Thesis submitted in partial fulfillment of the
M.Sc. in the Biodiversity and Taxonomy of Plants:

A taxonomic review of the yellow-flowered species of *Rhododendron* L. subsection *Maddenia* (Hutch) Sleumer

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20th August 2012

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DECLARATION

I declare that all of the original work presented in this study is my own. All external sources of information have been acknowledged fully by citation of the authors in the text and full publication details listed in the reference section. All illustrations included in this study that were not designed by F. Donald have been acknowledged with regard to the original place of publication. All photographs have been supplied by F. Donald.

Flora Donald

20th August 2012

Frontispiece: *R. burmanicum* x *R. valentinianum* by Stones, M. In: Taylor, G. ed., 1969. *Curtis's Botanical Magazine,* 177(2), p.546.

ABSTRACT

Rhododendron L. subsection *Maddenia* (Hutch) Sleumer primarily contains lepidote species with white flowers but nine (including one potentially new species) are yellow-flowered. The aim of this study was determine if these species were taxonomically distinct and formed a monophyletic subgroup within the subsection. These aims were investigated by the morphological characterisation of 64 herbarium specimens, including scanning electron microscopy of 15 specimens, and geo-referencing specimens to visualise the biogeographic relationships of the species. Twenty-six sequences of the *mat*K chloroplast region representing 17 taxa were generated using the Qiagen method. This data matrix was expanded by including *Rhododendron* sequences from GenBank and analysed using maximum parsimony and maximum likelihood phylogenetic techniques. These analyses found all eight yellow-flowered species investigated to be justifiable and a taxonomic account was written to present these findings. Three yellow-flowered species with crenulate leaf margins did not conform to the monophyletic clade of yellow-flowered taxa within subsection *Maddenia*. Flower colour is thought to have evolved several times within the subsection and is not necessarily shared by closely related species. Preliminary evidence suggested that *Rhododendron vanderbiltianum* Merr., sometimes included in section *Schistanthe* Schltr., may in fact belong in section *Rhododendron* subsection *Maddenia*.

ACKNOWLEDGEMENTS

I have so many people to thank for generously sharing their time in order to help me complete this research project:

Dr David Chamberlain for devising the project title, supervising and editing the work and sharing his extensive knowledge of *Rhododendron*.

Dr Richard Milne for his helpful comments on a number of the chapters presented here.

Dr Michael Möller for patiently sharing his expertise in conducting phylogenetic analyses and for making the undertaking seem much less daunting!

Peter and Kenneth Cox of Glendoick nurseries for allowing me access to their amazing *Rhododendron* collection.

Dr Laura Forrest, Dr Michelle Hollingsworth and Ruth McGregor for teaching DNA protocols, support in sequence editing and helping make the lab environment a little less stressful!

Maria Chamberlain for driving me all the way to Logan Botanic Garden and Glendoick!

Dr George Argent for sharing some of his vast knowledge of vireyas.

Melissa Simpson and Steve McNamara at the National Trust for Scotland for collecting leaf material of *R. valentinianum* for DNA sequencing.

Frieda Christie for help with SEM work.

Tony Conlon for assisting with the collection and drying of material from RBGE.

Adele Smith, Suzanne Cubey and Fiona Inches for help with BGBase and the creation of new specimens.

Leiden Nationaal Herbarium for lending me many specimens and allowing me to (carefully!) destructively sample some of them.

Finally I must thank my parents for their continual support, encouragement and enthusiasm about all things botanical and my sister for distracting me when necessary! Special thanks are also due to my grandmother without whose financial assistance I would never have realised my dream of studying rhododendrons at RBGE!

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ABBREVIATIONS

ANOVA	analysis of variance
bp	base pairs
CI	consistency index
cpDNA	chloroplast DNA
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
ddNTP	dideoxynucleotide
E	Royal Botanic Garden, Edinburgh, herbarium
GIS	geographic information systems
K	Royal Botanic Garden, Kew, herbarium
L	Nationaal Herbarium Nederland - Leiden
MP	maximum parsimony
ML	maximum likelihood
PCR	polymerase chain reaction
RBGE	Royal Botanic Garden, Edinburgh
RI	retention index
RNA	ribonucleic acid
Sect.	section
SEM	scanning electron microscope
Subsect.	subsection
subsp.	subspecies
SZ	Sichuan University herbarium
TBR	tree-bissection-reconnection
var.	variant

CHAPTER 1: INTRODUCTION

1.1 GENERAL OVERVIEW

The introduction of *Rhododendron* L. (Ericaceae) species into British gardens has had a profound and enduring effect. The legacy of introductions from the Himalayas and SE Asia during the mid 19th-20th centuries can be spectacularly observed along the length and breadth of the United Kingdom in both public and private collections, from Cornish gardens like Trewithen to those in the Scottish Highlands such as Inverewe. The attraction of rhododendrons to Victorian and Edwardian growers is easily understood given their large, brightly coloured inflorescences that are luxuriant during the flowering season and their vegetative characters such as colourful bark, buds and leaf indumentum that add interest outside of the flowering season. In addition, many species form large, dense shrubs, ideal for creating shelter belts and diversifying arboretums. Furthermore, the floristic diversity of rhododendrons created lucrative business opportunities for nurserymen and plant breeders to supply specialist collectors.

Current estimates suggest there are over 1,000 species of *Rhododendron* (Chamberlain *et al*., 1996). The genus occupies a wide geographical area extending from isolated pockets on the E and W Coast of N America, the Caucasus, NE Turkey and NE Asia (Japan, North and South Korea) to its centre of diversity in SE Asia and the Himalayas (Sleumer, 1958). Tectonic activity resulting in the uplift of the Himalayan mountain chain and the geographic isolation of islands in SE Asia facilitated extensive adaptive radiation of *Rhododendron* in these areas in response to niche creation and the isolation of populations (Milne, 2004; Milne *et al.*, 2010). Species barriers within *Rhododendron* are generally weak and hybridisation between even distantly related species is common (Chamberlain, 1982; Ma *et al*., 2010; Sleumer, 1966). Whilst this diversity has ensured the ecological and commercial success of the genus, it has caused much taxonomic deliberation over species concept definitions and the development of an appropriate classification system for *Rhododendron*.

1.2 THE CLASSIFICATION OF ERICACEAE

Plant taxonomy is a fundamental science. Understanding ecosystems, their components, functions and interactions would be impossible without the ability to recognise and communicate about a named entity. Delimiting the boundaries of that entity and how it relates to those which are similar is the pursuit of taxonomists.

Taxonomic process has undergone a rapid transformation during the last decade brought about by the increased availability and reliability of molecular techniques used to understand species relationships. The Angiosperm Phylogeny Group (APG) system currently used to classify plant species was developed by sequencing comparable genomic regions from species representing every known plant family (APG, 1998). From this the progression of angiosperm evolution could be determined and distilled into orders composed of families that formed monophyletic clades (APG, 1998). The principal of monophyly underlies all taxonomy but has to be used discriminatingly. An overly fragmented classification system that fails to reflect any morphological synapomorphies would not be a useful tool for communicating about species. Taxonomy, therefore, relies on the inclusion of data from all branches of research whether molecular, chemical, morphological or ecological in order to develop robust classification systems.

According to APG III the order Ericales is included in the basal asterids and consists of 23 families (poorly understood in terms of morphological synapomorphies), 353 genera and more than 11,000 species (Chase *et al.,* 2009; Schönenberger *et al.*, 2005). Ericaceae forms a monophyletic clade with Cyrillaceae, Clethraceae, Actinidiaceae, Sarraceniaceae and Roridulaceae (Anderberg *et al.,* 2002; Schönenberger *et al.*, 2005). 40% of genera found in the Ericales occur in Ericaceae, which is represented on every continent except Antarctica (Stevens, 1971). Synapomorphies include sympetaly, diplostemony, unitegmic ovules and cellular endosperm formation (Schönenberger *et al.*, 2005; Stevens, 1971). Kron *et al.* (2002) used *nr*18s, *rbc*L and *mat*K sequences to construct a phylogeny representing relationships within Ericaceae. Seven monophyletic subfamilies were determined (Figure 1). Ericoideae was found to be derived compared to Arbutoideae contrary to prior hypotheses postulating Ericoideae to be the most primitive lineage in Ericaceae (Kron, 1997; Kron *et al.*, 2002).

Within Ericoideae, *Rhododendron* was found to form a monophyletic subclade with *Therorhodion* (Maxim.) Small, *Ledum* L. and *Menziesia* Sm. (Kron *et al.*, 2002).

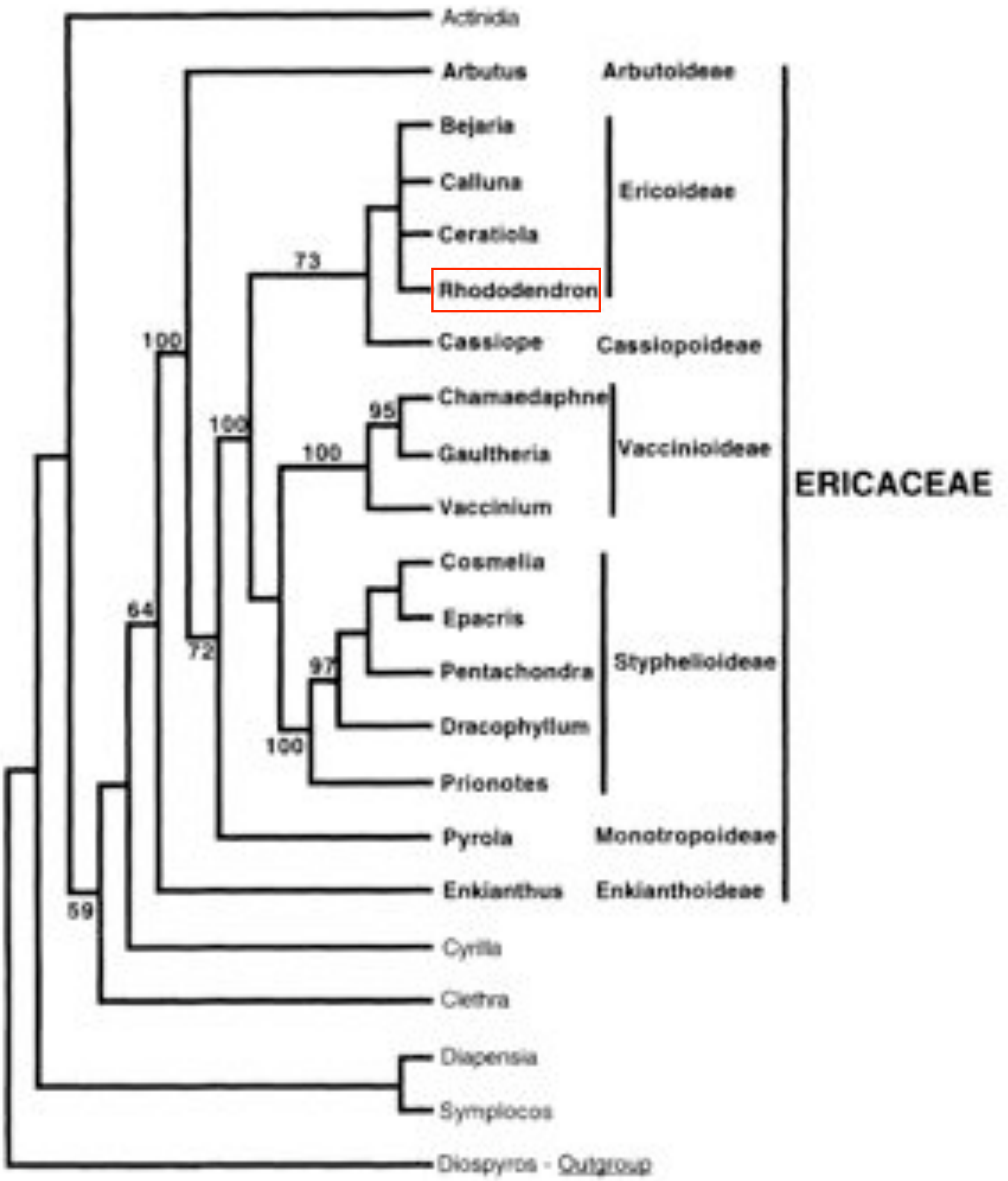


Figure 1: Subfamilies within Ericaceae determined from *nr*18s, *rbc*L and *mat*K DNA sequences for 22 taxa from Ericales. Phylogeny is strict consensus of 3 trees, bootstrap values given above or below branches and taxa in bold are representatives of Ericaceae. Placement of *Rhododendron* highlighted in red. After Kron *et al.*, 2002.

1.3. THE GENUS *RHODODENDRON* L.

1.3.1 A brief taxonomic history of *Rhododendron* L.

The name *Rhododendron* was published by Linnaeus in the first volume of the *Species Plantarum* with five different species (von Linné, 1753). Accounts of new species accumulated gradually over the next 150 years but remained below 100 until the botanical wealth of Asia was discovered and explored. Successive expeditions to China (predominantly Yunnan and Sichuan provinces) conducted by Forrest (1904-1952), Kingdon-Ward (1909-1956), Farrer (1914-1920), Rock (1920-1949) and Ludlow and Sherriff (1933-49) revealed hundreds of new species (Davidian, 1982; RBGE, 2012; Smithsonian Libraries, 2012). Many exotic specimens were also collected from SE Asia where approximately 300 species of endemic vireya rhododendrons occurred (Sleumer, 1966). Professor Bayley Balfour, the then regius keeper of the Royal Botanic Garden, Edinburgh (RBGE) and mentor to George Forrest, recognised the need to quickly devise a classification system for *Rhododendron* in order to cope with the sudden influx of specimens and assist growers and collectors in finding and cultivating new species (Cullen, 1980). The system he developed was aimed, therefore, at horticulturalists and often used cultivated material to describe new species. Similar species were grouped into series named after representative species. Series were not arranged hierarchically or according to shared common ancestry and were geographically biased, omitting species found outside the collecting localities of the explorers listed above (Cullen, 1980). Despite these flaws and the assertion by Bayley Balfour that his system was a temporary solution to the problem of *Rhododendron* taxonomy, this classification system persisted for many decades.

A more robust taxonomic classification had already been devised by Maximowicz (1870) who arranged species into sections based on the position of the inflorescence and the shape of the corolla. As fewer than 100 species were included in this system, it failed to achieve the widespread influence exerted by the Balfourian system and was largely overlooked until Sleumer (Rijksherbarium, Leiden) used it as a basis for his taxonomic review of *Rhododendron* in 1949 (Sleumer, 1980a). Sleumer adopted the use of subgenera, sections and subsections to create a hierarchical classification that brought together species information generated from a wide variety of different sources, including Balfourian series names where taxonomically appropriate (Sleumer, 1980b). Sleumer's system did not immediately supercede the Balfourian classification but gradually gained support, particularly after the International Rhododendron Conference convened in 1978. The conference was attended by taxonomists and horticulturalists mandated to investigate branches of research useful in developing a universal classification system for *Rhododendron* including studies of chemical taxonomy (Evans *et al*., 1980) and seed morphology (Hedegaard, 1980).

The classification currently in use was adapted by Cullen (1980) and Chamberlain *et al.* (1996) drawing together the findings of numerous morphological studies to update the classification published by Sleumer (1949 as cited by Sleumer, 1980b). Eight subgenera are recognised in this classification, defined using the characters displayed on Table 1. One of the most important characteristics is the position of the inflorescence buds: terminal in subgenus *Pentanthera* (G. Don) Pojarkova and *Hymenanthes* (Blume) K. Koch; lateral in subgenus *Therorhodion* (Maxim.) Drude, *Azaleastrum* Planch. ex K. Koch, *Mumeazalea* (Sleumer) W. R. Philipson and M. N. Philipson and *Candidastrum* Franch.; and arising from the same bud scales as the leaves in subgenus *Tsutsusi* (Sweet) Pojarkova. Another distinguishing character is the possession of scales on the leaves and inflorescences (subgenus *Rhododendron*).

Table 1: List of *Rhododendron* subgenera and important distinguishing characters. (Chamberlain 1982; Cox and Cox, 1997; Cullen 1980; Goetsch *et al.,* 2005).

Subgenus	**Characters**
Azaleastrum Planch. ex K. Koch	Evergreen shrubs. Inflorescence lateral. Leaves elepidote.
Candidastrum Franch.	Deciduous shrub. Leaves elepidote. Inflorescences axillary, scattered along branchlets.
Hymenanthes (Blume) K. Koch	Evergreen shrubs. Leaves elepidote. Inflorescences terminal.
Mumeazalea (Sleumer) W. R. Philipson and M. N. Philipson	Deciduous shrub. Leaves elepidote. Flowers lateral, solitary.
Pentanthera (G. Don) Pojarkova	Deciduous shrubs. Leaves elepidote. Inflorescences terminal.
Rhododendron L.	Leaves lepidote. Inflorescences terminal or axillary.
Therorhodion (Maxim.) Drude	Deciduous shrubs. Leaves elepidote. Inflorescences lateral.
Tsutsusi (Sweet) Pojarkova	Vegetative and inflorescence buds develop within the same bud scales.

Subgenus *Rhododendron* contains most of the lepidote (scale-bearing) species (Table 1). The former genus *Ledum* L. has been subsumed into *Rhododendron* and contains lepidote species, shown to be monophyletic with subgenus *Rhododendron* using nuclear but not chloroplast sequence data (Gao *et al*., 2002; Goetsch *et al*., 2005; Kron and Judd, 1990 and Kurashige *et al*., 2001). Subsection *Ledum* is, therefore, currently unplaced to subgenus. Breeding experiments have demonstrated hybridisation between lepidote and elepidote species is infrequent, supporting a distinction between subgenus *Rhododendron* and all of the other subgenera (Cullen, 1980).

Subgenus *Rhododendron* is divided into three sections (Table 2). Section *Pogonanthum* Aitch. & Hemsl. is characterised by lacerate scales and condensed terminal inflorescences (Table 2). Section *Schistanthe* Schltr. was previously known as section *Vireya* (Blume) Copel.f. so species are often informally referred to as ‘vireyas’. This section is unique in lacking blue pigments and in having tailed seeds and idioblast cells in the leaf (Table 2). Section

Rhododendron is morphologically variable, including taxa with tubular, campanulate or funnel-shaped flowers and crenulate, undulate or entire leaf scales (Cullen, 1980). These species are distributed between 27 subsections, one of which is the focus of this study: subsection *Maddenia* (Hutch.) Sleumer.

Table 2: List of sections in subgenus *Rhododendron* and important distinguishing characters (Argent, 2006; Cullen, 1980).

Section	**Characters**
Rhododendron L.	Leaves evergreen or deciduous. Scales entire, crenulate or undulate. Inflorescences terminal or axillary. Seeds ± winged, finned.
Pogonanthum Aitch. & Hemsl.	Leaves evergreen. Scales lacerate. Inflorescences terminal and condensed. Seeds unwinged, fins obscure.
Schistanthe Schltr.	Leaves with idioblast cells. Scales stellate, substellate or entire. Inflorescences terminal. Seeds tailed.

1.3.2 Subsection *Maddenia* (Hutch.) Sleumer

Rhododendron maddenii Hook.f. was discovered in Cheungtong in Sikkim by Joseph Hooker who named it after Major Madden, his friend in the Sikkim Civil Service (Hooker, 1849). When Bayley Balfour grouped all the large leaved, lepidote rhododendrons from India and China into a series, *R. maddenii* was used as the type species. The first taxonomic review of the Maddenii series was conducted by Hutchinson in 1919 who was at that time working as the Herbarium Assistant for India at the Royal Botanic Garden, Kew. Characters used by Hutchinson (1919) to delimit the series included the epiphytic habit of many species, evergreen leaves with a linear, obovate or oval shape that were often fringed with bristles. Leaves were always lepidote and papillate, and scales were often also found along the centre of the corolla lobes. The series was then further divided into three subseries based on the number of stamens, petiole shape and scale density (Hutchinson, 1919). The taxonomy of the group was revisited by Sleumer in 1958 who re-classified it as subsection *Maddenia*. Sleumer (1958) criticised the use of scale density and position to delimit species but offered few alternative characters.

The most recent revision was conducted by Cullen (1980) who included 36 species in the subsection. Cullen (1980) recognised new species where a minimum of two independent characters were present and a different geographical area was occupied. Hutchinson (1919) noted the broad geographic distribution of the series from Bhutan and the Indian provinces of Sikkim, Assam and Manipur, through S. China (Yunnan and Szechuan) to Myanmar and Thailand in SE Asia and remarked that many species were confined to one of these areas. This suggests speciation has occurred through the geographical isolation of species on Asian inselbergs accounting for the character variability exemplified in this subsection.

Cullen (1980) broadly divided the subsection into four informal alliances, which are summarised on Table 3. The Maddenii Alliance only contained *R. maddenii* which is morphologically variable in leaf shape, stamen and locule number and is the only species of subsection *Maddenia* found to exhibit polyploidy (Cubey, 2000). The Megacalyx Alliance was also monospecific, containing only *R. megacalyx* Balf.f. & Ward, which is unique in subsection *Maddenia* because the pedicels and calyx are covered in a frost-like powder (pruinose) (Cullen, 1980). Species with large, deeply lobed calyces, stamens varying in number from 10-15, divaricate pedicels when fruiting and capsules exceeding the length of the sepals were assigned to the Dalhousiae Alliance (Cullen, 1980). The remaining species were included in the Ciliicalyx/Johnstoneanum Alliance, sharing adaxially impressed midribs and rim-like calyces.

Table 3: List of Alliances in subsection *Maddenia* including the characters which define them, the species contained in each Alliance and the flower colour of each species. (Argent, 2006; Cullen, 1980).

Alliance	Characters	Species	Flower Colour
Maddenii	Calyx large, 5-lobed, glabrous; Stamens 17-35; Ovary 8-12 locular.	*R. maddenii* Hook.f. ssp. *maddenii* ssp. *crassum* (Franch.)	White flushed pink, yellow blotch
Megacalyx	Leaf scales sunken in pits; Pedicels and calyx pruinose; Stamens 10; Ovary 5-locular.	*R. megacalyx* Balf.f. & Kingdon-Ward	White, rarely flushed pink
Dalhousiae	Midrib raised adaxially; Calyx large, deeply lobed; Stamens 10-15; Ovary 5-locular; Capsule longer than sepals.	*R. dalhousiae* Hook.f. *var. dalhousiae* *var. rhabdotum* (Balf.f. & R. E. Cooper) Culllen *R. excellens* Hemsl. & E. H. Wilson *R. kiangsiense* Fang *R. levinei* Merr. *R. liliiflorum* H. Lév. *R. lindleyi* T. Moore *R. nuttallii* T. J. Booth ex Nutt. *R. taggianum* Hutch.	White with yellow blotch White White White White White, yellow blotch White, yellow blotch White, yellow blotch

Alliance	Characters	Species	Flower Colour
Ciliicalyx/Johnstoneanum	Midrib impressed adaxially; Calyx usually small, loriform; Stamens 10; Ovary 5-locular.	**Subgroup a:** styles impressed into the ovary	
		R. amandum Cowan	Yellow
		R. burmanicum Hutch.	Yellow
		R. changii (Fang) W. C. Fang	Yellow
		R. ciliatum Hook.f.	White, flushed pink
		R. crenulatum Hutch. ex Sleumer	Yellow
		R. cuffeanum Craib ex Hutch.	White, yellow blotch
		R. fletcherianum Davidian	Yellow
		R. formosum Wall.	White flushed pink ± yellow
		R. scopulorum Hutch.	White flushed pink ± yellow
		R. valentinianum Forrest ex Hutch. var. *valentinainum* var. *oblongilobatum* R. C. Fang	Yellow
		R. vanderbiltianum Merr.	Yellow
		R. ciliipes Hutch.	White, green blotch
		R. dendricola Hutch.	White, yellow/orange/ pink/green blotch
		R. johnstoneanum Watt ex Hutch.	White, flushed pink, yellow blotch
		R. rufosquamosum Hutch.	White
		R. walongense Kingdon-Ward	White, green blotch
		Subgroup b: styles tapered into the ovary	
		R. carneum Hutch.	Pink
		R. ciliicalyx Franch.	White or pink
		R. fleuryi Dop	White, yellow lines
		R. horlickianum Davidian	White, flushed pink, yellow
		R. leptocladon Dop	Yellow
		R. ludwigianum Hosseus	White and pink
		R. lyi H. Lév.	White
		R. pachypodum Balf.f. & W. W. Sm.	White, yellow blotch
		R. pseudociliipes Cullen	White, flushed pink
		R. roseatum Hutch.	White, flushed pink, yellow
		R. surasianum Balf.f. & Craib	Pale pink
		R. veitchianum Hook.f.	White, yellow blotch
		R. yungchangense Cullen	White, flushed pink

1.3.3 Taxonomic problems in subsection *Maddenia*

The majority of species within subsection *Maddenia* have white flowers ± pink or yellow blotches inside the corolla tube. However, eight of the currently recognised species have wholly yellow flowers: *R. amandum* Cowan, *R. burmanicum* Hutch., *R. changii* (Fang) W. P. Fang, *R. crenulatum* Hutch. ex Sleumer, *R. fletcherianum* Davidian, *R. leptocladon* Dop, *R. valentinianum* Forrest ex Hutch. and *R. vanderbiltianum* Merr.. Studies of floral pigmentation have shown this is produced by a flavonol called gossypetin (Harborne and Williams, 1971). Gossypetin is limited to a few sections within *Rhododendron* (Harborne, 1969; Spethmann, 1980) and within subsection *Maddenia* is confined to the Ciliicalyx/Johnstoneanum alliance (Table 3) (Cox and Cox, 1997; Cullen, 1980). This implies that species possessing yellow-flowers are likely to be closely related to each other.

The twenty-nine species of the Ciliicalyx/Johnstoneanum Alliance were divided into two subgroups: those with styles tapering into the ovary and those with styles impressed into the ovary (Table 3). All of the species with yellow flowers were included in the subgroup with impressed styles apart from *R. leptocladon*. Cullen (1980) did not recognise *R. leptocladon* as a separate species from the morphologically similar, although white-flowered, species called *R. lyi* H. Lév which has a style that tapers into the ovary. Holland (1997) showed that *R. leptocladon* should be considered as a distinct species from *R. lyi* but did not challenge its placement in the same subgroup. As the use of the style-ovary transition character separated *R. leptocladon* from all the other yellow-flowered species, doubt has been cast as to whether this was an important character with which to group species (Cullen, 1980).

The relationships of the yellow-flowered species in the subgroup with impressed styles are also unclear as a result of taxonomic uncertainty. *R. valentinianum* was collected in the southern Chinese province of Yunnan by George Forrest who named the bright yellow flowered species after a friend, Père Valentin, who assisted his explorations of the region (Hutchinson, 1919). There are two varieties of *R. valentinianum* in its current classification: *var. valentinianum* and *var. oblongilobatum* R. C. Fang. Material collected by Cox and Hutchinson during a 1995 expedition to Yunnan has been cultivated at RBGE as *R. valentinianum var. oblongilobatum*. Review of both living material and herbarium vouchers from this collection has indicated that it may be distinct species, provisionally named *R. valentinioides* (D. F. Chamberlain, February 2012, pers. comm.). Another taxon similar to *R. valentinianum,* called *R. changii,* was collected in Chinese Sichuan and originally included within the *R. valentinianum* species concept as a variety. W. P. Fang determined it to be a species in its own right in 1983. *R. burmanicum* was described from cultivated type material originally collected in Myanmar (Hutchinson, 1919) and also shares morphological similarities with *R. valentinianum*. A review of the species boundaries and discerning characters for this complex group of taxa is clearly needed.

R. fletcherianum was thought by Cullen (1980) to be similar to *R. valentinianum*, whereas Davidian (1982) found it to resemble *R. crenulatum* as both species had crenulate leaf margins. A further species, *R. vanderbiltianum*, was cited as being morphologically very similar to *R. crenulatum* (Argent *et al.*, 2008). *R. vanderbiltianum* is a Sumatran endemic placed in Section *Schistanthe* subsection *Pseudovireya* (C.B.Clarke) Argent by Sleumer (1966) but Argent *et al.* (1998) suggested that it might in fact belong to subsection *Maddenia*. A review of the characters shared by these three species needs to be undertaken to determine if they should be included in subsection *Maddenia* and if they are closely related to the yellow-flowered species with entire leaf margins.

Much molecular work has successfully been conducted within *Rhododendron*, particularly with regard to high level relationships between subgenera and sections e.g. section *Tsutsusi* using chloroplast and nuclear DNA (Kron and Powell, 2009) and section *Schistanthe* using chloroplast DNA (Brown *et al.*, 2006). As yet, few studies have investigated species phylogenies within subsections (Milne, 2004). Molecular analysis of the yellow-flowered species of subsection *Maddenia* may illuminate the relationships of these morphologically similar taxa and resolve some of the taxonomic uncertainties currently plaguing the subsection.

1.4 AIMS AND HYPOTHESES

The aims of this study are:

1. to establish how many of the yellow-flowered species in subsection *Maddenia* are justified and to provide full descriptions of them based on morphological observations.

2. to establish whether the yellow-flowered species form a monophyletic group within subsection *Maddenia*.

The following species have been investigated:

- *R. burmanicum* Hutch.
- *R. changii* (Fang) W. P. Fang
- *R. crenulatum* Hutch. ex Sleumer
- *R. fletcherianum* Davidian
- *R. leptocladon* Dop
- *R. valentinianum* Forrest ex Hutch.
- *R. valentinioides* sp. nov.
- *R. vanderbiltianum* Merr.

The yellow-flowered species *R. amandum* Cowan was intended to be investigated but unfortunately only one specimen (isotype (E), Ludlow and Sherriff 1365) could be located, prohibiting any analysis. In order to study *R. leptocladon*, its status as a separate species from *R. lyi* had to be determined (Cullen, 1980).

In pursuance of the project aims, the species listed above were investigated by:

1. collecting descriptions of morphological characters, including scale morphology, to develop a character matrix using herbarium specimens deposited at the Royal Botanic Garden, Edinburgh (E) and those loaned by the Nationaal Herbarium Nederland (L).

2. generating DNA sequences of target species, species from each Alliance within subsection *Maddenia* and species from closely related subsection *Boothia* (Hutch.) Sleumer using the *mat*K chloroplast region from material cultivated in Scottish gardens.

3. collecting voucher specimens of the species obtained at step 2 to verify the identification of the material used for DNA sequencing.

4. undertaking phylogenetic analyses of the resulting molecular data in order to establish the genetic relationship shared by the yellow-flowered species.

5. determining if the tentatively named *R. valentinioides* is indeed a different species from *R. valentinianum*.

6. geo-referencing available herbarium specimens to explore the biogeographic relationships of the recognised species.

7. writing full descriptions for the yellow-flowered species based on morphological observations combined with any new insights gained from molecular analysis.

CHAPTER 2: MORPHOLOGICAL ANALYSES

2.1 MATERIALS AND METHODS

2.1.1 Selection and acquisition of material

Morphological characters of yellow-flowered species of subsection *Maddenia* were examined from a total of 64 herbarium specimens: 51 from the herbarium at the Royal Botanic Garden, Edinburgh (E) and 13 were from the Nationaal Herbarium Nederland - Leiden (L) (Appendix 1). Material of wild origin and cultivated specimens was examined so as to note morphological changes occurring under different environmental pressures.

Twenty-four voucher specimens were collected alongside material for DNA analysis from cultivated plants grown at RBGE, Logan Botanic Garden and Glendoick nursery (Section 3.1). Sixteen of these were collected from target species and a further eight from species used as ingroups in the molecular phylogeny (Table 4). The same analysis of morphological characters was conducted on these specimens as for the herbarium specimens. The information collected for target species was then compared to that collected from all of the herbarium specimens to verify the identification of voucher material. The ingroup voucher specimens were verified by comparison with at least three herbarium specimens sourced from different geographical regions.

Table 4: List of plants leaf material and voucher specimens were removed from, including where the plant is cultivated, the corresponding accession number, cultivated collector number, DNA bank number (EDNA) and information pertaining to the wild collection event.

Name	Cultivated Location	Cultivated Collector Number	Accession	EDNA Number	Wild Collector	Year	Country
R. burmanicum	Logan	FLDO6	19802431*A	EDNA12-0025068	Unknown	Unknown	Unknown
R. burmanicum	Logan	FLDO7	20001490*A	EDNA12-0025069	Unknown	Unknown	Unknown
R. changii	Logan	FLDO3	20091273*A	EDNA12-0025065	Cox	1999	China
R. changii	Glendoick	FLDO20		EDNA12-0025365	Cox & Hutchison	1999	China
R. chrysodoron	Logan	FLDO8	20001557*A	EDNA12-0025070	Unknown	Unknown	Unknown
R. ciliatum	RBGE	FLDO27	20031365A	EDNA12-0025359	Bowes Lyon	2003	Bhutan
R. crenulatum	Logan	FLDO4	20091261*A	EDNA12-0025066	Cox	Unknown	Unknown
R. crenulatum	RBGE	FLDO15	20020810*A	EDNA12-0025225	Rushforth	2001	Vietnam
R. crenulatum	Glendoick	FLDO19		EDNA12-0025364	Rushforth	2001	Vietnam
R. dalhousiae	Glendoick	FLDO25		EDNA12-0025369	H.E.C.C.	2002	India
R. fletcherianum	RBGE	FLDO11	19754070*J	EDNA12-0025221	Rock	1932	Tibet
R. fletcherianum	Glendoick	FLDO22		EDNA12-0025366	B.A.S.E.	2000	China
R. johnstoneanum	Glendoick	FLDO26		EDNA12-0025361	H.E.C.C.	2002	India
R. leptocladon	Glendoick	FLDO18		EDNA12-0025363	Rushforth	Unknown	Vietnam
R. leucaspis	RBGE	FLDO10	19271007*A	EDNA12-0025220	Kingdon-Ward	1926	India
R. lyi	RBGE	FLDO17	19840942*C	EDNA12-0025362	Unknown	1984	Unknown
R. maddenii ssp crassum	RBGE	FLDO28	19391033J	EDNA12-0025360	Tse-tsun	1938	China
R. sulfureum	RBGE	FLDO13	20020811*A	EDNA12-0025223	Rushforth	2001	Vietnam
R. valentinianum	NTS Branklyn	BRG1		EDNA12-0025358	Unknown		Unknown
R. valentinianum var. oblongilobatum	Logan	FLDO5	19960621*E	EDNA12-0025067	K.Y.E.	1995	China
R. valentinianum var. oblongilobatum	Logan	FLDO9	19960619D	EDNA12-0025071	K.Y.E.	1995	China
R. valentinianum var. oblongilobatum	RBGE	FLDO12	19960621*F	EDNA12-0025222	K.Y.E.	1995	China
R. valentinianum var valentinianum	Glendoick	FLDO23		EDNA12-0025367	Forrest	1917	China
R. valentinioides	Glendoick	FLDO24		EDNA12-0025368	Cox & Hutchison	1995	China
R. vanderbiltianum	RBGE	FLDO16	19982483*A	EDNA12-0025072	Binney	1997	Indonesia
R. veitchianum	RBGE	FLDO14	19750211*D	EDNA12-0025224	Valder	1975	Thailand

2.1.2 Creation of voucher specimens

Voucher specimens were collected from the same individual as the leaf material collected for molecular analysis (Section 3.1.2). The accession number and location within the garden of each collected plant was recorded together with a brief description of characters pertaining to the general habit, width x height and corolla colour (if flowering) of the shrub. A small branch displaying representative leaves and flowers or capsules (where available) was carefully collected with secateurs so as not to damage the apical meristem of the shrub. The material was arranged in flimsies, layered between foam blotters and stacked in a wooden press that was tightened using straps and weighed down with iron bars. The specimens were left to dry in this way for one week in the RBGE drying room at 30˚C with the blotters changed after three days. Once dry, the specimens were transferred to a chest freezer, maintained at -20˚C for five days to sterilise the material. Collection data was entered into BGBase and labels detailing both the wild and cultivated collection events were printed. The specimens were mounted then deposited in the herbarium (E) as cultivated vouchers of each species.

2.1.3 Morphological characterisation

Characters were selected for analysis using descriptions and protologues obtained for species in subsection *Maddenia* and revised during specimen evaluation. All information was entered in to an Excel spreadsheet (v.12.2.3, Microsoft Corporation, USA). Collector, collector number, collection date, location, altitude (m) and plant habit were recorded from the specimen label. Specimens were then examined under a light microscope to record the characters outlined on Table 5. Entry fields were left blank where characters were lacking or ambiguous as a result of being obscured on the specimen. All measurements were taken using a ruler with cm and mm units. Due to time constraints only one measurement was taken for each character from organs judged to be representative of the specimen. Scale characters were recorded using descriptive categories following the methodology employed by Cubey (2000) so as to collect comparable information from all specimens. It was considered inappropriate to collect scale counts because only those recorded from wild material and leaves of similar ages would be comparable (Cullen, 1980).

Table 5: List of characters and associated methods used to describe specimen leaves.

Leaf character	Method of observation
Petiole length (mm)	Measured from base of leaf to where petiole attaches to stem
Petiole shape	Presence of wings, flattened, narrowed
Petiole pubescence	Presence and type of hairs
Petiole scales	Presence of scales
Leaf shape	Determined using illustrated examples in Davidian (1982)
Leaf apex	Determined using illustrated examples in Davidian (1982)
Leaf base	Determined using illustrated examples in Davidian (1982)
Leaf length (mm)	Measured from the base of the petiole to the lamina apex
Leaf width (mm)	Measured across the widest part of leaf
Leaf margin	Margin entire or crenulate
Leaf margin pubescence	Presence and type of hairs
Adaxial scales	Presence of scales
Abaxial scale density	Dense / intermediate / sparse
Abaxial scale distribution	Regular / touching / overlapping
Adaxial midrib	Prominent, sunken or planate adaxial midrib
Abaxial midrib	Prominent, sunken or planate abaxial midrib
Abaxial midrib pubescence	Presence and type of hairs on abaxial side of midrib
Adaxial primary veins	Visibility of primary veins

Where present, floral and fruiting characters were investigated using light microscopy in the same manner as the vegetative material (Table 6). Organs obscured by the corolla such as the filaments and ovary were described where possible and if, upon review, these characters had not yet been recorded for a species, one floral dissection was performed. Unfortunately, given the paucity of flowering material for some species these characters were sometimes only described from cultivated specimens.

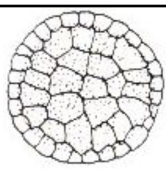
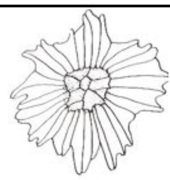
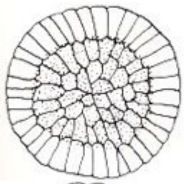
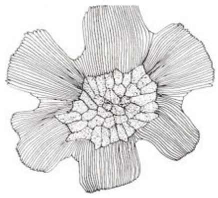
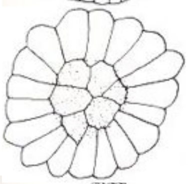
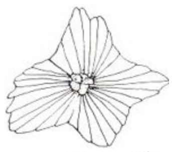
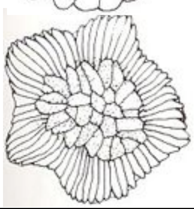
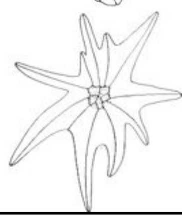
Table 6: List of characters and associated methods used to describe specimen inflorescences and capsules.

Inflorescence / Capsule Character	**Method of observation**
Number of flowers per inflorescence	Observed from persistent pedicels if flowers not present
Pedicel (mm)	Measured from the top of the stem to the base of the calyx
Pedicel pubescence	Presence and type of hairs
Pedicel scales	Presence of scales
Calyx length (mm)	Measured from the top of the pedicel to the tip of the calyx lobe
% calyx to flower length	(calyx length / flower length) x 100
Calyx pubescence	Presence of hairs
Calyx scales	Presence of scales
Corolla colour	Copied from specimen label if recorded
Corolla shape	Determined using illustrated examples in Davidian (1982)
Flower length (mm)	Measured from base of calyx to tip of uppermost corolla lobe
Tube length (mm)	Measured from base of calyx to base of corolla lobe incision
Corolla pubescence	Presence and type of hairs
Corolla scales	Presence of scales
Anther length (mm) (outer whorl)	Measured from tip of filament to top of anther
Anther description	Shape of anther
Filament lengths (mm) (both whorls)	Measured from base of filament to top of anther
Filament description (mm)	Presence, type and distribution of hairs
Style scales	Presence and distribution of scales
Ovary length (mm)	Measured from tip of ovary to base of style
Ovary description	Shape of ovary
Capsule length (mm)	Measured from the base of the pedicel to the capsule lobe apex
Capsule shape	Shape of capsule

2.1.4 Scanning Electron Microscopy

Light microscopy was sufficiently powerful to allow informative observations of scale morphology between subsections to be collected but differences between species within subsection *Maddenia* were difficult to observe. Scanning electron microscopy (SEM) was therefore conducted to study differences in the leaf epidermis between the yellow-flowered species of subsection *Maddenia*. Scales have three components: an inner zone consisting of irregularly sized cells which can be flattened or swollen; a rim that surrounds the centre, composed of elongated cells arranged in a regular pattern, varying in width and dissection between species; and a stalk attaching the scale to the surface of the organ, which is usually short and invisible from above (Cowan, 1950; Cullen, 1980). Cowan (1950) recognised five distinct scale types whereas Seithe (1980) distinguished between eight types (Table 7). Scales may be surrounded by papillae (epidermal cells with elongated outer walls) which are variously found in both subgenera and are “of little diagnostic significance” according to Cowan (1950). The function of scales in *Rhododendron* is still poorly understood, although it has been hypothesised that they secrete essential oils as a defence against insect herbivory (Doss, 1984) or repel water to prevent the waterlogging of stomata (Argent, 1988).

Table 7: Diagrammatic representation of scale types as defined by Seithe (1980).

Scale Type		Scale Type	
Vesicular		Lacerate	
Entire		Pleated	
Crenulate		Substellate	
Undulate		Stellate	

Specimens of wild origin only were selected for SEM study so as to be comparable. Due to the limited availability of material as well as time and financial constraints, a maximum of two specimens were examined per species (Table 8). Approximately 1 cm^2 of leaf material was removed from herbarium specimens and placed on aluminium stubs, which were then coated with platinum using the Emitech K575x Sputter coater. The material was often quite brittle so was further treated with Acheson Electrodag (Agar Scientific, UK) adding another conductive layer to insulate the material. Eight stubs were loaded into the specimen holder which was placed inside the LEOsupra 55VP SEM (Carl Zeiss Microscopy GmbH, Germany) and examined using the SmartSEM Image Navigation system (v.4.7., Carl Zeiss Microscopy GmbH, Germany). One overview and one close-up image was taken for each sample at 150 and 500 x magnification respectively. All images were added to the RBGE SEM analySIS database (Olympus Soft Imaging Solutions GmbH, UK).

The total diameter (mm) and the diameter of the inner zone (mm) of each scale was measured from the 150 x magnification image of each sample using a ruler. Statistical analysis was conducted using R (v.2.1.1., The R Foundation for Statistical Computing, USA). All data were visually assessed for normality (histograms) and equal variance (scatter graphs) prior to analysis and transformations were conducted where appropriate (Section 2.2.2). Data were compiled with one specimen for each species (randomly selected where two were available) and analysed using one-way ANOVA to test the null hypothesis that the mean total scale diameter (mm) and mean proportion of the diameter occupied by the inner zone (total diameter / inner zone diameter (mm)) were the same for each species. One-way ANOVA was further used to ascertain if these measurements varied significantly between the two specimens examined of *R. burmanicum*, *R. leptocladon*, *R. valentinianum var. valentinianum* and *R. vanderbiltianum.*

Table 8: List of herbarium specimens from which leaf material was examined using SEM. - indicates missing data.

Taxon name	Collector	Collector Number	Herbarium barcode	Year	Country
R. burmanicum	Cooper	5975	E00421855	-	Burma
R. burmanicum	Kingdon-Ward	21921	-	1956	Burma
R. changii	Cox & Hutchison	9001	E00087895	1999	China
R. crenulatum	Kerr	21044	L0007415	1932	Laos
R. fletcherianum	B.A.S.E.	9577	E00189931	2000	China
R. leptocladon	Rushforth	4416	E00073365	1993	Vietnam
R. leptocladon	Rushforth	2314	E00039871	-	Vietnam
R. lyi	Rushforth	2137	E00035298	1992	Vietnam
R. lyi	Cavalerie	7825	E00421853	1914	China
R. valentinianum var. oblongilobatum	Chui	53848	E00421857	1956	China
R. valentinianum var. valentinianum	Forrest	24138	E00421856	1924	China
R. valentinianum var. valentinianum	Ogisu	95310	E00053783	1995	Vietnam
R. valentinioides	Cox & Hutchison	7186	E00073235	1995	China
R. vanderbiltianum	de Wilde & de Wilde-Duyfjes	16071	L0442390	1975	Sumatra
R. vanderbiltianum	Argent & Aminin	99154	E00533156	1999	Sumatra

2.1.5 Geo-referencing

Fifty-two specimens were geo-referenced in order to map the distribution of the yellow-flowered species of subsection *Maddenia* (Appendix 2). Where the collection latitude/ longitude (lat/long) had been recorded on the specimen label it was copied to an Excel spreadsheet (v.12.2.3, Microsoft Corporation, USA). If no position had been recorded, location names were entered into Fuzzy Gazetteer (v.2.1, Joint Research Centre of the European Commission, 2012) to obtain lat/long information. This was then plotted on Google Earth (v. 5.2.1 Google Inc., USA) and cross-referenced with any further local information given on the specimen label. A number of specimens collected by Forrest could not be located by the gazetteer, but a further two specimens also collected by Forrest in the same locality had lat/long data recorded. These locations were plotted into Google Earth and the discrepancy between the Google Earth and the specimen altitude recordings was calculated. The specimens without lat/long localities were geo-referenced by subtracting the altitudinal difference, then searching for an area close to the recorded specimens that matched the calculated altitude. All the lat/long data were converted into decimal degrees using the Federal Communications Commission (2012) converter. The information was recorded to two decimal places for an accuracy of 1.11 km, considered to be suitably accurate for this study given the scale of the area over which the species occur. The resulting spreadsheet with species names and lat/long data was converted to a KML file and uploaded into Google Earth from which distribution maps were downloaded.

2.2 RESULTS

2.2.1 Morphological character descriptions

Thorough examination of herbarium specimens of the nine yellow-flowered taxa of subsection *Maddenia* rhododendrons highlighted the morphological diversity encompassed by the group. The only constant leaf characters were lepidote petioles, lepidote midribs and deciduous adaxial scales which often left scars. A summary of consensus vegetative character states for each species is listed on Table 9. Characters important for distinguishing between species are discussed below.

- Petiole length (mm): although this character was dependent on the number and age profile of specimens, some species such as *R. vanderbiltianum* and *R. fletcherianum* had consistently small petioles (1-3 and 3-6 mm respectively) whereas *R. burmanicum* and *R. valentinianum* showed a large range of petiole lengths (5-14 and 3-11 mm respectively).

- Petiole shape: the shape of the petioles was found to be a very important character for distinguishing between taxa. *R. valentinianum* and *R. valentinioides* had round petioles whereas petioles of the morphologically similar *R. changii* were adaxially flattened. *R. fletcherianum* had petioles which were conspicuously winged and so too did *R. leptocladon* and *R. vanderbiltianum* to a lesser degree.

- Petiole pubescence: the majority of species had setose petioles but *R. crenulatum* and *R. vanderbiltianum* had puberulent petioles and *R. leptocladon* petioles lacked hairs entirely. This is was found to be a reliable character separating *R. leptocladon* from *R. lyi* which was determined to have setose petioles.

- Leaf shape: this character was again variable as a result of the age profile of the leaves. The characterisation of leaf shapes is illustrated on Figure 2. Most species had oblong-elliptic leaves (Figure 2C) but *R. leptocladon* and *R. valentinioides* stood out from the other larger-leaved species in having elliptic (Figure 2A) and oblong (Figure 2F) leaves respectively. *R. valentinioides* leaves were also slightly cucullate, splitting when dried. The smaller leaved species *R. crenulatum* and *R. vanderbiltianum* had elliptic leaves.

- Leaf apex: mucronate was the most common character state described for the leaf apex but *R. crenulatum*, *R. leptocladon* and *R. vanderbiltianum* had acute apices whilst *R. burmanicum* had acuminate leaf apices.

- Leaf base: this character sometimes varied on individual specimens but the consensus showed *R. valentinianum*, *R. valentinioides* and *R. vanderbiltianum* to have obtuse leaf bases compared to *R. burmanicum* and *R. changii* which had attenuate bases. The remaining species had cuneate leaf bases.

- Leaf length (mm): most species had leaf lengths within the range of 30-60 mm although *R. fletcherianum* had a more conserved leaf length range of 30-48 mm and the specimen examined of *R. valentinioides* had leaves 50 mm long. *R. crenulatum* had leaves 25-35 mm long and *R. vanderbiltianum* had the smallest leaves (10-23 mm) of the group.

- Leaf margin: an interesting finding was the crenulate leaf margins of *R. crenulatum*, *R. fletcherianum* and *R. vanderbiltianum* (Figure 3B) as this is an uncommon condition in *Rhododendron* (D. F. Chamberlain, February 2012, pers. comm.). The remaining species all had entire leaf margins.

- Leaf margin pubescence: *R. fletcherianum*, *R. changii*, *R. valentinianum* and *R. valentinioides* all had leaf margins fringed with setose hairs (Figure 3A and 3B). *R. crenulatum* and *R. leptocladon* had hairless margins and *R. burmanicum* had sparsely loriform leaf margins (Figure 3A) on immature leaves which were later deciduous.

- Abaxial scale density: *R. crenulatum* and *R. fletcherianum* had a sparse covering of scales, *R. vanderbiltianum* and *R. valentinioides* had noticeable but thinly spread scales on the abaxial leaf surface whereas *R. burmanicum*, *R. changii* and *R. valentinianum* leaf surfaces were densely covered with scales.

- Abaxial scale distribution: two species had scales which conspicuously overlapped (*R. burmanicum* and *R. valentinianum*) whereas three species had scales whose rims sometimes touched (*R. changii, R. leptocladon, R. vanderbiltianum*) and three species had well-spaced scales (*R. crenulatum, R. fletcherianum, R. valentinioides*). These states were often difficult to assign given the continuous nature of the variable but were relatively consistent for each species.

- Abaxial midrib: the majority of species had midribs which were sunken adaxially (Figure 2E) and therefore abaxially protruded (Figure 2C). However, the midrib in leaves of *R. crenulatum*, *R. fletcherianum* and *R. vanderbiltianum* were planate on both leaf surfaces (Figure 2B).

- Adaxial primary veins: the primary veins of *R. burmanicum* and *R. changii* were difficult to observe whereas they could be seen adaxially in *R. leptocladon*. Primary veins were adaxially impressed in *R. fletcherianum*, *R. valentinianum* and especially so in *R. valentinioides* (Figure 2F), whereas in *R. crenulatum* and *R. vanderbiltianum* the veins were adaxially raised.

Table 9: Vegetative character consensus states that varied between eight yellow-flowered species of *Rhododendron* subsection *Maddenia*. N indicates number of specimens examined, () is number of *R. valentinianum var. oblongilobatum* specimens examined, * state applies to immature leaves only. A = acute, M = mucronate.

Character	*R. burmanicum*	*R. changii*	*R. crenulatum*	*R. fletcherianum*	*R. leptocladon*	*R. valentinianum*	*R. valentinioides*	*R. vanderbiltianum*
N	8	3	3	8	13	30(5)	3	11
Petiole length (mm)	5-14	3-4	2-4	3-6	1-9	3-11	10	1-3
Petiole shape	narrowly winged	flattened	flattened + winged	flattened + winged	narrowly winged	rounded	rounded	narrowly winged
Petiole pubescence	setose	setose	puber-ulent	setose	glabrous	setose	setose	puber-ulent
Leaf shape	obovate/ oblong-elliptic	obovate/ oblong-elliptic	elliptic	elliptic/ oblong-elliptic	elliptic	oblong-elliptic	oblong	elliptic
Leaf apex	A	M	A	M	A	M	M	A
Leaf base	attenuate	attenuate	cuneate	cuneate/ obtuse	cuneate	obtuse	obtuse	obtuse
Leaf length (mm)	40-70	30-50	25-35	30-48	45-65	32-60	50	10-23
Leaf margin	entire	entire	crenulate	crenulate	entire	entire	entire	crenulate
Leaf margin pubescence	loriform*	setose	glabrous	setose	glabrous	setose	setose	glabrous
Abaxial scale density	dense	dense	sparse	sparse	inter-mediate	dense	inter-mediate	inter-mediate
Abaxial scale distribution	overlap	touching	regular	regular	touching	overlap	regular	touching
Abaxial midrib	raised	raised	planate	planate	raised	raised	raised	planate
Adaxial primary veins	inconspi-cuous	inconspi-cuous	raised	impressed	± conspi-cuous	impressed	impressed	raised

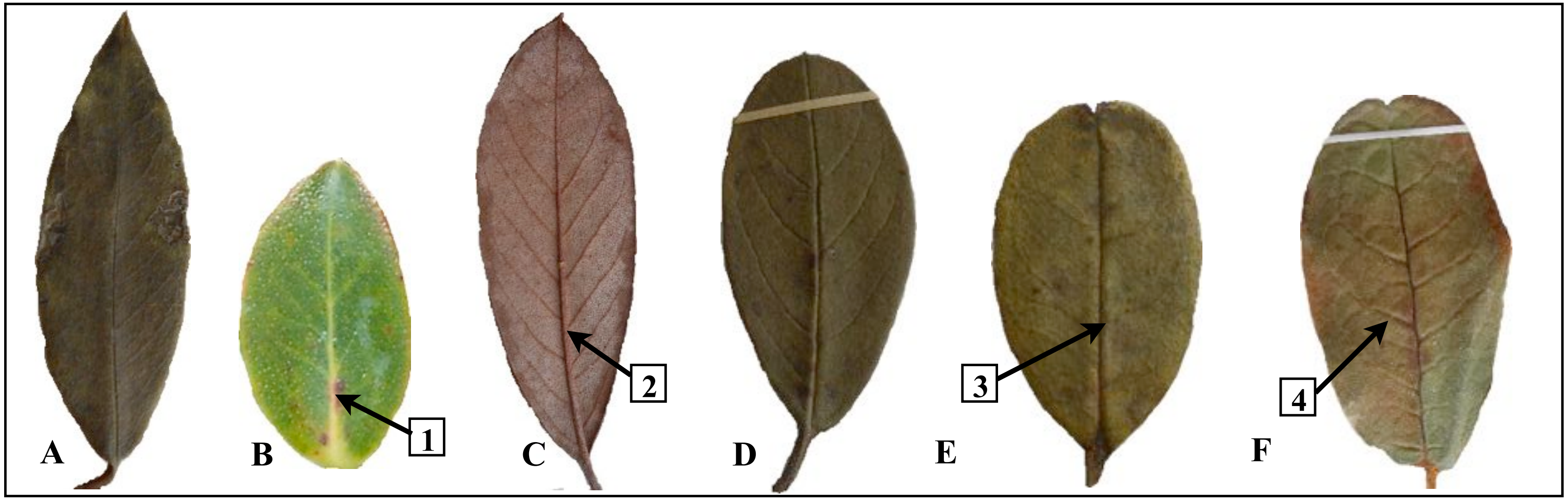


Figure 2: Characterisation of leaf shape, midribs and primary veins. **A.** Elliptic leaf shape **B.** Elliptic leaf shape **1.** Planate midrib **C.** Oblong-elliptic leaf shape **2.** Abaxially protruding midrib **D.** Obovate leaf shape **E.** Ovate leaf shape **3.** Adaxially impressed midrib **F.** Oblong leaf shape **4.** Impressed primary veins

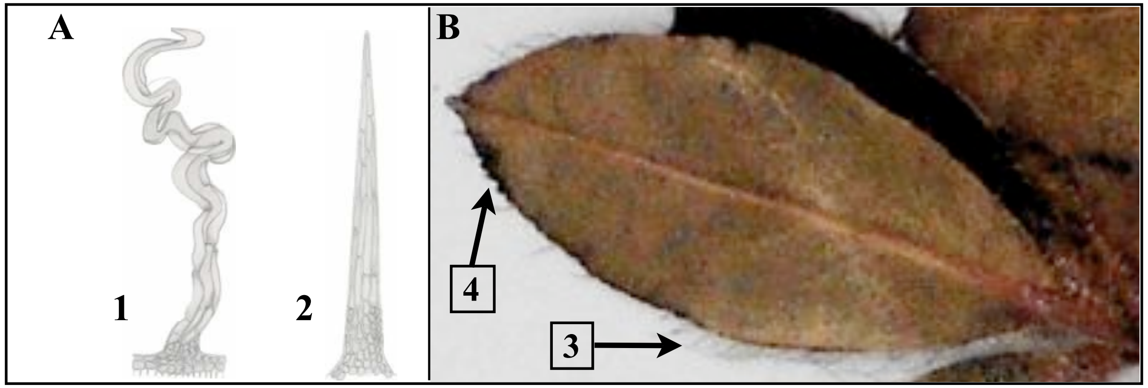


Figure 3: Characterisation of hairs and leaf margins. **A.** Hair types: **1.** loriform **2.** setose. **Source**: Henning's Rhododendron & Azalea pages, 2012 **B.** Leaf of *R. fletcherianum* demonstrating: **3.** setose hairs fringing leaf margin **4.** crenulate leaf margin.

A summary of states obtained for each species for floral and fruiting characters that varied between species is displayed on Table 10. Scales on the calyces and capsules were constant for all species. All species apart from *R. fletcherianum* had bands of scales running down the centre of each corolla lobe from the tip to the base of the corolla tube (Figure 4A), which was also puberulent. *R. fletcherianum* was also the only species to possess loriform hairs at the ovary apex and *R. leptocladon* was the only species examined where the ovary tapered into the style instead of being impressed into it.

- Number of flowers per inflorescence: most species had 2-4 flowers in each inflorescence but *R. burmanicum* was found to have a minimum of four flowers on every specimen examined.

- Pedicel length (mm): the majority of species had pedicels 5-12 mm long with the exceptions of *R. burmanicum* and *R. changii* which had pedicels 7-15 and 4-8 mm long respectively. However, when the length of the pedicel was considered in relation to the length of the flower, *R. crenulatum* (9-12/20-30 mm) and *R. vanderbiltianum* (5-11/9-19 mm) were seen as being long pedicellate, and *R. changii* (4-8/ 35-40 mm) as short pedicellate compared to the other species.

- Pedicel pubescence: all of the species had hairless pedicels apart from *R. fletcherianum* (hispid pedicels) and *R. valentinianum var. valentinianum* which sometimes had loriform hairs on the pedicels distinguishing it from *R. valentinianum var. oblongilobatum* where the hairs were always absent.

- Calyx length: species could be broadly divided into two groups - those which have a small calyx (1-3 mm) (Figure 4C) and those which have a large calyx (5-10 mm) (Figure 4B). *R. burmanicum*, *R. crenulatum*, *R. leptocladon* and *R. vanderbiltianum* had small, rim-like calyces whereas *R. changii, R. fletcherianum*, *R. valentinianum* and *R. valentinioides* had well-developed calyces.

- Calyx pubescence: *R. leptocladon* had glabrous margins in contrast to *R. lyi* that was found to have loriform calyx margins. *R. changii* also had a hairless calyx along with *R. valentinianum var. oblongilobatum*. All remaining species and *R. valentinianum var. valentinianum* were found to have calyces fringed with loriform hairs.

- Corolla shape: three corolla shapes were observed from the specimens. *R. crenulatum* was campanulate (Figure 5B), *R. burmanicum, R. changii*, *R. valentinianum* and *R. valentinioides* were funnel-campanulate (Figure 5A) and *R. fletcherianum*, *R. leptocladon* and *R. vanderbiltianum* all had wide funnelform corollas (Figure 5C).

- Flower length (mm): *R. vanderbiltianum* had the smallest flowers (9-19 mm) whereas *R. leptocladon* could have the largest flowers (30-65 mm). Most species had flower lengths between these two extremes (30-46 mm) although *R. crenulatum* and *R. valentinianum* could have smaller flowers at the lower end of their range (20-30 and 16-35 mm respectively).

- Corolla lobe / tube ratio: the length of the corolla lobes in relation to the corolla tube was very variable in *R. valentinianum* (0.30-0.90) and *R. crenulatum* (0.54-0.85). In *R. valentinioides* the corolla lobes of the specimen examined were ⅔ longer than the corolla tubes whereas the lobes could be from approximately ⅔ to equal the length of the tube in *R. burmanicum* (0.61-1.00). The lobes of *R. changii* flowers were slightly shorter than the length of the tube (0.85). The species with funnel-shaped corollas lacked a discernible corolla tube.

- Style scales: the position of the scales along the style was very variable between species. Scales were entirely absent from the style in *R. changii*, *R. fletcherianum* and *R. vanderbiltianum* and only covered the base of the style in *R. burmanicum* and *R. valentinianum*. In *R. leptocladon*, scales extended from the ovary along half the length of the style whereas in *R. crenulatum* they covered the whole length of the style.

- Capsule length: *R. fletcherianum* and *R. vanderbiltianum* had small capsules (9 mm) compared to *R. leptocladon*, *R. valentinianum* and *R. valentinioides* (7-16 mm). Unfortunately, no fruiting material was seen of *R. burmanicum*, *R. changii* or *R. crenulatum*.

Table 10: Floral characters and capsule character consensus states that varied between eight yellow-flowered species of *Rhododendron* subsection *Maddenia*. Number of flowering/fruiting specimens examined, () is number of *R. valentinianum var. oblongilobatum* specimens examined.
N flowers[1] = number of flowers per inflorescence. - indicates missing data, n/a is non-applicable,
* state applies to *R. valentinianum var. valentinianum* only.
FC = funnel-campanulate, C = campanulate, F = funnelform.

Character	*R. burmanicum*	*R. changii*	*R. crenulatum*	*R. fletcherianum*	*R. leptocladon*	*R. valentinianum*	*R. valentinioides*	*R. vanderbiltianum*
N flowering/ fruitng	7/0	1/0	2/0	5/1	6/2	19(3)/5	2/2	8/1
N flowers[1]	4-7	2-4	3-4	2-5	2-3	2-4	2	2-5
Pedicel length (mm)	7-15	4-8	9-12	6-10	5-10	5-11	-	5-11
Pedicel pubescence	glabrous	glabrous	glabrous	hispid	glabrous	loriform*	-	glabrous
Calyx length (mm)	1-2	10	1-2	7-9	1-2	5-10	5-6	1-3
Calyx pubescence	loriform	glabrous	loriform	lorifor m	loriform	loriform*	lorifor m	loriform
Corolla shape	FC	FC	C	F	F	FC	FC	F
Flower length (mm)	30-46	35-40	20-30	36-42	30-65	16-35	20	9-19
Corolla / tube length (mm)	0.61-1.00	0.85	0.54-0.85	n/a	n/a	0.30-0.90 (0.45-0.80)	0.67	n/a
Style scales	basal	absent	100% length	absent	50% length	basal	-	absent
Capsule length (mm)	-	-	-	9	13-16	7-15	14-15	9

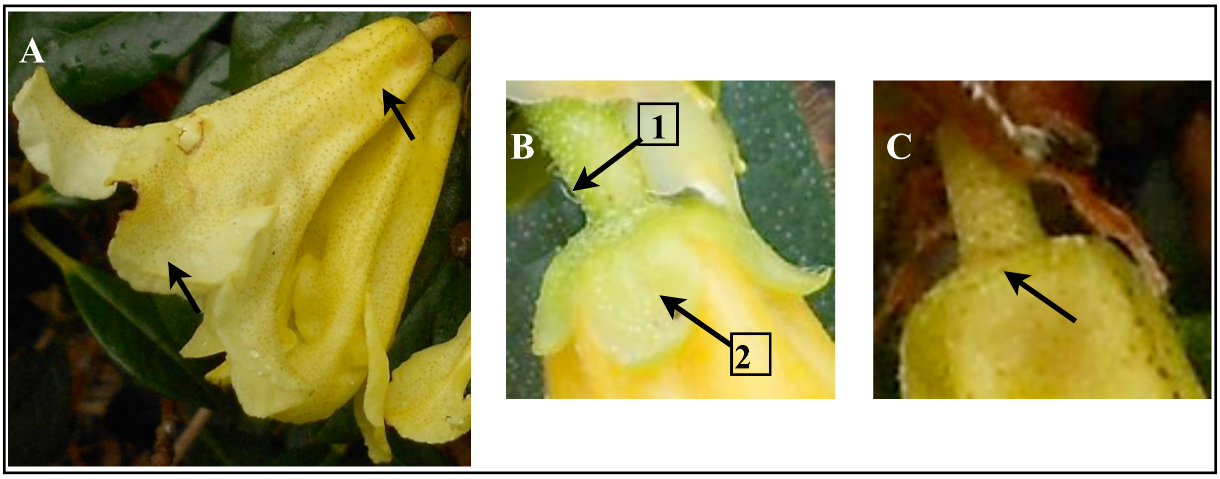


Figure 4: Characterisation of flower scales and calyces. **A.** Corolla scales extending from base of corolla tube to lobe apex **B. 1.** loriform pedicel **2.** Well-developed, deeply lobed calyx **C.** Poorly developed calyx

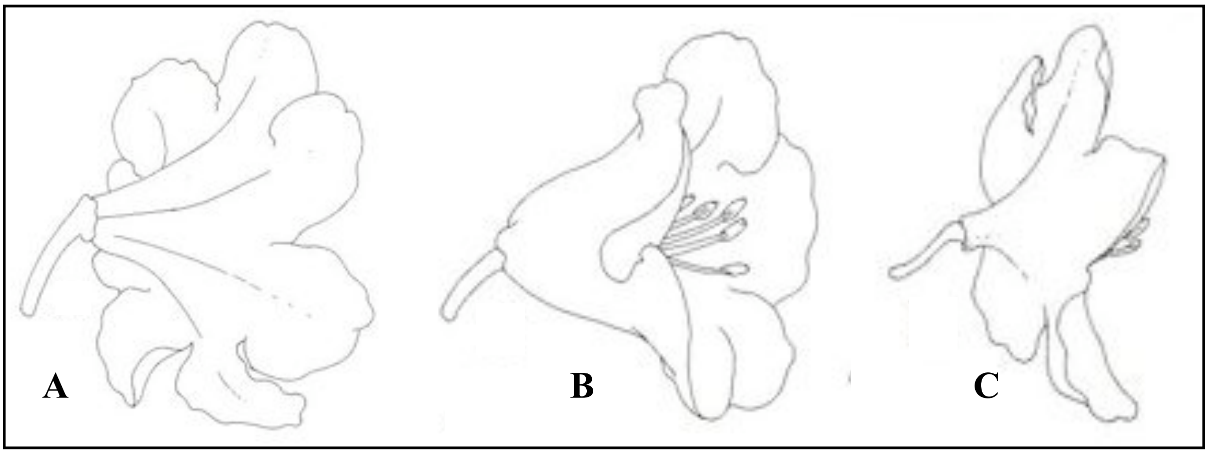
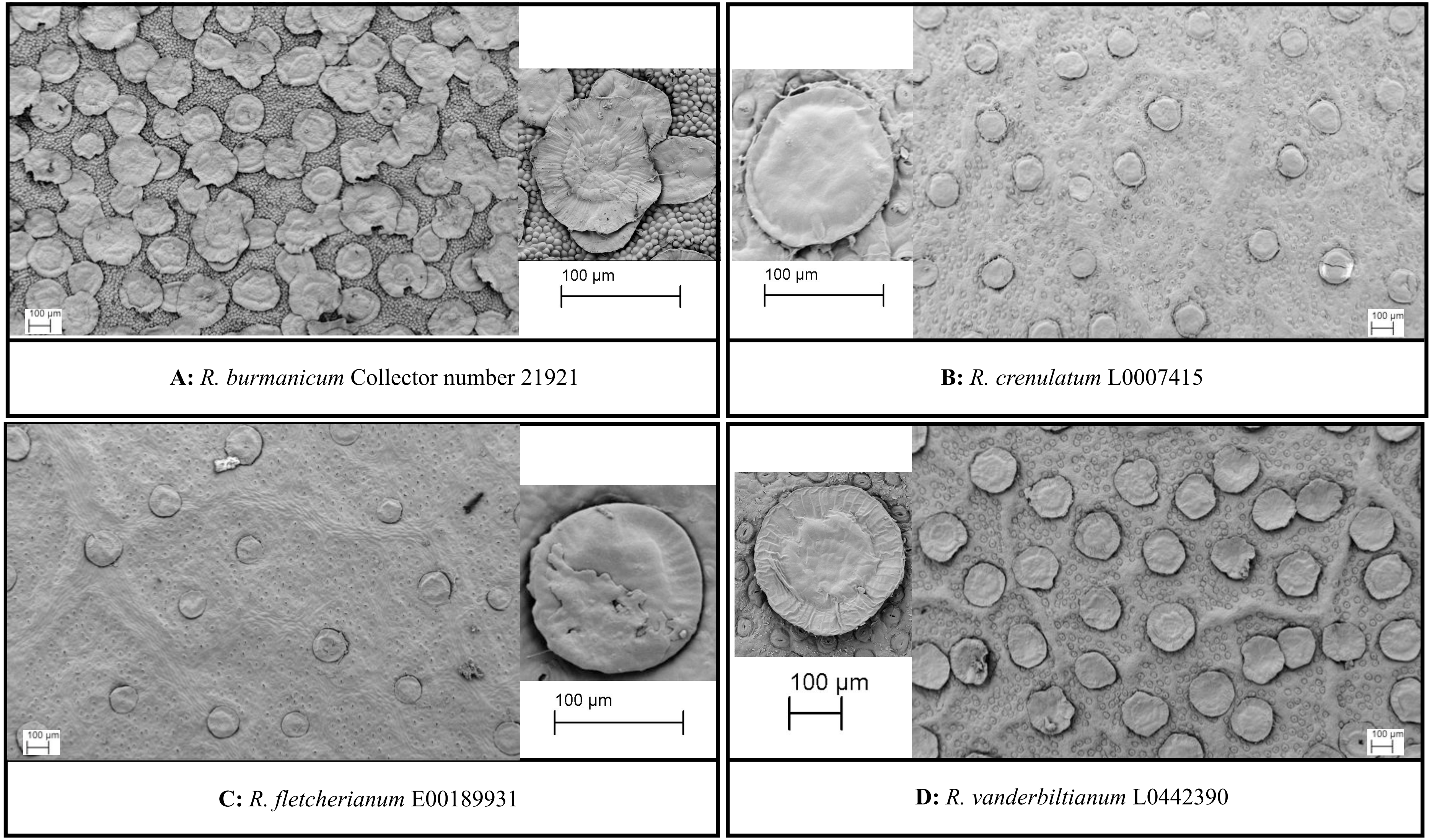


Figure 5: Characterisation of flower shape. **A.** Funnel-campanulate **B.** Campanulate **C.** Funnelform **Source:** Argent *et al*., 1997

In summary, morphological characters can be used to distinguish between all eight species. *R. burmanicum*, *R. changii*, *R. valentinianum* and *R. valentinioides* are the most similar species sharing setose petioles, oblong-elliptic leaves, raised abaxial midribs and funnel-campanulate corollas. Crenulate leaf margins and planate midribs are unique to *R. fletcherianum*, *R. crenulatum* and *R. vanderbiltianum* but are the only characters shared exclusively by these three species.

2.2.2 Scale morphology

A comparison of scanning electron micrographs obtained from an accession of each taxon is displayed on Figure 6. All the scales conformed to Seithe's (1980) description of entire scales, possessing "a narrow or broader entire rim and a large midfield consisting of high cells". *R. crenulatum*, *R. fletcherianum* and *R. vanderbiltianum* (Figure 6B, C and D) were immediately distinguishable by the lack of papillae. The rim of *R. fletcherianum* scales appeared to be rigid compared to the membranous rim of all the other species (Figure 6C). *R. burmanicum* (Figure 6A) scales were conspicuous in having misshapen, overlapping rims, whereas *R. valentinianum* (Figure 6F, G) had upturned rims. Scales of *R. leptocladon* (I) differed from *R. lyi* (J) in having a more irregular arrangement and shorter papillae.



A: *R. burmanicum* Collector number 21921

B: *R. crenulatum* L0007415

C: *R. fletcherianum* E00189931

D: *R. vanderbiltianum* L0442390

Figure 6: Scanning electron micrograph of one accession (herbarium barcode) of each sampled species: partial overview of material and close-up of individual scale at 500 X and 150 X magnification respectively. Working distance 11 mm, EHT 4.50 kV.

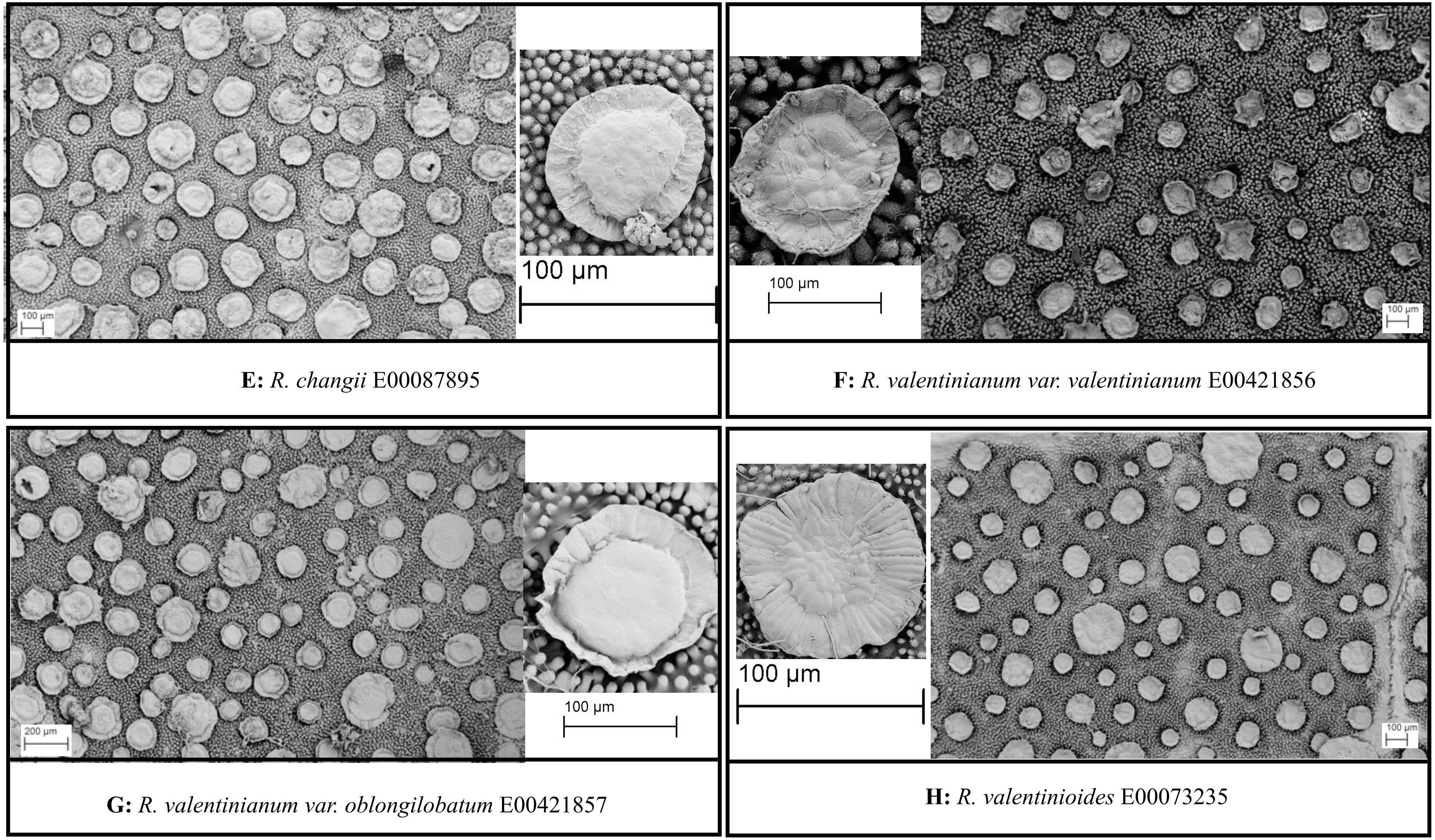
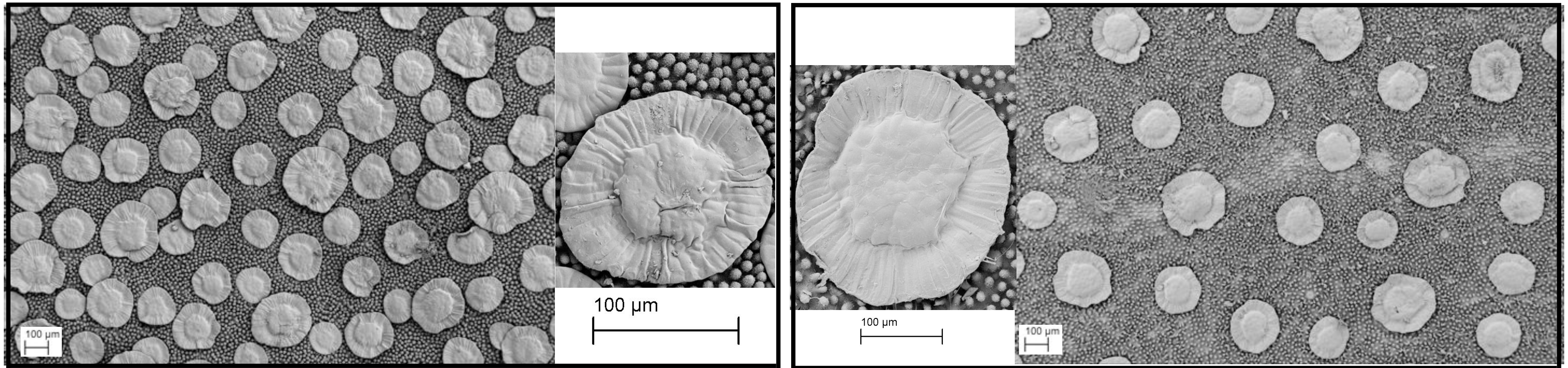


Figure 6: Scanning electron micrograph of one accession (herbarium barcode) of each sampled species: partial overview of material and close-up of individual scale at 500 X and 150 X magnification respectively. Working distance 11 mm, EHT 4.50 kV.



I: *R. leptocladon* E00073365

J: *R. lyi* E00421853

Figure 6: Scanning electron micrograph of one accession (herbarium barcode) of each sampled species: partial overview of material and close-up of individual scale at 500 X and 150 X magnification respectively. Working distance 11 mm, EHT 4.50 kV.

The data obtained for total scale diameter (mm) had a right-skew. A normal distribution and equal variance were obtained using a square root transformation. Total scale diameter (mm) was found to be significantly different ($P = 0.000$) between species by the one-way ANOVA analysis. Figure 7 shows the size distribution of scales for each species. Large variation in sizes between the lower and upper quartiles was found in *R. leptocladon* and *R. lyi*. The median scale size of *R. changii* and *R. leptocladon* were very similar but scale diameter was found to be significantly different between the species as the upper quartile size variation is smaller in *R. changii* (Figure 7). *R. crenulatum*, *R. fletcherianum* and *R. vanderbiltianum* had low numbers of scales with comparably little variation around the median. Scales of *R. crenulatum* were particularly uniform in size according to Figure 7. Both subspecies of *R. valentinianum* had the same median scale diameter but in this comparison *R. valentinianum var. oblongilobatum* had a greater number of scales larger than the median. All specimens were found to have highly significantly different mean scale diameters by one-way ANOVA (Figure 7) apart from *R. vanderbiltianum* ($P = 0.260$).

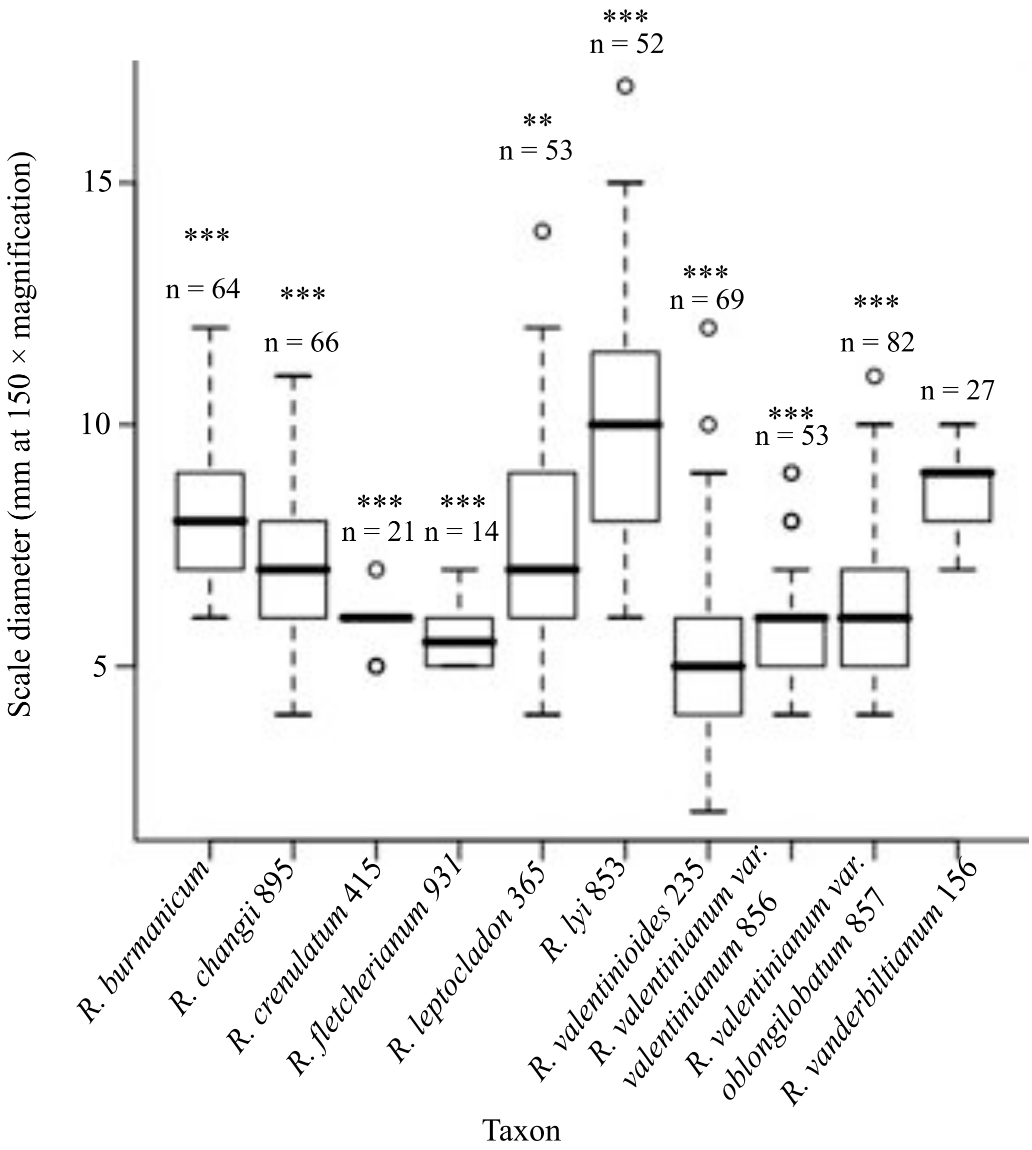


Figure 7: Box-plot of total scale diameter (mm) found for one specimen of each taxon examined using SEM. Specimen can be identified from last three digits of herbarium barcode, corresponding to Table 8. Solid lines indicate lower and upper quartiles and bold line represents median scale diameter; dashed lines indicate total range of values and circles indicate outlying values. n = number of scales measured.

P indicated by **0.001 - 0.009, ***0.000.

The one-way ANOVA output obtained from the comparison of total diameter (mm) measurements within species is displayed on Table 11. All data were normally distributed with equal variance apart from *R. burmanicum* which was transformed using a square-root calculation to correct for a right-skew. Scale diameter (mm) was found to differ very significantly between specimens of both *R. valentinianum var. valentinianum* and *R. vanderbiltianum*.

Table 11: Comparison of scale diameter (mm) within taxa. Two specimens examined per taxon. Mean diameter of scales ± 1 standard error, n = number of scales measured and P value obtained from one-way ANOVA analysis of each pair. *R. burmanicum* data were square-root transformed before ANOVA was performed. Significant P values highlighted in bold.

Taxon	Herbarium Barcode	n	Mean ± 1 s.e.	P
R. valentinianum var. valentinianum	E00053783	49	5.84 ± 0.19	**0.000**
	E00421856	32	7.81 ± 0.33	
R. burmanicum	-	64	8.22 ± 0.19	0.506
	E00421855	52	8.48 ± 0.29	
R. leptocladon	E00039871	70	7.76 ± 0.20	0.386
	E00073365	53	7.45 ± 0.30	
R. lyi	E00035298	52	10.00 ± 0.30	0.43
	E00421853	22	9.59 ± 0.36	
R. vanderbiltianum	E00533156	27	8.63 ± 0.12	**0.001**
	L0442390	17	7.88 ± 0.17	

Apart from the variation in scale size, Figure 6 shows marked differences in the proportion of the total diameter (mm) occupied by the inner zone. This variation between taxa was highly significant when tested using one-way ANOVA (P = 0.000). The data originally had a left-skew so were transformed using arcsin to obtain a normal distribution and equal variance. *R. crenulatum* is shown to have the largest inner zone for total scale diameter at 76% whereas *R. valentinioides* had the smallest with 51% of the scale diameter occupied by the inner zone (Table 12). *R. fletcherianum*, *R. leptocladon*, *R. valentinianum var. oblongilobatum* and *R. vanderbiltinaum* were not found by one-way ANOVA to have significantly different means. The inner zone proportion of *R. fletcherianum* was found to be especially variable with ± 30% and ± 10% standard error respectively (Table 12).

Table 12: Comparison of total / inner zone scale diameter (mm) of one specimen for each taxon examined. Mean ± 1 standard error; n = number of scales measured; P value obtained from one-way ANOVA with arcsin data transformation. P values significant between 0.000 and 0.005 highlighted in bold.

Taxon	Herbarium Barcode	n	Mean ± 1 s.e.	P
R. burmanicum	-	64	0.60 ± 0.01	**0.000**
R. changii	E00087895	66	0.68 ± 0.01	**0.000**
R. crenulatum	L0007415	21	0.76 ± 0.02	**0.000**
R. fletcherianum	E00189931	14	0.65 ± 0.34	0.047
R. leptocladon	E00073365	53	0.56 ± 0.01	0.031
R. lyi	E00421853	52	0.55 ± 0.01	**0.005**
R. valentinioides	E00073235	69	0.51 ± 0.01	**0.000**
R. valentinianum var. oblongilobatum	E00421857	82	0.66 ± 0.01	0.393
R. valentinianum var. valentinianum	E00421856	53	0.61 ± 0.02	**0.000**
R. vanderbiltianum	E00533156	27	0.62 ± 0.01	0.275

The possession of entire leaf scales by all the examined species may indicate that their placement in the same subsection is justified. A summary of all the characters detailed in this section for distinguishing between the yellow-flowered taxa is presented on Table 13. The presence or absence (correlated with crenulate leaf margins) of papillae divided the species into two groups. The proportion of the scale occupied by the inner zone was then an informative character for distinguishing between species within these groups. The only species found to have the exact same character scores in summary were *R. leptocladon* and *R. valentinioides*. However, referring to the detailed analyses it is obvious that *R. valentinioides* had smaller, more uniform scales (Figure 7) with smaller inner zones (Table 12) compared to *R. leptocladon*.

While these statistics offer insight into the differences between scales of different taxa, a more comprehensive study of herbarium leaf material would be required before any definite conclusions could be drawn about their comparative morphology. It was considered appropriate to use parametric statistics for the purpose of this study because criteria of normally distributed, independent values with equal variance were met. However, the number of specimens sampled was inadequate for robust statistical analysis and does not represent a true cross-section of the geographical and altitudinal distribution of species, which may be critical factors for interpreting scale morphology. Sampling also failed to account for differences in the age of leaf material and the part of the leaf examined, both of which are factors likely to exaggerate statistical differences in scale size and density between taxa. Interpretation of the above findings is, therefore, offered as tentative hypotheses pending thorough investigation.

Table 13: Summary of useful scale characters determined using SEM for yellow-flowered taxa of subsection *Maddenia*. n/a indicates data was not obtained for this taxon.

	Taxon								
Character	***R. burmanicum***	***R. changii***	***R. crenulatum***	***R. fletcherianum***	***R. leptocladon***	***R. valentinianum var. valentinianum***	***R. valentinianum var. oblongilobatum***	***R. valentinioides***	***R. vanderbiltianum***
Papillae (0 absent, 1 present)	1	1	0	0	1	1	1	1	0
Density (0 < 30, 1 ≥ 30 counted)	1	1	0	0	1	1	1	1	0
Shape (0 misshapen, 1 circular)	0	1	1	1	1	1	1	1	1
Rims (0 flattened, 1 upturned)	0	0	0	0	0	1	1	0	0
Total diameter range at x150 magnification (0 < 4 mm, 1 ≥ 4 mm)	1	1	0	0	1	0	1	1	1
Total diameter (mm) varies significantly between specimens (0 no, 1 yes)	0	n/a	n/a	n/a	0	1	n/a	n/a	1
Proportion of total scale diameter occupied by inner zone (0 < 65%, 1 ≥ 65%)	0	1	1	1	0	0	1	0	0

2.2.3 Geographic distribution

The collecting localities determined for specimens of *R. burmanicum*, *R. changii*, *R. crenulatum*, *R. fletcherianum*, *R. leptocladon*, *R. valentinianum* and *R. vanderbiltianum* are displayed on Figure 8. An accompanying list of geo-referenced specimens can be found on Appendix 2. The greatest concentration of specimens was found on Mount Fan Si Pan in N Vietnam along an altitudinal gradient of 1800-2475 m (Appendix 2). Mount Fan Si Pan also hosted the greatest species diversity (Figure 9). Seven specimens of *R. valentinianum var. valentinianum* were found there as well as all of the *R. leptocladon* specimens and one specimen of *R. crenulatum,* which was also found in an isolated locality in Phou Bia (Laos) again above 2700 m (Figure 8).

R. valentinianum had the broadest species distribution, extending from W Yunnan through into N Vietnam (Figure 10) and the longest altitudinal range from 1800-3600 m (Appendix 2). *R. valentinianum var. oblongilobatum* was only found in the eastern and central parts of the species' range in Yunnan. The specimen of *R. valentinioides* was collected near the Chinese border with Vietnam, within the range of *R. valentinianum var. valentinianum* (Figure 8).

Outside of Vietnam, species were found in small localised pockets quite remote from other yellow-flowered species. *R. fletcherianum* was found to be the most northerly distributed yellow-flowered species as a point endemic in NW Yunnan near the Xizang province border where it was found at the highest altitude (2900-4300 m) of any of the examined species. *R. changii* was also a point endemic and had the easternmost distribution of all the investigated species, with one specimen collected in the Chinese province of Sichuan at 2100 m (Figure 8). *R. burmanicum* was restricted to one locality in W Myanmar whereas *R. vanderbiltianum* was found on four different mountains ranging from 2100-3200 m on the Indonesian island of Sumatra, some 2000 km from all the other species (Figure 11). The three species sharing crenulate leaf margins were, therefore, found to be geographically isolated from each other as *R. vanderbiltianum* occured in Sumatra, *R. crenulatum* in Laos and Vietnam and *R. fletcherianum* in NW Yunnan.

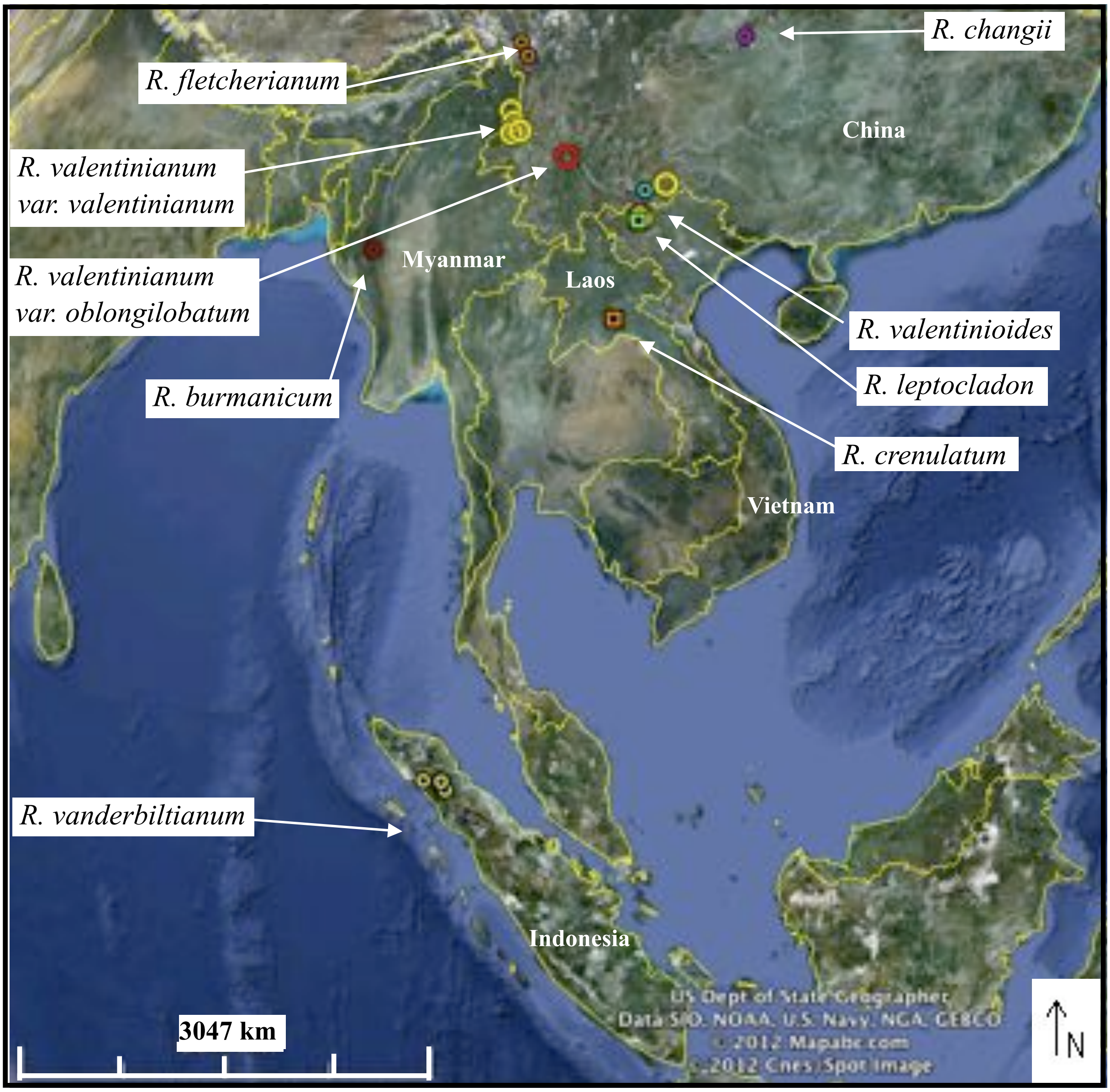


Figure 8: Overview of the distribution of yellow-flowered taxa from subsection *Maddenia*.

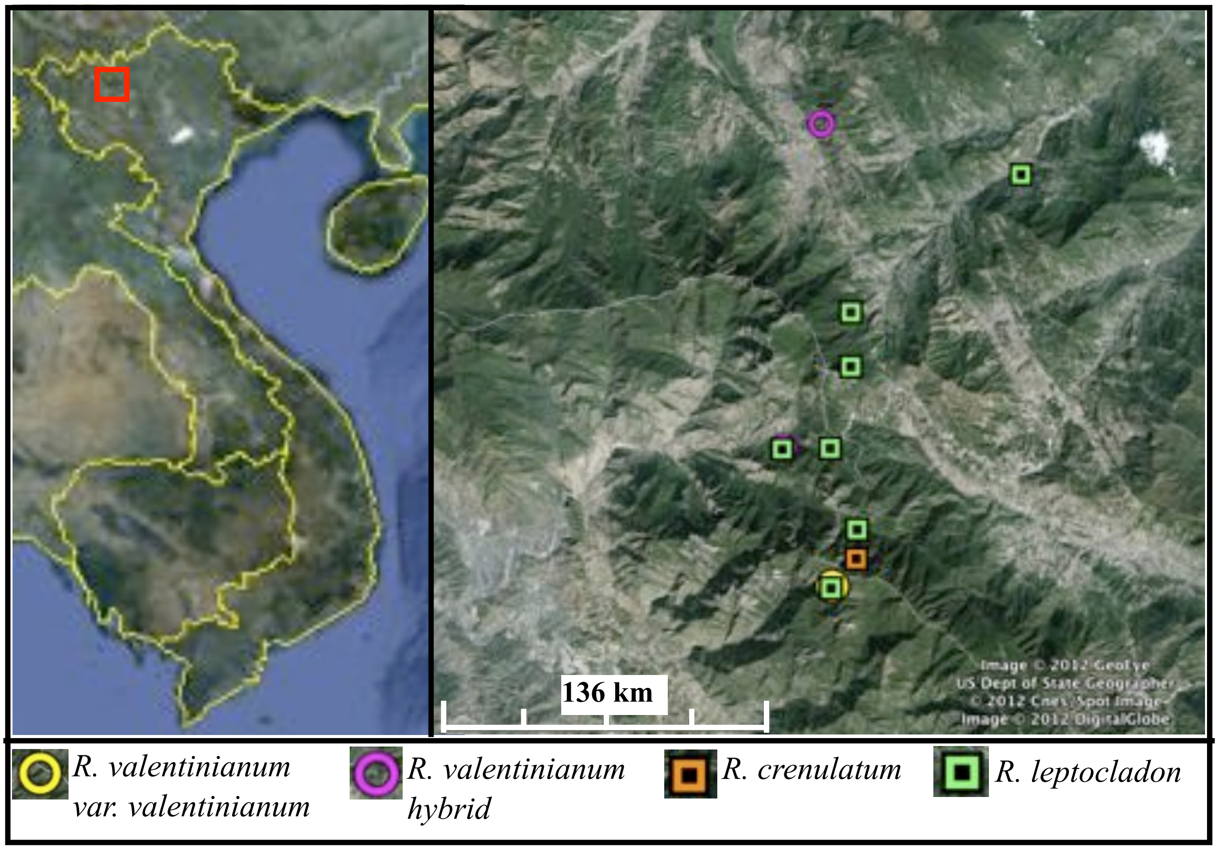


Figure 9: Distribution of species recorded at Mount Fan Si Pan in Vietnam.

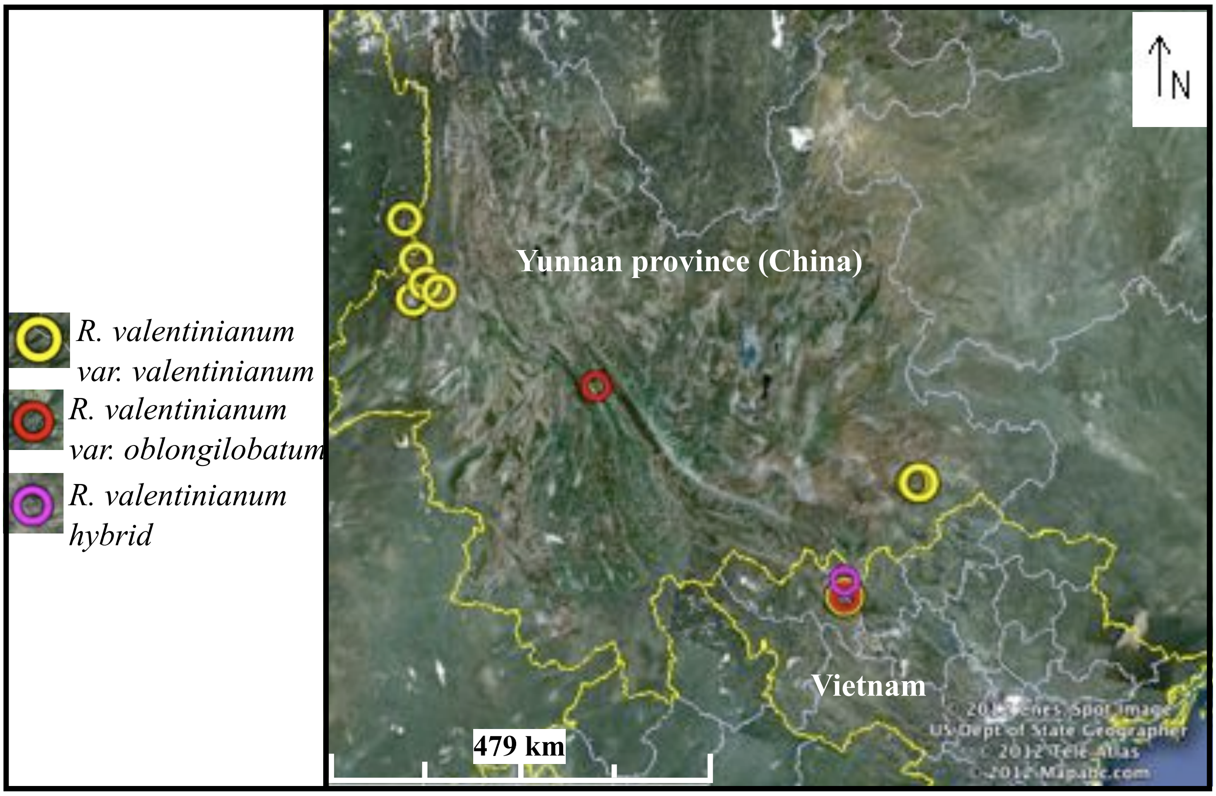


Figure 10: Distribution map of *R. valentinianum.*

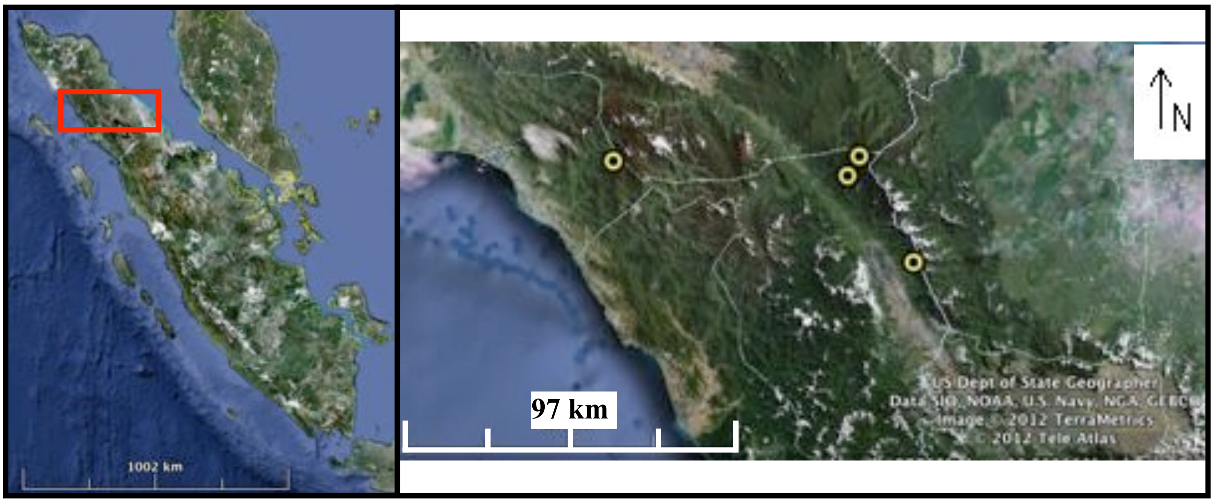


Figure 11: Distribution map of *R. vanderbiltianum* specimens.

CHAPTER 3: MOLECULAR ANALYSES

3.1 MATERIALS AND METHODS

3.1.1 Region selection and primer sequences

Choosing an appropriate gene region to sequence in order to build a phylogeny is vitally important because different genomes evolve at different rates (Wolfe *et al.,* 1987). Poor resolution of taxon relationships can be obtained if the genome investigated evolves too slowly (causing polytomies) or too quickly (long-branch attraction) as a result of increased homoplasy. In higher plants, the chloroplast genome evolves more slowly than nuclear DNA but more quickly than mitochondrial DNA (Wolfe *et al.,* 1987) and is frequently used to investigate genus and species level relationships (Taberlet *et al.*, 1991). The chloroplast gene *mat*K is a coding region of approximately 1550 base pairs located within the 2600 base pair intron between two *trn*K exons (Figure 12) (Johnson & Soltis, 1995). The *mat*K gene codes for a maturase that splices *trn*K group IIA introns from RNA transcripts (Zoschke *et al.,* 2010). It was chosen for the purposes of this investigation because it has already successfully been used for phylogenetic work both within Ericaceae (Kron, 1997; Li *et al.,* 2002) and *Rhododendron* itself for analysis of sectional and species relationships (Kurashige *et al.,* 2001; Milne 2004; Milne *et al.*, 2010).

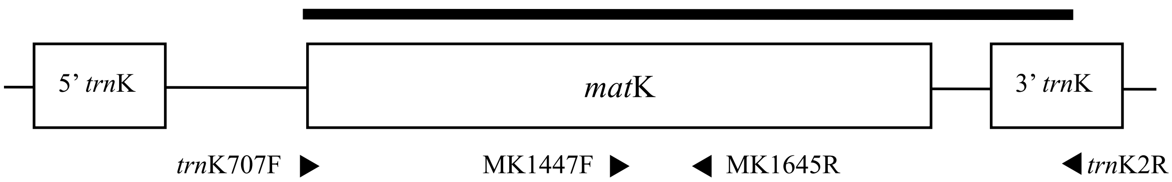


Figure 12: Diagram of the position of *mat*K within the *trn*K intron (Johnson and Soltis, 1995). Arrows indicate the location of forward and reverse primers used to amplify and sequence the region. Total area sequenced represented by the solid bar.

Sequence quality declines towards the end of long sequences due to the decreased probability of long chains of dNTPs being formed before a ddNTP is added, and the decline in accuracy of the Taq enzyme over time. Given the length of the *mat*K region, it was necessary to amplify it in two portions. The primers used to amplify the *mat*K region (Figure 12) were taken from Milne *et al.* (2010) and are presented in Table 14.

Table 14: Primer sequences

Primer name	Sequence
*trn*K707F	ACTGTATCGCACTATGTATC
MK1645R	AGCCAAAATGGCTTTTCCTC
MK1447F	CGCTCAATATCTTCTGAAACCTT
*trn*K2R	AACTAGTCGGATGGAGTAG

3.1.2 Collection of plant material

The species selected for molecular analysis from within subsection *Maddenia* are given on Table 4. Material was obtained for each of the yellow flowered species, including multiple accessions for all species apart from *R. leptocladon*, *R. valentinioides* and *R. vanderbiltianum*. In addition a single accession of at least one species from each Alliance within subsection *Maddenia* (apart from the monospecific Megacalyx alliance) was sampled and three species from subsection *Boothia* were selected as subsectional outgroups. These species were the white-flowered *R. leucaspis* Tagg and the yellow-flowered species *R. chrysodoron* Tagg ex Hutch. and *R. sulfureum* Franch.

Leaf material was collected for molecular analysis from cultivated plants grown at Logan Botanic Garden, the Royal Botanic Garden, Edinburgh and Glendoick nursery (Table 4). One further sample was collected from the National Trust for Scotland garden at Branklyn. Three young leaves were collected from each shrub. The midrib was removed from each leaf before the lamina tissue was deposited in a labelled 20 g bag of silica gel with indicator crystals. Gloves were worn whenever handling silica gel because it is a skin irritant. The material was left to dry for a minimum of seven days before DNA extractions were performed.

3.1.3 DNA extraction

DNA extractions were performed using the Qiagen Plant DNeasy mini kit (Qiagen 2003-2012). In order to extract total genomic DNA from the collected plant material, 10 - 20 mg of leaf material was removed using tweezers from the silica gel sachets and placed in a 2 ml Eppendorf tube with a 3 mm Retsch cone ball. Tweezers were cleaned using 70% ethanol between samples to prevent cross contamination. The tubes were placed in a Retsch MM300 mixer mill (Retsch GmbH, Germany) that ran two one minute cycles at 20 seconds frequency. This process ground the leaf material into a fine powder to which 400 µl of Buffer AP1 solution was added. The tubes were centrifuged for one minute at 8 000 rpm before being incubated for 30 minutes in a Grant QBD4 heat block maintained at 65˚C in order to lyse the cells (Qiagen, 2006). Upon removal from the incubator, 130 µl of Buffer AP2 was added to the tubes which were then left to stand in an ice bucket for a minimum of five minutes to encourage the precipitation of unwanted detergent, proteins and polysaccharides (Qiagen, 2006).

The tubes were centrifuged at 13 000 rpm for five minutes before the lysate was transferred using a pipette to QIAshredder Mini Spin Columns placed in 2 ml collecting tubes and centrifuged for two minutes at 13 000 rpm. The purpose of the spin column was to remove precipitates and debris from the lysate but a small amount of this was deposited as a pellet in the collection tubes. A 200 µl pipette was used to extract flow through from the collection tube in to fresh 2 ml tubes without disturbing the pellet. The volume of liquid removed varied between 400 and 450 µl to which 600 or 675 µl of Buffer AP3/E was added respectively. The solution was mixed using a pipette before 650 µl was pipetted into the DNeasy Mini Spin Columns sitting in 2 ml collection tubes. These tubes were centrifuged for one minute at 8 000 rpm from which the resulting flow through was discarded. This process was repeated using any remaining solution from the previous step.

The DNeasy columns were placed in new 2 ml collection tubes and 500 µl of Buffer AW was pipetted into the columns which were then centrifuged for one minute at 8 000 rpm. Flow through was discarded, a further 500 µl of Buffer AW was pipetted into the columns and these were centrifuged again for two minutes at 13 000 rpm. In order to dry the column, membrane flow through was discarded and the tubes were blotted gently on blue roll until dry. The tubes were replaced in the centrifuge and spun for one minute at 13 000 rpm to remove residual ethanol (Qiagen, 2006). The DNeasy column was placed in a new 1.5 ml collection tube and the extraction process was concluded by pipetting 100 µl of Buffer AE onto the column membrane which was left to stand at room temperature. The solution was eluted by centrifuging for one minute at 8 000 rpm and the concentration of extracted DNA increased by re-applying any flow through to the column and centrifuging at 8 000 rpm for one final minute.

3.1.4 Gel electrophoresis

Gel electrophoresis was used in order to visualise the results of the DNA extraction (Section 3.1.3) and the polymerase chain reaction (PCR) (Section 3.1.5) processes. A 1% agarose gel was made in a conical flask by adding 1 g of agarose powder (Bioline Reagents Ltd, UK) to 100 ml of 1 x Tri-Borate-EDTA (TBE) buffer (Sigma-Aldrich Company Ltd, UK), heated in a microwave until the solution became clear and left to cool for several minutes. 7 µl of Sybrsafe DNA gel stain (Invitrogen, USA) was pipetted into the flask and the solution was mixed by gently rotating the flask. The solution was left to cool for 30 minutes in a gel tray with gel combs and covered with black card in order to prevent the photosensitive Sybrsafe stain from degrading.

Once the gel had solidified, 5 µl of total genomic DNA or PCR product was added to 3 µl of gel loading solution. The gel loading solution contained glycerol to weigh the DNA down in the well and a blue dye to enable the progress of the samples on the gel to be visualised. The subsequent solution was pipetted into the gel wells and 5 µl of 1 kb+ ladder solution (Invitrogen, UK) was injected into a neighbouring well to enable size comparisons of the DNA products. The loaded gel was then placed in a tank with a positive and negative electrode at either end, filled with 1 x TBE buffer. The tank was connected to a Bio-rad power pack 300 (Bio-rad Laboratories Inc.) set to 80 V (400 mA) for 30 minutes. As DNA molecules are negatively charged at neutral pH they migrate through the gel towards the positive electrode when an electrical current is pulsed through the tank. The smaller the DNA fragment the faster it runs down the gel towards the positive electrode.

Once the electrophoresis procedure had been completed the gel was removed from the tank and placed on a lightbox in a Syngene Bioimaging system (Syngene, UK) which floods the lightbox with UV light causing the Sybrsafe stain to fluoresce where DNA bands were present. The resulting image was photographed using Genesnap software (v. 7.02., Synoptics Ltd, UK).

3.1.5 Polymerase Chain Reaction

Gel electrophoresis was conducted to check that DNA had successfully been extracted from the leaf material (Section 3.1.3). Once this had been confirmed, a PCR was run in order to amplify the *mat*K chloroplast region from the total genomic DNA. A reaction mixture was made to the recipe displayed on Table 15. All reagents were mixed in the vortex, added to the reaction mixture which was then also briefly vortexed and centrifuged, then aliquoted into 0.2 ml strips alongside 1 µl of genomic DNA. No DNA was added to the final tube of reaction mixture to act as a negative, ensuring no contamination of the reaction mixture had occurred.

Table 15: List and quantity (µl) of PCR reagents used per sample.

Reagent	Volume per sample (µl)
dH_20	12.05
dNTPs (2 mM) (Bioline Reagents Ltd, UK)	2.50
10 x NH_4 Buffer (Bioline Reagents Ltd, UK)	2.50
$MgCl_2$ (50 mM) (Bioline Reagents Ltd, UK)	1.25
Forward primer *trn*K 707F or MK 1447F (10 µM) (Invitrogen, UK)	0.75
Reverse primer MK 1645R or *trn*K2R (10 µM) (Invitrogen, UK)	0.75
Combinatorial Enhancer Solution (2.7 M betaine, 6.7 DMSO and 50 µg/ml BSA)	4.00
BioTaq polymerase (Bioline Reagents Ltd, UK)	0.20

Once mixed in the vortex and centrifuged, the tubes were placed in an Applied Biosystems GeneAmp PCR system 2700 with heated lid (Life Technologies, UK) programmed to run the PCR cycle displayed on Table 16. The cycle lasted for 2 hours 37 minutes after which the tubes were removed from the machine and the product was visualised using gel electrophoresis (Section 3.1.3).

Table 16: Display of PCR reaction cycle conducted in GeneAmp PCR system outlining temperature (˚C), duration (minutes) and process undertaken for each step (Brown, 2002). Steps 1 - 3 cycled 35 times.

Step	Temperature (˚C)	Time (minutes)	Process
1	94	2.5	Denatures hydrogen bonds to separate double stranded DNA
2	55	1.0	Primers anneal to single strands of DNA by complimentary base pairing
3	72	1.5	Taq polymerase binds free dNTPs to extend DNA strand by complimentary base pairing
4	72	7.0	Final extension phase
5	10	∞	Samples cooled and stored until removal from PCR machine

3.1.6 PCR purification

The product obtained from the PCR reaction contained surplus dNTPs and primers that needed to be removed so as not to interfere with the subsequent sequencing reaction. This was achieved by treating the product with the ExoSAP IT reagent (GE Healthcare, UK) which consisted of Exonuclease I and Shrimp Alkaline Phosphatase enzymes that degraded single-stranded DNA and hydrolysed dNTPs respectively. 2 µl of ExoSAP IT was added to 5 µl of PCR product and placed in a Peltier Thermal Cycler 200 (M J Research, USA) to incubate the samples at 37˚C for 15 minutes and then 80˚C for 15 minutes in order to denature the enzymes.

3.1.7 Sequencing PCR

A sequencing reaction mixture was made according to the recipe itemised on Table 17. Only one primer was added to amplify one strand of DNA in each reaction. To amplify *mat*K in its entirety, four sequencing reactions were conducted per DNA sample. The mixture was made in quantity then aliquoted to 0.2 ml strips to which 1 µl of purified PCR product was added. The mixture was vortexed, centrifuged and replaced in the GeneAmp PCR system which ran the same program outlined on Table 16. However, the reaction differs in that dNTPs with a fluorescent label are used by Taq polymerase to extend single strands of DNA. At any point during the extension of the strand a ddNTP may be added preventing the addition of any further dNTPs and thus terminating the extension (Brown, 2002). Upon completion of the reaction the samples were sent to The Genepool (University of Edinburgh, UK) where the sequences were read by an automated sequencer and returned via email.

Table 17: List and quantity (µl) of reagents used in sequencing PCR for each sample.

Reagent	Volume per sample (µl)
dH_20	6.18
BigDye (Life Technologies, UK)	0.50
5 x buffer	2.00
Primer (10 µM) (Invitrogen, UK)	0.32

3.1.8 Sequence editing

Sequences were edited using Sequencher (v. 5.0., Gene Codes Corporation, USA). Poor quality data and the primer sequences were trimmed from both ends of the sequence. The four sequences obtained for each taxon were assembled by name into a contig which was manually reviewed to ensure the sequences ran in the correct direction. Ambiguities detected between the forward and reverse strands were resolved by eye using chromatograms. Where both strands of sequence were ambiguous they were edited with reference to two or three good quality sequences obtained for other taxa. The consensus sequence for each taxon was exported as a nexus file (Appendix 3).

3.2 PHYLOGENETIC ANALYSES

3.2.1 Outgroup selection

In order to establish whether subsection *Maddenia* was monophyletic, a minimum of two outgroups were required. The PhyLoTa browser (2012) was searched for *Rhododendron* sequences of *mat*K. All of those found with ≥1700 characters were downloaded from GenBank (National Centre for Biotechnology Information, 2012) (Appendix 4) and imported with the consensus sequences obtained from fresh material into Mesquite (v.2.75., Maddison, W. and Maddison, D., USA). The 70 compiled sequences were were aligned manually by eye, trimmed so as to start and finish at the same character, and gaps were coded as 'N".

A maximum parsimony (MP) analysis was conducted using Paup* (v.4.0b10., Swofford, USA) to establish which species would be most appropriate to use as outgroups. A heuristic search with 10,000 replicates was conducted with unordered characters of equal weight, steepest descent and MulTrees switched on and tree-bissection-reconnection (TBR) off. The trees generated were filtered to include only the best score trees, which were saved to memory. A second heuristic search was conducted using the 546 best score trees with TBR and MulTrees in effect, branches collapsed if minimum branch length equalled zero and gaps treated as missing. The consistency (CI) and retention (RI) indices were calculated in Paup* including parsimony uninformative characters. The average number of steps per character was calculated by dividing the tree length of the most parsimonious trees by the number of characters in the matrix. A bootstrap heuristic search with 10,000 replicates, starting trees obtained via random stepwise addition with one tree held per addition, TBR on and MulTrees off was then conducted to add support to the parsimony trees obtained.

The results were used to select three species distantly related to subgenus *Rhododendron* to act as outgroups and to identify GenBank sequences which could be added to the subgenus *Rhododendron* matrix in order to improve the subsequent analysis of species relationships within subsection *Maddenia*.

3.2.2 Maximum parsimony

A second matrix including 34 ingroup and three outgroup sequences (Table 18) was aligned by eye using Mesquite (v.2.75., Maddison, W. and Maddison, D., USA) and imported into Paup* (v.4.0b10., Swofford, USA). This included four gap characters which were coded manually. MP analysis was conducted using a heuristic search of 10000 replicates with unordered characters of equal weight, gaps treated as missing, starting trees obtained via random stepwise addition, TBR and MulTrees in effect and branches collapsed if minimum branch length was zero. A bootstrap analysis was then conducted using the same parameters detailed in Section 2.2.1.

3.2.3 Maximum likelihood

A maximum likelihood (ML) analysis was also conducted using the sequences displayed on Table 18. The matrix was imported into Modeltest (v.3.7., Posada, D., Spain) to identify the most accurate model of nucleotide evolution for the ML analysis to use. The model determined for use by the Akaike information criterion was K81uf+G with six substitution types, assumed nucleotide frequencies of 0.32810, 0.1532, 0.1595 and 0.3592 for A, C, T, G respectively and the shape parameter of the alpha distribution 0.1492. The ML heuristic search was conducted in Paup* (v.4.0b10., Swofford, USA) using this model with starting trees obtained by stepwise addition and one tree held at each step, TBR and MulTrees in effect, branches collapsed if length was $\leq 1\ e^{-8}$. To assess the level of saturation of the data matrix, the ML K81uf+G model was executed in Paup* (v.4.0b10., Swofford, USA) to report the uncorrected (observed) and corrected (estimated) pairwise distances for the data matrix, which were then graphed using Excel (v.12.2.3, Microsoft Corporation, USA). Finally, a bootstrap analysis was conducted as a heuristic search with 1000 replicates using the K81uf+G model, TBR in effect, branches collapsed if length $\leq 1\ e^{-8}$ and MulTrees switched off so as to save only one tree.

Table 18: List of all sequences used for the maximum parsimony and maximum likelihood analyses of subsection *Maddenia* indicating subsectional taxonomic placement.

Taxon	Accession	Section	Subsection
Rhododendron albrechtii	AB012737	*Azaleastrum*	*Sciadorhodion*
Rhododendron canadense	AB012735	*Pentanthera*	*Rhodora*
Rhododendron edgeworthii	U61354	*Rhododendron*	*Edgeworthia*
Rhododendron ferrugineum	AB012741	*Rhododendron*	*Rhododendron*
Rhododendron javanicum	AB012742	*Vireya*	*Euvireya*
Rhododendron kawakamii	AM296053	*Vireya*	*Pseudovireya*
Rhododendron mucronulatum	AF454855	*Rhododendron*	*Rhodorastra*
Rhododendron pendulum	AF440429	*Rhododendron*	*Edgeworthia*
Rhododendron ponticum	AY494172	*Hymenanthes*	*Pontica*
Rhododendron primuliflorum	AB012740	*Pogonanthum*	-
Rhododendron santapaui	AB012743	*Vireya*	*Pseudovireya*
Rhododendron burmanicum	EDNA12-0025068	*Rhododendron*	*Maddenia*
Rhododendron burmanicum	EDNA12-0025069	*Rhododendron*	*Maddenia*
Rhododendron changii	EDNA12-0025365	*Rhododendron*	*Maddenia*
Rhododendron changii	EDNA12-0025065	*Rhododendron*	*Maddenia*
Rhododendron chrysodoron	EDNA12-0025070	*Rhododendron*	*Boothia*
Rhododendron ciliatum	EDNA12-0025359	*Rhododendron*	*Maddenia*
Rhododendron crenulatum	EDNA12-0025364	*Rhododendron*	*Maddenia*
Rhododendron crenulatum	EDNA12-0025066	*Rhododendron*	*Maddenia*
Rhododendron crenulatum	EDNA12-0025225	*Rhododendron*	*Maddenia*
Rhododendron dalhousiae	EDNA12-0025369	*Rhododendron*	*Maddenia*
Rhododendron fletcherianum	EDNA12-0025366	*Rhododendron*	*Maddenia*
Rhododendron fletcherianum	EDNA12-0025221	*Rhododendron*	*Maddenia*
Rhododendron johnstoneanum	EDNA12-0025361	*Rhododendron*	*Maddenia*
Rhododendron leptocladon	EDNA12-0025363	*Rhododendron*	*Maddenia*
Rhododendron leucaspis	EDNA12-0025220	*Rhododendron*	*Boothia*
Rhododendron lyi	EDNA12-0025362	*Rhododendron*	*Maddenia*
Rhododendron maddenii	EDNA12-0025360	*Rhododendron*	*Maddenia*
Rhododendron sulfureum	EDNA12-0025223	*Rhododendron*	*Boothia*
Rhododendron valentinianum var. oblongilobatum	EDNA12-0025358	*Rhododendron*	*Maddenia*
Rhododendron valentinianum var. oblongilobatum	EDNA12-0025067	*Rhododendron*	*Maddenia*
Rhododendron valentinianum var. oblongilobatum	EDNA12-0025071	*Rhododendron*	*Maddenia*
Rhododendron valentinianum var. valentinianum	EDNA12-0025367	*Rhododendron*	*Maddenia*
Rhododendron valentinianum var. valentinianum	EDNA12-0025222	*Rhododendron*	*Maddenia*
Rhododendron valentinioides	EDNA12-0025368	*Rhododendron*	*Maddenia*
Rhododendron vanderbiltianum	EDNA12-0025072	*Rhododendron*	*Maddenia*
Rhododendron veitchianum	EDNA12-0025224	*Rhododendron*	*Maddenia*

3.2.4 Analysis of codon position changes

The parsimony data and tree files were imported into MacClade (v.4.08a, Maddison D. and Maddison, W., USA) where a graph of the number of steps per character was created. Codon positions were then assigned for the first 1534 characters (all characters after this point were non-coding bases in the *trn*K intron). The number of base changes occurring at each codon position was calculated. To determine if particular base changes were associated with particular codon positions, all characters were traced on the cladogram and whenever a character had multiple states the character number, codon position, number of steps, CI, direction of change and type of change (autapomorphic, synapomorphic or homoplasious) was recorded. The data were analysed graphically using Excel (v.12.2.3, Microsoft Corporation, USA).

3.2.5 Morphological matrix

A morphological matrix was coded in Mesquite (v.2.75., Maddison, W. and Maddison, D., USA) for all 37 taxa included in the phylogenetic analyses. Characters were selected according to observations of useful distinguishing traits found during the review of herbarium specimens. The 26 characters and the corresponding states included in the matrix are displayed on Table 19. Missing or ambiguous characters were coded as '?'. Character states were coded for collected material from descriptions of the associated voucher specimens (Section 2.1.2). Where floral characters were absent, consensus data from other herbarium specimens was used. Character states of species included from GenBank were coded using descriptions from Argent (2006), Fang *et al*. (2005), Judd and Kron (2009), the Japanese Society for Plant Systematics (2012) and with reference to herbarium specimens (E). The character states determined for each species are presented in Appendix 5. The most parsimonious tree files obtained from the MP analysis (Section 3.2.2) were imported into Mesquite (v.2.75., Maddison, W. and Maddison, D., USA) and the character states were mapped on to them. Each character trait was observed on each most parsimonious tree to select useful synapomorphic character states for discussion (Section 3.3.). These characters were then mapped on to a 50% majority rule consensus tree for the purposes of the discussion.

Table 19: Character states coded for 26 morphological characters.

Number	Character	State 0	1	2	3	4	5	6	7	8
1	**Country**	China	Bhutan/Nepal	Vietnam/Laos	India	Thailand	Indonesia	Japan	Europe	N. America
2	**Altitude (m)**	0 - 1000	1001 - 2000	2001 - 3000	3001 - 4000	≥ 4001				
3	**Petiole length (mm)**	1.0 - 5.9	6.0 - 10.9	11.0 - 15.9	16.0 - 20.9					
4	**% petiole to leaf length**	0.0 - 4.9	5.0 - 9.9	10.0 - 14.9	15.0 - 19.9	20.0 - 24.9	25.0 - 29.9			
5	**Petiole scales**	Absent	Present							
6	**Petiole pubescence**	Glabrous	Setose	Loriform	Puberulent	Woolly				
7	**Leaf shape**	Ovate	Elliptic	Obovate-elliptic	Oblong	Lanceolate				
8	**Leaf apex**	Mucronate	Acuminate	Acute						
9	**Leaf base**	Attenuate	Cuneate	Obtuse						
10	**Leaf margin**	Entire	Crenulate							
11	**Leaf margin pubescence**	Glabrous	Setose	Loriform						
12	**Scale type**	*Boothia*	*Maddenia*	*Pseudovireya*	*Edgeworthia*	*Euvireya*	Absent	Crenate		
13	**Abaxial scale density**	Sparse	Intermediate	Dense	Absent					
14	**Abaxial scale distribution**	Regular	Touching	Overlapping	Absent					
15	**Midrib abaxial prominence**	Prominent	Planate							
16	**Number of flowers**	2 - 3	2 - 6	3 - 5	≥4					
17	**Pedicel hairs**	Glabrous	Pubescent	Setose	Loriform	Glandular	Woolly			
18	**Calyx length (mm)**	1.0 - 3.9	4.0 - 6.9	7.0 - 10.9	11.0 - 15.9					
19	**% calyx to flower length**	0.0 - 9.9	10.0 - 19.9	20.0 - 29.9	30.0 - 39.9	40.9 - 49.9				
20	**Calyx pubescence**	Glabrous	Hairy							
21	**Calyx scales**	Absent	Present							
22	**Corolla shape**	Campanulate	Tubular-campanulate	Funnel						
23	**Corolla colour**	Yellow	White & pink	White & yellow	Pink/purple	Orange				
24	**Flower size (mm)**	0.0 - 20.9	21.0 - 40.9	41.0 - 60.9	61.0 - 80.9	81.0 - 100.9	101.0 - 120.9			
25	**Flower scales**	Absent	Present							
26	**Flower pubescence**	Absent	Present							

3.3 PHYLOGENETIC RESULTS

3.3.1 Selection of outgroups

The characteristics of the data matrix compiled using 70 sequences of *Rhododendron* representing all eight subgenera, upon which a maximum parsimony analysis was conducted, are displayed on Table 20. The large number of constant characters (1524 of a total 1780 base pairs) indicated that the *mat*K region in *Rhododendron* is highly conserved. The CI of 0.8097 shows there were few homoplasious characters. A large number of most parsimonious trees was obtained (1558), one of which is displayed on Figure 13. This tree had a high RI value (0.9280) indicating a very good fit of the tree topology to the data.

Table 20: Characteristics of genus *Rhododendron* molecular matrix used for MP analysis and indices obtained from the most parsimonious tree displayed on Figure 13.

Parameter	Result
Total aligned matrix length (bp)	3318
Number of excluded characters (bp)	1538
Number of maximum parsimony trees	8850
Number of trees retained by filter	1558
Number of most parsimonious trees (bp)	546
Length of most parsimonious trees (steps)	352
Number of uninformative characters (bp)	129
Number of informative characters (bp)	127
Number of constant characters (bp)	1524
Average number of steps per character	0.3067
Consistency index (CI)	0.8097
Retention index (RI)	0.9280

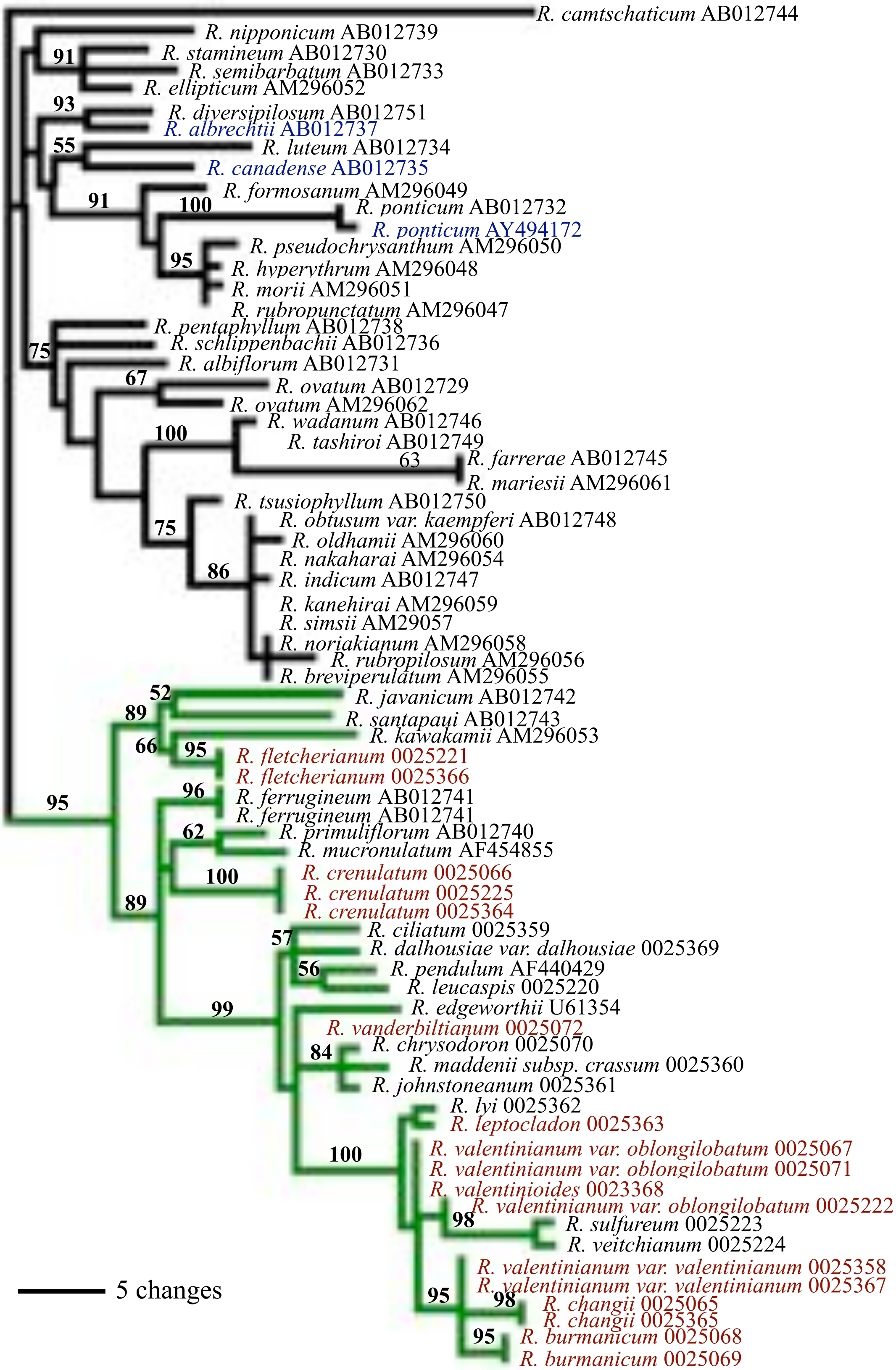


Figure 13: One of the most parimonious trees obtained from the MP analysis of 70 *Rhododendron* taxa. Taxon names indicate GenBank accession number (Appendix 4) or eight digits of the EDNA accession number (Table 4). Branch lengths represent number of changes. Branches of subgenus *Rhododendron* highlighted in green; yellow-flowered species of subsection *Maddenia* in red; species selected as outgroups in blue. Bootstrap values ≥50% above branches.

Subgenus *Rhododendron* was notable in forming a well supported monophyletic group (bootstrap 95%) if *R. diversipilosum* (Nakai) Harmaja from subsection *Ledum* was not included (Figure 13). All species accessions determined by this analysis to be in subgenus *Rhododendron* were selected for use in analysing the relationships of the yellow-flowered species of subsection *Maddenia*. This included three species from section *Schistanthe* and one species from section *Pogonanthum* (Table 18). *R. albrechtii* Maxim., *R. canadense* (L.) Britton, Sterns & Poggenb. and *R. ponticum* L. representing subgenus *Azaleastrum*, *Pentanthera* and *Hymenanthes* respectively were selected as outgroups.

3.3.2 Analyses of yellow-flowered species of subsection *Maddenia*

The output from the MP analysis of the subgenus *Rhododendron* data matrix is displayed on Table 21. The small average number of steps per character (0.1030) shows that there was a low level of homplasy, supported by the high CI value (0.8798). These measures therefore suggest that the data matrix was relatively unsaturated.

Table 21: Characteristics of the subgenus *Rhododendron* molecular matrix and parsimony indices obtained from MP analysis.

Parameter	Result
Total aligned matrix length (bp)	1775
Number of maximum parsimony trees	30
Number of trees retained by filter	14
Number of most parsimonious trees (bp)	14
Length of most parsimonious trees (steps)	183
Number of uninformative characters (bp)	77
Number of informative characters (bp)	73
Number of constant characters (bp)	1625
Average number of steps per character	0.1030
Consistency index (CI)	0.8798
Retention index (RI)	0.9360

The saturation level of the data matrix can be directly observed when the proportion of estimated substitutions generated by the ML model is plotted against the proportion of substitutions observed in the dataset (Figure 14). The data conformed to the expected linear relationship for approximately half its length before slowly diverging from it (Figure 14).

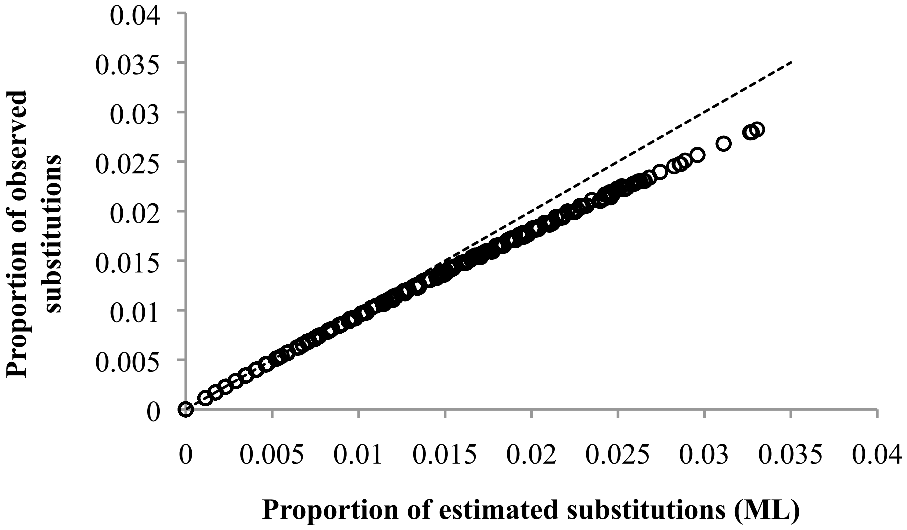


Figure 14: Scattergraph of the proportion of estimated (corrected) substitutions calculated using the K81uf+G ML model against the proportion of observed (uncorrected) substitutions found in the dataset. Linear relationship indicated by dotted line.

A high level of congruence between the dataset and the topology of the most parsimonious trees was found (RI 0.9360). The topology of the most parsimonious tree the CI and RI values (Table 21) were calculated from exactly matched the topology found by the ML analysis. The resulting ML tree is presented on Figure 15 with bootstrap support from both the ML and the MP analyses.

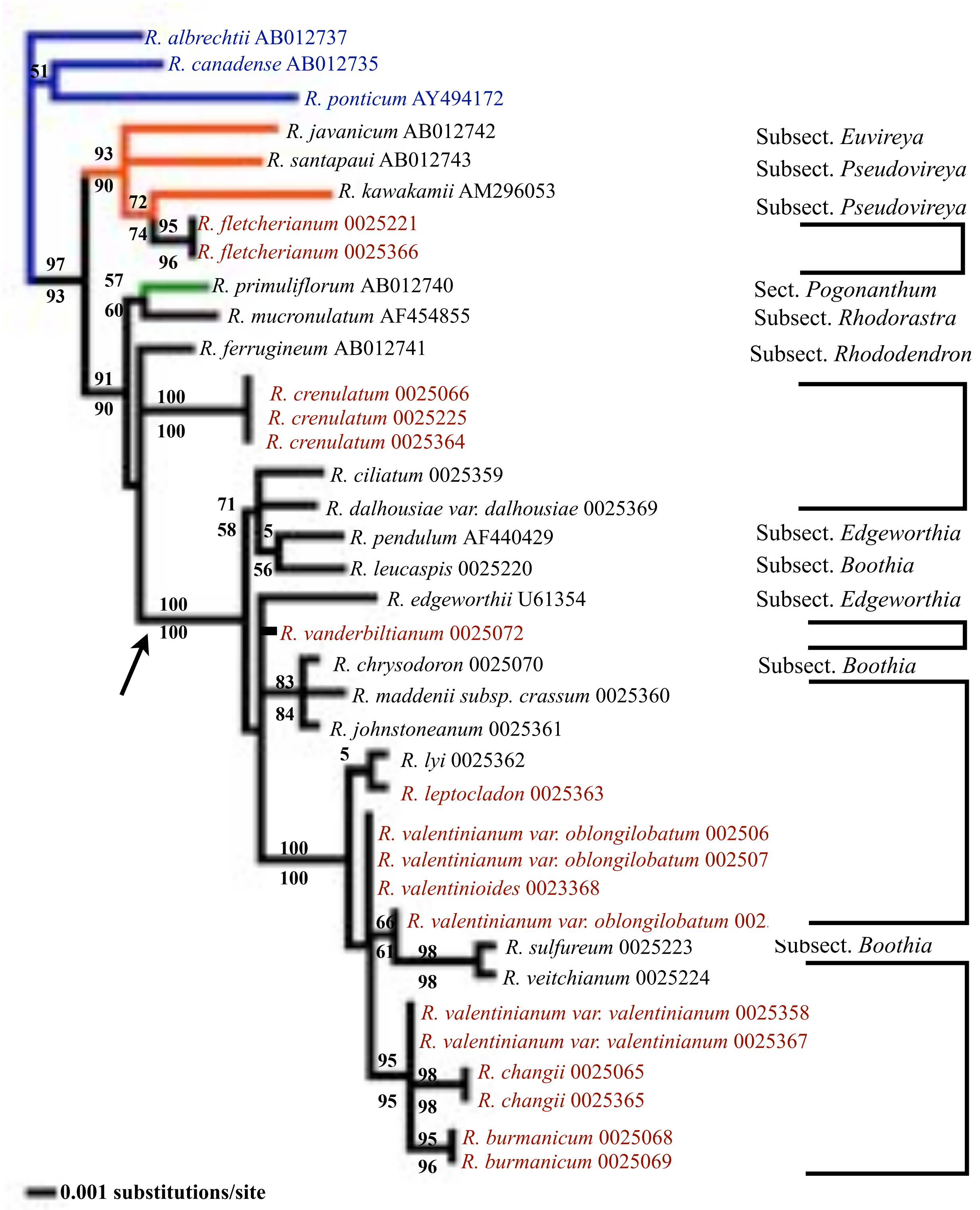


Figure 15: ML tree of 37 *Rhododendron* taxa. Bootstrap support ≥50% generated from the MP analysis written above, and the ML analysis written below, branches. Outgroups indicated in blue; branches of taxa from section *Schistanthe* highlighted in red, section *Pogonanthum* in green and section *Rhododendron* in black. Taxon names show EDNA or GenBank accession numbers and names highlighted in red indicate yellow-flowered taxa from subsection *Maddenia*. Arrow indicates “subsection *Maddenia* clade” referred to in text. Brackets indicate taxa currently placed in subsection *Maddenia*.

Figure 15 shows subgenus *Rhododendron* to be a well supported monophyletic group (bootstraps 97% MP and 93% ML). Section *Schistanthe* formed a strongly supported clade (bootstraps 93% MP and 90% ML) sister to section *Rhododendron.* Within the section *Schistanthe* clade, *R. fletcherianum* was included in a clade with *R. kawakamii* Hayata (72% MP and 74% ML bootstraps), sister to *R. javanicum* Benn. and *R. santapaui* Sastry *et al.*.

The section *Rhododendron* clade (including one species of section *Pogonanthum, R. primuliflorum* Bureau & Franch.) was well supported by bootstraps (91% MP and 90% ML) and shows the type species *R. ferrugineum* L. near the base of the group. *R. crenulatum* and *R. ferrugineum* were found between the clade composed of *R. primuliflorum* and *R. mucronulatum* Turcz. and the clade containing all of the other taxa tested from subsection *Maddenia*. The subsection *Maddenia* clade is strongly supported (MP and ML bootstraps 100%) advocating the assertion that *R. crenulatum* may not belong in subsection *Maddenia.* According to Figure 15 subsection *Maddenia* also has species from subsections *Edgeworthia* (Hutch.) Sleumer and *Boothia* (Hutch.) Sleumer nested within it. There is strong support for *R. chrysodoron* to be placed in subsection *Maddenia* as opposed to subsection *Boothia* given its inclusion in a clade with *R. maddenii* and *R. johnstoneanum* Watt ex Hutch. (bootstrap 83% MP, 84% ML). *R. vanderbiltianum* was not found to be closely related to any of the investigated taxa but is certainly included within the subsection *Maddenia* clade. The remaining yellow-flowered taxa of subsection *Maddenia,* all with entire leaf margins (*R. burmanicum, R. changii, R. leptocladon, R. valentinianum* and *R. valentinioides*), form a well supported clade (MP and ML bootstraps 100%) that is the furthest derived within the phylogeny (Figure 15).

Two accessions named *R. valentinianum var. oblongilobatum* (EDNA -067 and -071) appeared in the same clade as *R. valentinioides*, distinct from the *R. valentinianum* clade. The position of *R. valentinianum var. valentinianum* in a clade sister to *R. changii* and *R. burmanicum* with 95% MP and ML bootstrap support clearly indicates that *R. valentinioides* is a distinct species from *R. valentinianum*. The third accession of *R. valentinianum var. oblongilobatum* was placed by ML in a clade nested between *R. valentinioides* and *R. valentinianum var. valentinianum*, nearest to *R. sulfureum* and *R. veitchianum* Hook..

R. lyi and *R. leptocladon* were placed in the same clade but bootstrap support for this grouping was weak (50% MP and <50% ML), as was support for the branch separating them from the clade containing all of the yellow-flowered taxa with entire leaf margins (bootstraps <50%).

Finally, the placement of *R. sulfureum* in the same clade as *R. veitchianum* was strongly supported by bootstrap values (98% MP and ML).

3.3.3 Analyses of base pair changes

As *mat*K is a coding gene, it was expected that the majority of base pair changes would occur at the third codon position where changes are less likely to alter the amino acid coded for and are therefore less funtionally constrained (Bofkin and Goldman, 2006). Figure 16 illustrates that only 40% of changes occurred at the third position and 30% occurred at the second position which is the most functionally constrained (Bofkin and Goldman, 2006). The number of autapomorphic changes was highest at position three in accordance with expectations and the highest number of changes resulting in synapomorphies occurred at the first codon position (Figure 16).

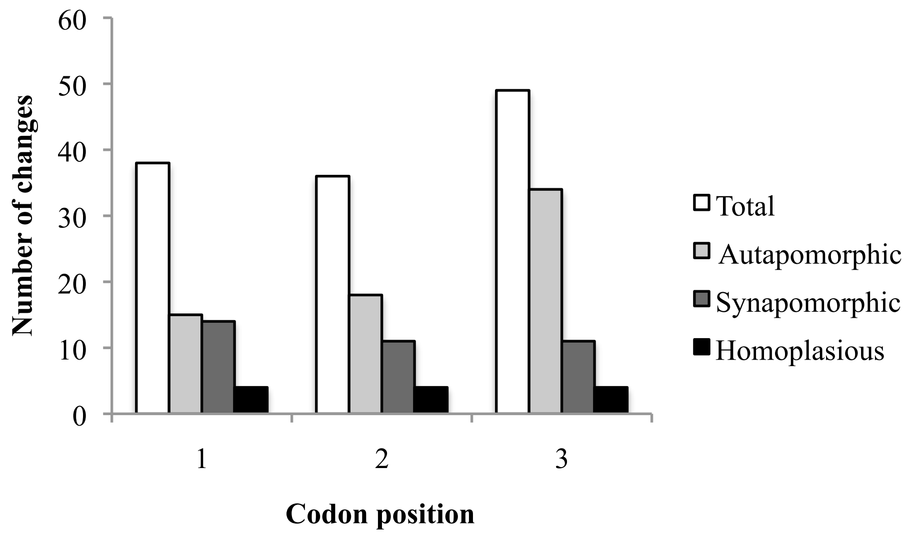


Figure 16: Histogram of the total number of base pair changes that occured at each of the codon positions of the *mat*K coding region and the number of changes at each codon position causing autapormophies, synapomorphies and homoplasies. N = 123.

The matrix was found to be rich in bases A (32.6%) and T (35.3%). When the base pair changes were counted it was revealed that a larger number of transitions than transversions had occurred (Figure 17). This is to be expected as transition mutations are less likely to result in amino acid substitutions (Brown, 2002). The most frequent transition mutation that occurred was a C-T base pair change (Figure 17).

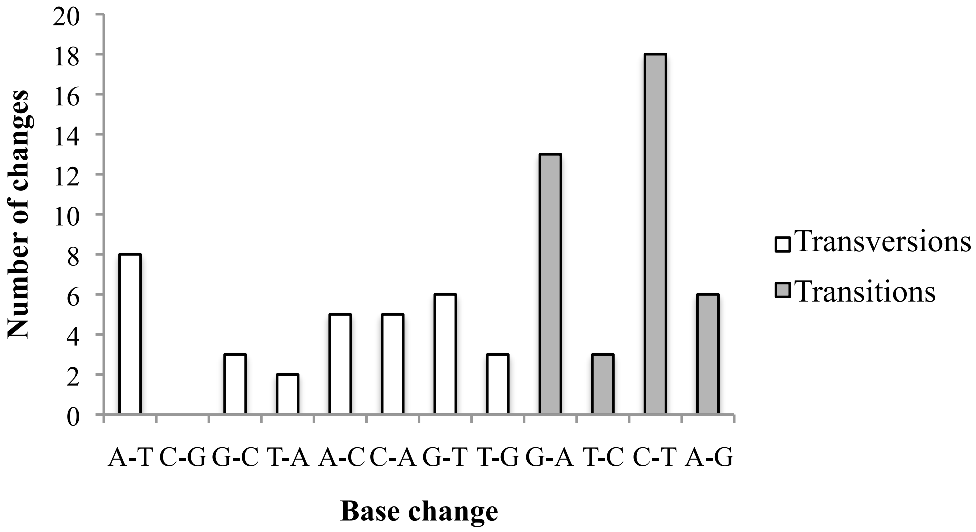


Figure 17: Histogram of the number of each type of base pair change that occured in the *mat*K coding region. N = 72.

The C-T base pair changes were analysed in order to ascertain if they resulted in a particular amino acid substitution but were discovered to occur across all three codon positions (Figure 18). A higher number of synaporphies resulted from C-T changes occurring at codon position one than at positions two or three (Figure 18).

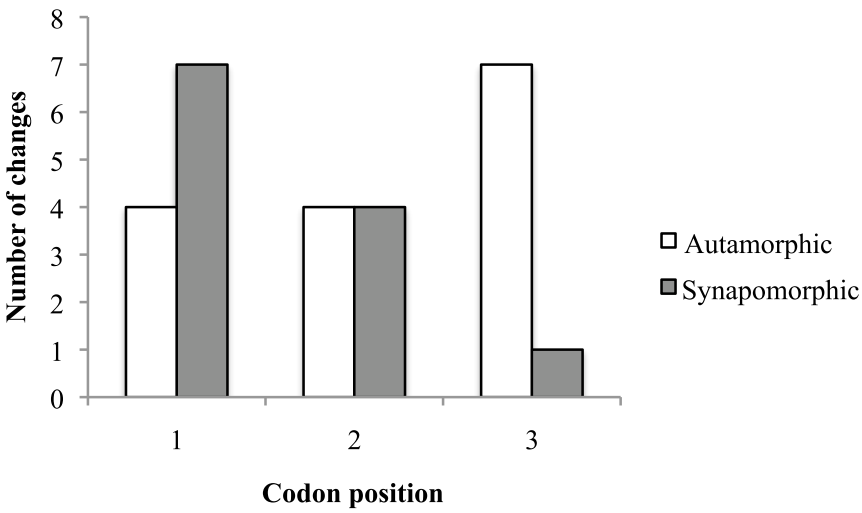


Figure 18: Histogram of the number of C-T base pair changes that occurred at each codon position, resulting in an autapomorphic and a synapomorphic character change. N = 27.

3.3.4 Maximum parsimony ancestral state reconstructions

Three of 26 morphological characters mapped to the 50% majority consensus tree obtained from 14 most parsimonious trees generated by the MP analysis are displayed on Figure 19. The basal node of the subsection *Maddenia* clade (Figure 19) was found to be white with yellow pigmentation on eight of the most parsimonious trees. This node was ambivalent on the remaining six trees for either yellow or white with yellow flowers.

Many of the other morphological characters mapped on to the molecular tree were seen to be homoplasious and did not help clarify species relationships. This partly resulted from insufficent taxon sampling across the subgenus and also from dividing continuous characters into arbitrary categories in order to include them in the analysis. However, calyx and petiole pubescence were found by the morphological review to be important characters in species identification (Section 2.2.1). Setose petioles was found to be a derived character in subsection *Maddenia* whereas loriform hairs on the calyx was found to be the ancestral state for all clades apart from section *Schistanthe* (Figure 19). All of the accessions of *R. valentinianum var. oblongilobatum* had setose petioles and loriform calyces, which is incongruent with the findings collected from herbarium specimens (Section 2.2.1).

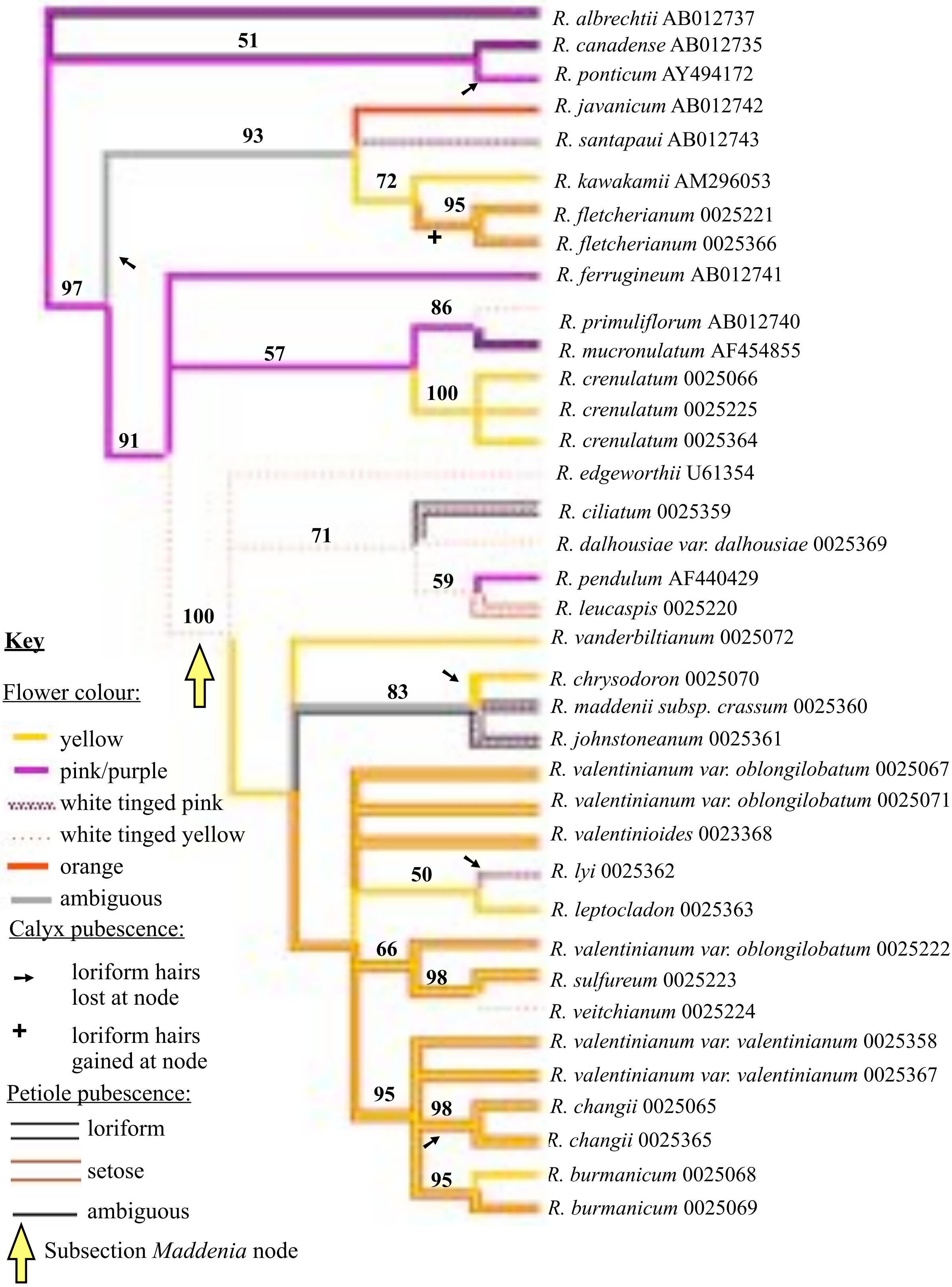


Figure 19: Reconstruction of ancestral states for flower colour, calyx pubescence and petiole pubescence mapped on to 50% majority rule consensus tree generated from MP analysis of 37 *Rhododendron* taxa. Ancestral state of all clades is a pubescent calyx unless otherwise indicated. Bootstrap support ≥50% generated from the MP analysis written above branches.

CHAPTER 4: SPECIES RELATIONSHIPS

4.1 OVERVIEW OF PHYLOGENETIC RESULTS

4.1.1 Evaluation of phylogenetic methods

The amplification of the *mat*K chloroplast region provided satisfactory resolution to determine species relationships within subsection *Maddenia.* The high number of constant characters in the data matrix showed that the region is under functional constraints, leading to the conservation of large areas of sequence in *Rhododendron*. The overwhelming majority of base changes observed were transitional (pyrimdine-pyrimidine or purine-purine) point mutations, which are usually silent (Brown, 2002). Yet the unusually high number of mutations at the first and second codon positions suggests that when these mutations did occur in the *mat*K region, the amino acid coded for was changed creating a sufficient difference in such a well conserved region to differentiate species from one another. The low level of data saturation allowed parsimony to effectively analyse species relationships and the high level of congruence between the most parsimonious trees and the ML tree show that the tree topology obtained represented the data in the best possible way. This was also demonstrated by the bootstrap support values which were generally high. Low bootstrap support was obtained for clades where taxon sampling within subsections was inadequate.

However, the reliability of single gene phylogenies is insufficient to draw incontrovertible conclusions from because the likelihood that a single gene represents the evolutionary history of the whole organism is very small (Aguileta *et al.*, 2008). This is especially true of chloroplast DNA (cpDNA) because it is maternally inherited and thus only represents the genome of one parent. The more independent loci found to converge on a single topology, the more confident it is possible to be about inferences of species relationships (Aguileta *et al.*, 2008). Rokas *et al.* (2003) showed using the yeast genus *Saccharomyces* that a small number of randomly selected genes had a high probability of supporting incorrect relationships and that a minimum of eight genes needed to be tested in order to return clades with 70% minimum bootstrap support at a 95% confidence interval. Aguileta *et al.* (2008) also used fungal sequences to suggest three to six genes should be sufficient to generate robust hypotheses about interspecific phylogenetic relationships, provided the genes were carefully selected. The results of the phylogenetic analyses conducted using the *mat*K gene region are therefore discussed below with caution and with the understanding that more genes and more taxa need to be sequenced in order to allow robust claims about species relationships to be made.

4.1.2 Subsection *Maddenia* monophyly

The MP and ML phylogenies indicated that subsection *Maddenia* may be paraphyletic. *R. ciliatum* Hook.f. and *R. dalhousiae* Hook.f. *var. dalhousiae* were found to be more closely related to *R. pendulum* Hook.f. (subsection *Edgeworthia*) and *R. leucaspis* (subsection *Boothia*) than any of the other sequenced species of subsection *Maddenia*. However, this finding was only weakly supported by bootstraps. Cullen (1980) wrote that the three subsections were clearly closely related, sharing seeds which are winged and finned, an epiphytic habit and a similar geographical range across N. India, China, Burma and Bhutan. Yet the morphological characters used to separate the three subsections are well recognised: species in subsection *Boothia* have broadly campanulate flowers with stamens that are not declinate and vesicular scales that are sunken into the lamina (Cullen, 1980; Seithe, 1980); subsection *Edgeworthia* is characterised by a unique indumentum of dense brown and white curled hairs that cover the adaxial leaf surface. A larger number of taxa from all three subsections across several loci would hence need to be tested before any conclusions could reasonably be drawn about the monophyly of subsection *Maddenia* and more still to convince taxonomists as to the merit of changing the subsectional classification.

4.1.3 The evolution of yellow flowers

Further accessions of species within the subsection need to be tested in order to conclusively discover the ancestral flower colour of subsection *Maddenia*. However, as all of these accessions would be white, it is possible to postulate that yellow flowers are a derived character within subsection *Maddenia.* Although yellow flower colour characterised a number of well-supported clades within the subsection, the yellow species investigated did not form a monophyletic group suggesting that yellow flower colour has been subject to numerous advances and reversals. This is unsurprising given the findings of Dunemann *et al.* (1999) who found flower colour was a complex quantitative trait involving up to six loci. The ancestral colour in subsection *Maddenia* was probably white and has changed to yellow several times, perhaps in response to changes in pollinators (as found in Bornean Zingiberaceae by Sakai *et al.*, 1999). Few studies of pollination syndromes have been conducted for species in subgenus *Rhododendron* (Escaravage *et al.*, 2001; Kjellsson *et al.*, 1985) but the white, highly scented flowers with an increased number of stamens found in species such as *R. maddenii* are likely to attract different pollinators to the non-scented yellow flowers. Stevens (1985) noted a strong correlation between flower colour and altitude in section *Schistanthe* where red corollas became more prevalent at higher elevations where bird pollination was more common. It is possible that a similar correlation occurs in subsection *Maddenia* (although given the flower-shape it is unlikely to be a result of bird pollination) and an interesting topic for further study would be to establish if yellow-flowered species are consistently found at higher altitudes than white-flowered species.

4.2 INTERSPECIFIC RELATIONSHIPS OF YELLOW-FLOWERED TAXA

4.2.1 Species with crenulate leaf margins

All three species with crenulate leaf margins, planate midribs, sparse abaxial scales and leaves lacking papillae were placed by the MP/ML trees outside of the major clade of yellow-flowered *Maddenia* species, posing interesting questions about their taxonomy. The phylogeny obtained by this study suggests that crenulate leaf margins might have resulted from parallel evolution as *R. crenulatum*, *R. fletcherianum* and *R. vanderbiltianum* did not form a monophyletic group and were geographically isolated from one another.

R. fletcherianum lacked a lepidote calyx and corolla tube which were constant characters for all the other yellow-flowered species of subsection *Maddenia* with or without entire leaf margins. It was also the only species to possess loriform hairs on the ovary. Furthermore, *R. fletcherianum* had the most northerly distribution and was found at higher altitudes than the other yellow-flowered species. It is difficult to state what bearing these differences in ecology have on the lack of corolla and calyx scales. Meta-analyses investigating the relationships between morphology and the environment are currently lacking in the wider literature and certainly with regard to *Rhododendron*. No correlation between climate and crenulate leaf margins is readily observable between *R. fletcherianum*, *R. crenulatum* and *R. vanderbiltianum* given the three different environments occupied by each species. These features are insufficient, however, to suggest that *R. fletcherianum* might belong to section *Schistanthe* as found by the MP/ML phylogeny.

Section *Schistanthe* is characterised by flowers that lack corolla spotting, posses rim-like calyces, ovaries that taper into the style and seeds that are tailed at both ends (Argent, 2006). *R. fletcherianum* seeds were briefly examined from the cultivated material collected and found to lack tails. The yellow pigment gossypetin was found to be absent from *Rhododendron* leaves in section *Schistanthe* whereas it has been found in *R. fletcherianum* leaves and is the likely source of the yellow flower colour (Harborne and Williams, 1971; Harborne, 1986). The centre of diversity for section *Schistanthe* is in Malesia (Stevens, 1985). Only ten species grow outside of this region on the SE Asian mainland in Nepal, Bhutan, China, Myanmar, Vietnam and N. India, all of which belong to subsection *Pseudovireya*. This includes *R. santapaui* and *R. kawakamii* which were used in the MP/ML analysis.

R. fletcherianum was shown to be closely related to *R. kawakamii* which has only been found in Taiwan. Given all the above information, it is unlikely that *R. fletcherianum* is closely related to the vireyas and its placement by the phylogenetic analyses in that clade is likely to be an anomaly, possibly caused by a sequencing error, that will be refuted were other loci to be sequenced.

R. crenulatum was not found by the MP/ML analyses to conform to the subsection *Maddenia* clade either but was shown to be within subgenus *Rhododendron*. Apart from crenulate leaf margins, lack of papillae, planate midrib and sparse covering of abaxial leaf scales, it differed from the other yellow-flowered species in possessing a campanulate corolla and scales along the length of the style. Puberulent petioles and raised abaxial primary veins were shared only with *R. vanderbiltianum* but despite these morphological similarities, the cpDNA did not support the hypothesis that the two species are closely related. The geographical separation of the two species also suggested a close relationship would be unlikely. The distribution of *R. crenulatum* with respect to that of subsection *Maddenia* is puzzling as it was the only one of the group found in Laos and its occurrence in Vietnam does not necessarily suggest it is closely related to the other yellow-flowered species found there as this region hosts a large number of endemic *Rhododendron* species (D. F. Chamberlain, August 2012, pers. comm.). Further investigation is needed to ascertain if Mount Fan Si Pan in Vietnam is truly the northernmost point of its range or if *R. crenulatum* occurs across the border into SE Yunnan which would suggest a closer affinity with species such as *R. valentinianum*.

It is possible that *R. crenulatum* should be included in subsection *Maddenia* and the species it is most closely related to have not been included in this study. This seems unlikely, however, as all Alliances apart from *R. megacalyx* have been sampled and found to form a well-supported monophyletic group. The evidence presented in this study is insufficient to assert that *R. crenulatum* should not be placed in subsection *Maddenia* but future studies investigating section *Rhododendron* should include *R. crenulatum* sequences to provide a clearer picture of its interspecific relationships.

The MP and ML analyses included *R. vanderbiltianum* in the subsection *Maddenia* clade but not as a close relative of any other species and outside of the clade containing all the yellow-flowered species with entire leaves. Certainly the occurrence of the species in Sumatra is highly incongruent with the other species of subsection *Maddenia* which are distributed throughout the Himalayas, Myanmar, Vietnam and Laos (Cullen, 1980). The majority of species found in Malesia belong to section *Schistanthe* and no other section of subgenus *Rhododendron* is represented in the *Flora Malesiana* (Sleumer, 1966). Geography may account for Sleumer's (1966) placement of *R. vanderbiltianum* in subsection *Pseudovireya,* a decision which was poorly supported by morphological characters (Argent *et al.*, 2008). The funnelform corolla, dimorphic stamens arranged in a complete circle within the corolla, leaf buds fringed with simple silver hairs and seeds that lack tails are characters that cannot be observed in section *Schistanthe* but are present in subsection *Maddenia*.

Phylogenetic analysis using the ITS nuclear region conducted by Argent *et al.* (2008) placed *R. vanderbiltianum* in a polytomy outside of section *Schistanthe*. In the MP phylogeny investigating subsectional relationships of section *Schistanthe* produced by Hall *et al.* (2006) using the chloroplast region RPB2-i, *R. vanderbiltianum* was not found to be closely related to any of the species and was placed between strongly supported clades of subsections *Discovireya* (Sleumer) Argent and *Pseudovireya*. *R. vanderbiltianum* was found to be closest to *R. santapaui* which was placed in subsection *Pseudovireya* with weak bootstrap support (53%) by Hall *et al*. (2006). Goetsch *et al*. (2011) conducted research using multiple nuclear genes. In both the MP and ML analyses *R. vanderbiltianum* was found between the species included from subgenus *Rhododendron* (*R. ferrugineum* and *R. minus* Michx.) and the *Schistanthe* subsection *Discovireya*.

It appears that no investigation of *R. vanderbiltianum* outside of section *Schistanthe* has been conducted prior to this study. The results of the MP/ML analyses found *R. vanderbiltianum* to be only distantly related to *R. ferrugineum* and may be the first indication that *R. vanderbiltianum* should indeed be included in subsection *Maddenia,* which would have interesting biogeographic implications. Inclusion of this species in broader investigations of species level relationships within subgenus *Rhododendron* using multiple gene regions is now necessary to draw holistic conclusions about the sectional and subsectional placement of *R. vanderbiltianum*. This task will become increasingly feasible as more rhododendron sequences are added to GenBank. Further investigation of the chemical composition of leaf and corolla pigments may also be informative as the pale yellow corolla of

R. vanderbiltianum is more comparable with those caused by gossypetin in subsection *Maddenia* than the carotenoid bright yellow of vireya species.

4.2.2 Species with entire leaf margins

All of the yellow-flowered species with entire leaf margins (*R. burmanicum*, *R. changii*, *R. leptocladon*, *R. valentinianum* and *R. valentinioides*) formed a monophyletic clade within the subsection *Maddenia* clade.

R. leptocladon was determined to be a separate species from the white-flowered *R. lyi* in accordance with the morphological findings of Holland (1997) and poor bootstrap support for the grouping of the two species on the MP/ML tree. Cullen (1980) separated *R. leptocladon* from the other yellow-flowered species of subsection *Maddenia* into a different subgroup because the style tapers into the ovary. Morphological examination of the species confirmed this, found scales to cover the basal half of the style and determined that the petioles were glabrous, all of which features were unique to *R. leptocladon*. However, in the MP/ML phylogeny *R. leptocladon* was found in a strongly supported monophyletic group with the other yellow-flowered species with entire leaf margins and the branch separating it from these species was poorly supported. Geographically the distribution of *R. leptocladon* overlaps with *R. valentinianum* on Mount Fan Si Pan in Vietnam and also in SE Yunnan, should a specimen collected under the name *R. nemorosum* R. C. Fang prove upon further examination to be synonym of *R. leptocladon* (Chapter 5). All of this information provides satisfactory evidence that despite some morphological differences *R. leptocladon* should not be maintained in a different informal subgroup to the other yellow-flowered species with entire leaf margins.

Two accessions of *R. valentinianum var. oblongilobatum* were found to possess setose petioles and loriform calyces and were found in the same clade as *R. valentinioides* rather than that of *R. valentinianum*. This suggests these accessions have been misnamed in cultivation and are in fact accessions of *R. valentinioides*. The placement of *R. valentinioides* outside of the clade containing *R. valentinianum var. valentinianum* demonstrates that it is a valid new species. *R. valentinioides* could be confused with *R. valentinianum* as it is such a widespread and variable species but *R. valentinioides* leaves were found to be cucullate and oblong compared to the oblong-elliptic leaves of *R. valentinianum*. More wild origin flowering material needs to be obtained for *R. valentinioides* in order to complete its taxonomic account and may reveal further characters that distinguish it from *R valentinianum*. This may also help identify *R. valentinianum var. oblongilobatum* (accession number 19960621*F) which was morphologically very similar to accessions of *R. valentinioides* grown in the research collection but was found to be more closely related to *R. sulfureum* and *R. veitchianum* in the MP/ML phylogeny. It would appear, therefore, that this taxon has also been cultivated under an inaccurrate name and may suggest it is a hybrid of a maternal parent which has not been sampled for this study. Further research needs to be conducted using this plant to identify if it has characters that suggest it might be a hybrid form of *R. valentinioides* and identify a likely maternal parent. Unfortunately, it appears that no true to name accessions of *R. valentinianum var. oblongilobatum* have been sequenced.

The confident grouping of the morphologically distinct *R. sulfureum* and *R. veitchianum* is extremely anomalous. The sequences were checked to ensure the correct sequences had been compiled from the correct material and the identification of voucher specimens was verified in comparison with herbarium material but the possibility of human error can never be entirely disproved. It is possible that this finding resulted from chloroplast capture, a type horizontal gene transfer, caused by the introgression of the chloroplast genome into a different species without the inclusion of any new DNA into the nuclear region (Stegemann *et al.*, 2011). Chloroplast capture has been found to occur frequently in *Rhododendron* in subgenus *Tsutsusi* (Tagane *et al.,* 2008), subgenus *Pentanthera* (Kron *et al.,* 1993) and subgenus *Hymenanthes* (Milne *et al*., 2010) and it is therefore likely to occur in subgenus *Rhododendron*. However, overlapping species distributions are necessary to facilitate horizontal gene transfer. The sampled plants of *R. sulfureum* and *R. veitchianum* were collected in Vietnam and Thailand respectively and it is unknown to what extent the species distributions overlap. The specimens should be re-sequenced to ascertain if this result is reproducible and then an investigation of nuclear DNA could be used to indicate if chloroplast capture was likely to have occurred.

It is interesting that *R. chrysodoron* was shown to be closely related to *R. maddenii* and *R. johnstoneanum* by the MP/ML analyses. Cullen (1980) noted that it displays many characteristics commonly found in subsection *Maddenia* including large flowers, a rim-like calyx and scales not sunken in pits unlike those which characterise subsection Boothia. Cullen (1980) maintained the position of *R. chrysodoron* in subsection *Boothia* having hypothesised that it may result from occasional hybridisation between a species of subsection *Boothia* and a species of subsection *Maddenia*. This study strongly suggests that at least the maternal parent of *R. chrysodoron* was from subsection *Maddenia* and further analysis of the species using nuclear DNA may suggest the species should be moved into the subsection.

R. burmanicum, *R. changii* and *R. valentinianum* shared a large number of morphological characters including setose petioles, oblong-elliptic leaves, a dense covering of abaxial scales, prominent midribs and funnel-campanulate corollas. It is unsurprising, therefore, that the MP/ML phylogeny found these species to be closely related to one another. However, the species ranges were not found to overlap as *R. changii* was distributed in SE Sichuan some considerable distance from *R. burmanicum* in NE Myanmar. *R. valentinianum* was found between the ranges of these two species in S Yunnan but was also isolated from them. The disjunct distribution of *R. valentinianum* may once have been a continuous corridor from N Vietnam to W Yunnan, which has possibly been fragmented by changes in climate. The morphological and molecular similarities of the three species indicate that they shared a common ancestor comparatively recently. This provides good evidence that speciation has occurred in this subsection as a result of geographic isolation on inselbergs associated with mountain building and subsequent climatic changes.

It is interesting that *R. burmanicum* and *R. changii* were found to be more closely related to each other than *R. valentinianum* given their geographical separation and morphological differences. *R burmanicum* was the most morphologically distinctive of the three species having overlapping, misshapen leaf scales, an acuminate leaf apex, loriform juvenile leaf margins that were otherwise glabrous and a large number of flowers per inflorescence. *R. changii* and *R. valentinianum* shared mucronate leaf apices, setose leaf margins and 2-4 flowered inflorescences but *R. changii* had no scales on the style, conspicuously flattened petioles and lacked the upturned rim found in *R. valentinianum* scales. These characters, the distribution of *R. changii* further NE of *R. valentinianum* and the MP/ML phylogeny support the changes made by W. P. Fang who elevated *R. changii* from the subspecific rank of *R. valentinianum var. changii*. Despite having been accidently excluded from the molecular analyses, *R. valentinianum var. oblongilobatum* was maintained as a variety because it differed from *R. valentinianum var. valentinianum* in lacking pedicel and calyx hairs and was found within the geographic range of *R. valentinianum var. valentinianum*.

4.2.3 Subsection *Maddenia* Alliances

It is interesting that species thought to be closely related within subsection *Maddenia* using morphological characters were not found to be so using molecular characters. Species grouped in the Ciliicalyx/Johnstoneanum Alliance were distributed throughout the phylogeny and were not monophyletic, partly because species from subsections *Boothia* and *Edgeworthia* were nested within the subsection. The placement of *R. ciliatum* with *R. dalhousiae var. dalhousiae* was weakly supported by bootstraps but suggests *R. ciliatum* was not closely related to any of the other species in the Ciliicalyx/Johnstoneanum Alliance. More species from the Dalhousiae Alliance need to be included in the data matrix, therefore, in order to ascertain if *R. ciliatum* is closely related to species in this Alliance and to find out if the Dalhousiae Alliance itself is monophyletic.

Little evidence was found to support the separation of the Ciliicalyx/Johnstoneanum Alliance using the style-ovary transition character. *R. ciliatum*, *R. johnstoneanum*, *R. crenulatum* and *R. vanderbiltianum* did not form a monophyletic group with *R. changii*, *R. burmanicum* and *R. valentinianum*, undermining the grouping of species with impressed styles. Conversely *R. veitchianum* was more closely related to these species that to *R. leptocladon* despite possessing a tapering style.

The Maddenii Alliance (represented by *R. maddenii*) was shown to be nested within the Ciliicalyx/Johnstoneanum Alliance. Cullen (1980) assigned *R. maddenii* to an alliance of its own because it is a variable species and has an increased number of stamens. This certainly justifies the separation of *R. maddenii* from the other species but the MP/ML findings suggest it should be at a subgroup rank as part of a wider Alliance.

The MP/ML phylogeny generated by this study clearly divides subsection *Maddenia* into two groups: one containing *R. lyi*, *R. leptocladon*, *R. valentinioides*, *R. valentinianum*, *R. changii* and *R. burmanicum* and a group containing the remaining species. More species of subsection *Maddenia* would need to be added to the phylogeny to examine if these groupings would still be supported when the full complement of species is examined. In addition no morphological characters have been identified that would support these groupings. The Alliances devised by Cullen (1980) do not reflect the relationships of species within subsection *Maddenia* but do facilitate species identification. Consequently, the Alliances should remain in use until such a time as groupings reflecting the entire species complement of the subsection can be defined matching morphological characters to closely related clades.

CHAPTER 5: TAXONOMIC ACCOUNT

All the data accumulated from this investigation was used to create a full taxonomic account of the yellow-flowered species of subsection *Maddenia*. All specimens are listed in Appendix 1. All measurements were taken from wild collected material only. Leaf measurements are presented as length × width and any solitary measurements are of length. Fruiting characters were extracted from species protologues if no material was available for consultation.

RHODODENDRON Linnaeus, Sp. Pl 1: 392. 1753.

Subgenus **Rhododendron**

Syn.: Subgenus *Lepidorrhodium* Koehne, Deutsche Dendrol. 449. 1853.

Subgenus *Eurhododendron* K. Koch, Dendrol. 2:157. 1852.

Section *Lepidorhodion* (Koehne) Rehder in Bailey, Standard Cycl. Hort. 5:2937. 1916.

Section **Rhododendron**

Syn.: Section *Lepipherum* G. Don, Gen. Hist. Dichlam. Pl. 3: 845. 1834.

Subsection **Maddenia** (Hutchinson) Sleumer, Bot. Jahrb. Syst. 74: 533. 1949.

Syn.: Series *Maddenii* sensu Hutchinson, Notes R. B. G. Edinb. 12: 1. 1919, and -

The Species of Rhododendron, 447. 1930.

Shrubs or small trees, 0.3-15.0 m tall; epiphytic or terrestrial. Young growth lepidote, often loriform-setose. Leaf sheaths lined with silver hairs. Leaves evergreen; adaxial surface sparsely lepidote when young, quickly becoming elepidote; abaxial surface covered in entire scales of unequal size and variable density; midrib (planate) or adaxially sunken and abaxially protruding. Inflorescences terminal, cymose or umbellate, 1-6(-10)-flowered. Calyx variable, from inconspicuous rim to conspicuous and 5-lobed, sometimes ciliate, usually lepidote. Corolla funnel-campanulate, funnelform or campanulate, yellow; outside of tube fluted with 5 grooves, often puberulent at base, usually with bands of scales extending from near base of tube to tips of corolla lobes. Stamens 8-10, dimorphic, filaments pilose to at least 1/3 from base. Ovary 5-locular, lepidote. Style longer than stamens, usually basally lepidote, impressed or tapering into the ovary. Capsule ovoid to cylindric, lepidote. Seeds winged and finned.

Type species: *R. maddenii* Hooker (K, M).

1a. Leaf margins crenulate **2**

1b. Leaf margins entire **4**

2a. Leaf margins and petioles setose **1. *R. fletcherianum***

2b. Leaf margins not setose, petioles ± puberulent **3**

3a. Leaf bases cuneate; corolla 20-30 mm from base of tube to tip of corolla lobes **2. *R. crenulatum***

3b. Leaf bases obtuse; corolla 9-19 mm from base of tube to tip of corolla lobes **3. *R. vanderbiltianum***

4a. Leaf margins not brown-setose, may have loriform bristles **5**

4b. Leaf margins brown-setose **6**

5a. Leaves obovate or oblong-elliptic, abaxial scales overlapping; corolla funnel-campanulate with tube 15-30 mm **4. *R. burmanicum***

5b. Leaves elliptic, abaxial scales rarely touching; corolla funnelform with no distinct tube **5. *R. leptocladon***

6a. Leaves obovate-elliptic with attenuate base, petioles flattened, primary veins inconspicuous **6. *R. changii***

6b. Leaves obovate-elliptic or oblong with obtuse base, petioles rounded, primary veins ± protruding abaxially **7**

7a. Leaves oblong, abaxial scales not touching and largest 5 × diameter of smallest scales, primary veins deeply adaxially impressed **7. *R. sp. nov.***

7b. Leaves obovate-elliptic, abaxial scales often overlapping with marked difference in size but not as pronounced as above, primary veins almost inconspicuous **8. *R. valentinianum***

1. R. fletcherianum Davidian, R. H. S. Rhodo. & Camellia Yearbook, 16: 103. 1961

Type: Rock #22302, Jun-Jul 1922, China: Xizang, Suola (E).
Illustr.: Cox, P. A., *The Smaller Rhododendrons*, Figure 467. 1985. Taylor, G., ed. *Curtis's Botanical Magazine,* 176(3), pp. 508. 1969.

Shrub, 0.6-1.2 m. Young growth sparsely setose. Leaf sheaths glabrous. Petioles 3-6 mm, flattened and narrowly winged, lepidote, setose. Leaves elliptic or oblong-elliptic, 30-48 × (5-)11-30 mm; apex mucronate; base cuneate or obtuse; margin crenulate, setose; lamina dark green above, pale below; adaxially glabrous; abaxial scales small, relatively evenly sized and distributed with a solid rather than membranous rim; papillae absent; midrib impressed above, planate below; all veins impressed and concave above. Inflorescence cymose, 2-5 flowered. Pedicels (3-)6-10 mm, hispid, sparsely lepidote. Calyx 7-9 mm, 5-lobed, oblong or oblong-ovate, loriform-ciliate, sparsely lepidote at base or lacking scales. Corolla broadly funnelform, (21-)36-42 mm, lobes rounded, glabrous. Stamens 10, longest equal length to corolla, outer whorl 18-26 mm, inner whorl 25-35 mm, pilose at base. Ovary 3 mm, oblong with loriform hairs at the apex. Style ± exserted from corolla, glabrous. Capsule 9 mm, cylindric. Fl. Apr-Jun.

CHINA (SE Xizang and NW Yunnan). Alpine regions and rocky outcrops, 2900-4300 m.

2. R. crenulatum Hutchinson ex Sleumer, Blumea Suppl. 4: 44. 1958

Type: Kerr #21044, 14th Apr 1932, Laos: Tranh-Ninh, Pu Bia (K).

Shrub to 1 m. Young growth loriform-setose. Petioles 2-4 mm, flattened and narrowly winged, sparsely lepidote, silvery puberulent. Leaves elliptic, 25-35 × 10-15 mm; apex abruptly acute or shortly acuminate; base cuneate; margin crenulate, glabrous; lamina dark green, shiny above; adaxial scales small and regularly spaced; abaxial scales small, of even size and distribution with a large inner zone occupying ± 75% total diameter; papillae absent; midrib planate; primary veins adaxially raised, puberulent. Inflorescence umbellate, 3-4 flowered. Pedicels 9-12 mm, densely lepidote. Calyx 1-2 mm, 5-lobed, rounded, loriform-ciliate, densely lepidote. Corolla campanulate, 20-30 mm, tube 13-20 mm, lobes obovate. Stamens 8-10, equal to or shorter than corolla, outer whorl 17-20 mm, inner whorl 10-13 mm, pilose at base. Ovary 4-5 mm, conical. Style ± exserted from corolla, lepidote along length with density decreasing from base to tip, impressed into ovary. Capsule 10-13 mm, cylindric. Fl. Apr.

LAOS (SE Vientiane), VIETNAM (W Lao Cai). 2000-2900 m.

R. crenulatum has a white corolla with a yellow flush inside the corolla tube and green speckles covering the centre of the three upper lobes when grown in cultivation. However, specimens collected in the wild describe the corolla as being ‘pale yellow’.

3. R. vanderbiltianum Merrill, Notul. Nat. Acad. Nat. Sci. Philadelphia 47: 5. 1940
Type: Ripley and Ulmer #81, 5^{th} May 1939, Indonesia: Aceh, Mount Leuser (PH).

Shrub, 0.2-1.5 m. Young growth minutely puberulent. Petioles 1-3 mm, flattened above, rounded below, narrowly winged, sparsely abaxially lepidote, puberulent. Leaves elliptic, 10-23 × 8-16 mm; apex shortly acute; base obtuse; margin crenulate, glabrous; lamina dark green, shiny above; adaxial scales small and regularly spaced if present; abaxial scales relatively large, little size variation and regularly distributed; papillae absent; midrib planate; primary veins adaxially raised, puberulent. Inflorescence umbellate, 2-5 flowered. Pedicels 5-11 mm, densely lepidote. Calyx 1-3 mm, occasionally loriform-setose, scales overlapping. Corolla funnelform, 9-19 mm, lobes acute. Stamens 10, equal to or shorter than corolla, outer whorl 14 mm, inner whorl 10 mm, pilose at base. Ovary 3 mm, conical. Style similar length to corolla, glabrous, impressed into ovary. Capsule 9 mm, cylindric. Fl. Mar-Jun.

INDONESIA (N. Sumatra - Aceh). Montane scrub, 2100-3200 m.

Although geographically distant, *R. vanderbiltianum* is morphologically very similar to *R. crenulatum*. Differences in scale size and distribution are only apparent under SEM. *R. vanderbiltianum* is generally smaller than *R. crenulatum*. The shape of the corolla is campanulate in *R. crenulatum* and funnelform in *R. vanderbiltianum*. Corolla lobes in *R. vanderbiltianum* are slightly crenulate and acute at the apex compared to being rounded and obtuse in *R. crenulatum*.

4. R. burmanicum Hutchinson, Bull. Misc. Inform. Kew 1914: 185. 1914

Type: Wheeler-Cuffe #5, 1st April 1917, Myanmar: Chin, Mount Victoria (K).

Shrub to 2 m. Young growth has dense indumentum of loriform setae. Petioles 5-14 mm, narrowly winged, lepidote, ± setose. Leaves obovate or oblong-elliptic, 40-70 × 20-40 mm; apex gradually acuminate; base attenuate; margin entire, loriform ciliate when young; adaxial surface may have remnants of desiccated scales; abaxial scales overlapping, misshapen, flattened, unequal sizes, inner zone occupying ± 60% total diameter; papillae dense, spherical; midrib abaxially lepidote; primary veins barely visible. Inflorescence cymose, 4-7(-11) flowered. Pedicels 7-15 mm, densely lepidote. Calyx 1-2 mm, indistinct, lepidote, margins loriform-ciliate. Corolla funnel-campanulate, 30-46 mm, tube 15-30 mm, lobes ovate. Stamens 10, shorter than corolla, filaments outer whorl 20-35 mm and inner whorl 18-25 mm, pilose to 1/2 length. Ovary 5 mm, obovoid. Style exserted from corolla, lepidote shortly at base, impressed into ovary. Fruit unknown. Fl. Apr.

MYANMAR (S Chin). Forest fringes, 2700-3000 m.

Originally described from cultivated material but has since been re-discovered in the wild.

5. R. leptocladon Dop in Lecomte, Fl. Indo-Chine 3: 745. 1930

Syn.: *R. nemorosum* R. C. Fang, Acta Bot. Yunnan. 6(3): 290. 1984.
Type: Poilane #12680, 19^{th} July 1926, Vietnam: Lao Cai, Lo Sui Tong (P).
Illustr.: Wu, Z. Y., Raven, P. H. and Hong, D. Y., eds. 2005. *Flora of China. Vol. 14: Apiaceae through Ericaceae.* Frontispiece.

Shrub to 4 m. Young growth not seen. Petioles 1-9 mm, rounded ± narrowly winged, densely lepidote, not setose. Leaves elliptic, 45-65(-100) × 19-38 mm; apex acute; base cuneate or truncate; margin entire, glabrous; adaxial scales very sparse if present; abaxial surface grey-green, scales dense but rarely touching, flattened, extremely variable in size; papillae dense, spherical; midrib abaxially lepidote; primary veins variably conspicuous. Inflorescence umbellate, 2-3 flowered. Pedicels 5-10 mm, densely lepidote. Calyx 1-2 mm, 5-lobed, ovate, loriform-ciliate, lepidote. Corolla funnelform, 30-65 mm, lobes ovate. Stamens 10, shorter than corolla, outer whorl 30-40 mm, inner whorl 22-33 mm, pilose at base. Ovary 5 mm, conical. Style shortly exserted from corolla, lepidote to 1/2 length, tapering into ovary. Capsule 13-16 mm, oblong-ovoid. Fl. Mar-Apr.

VIETNAM (W Lao Cai). Roadsides and cliffs, 1600-2500 m.

Morphologically very similar to *R. lyi* but distinguishing characters are yellow flowers with glabrous calyx, setose petioles that lose hairs at maturity, glabrous leaf margins except in new leaves and shorter, rounder and denser papillae in *R. leptocladon.*

R. nemorosum R. C. Fang is suspected to be synonym of *R. leptocladon* (Holland, 1997) but the type material has not been seen in order to verify this. If it is indeed a synonym, this would expand the geographic distribution of *R. leptocladon* across the border of Vietnam into China as *R nemorosum* was found at Jinping Xian in SE Yunnan (Fang, 1984).

6. R. changii (Fang) W. P. Fang, Acta. Phytotax. Sin. 21(4): 465. 1983

Syn.: *R. valentinianum var. changii* Fang, Contr. Biol. Lab. Sci. Soc. China 12: 71. 1939.
Type: Chang #158, 21st April 1930, China: Sichuan, Jinfo Shan (SZ).
Illustr.: Wu, Z. Y., Raven, P. H. and Hong, D. Y., eds. 2005. *Flora of China. Vol. 14 Illustrations: Apiaceae through Ericaceae*. Figure 468.

Shrub, 1.0 - 1.5 m. Young growth setose. Petioles 3-4 mm, flattened, lepidote, setose. Leaves obovate or oblong-elliptic, 30-50 × 20-39 mm; apex mucronate; base attenuate; margin entire, usually setose; adaxial scales soon deciduous; abaxial scales dense, flattened, very variable in size, inner zone occupying ± 70% total diameter; papillae dense, spherical; midrib abaxially lepidote and occasionally loriform-setose; primary veins inconspicuous. Inflorescence cymose, 2-4 flowered. Pedicels 4-8 mm, densely lepidote. Calyx 10 mm, 5-lobed, ovate, lepidote. Corolla funnel-campanulate, (25-)35-40 mm, tube 13-20 mm, lobes ± rounded. Stamens 10, almost equal or shorter than corolla, outer whorl 23-25 mm, inner whorl 18-20 mm, densely pilose to ≥1/2 length. Ovary 5 mm, ovoid. Style exserted from corolla, shortly hairy along length, impressed into ovary. Capsule 12-15 mm, cylindric-ovoid. Fl. Apr-May.

CHINA (SE Sichuan). Sheltered rocks and thickets, 1600-2100 m.

Very similar to *R. valentinianum* but differs in having scales with a smaller rim that is flattened rather than upturned, larger flowers and no hairs on the calyx and pedicels.

7. R. sp. nov. (tentatively named **R. valentinioides** by Dr. D. F. Chamberlain)

Type: Cox and Hutchison #7186, 17th October 1995, China: Yunnan, Lao Jing Shan (E).

Shrub to 2 m. Young growth not seen. Petioles 10 mm, rounded, densely lepidote, setose. Leaves oblong, 50 × 32 mm; apex mucronate, cucullate; base obtuse; margin entire, setose; adaxial scales inconspicuous; abaxial scales dense but well spaced, flattened, extreme size variation with largest scales 5 × diameter of smallest, inner zone consistently occupies 50% of the total scale diameter, somewhat sunken into lamina; papillae subdense, cylindrical; midrib abaxially lepidote, setose when immature; primary veins deeply adaxially impressed and abaxially protruding. Inflorescence umbellate, 2-4 flowered. Pedicels not seen. Calyx 5-6 mm, 5-lobed, ovate, lepidote and loriform-ciliate. Corolla funnel-campanulate, 20 mm, lobes ovate. Stamens shorter than corolla. Ovary not seen. Capsule 14-15 mm, ovoid. Fl. unknown in wild.

CHINA (SE Yunnan). Mixed evergreen, deciduous and bamboo low forest, 2700-2900 m.

No illustration could be found for this taxon. However, photographs of *R. valentinianum var. oblongilobatum* given in Yang and Feng (1999) on Plate 66 3-5 exhibit large, cucullate leaves, which are very similar to specimens examined of *R. valentinioides*. The photographs were taken from plants found in the same locality in SE Yunnan as these specimens. It is likely, therefore, that these are photographs of *R. valentinioides*.

8. R. valentinianum Forrest ex Hutchinson, Notes R. B. G. Edinb. 12(56): 45. 1919

Type: Forrest #15899, May-June 1917, China: Yunnan, Shweli-Salween divide (E).
Illustr.: Cox, P. A., 1985. *The Smaller Rhododendrons*, Pl II.; Wu, Z. Y., Raven, P. H. and Hong, D. Y., eds. 2005. *Flora of China. Vol. 14 Illustrations: Apiaceae through Ericaceae*. Figure 468.

Shrub, 0.3-1.3 m. Young growth densely loriform-setose. Petioles stout, 3-11 mm, rounded, lepidote, setose. Leaves ± oblong-elliptic, rugose, (15-)32-60 × (11-)18-42 mm; apex mucronate; base obtuse; margin entire, setose; adaxial surface may have remnants of desiccated scales; abaxial scales usually touching or overlapping, unequal sizes with rim often upturned; papillae dense, cylindrical; midrib setose when young, usually abaxially lepidote; primary veins abaxially raised. Inflorescence cymose, 2-4(-6) flowered. Pedicels (3-)5-11 mm, lepidote, occasionally with loriform hairs. Calyx 5-10 mm, 5-lobed, acute or obtuse, lepidote, margins ciliate or glabrous. Corolla funnel-campanulate, 16-35 mm, tube 11-23 mm, lobes orbicular. Stamens 10, shorter than corolla, filaments outer whorl 20-22 mm and inner whorl 12-18 mm, pilose to 1/3 length. Ovary 3-5 mm, ovoid. Style exserted from corolla, variably lepidote at base, impressed into ovary. Capsule 7-15 mm, ovoid, densely lepidote. Fl. Apr-Jun.

CHINA (NW and SE Yunnan), Vietnam (W Lao Cai). Open scrub and rocky outcrops, 1800-3660 m.

1a. Pedicels sometimes have loriform hairs, calyx margins have loriform hairs

8a. var. valentinianum

1b. Pedicels never have loriform hairs, calyx margins never have loriform hairs

8b. var. oblongilobatum

8a. Rhododendron var. valentinianum

CHINA (NW and SE Yunnan), Vietnam (W Lao Cai). Open scrub and rocky outcrops, 1800-3660 m.

8b. Rhododendron var. oblongilobatum R. C. Fang, Acta Bot. Yunnan. 4(3): 250. 1982.

CHINA (SE Yunnan), Vietnam (W Lao Cai). Open scrub and rocky outcrops, 2600-3050 m.

Subspecies cannot be distinguished vegetatively. Results of SEM study (Section 2.2.2) indicate that scale density and inner zone diameter may differ between varieties but may simply result from insufficient sampling of each variant.

CHAPTER 6: TAXONOMIC CONCLUSIONS

The yellow-flowered species in subsection *Maddenia* are a complex group sharing similar morphologies and offering few subtle characters that can be used to define species. Many of the species occupy montane habitats that are geographically isolated from each other by the deep river valleys that dissect the region, facilitating speciation (Chamberlain, August 2012, pers. comm.). The taxonomic review of the species undertaken by this study using morphological, molecular and geographical observations has determined that *R. burmanicum*, *R. changii*, *R. crenulatum*, *R. fletcherianum*, *R. leptocladon*, *R. valentinianum*, *R. valentinioides* and *R. vanderbiltianum* are all justifiable species. Despite being the only species within the subsection to possess yellow flowers they were not found to form a monophyletic group, suggesting that flower colour has evolved and been lost several times during the evolution of the subsection. Investigations of the pollination biology of the subsection may be very interesting and add to our current understanding of the evolution of flower colour within the genus.

It is hoped that this research will be viewed as a pilot study for a thorough taxonomic investigation of species relationships within subsection *Maddenia*. Further analysis is required to ascertain the relationships of the crenulate leaved species *R. fletcherianum*, *R. crenulatum* and *R. vanderbiltianum* within section *Rhododendron* as this character was found to be more informative in understanding species relationships than flower colour. Preliminary evidence indicates that *R. vanderbiltianum* may not belong in section *Schistanthe* and could possibly be placed in subsection *Maddenia*. Larger molecular investigations of section *Rhododendron* would also be useful to determine the monophyly of subsections *Boothia, Edgeworthia* and *Maddenia*.

The genus *Rhododendron* is important both from an ecological and an economic standpoint. Subsection *Maddenia* represents both of these concerns as it contains a large number of species, some of which are horticulturally lucrative because of their large, scented flowers. These factors make the conservation of the species very important but to achieve this, species need to have well-defined boundaries enabling them to be readily identified. Further taxonomic investigation of subsection *Maddenia* is therefore warranted.

17 591 words

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Appendix 1: List of all herbarium specimens consulted, including those for molecular ingroup species. *R. valentinianum* corresponds to *R. valentinianum var. valentinianum*.
- indicates missing data.

Species	Herbarium barcode	Collector	Collector number	Date (dd mmm yyyy)
R. burmanicum	E00010149	Wheeler Cuffe	-	01 APR 1917
R. burmanicum	-		30	16 Apr 1929
R. burmanicum	E00421855	Cooper	5957	-
R. burmanicum	-	Unwin	3064	17 Apr 1926
R. burmanicum	-	Kingdon-Ward, F.	21921	03 Apr 1956
R. changii	E00087895	Cox, P. & Hutchison, P.	-	20 May 1999
R. chrysodoron	-	Unknown	5	27-Feb-34
R. chrysodoron	-	Unknown	6	30-Mar-34
R. chrysodoron	E00010280	Forrest, G.	5	24-Mar-31
R. ciliatum	-	Ludlow, F., Sherriff, G. & Elliot, H. H.	16019	06 Apr 1949
R. ciliatum	-	Ludlow, F., Sherriff, G. & Elliot, H. H.	15835	14 Apr 1948
R. ciliatum	E00269044	Hedegaard, J. B.	19831843*A	Oct 1981
R. crenulatum	L0007415	Kerr	21044	14 Apr 1932
R. fletcherianum	E00010126	Rock, J. F.	22302?	May-Jun 1932
R. fletcherianum	E00327153	Rock, J. F	-	Aug-Oct 1932
R. fletcherianum	E00189931	B.A.S.E.	9577	25 May 2000
R. fletcherianum	-	Rock, J. F.	C10097	May 1974
R. fletcherianum	-	Rock, J. F.	C12426	May 1979
R. fletcherianum	-	Rock, J. F.	C9082	April 1971
R. johnstoneanum	E00010130	Watt, G.	6401	April 11 1882
R. johnstoneanum	-	Unknown	-	-
R. johnstoneanum	-	Kingdon-Ward, F.	93	-
R. leptocladon	-	Rushforth, K. D.	4480	30 Mar 1997
R. leptocladon	-	Rushforth, K. D.	4511	01 Apr 1997
R. leptocladon	E00073365	Rushforth, K. D.	4416	29 Mar 1993
R. leptocladon	E00076254	Rushforth, K. D.	1877	11 Nov 1991
R. leptocladon	-	Rushforth, K. D.	4416	29 Mar 1997
R. leptocladon	E00076257	Rushforth, K. D.	1970	19 Nov 1991
R. leptocladon	E00064251	Rushforth, K. D.	4397B	29 Mar 1997
R. leptocladon	E00038774	Rushforth, K. D.	-	26 Apr 1996
R. leptocladon	E00269065	Rushforth, K. D.	1929	18 Nov 1991
R. leptocladon	E00039871	Rushforth, K. D.	2314	-
R. leptocladon	L0007590	Poilane	-	-
R. leptocladon	E00076167	Rushforth, K. D.	2932	24 Oct 1994
R. leucaspis	-	Ludlow, F., Sherriff, G. & Elliot, H. H.	13549	27 Apr 1947
R. leucaspis	E00269952	Kingdon-Ward, F.	171	23 Jul 1926
R. leucaspis	E00327632	Ward, J.	6273	09 Apr 1929
R. lyi	L0007584	Cavalerie, J.	3883	Apr 1912
R. lyi	E00035298	Rushforth, K. D.	2137	03 May 1992
R. lyi	-	Valder, P. G.	750207	-
R. maddenii subsp. crassum	E00190086	Bowes Lyon, S.	15028A	19 Jul 2002
R. maddenii subsp. crassum	-	Ludlow, F. & Sherriff, G.	2332	-
R. sulfurem	-	Unknown	813	Apr-19
R. sulfureum	E00010284	Forrest, G.	11910	Apr-13
R. sulfureum	-	Forrest, G.	12114	Dec-13

R. valentinanum	-	Rock, J. F.	22032	1934
R. valentinianum	E00314427	Forrest, G.	-	May - Jun 1917
R. valentinianum	E00314428	Forrest, G.	15899	May - Jun 1917
R. valentinianum	E00010125	Forrest, G.	15899	May - Jun 1917
R. valentinianum	-	Ward, J.	3191	06 Jun 1919
R. valentinianum	-	Yang, C. A.	101614	16 Nov 1963
R. valentinianum	E00421856	Forrest, G.	24138	May 1924
R. valentinianum	-	Forrest, G.	27715	Nov 1925
R. valentinianum	-	Forest, G.	24347	June 1924
R. valentinianum	E00053783	Ogisu, M.	95310	15 Mar 1995
R. valentinianum	E00039875	Rushforth, K. D.	2279	09 May 1992
R. valentinianum	E00212738	K. Y. E.	1230	15 Oct 1995
R. valentinianum	-	Forrest, G.	18507	Sept 1919
R. valentinianum	-	Forrest, G.	24347	25 Apr 1979
R. valentinianum	L0790515	Forrest, G.	15899	May - June 1917
R. valentinianum	-	Forrest, G.	26112	June 1924
R. valentinianum	-	Forrest, G.	17750	01 Oct 1918
R. valentinianum	-	Forrest, G.	17596	01 Jun 1918
R. valentinianum	E00421022	Rushforth, K. D.	2247	08 May 1992
R. valentinianum	E00212737	K. Y. E.	1258	15 Oct 1995
R. valentinianum hybrid	E00039854	Rushforth, K. D.	2287	09 May 1992
R. valentinianum hybrid	E00039857	Rushforth, K. D.	2319	10 May 1992
R. valentinianum hybrid	-	Rushforth, K. D.	4531	02 Apr 1997
R. valentinianum var. oblongilobatum	E00421857	Chui, P. Y.	53848	19 Nov 1956
R. valentinianum var. oblongilobatum	-	Wu, C. A.	9086	12 May 1963
R. valentinianum var. oblongilobatum	-	Unknown	2960	-
R. valentinianum var. oblongilobatum	-	Rushforth, K. D.	4532	02 Apr 1997
R. valentinioides	E00073235	Cox, P. & Hutchison, P.	7186	17 Oct 1995
R. vanderbiltianum	E00533156	Argent, G. & Aminin	99154	04 Mar 1999
R. vanderbiltianum	L0904468	de Wilde, W. J. J. O. & de Wilde-Duyfjes, B. E. E.	16204	08 Apr 1975
R. vanderbiltianum	L0904467	de Wilde, W. J. J. O. & de Wilde-Duyfjes, B. E. E.	16526	07 Apr 1975
R. vanderbiltianum	L0904465	de Wilde, W. J. J. O. & de Wilde-Duyfjes, B. E. E.	15248	27 Feb 1975
R. vanderbiltianum	L0904466	de Wilde, W. J. J. O. & de Wilde-Duyfjes, B. E. E.	16885	14 May 1975
R. vanderbiltianum	L0442391	Iwatsuki, K., Murata, G., Dransfield, J. & Saerudin, D.	s/ 1209	24 Aug 1971
R. vanderbiltianum	L0442390	de Wilde, W. J. J. O. & de Wilde-Duyfjes, B. E. E.	16071	04 Apr 1975
R. vanderbiltianum	L0904462	van Steenis, C. G. G. J.	9039	20 Feb 1937
R. vanderbiltianum	L0904463	de Wilde, W. J. J. O. & de Wilde-Duyfjes, B. E. E.	13301	24 Jun 1972
R. vanderbiltianum	L0904464	de Wilde, W. J. J. O. & de Wilde-Duyfjes, B. E. E.	13186	22 Jun 1972
R. vanderbiltianum	L0904461	van Steenis, C. G. G. J.	8442	29 Jan 1937
R. veitchianum	-	Valder, P.G.	750211	08 Feb 1979
R. veitchianum	-	Fields Clarke, V. H. T.	35	Apr 16
R. veitchianum	-	van Beusekom, C. F. & Phengkhlai, C.	350	04 Apr 1968

Appendix 2: List of geo-referenced herbarium specimens including collecting country and locality, long/lat position (decimal degrees) and reported altitude (m). - indicates missing data.

Herbarium barcode	Taxon name	Collector	Number	Country	Description	Decimal latitude	Decimal longitude	Altitude (m)
E00010149	*R. burmanicum*	Wheeler Cuffe		Myanmar	Mount Victoria	21.23	93.92	-
-	*R. burmanicum*	Cooper	5957	Myanmar	Mount Victoria	21.23	93.92	-
-	*R. burmanicum*	Unwin	3064	Myanmar	Mount Victoria	21.23	93.92	-
-	*R. burmanicum*	Kingdon-Ward	21921	Myanmar	Mount Victoria	21.23	93.92	2743
E00087895	*R. changii*	Cox & Hutchison	-	China	Jinfo Shan	29.03	107.22	2100
L0007415	*R. crenulatum*	Kerr	21044	Laos	Phou Bia	18.97	103.15	2800
-	*R. crenulatum*	Rushforth	7369	Viet Nam	Mount Fan Si Pan	22.31	103.78	2074-2900
E00189931	*R. fletcherianum*	B.A.S.E.	9577	China	Qi Na Valley	28.33	98.83	2910-3000
E00010126	*R. fletcherianum*	Rock	22302	Tibet	Solo-La (Suola)	28.87	98.45	4268
E00327153	*R. fletcherianum*	Rock	22302	Tibet	Solo-La (Suola)	28.87	98.45	4114
E00076257	*R. leptocladon*	Rushforth	1970	Viet Nam	Mount Fan Si Pan	22.30	103.77	2255
E00269065	*R. leptocladon*	Rushforth	1929	Viet Nam	Mount Fan Si Pan	22.30	103.77	2200
E00039871	*R. leptocladon*	Rushforth	2314	Viet Nam	Mount Fan Si Pan	22.30	103.77	2255
-	*R. leptocladon*	Rushforth	4511	Viet Nam	Mount Fan Si Pan	22.32	103.78	2475
E00076167	*R. leptocladon*	Rushforth	2932	Viet Nam	Mount Fan Si Pan	22.32	103.78	2130
E00073365	*R. leptocladon*	Rushforth	4416	Viet Nam	Mount Fan Si Pan	22.35	103.75	2060
-	*R. leptocladon*	Rushforth	4416	Viet Nam	Mount Fan Si Pan	22.35	103.75	2060
E00064251	*R. leptocladon*	Rushforth	4397B	Viet Nam	Mount Fan Si Pan	22.35	103.77	2060
-	*R. leptocladon*	Rushforth	4480	Viet Nam	Mount Fan Si Pan	22.38	103.78	1900
E00038774	*R. leptocladon*	Rushforth	-	Viet Nam	Mount Fan Si Pan	22.40	103.78	2255-2377
L0007590	*R. leptocladon*	Poilane	12680	Viet Nam	Lo-sui-tong	22.45	103.85	-
E00039854	*R. valentinianum hybrid*	Rushforth	2287	Viet Nam	Mount Fan Si Pan	22.30	103.77	2900-3050
E00039857	*R. valentinianum hybrid*	Rushforth	2319	Viet Nam	Mount Fan Si Pan	22.35	103.75	2255
-	*R. valentinianum hybrid*	Rushforth	4531	Viet Nam	Mount Fan Si Pan	22.47	103.77	3030
-	*R. valentinianum var. oblongilobatum*	Rushforth	4532	Viet Nam	Mount Fan Si Pan	22.30	103.77	3030
-	*R. valentinianum var. oblongilobatum*	Chui	53848	China	Jingdong Xian	24.47	100.90	3100
-	*R. valentinianum var. oblongilobatum*	Yang	101614	China	Jingdong Xian	24.47	100.90	2600-2900
-	*R. valentinianum var. oblongilobatum*	Wu	9086	China	Jingdong Xian	24.47	100.90	2640

Appendix 2: List of geo-referenced herbarium specimens including collecting country and locality, long/lat position (decimal degrees) and reported altitude (m). - indicates missing data.

Herbarium barcode	Taxon name	Collector	Number	Country	Description	Decimal lat	Decimal long	Altitude (m)
E00212737	*R. valentinianum var. valentinianum*	K.Y.E.	1258	China	Xichou	23.50	104.63	1800
L0790515	*R. valentinianum var. valentinianum*	Forrest	15899	China	Shweli-Salween	25.33	98.65	3352
E00053783	*R. valentinianum var. valentinianum*	Ogisu	95310	Viet Nam	Mount Fan Si Pan	22.30	103.77	2905
E00039875	*R. valentinianum var. valentinianum*	Rushforth	2279	Viet Nam	Mount Fan Si Pan	22.30	103.77	2468
E00421022	*R. valentinianum var. valentinianum*	Rushforth	2247	Viet Nam	Mount Fan Si Pan	22.30	103.77	2194
E00212738	*R. valentinianum var. valentinianum*	K.Y.E.	1230	China	Laujunshan	23.50	104.58	2600
E00314427	*R. valentinianum var. valentinianum*	Forrest	15899	China	Shweli-Salween	25.33	98.65	3352
E00314428	*R. valentinianum var. valentinianum*	Forrest	15899	China	Shweli-Salween	25.33	98.65	3352
E00010125	*R. valentinianum var. valentinianum*	Forrest	15899	China	Shweli-Salween	25.33	98.65	3352
-	*R. valentinianum var. valentinianum*	Forrest	24138	China	Shweli-Salween	25.42	98.97	3352
-	*R. valentinianum var. valentinianum*	Forrest	27715	China	Shweli-Salween	25.50	98.80	3352-3657
-	*R. valentinianum var. valentinianum*	Forrest	24347	China	Shweli-Salween	25.75	98.67	3352-3657
-	*R. valentinianum var. valentinianum*	Ward	3191	China	Chawng-maw-hka	26.17	98.50	2740-3050
E00073235	*R. valentinioides*	Cox & Hutchison	7186	China	Lao Jing Shan	23.35	103.92	2750-2850
L0904462	*R. vanderbiltianum*	van Steenis	9039	Indonesia	Goh Lemboeh	3.48	97.92	3000
L0904461	*R. vanderbiltianum*	van Steenis	8442	Indonesia	Goh Lemboeh	3.48	97.92	2100-2250
E00533156	*R. vanderbiltianum*	Argent & Aminin	99154	Indonesia	Mount Kemiri	3.70	97.75	2700
L0904468	*R. vanderbiltianum*	de Wilde	16204	Indonesia	Mount Leuser	3.73	97.15	3150-3200
L0904467	*R. vanderbiltianum*	de Wilde	16526	Indonesia	Mount Leuser	3.73	97.15	2900-3000
L0442390	*R. vanderbiltianum*	de Wilde	16071	Indonesia	Mount Leuser	3.73	97.15	2300
L0442391	*R. vanderbiltianum*	Iwatsuki *et al.*	1209	Indonesia	Mount Kemiri	3.73	97.50	2600-2900
L0904465	*R. vanderbiltianum*	de Wilde	15248	Indonesia	Mount Bandahara	3.75	97.78	2800-3000
L0904463	*R. vanderbiltianum*	de Wilde	13301	Indonesia	Mount Bandahara	3.75	97.78	2600-2700
L0904464	*R. vanderbiltianum*	de Wilde	13186	Indonesia	Mount Bandahara	3.75	97.78	2400

Appendix 4: List of all sequences downloaded from GenBank and used to run MP analysis to choose outgroups for phylogenetic analyses.

Taxon	GenBank Accession	Sequence length (bp)	Authors	Year
R. albiflorum	AB012731	2192	Kurashige *et al.*	2009
R. albrechtii	AB012737	2187	Kurashige *et al.*	2009
R. breviperulatum	AM296055	2303	Hwang *et al.*	2007
R. camtschaticum	AB012744	2078	Kurashige *et al.*	2009
R. canadense	AB012735	2182	Kurashige *et al.*	2009
R. diversipilosum	AB012751	2138	Kurashige *et al.*	2009
R. edgeworthii	U61354	1521	Kron, K. A.	2003
R. ellipticum	AM296052	2306	Hwang *et al.*	2007
R. farrerae	AB012745	2158	Kurashige *et al.*	2009
R. ferrugineum	AB012741	2159	Kurashige *et al.*	2009
R. formosanum	AM296049	2307	Hwang *et al.*	2007
R. hyperythrum	AM296048	2306	Hwang *et al.*	2007
R. indicum	AM296047	2307	Hwang *et al.*	2007
R. javanicum	AB012742	2169	Kurashige *et al.*	2009
R. kanehirai	AM296059	2303	Hwang *et al.*	2007
R. kawakamii	AM296053	2317	Hwang *et al.*	2007
R. luteum	AB012734	2190	Kurashige *et al.*	2009
R. mariesii	AM296061	2307	Hwang *et al.*	2007
R. morii	AM296051	2307	Hwang *et al.*	2007
R. mucronulatum	AF454855	1521	Gao *et al.*	2003
R. nakaharai	AM296054	2303	Hwang *et al.*	2007
R. nipponicum	AB012739	2179	Kurashige *et al.*	2009
R. noriakianum	AM296058	2303	Hwang *et al.*	2007
R. obtusum var. kaempferi	AB012748	2165	Kurashige *et al.*	2009
R. oldhamii	AM296060	2302	Hwang *et al.*	2007
R. ovatum	AM296062	2313	Hwang *et al.*	2007
R. ovatum	AB012729	2195	Kurashige *et al.*	2009
R. pendulum	AF440429	1423	Kron, K. A.	2003
R. pentaphyllum	AB012738	2196	Kurashige *et al.*	2009
R. ponticum	AB012732	2189	Kurashige *et al.*	2009
R. ponticum	AY494172	1775	Milne, R. I.	2004
R. primuliflorum	AB012740	2126	Kurashige *et al.*	2009
R. pseudochrysanthum	AM296050	2307	Hwang *et al.*	2007
R. rubropilosum	AM296056	2302	Hwang *et al.*	2007
R. rubropunctatum	AM296047	2307	Hwang *et al.*	2007
R. santapaui	AB012743	2205	Kurashige *et al.*	2009
R. schlippenbachii	AB012736	2113	Kurashige *et al.*	2009
R. semibarbatum	AB012733	2191	Kurashige *et al.*	2009
R. simsii	AM296057	2303	Hwang *et al.*	2007
R. stamineum	AB012730	2157	Kurashige *et al.*	2009
R. tashiroi	AB012749	2191	Kurashige *et al.*	2009
R. tsusiophyllum	AB012750	2182	Kurashige *et al.*	2009
R. wadanum	AB012746	2193	Kurashige *et al.*	2009

Appendix 5: Morphological character states for 26 characters, determined from voucher specimens of all species sequenced at RBGE (EDNA numbers) and from printed sources for sequences obtained from GenBank. State codes correspond to those on Table 19.

EDNA Number	**Species**	**Character Number** **00000000011111111112222222** **12345678901234567890123456**
EDNA12 0025068	*R. burmanicum*	??15102100012203011110021 1
EDNA12 0025069	*R. burmanicum*	??13112100112203011111021 1
EDNA12 0025065	*R. changii*	02141130001121011220120111
EDNA12 0025365	*R. changii*	02131100001122011220120111
EDNA12 0025070	*R. chrysodoron*	??131120000111020000110111
EDNA12 0025359	*R. ciliatum*	1?111210002100111211111101
EDNA12 0025225	*R. crenulatum*	22011011110110120001100110
EDNA12 0025364	*R. crenulatum*	22010012110100120001100010
EDNA12 0025066	*R. crenulatum*	????0012110100120001100010
EDNA12 0025369	*R. dalhousiae var. dalhousiae*	3?231040200110101311122400
EDNA12 0025221	*R. fletcherianum*	04130111111100111221020100
EDNA12 0025366	*R. fletcherianum*	02011120112100111221020100
EDNA12 0025361	*R. johnstoneanum*	3?231220102121100001121211
EDNA12 0025363	*R. leptocladon*	2?011012100111000001110210
EDNA12 0025220	*R. leucaspis*	32121110001021021241102011
EDNA12 0025362	*R. lyi*	??121210100110010001111111
EDNA12 0025360	*R. maddenii subsp crassum*	01131221002110100210021310
EDNA12 0025223	*R. sulfureum*	22011100201010030320110?11
EDNA12 0025067	*R. valentinianum var. oblongilobatum*	01231100201121010231110111
EDNA12 0025071	*R. valentinianum var. oblongilobatum*	02131100201121032221120111
EDNA12 0025222	*R. valentinianum var. oblongilobatum*	011311002011210?33?1010?11
EDNA12 0025358	*R. valentinianum*	0?14112000112202112?120111
EDNA12 0025367	*R. valentinianum var. valentinianum*	03141120001122001121120111
EDNA12 0025368	*R. valentinioides*	0223110020111001???11?0?11
EDNA12 0025072	*R. vanderbiltianum*	5?011312210111010001120011
EDNA12 0025224	*R. veitchianum*	400010211001100?0001122211
AB012737	*R. albrechtii*	6?001332012533014001003100
AB012735	*R. canadense*	8?030322202533034001023000
U61354	*R. edgeworthii*	0?320411200300005301122110
AB012741	*R. ferrugineum*	1?151020110122030011103011
AB012742	*R. javanicum*	51211012000400030100124100
AM296053	*R. kawakamii*	62131020100210020010100011
AF454855	*R. mucronulatum*	0?001312112111100001123101
AF440429	*R. pendulum*	1?130420200310005101123010
AY494172	*R. ponticum*	7?330012100533030000023500
AB012740	*R. primuliflorum*	0?031040100622030101112000
AB012743	*R. santapaui*	31021022100211010000101011