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Phylogenetic relationships of *Rhododendron* section *Vireya* (Ericaceae) inferred from the ITS nrDNA region

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Abstract. *Rhododendron* L. taxonomy has been tested in recent times by molecular phylogenies based on several DNA regions. Most of these studies have aimed at higher-level relationships, despite the importance of lower ranks, such as sections, to most workers on the genus. Almost one-third of the species of *Rhododendron* are placed in one of the lepidote (scaly) sections, section *Vireya* (Blume) Copel.f. Results of phylogenetic analyses of the ITS region (ITS-1, 5.8S and ITS-2) for the genus *Rhododendron*, with sampling concentrated on section *Vireya*, are presented. The results of Bayesian and parsimony analyses were predominantly congruent. Subgenus *Rhododendron* is inferred to be monophyletic, while two of the three sections, *Rhododendron* and *Vireya*, are polyphyletic; the monophyly of section *Pogonanthum* Aitch. & Hemsl. was not tested in this study. Relationships between the species of section *Vireya* do not correspond to the traditional classification based on morphology, instead correlating strongly with geographic areas, with a disjunction between an Australian–New Guinea clade and clades of west and middle Malesian taxa. The phylogeny also indicates that the ITS region may not undergo complete homogenisation in all species of *Rhododendron*.

Introduction

Rhododendron L. is one of the most speciose genera of the family Ericaceae Juss. comprising over 1000 species (Chamberlain *et al*. 1996), differentiated from other genera by a combination of characters: inflorescences with scarious perulae, chromosome number of $x = 13$, fruit with a septicidal capsule, ovary superior or nearly so, stamens without appendages, and agglutinate pollen (Sleumer 1966; Stevens 1971; Kores and van Royen 1982; Kron and Judd 1990). The genus is widely distributed, primarily encompassed between the latitudes of 80°N and 20°S, extending from North America in the west, Russia in the east, Greenland in the north and to northern Australia (Queensland) and the Solomon Islands in the south (Cowan 1949).

The taxonomic history of *Rhododendron* since, and throughout, its inception has been complex, and the large number of species currently recognised and diverse range of morphological variation have sustained this complexity (see Cowan 1949; Sleumer 1949, 1980; Philipson and Philipson 1996). The presently accepted classification of the genus is that of Chamberlain *et al*. (1996), based primarily on the classification of Sleumer (1966), with alterations in light of recent revisions and phylogenetic studies (Philipson and Philipson 1975, 1986; Cullen and Chamberlain 1978, 1979; Cullen 1980*a*; Chamberlain 1982; Chamberlain and Rae 1990; Kron and Judd 1990; Kron 1993; Judd and Kron 1995). As currently circumscribed, *Rhododendron* includes the well known 'Azaleas' and all species of the previously segregate genera *Ledum* L. (Kron and Judd 1990) and *Tsusiophyllum* Maxim. (Kron 1997).

Rhododendron is currently divided into eight subgenera—*Azaleastrum* Planch., *Candidastrum* Franch., *Hymenanthes* (Blume) K. Koch, *Mumazalea* (Sleumer) W.R. Philipson & M.N. Philipson, *Pentanthera* (G. Don) Pojarkova, *Rhododendron*, *Therorhodion* (Maxim.) A. Gray and *Tsutsusi* (Sweet) Pojarkova—on the basis of presence or absence of scales, leaf deciduousness, and floral and vegetative branching patterns (Sleumer 1980). There has long been a distinction between lepidote and elepidote species and this is reflected in the current classification, with all lepidote species considered to be related and placed in the one group, subgenus *Rhododendron*. This subgenus includes almost half the number of all *Rhododendron* species and is divided into three sections: *Rhododendron*, *Pogonanthum* Aitch. & Hemsl. and *Vireya* (Blume) Copel.f.

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Section *Vireya* is the largest section, consisting of ~300 species. This large group of morphologically diverse taxa is widespread across the Malesian Archipelago and defined by the possession of seeds with tailed appendages, leaf idioblasts and capsule valves that twist after opening (Cullen 1980*b*; Sleumer 1980; Nilsen 2003). However, vireyas differ widely in floral and vegetative characteristics among species.

The phylogeny of section *Vireya* elucidated from two cpDNA regions showed that clades strongly correlated with geographic areas (Brown *et al*. 2006*a*) rather than reflecting the traditional classification of Sleumer (1966) based on morphology, with a general split between eastern Malesian taxa, and those from western and middle Malesia.

To test the relationships uncovered with the cpDNA data, a nuclear region was sequenced. Sequences of the nuclear rDNA (nrDNA) have been widely exploited as markers in molecular systematics of angiosperms because of their ease of isolation and amplification owing to their large number of copies in the genome (Palmer 1987; Hamby and Zimmer 1992; Hershkovitz *et al*. 1999), with the ITS being the most popular (e.g. Kim and Jansen 1994; Baldwin *et al*. 1995; Bena *et al*. 1998; Alice and Campbell 1999; Hershkovitz *et al*. 1999; Brown *et al*. 2001; Denduangboripant *et al*. 2001; Valiejo-Roman *et al*. 2002; Eriksson *et al*. 2003).

Favourable properties resulting in ITS being the most widely used nrDNA region in angiosperm molecular phylogenetics include its small size, highly conserved flanking regions and the assumption that this gene family undergoes rapid concerted evolution (Baldwin *et al*. 1995). Some studies (e.g. Ritland *et al*. 1993; Suh *et al*. 1993; Buckler and Holtsford 1996), however, have indicated that concerted evolution does not completely homogenise paralogous loci in the ITS region and polymorphisms have been detected, indicating paralogous copies.

While sequences of nrDNA have several potential drawbacks for recovering phylogeny, such as paralogy (Patterson 1987), they are at present, the best independent data available to test molecular phylogenies produced from cpDNA. Therefore, sequences of nrDNA, such as the ITS, will continue to be widely utilised in plant systematics because they are universally amplifiable, and the potential alternatives of low-copy nuclear DNA sequences, at present, are difficult and expensive to work with (Álvarez and Wendel 2003; Bailey *et al*. 2003; Small *et al*. 2004).

Several studies of *Rhododendron* phylogeny have used ITS sequence data but sample sizes in these studies were quite small for such a large genus (26 species, Chamberlain and Hyam 1998; 16 species, Scheiber *et al*. 2000; 27 species, Gao *et al*. 2002; 12 species, Gao *et al*. 2003; 20 species, Tsai *et al*. 2003); over the five studies only four accessions of species of section *Vireya* were included. Two studies were at the sectional level (Scheiber *et al*. 2000; Gao *et al*. 2003), two investigated the infrageneric relationships of the genus (Chamberlain and Hyam 1998; Gao *et al*. 2002), while another focused on rhododendrons found on the island of Taiwan (Tsai *et al*. 2003). This present study aims to increase the sample number of taxa for ITS from the diverse, and largest section, *Vireya*, and use these data to elucidate the phylogeny of the section and its relationships within subgenus *Rhododendron*, as an independent dataset for comparison with the cpDNA phylogeny of Brown *et al*. (2006*a*).

Materials and methods

Plant material

Ingroup taxa were selected to maximise the sampling of morphological diversity of section *Vireya* over the total distributional range. Sampling included representatives from all seven subsections, although not all islands were thoroughly represented for each subsection; in total the ingroup included 39 accessions representing 32 taxa (Table 1). The species sampled in this study represent a subset of the taxa sampled for the chloroplast phylogeny presented by Brown *et al*. (2006*a*).

Outgroup species were selected from within the genus because several studies indicate *Rhododendron*, and subgenus *Rhododendron* to be monophyletic (Chamberlain and Hyam 1998; Kurashige *et al*. 1998, 2001; Gao *et al*. 2002). Five of the eight subgenera of *Rhododendron* were represented, including species from sections *Rhododendron* and *Pogonanthum* (subgenus *Rhododendron*; Table 1).

Leaf tissue for extraction came from cultivated collections and also from field collections in Sulawesi (Indonesia; Table 1). Where the use of fresh leaf material for isolation of DNA was not possible, leaves were collected in a saturated NaCl–CTAB solution (Rogstad 1992) or fine silica gel combined with blue indicator silica gel.

Voucher herbarium specimens of leaf material utilised were collected to confirm the identification of species. These vouchers are lodged at the Australian National Herbarium (CANB), except vouchers for the species collected from the Royal Botanic Garden Edinburgh (RBGE), which are held at the RBGE herbarium (E).

Isolation of DNA and amplification

Prior to extraction of DNA, leaf indumentum was removed by shaving with a razor blade because it inhibits polymerase chain reactions (PCR) in species of *Rhododendron* (A Denton pers. comm.). Genomic DNA was isolated either by the CTAB method of Thomson and Henry (1993) with a high-salt wash (Mackenzie *et al*. 1998), because initial extractions were extremely viscous and amplification proved to be problematic, or with the Plant DNAzol Reagent (GibcoBRL Life Technologies, New York, NY). Extracted DNA was purified with the QIAquick PCR purification column (Qiagen, Doncaster, Vic.) or by Protocol A of the Geneclean spin kit (Qbiogene, Illkirch Cedex, France), before quantification with a fluorometer (DyNA Quant™ 200, Hoefer, Inc., San Francisco, CA).

The ITS region, including ITS-1, 5.8S and ITS-2, was amplified from genomic DNA with the primers ITS1 and ITS4 (White *et al*. 1990) or 17SE and 26SE (as ABI 102R; Sun *et al*. 1994).

PCR mixtures were prepared with Applied Biosystems (Norwalk, CT) reagents and Fisher Biotec (West Perth, WA) *Taq* Polymerase. The 50-μL reactions contained 5 μL 10× PCR buffer, 3 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μM forward primer, 0.5 μM reverse primer, 1.25 units *Taq* polymerase and 50–100 ng of DNA. All amplifications were performed on a Hybaid PCR Express Thermal Cycler or a Hybaid Touchdown Thermal Cycler (Hybaid, Middlesex, UK). For each set of PCR reactions, a negative control (no template) was run.

Table 1. Plant material

Species are listed in alphabetical order by subgenus (bold), then section (italic) and subsection where applicable. The total distribution for the species is listed with the sampled location indicated with *, when known, where distributed over more than one area. Sources for the molecular material are also listed: MTBG, Mt Tomah Botanic Garden; LC, Lyn Craven's private collection; RBGE, Royal Botanic Garden Edinburgh; JR, the late John Rouse's private collection; DB, David Binney's private collection; BC / BCJ / BCJR numbers field collection numbers (B = Brown, G.K.; C = Craven, L.A.; J = Juswara, L.; R = Ramadhanil, P.); GenBank, sequences obtained from the web-based GenBank database. GenBank accession numbers are listed for each taxon. Voucher information for taxa sequenced in this study is associated with the GenBank numbers

Subgenus *Section*	Subsection	Rhododendron species	Distribution	Source	GenBank acc. no.
Azaleastrum					
Choniastrum		*championae*	China (Hunan, Guangxi, Guangdong, Fujian, Zhejiang, Jiangxi, Hong Kong)	GenBank	AF393426
		moulmainense	Burma, China (Xizang, Yunnan, Guizhou, Guangxi, Hunan, Guangdong, Hong Kong), Taiwan, Japan, India (Arunachal Pradesh), Thailand, Laos, Cambodia, Vietnam, Malay Peninsula	GenBank	AF393432
		stamineum	Burma, China (Yunnan, Sichuan, Guizhou, Hubei, Hunan, Guangxi, Anhui)	GenBank	AF393435
		stamineum	As above	GenBank	AF452226
Hymenanthes					
Ponticum	*Pontica*	*ponticum*	Spain, Portugal, Bulgaria, Turkey, Gruziya, Armeniya, Abkhasiya, Lebanon	GenBank	X97415
Mumazalea					
		semibarbatum	Japan	GenBank	X96812
Pentanthera					
Pentanthera		*molle*	Japan	GenBank	X97425
Rhododendron					
Pogonanthum		*anthopogon*	India (Jammu-Kashmir, Himachal Pradesh, Uttar Pradesh, West Bengal, Arunachal Pradesh), Nepal, Bhutan, China (Xizang)	GenBank	X97418
Rhododendron	*Campylogyna*	*campylogynum*	India (Arunachal Prasdesh), Burma, China (Yunnan, Xizang*)	MTBG	AY877280
	Maddenia	*maddenii*	Vietnam*, India	MTBG	AY877281
	Rhododendron	*ferrugineum*	Austria, France, Germany, Italy, Spain, Switzerland	GenBank	AF393415
		ferrugineum	As above	GenBank	X97420
		ferrugineum	As above	GenBank	X97419
		mucronulatum	Amur, China (Hubei, Shandong), Mongolia, Korea, Japan	GenBank	AF393412
	Scabrifolia	*spinuliferum*	China (Yunnan, Guizhou)	GenBank	AF452233
Vireya	*Albovireya*	*aequabile*	Sumatra	RBGE	AY877284
		aequabile	Sumatra	JR	AY877268
		lagunculicarpum	Sulawesi	DB	AY877287
		lagunculicarpum	Sulawesi	BCJ30	AY877289
		zollingeri	Sulawesi	DB	AY877296
		zollingeri	Sulawesi	BCJR125	AY877297
	Euvireya	*christi*	PNG	LC	AY877269
		gracilentum	PNG	LC	AY877271
		javanicum	Java*, Sumatra, Sulawesi, Philippines, Bali, Malay Peninsula	RBGE	AY877274
		javanicum	As above	GenBank	X97424
		lochiae	Australia	LC	AY877279
		luraluense	North Solomons	LC	AY877277
		rhodopus	Sulawesi	BCJR129	AY877293
		rousei	Philippines (Sibuyan Island*)	DB	AY877291
		rubineiflorum	PNG	LC	AY877294
		saxifragoides	PNG*, IJ	DB	AY877299
		viriosum	Australia	LC	AY877288
		zoelleri	Moluccas, IJ*, PNG*	LC	AY877302

Table 1. (*continued*)

Subgenus *Section*	Subsection	Rhododendron species	Distribution	Source	GenBank acc. no.
	Malayovireya	*apoanum*	Philippines (Mindanao*)	RBGE	AY877267
		malayanum	Sumatra*, Sulawesi, Malay Peninsula, Java	DB	AY877278
	Phaeovireya	*leptanthum*	PNG	LC	AY877275
		leptanthum	PNG	GenBank	X97421
		rarum	PNG	LC	AY877285
	Pseudovireya	*ericoides*	Borneo (Sabah*)	LC	AY877270
		kawakamii	Taiwan	GenBank	AF432450
		kawakamii	Taiwan	GenBank	AF432420
		nanophyton	Sulawesi	BCJ 46	AY877290
		quadrasianum var. *rosmarinifolium*	Philippines (Luzon*)	DB	AY877292
		retusum	Sumatra*, Java	LC	AY877286
		santapaui	India- Arunachal Pradesh	LC	AY877298
		santapaui	As above	GenBank	AF452229
		vaccinioides	Bhutan, India (Sikkim, West Bengal), China (Xinzang), Nepal	LC	AY877301
	Siphonovireya	*herzogii*	PNG	LC	AY877272
	Solenovireya	*alborugosum*	Borneo (Kalimantan*)	DB	AY877283
		jasminiflorum var. *heusseri*	Malay Peninsula, Philippines, Sumatra*, Borneo	LC	AY877273
		loranthiflorum	PNG (Bismark Archipelago*), Solomons	LC	AY877276
		edanoi ssp. *pneumonanthum*	Borneo (Sarawak*)	DB	AY877282
		ruttenii	Moluccas (Ceram*)	LC	AY877295
		tuba	PNG	LC	AY877300

The cycling parameters for amplification were an initial denaturation at 94°C for 3 min, 30 cycles of 94°C for 1 min, 48°C for 1 min and 72°C for 2 min, followed by a final extension step of 72°C for 7 min. After the initial 3 min denaturation period at 94°C, a hold was included to allow for the addition of the *Taq* polymerase. This 'hotstart' procedure was included to prevent polymerase activity in the initial denaturation phase, thereby minimising non-specific priming and formation of primer dimers. PCR products were purified with QIAquick PCR purification columns (Qiagen) and then quantified with a fluorometer (Hoefer® DyNA Quant™ 200).

DNA sequencing and alignment

Direct sequencing was completed for the forward and reverse primers with the recommended half reactions of the ABI Prism® Big DyeTM Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems), with ~50–100 ng of PCR product. Amplifications were performed on a Hybaid PCR Express Thermal Cycler with the cycling parameters: 30 cycles of denaturation at 96°C for 10 s, annealing at 57°C for 5 s and extension at 60°C for 4 min. Sequencing reactions were purified by an ethanol / sodium acetate precipitation method (ABI Prism®), before being analysed on an ABI–Perkin Elmer 377XL sequencer.

Contiguous sequences were edited in Sequencher *v.*3.0 (Gene Codes Corporation, Ann Arbor, MI) and because of the conserved nature of the sequences, were manually aligned in BioEdit sequence alignment editor *v.*4.8.6 (Hall 1999). To achieve the most parsimonious alignment, gaps were incorporated according to the alignment method of Kelchner and Clark (1997). Sequences are lodged with GenBank, accession numbers AY877267 to AY877302 (Table 1). Informative multi-residue gaps were coded as indels and added to the data matrix as binary characters (see Table 2).

Analyses

Parsimony and Bayesian inference were employed to analyse the ITS data. Parsimony analysis was conducted with the computer package PAUP* version 4.0b10 (Swofford 2003). The heuristic search option was employed, using tree bisection-reconnection (TBR), with random stepwise addition replicated 1000 times. Uninformative characters were excluded from the analysis and a maximum of 20 000 trees were saved. A strict consensus tree was calculated for equally parsimonious trees. Trees were rooted by inclusion of an outgroup: *Rhododendron molle* (Blume) G.Don (subgenus *Pentanthera*). This was based on previous studies, which show that subgenus *Pentanthera* is clearly outside the monophyletic subgenus *Rhododendron* (Chamberlain and Hyam 1998; Kurashige *et al*. 2001; Gao *et al*. 2002). The support for nodes was measured by the bootstrap (Felsenstein 1985) employing a full heuristic search of 1000 replicates, each with 100 random stepwise addition replicates, and saving no more than 200 trees $\geq$ score 1 per addition replicate.

Erroneous inferences can be drawn from phylogenetic analysis when the model is a poor fit to the data (Huelsenbeck *et al*. 2001). For the Bayesian analyses, the simplest evolutionary model that adequately explained the data was selected under the Akaike Information Criterion (AIC) with Modelltest version 3.06 (Posada and Crandell 1998; Posada and Buckley 2004). Indels were excluded from the model selection process because gaps may evolve under different evolutionary constraints from nucleotides, and the processes of different insertion and deletion events are still unclear (Simmons and Ochoterena 2000; Steel and Penny 2000).

The AIC test selected SYM+Γ (SYM plus gamma) as the most appropriate model for the nucleotide data. The SYM model allows for transitions and transversions to occur at different rates, but equal base frequencies; the Γ term models rate heterogeneity among sites

Table 2. Indel characters of the ITS sequences

Position, type (insertion or deletion with the bases indicated in parentheses), and the species that possess each indel character are listed. All accessions without GenBank numbers were sequenced as part of this project (see Table 1 for their GenBank numbers). Abbreviations in parentheses indicate the accession that possess indel, where multiple accessions exist: LC, Lyn Craven's private collection; RBGE, Royal Botanic Garden Edinburgh; JR, the late John Rouse's private collection; DB, David Binney's private collection; BC / BCJ / BCJR numbers field collection numbers (B = Brown, G.K.; C = Craven, L.A.; J = Juswara, L.; R = Ramadhanil, P.). – Represents a gap in the alignment. N represents that the base was unknown at this position of the sequence, and was coded as ? for analysis

Indel	Position (base no.)	Type (bases)	Species
1	42–43	Insertion	
		(– –)	*Rhododendron championae*, *R. ferrugineum* (AF393415, X97420, X97419), *R. molle*, *R. moulmainense*, *R. ponticum*, *R. semibarbatum*, *R. stamineum* (AF393435, AF452226)
		(T–)	*R. aequabile* (JR), *R. ericoides*, *R. campylogynum*, *R. anthopogon*, *R. javanicum* (RBGE, X97424), *R. maddenii*, *R. kawakamii* (AF432420, AF432450), *R. edanoi* ssp. *pneumonanthum*, *R. mucronulatum*, *R. santapaui* (LC, AF452229), *R. alborugosum*, *R. spinuliferum*, *R. jasminiflorum*, *R. retusum*, *R. javanicum*, *R. malayanum*, *R. lagunculicarpum* (DB, BCJ30), *R. nanophyton*, *R. rousei*, *R. quadrasianum*, *R. rhodopus*, *R. ruttenii*, *R. zollingeri* (DB, BCJR125), *R. vaccinioides*
		(TT)	*R. rarum*, *R. apoanum*, *R. viriosum*, *R. christi*, *R. leptanthum* (LC, X97421), *R. rubineiflorum*, *R. gracilentum*, *R. saxifragoides*, *R. herzogii*, *R. tuba*, *R. zoelleri*, *R. loranthiflorum*, *R. luraluense*, *R. lochiae*, *R. aequabile* (RBGE)
2	639–641	Insertion	
		(– – –)	*Rhododendron championae*, *R. ferrugineum* (AF393415, X97420, X97419), *R. molle*, *R. moulmainense*, *R. ponticum*, *R. semibarbatum*, *R. stamineum* (AF393435, AF452226)
		(CAG)	*R. christi*, *R. leptanthum* (LC, X97421), *R. gracilentum*, *R. herzogii*, *R. loranthiflorum*, *R. luraluense*, *R. lochiae*, *R. rarum*, *R. viriosum*, *R. saxifragoides*, *R. tuba*, *R. zoelleri*
		(CAA)	*R. apoanum*, *R. aequabile* (RBGE, JR), *R. ericoides*, *R. anthopogon*, *R. javanicum* (RBGE, X97424), *R. kawakamii* (AF432420, AF432450), *R. mucronulatum*, *R. santapaui* (LC, AF452229), *R. spinuliferum*, *R. jasminiflorum*, *R. malayanum*, *R. campylogynum*, *R. maddenii*, *R. edanoi* ssp. *pneumonanthum*, *R. alborugosum*, *R. retusum*, *R. lagunculicarpum* (DB, BCJ30), *R. nanophyton*, *R. rousei*, *R. quadrasianum*, *R. rhodopus*, *R. ruttenii*, *R. zollingeri* (DB, BCJR125), *R. vaccinioides*
		(NNN)	*R. rubineiflorum*

as a gamma distribution. The substitution model rate matrix (A–C, 0.7246; A–G, 2.8570; A–T, 1.2361; C–G, 0.4262; C–T, 4.8720; G–T, 1.0000) and gamma distribution shape parameter (0.2710) estimated in Modelltest were implemented in the Bayesian analyses, conducted with the computer program MrBayes version 3.0 (Ronquist and Huelsenbeck 2003). The JC + Γ (Jukes Cantor plus gamma) model, which is the simplest evolutionary model that allows for rate heterogeneity, was applied to the indel characters.

Ten Markov Chains were run for 2 000 000 generations, sampling a tree every 100 generations. These values were chosen from a survey of recent literature (Soltis *et al*. 2002; Valiejo-Roman *et al*. 2002; Eriksson *et al*. 2003; Salazar *et al*. 2003; Vargas *et al*. 2004) to maximise mixing of the chains, therefore increasing the potential for convergence. Once the analysis had run to completion, the log-likelihood values were used to assess the burnin value and, therefore, the number of trees to disregard when creating the consensus tree, a 50% majority rule tree (Larget and Simon 1999; Huelsenbeck *et al*. 2002). The Bayesian analysis was repeated five times, and the consensus trees compared to check that each run converged on the same log-likelihood values and tree.

Results

Fifty-five accessions, representing 44 species of *Rhododendron* including 32 ingroup and 12 outgroup taxa, were sampled for the ITS dataset; eighteen of these accessions were obtained from GenBank (Table 1). Five of the eight *Rhododendron* subgenera were sampled, including subgenus *Rhododendron*. Representatives of all three sections of subgenus *Rhododendron* were included: one from section *Pogonanthum*, five from section *Rhododendron* (representing four of the 28 subsections), and 32 from section *Vireya*. All seven subsections of section *Vireya* were sampled but due to amplification difficulties, only one representative of subsection *Siphonovireya* Sleumer was sequenced, *R. herzogii* Warb. (Table 1).

The base composition of ITS was relatively constant: 45–47% AT and 53–55% GC (Table 3). *Vireya* sequences diverged by up to 5.25%, while the maximum sequence divergence between ingroup and outgroup taxa was slightly higher at 6.61%. The ITS region ranged from 570 to 657 bp long, unaligned, while the final alignment was 668 bp long. The aligned length of ITS-1 was 265 bp, 5.8S was 164 bp and ITS-2 was 239 bp (Table 3). Two potentially informative indels (Table 2), one in each of the ITS spacers, were identified and coded as binary characters, resulting in 670 characters, of which 78 were potentially parsimony informative. ITS-1 contained slightly more informative sites than ITS-2: 41 (15.47%) compared with 33 (13.81%) (Table 3).

Table 3. Sequence characteristics of the ITS region

Sequence lengths, sequence divergence, base composition, number of parsimony informative characters (excluding indels), number of indels are listed for ITS of all taxa, unless otherwise indicated

Sequence characteristics	Total ITS	ITS-1	5.8S	ITS-2
Aligned length (bp)	668	265	164	239
Unaligned length range (bp)				
vireyas[A]	(570) 645–657	(231) 253–257	164	(175) 226–237
non-vireyas	651–655	253–255	164	233–236
Sequence divergence percentage				
between vireyas	5.25	8.38	1.24	8.05
between vireyas and non-vireyas	6.61	9.39	1.24	9.18
between non-vireyas	4.45	6.62	0.60	6.30
No. of parsimony informative sites (%)	76 (11.38%)	41 (15.47%)	2 (1.21%)	33 (13.81%)
Base composition				
AT%	45.16–47.18	47.06–51.17	47.24–47.85	40.77–43.43
GC%	52.59–54.84	48.41–52.94	52.15–52.76	56.57–59.23
No. of indels (synapomorphic)	2	1	0	1
Indel size range (bp)	2–3	2	n/a	3

[A]The lowest value, in parentheses, indicates the sequence lengths for *R. rubineiflorum*; it is shorter than the other vireyas because of poor sequence reads at each end.

An heuristic search in PAUP revealed 855 equally parsimonious trees of length 134, CI = 0.72, RI = 0.93, HI = 0.28, RC = 0.67. This tree length was detected in all replicates. The strict consensus tree (not shown) had 24 nodes, all of which were supported by bootstrap values >50%.

The log-likelihood in all five Bayesian iterations converged to a stable range of −2470 to −2490, after ~33 500 generations. These generations were considered the burnin period. The 50% majority rule tree is shown in Fig. 1 with all but two of the 29 nodes supported by posterior probabilities greater than 80. Bootstrap values from the parsimony analysis are also included in Fig. 1.

The parsimony and Bayesian analyses were congruent, although the Bayesian analyses resolved six nodes that parsimony did not (Fig. 1). The species of subgenus *Azaleastrum* Planch. (node 2) are strongly supported as monophyletic, with the representative of subgenus *Mumazalea* (Sleumer) Philipson & M.N. Philipson moderately supported as sister to this clade. Subgenus *Rhododendron* is also well supported as monophyletic at node 3 (Fig. 1).

Within subgenus *Rhododendron*, section *Rhododendron* is polyphyletic. The representative of section *Pogonanthum* (*R. anthopogon* D.Don) is grouped with four representatives of section *Rhododendron* (node 6), excluding a fifth representative of this section, *R. ferrugineum* L. The three accessions of *R. ferrugineum* cluster together with strong support (bootstrap of 97% and posterior probability of 100; node 4, Fig. 1) and are sister to the rest of subgenus *Rhododendron* (node 3, Fig. 1).

Section *Vireya* is resolved by the Bayesian analyses as related to the rest of subgenus *Rhododendron* (node 8, posterior probability of 83). However, the strict consensus tree from the parsimony analysis does not resolve this node or node 5 (Fig. 1), instead resulting in a polytomy of section *Rhododendron* taxa from node 6 with the clades of section *Vireya* at nodes 9, 12, 13 and 15. Based on the ITS data, the monophyly of section *Vireya* is not well supported.

Parsimony does not resolve all deep nodes resolved by Bayesian analysis. However, many of the clades within section *Vireya* are recovered by both analytical methods. In this study none of the subsections of section *Vireya*, excluding subsection *Siphonovireya*, which is represented here by only one species, is shown to be monophyletic. Subsection *Pseudovireya* Clarke, while not resolved as monophyletic, is shown as related to the rest of section *Vireya* (node 8, Fig. 1). The two mainland Asian species of *Pseudovireya—R. santapaui* Sastry *et al*. (both accessions) and *R. vaccinioides* Hook.f.—are well supported as sister taxa (node 9). Node 11 groups the Malesian species of subsection *Pseudovireya—R. ericoides* Low ex. Hook.f., *R. nanophyton* Sleumer, *R. quadrasianum* Vidal and *R. retusum* (Blume) Benn.—with the two accessions of *R. kawakamii* Hayata from Taiwan, although this relationship is not resolved in the parsimony strict consensus tree. Within this clade, *R. ericoides*, *R. nanophyton* and *R. quadrasianum* are clustered as a polytomy (node 14), sister to *R. retusum* (node 13); branch lengths leading to each of these two nodes are both very short (see Fig. 2).

The six other subsections of section *Vireya* form a well-supported clade, 'Euvireya' (node 15, bootstrap of 90% and posterior probability of 100; Fig. 1). All species sampled from western and middle Malesia (Borneo, Moluccas, Philippines and Sumatra) from

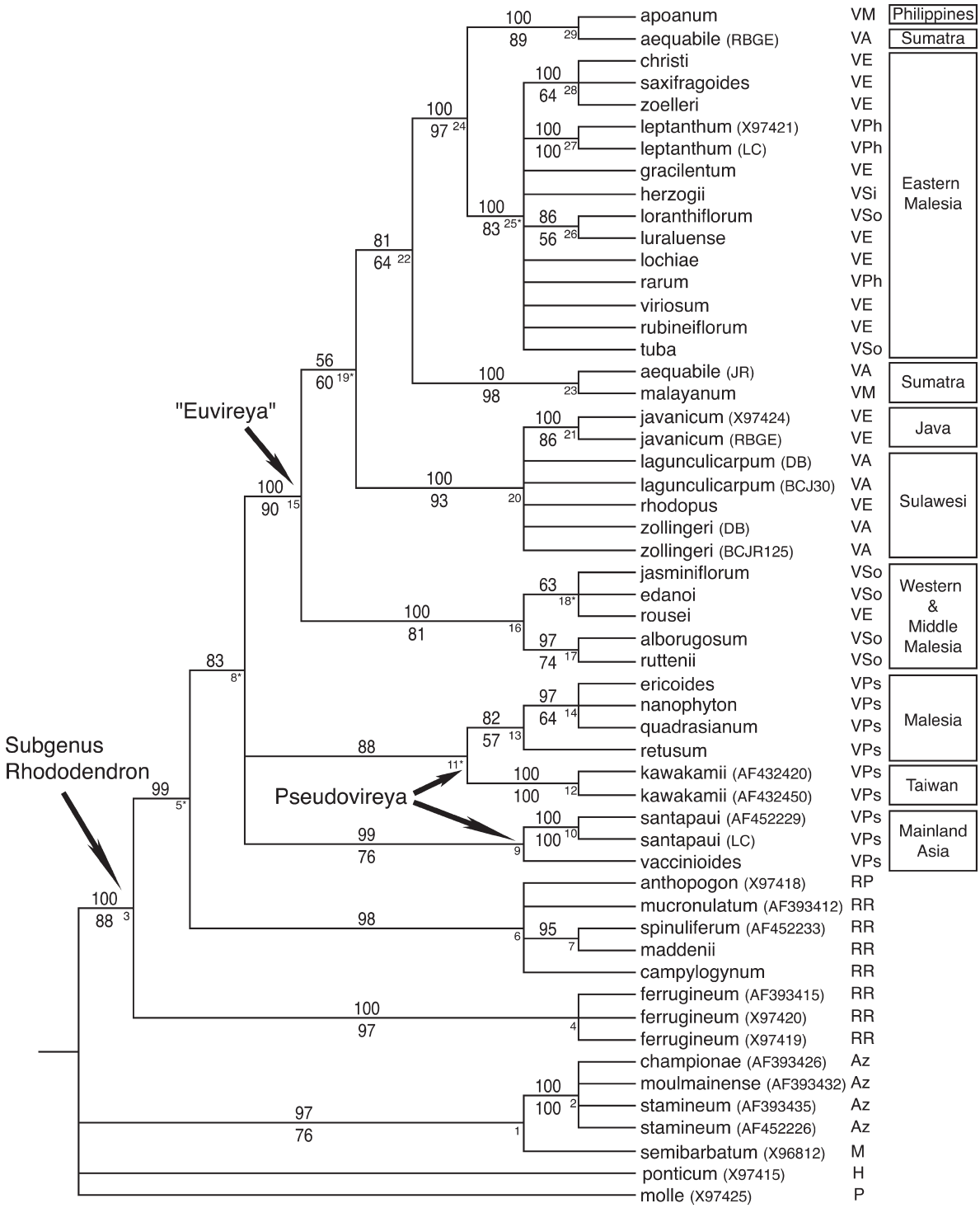

Fig. 1. ITS 50% majority rule tree from Bayesian analysis (iteration number 5); log-likelihood range −2470 to −2490. Nodes that are not resolved by the parsimony analysis (tree length 135, CI = 0.72, RI = 0.93) are marked with *. Posterior probability values are shown above the node and bootstrap values are shown below the nodes they support. All species of section *Vireya* are indicated by a V and an abbreviation of the subsection: *Albovireya*, VA; *Euvireya*, VE; *Malayovireya*, VM; *Phaeovireya*, VPh; *Pseudovireya*, VPs; *Siphonovireya*, VSi; *Solenovireya*, VSo. The subgenus, or section of subgenus *Rhododendron* taxa, and outgroup taxa are also indicated: Subgenus *Rhododendron*, section *Rhododendron*, RR; Subgenus *Rhododendron*, section *Pogonanthum*, RP; Subgenus *Azaleastrum*, Az; Subgenus *Mumazalea*, M; Subgenus *Hymenanthes*, H; Subgenus *Pentanthera*, P. Parentheses are used to identify the different sequence accession (see Table 1) or the GenBank numbers of sequences sourced from the GenBank database. General area distributions are also shown.

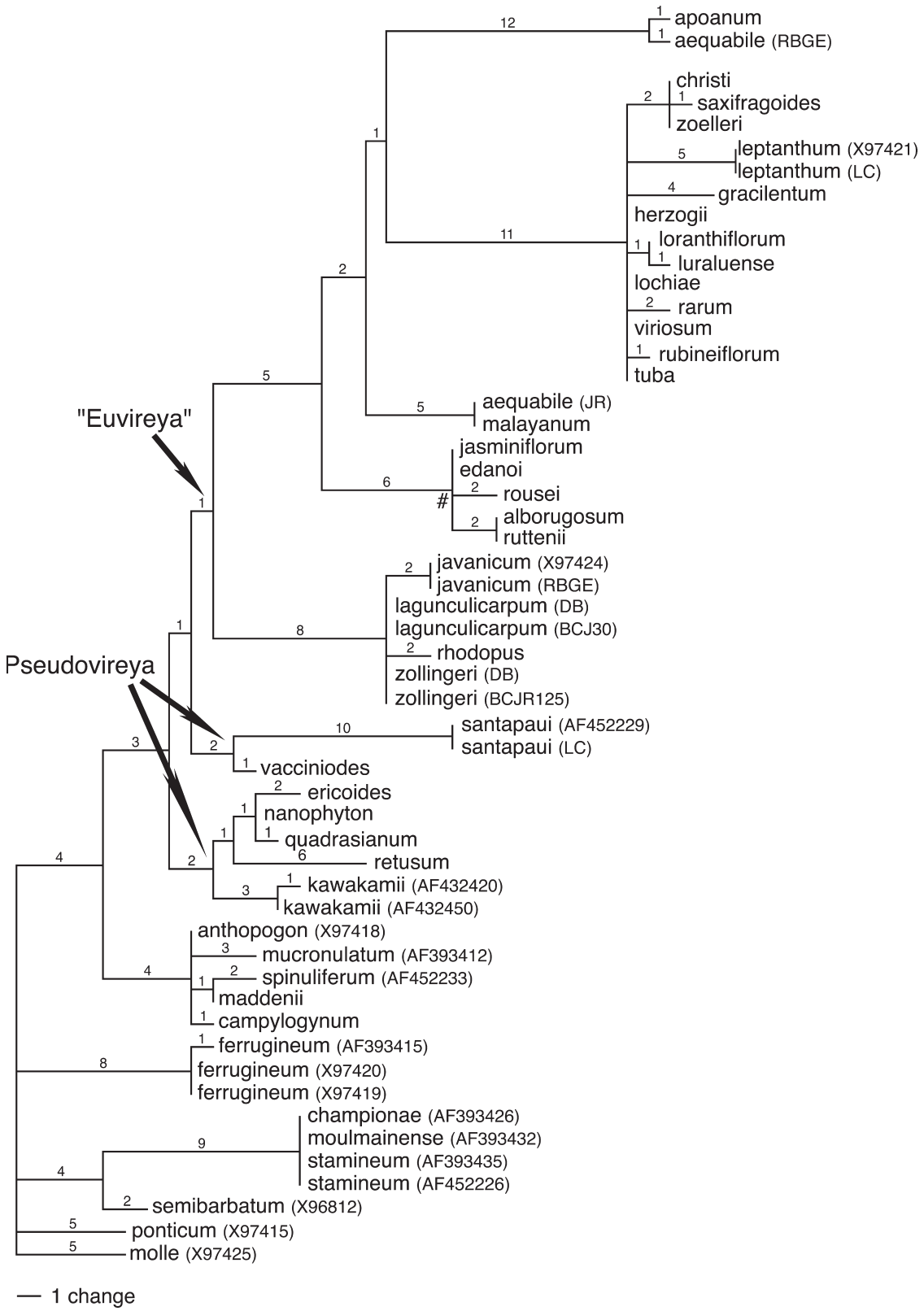

Fig. 2. Phylogram of one of the ITS parsimony trees. Tree number 1139 (selected at random) of 1709 trees recovered in the parsimony analysis of the ITS dataset is shown. Branch lengths are indicated above the node. Node 16 of Fig. 1 is indicated with # because its position alternates between that shown in Fig. 1 and that shown here.

subsection *Solenovireya* Copel.f.—*R. jasminiflorum* Hook., *R. edanoi* ssp. *pneumonanthum* (Sleumer) Argent, *R. alborugosum* Argent & J. Dransf., *R. ruttenii* J.J.Sm.—are grouped together at node 16, with the Philippine species *R. rousei* Argent & Madulid of subsection *Euvireya* Copel.f. The position of this clade at node 16 within the 'Euvireya' is ambiguous. In the Bayesian analysis it is placed as sister to the rest of 'Euvireya' with a low posterior probability (56) and bootstrap value (60, node 19; Fig. 1). In the parsimony analysis, the clade at node 16 is placed either as in the Bayesian analyses (see Fig. 1), or as sister to the clade at node 22 (for example see Fig. 2). This ambiguity is represented in the parsimony strict consensus tree (not shown) with node 19 not resolved (marked with an asterisk in Fig. 1).

The clade at node 20 (Fig. 1) is well supported and contains all accessions of species sampled from Sulawesi—*R. lagunculicarpum* J.J.Sm., *R. rhodopus* Sleumer and *R. zollingeri* J.J.Sm.—along with the two accessions of *R. javanicum* (Blume) Benn. from Java (Fig. 1). The ITS sequences of *R. lagunculicarpum* and *R. zollingeri* accessions are identical (AY877287, AY877289, AY877296, AY877297). Accessions of *R. lagunculicarpum* were sampled from the same population on Gunung Rantemario (Sulawesi Selatan), whereas accessions of *R. zollingeri* were from two separate populations, one from G. Rorekatimbu (Sulawesi Tengah) and the other from G. Sesean (Sulawesi Selatan).

The two accessions of *R. aequabile* J.J.Sm. are not in the same clade; one accession (JR) is placed sister to *R. malayanum* Jack (node 23) with high bootstrap and posterior probability support (98 and 100 respectively), and the other (RBGE) is shown, again with good support (100 posterior probability and 98% bootstrap, node 29), to be closely related to *R. apoanum* Stein.

Node 25 represents all species sampled from Eastern Malesia. The two accessions of *R. leptanthum* F. Muell have 100% support as a clade by both posterior probability and bootstrap values (node 27; Fig. 1). The other relationships resolved in this clade are the sister pairing of *R. loranthiflorum* Sleumer and *R. luraluense* Sleumer (node 26), and the polytomy of *R. christi* F. Först, *R. saxifragoides* J.J.Sm. and *R. zoelleri* Warb. (node 28). These branch lengths are relatively short with only one or two character state changes (Fig. 2); both nodes are supported by high posterior probabilities, yet low bootstrap values (Fig. 1).

The indel events identified for the ITS (Table 2) are synapomorphies. At node 5 the state T– appears for indel 1, and the state CAA is gained for indel 2. At node 25, the Eastern Malesian clade, all taxa gain another T in indel 1, resulting in the state TT, and for indel 2 there is a transition from A to G in the third position, resulting in the state CAG.

Discussion

Phylogenetic utility of ITS

As previous studies have found (see references in Baldwin *et al*. 1995), variation in the ITS was mainly due to point mutations rather than indel events. The length and sequence composition of ITS in *Rhododendron* are within the previously reported range (Baldwin *et al*. 1995), while the total percentage of informative sites (11.38%) was relatively low. ITS resolved some of the deeper relationships in the vireyas; however, it does not appear to be sufficiently variable to resolve the relationships between all terminal taxa. Previous studies utilising ITS to investigate sectional relationships within *Rhododendron* sections *Azaleastrum* (Planch.) Maxim (Gao *et al*. 2003) and *Pentanthera* (Scheiber *et al*. 2000) have found similar patterns of resolution, with internal nodes well resolved but terminal nodes unresolved as polytomies. This lack of resolution suggests that ITS is not a suitable marker to resolve relationships within section *Vireya*. Alternately, it may indicate that the group has undergone a relatively rapid radiation, as Hershkovitz *et al*. (1999) suggested a region may appear to have poor resolving power yet still be the best sequence for that problem if the divergence pattern reflects a rapid radiation.

Comparison of analytical methods

The two analytical methods used in this study, parsimony and Bayesian inference, gave congruent results, however, Bayesian analyses resulted in trees with greater resolution, particularly for internal nodes, than did parsimony. A similar higher resolution for Bayesian inferred phylogenies was found in an ITS study of the Sino-Himalayan Apioideae (Umbelliferae) by Valiejo-Roman *et al*. (2002), and also in the cpDNA phylogenies inferred for section *Vireya* (Brown *et al*. 2006*a*). The other difference noted between the two methods of analysis was an incongruence between the measures of support for some nodes, for example, nodes 13 and 26 (Fig. 1). This phenomenon has been reported previously (Soltis *et al*. 2002; Valiejo-Roman *et al*. 2002; Kimball and Crawford 2004; Vargas *et al*. 2004). This difference is not surprising, given that bootstrap and posterior probabilities are not considered interchangeable (Alfaro *et al*. 2003; Douady *et al*. 2003). Nodes with greatly differing bootstrap and posterior probability values are not viewed with a high level of confidence.

Phylogeny of Rhododendron

Based on the ITS data, subgenus *Azaleastrum* is monophyletic and sister to subgenus *Mumazalea*. It should be noted, however, that only members of *Azaleastrum* section *Choniastrum* Franch. were sampled in this study. Previous studies have found the two sections of *Azaleastrum* to be monophyletic, while the subgenus as a whole is polyphyletic (Chamberlain and Hyam 1998; Kurashige *et al*. 1998, 2001;

Gao *et al*. 2002, 2003). Strong support for the monophyly of subgenus *Rhododendron*, the lepidote rhododendrons, is found in all ITS analyses. This close relationship of the lepidote rhododendrons has long been hypothesised on the basis of morphological similarities and, more recently, has been supported by data from several sources, including breeding, grafting and molecular phylogenetic studies (Kehr 1977; Williams *et al*. 1985, 1990; Rouse *et al*. 1993; Rouse and Williams 1994; Kurashige *et al*. 2001; Goetsch and Hall 2003).

While subgenus *Rhododendron* is a natural group, the three currently recognised sections are not. This conclusion is supported by results from a low copy nuclear gene, RPB2 (Goetsch and Hall 2003). ITS, RPB2 (Goetsch and Hall 2003) and several cpDNA studies, including *mat*K (Kurashige *et al*. 2001) and *trn*T–*trn*L (Brown *et al*. 2006*a*), suggest that sections *Vireya* and *Rhododendron* are not monophyletic, with species of section *Rhododendron* nested within section *Vireya*. Owing to limited sampling in this study, the monophyly of section *Pogonanthum* and the many subsections of section *Rhododendron* remain unknown, and the relationships between them unclear. Investigations with the RPB2 gene are currently underway in an attempt to answer these outstanding questions of sections *Pogonanthum* and *Rhododendron* (L. Goetsch and B. Hall pers. comm.).

The phylogenetic relationships within section *Vireya* do not correlate with the traditional classification based on morphology (Sleumer 1966), with none of the seven subsections inferred as monophyletic, although the monophyly of subsection *Siphonovireya* was not tested here because only one species was sequenced owing to amplification difficulties. The polyphyletic nature of the subsections was not unexpected, because phylogenies from cpDNA data (Brown *et al*. 2006*a*) show comparable findings, with only one of the seven subsections, *Siphonovireya*, confirmed as monophyletic, and subsections of section *Vireya* are defined on the basis of only two homoplasious (Brown *et al*. 2006*a*) morphological characters: leaf scale type and corolla shape (Sleumer 1966).

Pseudovireyas

Evidence that species of subsection *Pseudovireya*, quite aptly named, are paraphyletic and related to ‘Euvireya’ has also been suggested by cpDNA (Kurashige *et al*. 2001; Brown *et al*. 2006*a*), morphology (Philipson and Philipson 1996) and sexual traits (Rouse *et al*. 1993; Rouse and Williams 1997). Capsule valves of *Pseudovireya* do not twist after dehiscence as they commonly do in the remainder of the section and species are incompatible with species of ‘Euvireya’ based on pollination and grafting studies.

The relationships between the clades of *Pseudovireya* species are not resolved, but each of the clades corresponds to the broad geographic areas of Malesia (node 13), Taiwan (node 12), and mainland Asia (node 9) as they do in ‘Euvireya’. The same pattern was also uncovered by the cpDNA data (Brown *et al*. 2006*a*), however the polytomy at node 14 (Fig. 1) was resolved in the combined cpDNA analyses to show *R. ericoides* and *R. nanophyton* as sister taxa, and *R. quadrasianum* sister to them.

The area relationships in the clades of *Pseudovireya* and ‘Euvireya’ are not identical, although they do suggest that the currently circumscribed section *Vireya*, including all species of *Pseudovireya*, has evolved within at least two different lineages throughout Malesia. More detailed sampling of the pseudovireyas, particularly representatives from the islands of New Guinea and Moluccas, is required to test whether or not the geographic patterns are similar to ‘Euvireya’.

‘Euvireya’

The remaining six subsections—*Albovireya* Sleumer, *Euvireya*, *Malayovireya* Sleumer, *Phaeovireya* Sleumer, *Siphonovireya* and *Solenovireya*—are well supported as a monophyletic group, ‘Euvireya’, as they were in the cpDNA analyses (Brown *et al*. 2006*a*). The close relationships of ‘Euvireya’ taxa are reflected by their ease of interspecific fertilisation, regardless of their subsectional affiliation, when the parent plants have similar style lengths (Williams and Rouse 1988). However, this ability to reproduce across subsectional boundaries could represent the plesiomorphic condition.

Geographic clades

Within the ‘Euvireya’ clade (node 15) taxa are resolved into broadly geographic clades (Fig. 1). The close relationship between all the sampled Sulawesi taxa was predicted by the cpDNA results (see Brown *et al*. 2006*a*). It was surprising, however, that based on ITS, *R. rhodopus* was not distinct from *R. lagunculicarpum* and *R. zollingeri*. *R. rhodopus* is morphologically different from the other two species, which share numerous morphological and genetic similarities (Sleumer 1966; Brown *et al*. 2006*a*). Within and among populations, ITS has been considered to underestimate genetic diversity (Soltis and Kuzoff 1993; Utelli *et al*. 2000), with genetic variation identified through allozyme and isozyme data but not sequences of ITS-1. This may explain why the two accessions of *R. lagunculicarpum* were not resolved as a monophyletic group, as was the case with *R. zollingeri*, but it does not explain the lack of resolution between apparently distinct species. Therefore these results may indicate that there has been relatively recent speciation of *Rhododendron* on the island of Sulawesi.

All New Guinea, Solomon Island and Australian species of ‘Euvireya’ form a well supported clade (node 25, Fig. 1), as was found in the cpDNA phylogeny (Brown *et al*. 2006*a*). As with the Sulawesi clade mentioned above, the relationships between the terminal taxa are not well resolved with strong support, with the exception of the two accessions of *R. leptanthum*. The lack of variation in the ITS

region for resolving the relationships of these 'Euvireya' taxa from Eastern Malesia may indicate that they have undergone recent speciation events or a rapid divergence. Of the other resolved nodes, albeit poorly supported, in the Eastern Malesian clade one is well supported in the cpDNA analyses (node 26: *R. loranthiflorum* and *R. luraluense*), while the other (node 28: *R. christi*, *R. saxifragoides* and *R. zoelleri*) appears, at first glance, to contradict the cpDNA results (compare Fig. 1, ITS, with fig. 3 of Brown *et al*. 2006*a*). However, a closer look at the support values of the contradictory nodes (34, 36 and 38, fig. 3 Brown *et al*. 2006*a*) show great variation between bootstrap and posterior probability values and were therefore not viewed with a high level of confidence.

One clade that showed greater resolution among terminal taxa is that at node 16 (Fig. 1), which includes all representatives of subsection *Solenovireya* from western and middle Malesia (Borneo, Moluccas and Sumatra) and a *Euvireya* taxon *R. rousei* from the Philippines. A similar relationship between the western and middle Malesian representatives of *Solenovireya* and a Philippine *Euvireya* was elucidated by cpDNA data (Brown *et al*. 2006*a*), although *R. rousei* was placed in a sister clade. This latter difference is most likely an artefact of sampling, since the ITS dataset represents only a subset of the taxa included in the cpDNA dataset and phylogeny.

The geographic clades within 'Euvireya' provide an opportunity to analyse further biogeographic, historical area relationships (Brown 2004; Brown *et al.* 2006*b*).

Taxon irregularities

The relative position of the two accessions of *R. aequabile* was unexpected. They are not shown to cluster together as for other multiple accessions in this study. Instead they are inferred to be related to two different species from subsection *Malayovireya* (*R. malayanum* and *R. apoanum*; nodes 23 and 29, Fig. 1), a subsection that is possibly monophyletic based on the combined evidence from morphological and cpDNA data (see Brown *et al*. 2006*a*). The identifications and chromatograms of both *R. aequabile* accessions have been double checked and confirmed, with the cpDNA results supporting them as conspecific, placing both *R. aequabile* accessions in the same clade (Fig. 2 in Brown *et al*. 2006*a*). This suggests that two different copies of ITS may exist in *Rhododendron*, as has been found in taxa of Winteraceae Lindl. (Suh *et al*. 1993), *Mimulus* L. (Ritland *et al*. 1993) and *Zea* L. (Buckler and Holtsford 1996). This has never been reported for the genus *Rhododendron* before (Chamberlain and Hyam 1998; Scheiber *et al*. 2000; Gao *et al*. 2002, 2003; Tsai *et al*. 2003). When two types of ITS occur, the two paralogues can show the same phylogenetic relationships (Suh *et al*. 1993). This may be the case here with one type of ITS sequenced for *R. malayanum* and the other for *R. apoanum*. If so, *R. aequabile* would be considered closely related to both *R. malayanum* and *R. apoanum*, and this clade would be the sister group to the Eastern Malesian vireyas (node 25); this relationship would not conflict with phylogenies inferred from cpDNA (Brown *et al*. 2006*a*). Sequencing multiple clones of the *Malayovireya* taxa, and other vireya species, is required to test this hypothesis, but from these initial results, the presence of multiple ITS paralogues is a possibility.

Alternately, hybridisation could explain the incongruence between the two accessions of *R. aequabile* in the cp- and nrDNA phylogenies. After a hybridisation event ITS sequences can clearly look like one of its two parents depending on the mechanisms of concerted evolution (Álvarez and Wendel 2003; Chase *et al*. 2003). Therefore, it is possible that the monophyletic cpDNA sequences of *R. aequabile* (Brown *et al*. 2006*a*) represent the relationships of the same maternal lineage, while their ITS sequences represent the different paternal lineages.

Hybridisation is common in vireyas, although breeding barriers have been recorded for species with disparate style lengths (Rouse *et al*. 1993). Such a barrier would not exist between *R. aequabile* and the two *Malayovireya* species, *R. apoanum* and *R. malayanum*, as their styles are similar in length (all less than 2 cm; Sleumer 1966). A hybrid origin for the RBGE accession of *R. aequabile* is unlikely, as the distributions of *R. aequabile* (west coast of Sumatra) and *R. apoanum* (Mindanao, Philippines) do not overlap. However, the distribution of *R. aequabile* does overlap with that of *R. malayanum* (widespread throughout Malesia), with *R. malayanum* found on all Sumatran mountains where *R. aequabile* is found (Gunung Singalan, G. Kerintji and G. Pesagi).

Conclusions

The ITS data indicate that subgenus *Rhododendron* is monophyletic, as is section *Choniastrum*, subgenus *Azaleastrum*. Two of the three sections of subgenus *Rhododendron*—*Rhododendron* and *Vireya*—are indicated to be polyphyletic, while the monophyly of the other section, *Pogonanthum*, was not tested here. Six of the seven subsections of section *Vireya* are inferred to be polyphyletic, confirming results from cpDNA, while the seventh subsection, *Siphonovireya*, was not tested. All representatives of subsections *Albovireya*, *Euvireya*, *Malayovireya*, *Phaeovireya*, *Siphonovireya* and *Solenovireya* are strongly supported as a clade, 'Euvireya', while the representatives of subsection *Pseudovireya*, are related (in several clades) to 'Euvireya'. Within the 'Euvireya' a broad geographic pattern is inferred with a group endemic to Australia–New Guinea sister to clades of taxa from western and middle Malesia. The ITS phylogeny is not identical to that elucidated by cpDNA, but well-supported nodes from analyses of each of these genomes are not contradictory.

The ITS region may not undergo total homogenisation in section *Vireya* and a more variable nrDNA marker is required to elucidate the relationships between some terminal taxa.

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