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A SCANNING ELECTRON MICROSCOPE SURVEY OF VIREYA RHODODENDRONS
I: Preparation techniques

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ABSTRACT. The preparation of herbarium and fresh leaf material of species of *Rhododendron* section *Vireya* (Ericaceae) for scanning electron microscopy is described. Herbarium material was found to be comparatively easier to prepare than fresh material which required long exposures to osmium tetroxide vapour to ensure good results.

INTRODUCTION

There are c.300 species of Vireya rhododendrons which represent almost a third of the genus. They are tropical species occurring in SE Asia, mainly in the Malesian region and are characterized by their long-tailed seeds and the scales on the undersurface of their leaves. The scales which are important in subsectional classification are multicellular epidermal trichomes consisting of a central area and a marginal flange, and may be either sessile or stipitate. Vireya rhododendrons are divided into two groups (Argent, 1989): scales of those in subsection *Vireya* with a small point-like centre and of those in subsection *Pseudovireya* with a large cushion-like centre.

The Royal Botanic Garden Edinburgh has one of the largest collections of Vireya rhododendrons in cultivation and in addition houses a comprehensive herbarium collection. With these valuable resources to hand an investigation of Vireya rhododendron scales was initiated in 1980 using a scanning electron microscope (SEM). The investigation had originally concentrated on Bornean species, but was widened to include species from New Guinea and other areas of Malesia. The first micrographs showed evidence of charging and dust contamination and further study was required to eliminate these problems.

The aim of the present study was to prepare specimens in a way that they could be examined with the SEM without showing signs of charging, distortion or any other damage or artefact. Preliminary experiments with rhododendron leaf scales showed that fixation techniques involving solutions of osmium tetroxide (OsO_4) resulted in excessive damage to their delicate structure (Helfer & Warwick, 1989) and as a result OsO_4 fumigation rather than solution was chosen for use with fresh material. This technique has already been reported for the preparation of fungal specimens (Dowsett *et al.*, 1977; Quattlebaum & Carner, 1980; Elad, 1988; Weidenbörner *et al.*, 1989).

MATERIALS AND METHODS

Herbarium specimens: A mature leaf was removed from herbarium specimens and a c.1cm^2 portion cut from about halfway down the leaf close to the mid-vein. Whenever possible it was taken from the flattest area where no lateral veins were present. Loose dust was removed with an airblower taking care not to damage or remove the often fragile scales. The sections were mounted on electron microscope stubs using double sided adhesive tape and colloidal silver. The specimens examined are listed in the Appendix (p. 373).

Specimens which showed considerable dust contamination (mainly due to lengthy herbarium storage) required pretreatment prior to mounting. They were cleaned by immersion in either water, ethanol or acetone in an ultrasonic cleaner for one minute. The results of these three pretreatments are listed in Table 1.

The only other pretreatment required was to remove gum from a specimen which had been accidentally coated and three procedures were tried with varying success (see results below): portions immersed in boiling water for 30sec; portions immersed in boiling water for 1min.; portions immersed in water of c.40°C for 2min.

All specimens were Au/Pd coated for 2min. at 20mA except those species with thick coverings of dendroid scales or layers of large overlapping scales which required 4min. coating at the same intensity. These coating times correspond to deposits of 12 and 24nm respectively and did not appreciably reduce the morphological detail seen at the magnifications used in this study

Fresh specimens: A mature leaf with no sign of pest or disease was chosen and a portion of c.1cm^2 was removed from an area similar to that described for herbarium specimens.

The following five treatments were used: i, freeze dried, Au/Pd coated for 2min. at 20mA; ii, freeze dried, Au/Pd coated for 4min. at 20mA; iii, freeze dried, OsO_4 fumigation for 6hr, Au/Pd coated for 2min. at 20mA; iv, freeze dried, OsO_4 fumigation for 6hr, Au/Pd coated for 4min. at 20mA; v, OsO_4 fumigation of hydrated specimens overnight (approximately 16hr), freeze dried, Au/Pd coated for 4min. at 20mA.

The results of these treatments are listed in Table 2.

Results and Discussion

Herbarium specimens (see Table 1): Of the 39 specimens treated with an air blower, 20 produced good results (clean surface without charging), eight produced adequate results although there was some evidence of dust contamination, two specimens (of *R. malayanum*) showed evidence of charging which was eliminated by a further 2min. sputter coating, and nine were too contaminated by dust to be acceptable. Specimens which required cleaning were most successful when treated with ethanol, although *R. rarilepidotum* and *R. sumatranum* lost many of their scales in the process. The use of acetone was unsuccessful as it did not clean the specimens and some species (*R. buxoides, R. nummatum, R. konori* and *R. yelliottii*) sustained considerable damage and distortion to their scales (see Figs 1–3).

To remove gum from dried specimens pretreatment with water at c.40°C was found to be most successful in dissolving the gum and preserving the specimen (Fig. 4).

Fresh specimens (see Table 2): Of the 62 specimens given treatment i, 32 produced good results, 14 produced adequate results, and 16 (comprising 13 species) produced evidence of charging. These 16 specimens were given a further 2min. sputter coating and all produced good or adequate results with the exception of *R. phaeochitum, R. durionifolium, R. beyerinkianum* and *R. malayanum* (Figs 5b & 6b). It was noted that the greatest degree of charging

TABLE 1.

Results of pretreatments on herbarium specimens

	Airblower	Water	Ethanol	Acetone
SUBSECTION VIREYA				
R. aequabile	++			
R. alborugosum	++			
R. aurigeranum	++			
R. bagobonum	+	+	++	–
R. beyerinkianum	++			
R. brookeanum var. cladotrichum	+	+	++	–
R. comptum	+			
R. comptum var. comptum	+			
R. comptum var. trichodes	++			
R. goodenoughii	++			
R. konori	+	+	++	–
R. pauciflorum	++			
R. phaeochitum	–	–	++	–
R. pleianthemum	++			
R. pubigermen	+	+	++	–
R. rarilepidotum	+	+	++	–
R. rubineiflorum	++	–	++	
R. ruttenii	–	–	++	–
R. sessilifolium	–	–	++	–
R. stenophyllum	++			
R. stevensianum	++			
R. sumatranum	+	–	++	–
R. sumatranum (scales removed)	++			
R. tuba	++			
R. yelliottii	–	–	–	–
SUBSECTION PSEUDOVIREYA				
R. acuminatum	++			
R. borneense subsp. villosum	++			
R. buxoides *Lamb* 209/85	–	–	++	–
R. buxoides *Arg. & Kerby* 832a	–	–	++	–
R. gaultheriifolium	–	+	++	–
R. malayanum C8798	+	+	++	–
R. malayanum *Comber et al.* 1	+	+	++	–
R. meliphagidum	++			
R. nummatum *Reeve* 6768	–	+	++	–
R. pulleanum	++			
R. quadrasianum var. rosmarinifolium	++			
R. retusum 67.2708	++			
R. retusum AND 467	++			
R. retusum *Comber et al.* 2	–	+	++	–

– denotes poor result; + denotes adequate result; ++ denotes good result

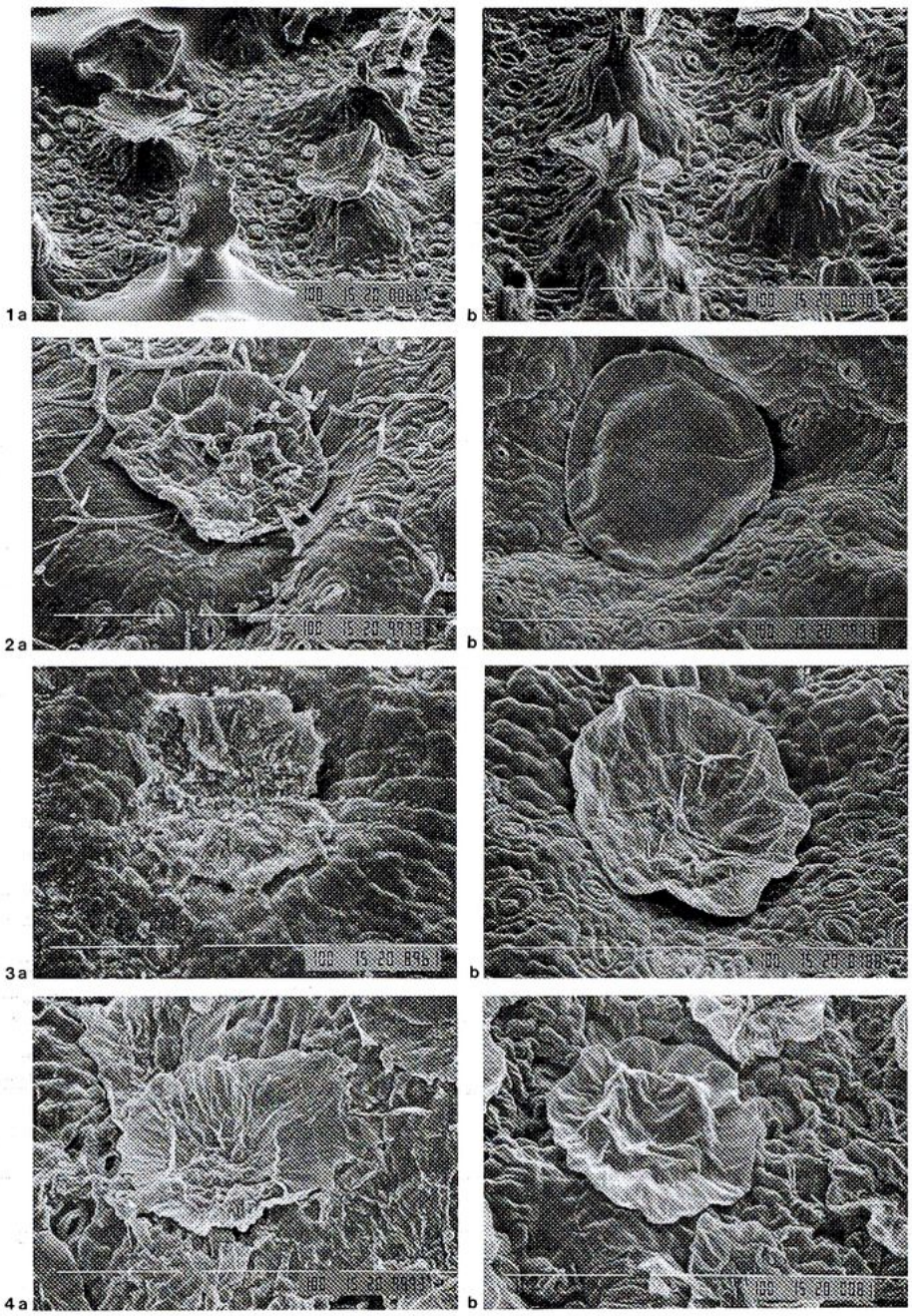
TABLE 2.

Results of treatments on fresh specimens

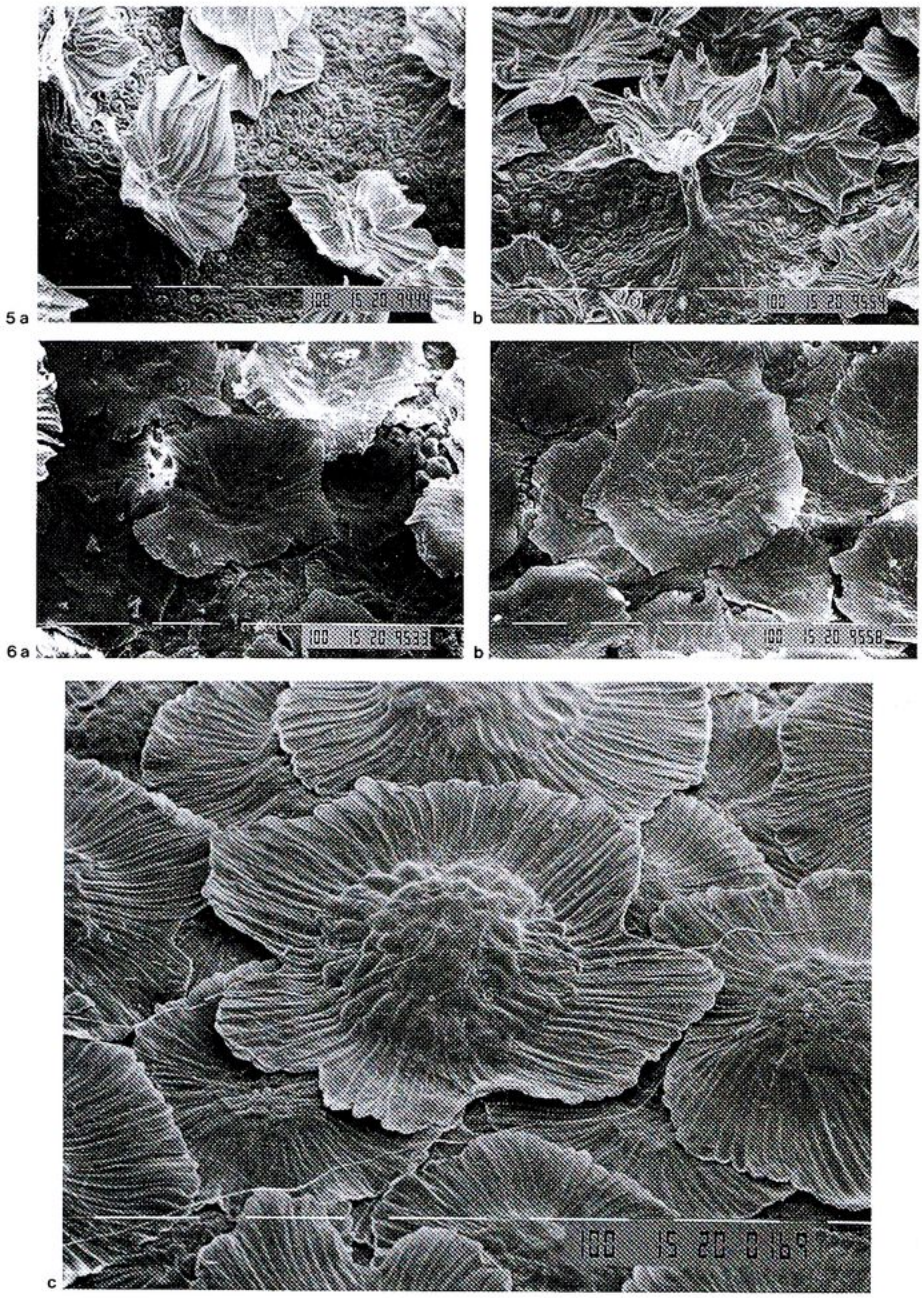
	i	ii	iii	iv	v
SUBSECTION VIREYA					
Rhododendron aequabile	–	+	+		++
R. album	–	+			++
R. album (scales removed)	++				
R. alticolum	++				
R. armittii	–	+	+		++
R. aurigeranum 83.2076	–	++			

R. aurigeranum 84.0612	–	++			
R. beyerinkianum	–	–		++	
R. blackii	++				
R. brookeanum 80.1291	+				
R. brookeanum 83.1219	–	++			
R. brookeanum 85.1901	–	++			
R. brookeanum var. gracile	–	++			
R. christiane 73.1628	++				
R. christiane 75.2617	++				
R. citrinum	++				
R. crassifolium	++				
R. cruttwellii	++				
R. exuberans	+	++			
R. goodenoughii	–	+		++	
R. javanicum	+	++			
R. konori	++				
R. leptanthum	++				
R. leucogigas	++				
R. longiflorum	++				
R. lowii	+				
R. macgregoriae 74.1767	+				
R. macgregoriae 61.4242	+			++	
R. maius	++				
R. moultonii	++				
R. multicolor	++				
R. orbiculatum	–	++			
R. pauciflorum	+				++
R. phaeochitum 65.0269	–	–		++	
R. phaeochitum 74.1770	–	–		++	
R. polyanthemum (juv. leaf)	+	++			
R. polyanthemum (mature leaf)	+	++			
R. pneumonanthum	++				
R. rarum 61.4142	++				
R. rarum 74.1174	++				
R. rhodoleucum	++				
R. robinsonii	+	++			
R. rugosum × buxifolium	++				
R. salicifolium	++				
R. scabridibracteum 74.1175	++				
R. scabridibracteum 74.1772	++				
R. stapfianum	++				
R. stenophyllum	++				
R. suaveolens	++				
R. tuba	+	+			++
SUBSECTION PSEUDOVIREYA					
R. durionifolium	–	–			
R. ericoides	++				
R. herzogii 61.4126	++				
R. herzogii 76.1321	++				
R. lineare	+				
R. malayanum 82.0746			+	+	++
R. malayanum 85.1900	–	–		+	
R. quadrasianum var. rosmarinifolium	++				
R. santapauii 83.0536	+				
R. santapauii 83.0996	++				
R. taiwanianum	++				
R. vaccinioides	–	+		++	

– denotes poor result; + denotes adequate result; ++ denotes good result



FIGS 1–4. FIG. 1. *Rhododendron konori*: a, cleaned in acetone; b, cleaned in ethanol. FIG. 2. *Rhododendron nummatum*: a, cleaned with air blower; b, cleaned in ethanol. FIG. 3. *Rhododendron retusum*: a, cleaned in water; b, cleaned in ethanol. FIG. 4. *Rhododendron yelliottii*: a, coated with gum; b, gum removed. Scale-bars = 0.1mm.



FIGS 5–6. FIG. 5. *Rhododendron beyerinkianum*: a, freeze dried, Au/Pd coated for 4min.; b, freeze dried, OsO_4 fumigation for 6hr, Au/Pd coated for 4min. FIG. 6. *Rhododendron malayanum*: a, freeze dried, OsO_4 fumigation for 6hr, Au/Pd coated for 2min.; b, freeze dried, OsO_4 fumigation for 6hr, Au/Pd coated for 4min.; c, OsO_4 fumigation overnight, freeze dried, Au/Pd coated for 4min. Scale-bars = 0.1mm.

occurred in species with dendroid or large overlapping scales (Figs 5a & 6a). *R. aequabile* and *R. aurigeranum* with treatment iii produced adequate micrographs but were most successful with treatment v. Species which had produced poor or adequate micrographs with treatment ii were additionally given treatment iv (Fig. 5b) which proved successful with the exception of *R. malayanum* (Fig. 6b)—the most difficult species of the fresh specimens used. In treatment v, which had a longer OsO_4 fumigation before the freeze drying process, all six species were very successful (Fig. 6c).

These results indicate that species with dendroid or large overlapping layers of scales are likely to show evidence of charging in the SEM unless they receive special treatment. This applies to both herbarium and fresh specimens.

When adequately clean, a herbarium specimen requires 4min. sputter coating to eliminate charging, whereas freeze-dried specimens may require treatment with OsO_4. Species with dendroid scales when freeze dried, treated for 6hr with OsO_4 and sputter coated for 4min. are likely to produce good micrographs, but those with layers of large scales require a longer OsO_4 treatment before the freeze drying process and 4min. sputter coating. Species with other types of scales are likely to produce good micrographs after simply freeze drying and sputter coating for 2 or 4min.

As a result of the problems of excessive contamination with dust and gum on herbarium specimens encountered in this investigation the current practice of herbarium storage for Vireya rhododendrons at Edinburgh is now to put a quantity of suitable leaf material in a separate cellophane packet before mounting.

Scanning electron micrographs provide a convenient visual record of the scale structure, density and distribution on a leaf. This is invaluable to the taxonomy of Vireya rhododendrons since at the low magnifications used the pictures can be matched with observations using a hand lens or binocular dissection microscope.

Few scanning electron micrographs have been used in publications on Vireya rhododendrons. Spady & Averill (1984) produced 14 micrographs which were prepared 'to satisfy our own curiosity' though they doubted whether they would add much to scientific studies. Argent *et al.* (1988: 118–142) used micrographs to more fully illustrate new information about Bornean rhododendrons although a number of the micrographs published there showed the typical signs of charging encountered with inadequate preparation. Argent (1989) expands on the classification of Vireya rhododendrons using micrographs of both leaf and bud scales as a significant tool to demonstrate surface morphology. Philipson & Philipson (1975) illustrated their revision of *Rhododendron* section *Lapponicum* with micrographs of lower leaf surfaces, and H. B. Yang (1984) also made use of micrographs in her research into *Rhododendron* section *Pogonanthum* in China.

Conclusion

The problems encountered in preparing Vireya rhododendrons for examination in a SEM can usually be simply overcome. The species which are particularly difficult, usually those with dendroid scales or layers of large overlapping scales, can be prepared more easily and quickly from herbarium

specimens than from fresh material. This eliminates both their long exposure to OsO_4 vapour, which is a very hazardous chemical, and the freeze drying process. As there seems to be no significant loss of detail in using only herbarium specimens we recommend their use when examining the more difficult species of Vireya rhododendrons.

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Appendix

Species of *Rhododendron* section *Vireya* examined

Species:	Collector/ Accession no.:	Subsection after Argent (1989):
Herbarium Specimens		
R. acuminatum	*Arg.* 1602	Pseudovireya
R. aequabile	75.0002	Vireya
R. alborugosum	*Dransfield* 2910	Vireya
R. aurigeranum	66.1921	Vireya
R. bagobonum	C8796	Vireya
R. beyerinkianum	68.2536	Vireya
R. borneense subsp. villosum	*MW*/23	Pseudovireya
R. brookeanum var. cladotrichum	*Comber et al.* 3	Vireya
R. buxoides	*Lamb* 209/85	Pseudovireya
R. buxoides	*A & K* 832a	Pseudovireya
R. comptum	LAE 61425	Vireya
R. comptum var. comptum	*Kores* WE11568	Vireya
R. compton var. trichodes	LAE 61780	Vireya
R. gaultheriifolium	*Sandham* s.n.	Pseudovireya
R. goodenoughii	77.2400	Vireya
R. konori	75.0101	Vireya
R. malayanum	C8798	Pseudovireya
R. malayanum	*Comber et al.* 1	Pseudovireya
R. meliphagidum	C8797	Pseudovireya
R. nummatum	*Reeve* 6768	Pseudovireya
R. pauciflorum	75.0119	Vireya
R. phaeochitum	77.2884	Vireya
R. pleianthemum	*Reeve* 1278	Vireya
R. pubigermen	*Comber et al.* 4	Vireya
R. pulleanum	*Reeve* 1735	Pseudovireya
R. quadrasianum var. rosmarinifolium	83.0535	Pseudovireya
R. rarilepidotum	*Comber et al.* 5	Vireya
R. retusum	67.2708	Pseudovireya
R. retusum	AND 467	Pseudovireya
R. retusum	*Comber et al.* 2	Pseudovireya
R. rubineiflorum	*Reeve* 1352	Vireya
R. ruttenii	C8786	Vireya
R. sessilifolium	*Comber et al.* 6	Vireya
R. stenophyllum	67.2546	Vireya
R. stevensianum	*Reeve* 3483	Vireya
R. sumatranum	*Comber et al.* 7	Vireya
R. tuba	83.0538	Vireya
R. yelliottii	LAE 74824	Vireya
Fresh Specimens		
R. aequabile	75.0002	Vireya
R. album	88.2544	Vireya
R. alticolum	76.0988	Vireya
R. armittii	68.2068	Vireya
R. aurigeranum	83.2076	Vireya
R. aurigeranum	84.0612	Vireya
R. beyerinkianum	60.2616	Vireya
R. blackii	83.2074	Vireya
R. brookeanum	80.1291	Vireya
R. brookeanum	83.1219	Vireya
R. brookeanum	85.1901	Vireya
R. brookeanum var. gracile	85.1957	Vireya
R. christiane	73.1628	Vireya
R. christiane	75.2617	Vireya

R. citrinum	84.2321	Vireya
R. crassifolium	80.1206	Vireya
R. cruttwellii	65.0268	Vireya
R. durionifolium	77.2458	Pseudovireya
R. ericoides	87.1805	Pseudovireya
R. exuberans	84.1213	Vireya
R. goodenoughii	77.2400	Vireya
R. herzogii	61.4126	Pseudovireya
R. herzogii	76.1321	Pseudovireya
R. javanicum	68.0840	Vireya
R. konori	75.0101	Vireya
R. leptanthum	63.0476	Vireya
R. leucogigas	68.2431	Vireya
R. lineare	82.0847	Pseudovireya
R. longiflorum	80.1313	Vireya
R. lowii	82.0912	Vireya
R. macgregoriae	74.1767	Vireya
R. macgregoriae	61.4242	Vireya
R. maius	65.0267	Vireya
R. malayanum	82.0746	Pseudovireya
R. malayanum	85.1900	Pseudovireya
R. moultonii	78.1745	Vireya
R. multicolor	67.0827	Vireya
R. orbiculatum	80.1407	Vireya
R. pauciflorum	75.0119	Vireya
R. phaeochitum	65.0269	Vireya
R. phaeochitum	74.1770	Vireya
R. polyanthemum	78.0969	Vireya
R. pneumonanthum	67.2550	Vireya
R. quadrasianum var. rosmarinifolium	83.0535	Pseudovireya
R. rarum	61.4142	Vireya
R. rarum	74.1174	Vireya
R. rhodoleucum	75.0103	Vireya
R. robinsonii	73.1358	Vireya
R. rugosum × buxifolium	76.2796	Vireya
R. salicifolium	82.0723	Vireya
R. santapauii	83.0536	Pseudovireya
R. santapauii	83.0996	Pseudovireya
R. scabridibracteum	74.1175	Vireya
R. scabridibracteum	74.1772	Vireya
R. stapfianum	80.1292	Vireya
R. stenophyllum	80.1190	Vireya
R. suaveolens	79.2885	Vireya
R. taiwanianum	71.0098	Pseudovireya
R. tuba	83.0538	Vireya
R. vaccinioides	87.2104	Pseudovireya