron* in Taiwan plus a single species, *Gaultheria itoana* from another genus, as an outgroup, were used in this study (summarized in Table I). Total cellular DNA was extracted from fresh or silica-gel dried (Chase and Hillis, 1991) leaves, using the method of CTAB (cetyltrimethylammonium bromide) (Doyle and Doyle, 1987). Approximate DNA yields were determined using a spectrophotometer (Hitachi U-2001), and the DNA samples were then stored in a freezer at −20°C.

Primer design and PCR amplification

One primer was designed from the conserved regions of the 5' end of 18S rDNA of rice (Takaiwa *et al.*, 1984) and tomato (Kiss *et al.*, 1989a). Another primer was designed from their complementary conserved regions of the 3' end of 26S rDNA. In the present study, two sets of primers, i.e. IT1: 5'TCGTAACAAGGTTTCCGTAGGT3' and IT2: 5'GTAAGTTTCTTCTCCTCCGCT3' were designed to amplify the internal transcribed spacer (ITS) of rDNA in azalea plants (Figure 1). PCR was performed as follows: we used a 50 μl mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2 mM $MgCl_2$, 0.01% BSA, with four dNTPs (0.2 mM each), primers (0.5 μM each), 2.5 units of *Taq* DNA polymerase (Virogene), 10 ng genomic DNA, and a 50 μl volume of mineral oil. Amplification reactions were performed in a dry-block with two-step thermal cycles (Biometra). In the first step, the mixture was incubated at 94°C for 3 min, then it underwent 10 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 1 min. The second step was carried out by the following process: 30 cycles of denaturation at 94°C for 45 s, annealing at 54°C for 45 s, extension at 72°C for 1 min, with a final extension for 10 min at 72°C. These PCR products were detected by agarose gel electrophoresis (1.0%, w/v in TBE), and staining by 0.5 $\mu g/ml^{-1}$ of ethidium bromide, and were finally photographed under UV light exposure.

DNA sequencing

PCR products of ITS sequences from the 20 *Rhododendron* species were recovered by glassmilk (BIO 101, California) and directly sequenced by the dideoxy chain-termination method using an ABI377 automated sequencer with a BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, California). Sequencing primers were the same as those used for PCR. Each sample was sequenced two or three times to confirm the sequences. These reactions were performed based on the recommendations of the manufacturers.

Data analysis

Genetic relationships were determined by using the program MEGA version 2.1 (Kumar *et al.*, 2001). The genetic distance matrix was calculated by the two-parameter method of Kimura (1980). These data were then used to construct a phylogenetic tree using the unweighted pair-group method analysis (UPGMA) (Rohlf *et al.*, 1982).

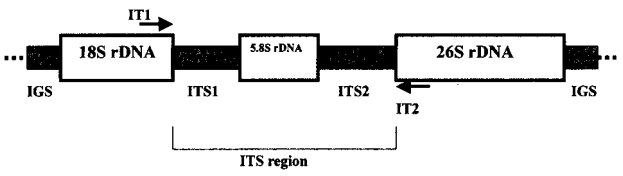
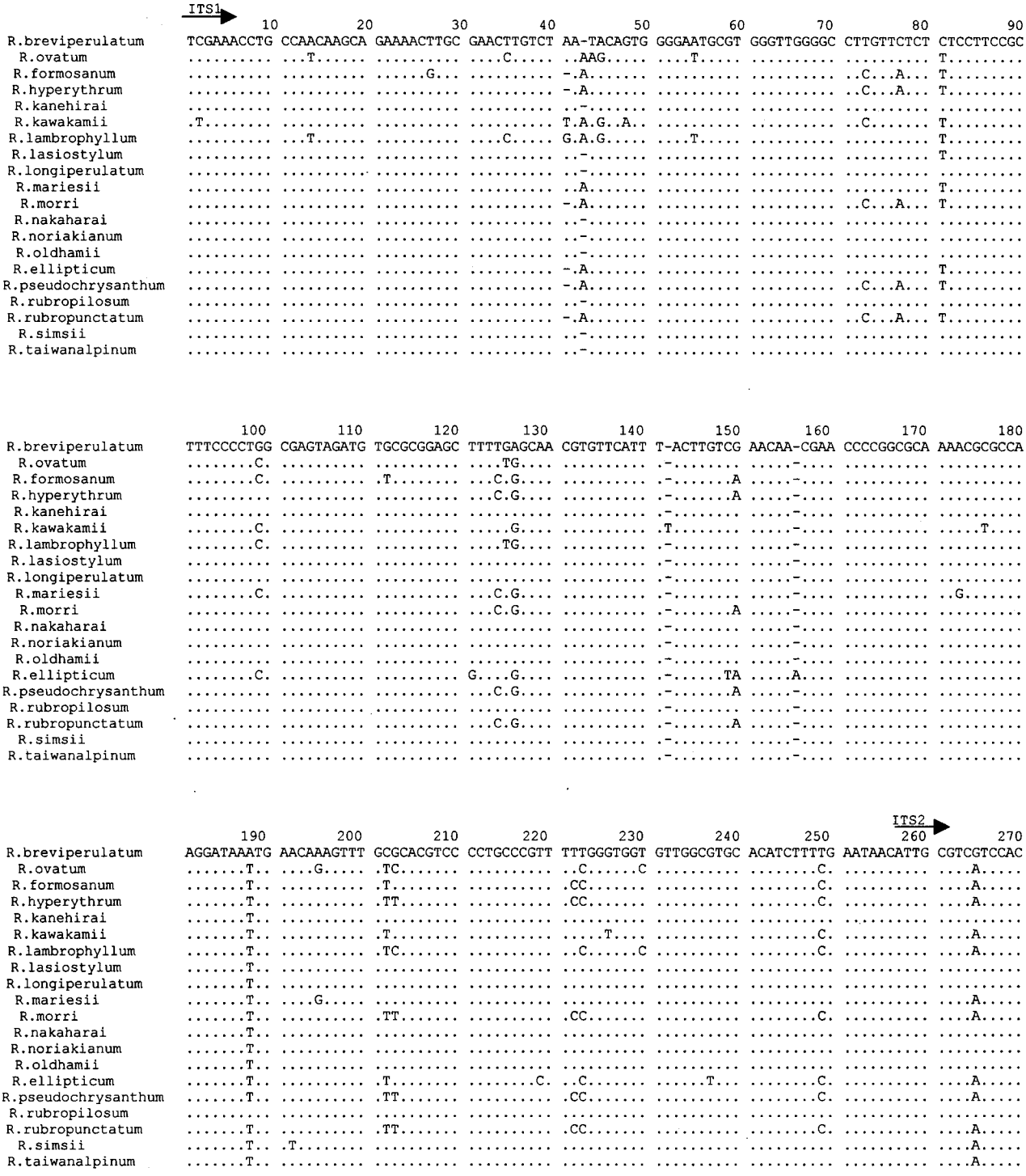


FIG. 1
The structure of ribosomal DNA. Positions of internal transcribed spacer (ITS) regions relative to 18S, and 26S rRNA genes and the intergenic spacer (IGS) are shown. The relative position of the primers used for PCR is indicated.

RESULTS AND DISCUSSION

Each PCR product of the 20 *Rhododendron* and one outgroup species, *Gaultheria itoana*, was separately analysed by agarose gel electrophoresis, and a single band appeared. Each PCR product was then taken for sequencing using PCR amplification primers. The nucleotide sequences among the 20 *Rhododendron* species in Taiwan were further determined. We found that they comprised a partial sequence of 70 bp for the 26S rRNA gene, 43 bp for the 18S rRNA gene, and a total sequence of 642~648 bp for the ITS region. The ITS sequences were composed of 253~255 bp for ITS1, 164 bp for the 5.8S rRNA gene, and 225~229 bp for ITS2. ITS sequences of *Rhododendron* in this study were aligned and submitted to GenBank (Table I). Among the 20 *Rhododendron* species, totally 72 polymorphic sites were found within ITS1 and ITS2, of which 37 bp were in ITS1 and 35 bp in ITS2 (Figure 2). The polymorphic sites in this study were higher than the variation of the ITS sequence in members of the section *Pentanthera* (genus *Rhododendron*), of which 38 polymorphic sites in total were found (Scheiber *et al.*, 2000). This may have resulted from samples used in the study



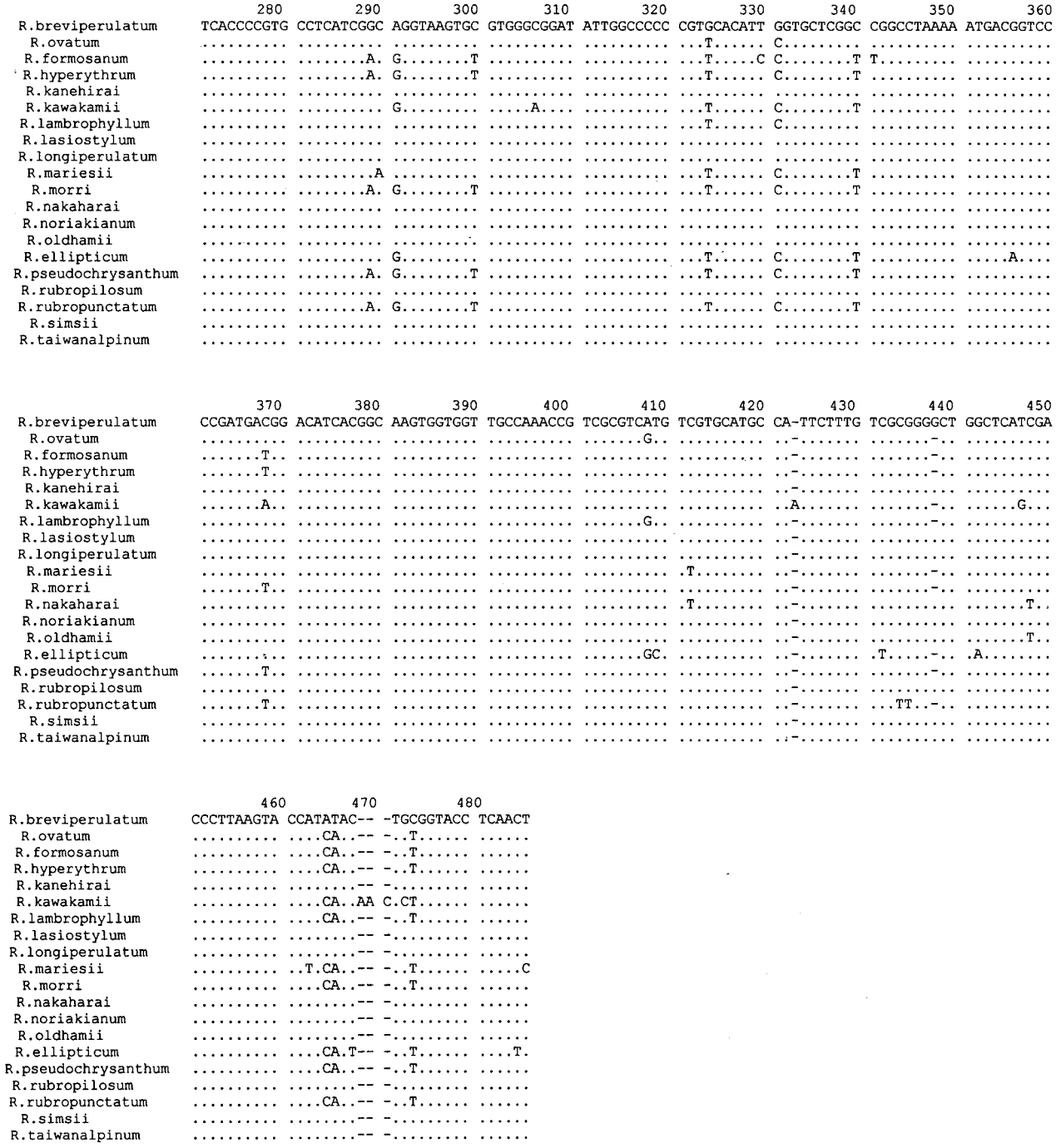


FIG. 2
Sequence comparison of the ITS1 and ITS2 regions from 20 taxa of the genus *Rhododendron* and *G. itoana* in Taiwan. Dots (.) indicate the same nucleotides, and gaps (-) are introduced to maximize homology.

being more diverse, and the group used including members of four subgenera of *Rhododendron*.

Aligned sequences of ITS1 and ITS2 from the 21 taxa resulted in 493 characters. A distance matrix of genetic divergence values among the 21 taxa in this study is given in Table II. Genetic distances among the 20 *Rhododendron* species are in the range from 0 to 0.062 as measured by the two-parameter method described by Kimura (1980). In comparison with other species, extremely high divergence values in the range of from 0.190 to 0.204 were found among species of the genus *Rhododendron* and the outgroup *G. itoana*. The highest genetic difference value of 0.204 was found between *G. itoana* and *R. ovatum* (Table II).

A dendrogram was obtained from ITS sequence comparisons. According to the dendrogram, the 20 *Rhododendron* species in Taiwan were grouped into six clusters. *Rhododendron olhamii*, *R. nakaharai*, *R. taiwanalpinum*, *R. simsii*, *R. lasiostylum*, *R. rubropilosum*, *R. breviperulatum*, *R. kanehirai*, and *R. noriakianum* were grouped with *R. longiperulatum* in cluster I. *Rhododendron lamprophyllum* was grouped with *R. ovatum* in cluster II. *Rhododendron pseudochrysanthum*, *R. morri*, *R. hyperythrum*, and *R. rubropunctatum* were grouped with *R. formosanum* in

TABLE II
Genetic distance of the ITS sequence among 20 species of the genus Rhododendron *plus* Gaultheria itoana *according to the two-parameter method of Kimura*

	R. formosa-mum	*R. hypery-thrum*	*R. morri*	*R. pseudo-chrysan-thum*	*R. rubro-punctac-tum*	*R. breviper-ulatum*	*R. kanehirai*	*R. lasiosty-lum*	*R. longiper-ulatum*	*R. naka-harai*	*R. noriakia-mum*	*R. oldhamii*	*R. rubropil-soum*	*R. simsii*	*R. taiwanal-pinum*	*R. mariesii*	*R. lampro-phyllum*	*R. ovatum*	*R. ellipticum*	*R. kawaka-mii*
R. formosomum																				
R. hyperythrum	0.013																			
R. morri	0.013	0.000																		
R. pseudochrysanthum	0.013	0.000	0.000																	
R. rubropunctatum	0.017	0.004	0.004	0.004																
R. breviperulatum	0.059	0.050	0.050	0.050	0.054															
R. kanehirai	0.057	0.048	0.048	0.048	0.052	0.002														
R. lasiostylum	0.054	0.046	0.046	0.046	0.050	0.004	0.002													
R. longiperulatum	0.057	0.048	0.048	0.048	0.052	0.002	0.000	0.002												
R. nakaharai	0.061	0.052	0.052	0.052	0.057	0.006	0.004	0.006	0.004											
R. noriakianum	0.057	0.048	0.048	0.048	0.052	0.002	0.000	0.002	0.000	0.004										
R. oldhamii	0.059	0.050	0.050	0.050	0.055	0.004	0.002	0.004	0.002	0.002	0.002									
R. rubropilosum	0.059	0.050	0.050	0.050	0.054	0.000	0.002	0.004	0.002	0.006	0.002	0.004								
R. simsii	0.057	0.048	0.048	0.048	0.052	0.006	0.004	0.006	0.004	0.004	0.008	0.004	0.006							
R. taiwanalpinum	0.054	0.046	0.046	0.046	0.050	0.004	0.002	0.004	0.002	0.006	0.002	0.004	0.004	0.002						
R. mariesii	0.046	0.041	0.041	0.041	0.046	0.039	0.037	0.034	0.037	0.037	0.037	0.039	0.039	0.036	0.034					
R. lamprophyllum	0.048	0.041	0.041	0.041	0.046	0.045	0.043	0.041	0.043	0.048	0.043	0.045	0.045	0.043	0.041	0.043				
R. ovatum	0.052	0.046	0.046	0.046	0.050	0.050	0.048	0.045	0.048	0.052	0.048	0.050	0.050	0.048	0.045	0.041	0.006			
R. ellipticum	0.048	0.043	0.043	0.043	0.048	0.059	0.057	0.055	0.057	0.062	0.057	0.059	0.059	0.057	0.055	0.050	0.043	0.048		
R. kawakami	0.043	0.039	0.039	0.039	0.043	0.052	0.050	0.048	0.050	0.054	0.050	0.052	0.052	0.050	0.048	0.045	0.043	0.047	0.050	
G. itiona	0.193	0.196	0.196	0.196	0.201	0.195	0.193	0.196	0.193	0.198	0.193	0.196	0.195	0.192	0.190	0.195	0.201	0.204	0.202	0.203

cluster III. In addition, *R. mariesii*, *R. ellipticum*, and *R. kawakamii* formed three independent clusters (Figure 3). Based on the systematics of *Rhododendron* (Yang *et al*., 1999; He *et al*., 1999), these 20 *Rhododendron* species in Taiwan were classified into four subgenera, of which *R. kawakamii* belongs to the subgenus *Rhododendron; R. lamprophyllum*, *R. ovatum*, and *R. ellipticum* belong to the subgenus *Azaleastrum; R. hyperythrum*, *R. morri*, *R. formosanum*, *R. pseudochrysanthum*, and *R. rubropunctatum* belong to the subgenus *Hymenanthes; and R. lasiostylum*, *R. rubropilosum*, *R. kanehirai*, *R. breviperulatum*, *R. oldhamii*, *R. noriakianum*, *R. nakaharai*, *R. taiwanalpinum*, *R. longiperulatum*, *R. simsii*, and *R. mariesii* belong to the subgenus *Tsutsusi. Rhododendron ellipticum* is separated from the other species and is closer to cluster II (including *R. lamprophyllum* and *R. ovatum*). This result supports *R. ellipticum* (section *Choniastrum*) and cluster II species (section *Azaleastrum*) all being under the subgenus *Azaleastrum.* In addition, *R. mariesii* (section *Brachycalyx*) is another unique species and more closely aligned to cluster I (section *Tsutsusi*). Both of these groups are under the subgenus *Tsutsusi* (Table I and Figure 3). Therefore, molecular data from ITS sequences in this study are in agreement with the traditional systematics of *Rhododendron* in Taiwan.

According to revisions of the genus *Rhododendron* in Taiwan described by Lu and Yang (1989) and Li *et al.* (1998), *R. hyperythrum*, *R. morri*, *R. rubropunctatum*, and *R. pseudochrysanthum* were combined into one species; *R. longiperulatum*, *R. simsii*, and *R. nakaharai* were combined into one species; and *R. breviperlatum* and *R. lasiostylum* were combined into one species. Basically, the findings of ITS sequence analysis support those revisions of the genus *Rhododendron* in Taiwan by Lu and Yang (1989) and Li *et al.* (1998). Molecular data from the *trn*F-*trn*L gene sequence of cpDNA also showed that *R. hyperythrum*, *R. morri*, *R. rubropunctatum*, and *R. pseudochrysanthum* should be combined into one species (Hwang and Hsu, 2001). Furthermore, from the phylogenetic tree, the genetic relationships among the ten *Rhododendron* species in cluster I (members of the section *Tsutsusi*) are very close, although these ten species of the section *Tsutsusi* in Taiwan still have questions involving their classification. Therefore, more species of the section *Tsutsusi* from other localities and more molecular data should be analysed to evaluate the genetic relationship within the section *Tsutsusi* in the future.

In conclusion, the genetic relationships of the 20 *Rhododendron* species in Taiwan could be established based on the sequence of the ITS of rDNA. The findings in this study agree with the traditional systematics of *Rhododendron* in Taiwan. In the future, the ITS sequence may be applied to evaluate the systematics of *Rhododendron* species in the world. Furthermore, this study also revealed that members of the section *Tsutsusi* of *Rhododendron* in Taiwan still have some questions at the species level.

We are grateful to Dr T. Y. Chou for his valuable comments and helpful discussions in the course of the study.

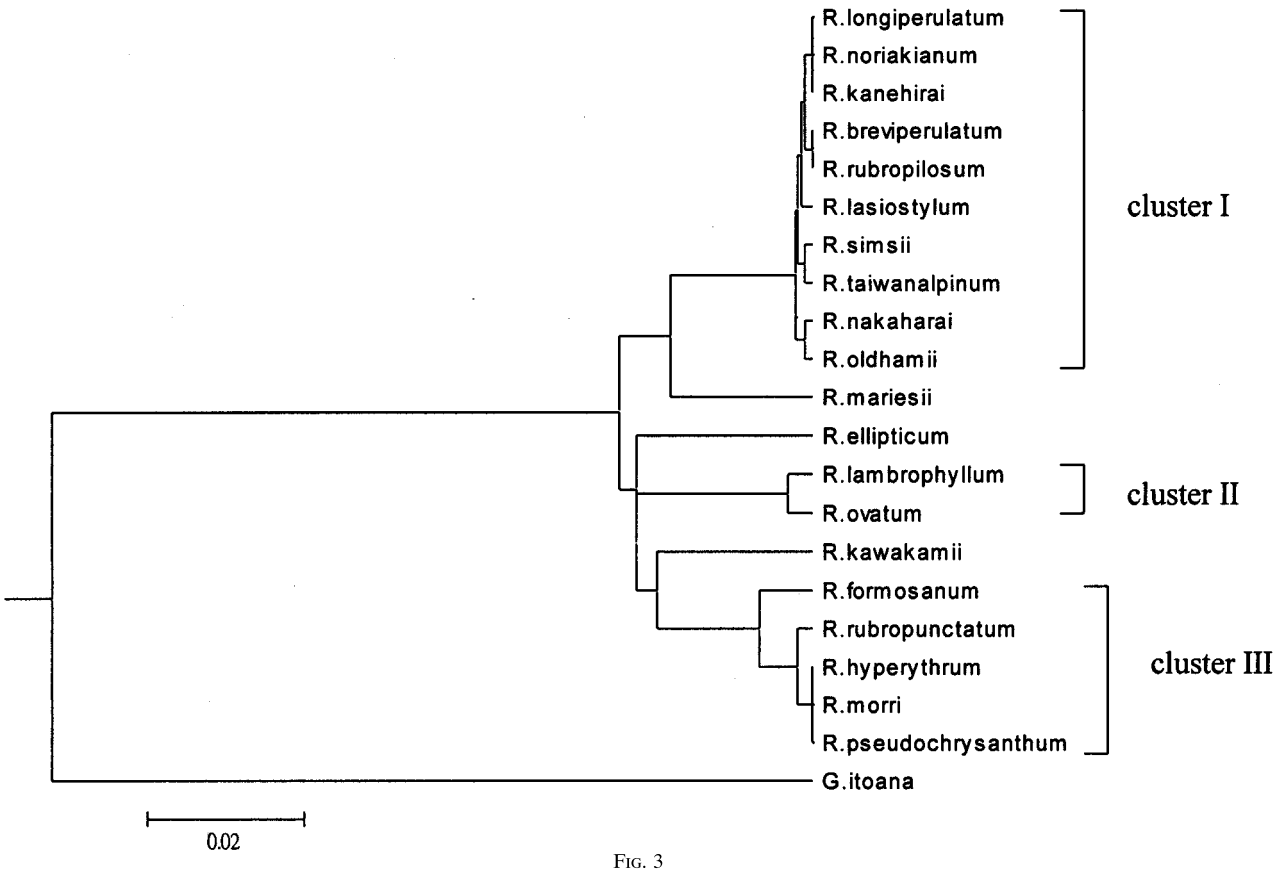


Fig. 3
A dendrogram of the 20 species of the genus *Rhododendron* in Taiwan obtained from sequence comparison of the ITS region of rDNA.

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