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MOLECULAR SYSTEMATICS OF *RHODODENDRON* SUBGENUS *TSUTSUSI* (*RHODOREAE*, *ERICOIDEAE*, *ERICACEAE*)

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Rhododendron subgenus *Tsutsusi* (*Rhodoreae*, *Ericoideae*, *Ericaceae*), commonly known as evergreen azaleas, includes approximately 117 deciduous and evergreen species from Japan, China and northeastern Asia. Subgenus *Tsutsusi* has been divided into two sections, *Brachycalyx* and *Tsutsusi*, based on characteristics of the leaves, young twigs and corolla. We obtained molecular data from three chloroplast (*matK*, *ndhF* and *trnS-trnG*) and two nuclear (nrITS and the third intron of *rpb2*) regions for 30 species of *Rhododendron* subgenus *Tsutsusi* and five species of *Menziesia*. Parsimony, Bayesian and likelihood analyses based on total evidence were used to assess the monophyly of the sections within *Rhododendron* subgenus *Tsutsusi* and relationships among the species sampled. In particular, the placement of the problematic *Rhododendron tashiroi* was addressed. Results support *Rhododendron tashiroi* as a member of the *Rhododendron* section *Brachycalyx* clade. Molecular evidence also supports a clade within *Rhododendron* section *Tsutsusi* containing *R. indicum*, *R. tsusiophyllum*, *R. tschonoskii* and *R. serpyllifolium*, species which were not previously considered closely related.

Keywords. ITS, *matK*, phylogeny, *Rhododendron*, *Tsutsusi*.

Introduction

The evergreen azaleas (*Rhododendron* subgenus *Tsutsusi*; *Rhodoreae*, *Ericoideae*, *Ericaceae*) are among the most popular of the cultivated azaleas. Native to Asia, many species of *Tsutsusi* azaleas were cultivated in China and Japan long before collectors such as Robert Fortune and Carl Maximowicz introduced them to European gardens (Wilson, 1921). Today the evergreen azaleas are a major component of the florist and nursery trades, being grown as landscape plants in areas with warm climates, bonsai, and potted plants. *Rhododendron* subgenus *Tsutsusi* is one of several (the number varies depending on the author) subgenera currently recognised within the genus *Rhododendron* by Chamberlain *et al.* (1996; Table 1). This group of azaleas can be recognised by the presence of terminal buds that contain both floral and vegetative shoots. Flattened ferrugineous hairs and/or pseudoverticillate leaves that are rhombic in outline are also common in some members of the group.

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Tsutsusi azaleas occur in temperate and subtropical zones at a variety of elevations in Mainland China and Japan. A few species also occur in Korea, and in Burma, Arunchal-Pradesh (India), Laos and Thailand (Chamberlain & Rae, 1990).

Rhododendron subgenus *Tsutsusi* has consistently been considered a distinctive group because most members possess flattened multicellular hairs that are often ferrugineous. In some species these hairs cover the leaves and stems and give the plant an almost coppery sheen. Other species have less dense coverage with hairs found only in the axils of the leaves or at the base of the floral buds. Sleumer (1949) considered the inclusion of both vegetative and floral buds within the same shoot a significant feature, but this character has apparently evolved at least twice in *Rhododendron*, occurring also in *R. schlippenbachii* and in *R. quinquefolium* (Judd & Kron, 1995; Goetsch *et al.*, 2005). Neither of these two species possess other characteristics of *Rhododendron* subgenus *Tsutsusi* and were not included in this study.

Most authors have recognised two groups within *Rhododendron* subgenus *Tsutsusi* – section *Tsutsusi* and section *Brachycalyx* (Wilson, 1921; Chamberlain & Rae, 1990). *Rhododendron* section *Brachycalyx* is characterised in these treatments as possessing deciduous leaves that are rhombic in outline and crowded at the shoot apex, appearing verticillate (i.e. pseudoverticillate). Most of the recognised species in *Rhododendron* section *Brachycalyx* are reported to lack the characteristic flattened and ferrugineous multicellular hairs found in *Rhododendron* section *Tsutsusi*. However, herbarium specimens of *Rhododendron weyrichii*, *R. wadanum*, etc. refute this as these hairs are present in the axils of leaves and flowers or flower buds in all specimens examined. Of the approximately 15–16 species currently recognised in *Rhododendron* section *Brachycalyx*, only *R. farrerae* and *R. mariesii* are native to China (*R. daiyunicum* also is reported from Mainland China but is known only from the type specimen) and not found in the Japanese archipelago.

Rhododendron section *Tsutsusi* is morphologically well defined by the distinctive hairs (but see above) and many species possess dimorphic leaves. In these taxa some leaves (usually the ones scattered along the stem) are annually deciduous whereas others (usually those crowded towards the stem apex) over winter and are retained on the plant for two seasons or more. Although occasionally *Rhododendron tashiroi* has been placed in *Rhododendron* section *Tsusiopsis*, as in the recent treatment in the Flora of China (He & Chamberlain, 2005), it was placed in *Rhododendron* section *Tsutsusi* by Chamberlain & Rae (1990) based on its possession of flattened brown hairs. Chamberlain & Rae (1990) noted that the leaves of *Rhododendron tashiroi* are similar to those of *Rhododendron* section *Brachycalyx* (i.e. monomorphic and pseudoverticillate), making *R. tashiroi* an anomalous member of *Rhododendron* section *Tsutsusi*. Recently, *Rhododendron tashiroi* was placed in a clade with species from *Rhododendron* section *Brachycalyx* in a molecular phylogenetic analysis of *Rhododendron* based on *matK* sequence data (Kurashige *et al.*, 1998). *Rhododendron* subgenus *Tsutsusi* has consistently been recognised as a group but evolutionary relationships have rarely been addressed with more than a few species (Kurashige *et al.*, 1998; Kron *et al.*, 2002; Goetsch *et al.*, 2005). Other published studies of the

evergreen azaleas include revisions by Chamberlain & Rae (1990) and by Yamazuki (1996). Others have been floristic, descriptions of new species or studies of population-level variation. Molecular work on the *Tsutsusi* group (*Rhododendron* subgenus *Tsutsusi*) has been published regarding population-level variation in selected cultivars and in selections from the wild (e.g. De Riek *et al.*, 1999; Scariot *et al.*, 2007). Phylogenetic studies have been limited to a few representative species of *Rhododendron* subgenus *Tsutsusi* (Kurashige *et al.*, 1998; Kron *et al.*, 2002; Goetsch *et al.*, 2005) as part of a larger analysis of *Rhododendron* as a whole. This study tests evolutionary relationships in the *Tsutsusi* group using two nuclear and three chloroplast molecular markers.

Material and Methods

We used DNA sequence data from five regions, three chloroplast (*matK*, *ndhF* and *trnS-trnG*) and two nuclear (nrITS and the third intron of *rpb2*), in order to investigate relationships in *Rhododendron* subgenus *Tsutsusi* (Chamberlain & Rae, 1990; Chamberlain *et al.*, 1996) and the relationships of members representing *Rhododendron* sections *Brachycalyx* and *Tsutsusi*.

Taxon sampling

DNA sequence data were collected for 30 species of *Rhododendron* subgenus *Tsutsusi* and five species of *Menziesia* (Table 1). Previous analyses have shown *Rhododendron* subgenus *Tsutsusi* to be monophyletic (Kurashige *et al.*, 1998; Goetsch *et al.*, 2005) even though different taxa were used as representatives of the subgenus. This molecular evidence combined with the distinctive morphological features provide a well-supported argument for the monophyly of *Rhododendron* subgenus *Tsutsusi*. Therefore this study focused on the relationships within the *Tsutsusi* group.

Many of the species described in *Rhododendron* subgenus *Tsutsusi* are known from few specimens (Chamberlain & Rae, 1990), are not in cultivation or are otherwise difficult to obtain. Of the 76 described species listed in *Rhododendron* section *Tsutsusi* by Chamberlain & Rae (1990), 27 were known from nine or fewer localities within China and of these 13 were known only from a single locality (8 of these only from the type specimen). The Chamberlain *et al.* (1996) classification lists 22 recognised species in *Rhododendron* section *Brachycalyx* and 86 species in *Rhododendron* section *Tsutsusi* for a total of 108 described species. The more recent treatment of *Rhododendron* in the Flora of China (He & Chamberlain, 2005) indicates 85 recognised species in *Rhododendron* section *Tsutsusi* and 23 recognised species in *Rhododendron* section *Brachycalyx* (*R. tashiroi* is placed in a separate section – *Tsusiopsis*) for a total of 109 species. It should be noted that there is significant morphological diversity among the species of *Rhododendron* section *Tsutsusi* within Mainland China that is not represented in this study. Unfortunately this material was unavailable at the time of our investigation.

TABLE 1. Taxa sampled in the analysis of nrITS, *matK*, *ndhF*, *rpb2* intron3 (*rpb2i3*) and *trnS-G* (see text) sequence data for species of *Rhododendron* and *Menziesia*

Taxon	Geographic range	Herbarium and accession	GenBank Accession Number				
			nrITS	*matK*	*ndhF*	*rpb2i3*	*trnS-G*
Rhododendron L.							
Subgenus *Tsutsusi* (Sweet) Pojarkova Section *Brachycalyx* Sweet (6/15)							
Rhododendron dilatatum Miq.	Japan	E 1975-0766B	EU855852	EU855886	EU855942	EU855916	EU855971
R. farrerae Tate ex Sweet‡	China	RSF 78-037	EU855854	EU855888	EU855944	EU855917	EU855973
R. kiyosumense (Makino) Makino	Japan	RSF 77-027	EU855858	EU855892	EU855948	EU855921	EU855977
R. lagopus Nakai var. *lagopus*	Japan	RSF 03-432	EU855859	EU855893	EU855949	EU855922	EU855978
R. mayebarae Nakai & Hara	Japan	E 1995-0441A	EU855860	EU855895	EU855950	EU855923	EU855979
R. mariesii Hemsl. & E.H.Wilson	China	RSF 76-352	NA	AF454860	EU855950	NA	NA
R. reticulatum D.Don ex G.Don	Japan	E 1975-2245	EU855864	EU855899	EU855854	EU855926	EU855983
R. sanctum Nakai	Japan	RSF 73-250	EU855866	EU855900	EU855956	EU855927	NA
R. viscistylum Nakai	Japan	RSF 77-028	EU855876	EU855908	EU855963	EU855932	NA
R. wadanum Makino	Japan	E 1976-1072D	EU855877	EU855909	EU855964	EU855933	EU855989
R. weyrichii Maxim.	Japan	E 1994-2387A	EU855878	EU855910	EU855965	EU855934	EU855990
Subgenus *Tsutsusi* (Sweet) Pojarkova Section *Tsutsusi* Sweet (8/66)							
Rhododendron breviperulatum Hayata	Taiwan	RSF 82-088	EU855851	EU855885	EU855941	EU855915	EU855970
R. eriocarpum (Hayata) Nakai	Japan	E 1988-0983	EU855853	EU855887	EU855943	NA	EU855972
R. indicum (L.) Sweet‡	Japan	E 1995-1015A	EU855855	AB012747	EU855945	EU855918	EU855971
R. kaempferi Planch.	Japan/Korea	E 1976-1898	EU855856	U61350	EU855946	EU855919	EU855975
R. kiusianum Makino	Japan	RSF 79-059	EU855857	U61332	EU855947	EU855920	EU855976
R. mucronatum (Blume) G.Don var. *ripense* (Makino) E.H.Wilson	Japan	RSF 98-244	EU855861	EU855896	EU855951	NA	EU855980
R. nakaharae Hayata	Taiwan	RSF 74-85	EU855862	EU855897	EU855952	EU855924	EU855981
R. oldhamii Maxim.	Taiwan	E 1971-0104	EU855863	EU855898	EU855953	EU855925	EU855982

TABLE 1. (*Cont'd*)

R. rubropilosum Hayata	Taiwan	RSF 73-242	EU855865	NA	EU855955	NA	EU855984
R. scabrum G.Don	Japan	RSF 87-062	EU855867	EU855901	EU855957	NA	NA
R. serpyllifolium (A.Gray) Miq.	Japan	RSF 76-356	EU855868	EU855902	EU855958	NA	NA
R. simsii Planch.	China	NA	EU855869	AM296057	NA	NA	AB105288
R. stenopetalum (Hogg) Mabb.	Japan	RSF 37-34	EU855870	NA	NA	NA	NA
R. subsessile Rendle	Philippines	RSF 99-310	EU855871	EU855903	EU855959	NA	NA
R. tashiroi Maxim.	Japan	RSF – s.n.	EU855872	EU855904	NA	EU855928	EU855986
R. tosaense Makino	Japan	RSF 98-585	EU855873	EU855905	EU855960	EU855929	NA
R. tschonoskii Maxim.	S Korea, Japan, Kamchatka	E 1975-0766B	EU855874	EU855906	EU855961	EU855930	EU855987
R. tsusiophyllum Sugimoto	Japan	K 1985-4676	EU855875	U61357	EU855962	EU855931	EU855988
R. yedoense Maxim. var. *poukhanense* (H.Lév.) Nakai	Korea, Japan	E 1977-2159B	EU855879	EU855911	EU855966	EU855935	EU855991
Menziesia J.E.Smith*							
M. ciliicalyx (Miq.) Maxim.	Japan	E 1969-5350	EU855846	U61331	EU855936	EU855912	EU855967
M. ferruginea J.E.Smith	NW North America	UTW – Olmstead	EU855847	EU855937	EU855937	EU855913	EU855968
M. lasiophylla Nakai	Japan	RSF – s.n.	EU855848	EU855882	EU855938	NA	NA
M. pilosa (Michx.) Juss.	E North America	WFU – Thornton	EU855849	U61351	EU855939	EU855914	EU855969
M. purpurea Maxim.	Japan	E – s.n.	EU855850	EU855884	EU855940	NA	NA

Herbarium acronyms follow *Index Herbariorum*, except RSF = Rhododendron Species Foundation, and are followed by accession numbers. Asterisk indicates outgroup taxon. ‡ indicates type species for *Rhododendron* sections *Brachycalyx* and *Tsutsusi*. NA = not available.

We have sampled from both *Brachycalyx* and *Tsutsusi* groups based on morphological variation, geographic distribution, and the availability of reliably identified material. Additionally we took care to sample from material that could be verified as naturally occurring such as in the case of *Rhododendron yedoense* var. *poukhanense*, *R. mucronatum* var. *ripense*, and *R. lagopus* var. *lagopus*. Sampling from the *Brachycalyx* group included 11 species, two (of the four) from Mainland China (*Rhododendron farrerae*, *R. mariesii*) and one species that occurs in southern Korea and Japan (*R. weyrichii*). The remaining eight species sampled are endemic to Japan. Sampling within *Rhododendron* section *Tsutsusi* included 19 species: one endemic to the Philippines (*R. subsessile*), four species endemic to Taiwan (*R. breviperulatum*, *R. nakaharae*, *R. oldhamii* and *R. rubropilosum*), 11 species endemic to Japan, two species that occur in northern Japan and southern Korea (*R. tschonoskii*, *R. yedoense*), and the widespread *R. simsii*. Material for representatives of *Rhododendron* section *Tsutsusi* from mainland Asia was limited to *R. simsii* for this study. This species has a wide geographic range and occurs in China, northern Burma, Thailand, Laos, Taiwan and Japan. Examples of the range of morphological sampling included species with very small flowers as well as those with large ones, those species densely covered with flattened hairs and those sparsely covered, etc. Although the species sampling was not comprehensive it is sufficient to test the monophyly of the sections and for an initial investigation into relationships among species within *Rhododendron* subgenus *Tsutsusi*.

DNA extraction and sequencing

Fresh or silica gel-dried leaf tissue was broken up using liquid nitrogen and a mortar and pestle or a mini-beadbeater and 2.5 mm zirconia/silica beads (Biospec Products, Inc., Bartlesville, OK, USA; M. Kallersjö, Swedish Museum of Natural History, pers. comm.). Total genomic DNA was extracted using the Epicentre MasterPure Plant Leaf DNA Purification kit (Epicentre Technologies, Madison, WI, USA) or a modification of the CTAB method (Doyle & Doyle, 1987). Five DNA regions (6602 base pairs total) were amplified for this study: *matK*, *ndhF* and *trnS-trnG* from the chloroplast genome and nuclear ribosomal internal transcribed spacer (nrITS) and the third intron of *rpb2* (*rpb2i3*) from the nuclear genome. All DNA regions were amplified from the same accession for each species. PCR protocol for the ribosomal genes *trnS* and *trnG* spacer region follows Shaw *et al.* (2005), those for *ndhF*, *matK* and ITS follow Kron *et al.* (2002) and those for *rpb2i3* follow those from Goetsch *et al.* (2005). Products were cleaned using the Qiagen Qiaquick PCR Cleanup Kit (Qiagen, Santa Clarita, CA, USA) and sequenced on an Applied Biosystems Model 3100 Genetic Analyzer at the Wake Forest University DNA Sequencing and Gene Analysis Laboratory at the Wake Forest University School of Medicine. Sequences were edited using Sequencher 3.1.1 (Gene Codes Corp. Inc. 1998) and were aligned manually with MacClade 4.0 (Maddison & Maddison, 2000).

Twenty-nine *matK* sequences and all *rpb2i3*, *trnS-trnG*, *ndhF* and nrITS data were produced for this study (Table 1). Kron (1997) and Kurashige *et al.* (1998) have shown that *matK* is phylogenetically informative in the *Rhodoreae*. Kurashige *et al.* (1998) showed that *matK* contained sufficient phylogenetic signal to support the monophyly and sister relationship of *Rhododendron* subgenus *Tsutsusi* sections *Tsutsusi* and *Brachycalyx* (if the problematic *Rhododendron tashiroi* was excluded). *RPB2* encodes the second-largest subunit of nuclear RNA polymerase II (Sawadogo & Sentenac, 1990) and is involved in the transcription of protein encoding genes. Oxelman & Bremer (2000) found two paralogs of *RPB2*, *RPB2*-i and *RPB2*-d, in taxa from the *Gentianales*. Goetsch *et al.* (2005) used several portions of *rpb2* to investigate phylogenetic relationships within *Rhododendron*. For this study we used Benjamin Hall's primers (19F and 20R – unpublished, used with permission, B. Hall, University of Washington, Seattle, WA) to amplify *rpb2i3* and assess its phylogenetic utility within *Rhododendron* subgenus *Tsutsusi*. The *trnS-trnG* region is found in the large single-copy region of the chloroplast genome and includes the *trnS*^{GCU}-*trnG* intergenic spacer (exon 1 of *trnG*^{UCC}), and the *trnG*^{UCC} intron (Shaw *et al.*, 2005; shortened to *trnS-G* in this study). Shaw *et al.* (2005) showed that this region contained phylogenetically useful variation at the interspecific level and should be helpful in elucidating phylogenetic relationships among closely related taxa. This is the first study to assess the phylogenetic utility of *trnS-G* in the *Ericaceae*. Primers for *trnS-G* follow Shaw *et al.* (2005). The aligned length and number of informative characters for each data set are shown in Table 2.

Phylogenetic analyses

Phylogenetic analyses were conducted using PAUP* 4.0b8 (Swofford, 2002) and trees were rooted with *Menziesia ciliicalyx* based on previous studies that show

TABLE 2. Aligned length, number of phylogenetically informative characters, tree statistics and models used for phylogenetic analyses of *Rhododendron* subgenus *Tsutsusi*

DNA region	Aligned length (bp)	No. of phylogenetically informative characters (%)	CI	RI	MPT length	Model used in Bayesian and maximum likelihood analyses
matK	1254	21 (1.7)	0.92	0.99	24	TVM+I
nrITS	614	41 (6.7)	0.69	0.90	68	K80+G
ndhF	2081	57 (2.7)	0.74	0.90	88	HKY+G
rpb2i3	721	43 (5.6)	0.94	0.89	51	GTR+G
trnS-trnG	1502	53 (3.5)	0.61	0.91	88	GTR+I+G
Nuclear	1335	84 (6.3)	0.75	0.91	118	HKY+gamma
Chloroplast	4837	131 (2.7)	0.69	0.88	204	GTR+gamma
Combined	6172	215 (3.5)	0.69	0.88	334	GTR+gamma

CI = consistency index; RI = retention index; MPT = most parsimonious tree.

a close relationship between *Rhododendron* subgenus *Tsutsusi* and *Menziesia* (Kron, 2003; Goetsch *et al.*, 2005). The five individual data sets, the chloroplast data partition, and the nuclear data partition were analysed using maximum parsimony, Bayesian analysis (MrBayes 3.2), and maximum likelihood (implemented in GARLi 0.951) (Table 2). Model estimates were performed in Modeltest 3.7. Trees were examined to look for well-supported topological incongruence (bootstrap $>$ 85%) among the data partitions. The nuclear and chloroplast data sets were each combined and analysed as above. Subsequently all five data partitions were then combined (Combined; Table 2) and analysed using parsimony, Bayesian and likelihood methods.

For all analyses characters were unordered and equally weighted, and gaps were treated as missing data. Relative clade support was assessed using 1000 heuristic bootstrap replicates (Felsenstein, 1985) or posterior probabilities (Bayesian analyses). Phylogenetically informative indels were scored (present/absent) and were mapped onto a single most parsimonious tree (MPT) obtained from the combined data analysis using MacClade 4.0 (Maddison & Maddison, 2000).

Results

The aligned length of the *matK* region is 1254 base pairs (bp) among the taxa [*Menziesia* (5 spp.) and *Rhododendron* subgenus *Tsutsusi* (30 spp.)] sampled and 1.7% of the sites are phylogenetically informative. This region contains the least percentage of potentially informative sites compared with the nuclear *rpb2i3* region at 5.6% and ITS at 6–7%. Variation in both the individual (Figs 1, 2) and combined analyses (Fig. 3) appear to be confined to the deeper nodes of the tree as none of them resolves the relationships of most of the species of *Rhododendron* subgenus *Tsutsusi*. Some of this lack of resolution may be due to missing data in some species of *Rhododendron* section *Tsutsusi* (5 species). Differences among several species in the intron of *rpb2i3* were so minor (1–2 bases) that *rpb2i3* was not sequenced for the remaining taxa.

Likewise, individual analyses of *matK*, *ndhF* and *trnS-G* data showed no topological conflict (trees not shown) and these data generally supported the results obtained from the nuclear data. There was no strongly supported conflict between the different chloroplast data partitions so all data were combined (Fig. 2). Maximum parsimony and maximum likelihood analyses of the combined chloroplast data partitions often resulted in low or no support for clades with at least some support in the nuclear data analyses (Fig. 2). This may be due to the lack of variation in the chloroplast regions used for this study. Bayesian posterior probability support was high for the *Tsutsusi* and *Brachycalyx* clades as found in the combined nuclear analyses (Fig. 1). Bayesian analysis also supported the clade containing *Rhododendron indicum*, *R. tsusiophyllum* and *R. tschonoskii* in agreement with the nuclear tree (Fig. 1). Both the combined nuclear data analyses and combined chloroplast analyses include the placement of *Rhododendron tashiroi* within the *Rhododendron* section *Brachycalyx* group. The clade containing members of *Rhododendron* section

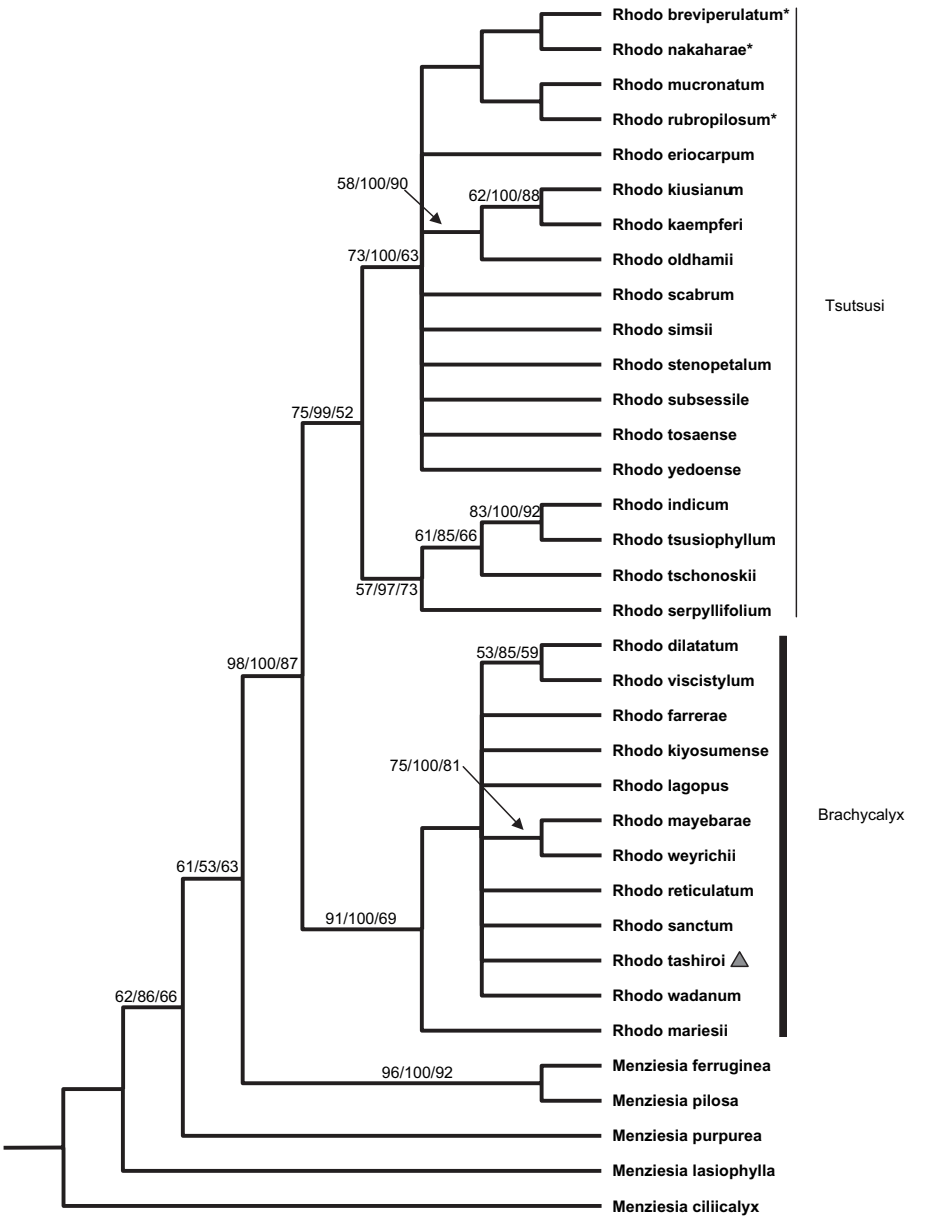

FIG. 1. Strict consensus of > 80,000 most parsimonious trees (L = 118, CI = 0.75, RI = 0.91) found in the maximum parsimony analysis of the combined data set (ITS, *rpb2i3*). Support values are shown above the branches in the following order: parsimony bootstrap (BS)/posterior probability (PP)/maximum likelihood bootstrap (ML). Terminal names are shortened to species in the case of *Rhododendron yedoense* var. *poukhanense*, *R. lagopus* var. *lagopus*, and *R. mucronatum* var. *ripense*. Tree is rooted with *Menziesia ciliicalyx*. Triangle = formerly placed in *Rhododendron* section *Tsutsusi*. Asterisk = Bayesian analysis placed these taxa in a clade but with very low posterior probability.

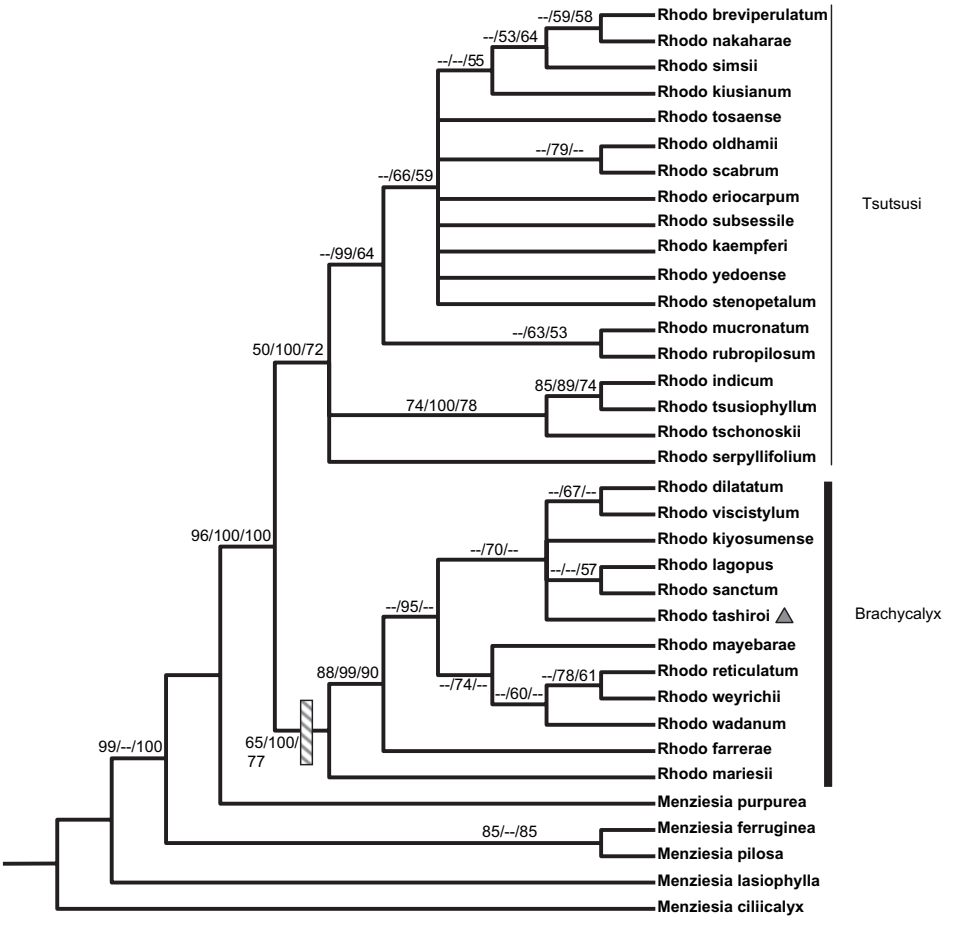

FIG. 2. Strict consensus of > 77,000 trees (L = 204, CI = 0.69, RI = 0.88) obtained in the maximum parsimony analysis of combined (*matK*, *ndhF*, *trnS-G*) chloroplast data. Support values as in Fig. 1 (BS/PP/ML). Terminal names are shortened to species in the case of *Rhododendron yedoense* var. *poukhanense*, *R. lagopus* var. *lagopus*, and *R. mucronatum* var. *ripense*. Tree is rooted with *Menziesia ciliicalyx*. Triangle = formerly placed in *Rhododendron* section *Tsutsusi*. Hatched bar = 17 base-pair insertion in *trnS-G* spacer.

Brachycalyx and *R. tashiroi* (also contains the type species of *Rhododendron* section *Brachycalyx*, *R. farrerae*) shows some structure with the two sampled Mainland China species falling at the deeper nodes within the group and the Japanese species placed in more derived positions within the group. Also within the *Brachycalyx* clade the *trnS-G* intergenic region has a 17-base insertion (11 bases in *Rhododendron weyrichii*) compared with the rest of *Rhododendron* subgenus *Tsutsusi* and *Menziesia*. This insertion can be mapped onto the branch leading to *Rhododendron mariesii* at the base of the *Brachycalyx* clade (Figs 2, 3). The insertion is present in all sampled members of *Rhododendron* section *Brachycalyx* including *R. tashiroi*. [Due to

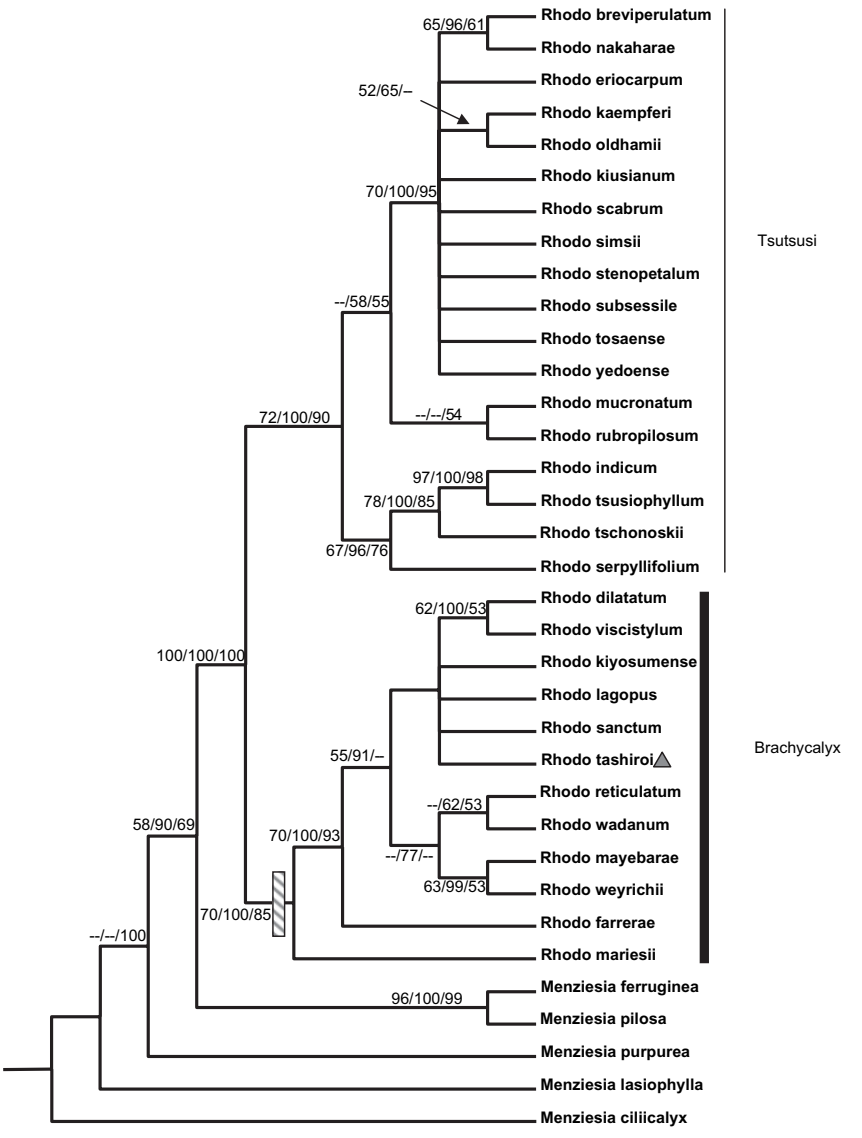

FIG. 3. Strict consensus of > 46,000 trees (L = 334, CI = 0.69, RI = 0.88) obtained in the maximum parsimony analysis of combined chloroplast and nuclear data (*matK*, *ndhF*, *trnS-G*, ITS, *rpb2i3*). Support values as in Fig. 1 (BS/PP/ML). Terminal names are shortened to species in the case of *Rhododendron yedoense* var. *poukhanense*, *R. lagopus* var. *lagopus*, and *R. mucronatum* var. *ripense*. Tree is rooted with *Menziesia ciliicalyx*. Triangle = formerly placed in *Rhododendron* section *Tsutsusi*. Hatched bar = 17 base-pair insertion in *trnS-G* spacer.

technical difficulties we were not able to completely sequence through the *trnS-G* region of *Rhododendron viscistylum* and *R. sanctum* to verify the possession or lack of this insertion, but predict its presence in these species.]

Phylogenetic relationships

Rhododendron subgenus *Tsutsusi* is supported as monophyletic (Fig. 1). Within *Rhododendron* subgenus *Tsutsusi* neither sections *Tsutsusi* nor *Brachycalyx*, as currently recognised, are monophyletic. This is due to the position of *Rhododendron tashiroi*, currently placed in *Rhododendron* section *Tsutsusi* by Chamberlain & Rae (1990), which is strongly supported (100%) as a member of *Rhododendron* section *Brachycalyx* (Figs 1, 3) in this study. *Rhododendron tashiroi* is closely related to species from *Rhododendron* section *Brachycalyx* in both the chloroplast and nuclear analyses and is not closely related to other taxa from *Rhododendron* section *Tsutsusi* sampled in any phylogenetic analysis conducted. Within *Rhododendron* section *Brachycalyx*, *R. tashiroi* is placed within a large polytomy that includes a few moderately supported pairs of species. These species pairs are *Rhododendron mayebarae* and *R. weyrichii*, as sister to *R. wadanum* and *R. reticulatum* (Fig. 3). *Rhododendron dilatatum* and *R. viscistylum* also form a moderately supported clade (Fig. 3). Within *Rhododendron* section *Tsutsusi* (Fig. 3), two clades are indicated. *Rhododendron serpyllifolium* is sister to *R. tschonoskii*, plus *R. indicum* plus *R. tsusiophyllum*. These relationships are well supported especially by Bayesian and maximum likelihood results. Another well-supported clade within *Rhododendron* section *Tsutsusi* contains 12 of the 18 species sampled in *Rhododendron* section *Tsutsusi*. However, of these 12 species only four show minor resolution: *Rhododendron breviperulatum* sister to *R. nakaharae* and *R. kaempferi* sister to *R. oldhamii*. The remaining eight species are unresolved with respect to each other and to the two clades mentioned above. For each parsimony analysis (individual or combined) thousands of trees were obtained and searches were stopped due to lack of memory. Random inspection of individual trees from each of these analyses showed trees with branch lengths of one or zero among terminals within the majority of *Tsutsusi* clade taxa (excluding *Rhododendron serpyllifolium*, *R. tschonoskii*, *R. tsusiophyllum* and *R. indicum*) and very short branch lengths among the *Brachycalyx* representatives as well.

Discussion

Although each section within *Rhododendron* subgenus *Tsutsusi* is well supported as a clade (as above), within each section relationships are not well supported. The lack of resolution at the terminal nodes of the trees obtained may be due to lack of variation in the genes or regions chosen, but it is also possible that many of the species represented in this study are very closely related. In the revision of *Rhododendron* subgenus *Tsutsusi* Chamberlain & Rae (1990) noted only slight differences in morphology between numerous species. More recent studies of *Tsutsusi* azaleas have addressed variations in morphology through investigations of the leaf epidermis (Wang Yu-Guo *et al.*, 2007) and indumentum (Jin *et al.*, 2007). Jin *et al.* (2007) also used style indumentum, anther length and indumentum and whether or not the lateral veins of the adaxial leaf surface were obvious or obscure as

taxonomic indicators of relationship. In most of these studies many character differences were either continuously varying among the species sampled or only slightly different between recognised taxa. Scariot *et al.* (2007) addressed relationships using DNA fingerprinting techniques among a selected group of horticultural forms within *Rhododendron* subgenus *Tsutsusi*. This included some selections of known hybrid origin and some listed only under a species name. Using data from AFLP, STMS and EST markers their results placed *Rhododendron tashiroi* in the *Rhododendron* section *Brachycalyx* clade with *R. mariesii*. Even with these fingerprinting data, branch lengths among exemplars (terminals) used in the Scariot *et al.* (2007) study were not very long. However, the Scariot *et al.* (2007) study was limited in its sampling of (assumed) naturally occurring species with many terminals representing selected cultivated varieties from possibly a single species or hybrid. The systematics of *Rhododendron* subgenus *Tsutsusi* may thus be complicated by its long history of horticulture, especially in Japan. Many forms and varieties have been given 'legitimate' names that are only later found to be of hybrid origin. An example of this is *Rhododendron yedoense*. *Rhododendron yedoense* is recognised in two varieties: *R. yedoense* var. *yedoense* and *R. yedoense* var. *poukhanense*. In this example, the second name is native to southern Korea, but the first is of cultivated origin. Therefore, caution must be exercised when obtaining material for DNA studies and identification should be verified by an expert before proceeding. Even with these precautions many very old cultivated varieties undoubtedly exist within the *Tsutsusi* group and as the Scariot *et al.* (2007) study shows, numerous lines of data will be necessary before relationships among this complex group can be fully resolved.

In this study we sampled four species native to Taiwan (*Rhododendron breviperulatum*, *R. nakaharae*, *R. oldhamii* and *R. rubropilosum*). *Rhododendron breviperulatum* and *R. nakaharae* are likely closely related and this relationship is moderately supported by the combined evidence (Fig. 3). Chamberlain & Rae (1990) consider *Rhododendron oldhamii* to be an isolated species with no close relatives within *Rhododendron* section *Tsutsusi*. *Rhododendron oldhamii* has spreading hairs, a large corolla, and 8–10 stamens. This combination of characteristics is found in only one other species within *Rhododendron* section *Tsutsusi*, *R. macrosepalum* (not available for this study) that is native to the Japanese islands of Honshu and Kikoshu. Chamberlain & Rae (1990) consider *Rhododendron kaempferi* to be more closely related to *R. indicum* and possibly conspecific with it. The results of this study do not support such a relationship. *Rhododendron indicum* is sister to *R. tsusiophyllum* and this clade is sister to *R. tschonoskii*. These relationships indicate that often neither flower colour, corolla size, nor whether leaves are mono- or dimorphic, appear to be very reliable as phylogenetic characters within *Rhododendron* section *Tsutsusi*. However, the taxon sampling in this study is primarily of Japanese species. A more comprehensive study of species relationships requires better sampling of the 62 species in Mainland China within the section. In the *Brachycalyx* clade, *Rhododendron mariesii*, then *R. farrerae*, branch sequentially to remaining members of the section sampled. *Rhododendron mariesii* is a shrub or small tree and *R. farrerae* is a dwarf shrub. Both are widespread

and represent the only members (with the exception of *Rhododendron daiyunicum* which is known only from the type) of *Rhododendron* section *Brachycalyx* that occur in Mainland China (Chamberlain & Rae, 1990). The rest of the species recognised within *Rhododendron* section *Brachycalyx* are Japanese. *Rhododendron dilatatum* is the only species within *Rhododendron* section *Brachycalyx* that possesses five stamens instead of the usual 10. The relationships among the sampled members of *Rhododendron* section *Brachycalyx* in the study are not strongly supported. Only *Rhododendron mayebarae* and *R. weyrichii* are moderately supported as sister taxa, but in Chamberlain & Rae's (1990) revision these two species were not considered closely related. The placement of *Rhododendron tashiroi* within *Rhododendron* section *Brachycalyx* in this study supports the results of Kurashige *et al.* (1998) and Scariot *et al.* (2007), but not Chamberlain & Rae's (1990) decision to put *R. tashiroi* in *Rhododendron* section *Tsutsusi*. The presence of a 17-base insertion in the *trnS-G-G* sequence of *Rhododendron tashiroi* also supports the placement of this species within *Rhododendron* section *Brachycalyx*. Based on the evidence of this study *Rhododendron tashiroi* should likely be placed in *Rhododendron* subgenus *Tsutsusi* section *Brachycalyx*. However, additional sampling from plants of Mainland China will undoubtedly increase our understanding of the evolution of the evergreen azaleas.

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