Molecular and Conventional Data Sets and the Systematics of *Rhododendron* L. Subgenus *Hymenanthes* (Blume) K.Koch

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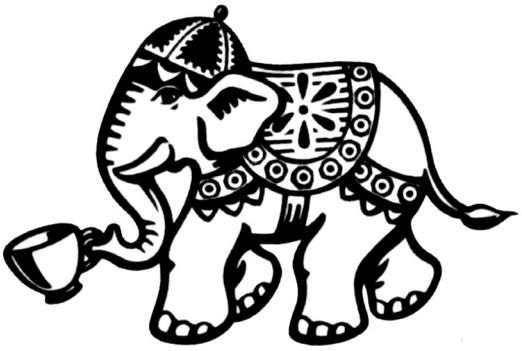
This edition has been rapidly prepared from the word processor files that were archived in 1997 on completion of the thesis so as to at least make the text available on-line. Not all the figures are presented and some figures have character encoding errors. It is worth noting that computers are over 200x more powerful today than when the first files were produced so combining and updating the documents was technically quick – but there has been no proof reading as human time seems to be more expensive today than it was 13 years ago.



"Formerly this mind wandered about where it liked, wherever it willed, as it pleased; today, with wisdom, I shall control it, as a mahout controls an elephant in rut."

(Buddha, the Dhammapada)

(see also Schaller 1983)

ABSTRACT

The 250 to 300 species of subgenus *Hymenanthes* are divided into 24 subsections that are contained within a single section, *Ponticum*. The subgenus as a whole has a clear identity and would appear to be a monophyletic group. Evidence includes three characters that appear to be synapomorphic. A number of the subsections are thought to represent natural groups others are considered to be assemblages that may or may not have evolutionary significance. Hybridisation occurs both within and between the subsections. Morphological, RAPD, PCR-RFLP and ITS sequence data was used to assess whether there was a phylogenetic structure within the subgenus that could form the basis of a sectional treatment. In the RAPD study difficulties were encountered in producing reproducible, scorable fingerprints and, in particular, in ascertaining the homology of different amplification products. A PCR-RFLP study of the ITS region failed to reveal sufficient polymorphic sites and a sequencing approach was therefore adopted. The ITS regions of 27 species from across the morphological range of diversity were sequenced but very little variation was encountered. There were a total of 27 variable base positions and of these 13 were autapomorphic for individual species leaving just 14 potentially informative characters. The final analysis, combining morphological and molecular data showed no congruence between the groups produced by the different methods although one well supported molecular grouping appeared to have a western Himalayan phytogeographical element. Whether the lack of ITS variation was due to the rapid evolution that is occurring in the group or the lack of breeding barriers or a combination of the two was debated. Random dominant marker methodologies such as RAPD appear inappropriate for use in the construction of hierarchical relationships. The results caste doubt on how large complex groups such as subgenus *Hymenanthes* may be adequately described without abandoning the hierarchical paradigm.

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CHAPTER 1: INTRODUCTION

The science of systematics is concerned with the classification of living things in accordance with their natural relationships. This thesis uses conventional and the more recently developed molecular techniques to examine the relationships of species within *Rhododendron* subgenus *Hymenanthes*. The current common usage for the term ‘molecular’ has been adopted here. It is taken as refering to the techniques involving the analysis of DNA directly. The only exception being in Chapter 3 where isozymes studies are dealt with along side the molecular techniques. The term ‘conventional’ is used to describe non-molecular techniques that were common place prior to the 1980s. The terms 'systematic' and 'taxonomic' will be used interchangeably although taxonomic is taken to refer to classifications that do not have to reflect the natural relationships of the groups.

The work was funded as part of the EC Framework III Biotechnology Program, in the project PL920295 "Development of rapid novel molecular and cellular tools for the screening and evaluation of genetic diversity in plants". There were two parts of this project that were concerned with the genus *Rhododendron*. One part dealt with collaboration between laboratories and comparison of molecular techniques, concentrating on the 'Azalea' subgenera (see below). The author was involved in administration, sampling, and data analysis for this part of the project but all practical work was carried out by participating laboratories. The study presented here constitutes the second part of the project that was concerned with *Rhododendron* and was carried out entirely by the author. Throughout this report the collaborative project is refered to as the 'Azalea' project. The initial results of the 'Azalea' project are summarised in Appendix G.

This thesis can be split into three parts. The first four chapters deal with the theoretical and historical aspects of the problem and provide a justification for the practical work, chapters 5 to 8 deal with the practical study whilst Chapter 9 deals with the final analysis and draws conclusions based on all the previous eight chapters. Details of data and synonomy are dealt with in the appendices. Given below is an overview of the classification of *Rhododendron* and subgenus *Hymenanthes* so as to place the study group in context.

General Taxonomic Considerations

Rhododendrons are evergreen and deciduous shrubs and trees with racemes of funnel-, bell-, or salver-shaped flowers. They are found throughout temperate regions of the northern hemisphere extending South through tropical Asia to just reach northern Australia. There are two centres of diversity, Southwest China and tropical South East Asia. Although fairly distinct as a genus *Rhododendron* shows a great deal of internal variation, ranging from 30 metre high trees to mat-forming, alpine shrubs and tropical epiphytes. The genus is split into two large subgenera, *Rhododendron* and *Hymenanthes*, plus a number of smaller subgenera some of which are referred to as 'Azaleas'.

This section deals with general considerations regarding the status of the genus *Rhododendron* and subgenus *Hymenanthes*. A brief outline of the history of the genus is given followed by a description of its current placement and taxonomy.

History of the classification of *Rhododendron*

In 1753 Linnaeus recognised just 5 species in the genus *Rhododendron*. Today almost 1,000 species are known from both temperate and tropical regions and *Rhododendron* is recognised as the largest genus of woody plants in Asia. Numerous accounts of how the taxonomy and cultivation of this group has developed over the intervening 240 years have been produced notably those by Sleumer (1980) and Postan (1996) (but see also Hyam 1996). A summary of the major points is given below.

By 1834 the genus had grown to 57 species and was split into 8 sections by George Don in his 'General System of Dichlamydeous Plants' (Don 1834). After many more species had been

collected, notably by J.D. Hooker, the genus was reviewed again in 1870 by Maximovicz, Curator of St. Petersburg Botanic Gardens. Using living material from the gardens as well as herbarium sheets Maximovicz developed a whole new series of diagnostic characters that broadly supported Don's work but greatly refined it (Maximovicz 1870). The influence of Don/Maximovicz revisions can clearly be seen in the classifications we use today.

The work of the collectors Forrest, Rock and Kindon-Ward in the first part of the 20th Century lead to another dramatic rise in the number of species known. Living material of many plants came to The Royal Botanic Garden, Edinburgh where an artificial system of classification was established, under The Regius Keeper, Bayley Balfour, to catalogue the new taxa. This culminated in the publication of 'Species of Rhododendron' (Stevenson 1930) in which all the temperate and subtropical species known were split into 39 series and numerous subseries. This book became a standard for the English speaking world for the next 50 years.

The period prior to the 2nd World War therefore saw the development of two separate systems, a 'natural' system based on the work of Don and Maximovicz and the 'artificial' system developed in Edinburgh. These two systems were united in the first modern revision just after the second world war (Sleumer 1949). Unfortunately this treatment was not widely adopted by horticulturists who had by now become familiar with Stevenson (1930).

Since 1978 the Royal Botanic Garden Edinburgh has been coordinating a modern review of the genus and 1996 saw the completion of the latest and most accurate treatment of the temperate species with the publication of the penultimate parts of the monograph. This work more-or-less completes the alpha-taxonomy of *Rhododendron*. Large areas of the taxonomy still remain unclear however. Species boundaries are often blurred and no formal infra-generic phylogenetic studies have been published. New species are still being described (examples are Hu Lin-Cheng 1992 and Fang Wen-Pei 1983) and there are still areas that have not been well collected particularly in Tibet and Papua New Guinea.

The Family Ericaceae

Traditionally the family Ericaceae is held to consist of around three and a half thousand species spread throughout the world except for Australasia where it is replaced, almost completely, by the family Epacridaceae (Mabberley 1987). These two large families have long been considered as very closely related (Takhtajan 1980) having no characters that uniquely separate them (Stevens 1971; Judd & Kron 1993). Other smaller families that also appear to be related to Ericaceae *sensu stricto* are Empetracaeae (which Judd & Kron (1993 p.106) refer to as "merely reduced-flowered, wind-pollinated ericads"), Pyrolaceae (perhaps merely herbaceous ericads?) and Monotropaceae (merely saprophytic ericads?).

Recent morphological (Anderberg 1992, Judd & Kron 1993), biochemical (Harborne 1986), geographical (Moore *et al* 1970) and molecular (Kron & Chase 1993) studies all point to a wider circumscription of the family so as to include these closely related, and probably derived, members. This is the view that will be taken here.

Ericaceae *sensu lato* consists of around 4,000 species in about 150 genera. A sub-familial treatment is not yet available but would probably consist of the following seven subfamilies. Ericoideae, Epacridoideae, Empetroideae, Vaccinioideae, Pyroloideae, Monotropoideae, Wittsteinioideae, (After Stevens 1971 Henderson 1919 & Copeland 1941 & 1947 with newly incorporated families being treated as sub-families.)

The genus *Rhododendron* is in tribe the Rhodoreae D.Don of subfamily Rhododendroideae, a group of shrubs and trees marked by typically possessing large leaves, perulate winter buds, flowers that bear the odd sepal adaxially and anthers that lack resorption tissues. This tribe is considered to be monophyletic; the perulae acting as an synapomorphy.

Circumscription and subgeneric classification of *Rhododendron*

The wide diversity of forms within the genus *Rhododendron* has lead, in the past, to different authors recognising different internal divisions of the genus as well as different segregate genera. These changes are briefly outlined below, for simplicity, authorities of taxa are omitted from the text but given in the synopsis that follows.

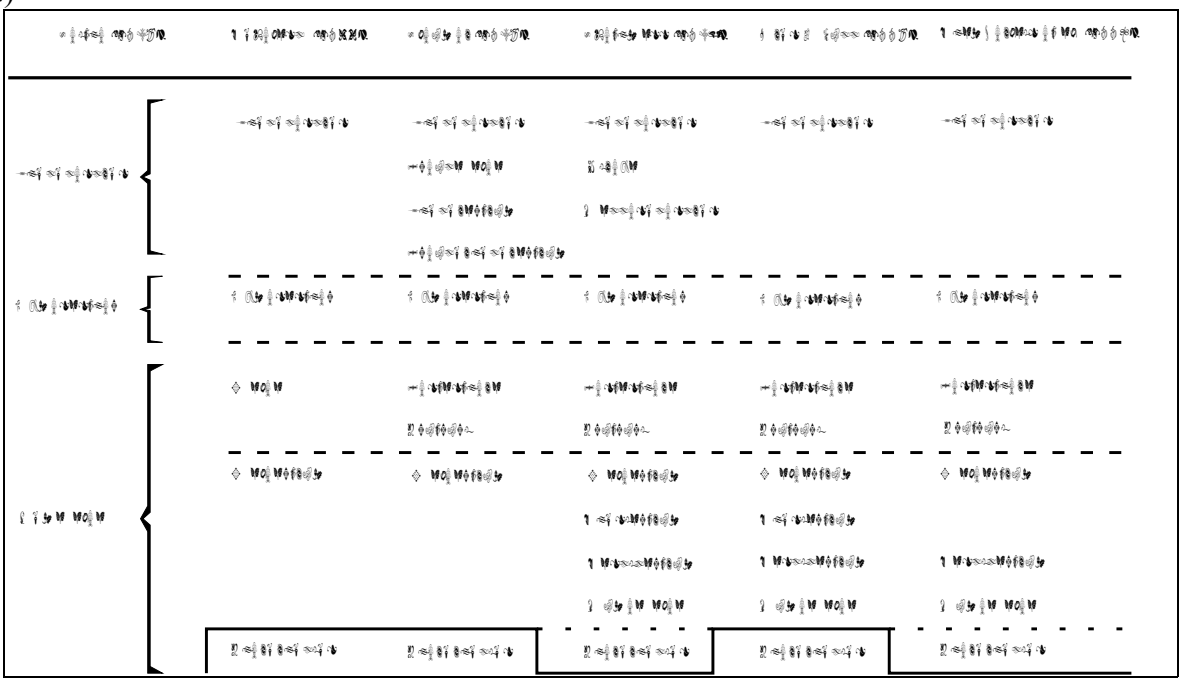
The first controversy to arise surrounded the delimitation of *Azalea* as a separate entity from *Rhododendron*. The original circumscription of *Azalea* was based on the species then known from North America, Europe and the Near East which were all deciduous. When semi-evergreen and deciduous species from eastern Asia became known there no longer appeared to be a clear phenetic gap between the two genera (Wilson & Rehder 1921) and *Azalea* was sunk into *Rhododendron*. Although the debate amongst taxonomists in the latter half of the 20th century has moved on, the fate of *Azalea* it is still a matter of some debate in the horticultural community. The name 'Azalea' is kept alive because it provides the ability to differentiate between the numerous cultivars available of leathery leaved, evergreen shrubs (from subgenus *Hymenanthes* and section *Rhododendron*) and the deciduous or semi-deciduous, papery-leaved shrubs (from subgenera *Pentanthera*, *Tsutsusi* and their allies). Quite how the horticultural 'Azalea' can be circumscribed in strict, taxonomic terms is uncertain. The best cause of action is probably to assume that it refers to all member of the genus *Rhododendron* not found within the subgenera *Rhododendron* and *Hymenanthes*.

The modern debate over the delimitation of *Rhododendron* could be said to have started with the account of Copeland (1943) who split the genus into five segregate genera. *Rhododendron*, *Hymenanthes*, *Azalea*, *Azaleastrum*, and *Therorhodion*. These five genera have appeared, in some form, in all subsequent classifications, although frequently as subgenera and sometimes split into segregate subgenera. None of the subsequent treatments have suggested taxa that contain members from more than one of Copeland's genera. Sleumer (1949,1980) sank all but *Therorhodion* to the rank of subgenus whilst splitting *Rhododendron* into four subgenera, (*Rhododendron*, *Pseudazalea*, *Rhodorastrum*, *Pseudorhodorastrum*) and *Azalea* into two (*Pentanthera* and *Tsutsusi*). Spethmann (1987) also represented *Azalea* as two subgenera but split Copeland's *Rhododendron* into three (*Rhododendron*, *Maddenodendron* and *Vireya*) on a different base to that used by Sleumer. He also recognised *Azaleastrum* as four different subgenera (*Azaleastrum*, *Choniastrum*, *Candidastrum* and *Mumeazalea*). Seithe (1980) suggested the addition of another rank to the hierarchy, that of chorus subgenerum. She proposed three taxa at this rank, *Rhododendron*, *Hymenanthes* (both monosubgeneric) and *Nomazalea* (which would be equivalent to the modern horticultural 'Azalea' mentioned above). Seithe's work was based largely on hair types and is discussed in more detail in below (see Trichomes on page 58). In the monograph coordinated by Royal Botanic Garden Edinburgh and summarised by Chamberlain (1996) *Rhododendron* was kept as a single taxon, divided in to three sections. *Azalea* was divided into two, and *Azaleastrum* into three, (as for Spethmann except that *Choniastrum* remaining a section within subgenus *Azaleastrum*).

Kron and Judd's (1990) carried out the first cladistic phylogenetic study of the tribe Rhodoreae and concluded that *Therorhodion* should remain as a separate genus to *Rhododendron* but that the genus *Ledum* L should be sunk into subgenus *Rhododendron*. The latter of these two suggestions has been taken on board by the latest version of the Edinburgh treatment (Chamberlain 1996). Figure 1 summarises the above paragraphs and Figure 2 and Figure 3 illustrate part of the range of variation found within the genus.

Figure 1: *Recently recognised subgenera and chora subgenera within Rhododendron.*

(The taxon *Therorhodion* is sometimes excluded from the genus *Rhododendron.* Full explanation in text.)



Synopsis of the current "Edinburgh" classification of the genus *Rhododendron*.

An outline of the current classification of the genus *Rhododendron* to the level of Section is given below. This treatment follows those given in the monographs published through the Royal Botanic Garden Edinburgh and Sleumer's treatment of the tropical species (Sleumer 1958, 1960, 1968). Chamberlain (1996) gives a fully synonymised species list according to this classification.

Genus ***Rhododendron*** L.

Subgenus ***Rhododendron***

A distinct subgenus characterised by the presence of scale-like trichomes at least on the young growth. Hairs may be present or absent.

Section ***Vireya*** (Blume) H.F. Copeland.

Around 310 species of evergreen, epiphytic and terrestrial shrubs and small trees currently arranged into 7 subsections. Separated from the other sections of this subgenus on the basis of its soft capsules, long seed appendages and largely tropical distribution.

Latest Monograph: Sleumer (1968)

Section ***Pogonanthum*** G. Don

Around 21 species of small, evergreen shrubs with a characteristic pineapple-like smell and lacerate scales. (Figure 2B).

Distribution: Eastern Himalaya as far West as Afghanistan.

Latest monograph: Cullen (1980)

Section ***Rhododendron***

Around 211 species of evergreen or deciduous shrubs and small trees divided amongst 27 intricately related subsections. (Figure 2A & E).

Distribution: Much of temperate Asia to North America and Europe.

Latest monograph: Cullen (1980).

Subgenus ***Hymenanthes*** (Blume) K. Koch

A distinctive subgenus that contains only a single section. See discussion below and

Figure 3.

Section ***Ponticum*** G. Don

Around 302 species of evergreen, dwarf shrubs to large trees that are characterised by their complex, branching dendritic hairs and lack of scales. The section contains 24 subsections.

Distribution: Much of temperate Asia to North America and Europe.

Latest monograph: Chamberlain (1982)

Complete synonomy presented in the Appendix A.

Subgenus ***Tsutsusi*** (Sweet) Pojarkova

A distinctive subgenus of around 117 species of evergreen or deciduous shrubs that are characterised by having there inflorescence and vegetative buds surrounded by the same bud scales.

Section ***Tsutsusi*** Sweet

94 species of shrubs with leaves of two kinds. Those produced in the spring are larger and deciduous whilst those produced in the summer are smaller and persist through the summer. In some species only the persistent leaves are produced. (Figure 2G).

Distribution: China and Japan

Latest monograph: Chamberlain & Rae (1990)

Section ***Brachycalyx*** Sweet

23 species of deciduous shrubs with leaves in pseudowhorls of 3 or occasionally 2.

Distribution: China and Japan

Latest monograph: Chamberlain & Rae (1990)

Subgenus ***Pentanthera*** (G.Don) Pojarkova

A complex subgenus of shrubs containing four quite different sections.

Section ***Pentanthera*** G.Don

23 species of deciduous shrubs with alternate leaves and five stamens.(Figure 2C).

Distribution: Chiefly eastern North America but also 1 species western North America, 1 species eastern Asia and 1 species in the Caucasus.

Latest monograph: Kron (1993)

Section ***Rhodora*** (L.) G.Don

2 species of deciduous shrubs with zygomorphic flowers bearing 10 or 7 (rarely 5) stamens.

Distribution: Eastern North America

Latest monograph: Judd & Kron (1995)

Section ***Viscidula*** Matsum. & Nakai

A monotypic section of deciduous shrubs with regular flowers bearing 10 stamens. (*R. nipponicum*)

Distribution: Japan

Latest monograph: Judd & Kron (1995)

Section ***Sciadorhodion*** Rehder & Wilson

4 species of deciduous shrubs with more or less zygomorphic flowers bearing 10 stamens.

Distribution: Japan, Korea and adjacent Russia.

Latest monograph: Judd & Kron (1995)

Subgenus ***Azaleastrum*** Planch.

A subgenus of two small, sympatric sections characterised by bearing their flowers laterally.

Section ***Azaleastrum*** (Planch.) Maxim.

11 species of evergreen shrubs with solitary flowers that bear 5 stamens.

Distribution: China

Latest Monograph: Philipson & Philipson (1986)

Section ***Choniastrum*** Franch.

19 species of evergreen shrubs and small trees with flowers carried singly or in clusters and bearing 10 stamens.
Distribution: China to the Malay Peninsula.
Latest Monograph: Philipson & Philipson (1986)

Subgenus ***Therorhodion*** (Maxim.) A.Gray

2 species of evergreen or deciduous shrubs or small trees with terminal inflorescences and corollas divided to the base on one side. (Figure 2H).
Distribution: North East China, Northern Japan, North East Siberia and Alaska.
Latest Monograph: Philipson & Philipson (1986)

Subgenus ***Mumeazalea*** (Sleumer) W.R. Philipson & M.N. Philipson

1 species of deciduous shrub with lateral, solitary flowers and distinctive, dimorphic stamens (3 long and 2 short).
Distribution: Japan.
Latest Monograph: Philipson & Philipson (1986)

Subgenus ***Candidastrum*** Franch.

1 species of deciduous shrub with bowl shaped, almost actinomorphic flowers.
Distribution: Western Canada
Latest Monograph: Philipson & Philipson (1986)

Colour Plate No 1 Rhodos.

Figure 2: *A colour plate illustrating the variation within the genus Rhododendron.*

A) Rhododendron campylogynum (Hutchinson) Sleumer. (Subgenus Rhododendron section Campylogyna).

B) *Rhododendron primuliflorum* Bureau & Franch. (Subgenus *Rhododendron,* Section *Pogonanthum*).

C) *Rhododendron luteum* Sweet (Subgenus *Pentanthera,* Section *Pentanthera*).

D) *Rhododendron meddianum* Forrest (Subgenus *Hymenanthes*).

E) *Rhododendron groenlandicum* (Oeder) Kron & Judd (Subgenus *Rhododendron*, Section *Rhododendron*).

F) *Menziesia lasiophylla* L. (A putative *Rhododendron* according to evidence from the 'Azalea' study).

G) *Rhododendron kiusianum* Makino (Subgenus *Tsutsusi* Section *Tsutsusi*.)

I) *Therorhodion camtschaticum* Pall. (A former *Rhododendron*)

Subgenus *Hymenanthes*

The 310 species of subgenus *Hymenanthes* are divided into 24 subsections that are contained within a single section, *Ponticum*. The subgenus as a whole has a clear identity and would appear to be a monophyletic or natural group (Kron & Judd 1990). Evidence includes three characters that appear to be synapomorphic (i.e. only occurring within the group). These are complex dendritic hair types (Siethe 1980), a complex nodal anatomy (Philipson & Philipson 1968) and presence of caryatin in the leaves (Harborne & Williams 1971). A number of the subsections are thought to represent natural groups, others are considered to be assemblages that may or may not have evolutionary significance. Most of the subsections first arose as artificial constructs under the Balfournian system (Stevenson 1930) that were later given the formal taxonomic rank of subsection by Sleumer (1949). (It is important to remember that these groups were never intended to have a formal taxonomic ranking, see discussion in Sleumer 1980, pp20-21). Many were then adopted and adapted by Chamberlain (1982) who did not feel there was sufficient data available to arrange the subsections into any formal groupings:

> "The 24 subsections recognised in this account are related to one another in a complex manner. The distinctions between them may well be obscured by hybridisation. In cultivation species from different subsections will cross freely and hybrids clearly also occur in the wild. Furthermore, the taxonomic significance of the morphological differences on which the classification is based is not always clear." (Chamberlain 1982. page 459).

Figure 4 is a reproduction of the figure give by Chamberlain (1982) to summarise relationships amongst the subsections. Table 1 summarises the relationships of the series and subseries of Stevenson (1930) to Sleumer's classification and to Chamberlain's. A full, synonomised list of the species of subgenus recognised by Chamberlain *et al* (1996) is given in Appendix A.

Table 1: *Relationship of the Series and Subseries of Stevenson (1930) to the subsections of Sleumer (1949, 1980) and Chamberlain (1982)(ss = subseries, pp = pro parte; sub series are only included where they add to clarity)*

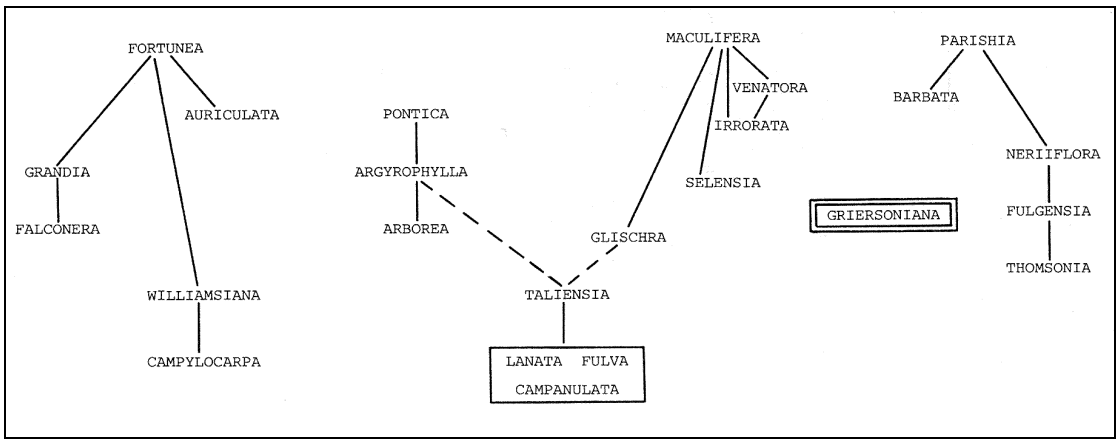
Stevenson 1930 Series & subseries	Sleumer 1949 & 80 Subsections	Chamberlain 1982 Subsections
Arboreum ss Arboreum	Arborea	Arborea
Arboreum ss Argyrophyllum	Argyrophylla	Argyrophylla
	Floribunda	Argyrophylla
Auriculatum	Auriculata pp	Auriculata
-	Auriculata pp	Griersoniana
Barbatum ss Barbatum	Barbata	Barbata
Barbatum ss Glischrum	Barbatum pp	Glischra
Barbatum ss Maculiferum	Maculifera	Maculifera
Campanulatum	Campanulata pp	Campanulata
	Campanulata pp	Lanata
Falconeri	Falconera	Falconera
Fortunei	Fortunea	Fortunea
Fulvum	Fulva	Fulva
Grande	Grandia	Grandia
Irroratum ss Irroratum	Irrorata	Irrorata
Irroratum ss Parishii	Parishia	Parishia
Lacteum	Lactea	Taliensia
Neriiflorum	Neriiflora	Neriiflora
Ponticum ss Ponticum	Ponticum	Ponticum
Ponticum ss Caucasicum	Ponticum	Ponticum
Taliense	Taliensia	Taliensia
Thomsonii ss Campylocarpum	Campylocarpa	Campylocarpa
Thomsonii ss Cerasinum	Thomsonia	Thomsonia
Thomsonii ss Souliei	Souliea	Campylocarpa
Thomsonii ss Selensia	Selensia	Selensia
Thomsonii ss Martinianum	Martiniana	Selensia
Thomsonii ss Thomsonii	Thomsonia	Thomsonia
-	-	Venator
-	-	Fulgensia
-	-	Williamsianum

Colour Plate No 2 Hymenanthes.
Some variation within subgenus *Hymenanthes*.

Figure 3: *A colour plate illustrating variation within subgenus Hymenanthes.*

A) R. uvarifolium (Subsection Fulva).
B) R. rex (Subsection Falconera)
C) R. principis (Subsection Taliensia)
D) R. wasonii (Subsection Taliensia)
E) R. ponticum (Subsection Pontica)
F) R. adenogynum (Subsection Taliensia)
G) R. forrestii (Subsection Neriiflora)
H) R. williamsianum (Subsection Williamsiana)
I) R. argipeplum (Subsection Barbatum)

Figure 4: *Figure given by Chamberlain (1982 page 462) to summarise relationships amongst the subsections of subgenus Hymenanthes*



Spethmann (1980 & 1987) is the only worker to have tried to create a hierarchical treatment of the subgenus. He did this in his somewhat controversial classification of the genus presented at the 1978 International Rhododendron Conference in New York and fully published in 1987. The treatment has not been widely accepted. It is marked by the use of a unique system of diagrammatic representation that is not fully explained (Figure 5). The overall treatment of the genus is included in Figure 1. Within *Hymenanthes* he suggests the following changes:

1) Subsections *Grandia*, *Falconera*, *Lactea* and *Taliensia* should be separated as a new section (section *Lactanthes* Spethmann) on the basis of their hair types, number of epidermal cells, numerous occurrences of yellow flower colour and similarity of flavonoid compounds.

2) Section *Lactanthes* should contain two subsections *Falconera* and *Taliensia*

3) Series Neriiflorum subseries Sanguineum should be separated out of subsection *Neriiflora* as section *Sanguinea* on the basis of the carotenoids in the flower colouring.

4) The subseries Ponticum and Caucasicum of series Ponticum should be treated as separate subsections on the basis of flower colour, water tissues and chromatogram patterns.

5) Subseries Fortunei should be treated separately from the other subseries of Series Fortunei on the basis of flavonoid compounds.

The subgenus is considered the most rapidly evolving and most taxonomically complex group within *Rhododendron*. Many of the species appear to hybridise in the wild and most do so in cultivation (although no formal study has been carried out). Many species are only known from one or a few locations that are typically associated with the more accessible, well worn mountain passes. Exploration away from these sites is time consuming and expensive, even so species recognised within the group have increase in number by almost 38% since the last monograph. Characters which are variable, and taxonomically useful in the rest of Rhododendron are constant within all taxa in *Hymenanthes* (e.g. seed appendages, cotyledon types and ploidy levels.)

Aims

As can be seen from the previous discussion there are many problems still to resolve in the systematics of Rhododendron and particularly in subgenus Hymenanthes. The main aim of this thesis was to investigate whether molecular techniques could help resolve these issues. More specifically the study had the following aims:

- To examine whether either conventional or molecular techniques will produce evidence of an underlying phylogenetic pattern to the variation with the subgenus.

- To establish whether the subsections proposed by Sleumer (1980) and Chamberlain (1982) can be arranged into a hierarchy.
- To assertain whether the sectional treatment proposed by Spethmann (1987) is valid and so should be more widely applied.
- To suggest subsets of taxa that could be studied in more detail.

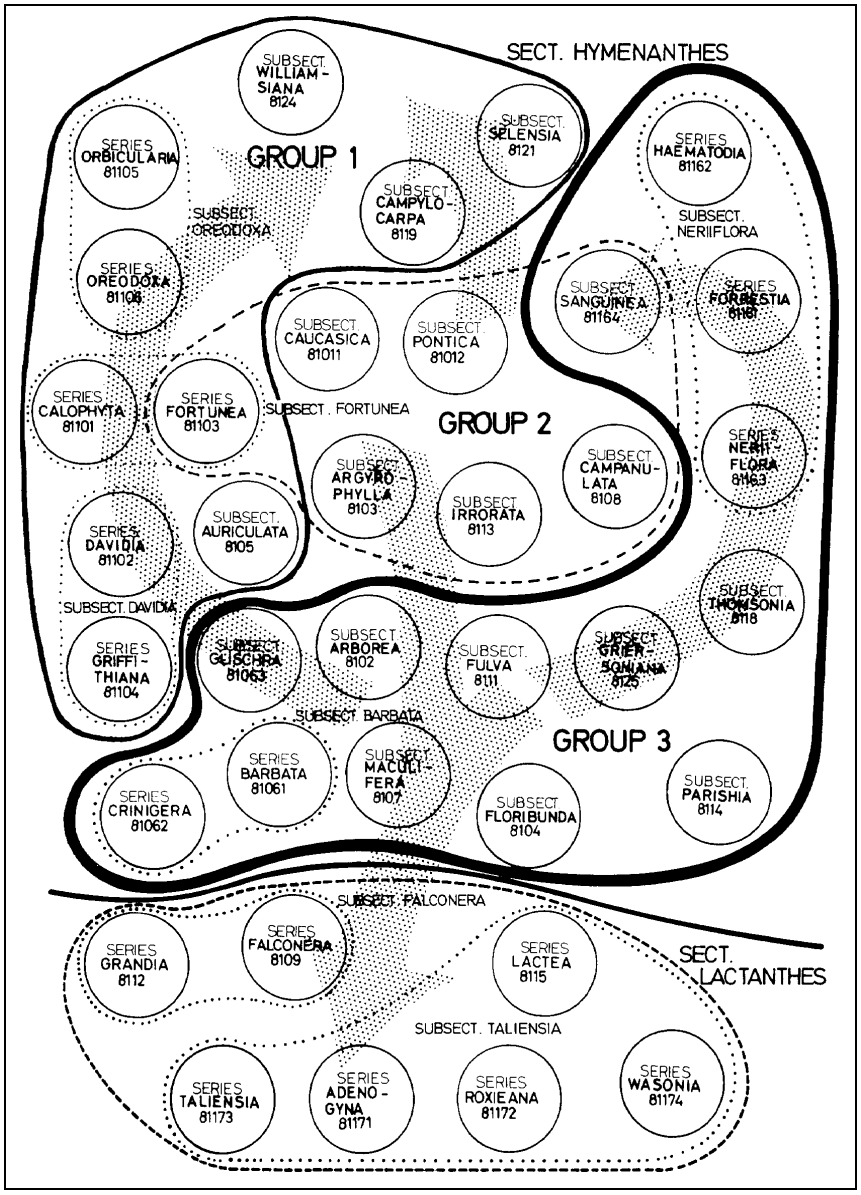
In order to meet these objectives the following actions will be taken:

- Address the theoretical questions surrounding what previous authors (i.e. Sleumer, Chamberlain and Spethmann) meant when they erected groups of different rank. (Chapter 2).
- Establish criteria for erecting new taxonomic groups. (Chapter 2).
- Establish criteria as to what constitutes suitable data for construction of said groups. (Chapters 2 and 3).
- Gather and analyse data on the basis of the criteria established. (Chapters 4 to 8).
- Draw conclusions on the basis of the theoretical arguments and data. (Chapter 9)

Chapter Summary

- An overview of the genus *Rhododendron* was given.
- Subgenus *Hymenanthes* was introduced and placed in contexted within *Rhododendron*.
- Several different classification schemes were reviewed.
- The five main aims of the project were stated.

Figure 5: *Diagram summarizing the relationships within subgenus Hymenanthes Spethmann (1987, page 23)*



CHAPTER 2: BIODIVERSITY AND TAXONOMIC DATA

In a study concerned with the delimitation of systematic groups, it is very important to establish an understanding of how the present groups were identified and to examine the theoretical basis for challenging these groups and forming new ones. It is also important to have an understanding of the data used to produce such groups. This chapter addresses these issues.

Units in Biodiversity

Solid, theoretical ground work is required to lay the foundations for decisions which need to be taken later in the study as to which specimens are (or are not) examined and which species are (or are not) sampled. Given below is a discussion of the most commonly used taxonomic rank, the species. This rank is often thought of as having more importance than other ranks and so is the one chosen for discussion here. It should be borne in mind, however, that what is concluded about the species here is also applicable to other taxonomic groups. Taxonomic groups, of whatever rank, may be thought of as essentially similar in this context. This exploration therefore starts with a discussion of the criteria that have been used to delimit *Rhododendron* species then moves on to other species concepts that have been proposed and their relevance to this study and then on to the nature of rank itself before coming to some workable conclusions of relevance here.

We are highly influenced in this area of botany by the evolution of ideas since the Renaissance. Although this treatment will go into some areas in depth no attempt will be made to review the history of biological thought or provide an in-depth analysis of all the philosophical concepts of biology; these have been well discussed in many previous publications (e.g. Mayr 1982; Minelli 1993, Stevens 1994).

Species criteria within the study group.

Cullen (1980) provided three basic criteria for delimiting species in his monographic study of subgenus *Rhododendron* excluding section *Vireya*.

> " a) species described and known only from cultivated material have not been accepted unless they are very distinct (even then, the possibility that they are of accidental hybrid origin must be borne in mind); b) species should differ from each other in at least two independent but correlatedly varying characters, and have geographical or ecological distributions different from those of their closest allies; c) if two (or more) taxa appear to intergrade, then the resulting treatment depends on the proportion of intermediate specimens. If these are very few in number, two (or more) species are recognised, which are considered to hybridise to a small extent. If the proportion of intermediate specimens is larger (up to c. 25% of the total), but the units are geographically discriminable with the morphological intermediates in a geographically intermediate area, then one species is recognised with two (or more) subspecies within it. Alternatively, if the various units are geographically indiscriminate, then one species is recognised, either undivided, or if the morphological variation is appropriate, divided into two (or more) varieties." (Cullen 1980 pages 3-4)

Chamberlain (1982), Chamberlain & Rae (1990) and Philipson & Philipson (1986) do not mention the species criteria adopted in their studies, but it may be assumed that they adopted that outlined by Cullen (1980) in the first part of the Edinburgh monograph published. (See also Cullen 1978). Cullen adopted the pragmatic approach used by herbarium and field botanists undertaking alpha taxonomic treatments for floras and monographs (c.f. Heywood 1967). It is not suitable, however, for beta and delta taxonomies, and within subgenus *Hymenanthes* the adoption of these criteria has proved problematic even at the alpha level. This is a monothetic species concept (*sensu* Sneath 1961), incurring a number of basic faults outlined under the *Typological Concept* below. Although

the handling of clinal variation by Cullen appears arbitrary it is actually a justifiable pragmatic way of dealing with what may be an unsolvable problem. See *Clinal variation and hybridisation* (Paragraph 2.1.6) below.

Judd & Kron (1995) adopt a definition used in an earlier monograph of the genus *Lyonia*:

> "Morphological entities that show a consistent complex of character states (i.e. have no major internal discontinuities) and that are separated from other similar entities by a consistent morphological gap are considered to be species. They have definite geographic distributions and ecological preferences and are often rather uniform in flower and/or fruit morphology. Species are isolated by differences in their ecology and/ or altitudinal characteristics, geographic distribution, flowering time and floral morphology and/or genetic composition." (Judd 1981 page 68)

by adding:

> "...The species recognized within *Rhododendron* sects. *Sciadorhodion*, *Rhodora* and *Viscidula* also possess unique derived characters (or at least unique combinations of derived features) and are, thus, presumed to be monophyletic."(Judd and Kron 1995, page 2).

The definition used in the genus *Lyonia* is a practical, polythetic definition (*sensu* Sneath 1961): individuals are grouped on the basis of sharing large numbers of features and yet are not required to possess set features in order to be members of the group. The weakness of the definition lies in the vague nature of the terms 'consistent complex of character states', 'major internal discontinuities' and 'consistent morphological gap'. The additional phrases added for the *Rhododendron* monograph turn the definition into a monothetic one and result is a definition that has the worst of both worlds.

Kron (1993) gives a succinct account of her approach to species in the monograph of section *Pentanthera*:

> "The topological view of a species is discarded because it does not adequately deal with variation within and among natural populations. Phenetic approaches (Sneath & Sokal 1973; Wishart, 1975) are used in species delimitation and cladistic approaches in the development of hypothetical evolutionary relationships [of these species]."(Kron 1993), page 250).

Despite detailing her cladistic analysis and including the data matrix, she does not stipulate which phenetic criteria from Sneath & Sokal or Wishart were used to define the species.

The other major western *Rhododendron* monographs (Sleumer 1966; Stevenson, 1930, 1947; Davidian 1982, 1989, 1995) give no criteria for the delimitation of species. There does not, therefore, appear to have been a clear definition of a species given specifically for *Rhododendron.*

A review of species concepts used for other organisms and their suitability to subgenus *Hymenanthes*

The species problem has been debated more-or-less continuously for the last 200 years and this is reflected in the vast body of literature available on the subject. A brief overview of the major concepts that have been put forward is outlined below, along with an appraisal of how applicable each would be to the study group.

Typological (essentialist): Every species has a unique essence and any variation within species is purely accidental. This is an interpretation of the Aristotelian approach to classification of all objects in which there are five classes of predicate: definition, genus, differentia, species and accident. The definition of the species is a statement of what the species must be to be that species and not something else. This statement is therefore equal to the essence of the subject and cannot

change without the species also changing. The genus is the part of the essence that is a predicable of another species. The differentia is the part of the definition that is not predicable of another subject and so expresses the difference between a particular species and the others in the genus. The property and the accident are other attributes of the species, the property following directly from the essence and the accident being any attribute that bears no relation to the essence. Cain (1958) discusses more fully Linneaus' application of this logic and its implications on modern taxonomy.

This is a monothetic species definition in the sense of Sneath (1961); groups are formed by rigid and successive divisions so that each group possesses a unique set of features. The chief weakness of the system is that if a single individual is aberrant in one or more of the qualifying characters (the definition) it should automatically be placed in a separate group. This leads to either the rules being bent so as to allow inclusion of aberrants or, if the rules are rigidly adhered to, an awkward classification in which similar and possibly closely related individuals can be placed in distant groups (Sneath & Sokal 1973). With the vast number of characters becoming available through the use of molecular biology it would in theory be possible to define many overlapping groups on the basis of essential characters.

Nominalist: Species do not exist in reality but are merely constructs existing solely in the mind of the observer. It is human nature to classify objects (Heywood 1967) and humans have become highly adept at detecting patterns in most situations. It is therefore a function of perception that on being faced with a large amount of complex diversity the taxonomist resolves it into manageable units. It is simpler to discuss supposedly discrete entities than continua. The result is that taxonomists will see discrete species in nature, whether or not they exist. This concept has been proposed by several authors (see Mayr 1970). Its major weakness is the fact that much of natural diversity occurs in demonstrably discrete units at any moment in time. This does not mean that the concept is totally bankrupt, indeed Darwin did not support the occurrence of species in reality:

> "I look at the term species, as one arbitrarily given for the sake of convenience to a set of individuals closely resembling each other, and that it does not essentially differ from the term variety, which is given to less distinct and more fluctuating forms. The term variety, again, in comparison with mere individual differences, is also applied arbitrarily, and for mere convenience sake." (Darwin 1859, p.52)

Darwin did, however, publish a large number of novel species names.

With the level of debate between taxonomists as to the species boundaries within subgenus *Hymenanthes* it is clear that some of the species being proposed are nominalistic. They cannot all exist in nature as they are often contradictory.

Biological: Species are breeding groups. This is the most widely accepted species concept amongst zoologists. It's chief proponent has been Mayr (see Mayr 1963, 1969, 1970, 1982) although it had been advanced by many others (see Sokal and Crovello 1970). This is a fairly practical species concept among higher animals but it breaks down in lower animals, fungi, bacteria and plants. Mayr defines the biological species as follows. "groups of actually or potentially interbreeding populations which are reproductively isolated from other such groups." Mayr (1963, p. 19).

On the theoretical level there are several problems with this concept. As an example, if A and B were near-identical organisms they would be considered to belong to separate species so long as they were not sexually compatible, even if this incompatibility were caused by a mutation at a single base-pair locus. If on the other hand they were distinctly different, perhaps by many hundreds of thousands of base-pair mutations, but were capable of sexual reproduction then they would be considered to belong to the same species. Further, if A does not breed with B it is considered a separate species but if they both breed with C the case becomes less clear. If D and E are introduced into the scenario the concept soon becomes unworkable. Breeding barriers are rarely definite and no limit is given as to how often organisms must exchange genes to be considered the same species. The concept implies a causal relationship between breeding barriers and morphology that does not

necessarily exist, for further discussion see Donoghue (1985). The biological species concept also falls down in numerous practical situations, typically in groups that have 'unusual' breeding systems such as apomicts. A further discussion of this concepts weaknesses is given by Sokal and Crovello (1970).

It is widely accepted that within subgenus *Hymenanthes* there are few if any breeding barriers among the currently recognised taxa. Apparent hybrids between morphologically distinct forms are often encountered in nature (*pers. obs*.) and breeders have little difficulty in producing crosses in cultivation (P. Cox pers. com. 1995). If the strict biological species concept were adopted for this group the whole subgenus would be considered a single species. Moreover as crosses with other subgenera are not unknown, even the boundaries of the subgenus would be questionable.

Phenetic: Species are groups of individuals that closely resemble each other and are separated from other such groups by discontinuities. This is a very widely applied concept although it is usually applied implicitly rather than explicitly. It is a polythetic concept in that species are not defined by essential characters or even essential combinations of characters. Two individuals could, theoretically, belong to the same species without having any characters in common but only share them with their intermediates (this is not likely to occur in nature). In a strict sense this may be taken as a nominalistic species concept in that the boundaries of the groups may be arbitrary and need not have any biological significance.

Sokal and Crovello (1970) demonstrate that most species concepts, when practically applied, have "phenetic bottle-necks" which has resulted in this concept being the most widely adopted one although usually closely associated with, and often combined with, a typological approach.

There are several weaknesses to the phenetic concept. First, it is dependent on a functional definition of the individual. In dioecious organisms males and females are likely to cluster separately despite their biological association. Different stages in life cycles, such as the sporophytes and gametophytes of ferns, would also cluster separately, despite their biological interdependence. This is closely related to questions of homology between the characteristics of different organisms, as discussed in the following chapter.

The greatest strength of the phenetic species concept is its broad utility. It has been used intuitively in combination with the typological approach throughout *Rhododendron* and is, at present, the only practical concept available for use in *Hymenanthes*.

Cladistic (Phylogenetic, Ontogenetic or Evolutionary): Species are the smallest monophyletic groups of organisms; they are separate ancestor-descendent lineages with their own evolutionary roles, tendencies and fates (Simpson 1961). Donoghue (1985) proposes a phylogenetic model in some detail. With the rise in the popularity of cladistic analysis since the translation of Hennig's major work into English (Hennig 1966) attempts have been made to use this methodology to define species. The current understanding of the variations on this concept were reviewed at a recent symposium (Davis 1995). Most workers (including Kron 1993) have, however, restricted the use of the technique to the arrangement of species that have been delimited by other means.

There are a number of theoretical and practical problems in adopting this approach to species delimitation. Cladistic methodology relies on having terminal taxa to analyse and so suffers from the same problems of defining the individual and homologous characters as the phenetic method. It also relies on the basic premise that the process of evolution results in a hierarchy of monophyletic groups, a premise that becomes more and more of an approximation at the lower levels of the organismal hierarchy. It is difficult to think of a mechanism where by interbreeding organisms form a nested hierarchy or indeed why they should. The polytopic origin of groups that it would be convenient to recognise as species has been demonstrated on numerous occasions. (e.g. Abbot 1993)

Problems of the phylogenetic species concept are illustrated in Donoghue (1985): "A species is monophyletic if it includes all and only the descendants of a common ancestor, even if that ancestor

was the product of hybridization. The parent species of a hybrid species are, of course, paraphyletic. However when a cladogram is constructed, positive paraphyly may not be discerned - instead the parent species may appear as unresolved and therefore acceptable groups." Clearly Hennig's principle of reciprocal illumination becomes flawed when taken to the level of defining species (see discussion on hybridisation below). No studies were encountered during the course of this study, in which a phylogenetic approach to delimiting species of higher plant had been used.

Subgenus *Hymenanthes* appears to contain numerous hybridising populations and much clinal variation. Groups are often delimited by a large number of qualitative characters that are unsuitable for cladistic analyses. No cladistic analyses have been carried out within the group. It seems unlikely, therefore, that it will ever be feasible to use cladistic analyses to define taxa at finer levels in this group. This does not rule out, however, the possibility of defining groups at a coarse level, perhaps for a sectional treatment.

A consensus of opinion

It would appear that no species concept is applicable to all organisms. Each concept appears to work because morphological discontinuities often coincide with breeding barriers at the boundaries of functionally monophyletic groups, thus producing discrete natural groups supported by discontinuities. The three criteria of morphology, breeding and monophyly occur together sufficiently often to enable a portion of the diversity of larger living organisms to be attributed to species with relative ease. For what percentage of all species these three criteria coincide is not clear. What *is* clear is that there are a number of groups in which the three do not occur together and subgenus *Hymenanthes* may well be one of these groups. The role of the taxonomist is to describe and catalogue the diversity of *all* living things including these 'difficult' groups, and it is therefore important to understand why such weight is placed on the species as a special (almost sacred) unit. This is probably due to the logic of modern thought and on the purely pragmatic requirement for units of diversity for the global biodiversity catalogue. What other evidence is there that species are a universal unit of diversity?

Evidence of the reality of species from non-European taxonomies

Workers have suggested that because people of vastly differing cultures recognise similar or identical taxa at the species level species must exist in reality (Berlin (1973), Berlin *et al* (1966 and 1974) Bulmer & Tyler (1968), Mayr (1969), Diamond (1966), Gould (1980)). This view is strongly expounded by Mayr:

> "I have always thought that there is no more devastating refutation of the nominalistic claims than the fact that primitive natives in New Guinea, with a stone age culture, recognize as species exactly the same entities of nature as western taxonomists. If species were something purely arbitrary, it would be totally improbable for representatives of two drastically different cultures to arrive at the identical species delimitations." (Mayr 1988)

Many of these studies are based on organisms that may have relatively simple breeding systems and so form 'good species'; for example, birds (Diamond 1966) and frogs (Bulmer 1968). They are often carried out on groups of people living in one location, who are rarely troubled by the clinal variation and hybridisation of partially sympatric species that are common place when working on a global scale. Diamond (1966) moved men to unfamiliar areas where they encountered species they had not seen before. In this case they frequently placed the new species with species from their own classification that are closely related to it under the Linnean system. This does not support the existence of species as natural groups but suggests that there may be higher taxa that exist as real entities. Further support for this position is given by Berlin *et al* (1974) in which 37% of native names referred to groups of higher rank than species (c.f. Gould 1980). If higher taxa are real, what is so special about species?

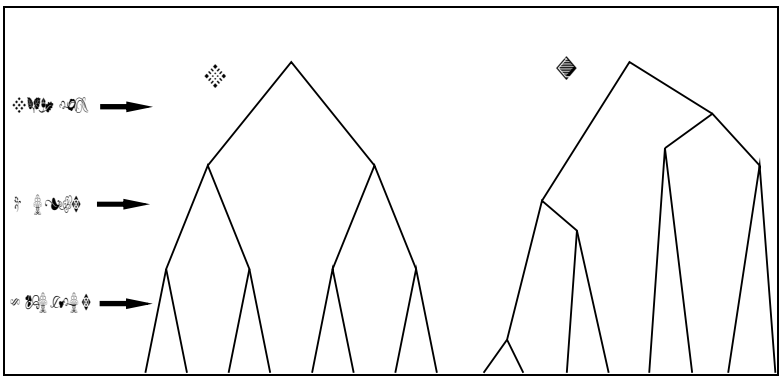
In summary, the comparison of folk and Linnean taxonomies provides evidence for the existence of discontinuities in nature and perhaps the occurrence of Aristotelian logic within primitive cultures. It does not demonstrate the existence of species as the unique unit of biological diversity.

'Species level' and the nature of rank

The term 'species level' is frequently encountered in the literature. Molecular techniques are described as being useful above or below the species level and cladistic (parsimony) analysis is often abandoned at this point, being replaced by phenetic techniques. Here population genetics meets systematics. From the evidence given above this term would seem to refer to the point above which gene exchange becomes much rarer and morphological discontinuities tend to occur, and below which lineages tend to become strongly reticulate in nature. It is a useful term but has no absolute meaning because of the nature of rank.

The taxonomic system arranges taxa into a nested hierarchy of sets. The position of each taxon in this hierarchy (its rank) can only be defined by its relative vertical position; that is, its relationship to the taxon that encompasses it and the taxon (or taxa) that it in turn contains. Lateral comparisons across the hierarchy are meaningless because there are no vertical bench marks. Although a hierarchy is often drawn as in Figure 6A it is more accurately represent by Figure 6B. Figure 6A represents the hierarchy of name types. However there is little evidence that this has any correlation with the actual hierarchy found in nature, which is more like that in Figure 6B. Although within each lineage of hierarchical sets there may be disjunctions that suggest where formal ranks should be placed there is no reason to suggest that these ranks should be in the same position on each lineage or any mechanism available where by they could be place in homologous positions. Overall, rank is therefore a continuously variable quality. Taxa are only placed at certain ranks in the naming system as a matter of convenience, though rules-of-thumb are sometimes followed within certain taxa.

Figure 6: *Illustration of lateral comparisons across hierarchy.*



Clinal variation and hybridisation.

Cullen (1980) suggests an arbitrary but practical way for dealing with clinal variation in *Rhododendron*. (This is possibly the only way that this can be dealt with.) Figure 7 represents a cline over which plants in region A begin to resemble those in region C as the observer passes through region B. There are two contrasting ways of describing the diversity represented by this hypothetical case:

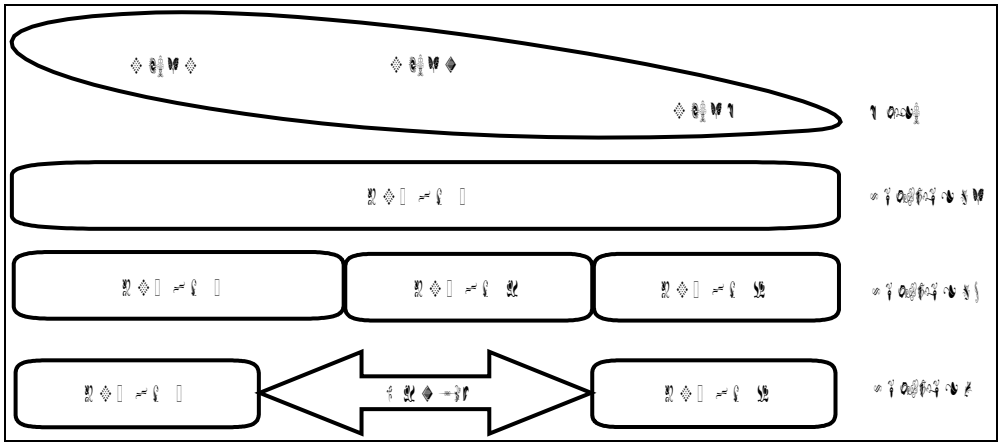
(1). All the plants in A, B and C can be placed in the same species and either (a) a note added to the species description detailing the nature of the clinal variation or (b) the plants from the different areas can be described as subspecific taxa or even separate species.

(2) Plants in areas A and C can be described as two separate species and the intermediate plants of area B described as a hybrid.

Provided the diversity is described there is no reason to favour either approach until more evidence is available about the relationships of the extremes of the cline.

If it can be shown, in an analysis of the next highest monophyletic group in which intermediate taxa are excluded, that the plants from A and C are sister taxa then it is reasonable to suggest that they may belong to the same species by virtue of having a large number of intermediates. If A and C form a paraphyletic group it is reasonable to suggest that the plants found in B are of hybrid origin and represent the merging of two separate lineages.

Figure 7: *Illustration of different taxonomic approaches to clinal variation.*



Thus often it is not possible to solve hybridisation problems in isolation from the taxonomy of the larger group in which the putative hybrids reside. It is also necessary to carry out both phenetic and cladistic analyses in order to solve such problems. The cline could not be recognised in a cladistic analysis as it would appear as an unresolved group, depending on where and how the terminal taxa were selected from within the spectrum of variation. A phenetic analysis would not provide an explicit relationship for the extremes of the cline and inclusion of intermediates would force the extremes of the variation closer together on a dendrogram masking any genealogical relationship (Minelli 1993).

Initial conclusions on the nature of taxa.

It would appear that much of natural diversity can be broken up into discrete units but that much cannot. Current policy is to describe all of biodiversity by dividing it into discrete units. There is much evidence to support the non-universal nature of the species taxon. There is little or no evidence to support the contrary view.

The following definition is proposed as recognising these views and combining the relevant concepts discussed above:

> *Species are groups of organisms that are delimited by the coincident occurrence of perceived morphological discontinuities and breeding barriers at the boundaries of functionally monophyletic groups during a given time-frame and, as such, are separate from considerations of rank. The totality of biodiversity can not be divided into species, nor is there any reason why it should.*

As it has been established that different ranks of taxa are not directly comparable it should be possible to apply this definition equally well to groups such as subsections and sections.

Summary: approach taken in the current study

None of the orthodox species concepts will suffice in fully describing the variation in subgenus *Hymenanthes*. Although some species are describe by Chamberlain (1982) as being discrete entities

there are many that have very close affinities with other species and frequently hybridise. There are also many 'species' that are not supported by other authors, notably Davidian (1982, 1989, 1995). This is true of any critical group studied by more than one taxonomist.

It is clear that some species complexes form intricate clinal patterns that are not resolvable into simple groups, and may have to be treated as aggregate or ochlo-species (sensu White 1962) for the purposes of formal taxonomy. It is also clear that it may be possible to resolve some of these clines into discrete but hybridising taxa. The only way that these two alternatives will be resolved is by performing an overall analysis of the variation of the subgenus taking, as exemplars, the extremes of clinal variation where it occurs. Until this analysis has been carried out it will not be possible to make explicit and defensible decisions regarding lower taxonomic levels. This is the justification for the form that the rest of the study takes.

Taxanomic Data

Biodiversity is typically assessed by some method of sampling (i.e. a sampling of individual organisms from the total population of organisms and a sampling of the features of those organisms). The formalisation of this process in taxonomy has lead to the compartmentalisation of such data into units that are usually termed characters. The division of sensory data into distinct characters is closely related to, and integrally linked with, the process of splitting diversity into units and suffers from many of the problems dealt with above. The subject will be dealt with in a purely pragmatic manner here in order to reach a working approach for the present study.

Philosophical Matters

Characters are always treated as Aristotalian entities. Each has a definition made up of a genus and differentia and may have properties and accidents. In the classic hypothetical case of a group of plants with different coloured flowers the character 'Flower Colour' is the *genus* and the colours of the flowers in the different plants are the *differentia* of that genus. Together 'Flower Colour' and 'Blue' for example make up one of the *species* of that character. A *property* of that species may be the pigment that causes the flower colour and the amount of the pigment could be considered an *accident.* Clearly if the 'Flower Colour Blue' can be caused by two different pigments then the definition is predicable of two different entities which may not be desirable. The definition of the character could be changed so that 'Flower Colour Pigment' was the genus (character) and the differentia (states) were the different pigments. In another case the genus (character) could be the pigment and the amount present be the differentia (states).

Some phylogenetisist use a slightly different terminology for characters and character states. (e.g. Wiley *et al* 1991). They replace character with the term 'transition series' and character state with 'character'. In this way 'Flowers Blue', 'Flowers Red' and 'Flowers Purple' may all be characters in a transition series of 'Flower Colours'. This is a useful way of visualising data when carrying out phylogenetic analyses, especially using parsimony techniques, but is logically the same as the case given above. The transition series is the genus and the characters are the differentia. In this study the term transformation series will only be used in place of conventional character terminology when it is believed that the character states are homologous and form an evolutionary series. Other systematists use a third convention, basing their classification on the presents or absents of 'homologies'. See Stevens (1984). This convention is confusing as it can not be applied to quantitative data and will not be used in this study.

Homology

The sampling of organisms for taxonomic characters involves making decisions concerning the evolutionary homology of those characters. Are the same attributes being measured in each organism? Are the *differentia* predicable for the *genus*? This subject is a complex one and has been debated extensively in the literature. (e.g. Patterson 1982; Roth 1988; Stevens 1984 Sneath and

Sokal 1973). A pragmatic approach will be taken here. Structures in different organisms will be considered as homologous if they are similar in position and appearance and do not occur in more than one state in the same organism. This hypothesis will then be test by the congruence between different characters in subsequent analyses. These are the three criteria (similarity, conjunction and congruence) suggested by Patterson (1982).

Character States

It is customary to distinguish two main classes of taxonomic character; qualitative and quantitative. Quantitative is further divided into two more classes; discrete-quantitative (or meristic) and continuous-quantitative. (Stevens 1991). The terms 'hard' and 'soft' have been used to describe the kinds of data produced by these characters (Bateman 1995 pers comm.): Discrete-quantitative being harder than continuous-quantitative and softer than qualitative.

In qualitative characters organisms are perceived to posses one or more of a number of possible discrete states. The possession of particular hair types is a good example. Qualitative characters are particularly useful in cladistic analyses. Discrete-quantitative characters are similar to qualitative characters in that an organism is scored for possession of one or more of a series of discrete states even though those states may include all possible integers. Examples of a discrete-quantitative characters include petal number and leaflet number. Continuous-qualitative characters differ from the previous two types. Leaf length is an example, this character could, effectively, have any value dependent on the accuracy of measurement. Continuous-qualitative characters are not suitable for parsimony analyses and have to be broken up into a number of key states. The decisions taken in arriving at the boundaries of these key states may have a considerable effect on the resulting analysis. This is unfortunate as these characters are typically more common in critical groups than the other two character types. Stevens (1991) gives a detailed review of the problems associated with this matter. For the purposes of this study each character will be discussed independently. Characters will only be split into key states for the purposes of particular analyses.

Independence and Convergence

Classifications are based on congruence; characters having similar distributions of their states through the study group as a result of the relationships of the taxa. There are, however, other reasons for character congruence. Characters may be functionally dependent on one another, (examples are corolla and stamen length or base pairs that are important for secondary structure in non-coding sequences.) or they may have similar distributions through convergence due to evolutionary pressures. Whole character suites may be effect by changes in climate or altitude that bear no relation to the relationships of the species.

In constructing a classification that reflects the 'true' relationships of the taxa it is important to minimise the noise (homoplasy or non-congruent data) in the analysis by choosing characters that are not likely to be effected by the factors mentioned above. For this reason selectively neutral characters are often favourable for reconstruction of phylogenies as they minimize the influence of convergence and are more likely to act as independent markers of evolutionary relationships. There is no way of allowing for convergence due to genetic drift. Where morphological characters are being used care must be taken that they are not structurally or functionally dependent.

Functionality

Characters that do not vary in the study group do not convey information regarding the relationships of the taxa. Characters that have a single state for all but one of the study taxa (autapomorphies) only serve to define that taxon and do not provide information regarding other possible groupings. It therefore follows that characters for investigating the relationships between taxa should have states that are shared by two or more them. It is also important that characters should be available in large enough numbers and states to produces a classification of suitable resolution. It takes N-2 fully informative binary characters to draw a fully resolved cladogram for example (where n is the number of terminal taxa). Molecular markers that are dominant do not convey any information concerning heterozygosity of individual taxa and convey limited information regarding the frequencies of alleles within populations. Characters should be of an explicit nature. This enables taxa to be scored consistently throughout the duration of the study and across the range of the study

group. It also allows characters to be approached as hypotheses that may be tested and possibly rejected.

Summary: Five criteria for accessing taxonomic data

The following chapters deal with the selection of molecular techniques and the compilation of a morphological data set for the study. These possible sources of data will be assess for the five qualities listed here:

1. **Selective** - Characters must convey some information about relationships within the study group.

2. **Homologous** -Character states must be predicable of the character and agree with the recommendations of Patterson (1982).

3. **Independent.** - Correlation between characters should be related to the 'relationship' between the OTUs and not to other factors.

4. **Verifiable** - Characters are mini-hypothesis and as such should be testable. Are the characters demonstrably homologous and independent either on practically or theoretically grounds? Is it possible to score for the presence of the character state in question by more than one means?

5. **Available.** - Characters must be available in sufficient quantity and quality from all the OTUs to produced a resolved classification.

Chapter Summary

- Theoretical aspects of the division of biodiversity into units were considered and a working species definition devised.
- The nature of taxonomic data was discussed.
- A set of criteria were established that could be used to assess potential sources of taxonomic data. These criteria comprise the acronym SHIVA.

CHAPTER 3: POTENTIAL MOLECULAR TECHNIQUES

Molecular biology is a wide ranging subject and there are many techniques that could be used to generate data for phylogenetic analysis. The aim of this chapter is to assess the applicability of the some of the major tools to this form of analysis in general and to the study group in particular.

ISOZYMES

Isozyme electrophoresis has been one of the major tools for examining genetic relationships at the population and species level since the development of starch gel electrophoresis and the histochemical visualisation of enzymes in the late 1950's. It has been used successfully in studies on population structure, breeding, paternity and to reveal cryptic species in animals. (see Murphy *et al* 1990 for examples). Its use in phylogenetic systematics, along with other techniques providing gene frequency data, is more controversial (Felsenstein 1981). A detailed review and technical description of the technique is given by Murphy *et al* (1990) The advent of PCR base technologies may lead to a decrease in its use.

Description

Isozymes are functionally similar proteins that have different forms and are produced by different alleles. They are termed allozymes when they are presumed to be produced by genes at the same locus. Proteins are composed of combinations of twenty possible amino acids joined together by covalent peptide bonds to form polypeptides. They are two steps removed from DNA in that they are the formed when DNA has first been transcribed into RNA and the RNA translated into the protein structure. They are, therefore, part of the phenotype.

The twenty amino acids are characterised by their different side chains. Five of these side chains carry either a positive or a negative charge. By producing a protein extract, typically using a detergent such as SDS, and running it in an electrophoresis matrix it is possible to separate different protein molecules on the basis of their rate of migration. The migration rate is dependent on the net charge of the molecule, its size and shape. The net charge is dependent on the pH of the buffer system used. Buffer conditions can also affect the shape of the molecule. Histochemical staining is used to detect the position of the proteins on the gel at the end of the electrophoresis, usually a substrate for the reaction which the enzyme catalyses is provided and then staining is used to detect the absence of this substrate or the presence of reaction products.

Assessment of criteria for good taxonomic data

Selective: As codominant markers isozymes are well suited to use in studies at and below the species level where estimates of population genetic parameters such as F_{ST}, F_{IS} and F_{IT} are to be made. They differ from other codominant markers in that for detection of both alleless in heterozygotes it is required that both alleles are expressed They are also of use as phylogenetic markers although two major problems may be encountered. Either polymorphisms at the study loci may be too low so that the data are not selective (c.f. the PCR-RFLP study) or the genomes could have diverged to such an extent that shared alleles are more likely to be the result of convergence than of shared relationship. Nei (1987) suggests that allozymes should not be used where true genetic distance is greater that 1.0. (c.f. Clark and Lanigan's recommendations in the section on RAPD given below.)

Homologous: The principle assumption of allozyme analysis is that similar changes in the mobility of the proteins are the result of similar changes in the DNA encoding for that enzyme. This may not be the case and becomes less likely as the taxa become more dissimilar. Another assumption made is that a band on the gel is the product of a single allele i.e. that comigrations have not occurred. This can be tested by carrying out the electrophoresis and detection under different conditions for

the same enzyme (Johnson 1976 & 1979) although any such investigation is likely to detract from the availability criteria below.

Independent: The independence of allozymes as taxonomic characters depends greatly on how the data is analysed. If each allele is treated as a character then they are clearly not completely independent of each other as it would frequently be lethal to possess none of the possible alleles. A similar situation occurs when using allele frequency data for each taxon, the total frequency at a locus must always sum to one and so the frequency of each allele is dependent on all the others. If loci are treated as characters and alleles as character states, different problems arise similar to those found with multi-state morphological data. If the characters are left unordered they may convey little or no information, simply being mapped onto a tree supported by the other characters in the analysis. If, on the other hand, they are ordered they are more likely to produce a tree that is a function of the *a priori* judgments that have been made rather than the 'true' relationships of the taxa. These factors have been much discussed in the literature, notably by Swofford and Olsen (1990).

Verifiable: The use of allozymes is so well established that they have gained the reputation of being reproducible markers between labs provided similar extraction and electrophoresis conditions are used. It has been demonstrated, though, that if different conditions are used many cryptic alleles may be uncovered. (Johnson 1976 & 1979). Methods of scoring for the presence of a particular allele by means other than those described above, such as sequencing DNA or RNA, are long winded and require a very different approach.

Available: Allozymes are one of the cheapest 'molecular' assays to set up, requiring relatively simple equipment that, if need be, can be fabricated in the laboratory. It may take some time however to optimise extraction and electrophoresis conditions for a specific study group even in a well established laboratory and there is always the danger that conditions that are optimal for some members of the group may be sub-optimal for others, especially in taxonomic studies. These conditions can only be optimised empirically and problems may be magnified if similar tissues of similar ages are not available from all the study taxa. There are now detection systems for numerous loci although those which will be sufficiently variable for a particular study can only be determined empirically much as with choosing specific regions for a sequencing or PCR-RFLP study.

Application to the current study.

The current study is chiefly concerned with the phylogenetic relationships within the group, an area for which allozyme data is least appropriate. There are no reports of the use of allozymes in the genus *Rhododendron* in the literature and so any study would have to start from scratch in establishing protocols. Further to this the plants to be studied exhibit a wide morphological range and so may have different requirements for extraction and buffering systems. Allozymes were therefore deemed inappropriate for this study.

Restriction Site Analysis (RFLP, PCR-RFLP, CAPS)

Examination of restriction fragment length polymorphisms has been the major technique for direct assessment of genetic diversity over the past 15 years. A practical review of this technique is given by Dowling *et al* (1990).

Description

RFLPs are based on the action of Restriction Endonucleases (REs). These enzymes cleave DNA at specific positions that have particular recognition sequences, typically 4-6 base pairs long. Over 500 REs have been isolated from bacteria where their role is to protect the organism by cleaving foreign DNA. The bacteria's own DNA is protected by methylation of the RE recognition sites. Variation in the occurrence of the restriction sites is used as a measure of base pair changes in the DNA of the

study taxa.

An extraction of either total genomic DNA (or one enriched for organellarDNA) is made and then cleaved using one or more restriction enzymes. The resulting DNA is fractionated on an electrophoresis gel and viewed using a stain such as ethidium bromide. A successful digestion will appear as a smooth smear down the gel with, perhaps, a few bands representing high copy number organellarDNA. A selective method of detection is then used to identify the size of particular fragments in this smear. The most commonly used method is Southern Blotting (Southern 1975). This method involves chemically denaturing the DNA on the electrophoresis gel and then transferring it to a nitrocellulose or nylon membrane where it is fixed. Transfer is typically carried out by capillary action although it is also possible to use the process of electro-blotting where a potential is placed between the gel and the membrane. The blot is exposed to a single stranded, labeled DNA probe that binds to its complimentary sequence on the membrane. The probes are typically radioactively labeled and detected by autoradiography. The probe may be dissociated from the membrane and another hybridised as many as 20 times. It is becoming more common to use biotin labeled probes and to detect their presence by staining. Using staining techniques it is possible to apply different probes to the same membrane at the same time using different colours.

Analysis

There are two approaches that can be taken to analysing RFLP banding patterns. Taxa may be scored for the presence/absence of polymorphic bands or an attempt can be made to identify the reasons for these polymorphisms (typically mutations and insertion/deletion) and these events scored. If the former method is adopted then the data has to be regarded as similar in nature to the fingerprint data such as that produced by RAPD and AFLP. (Thormann *et al* 1994 compares RFLP and RAPD approaches, see also those sections of this chapter for a discussion). If, however, the latter method of scoring is adopted and an attempt made to score the gain and loss of restriction sites and indels the technique is far more suitable for phylogenetic analysis. It is presumed below that this approach has been taken.

Assessment of criteria for good taxonomic data:

Selective: This technology enables large areas of the DNA to be scanned rapidly and cheaply enabling a set of informative restriction sites to be derived relatively quickly. The markers are also codominant and so convey information regarding heterozygosity, although this is rarely an issue as most studies only examine organellar DNA. (see below.)

Homologous: It is possible to make clear statements of homology about restriction sites and indels although the statements may be asymmetric due to it being more likely that a restriction is lost than gained. These asymmetries in confidence can be corrected for during the subsequent analysis. (Dowling *et al* 1990)

Independent: Restriction sites may be assumed to behave independently, subject to the normal conditions as sequence data (see sequencing section below). Although large indel events may cause numerous restriction site changes it is unlikely that these would not become clear during the mapping of the restriction sites.

Verifiable: Possession of a restriction site may be scored by other means, such as the use of isoschizomers, (enzymes with the same recognition site), overlapping enzymes and by sequencing.

Available: Restriction *fragment* data can be produced rapidly and cheaply, however converting this into restriction *site* data may be far more problematic and time consuming. For this reason organellar DNA is frequently studied because its overall structure is so well known and conserved probes are available that enable the rapid production of a restriction site map. This is a severe restriction on the applicability of this technique. Other factors affecting the availability are the need for relatively large (in the region of 1 - 2ug) amounts of DNA as compared to PCR base techniques

and the need for this DNA to be free from contaminants that may affect the actions of the restriction enzymes.

Application to current study

RFLP analysis has been used in a parallel study carried out at the University of St Andrews by Richard Miln examining the origins of *Rhododendron ponticum* in the British Isles. This study has found that infraspecific cpDNA RFLP variation existed in *R. ponticum*, with several mutations separating the Iberian populations from those around the Black Sea. *R. catawbiense* and *R. macrophyllum* also displayed variation. These results were used to show that the *R. ponticum* naturalised in the British Isles may be of wholly Iberian origin. Nuclear RFLP markers showed some accessions may have *R. catawbiense* in their parentage, especially in the north.

Because of the difficulty in mapping non-organellar regions it was felt that this technique was not suitable for this study. (See discussion on organellar DNA in chapter 8.)

Associated techniques

Restriction site analysis of PCR amplified fragments. (PCR-RFLP): The difficulties in mapping restriction sites from fragment data may be overcome by carrying out restriction site analyses of known regions amplified using conserved primers. Examples of such studies include Taberlet *et al* (1991) and Demesure (1995)

The advantages over the traditional study outlined above include:

- The ability to look at nuclear as well as organellar genomes.
- The requirement for only picogram amounts of DNA.
- The potential of validating sites by a use of double digests, partial digests (with end-labeling of fragments) or sequencing of some fragments.
- The ease of combining data from such a study with sequence data.

The disadvantages of such a study include:

- The relatively small amounts of data produced.
- The small proportion of the genome that is covered by the study.
- The risk of not choosing variable enough regions.

Application to current study

Because of the advantages of this technique and the availability of amplified fragments a pilot study was carried out on a nuclear and an organellar region. These studies are detailed in chapter 7.

Random Amplified Polymorphic DNA

RAPD (Random amplified polymorphic DNA) is a widely applied technique that is typically perceived as being quick and easy to implement. It arose at a time when three very similar techniques that were developed contemporaneously by Welsh and McClelland (1990), Williams *et al* (1990) and Caetano-Anolles *et al* (1991). It has grown in popularity very rapidly since its inception (as illustrated in Table 2) but is now often viewed as controversial for some applications.

Table 2: *Number of publications mentioning the term RAPD on the BIDS database. (After Arnold 1995)*

Year	1990	1991	1992	1993	1994	1995	1996
Number	1	9	52	170	292	458	568

Description

The technique is a variation on the polymerase chain reaction except that only a single primer,

typically of around ten base pairs in length, is included in the reaction mixture. This results in the polymerase enzyme amplifying fragments of DNA that are bounded by two inverted repeats of the primer sequence. In this way numerous amplification products are synthesised which are subsequently visualised on agarose or polyacrylamide electrophoresis gels. In order to produce successful reactions it is necessary to use relatively low annealing temperatures: around 36˚C instead of the temperatures of between 45˚C and 55˚C commonly used in specific PCR. This decreases the selectivity of the primer allowing it to bind to more sites and so increasing the total number of amplification products. The upper and lower limits on the sizes of fragments that can be produced are thought to be governed by the secondary structures formed by the primers and template, the duration of the extension period of the cycle and the efficiency of the polymerase enzyme.

The three variations originally proposed to the basic technique are:

- Arbitrary Primed PCR (AP-PCR: Welsh and McClelland 1990) in which primers of around ten base-pairs in length are used and the first two cycles of the PCR reaction have a lower annealing temperature followed by ten cycles with a much higher annealing temperature. The resulting amplification products are visualised on agarose gels with ethidium bromide staining.
- Random Amplified Polymorphic DNA (RAPD: Williams *et al* 1990) in which primers of around ten base pairs in length are used but a consistent, low annealing temperature is applied throughout the PCR reaction. The resulting amplification products are visualised on agarose gels with ethidium bromide staining.
- DNA Amplified Fingerprinting (DAF: Caetano-Anolles *et al* 1991) in which much shorter primers (typically less than 6 base pairs) are used. The amplification products are visualised on polyacrylamide gels with silver staining.

The term RAPD has taken on common parlance to mean almost any variation on this basic model and will be the one used here (excepting Semi-Random PCR) although some authorities may dissagree with this. The technique has been reviewed by Hadrys *et al* (1992) and, from a theoretical point of view, by Clark & Lanigan (1993).

Marker characteristics

Theoretically if suitable primer sites are present in a total genomic DNA extraction they will produce an amplification product, whether they come from either of the two genomes or from the organellar genomes. RAPD bands are therefore dominant, anonymous markers and it is impossible to detect heterozygotes or hybrids without either breeding trials or comparison with closely related taxa. As the quantity of product is positively effected by the quantity of template it is to be expected that the brighter bands on RAPD fingerprint are the product of repetitive DNA. This has been confirmed experimentally by Southern blotting and probing of fingerprints. (Williams *et al* 1990 and Hilu & Stalker 1995). It also follows that those bands produced by single copy heterozygous amplification sites should be proportionately less intense than those sites produced by homozygotes. No studies were encountered in which this has been shown empirically.

Assessment of criteria for good taxonomic data:

Selectivity: RAPD markers are highly polymorphic and as such appear to be selective. However, being dominant markers they lack the ability to select between homozygotes and heterozygotes.

Homology: The importance of comparing homologous characters in both cladistic and phenetic analyse cannot be over stressed and this is the area of greatest weakness for RAPDs. Direct comparison of band sizes across a number of lanes of a gel gives a crude estimate of homology; each character being represented by the presents or absence of a band. In many studies this is the only assessment of homology that is carried out. This estimation requires assumptions to be made:

- The band is not the product of repetitive DNA and if it is then the DNA that it comes from is homologous within the genome.
- When the band cannot be detected on the gel the amplification site is absent from the genome. This has been shown not to be the case. Bands that are not visible in ethidium bromide or silver stained gels may appear of Southern blots (Iqbal *et al* 1995) and the amplification of one site may be inhibited by that of another. (Smith *et al* 1994).
- Comigration of unrelated bands has not occurred.

There are numerous reasons why an amplification site may not be present in the genome these include: mutations at any of the critical base-pairs within the primer annealing sites, nesting of primer sites, insertion/deletion events within the fragment and changes in secondary structure that effect primer annealing. Band absence cannot, then, be deemed equivalent to band presence, the homology is asymmetric, much as with restriction sites.

Steps can be taken to check the homology of bands, these include excising and re-amplifying a band then using it to probe a Southern blot or sequencing it so as to design specific primers, restriction site analysis of a band and mixing of DNA samples. If any of these steps are taken, however, they dramatically effect the advantages of the technique. Smith *et al* (1994) discusses the practical problems in the use of RAPD markers for phylogenetic analysis.

If RAPD markers are not good homologous markers and ineffective as traditional taxonomic characters what use are they at providing an estimate of overall genomic similarity? Clark & Lanigan (1993) reviewed the use of RAPD markers for estimating nucleotide divergence and found that they may be useful if nine criteria are satisfied. These criteria are:

1) Primer selection must not be biased in favour of those that reveal the most polymorphism. Commercially available primers tend to have a G+C content of 60%-80%, which may result in an overestimate of human nucleotide diversity, because of the high degree of polymorphism at CpG sites.

2) All polymorphic and monomorphic bands must be carefully scored. If some bands are not scored, then there must be no bias in scoring monomorphic bands versus polymorphic bands.

3) Polymorphic bands must be shown to behave as Mendelian factors.

4) Allelism of bands must be ascertained by Southern blotting or segregation analysis. If two or more bands are allelic, only one should be scored.

5). Homology of bands of the same size in different species should be demonstrated, e.g., by Southern blotting.

6). For diploids, a population sample must be examined to determine band frequencies.

7). True nucleotide sequence divergence should not exceed ~10%.

8). Single nucleotide substitutions are assumed to result in a loss of amplification. We assume that amplification at imperfectly matching primer sites is rare, but further experimental work on both of these issues is desirable.

9). Insertion/deletion variation that results in variation in band presence/absence is assumed to be rare. Insertion/deletion variation that results in variation in band size must be identified and analyzed appropriately (see 4).

Clark & Lanigan concluded:

> "Although RAPDs can be an efficient means of collecting large quantities of nucleotide divergence data, we emphasize that, unless these conditions are met, inference of phylogenetic relationships on the basis of RAPDs can be highly error prone."

Independence: It has been shown above that RAPD bands may not be independent and it is difficult to ascertain with what confidence the independence of bands may be assumed. If steps are

taken to clarify their independence RAPD will score lower on the availability criterion

Verifiable: This technique has gained a reputation for not being reproducible between different studies, laboratories and workers. Two organisms may produce approximately the same size band with the same primer but there is no way of knowing whether these bands are homologous without further testing. It is also difficult to standardise measures of whether a band is present or not. It has not proved possible to communicate results explicitly between workers even when most of the reaction variables are standardised.

Availability: RAPD has a number of perceived advantages over other techniques and particularly over techniques that were available at its conception. Only small amounts of DNA are required, typically in the region of 1-10ng as opposed to the 1-5mg frequently used in RFLP studies. This facilitates the scaling down of extraction procedures and enables larger number of samples to be screened. No previous knowledge of the genome is required and so this technique may be quickly applied to novel plant groups and, as no project-specific materials or radio isotopes are required, costs are kept to a minimum. These are the great strengths of RAPD markers but because of the sensitivity of the technique to DNA purity and the need to repeat amplifications several times, problems are often encountered. When looking at different species with different morphologies and secondary metabolites it may be very difficult to produce consistent standard of extractions. This is confirmed by Williams *et al* (1990)

> "Even if one can identify bands that segregate as good Mendelian markers, DNA preparations of low quality may result in higher rates of mispriming, making it impossible to get an accurate count of monomorphic bands"

If steps are taken to clarify the homology of the bands this technique scores lower on the availability criterion.

Application to the current study

Five studies have been published that make use of RAPD markers for analysis of variation in *Rhododendron*. Four of these studies have been carried out by a research group in the Department of Agronomy at the University of Illinois, USA and one by group in Merelbeke, Belgium. Because of the relevance of this work to the current study an outline and critique of these papers is given here.

Positive Identification of Rhododendron through DNA finger printing (Rayburn *et al* 1993): DNA was extracted from 11 plants purchased from The Rhododendron Species Foundation and RAPD fingerprints produced from four of them. Results from two of these species amplified by three different primers are presented in the paper. No mention is made of the sequences of the primers and no attempt is made to score the banding pattern. The paper acts as a short communication to show that it is possible to produce RAPD fingerprints in *Rhododendron*. Under the heading "Future Research" it is claimed that RAPD patterns may be used in future to delimit species, a claim that cannot be met (see discussion above). It is also claimed that RAPD fingerprints may help revealing the parentage of known hybrids and clonal identification, uses for which the technique may be practical.

Research Note: Feasibility of Rhododendron DNA profiling by RAPD. (Iqbal *et al* 1993): RAPD reactions were carried out with 20 primers and template DNA from *Rhododendron atlanticum*. Seven primers that gave "very consistent and scorable markers" were chosen for a wider study that included one representative of each of four different species; two deciduous and two evergreen. Bands were assessed visually on ethidium bromide stained agarose gels and it was hypothesised that one band was common to all four species and one band was common to the two deciduous species. It was suggested that the latter common band was the result of their evolutionary relationship. All four plants otherwise produced notably different banding patterns. It was concluded that RAPD is a useful tool in the study of genetic diversity in *Rhododendron* and can be used to make a RAPD

profile bank as a reference for the identification of species.

Clonal stability of RAPD markers in three Rhododendron species. (Iqbal *et al* 1995): RAPD fingerprints were produced, using ten different primers, for ten clones from each of three plants representing three different species and from thirteen plants representing two inter-specific F2 crosses. There was no variation in banding pattern between clones for a particular plant although there were differences between clones from different plants. The plants representing the F2 crossed showed considerable variation. Four conclusions are drawn from these results: 1) "The insignificant variability among Rhododendron plants signifies their use as a tool for varietal identification in nurseries". 2) Polymorphism within the hybrids shows that RAPDs are able to detect genetic variation. 3) RAPD provide excellent markers for identification of genetically distinct plants. 4) Such markers would be useful in varietal identification by nurseries etc.

Assessment of genetic relationships among Rhododendron species. varieties and hybrids by RAPD analysis. (Iqbal *et al* 1995): RAPD patterns were produced, using ten primers, with four species and nine hybrids and cultivars. 183 different RAPD products were produced and on the basis of presence and absence of these bands a cluster analysis was carried out that clustered hybrids with similar parentage closely as opposed to those with different parentage. An assessment of homology probes were made from two of the bands and Southern blots of the RAPD fingerprints were probed with them. The probes were made by cutting the bands from the gels and PCR amplifying them using a biotin 7-dATP. The assumption was made, but not stated, that the bands represented single products and that the resulting probes were therefore a single product. One of the probes only hybridised to the product in the species it had been excised from, the other hybridised to more than one species but also to more than one product including products that were not visible in the ethidium bromide stained gel. The first probed band is therefore uninformative about the relationships of the organisms the second represents highly repetitive DNA. This is not mentioned in the paper in which the similarity analysis is based on the bands visible in the ethidium bromide stained gel.

The application of RAPD markers for the identification of Rhododendron species. De Reik *et al* (1996). Six species of 'Azalea' were examined using ten commercially available primers. The scorings from the six primers that provided the most polymorphic patterns were combined to give a total of 50 different markers. These markers were then used to compare the species utilising the Jaccard coefficient of similarity and UPGMA clustering algorithm. The highest coefficient of similarity observed between two species was 62%. It was concluded that although not enough bands were present to make judgments regarding the phylogenetic relationships of the species the banding patterns were sufficiently different to enable identification of individual species. It was proposed that further RAPDs and AFLPs could be used to generate a library of DNA fingerprints for discriminating 'Azalea' varieties. No reproducibility experiments were mentioned, neither was any attempt made to assess the variability within species or cultivars.

Although they have been applied in very many different studies, including some on *Rhododendron*, RAPD markers appear to have very many weaknesses. Clark & Lanigan (1993) warn about their use without extreme care, although they do suggest they may be useful at lower levels of diversity, where true genetic divergence is less that 10%. RAPD could therefore be an appropriate technique to apply in the rapidly evolving study group where there may not be a great deal of divergence at the sequence level and where little is known about the genome. An RAPD study was therefore attempted.

Associated techniques

Semi-random PCR: When steps are taken to reduce the random nature of the RAPD amplification products the method is termed semi-random PCR. Weining and Langride (1991), for example, used the conserved sequences present at the intron splice junctions in combination with random and non-random primers to give reproducible molecular markers in cereal DNA. Due to low annealing

temperatures required this technique has many of the draw backs of conventional RAPDs but may prove a powerful investigative tool with closely allied plants such as cereal cultivars.

Amplified Fragment Length Polymorphisms (AFLP): This technique is a relatively new development, (Keygene 1992, Vos *et al* 1995).

Typically around 0.5ug of sample DNA is digested by two restriction enzymes (one a frequent cutter and the other a less frequent cutter). This produces a restriction fragment mixture that contains three populations of fragments. Those with a frequent cutter site at both ends, those with a rare cutter site at both ends and those with a frequent cutter at one end and a rare cutter at the other end. A ligation reaction is carried out on this mixture to attach adaptor fragments of known sequence to the ends of the cut fragments. Different adaptors are used for the frequent and rare sites. A round of PCR amplification is then carried out using primers designed for these adaptors. This is termed a non-selective PCR as it does not select between the frequent-rare fragments. It does, however, select these over the frequent-frequent fragments and the rare-rare fragments. The resulting fragment mix therefore largely consists of frequent-rare fragments. Another round of PCR amplification is then carried out on a diluted aliquot of the second mix using labeled, selective primers. These primers are longer than those previously used by between one and three bases at the three primed end. This means that only those fragments that possess the additional base(s) next to the original cut site are amplified. These fragments are then visualised using a one base pair resolution electrophoresis system. The number of fragments seen and the level of polymorphism in those fragments is dependent on the genome, and the choices of enzymes and selective primers used but may be very high.

Although this technique is based primarily on restriction site analysis it is placed here as the markers it produces are closest in nature to those produced by RAPD analysis. AFLP markers are theoretically dominant markers. If the two restriction sites are present in one of the haplotypes then a band will be produced on the final gel. Much commercial effort is being placed into making them perform as codominant markers by using a form of quantitative PCR. This has yet to be achieved and may never be feasible in organisms with poorly known genomes. Other than for their high resolution and the large amount of data that they produce all the theoretical comments mentioned for RAPD above apply here.

SEQUENCING

The basic unit of variation within the genome is the linear order of nucleotide bases that constitute the DNA. Ascertaining the order of these nucleotide bases is the most accurate way of sampling the genome for molecular characters. It therefore appears to be to be one of the most desirable molecular techniques.

Description

All modern techniques for sequencing DNA rely on the principle of producing four nested sets of single stranded DNA fragments. A nested set consists of a collection of fragments that all have a common starting point (the fixed end) but which terminate at different distances from this point (at the variable end). The position of the fixed end is governed by the sequence of the primer used. The position of the variable end is a function of one of the nucleic acid bases. By separating these fragments on the basis of their size and then detecting them using either radio isotopes or florescence techniques it is possible to deduce the sequence for the entire fragment. Figure 8 is an example of the four nested sets that may be produced from a 20 base pair fragment. There are two basic techniques for the production of nested sets, dideoxy sequencing and sequencing by partial chemical degradation.

Dideoxy Sequencing:- (Sanger & Coulson 1975) This technique is similar, in some respects, to that of the Polymerase Chain Reaction in that it relies on the action of DNA polymerase enzymes. These

enzymes function by adding a 5'-mononucleotide to the recessed 3'-OH end of an incomplete DNA molecule thus providing another 3'-OH end for subsequent additions. The complimentary strand dictates which deoxynucleotide must be added. In normal PCR reactions the four different deoxynucleotide triphosphates (dNTP) are provided in the reaction mixture but in a sequencing reaction a proportion of one of the nucleotides is replaced with 2'-3'-dideoxynucleotide triphsosphate which the DNA polymerase will incorporate into the growing DNA molecule but which does not provide a 3'-OH end for subsequent extension of that fragment. Because the ddNTP is only present as a proportion of its corresponding dNTP fragments will be produced that represent each of the possible stop positions for the target nucleotide. If four reactions are carried out in parallel, one for each nucleotide, then a completely informative collection of nested sets are produced.

Partial Chemical Degradation:- This method for the preparation of nested sets was developed by Maxam & Gilbert (1977) and is used less frequently than the dideoxy method. The technique relies on chemical reactions that specifically alter the purine and pyrimidine bases. The extent of the reaction is limited to less than one base per molecule. Modified bases are destroyed under conditions that preserve the glycoside bonds between unmodified bases. When the products of these reactions are visualised they represent a negative of that produced by the dideoxy process; thus bands represent the absence of a target base.

Figure 8: *Examples of four nested sets that may be produced from a 20 base pair fragment from the beginning of the ITS1 of Rhododendron yedoense.*

Original fragment	TTTCCGTAGGTGAACCTGCG
T nested set	T
	TT
	TTT
	TTTCCGT
	TTTCCGTAGGT
	TTTCCGTAGGTGAACCT
A nested set	TTTCCGTA
	TTTCCGTAGGTGA
	TTTCCGTAGGTGAA
C nested set	TTTC
	TTTCC
	TTTCCGTAGGTGAAC
	TTTCCGTAGGTGAACC
	TTTCCGTAGGTGAACCTGC
G nested set	TTTCCG
	TTTCCGTAG
	TTTCCGTAGG
	TTTCCGTAGGTG
	TTTCCGTAGGTGAACCTG
	TTTCCGTAGGTGAACCTGCG

Once nested sets have been produced they must be fractionated by size and detected. Fractionation may be problematic as fragments may vary in size by only a single base pair, this may represent only a portion of one percent of the length of the fragment. Each fragment is also only present in very small (picogram) amounts and so detection is critical.

Fractionation:- Denaturing polyacrylamide slab gels are almost universally used as the method of fractionation of nested sets although the use of capillary electrophoresis columns is becoming established.

Detection:- There are two possible approaches to detection of fractionated nested sets. In post-electrophoresis methods the polyacrylamide gel is fixed after a set run time and the positions of the fragments detected either with the use of radio isotopes or chemiluminescence. These methods have been the most popular until recently typically using labeled nucleotide analogs to label the

fragments and then visualising them by exposing on photographic emulsions.

Real time detection methods rely on the fragments being detected during electrophoresis as they pass a detector positioned on the gel, typically using florescent labeling techniques. The real-time detection methods have made it possible to automate the fractionation/detection phases of the sequencing process. There are three possible approaches to the production and fluorescent detection of nested sets in real time .

Ansorge *et al* (1986) proposed a method whereby the primer is labeled with dye and four separate sequencing reactions are carried out in order to produce the four nested sets. The products of each of these reactions is then run in a separate lane on the gel. Smith *et al* (1986) proposed the use of four primers each tagged with a different colour dye. The products of the four separate sequencing reactions are then combined and run on a single lane of the gel. This has the advantage of increasing the number of samples that can be run on a single gel by a factor of four. Another approach was proposed by Prober *et al* (1987) in which instead of labeling the primer four differently labeled ddNTP were used. This method had a number of advantages. It enabled the four different nested sets to be produced in a single reaction tube, it reduced noise because chain termination events not caused by presence of the target nucleotide are not labeled and it removed the need to produce specific primers for each region to be sampled. The problem with this system is the uncertainty concerning the efficiency with which DNA polymerase enzymes will incorporate precursors that have been modified both to terminate the chain and to carry a labeling dye.

Marker Characteristics:

Base substitutions in sequences are codominant markers but heterozygosities may not always be detected when consensus sequences are being made directly from PCR products, especially when base pairs are called automatically. These problems may be compounded by copy errors made by the polymerase enzymes either at the initial amplification stage or during the sequencing reactions. Heterozygosity can be more readily detected if fragments are cloned and a number of clones sequenced but this is time consuming and costly. Insertion/deletion mutations may, however, make it difficult or impossible to produce consensus sequences forcing a cloning step. In practice, sequencing markers are therefore treated as dominant unless there is reason to suspect heterozygosity in which case further investigation will be required.

Assessment of criteria for good taxonomic data:

Selective: Sequence data will only be selective if the correct region is sequenced. This is discussed more fully in the practical section on sequencing. Unless great care is taken and control experiments carried out base pair substitutions should be treated as codominant markers.

Homologous: The homology of sequence data can be clear, provided problems are not encountered with multicopy genes, pseudo-genes or heterozygosity. Clearly, examining specific genomic regions has an advantage over anonymous marker techniques (such as RAPD and AFLP) in that the genes should have a single phylogeny that can then be interpreted in terms of species evolution (Doyle 1992). Problems may be encountered if the base substitution rates are too high to allow unambiguous alignments to be made, this is especially so in non-coding regions. These matters aside sequencing appears to offer the most reliably homologous taxonomic data of the techniques surveyed here.

Independent: Taxonomic studies frequently treat base-pair substitutions as independent characters (e.g. Johnson & Soltis 1994, Steele & Vilgalys 1994 and Johnson & Soltis 1995). This is an assumption that may not be valid. It is clearly not the case in coding regions and in non coding regions secondary structure may be of great importance, even in those areas that are not transcribed. Insertion/deletion events of greater than a single base pair in length may have to be scored separately to the rest of the sequence data; individual indels may be the result of single evolutionary events and should not be weighted above base-pair substitutions.

Verifiable: The data produced by this technique is digital in nature and can easily be transferred from one study to another. The same data should be obtained for the same organism in different studies whether they are separated in time or space (c.f. RAPD). Errors do occur in completed sequences, however, and an estimation of error should be taken into account during subsequent analyses. This rarely occurs in the literature.

Available: As with other direct PCR-based techniques sequencing has the advantage of requiring very small amounts of DNA and, as only one or a few successful PCR reactions are required from each extraction, small crude extractions will often suffice. If direct sequencing of the PCR product is possible taxa may be rapidly scored for a large number of characters. There are, however, a number of factors that may restrict this. Production of sequencing reactions and visualisation of products is a complex procedure during which there are numerous opportunities for error. (These can be overcome to some extent by automation.) If the region to be sequenced is present in multiple copies and these copies differ significantly, through the occurrence of pseudo-genes or heterozygosity, then it may be necessary to clone the fragments and sequence a number of clones with the attendant increases in cost, time and error factors. Increases in the consumption of resources are likely to lead to fewer taxa being sequenced and an overall decrease in utility of the technique. Further problems may be encountered in selecting a suitable region to sequence and in finding conserved primer to amplify this region. If substitution rates are too high or too low or if there have been numerous insertion/deletion events then the chosen region may be inappropriate leading to a further waste of resources.

Application to current study

The study group contains around 300 putative species. To sequence a nuclear and organellar region from all of these species, or even from a hundred exemplar species is currently a prohibitive task (c.f. Chase *et al* 1993) although sequencing technology is rapidly advancing and it is likely that this kind approach will be feasible in the foreseeable future. It may be more practical to use a sequencing approach combined with PCR-RFLP or Dideoxy fingerprinting. A sequencing approach was adopted in this study and is outlined later.

Associated techniques

If rates of variation in a target region are low it is possible to increase the speed of screening by only visualising one of the four nested sets. This techniques, **dideoxy fingerprinting**, has an advantage over normal sequencing in that it can increase throughput by four fold. It has the disadvantages of not having 100% resolution (changes not involving the visualised base will not be seen) and polymorphic markers not being demonstrably homologous (when a band appears or disappears it is not known with which of the other three bases the swap was made). A variation on the technique that goes some way to overcoming this problem, by combining the screening with an SSCP analysis (see page 44.), has been suggested (Haavik *et al* 1996) but is unlikely to become widely used as sequencing technology becomes more automated. Dideoxy fingerprinting was used in this study and is discussed later.

Simple Sequence Repeats (SSR)

Tautz & Renz (1984) established that simple sequences are ubiquitous repetitive components of eukaryotic genomes. SSRs (Simple Sequence Repeats, also referred to a Variable Number Tandem Repeats and microsatellites) are a subset of this repetitive DNA consisting of tandemly repeated motifs of less than six base pairs in length (Tautz 1989). It has been shown, through the use of the polymerase chain reaction (PCR), that the length of these sites is highly variable and that they can therefore be used as highly polymorphic, codominant DNA markers. These markers have been used widely in human genetics (e.g. Weber & May 1989) and animal population genetics. Their use in plants is still rare and somewhat tentative. (Akkaya *et al* 1992, Morgante & Oliveri 1993, Kung-

Sheng & Tanksley 1993, Saghai-Maroof *et al* 1994, Powell *et al* 1995a & 1995b).

SSRs have advantages over most conventional molecular markers.

Assessment of criteria for good taxonomic data:

Selective: Unlike RAPD and AFLP markers SSRs are codominant and so are more suitable for producing estimates of F_{ST}, F_{IS} and F_{IT} or even measures of genetic distance (Clark and Lanigan 1993). They are more informative than unmapped RFLP markers, (both alleles always being visible) and more efficient to produce than mapped RFLP markers in unknown genomes.

Homologous: Loci amplified by 5LR primers are considered to be homologous. It is assumed that homoplasious regions bounded by identical primer sites are rare and that where they do occur they are easily detected. The homology of alleles is another matter. Length variation in microsatellites are the result of little understood slippage and looping events at replication. As these events occur independently it is possible to arrive at the same length (i.e. allele) by a number of different routes. Hypotheses of allele homology therefore have to be made with great caution but can be aided by making assumptions as to the mechanisms of length change such as assuming small changes are more likely than large (Slatkin 1995 & 1996).

Independent: SSRs are thought to be independent and frequently presumed not to have a function. Length variation in a human SSR has been shown to be responsible for a genetic disorder however.

Verifiable: There are a number of internal controls within a microsatellite survey. Nuclear loci are expected to possess pairs of alleles in diploid individuals for example. These markers are also robust, slight variations in PCR conditions or in non 3' primer sequences are not expected to produce dramatically different results.

Available: The major drawback of SSRs is the need to generate a set of PCR primers for the study organism. This is typically done by producing an enriched library of genomic clones and probing this library with known simple sequence repeats. The clones identified as possessing SSRs in this screening are then sequenced and primers designed to amplify this region in the genomic DNA (Edwards *et al* 1996). Each locus amplified in this way must then be screened to see if it is variable within the study group.

Application to current study

At the beginning of the study no microsatellite libraries were available for *Rhododendron* and the skills or facilities were not available to build one. Towards the end of the project a library was prepared for use in the 'Azalea' study. This library has not been tested in subgenus *Hymenanthes*. The nature of these markers is such that they are not ideally suited to reconstruction of phylogenies unless this is carried out as part of a population sampling study (c.f. Felsenstein 1985)

Physical Measures of Polymorphisms between DNA Molecules

There are methods of directly screening for mutations at specific loci amplified by PCR. These rely on the behavior of the DNA molecule under electrophoresis and are dealt with together here. These techniques have been reviewed in more detail by Lessa & Applebaum (1993)

Single Stranded Confirmation Polymorphisms (SSCP): This is a simple technique for detecting sequence variation at given loci pioneered by Orita *et al* (1989). PCR amplified fragments are denatured at high temperature in formamide or NaOH before being snap cooled and run on a native (i.e. non-denaturing) polyacrylamide electrophoresis gel. Slight differences in the sequence of the resulting single strands cause them to form different secondary structures and so to migrate at different rates.

Heteroduplex analysis: If an individual is heterozygous at a particular locus and the PCR products

from that locus are denatured and then allowed to renature four different products will be formed: the original homoduplex alleles (AA' and BB') plus two different heteroduplex combinations of strands (BA' and AB'). These four products will have slightly different structures and so slightly different mobilities in electrophoresis. Hetereoduplex reactions are typically run over a long period on high sieving polyacrylamide gels.

Denaturing Gradient Gel Electrophoresis (DGGE): If double stranded DNA is run on in an electrophoresis gel in which there is a steadily increasing concentration of a denaturant, such as urea, a point will be reached at which the concentration of the denaturant is high enough to cause the molecule to melt (i.e. denature). At this point the fragment's effective size increases dramatically causing a spectacular reduction in its migration rate in relation to molecules of similar molecular weight and charge that are not melting. The melting point of the molecule is dependent on its sequence, thus fragments of the same molecular weight but of different sequences can be separated (Myres *et al* 1986).

Temperature Gradient Gel Electrophoresis (TGGE): This technique is similar in nature to DGGE but relies on a temperature gradient being maintained across the gel as the denaturing agent. (Wartell *et al* 1990) This requires specialist equipment that is difficult to construct and is not currently manufactured. It is not therefore currently a practical tool.

Assessment of criteria for good taxonomic data:

Selective: As with the sequence data the selectivity of these techniques depends very much on the level of variation in the chosen PCR fragment. They all have the potential to produce selective markers and have the strength of not only being able to identify heterozygotes but also give some indication of which alleles are present within the heterozygote.

Homologous: Bands that migrate similar distances with these methods may have some physical property in common but could be composed of fragments with different sequences. Care should be taken in assuming that bands are totally homologous.

Independent: As with sequence data the independence of the sequence of a fragment must depend on a large number of factors and should be treated with caution. In addition, conserved secondary structures may play a role in the structures produced by molecules during electrophoresis and so effect migration patterns.

Verifiable: These methods are highly verifiable in that bands may be excised from the gels and sequenced or cloned from the PCR product and sequenced.

Available: All these methods require a period of optimisation and some require specialist equipment and are technically demanding. Bearing in mind the problems associated with homology of bands they are best used in conjunction with a sequencing study so that the identity of fragments may be established and a series of standards produced.

Application to the Current study.

This study is at an early stage in the molecular analysis of the group and it was felt that no fragments well enough known for one of these techniques to be used. They may, however, prove useful in the future, see discussion on sequencing results.

Chapter Summary

- The major molecular techniques were reviewed.
- Each technique was assess using the SHIVA criteria established in chapter 2.
- The applicability of the techniques to the current study was discussed.

CHAPTER 4: CONVENTIONAL CHARACTERS

Detailed descriptions of both *Rhododendron* biology and of the taxonomic characters used are pertinent to the present study. As these two items are closely associated it was felt that they should be combined within a single chapter. This chapter therefore takes the form of a descriptive tour of the genus from its geography to a brief description of its chromosome number with particular attention being paid to subgenus *Hymenanthes* and the selection of characters for scoring and subsequent analysis. Sampling procedures, analysis and re-scoring of the data are outlined in the next chapter along with a summary of all the characters used. The same criteria used in assessing the quality of molecular markers as good taxonomic characters are applied here (see "Summary: Five criteria for accessing taxonomic data" above) although not as explicitly because of the range and number of characters available.

GEOGRAPHY

The genus Rhododendron is almost entirely restricted to the northern hemisphere with its largest centres of endemism in southeastern Asia (at the eastern end of the Himalaya) and in southern Asia, (particularly New Guinea). It is considered a good example of a genus with massive center of endemism because more than half the species are restricted to what amounts to only part of a floristic region (Takhtajan 1986). *Rhododendron* has a degree of specific endemism possibly unique for a genus of its size (Good 1974).

From these centres the genus spreads to northeastern Asia, North America and Europe. A full treatment of the current distribution of the genus, to BRU level four (Hollis & Brummit 1992), is given by Chamberlain *et al* (1996) and for subgenus *Hymenanthes* in Appendix B. The distribution is consistent with the genus having an origin on the Laurasian super-continent after it had been separated from Gondwanaland by the Tethys sea at the end of the Triassic Period, around 200 million years ago. Members of the genus are widespread in all areas that formerly belonged to that land mass (North America, Europe and Asia) but scarce or absent in all areas that were formed from the Gondwanan land mass (principally Central and South America, Africa Polynesia, Australia). Two species of section *Vireya* (*R. lochiae* F.Muell. and *R. notiale* Craven),are found as far South as Northern Queensland but it is felt that these are migrants from the Malesian Archipelago (Leppik 1974, Hutchinson 1947). The Indian peninsula was formerly part of Gondwanaland but migrated North to make contact with Asia. Rhododendrons are restricted to the North in this area although it is directly adjacent to the largest centre of endemism for the genus, the eastern Himialaya. These observations are further supported by the distribution of heteroecious rust fungi that closely link the distributions of *Rhododendron* and *Picea* and *Tsuga* (Leppik 1975).

Subgenus *Hymenanthes* is an almost totally temperate genus with the majority of species occurring in The Chinese provinces of Yunnan and Sechuan and in eastern Tibet (Xizang). Table 3 is a summary of the distribution of species by Biological Recording Unit (Hollis & Brummitt 1992).

Table 3: *Distribution of subgenus Hymenanthes by BRU (Data from Royal Botanic Garden Edinburgh database November 1996)*

BRU Code	Region	No. of Species
CHC-YU	Yunnan	210
CHT-XI	Xizang	150
CHC-SI	Sichuan	143
BMA-OO	Burma	72
CHC-GU	Guizhou	43
ASS-AP	Arunachal Pradesh	39
BHU-BH	Bhutan	36
CHS-GX	Guangxi	29

BRU Code	Region	No. of Species
BHU-SI	Sikkim	25
NEP-OO	Nepal	22
CHS-HA	Hunan	17
JAP-OO	Japan	17
CHN-GA	Gansu	15
IND-WB	West Bengal	15
CHC-HU	Hubei	14
CHN-SA	Shaanxi	10
CHS-AN	Anhui	8
CHS-GD	Guangdong	7
CHS-JX	Jiangxi	7
ASS-MA	Manipur	6
CHT-QI	Qinghai	6
CHS-ZH	Zhejiang	5
IND-UP	Uttar Pradesh	5
CHS-FU	Fujian	4
IND-HP	Himachal Pradesh	4
JMK-OO	Jammu-Kashmir	4
TCS-GR	Gruziya	4
AMU-OO	Amur	3
ASS-AS	Assam	3
ASS-ME	Meghalaya	3
CHS-HN	Henan	3
KOR-SK	South Korea	3
SUM-OO	Sumatra	3
ALT-OO	Altay	2
CHH-OO	Hainan	2
CHM-JI	Jilin	2
CHS-HK	Hong Kong	2
CTA-OO	Chita	2
IND-TN	Tamil Nadu	2
KAM-OO	Kamchatka	2
KHA-OO	Khabarovsk	2
KOR-NK	North Korea	2
KRA-OO	Krasnoyarsk	2
KUR-OO	Kuril Is	2
PRM-OO	Primorye	2
SRL-OO	Sri Lanka	2
TCS-AB	Abkhasiya	2
TCS-AR	Armeniya	2
BRC-OO	British Columbia	1
BUL-OO	Bulgaria	1
CHS-JS	Jiangsu	1
LAO-OO	Laos	1
LBS-LB	Lebanon	1
MLY-PM	Peninsular Malaysia	1
NBR-OO	New Brunswick	1
NSC-OO	Nova Scotia	1
POR-OO	Portugal	1
SPA-SP	Spain	1

The reasons for the high level of species diversity in the eastern Himalaya are uncertain but could be due to the fragmented mountainous terrain and the numerous geological events that have occurred in recent history combined with the breeding system of these plants (see below). The dry valleys of this region are hostile to *Rhododendron* with few if any species being recorded at lower elevations. At higher altitudes, where there is more year round moisture, there is stratification of species into relatively narrow altitudinal bands all the way to the permanent snow line (pers. obs.). Higher altitude areas may act as a mosaic of islands; species from these different islands being brought into contact with each other as the result of climatic, geological and chance events. (see Figure 9A, B & C) At the moment these hypotheses must be treated as conjecture as the sampling of the area is far from adequate.

The Fossil Record

Modern Rhododendrons typically grow at high altitude, in areas of erosion rather than of deposition. If this has always been the case then it would explain the relatively small number of macro fossils that have been reported. The large sticky pollen of these entomophilous plants occur rarely in the pollen record. Leppik (1974) found just 40 macro fossil records in the Compendium Index of the Paleobotanical Library at the Smithsonian Institute in Washington. (summarised below) The majority of these records are from Japan, Europe and North America. Undoutedly fossils exist in China but they are not currently represented in western literature.

Table 4: *Fossils Mentioned in Leppik (1975)*

Period	Specimens
Late Cretaceous	1 species
	A doubtful record from the former USSR
Eocene	5 species
	A number of doubtful records from Europe.
Oligocene	3 species
	2 Europe, 1 North America.
Miocene	13 species
	5 Europe, 6 North America 1 Japan.
Pliocene	6 species
	3 North America, 1 Japan, 1 Europe, 1 former USSR.
Pleistocene	9 species
	5 Europe, 4 Japan
Quaternary	2 species
	1 North America, 1 Europe.

Morphology

Habit and Branching

There are two modes of branching in mature Rhododendrons; sympodial, in which the inflorescence is borne terminally and the following years shoots emerge from the buds that subtend the inflorescence and monopodial in which the inflorescences are borne laterally and subsequent years growth continue along the axis of the branch. Monopodial growth only predominates in subgenus *Azaleastrum* and acts as a autapomorphic character for that subgenus. (Kron & Judd 1990). Sympodial growth is found in all other members of the tribe Rhodoreae including all members of subgenus *Hymenanthes* although it has been suggested that in some species the main axis may continue to grow monopodially leading to an arboraceous habit (Philipson 1985). This trait has not been observed in this study and it is felt that arborescense in *Hymenanthes* is caused by one of the sub-inflorescence buds becoming dominant over the others. There are, however, distinct growth forms within the subgenus, some species being creeping shrubs whilst others are medium to

large trees. Although very variable there must be some genetic component to this trait as even when grown under similar conditions in cultivation all Rhododendrons do not adopt the same habit. It is therefore desirable to attempt to score this character for analysis.

Nodes

The nodal anatomy of Rhododendron has been examined in some detail by Philipson & Philipson (1968) who looked at the leaf trace anatomy of some 264 different species. They recognised five patterns of vascular traces.

1. **Simple Unilacunar**. A single trace departs the stele leaving a single gap. found in 55% of all the species examined including all of subgenera *Rhododendron*, *Pentanthera* and *Tsutsusi*.

2. **Intermediate**. The leaf trace arises from a single gap in the stele and consists of a number of bundles. Accessory bundles arise close to the abscission layer and then turn and run along the petiole. This type is very similar to the complex types listed below but the authors considered it a separate type on the grounds that separate portions of the central trace do not diverge. This type is only found in subgenus *Azaleastrum* section *Choniastrum*.

3. **Three-trace Unilacunar**. A three stranded leaf trace arises from a single gap in the stele. This type is only found in the leaf bracts of a *R. camtschaticum.*

4. **Complex Unilacunar**. A number of strands leave the margin of a single gap in the stele and splay out as they pass through the abscission layer. Accessory bundles arise on the leaf side of this layer and run parallel to it before turning along the petiole. This type was observed in all subdivisions of subgenus *Hymenanthes* examined. None of the first three types of bud were encountered in this subgenus. Considerable variation on this basic pattern was found both between and within species.

5 **Complex Trilacunar**. Similar to the complex unilacunar type but with the accessory bundles arising directly from the stele and leaving their own gaps. This type only occurs in subgenus *Hymenanthes* notably in all species examined in subsection Grandia and some species of section Barbata, Falconera, Fortunea, Lactea, Thomsonia and Campanulata. Within any plant this type was often found with the Complex Unilacunar type or as an intermediate form.

The complex nodal types four and five were found in every member of *Hymenanthes* examined and in no other member of the genus (perhaps with the exception of section *Choniastrum*). This is therefore an autapomorphy for the subgenus. The fact that the complex type can be divided into two broad categories suggests that it may be a useful source of data within the study group but on closer examination this does not appear to be the case. Philipson & Philipson (1968) report that these two nodal types may occur together in different species of the same taxonomic group, in different plants of the same species or even in different nodes of the same shoot. Occurring in more than one state in an individual breaks the second test of homology mentioned above. Intermediate forms also occur. Separating the two types outlined by these authors as taxonomic characters for this study does not, therefore meet the criteria listed above and so they are not adopted as characters here. They may, however, be of use in future studies at lower taxonomic levels.

Buds

There have been numerous studies of bud morphology and biochemistry principally through the interest of horticulturists in breeding for hardiness (Sinclair 1937, Badola and Paliwal 1987, Badola *et al* 1987, Foster 1937). There are two significant taxonomic characters associated with vegetative and floral buds, these are the rolling of the leaf before bud break and the persistence of bud scales.

The leaves of all Rhododendrons are revolute in bud with the exception of subgenus *Rhododendron* which has imbricate leaves (Sinclair 1937). This character is therefore an autapomorphy for that subgenus along with its unique trichome type. No variation of vernation was found in the literature for *Hymenanthes* and none observed in the few species examined. It was therefore discarded as a

potential character.

Perulae/Cataphylls

The term 'perulae' has been used in two different senses in *Rhododendron*. Chamberlain (1982) defined them as scales of vegetative buds whilst Sleumer (Philipson 1985) used the term to describe the scales of flowering buds. It appears that Chamberlain (1982) has the more orthodox usage (see Stern 1983 and Daydon Jackson 1949), however, as this term has caused some confusion it will not be used here. Argent (pers com 1996) has suggested the use of the term cataphyll (the early leaf-forms of a plant or shoot such as bud scales and rhizome scales.) as used by Copeland (1943). These structures are ubiquitous in the group (see Figure 9F) forming intermediaries between true bud scales and leaves and occurring both in vegetative and floral buds. The only clear cut variability that has been observed in the study group is the longevity of these structure in vegetative shoots. In most species they are shed within one year of bud break but in several species, for example *R. forrestii*, they persist for a number of years. This characteristic has been used taxonomically before (Chamberlain 1982) and so was scored as a character here. A correlation between internode length and persistent cataphylls was noted and there is a possibility that this trait is governed by the slow growth of alpine plants rather than purely genetically.

Leaves

Rhododendrons possess 'normal' dorsiventral leaves. The upper epidermis may consist of one or two layers of cells and is often coated with a thick cuticle. This layer entirely lacks stomata. The mesophyll is very variable with the number, size and shape of cells within the palisade layer being variable within and between species. The spongy mesophyll may be dense or lax and may or may not contain large, thin wall water storage cells (Hayes *et al* 1951). This layer is often interrupted by reinforcing girders of sclerose cells. The lower epidermis is typically single celled and densely coated with stomata which are borne on raised mounds in *Hymenanthes* but typically level with the surface in the other subgenera, notably subgenus *Rhododendron*. The lower surface is often papillate.

The leaves may be evergreen or deciduous, leathery or papery in texture and are arranged in alternately or in pseudo-whorls. (see introduction for the distribution of these characters within the genus). All species of subgenus *Hymenanthes* have evergreen, more or less leathery, alternate leaves.

Bocher (1981) carried out a survey of evolutionary trends within leaf structure for the whole of the Ericales but did not deal specifically with *Rhododendron*. Copeland (1943) surveyed the anatomy of the Rhododendroideae including the genus *Rhododendron* (although as a number of separate genera) but failed to identify characteristics that may be of taxonomic use within *Hymenanthes*. Hayes *et al* (1951), however, produced a fairly detailed survey of the leaf anatomy of *Rhododendron* covering 587 species for five characters that they believed would be of taxonomic importance. These characters were:

1. Number of layers of cells in the upper epidermis.

2. The relative size of the cells in the different dermal layers.

3. The thickness of the cuticle in relation to the depth of the outer cells of the dermal layer.

4. The presence or absence of water tissue.

5. The presence or absence of papillae.

Their conclusion was that leaf anatomy is not a major criterion in the broad classification of the genus but may be useful in dealing with specific species and series delimitation problems. Indeed, it appeared that species that were widely accepted as being very closely allied were rarely homogeneous for the five characters surveyed and characters often varied within species. Hayes *et*

al (1951) published their entire data matrix and an attempt was made to include this in the morphological analysis. Unfortunately none of the specimens used in the study had been vouchered and so conformation of the identity of the samples was not possible. When it was found that the scoring of papillae was different from that observed in the material at Edinburgh the attempt was abandoned.

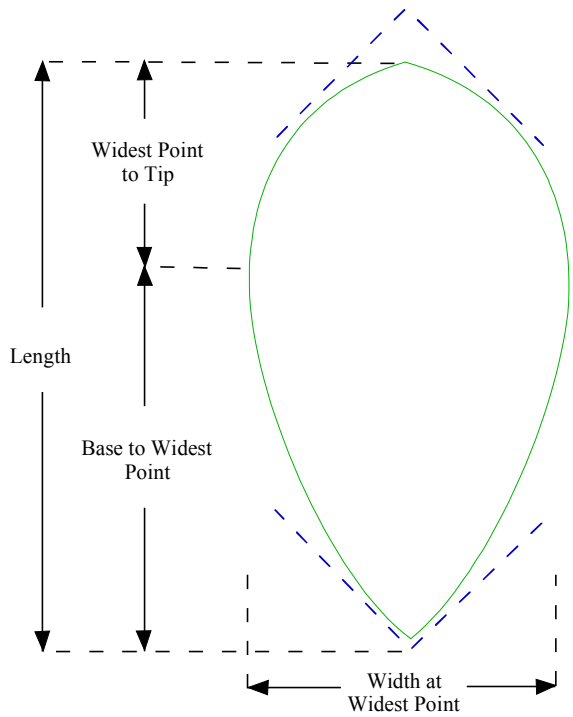
Plate 3 Characters

Figure 9: *A colour plate illustrating habitats and characters.*

A) The dry sides of Mekong valley: Approximately 28° 15' North 2,000m altitude.
B) Steep valley sides of Lungdre river. 4 miles East of previous figure 2,200m altitude.
C) Moist areas at higher altitude: Base of the Dokar La Pass, 4,000m altitude.
D) Large calyx of *R. adenogynum.*
E) Small calyx and of glandular hairs of a *R. cititriniflorum* hybrid.
F) Cataphylls of *R. irroratum*.
G) Cross section through red spot of the yellow flower of *R. wardii* (after Spethmann 1980).
H) Yellow flower colouring of *R.* 'Mrs John Millais' (after Spethmann 1980).

Leaf shape can be very elastic (one only needs to examine the shape of the leaves of *Hedera helix* to see how much variation on a theme is possible). Despite this leaf shape is frequently used in plant descriptions and keys and some measure of the size and shapes of the leaves is given in all the descriptions in the most recent *Rhododendron* monographs. Chamberlain (1982) uses the following adjectives to describe leaf shape in the exemplar species of *Hymenanthes*, lanceolate, oblanceolate, elliptic, oblong, linear, ovate, obovate, orbicular and oval. These words are often used with broad(ly) and narrow(ly) or with the prefix sub- and strung together with conjunctions and hyphens to convey an overall impression of the variation of leaf shape in the species. Thus the leaves of *R. campylocarpum* are "broadly ovate-elliptic or orbicular". Chamberlain also uses the terms acute, acuminate, apiculate and rounded to describe leaf apex shape and cuneate, rounded and cordate to describe leaf base shape. He gives the range of leaf lengths encountered in the species as well as a leaf length to width ratio. Although these are useful descriptive devices they are difficult to use in a comparative sense and in the design characters that meet the criteria of verifiability or availability. An alternative, simplified system was therefore devised that could be scored reasonably objectively. This system is summarised in Figure 10. Leaf length and leaf length-width ratio were maintained but a new measure was added, the distance from the base to the widest point. (and by default the distance from the widest point to the tip) This measure gives some indication of whether the leaf is ovate (having the widest point closer to the base of the leaf) obovate (having the widest point closer to the tip of the leaf.) or elliptic (in the botanical sense, with the widest point at the centre of the leaf). For leaves that tended towards the oblong and linear the centre of the widest portion of the leaf was taken as being the widest point. The terms used to described the tip and base of the leaf were also simplified. Both tip and base were scored as either acute (the edges meeting at an angle of less than 90 degrees) or obtuse (the edges meeting at an angle of greater than 90 degrees). In addition, some leaf bases were scored as cordate if the angle subtended was greater that 180 degrees. The apiculus (in the form of a slightly extended midrib), present in some of the obtuse-tipped leaves was found to be present to a greater or lesser extent in all leaves, though not so apparent in the acute tipped specimens. Although serving a useful descriptive function it was of no use in a comparative sense and so was not used in the study. This same structure may be of taxonomic importance in other sections of *Rhododendron* however, notably in *Vireya* where it is glandular in some species. (*pers. obs.*)

Figure 10: *Leaf shape metrics.*



Clearly there are other ways of describing shape in biology as is illustrated by the work on diatoms (Droop 1994) but these are not practical given the size of the current study.

A number of species have revolute margins in fully expanded leaves. This is especially true in those with densely hairy indumenta. In the specimens examined there seemed to be a clear delimitation between those with and without this feature and so it was scored as a potentially useful character;

bearing in mind that it may have a functional link with the density of the lower indumentum. Where revolute margins occur in acute tipped species the tip is often described as cucullate. This was considered an additive character, only occurring as a result of having the two other characteristics present, and so was ignored. Wavy margins were also encountered but only in *R. irroratum* within the study group and so were not considered.

Reproductive Biology

Before a description of the inflorescence and fruit is embarked on an overview of the reproductive biology of *Rhododendron* is worthwhile. Rhododendrons produce large numbers of small, wind dispersed seeds, often many thousands from a single pod. Each seed is the result of a single fertilisation event and so it is necessary for a large number of pollen grains to reach the stigma, to germinate and to have their tubes arrive at the ovules. In order for this to occur pollen is released in tetrads (the products of meiosis) and the pollen tetrads are held together by vicin threads so that the entire contents of a single anther chamber may be transported by a single pollination event. Pollen is transported from flower to flower by large insects and, in some cases, birds. The flowers appear to be fairly promiscuous, being pollinated by a wide range of bees (*Bombus*). No formal pollination study is known although there are number of studies that have been carried out into post pollination events. (e.g.Kenrick & Knox 1985, Padrutt *et al* 1992, Rouse & Williams 1985, Williams *et al* 1982, Williams *et al* 1984, Williams *et al* 1986). Stigmas are wet and the pollen tubes able to penetrate the style on mass. All the flowers observed during the study were protandrus thus reducing the likelihood of self-pollination. Anthesis appear to be synchronised within any one plant.

There appear to be few barriers to sexual compatibility within the subgenera of *Rhododendron* and intersubgeneric crosses are also known (Williams *et al* 1985). Commercial breeders experience little difficulty in producing fertile crosses between any members of subgenus *Hymenanthes* they choose and should difficulties be encountered it can usually be overcome by crossing with an intermediate specimen that is fertile with both the parent species (Cox pers. com. 1994). Incompatibility mechanisms within the genus have been studied in detail by Williams and Rouse (1990 & Williams papers cited above) with particular reference to section *Vireya*. The conclusion of their work was that although there are cases in which pollen tube growth is arrested in the style due to incompatible pollination subsystems (Williams *et al* 1982) the majority of infertile crosses may be due to incompatibility of pollen volume and pollen tube length. (Williams & Rouse 1988 & 1990). Species with longer styles producing pollen grains of larger volume and species with shorter styles producing smaller pollen. By carrying out a number of interspecific crosses it was shown that the pollen tubes from larger grains would over shoot the micropyles of species with shorter stigmas and tubes of smaller grains would not reach the micropyles of long styled species. It was concluded that a male/female style length ratio of between 0.2 and 6.0 was required in order cross species pollinations to be successful. In the light of this work an estimate was made of pollen volume and of style length for each species in the study.

Inflorescence/Infrutescence

Rhododendrons bear their flowers in racemes of varying length and flower number. In some species the rachis is much reduced in length so that the inflorescence appears umbelliform. (especially in section *Vireya*) In others the number of flowers is reduced to one (e.g. *R. forrestii*). Characters that have commonly been used in the monographs include, number of flowers, rachis length, pedicel length in flower and pedicel length in fruit. All these characters are accessible and possibly taxonomically useful and so were scored for in the study. It is difficult to assess whether or not they can be considered to behave independently, however, as acting together they affect a number of attributes of the inflorescence and its interactions with pollinators. The density of the inflorescence, for example, is a function of all of them plus the size and shape of the corolla. In exceedingly lax inflorescences the flowers probably act as independent units in attracting pollinators whilst in the more densely packed inflorescence the entire structure acts as a single unit. Likewise when the

inflorescence changes into an infructescence the relative sizes of the different organs may or may not require adaptation for the structure to perform effectively.

In Rhododendron as a whole, racemes may be borne terminally or laterally but those species that have lateral inflorescences are restricted to subgenera *Mumeazalea* and *Azaleastrum*. All species in *Hymenanthes* have terminal inflorescences.

In addition to the gross characteristics of the fully expanded inflorescence mentioned above, close attention was also paid to characters associated with the inflorescence bud scales and their senescence. It was not felt, though, that they exhibited any characteristics that warranted further investigation or scoring for all taxa.

Floral Characters

There are two basic flower types within subgenus *Hymenanthes* those that appear to be close to a bird pollinated flower with a slightly fleshy, five lobed, dark coloured corolla and those that take the form of a promiscuous insect pollinated flower with a five to seven lobed, membranous, pale coloured corolla. There are therefore numerous floral characters of evolutionary significance that will be of use in the morphological study. Detailed work has been published on gynoecium morphology (Palser *et al* 1985) and nectary morphology (Philipson 1985).

Zygomorphy: Recent work has outlined the importance of simple genetic control of zygomorphy in some species (Luo *et al* 1996). Control in *Rhododendron* appears to be somewhat more complex. It could be argued that *Rhododendron* flowers are never truly actinomorphic but all show some degree of bilateral symmetry although in the proportionate sizes of their parts rather in the arrangement or loss of any particular organs as in archetypal zygomorphy of families such as Scrophulariaceae and Labiate (see Figure 2 & Figure 3). Leppik (1974) argues that the flowers of subsections *Falconeri* and *Grande* are radially symmetrical and (having 7-10 corolla lobes and 12-18 stamens) are primitive compared to other rhododendrons that are strictly pentamerous (K(5) C(5) A5 G(5)) and show "a clear tendency for bilateralism or zygomorphism". Little other evidence for bilateralism is quoted but a casual study of the genus as a whole shows that the flowers of subsections *Falconeri* and *Grande* are no more or less bilateral than flowers of some other subsections and that the truly pentamerous flowers such as those in subgenus *Vireya* or of *R. forrestii* may appear symmetrical whilst still remaining bilateral in having up turned or declinate stigmas and stamens of differing lengths. Corolla with greater that five lobes also occur in subsections *Pontica* and *Taliensia.* It is feasible then that the zygomorphism of *Rhododendron* is not under direct genetic control but is the product of a combination of other genes that interact in a complex manner to produce the final result. As this form of zygomorphy also involves no gross structural adaptations it is difficult to assess and is therefore unlikely to be a good taxonomic character. It was felt, though, that some attempt should be made to assess zygomorphy both to test this hypothesis. To this end the maximum and minimum stamen length of the flowers were assessed. This was found to be the only measure that could be used both in fresh material and in a wide range of pressed material. Measurements of corolla shape were not feasible without having fresh or pickled material for all the taxa.

Calyx: The *Rhododendron* calyx is of two types. In the majority of species it is reduced to around 1-2mm in length, occasionally with slightly longer, acute of rounded lobes. These lobe characteristics tending to merge into one another. In a few species the calyx is large, irregular and more or less petaloid. (see Figure 9D & E).

Corolla: The corollas of Rhododendrons are tubular, campanulate, funnel-shaped or, rarely, flat and disc like. Sometimes the corolla tube is long and parallel sided with rotate, spreading lobes. They are typically 5 lobed although some species bear up to 8 lobes. (see discussion above on zygomorphy). The lobes may be shorter or longer than the tube and, depending on the level of zygomorphy, may be of different sizes. In some species the corolla is extended backward to form distinct cups that serve as nectar pouches. This character is useful descriptively but as intermediate

forms with more or less pouched corollas are frequent it is near impossible to score as part of a comparative study (see Figure 3).

From a horticultural point of view corolla colour is one of the most important characteristics of a species. Flowers have a background colour that may be white, yellow, red or any number of shades of pink and purple, in addition they are frequently marked inside with blotches on the upper (adaxial) corolla lobe and at the base. These blotches may also vary in colour from yellow through different shades of red and purple to a very dark, (almost black) red-purple (c.f. Figure 2 & Figure 3). A detailed study of the chemistry and anatomy of these colours was carried out by Spethmann (1987). He concluded that there were nine mechanisms that influence the colours as detailed in Table 5 and illustrated in Figure 9G & H.

Table 5: *Causes of flower colour given by Spethmann (1980)*

Colour	Cause
White	reflection of the visible light by the air enclosed in the mesophyll.
Red to violet	the vacuoles of epidermal cells are coloured by anthocyanins.
Red	weakly coloured mesophyll cells.
Lemon-yellow	the vacuoles of the epidermal cells are coloured by gossypetin.
Yellow-green	chloroplast or early stages of the change to chromoplasts are present in the mesophyll.
Yellow	Gossypetin is present in the epidermis and well developed chromoplasts occur in the mesophyll with an accumulation in the subepidermal layers. (Rare).
Golden Yellow	spindle shaped chromoplasts, well developed in the mesophyll and often the epidermis.
Orange	orange colours only occur if chromoplasts are present in the mesophyll at the same time as anthocyanins are present in the epidermis.
Red-brown to black-red	combination of anthocyanin-coloured epidermis and chloroplasts in the mesophyll.

It can bee seen from the above that the mechanisms controlling flower colour are complex and that simply observations of flower colour are not sufficient. The colours are not likely to be homologous, and if the necessary work is carried out to show that they are then they will no longer be available for a large number of individuals in the study. It is clear that they do not act independently and so are unlikely to have evolved independently.

Spethmann (1980 & 87) combined the information he had gathered in carrying out the survey outlined above with leaf anatomy survey of Hayes *et al* (1951) to produce his classification of the genus. Details of this classification are given in the introduction.

From the point of view of this study a number of characters were derived that gave some measure of the variation in corolla shape. These are outlined in the next chapter.

Androecium: A *Rhododendron* stamen consists of a filament, that may be flattened at its base, and a two lobed anther that dehisces via apical pores. Dehiscence occurs when the septum dividing the two pollen sacs within each lobe of the anther folds and collapses bringing with it the thin plate of tissue which covers the apical pore and so exposing the pollen. As mentioned above, the pollen grains are maintained in tetrads and bound loosely together with viscin threads. The material from which these viscin threads are derived is uncertain but it has been suggested that they are derived from small quantities of protoplasm left in the minute cavities which occur below each germ pore between the extine and intine of the pollen grain (Matthews & Knox 1926).

Observations of the gross morphology of the stamens of subgenus *Hymenanthes* during the course of the project did not reveal any marked characteristics that may be of use in a comparative study.

Often one or more anthers within a flower were much reduced in size but this always appeared to correspond with a reduction in length of the filament which was already being recorded as a character.

One of the most striking features of the ericoid stamen is that the anther is entirely inverted during development. (Matthews & Taylor 1926, and Matthews & Knox 1926). The vascular bundles of the filament are attached to the anther along the abaxial surface before entering the anther body and travelling down to the more pointed base. The pores through which pollen is released are therefore, in fact, basal rather than terminal. The original, derived terminology will be maintained here though to avoid confusion with other works.

Pollen: Pollen has not been used in taxonomic studies of *Rhododendron*, it has not shown significant variability to warrent analysis. Observations carried out during the cause of study confirmed this. The diameter of pollen tetrads was scored as an estimate of the pollen grain volume. Further comments are made in the results section.

Gynaecium: The superior ovary of *Rhododendron* is divided into a number of locules, (typically 5 but occasionally many as 17.). Placentation is axile. Each locule typically contains many ovules, (usually around a thousand although examples of 12, *R. micranthum*, and almost 2000, *R. nuttallii* are known. (Palser *et al* 1985). Towards its apex the ovary either tapers more or less abruptly into the style or more commonly the style arises from a depression in the apex. Tapering ovaries are most commonly observed in section *Vireya* and a few of the subsections of section *Rhododendron*. They have also been reported in *R. auriculata* and in subsection *Neriiflora* in subgenus *Hymenanthes*. Observations made throughout the study failed to identify clear divisions between species with and without tapering ovaries in *Hymenanthes* and so it was not scored for analysis.

Palser *et al* (1985) carried out a detailed study of the ovary, ovule and megagametophyte of the entire genus of *Rhododendron*. They concluded:

> "Although the indumentum types on the ovary show an essentially similar distribution among subgeneric taxa as do those on the leaves, it has not been possible to discern other features, singly or in clusters, which could serve to arrange groups of species in clearly distinguishable subgeneric taxa, whether newly erected ones or those presently recognised on other grounds." (Palser *et al* 1985)

A detailed sectioning study of the ovary was not therefore carried out.

The length of the style has been shown to be an important factor in sexual compatibility mechanisms (see above) and so was scored as was the diameter of the stigma, some species having dramatically larger stigmas than others.

The base of the ovary is surrounded by a ring of nectaries. These nectaries consist of a series of bulges that vary in size and shape between species. The bulges generally protrude between the bases of the filaments and thus vary in number according to the number of stamens. They may perform some role in maintaining the position of the stamens relative to each other. The anatomy of the ovary throughout the genus has been examined by Philipson (1985). She suggested a classification of these nectaries into three types, a rounded bulge (Type A), a broadly-base bulge (Type B) and an only slightly raised type Type C). She proposed that these different ovary types were of some taxonomic importance, (Type A only occurring in subgenus *Rhododendron*, some sections of *Tsutsusi* and section *Viscidula*: Type B only occurring in subgenus *Hymenanthes* and *Mumeazalea*: Type C only occurring in the other 'Azalea' subgenera.) Unfortunately Philipson (1985) does not give an indication of how many species were examined from each taxon and the classification of types appears to be very loose so that it would be hard to expand on her results. Likewise she describes the parenchymatous tissues of the nectaries as of being made up of two types of cell. One type that stains purple or red in safranin and is electron dense in her TEM studies (suggestive of a high phenolic or tanin content) and another that appears clear. These two types of cell are present in

different propotions in different species with a few species containing clear cells exclusively. She concludes that the distribution of the cell types may be of service taxonomically but that they integrade too much in her study to be useful. She notes, though, that nectaries containing the clear type of cell occurred exclusively in subgenus *Hymenanthes*. As with the nectary shape-types it was decided that the definition of types and the lack of clarity concerning the species and number of individuals in which they were found did not provide sufficient evidence for a complete histological survey of the subgenus to be carried out. It may, however, prove useful in future studies.

Some *Rhododendron* flowers are fragrant. Most notable among these being *R. luteum* in subgenus *Pentanthera*. Fragrance in subgenus *Hymenanthes* is rare but does occur. As smell is a highly objective sense a special sampling procedure was adopted and this character scored for analysis.

The nectar of Rhododendrons is a source of food not only for legitimate pollinators but also for a number of nectar raiding animals. These include wasps, small birds and even squirrels (pers obs. RBGE 1993-6). In some species the nectar has been the cause of poisoning (Schaller 1983), notably honey derived from *R. luteum* which poisoned the army of the Roman general Pompey in 67 B.C., possibly effecting the history of the Roman empire (Leach 1975).

Fruit & Seeds

There are two basic fruit types in *Rhododendron*. The majority of the genus posses hard woody fruits that dehisce from the top, the valves spreading into a star shape. Those in section *Vireya* are softer and not as woody, the valves tending to curl as the capsule dehisces. In Hymenanthes the fruit is comparatively uniform varying chiefly in size shape. It may also be straight or curved but this characteristic is highly variable within species and so could not be used for this study. The seeds of *Rhododendron* are small and may bear wing-like hylem that is extended towards the base and/or the tip; this characteristic being particularly notable in section Vireya where the seed bear long tails and wings at both ends. In subgenus *Hymenanthes* the wings are small but variable in shape and sometimes broken into finger-like projections at the tips. The variability in fruit and seed characters was surveyed by Hedegaard (1980a and 1980b) who concluded that although useful in delimiting species there was little taxonomic pattern to the distribution of seed characteristics. For this reason, and because time did not permit, seed characters were not used although they may prove useful in future studies.

Trichomes

Much work has been carried out on the trichomes and papillae in the genus *Rhododendron* and they have been regarded as being of great taxonomic importance. The hair types found in the genus are complex and a potential source of many taxonomic characters. The main division of the genus is on the basis of the those species with scales (lepidote) represented by subgenus *Rhododendron* and those without scales (elepidote). Of the elepidote species subgenus *Hymenanthes* is alone in possessing more complex, branched hairs. Two main systems of classification of hair types have been proposed; Cowan (1950) and Seithe (1980).

The classification of Cowan (1950) recognised 25 different hair types. That of Seithe (1980) is the more finely divided proposing 43 different hair types. She grouped these into two classes; 'secreting' and 'covering'. These two classes are further divided into subclasses; glandular hairs, scales, flock hairs and virgate hairs. Only three of the four possible combination of hair subclass occur.

1) scales and virgate hairs, found exclusively in subgenus *Rhododendron.*

2) glands and flock hairs, found exclusively in subgenus *Hymenanthes*.

3) glands and virgate hairs, found in all other subgenera (the Azalea complex).

She hypothesised that all trichomes within the genus begin development with an anticlinal division of an out growth of an epidermal cell. Multiseriate, straight, long celled trichomes develop from a

subsequent series of oblique periclinal divisions. These trichomes may be gland tipped or eglandular. In scales a second stage of oblique periclinal divisions occurs in which new cells are produced proximally whilst in branched trichomes these same periclinal divisions produce new cells distally. Seithe suggests that the common ancestor of the genus bore the two hair classes and that these are represented in the extant subgenera. From this she proposed the classification of the genus into three chora subgenera, as detailed in the introductory chapter and illustrated in Figure 11. Williams *et al* (1985), however, crossed a lepidote *Vireya* with an elepidote, eglandular *Tsutsusi* and noted that the seedlings bore stalked, round headed glands. This suggests that the situation is not quite as simple as is suggested by Seithe (1980). Kron and Judd (1990) also caste doubt on the interpretation of her results but not on the developmental evidence itself. They treat multicellular, unbranched trichomes as plesiomorphic and scales as derived but do not pass judgment on branched hairs as they are uninformative from the point of view of their particular study. Quite how this evidence is to be interpreted in terms of the overall phylogeny of the genus will probably only become clear when it is combined with other, independent data.

Figure 11: *Summary of phylogeny of chora subgenera and hair class. (Seithe 1980, page 115)*

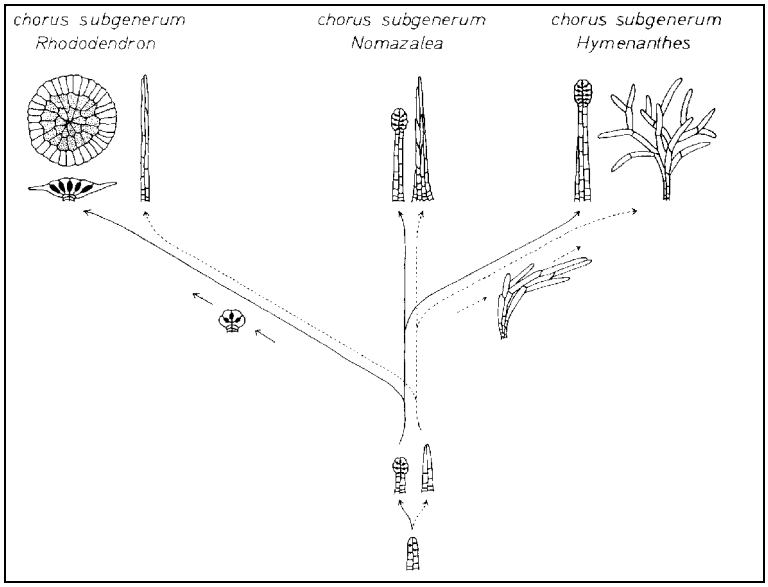


Figure 12: *Hair Types (see next page).*

A - 'Glandular' hair from *R. championae* (x150) after Cowan (1950).
B - Glandular hair from *R. griersonianum* (x150) after Cowan (1950).
C - Glandular hair from *R. fulvoides* (x200) after Cowan (1950).
D - Cup-shaped hair from *R. falconeri* (x100) after Cowan (1950).
E - Papillae from *R. pubescens* (x200) after Cowan (1950).
F - Folioliferous hair from *R. pachytrichum* (x100) after Cowan (1950).
G - Stalk-radiate hair from *R. fulgens* (x125) after Cowan (1950).
H - Ramiform hair from *R. insigne* (x75) after Seithe (1980).
I - Radiate hair from *R. lacteum* (x200) after Cowan (1950).
J - Dendroid hair from *R. bureavii* (x200) after Cowan (1950).

Plate 4 the Hairs one.

No captioning here as it is on the pervious page.

A possible interpretation is that there are three basic genetic traits. One is for the production (or not) of glands, one for the production of a second phase of distal cell division (essentially leading to branched hairs) and one that produces a second phase of proximal cell division(essentially leading to scales). When both traits for scales and glands are present they interact to produce glandular scales but the traits for branches and glands don't interact and so two structures are produced when they are both present. Care is taken here to use the term trait rather than gene as these factors may be the product of numerous discrete genes at many loci.

Within subgenus *Hymenanthes* the two classes of hair are represented by glandular tipped setose hairs, eglandular setose hairs, single celled hairs and numerous, complex forms of branched hair. The occurrence of hair types and their location on the plants has been of great importance for the taxonomy of the group and has been used in all monographic and other descriptions. The question is how to use them as analytical, comparative characters rather than diagnostic descriptors and how to design characters that fit the criteria of being selective, homologous, independent, verifiable and available. If the classification of Seithe (1980) is followed strictly then most species in a sample will tend to posses unique (or autapomorphic) hair types and so the selectivity of the system will be low. The hair type classes are very narrow statements of homology. It is clear that broader statements of homology must be made so that groups of individuals share character states. One solution to this problem could be to use the classification designed by Cowan (1950) but, as can be seen from **Table 6** this system does not agree with that of Seithe (1980) and was designed with no knowledge of the developmental evidence presented there. Another solution would be to use the higher resolution classification for scoring and then, at the analysis stage, experiment with combining the different characters. This would allow for the greatest flexibility. It was found on practical grounds, however, that it was impossible to reliably score actual specimens to Seithe's model. Intermediates appeared to occur between many of the hair types and Seithe's treatment is unclear, in parts, as to the differences between several of the forms. A third classification was therefore developed that took on board the aims of this project as well as the developmental evidence put forward by Seithe (1980) and the pragmatic approach of Cowan (1950). This classification is outlined below and its relationships to the other classifications is shown in Table 6.

Table 6: *Hair type classifications used by Seithe (1980), Cowan (1950) and in this study.*

Seithe (1980)	Cowan (1950)	This Study
1) pearled rosette	radiate hair/ovoid arms, vesicular hair	stalked-radiate
2) horned rosette	radiate hair with pyriform arms	stalked-radiate
3) tubular rayed rosette	radiate hair with sausage-shaped arms	stalked-radiate
4) plate rosette	rosulate hair (pp)	radiate
5) split rosette	rosulate hair (pp)	radiate
6) closed rosette	rosulate hair (pp)	radiate
7) pearled hair	-	radiate
8) horned hair	long-rayed hair (pp)	radiate
9) tentacle hair	ramiform hair (pp); dendroid hair (pp)	dendroid
10) snake hair	ramiform hair (pp)	dendroid
11) funnel hair	funnel-shaped hair; cup-shaped hair	cup-shaped
12) mop hair	capitellate hair	radiate
13) whip hair	-	stalked-radiate
14) stellate hair	stellate hair	stalked-radiate
15) bunchy hair	long-rayed hair (pp)	radiate
16) tuft hair	ramiform hair (pp); dendroid hair (pp)	dendroid
17) penicillate hair	dendroid hair (pp)	dendroid
18) tree hair	dendroid hair (pp)	dendroid
19) branch hair	flagellate hair, folioliferous hair (pp)	dendroid

Seithe (1980)	Cowan (1950)	This Study
20) cluster hair	folioliferous hair (pp)	folioliferous
21) sheaf hair	fasciculate hair	radiate
22) tress hair	dendroid hair (pp), ramiform hair (pp)	dendroid
23) fringed hair	strigose hair (pp)	gland
24) candelabra hair	-	not seen
25) loriform hair	loriform hair	not seen
26) setiform hair	strigose hair (pp); setose hair (pp)	gland
27) ampullate hair	-	not seen
28) articulate hair	-	filament hairs
29) tubiform hair	-	filament hairs
30) filiform hair	filiform; acicular hair	not seen
31) ampullate gland	-	gland (pp)
32) stipitate gland	simple gland	gland (pp)
33) setiform gland	setose glandular hair	gland (pp)
34) dentiform gland	-	gland (pp)
35) globular gland	-	gland (pp)
36) vesicular scale	vesicular scale	not in *Hymenanthes*
37) entire scale	entire scale	not in *Hymenanthes*
38) undulate scale	undulate scale	not in *Hymenanthes*
39) pleated scale	-	not in *Hymenanthes*
40) crenulate scale	crenulate scale	not in *Hymenanthes*
41) lacerate scale	lacerate scale	not in *Hymenanthes*
42) substellate scale	-	not in *Hymenanthes*
43) stellate scale	-	not in *Hymenanthes*

1) Glands: Glandular trichomes appear in a number of forms within the group, ranging from almost sessile pin heads to gland tipped setose hairs several millimetres long. All these structures are assumed to be homologous and included here. In addition to these glandular structures non-glandular setose hairs are also included. It was observed that these structures only occurred in individuals that produced at least some glands and that glands with reduced heads were not uncommon. It was therefore concluded that these were also homologous structures. Seithe's developmental evidence suggests that these trichomes have not undergone a second round of distal or proximil cell division. (Figure 12; A, B and C).

2) Radiate hairs: Seithe(1980) recognises nine hair types which have no apparent stalk but radiate outwards from a central point. Cowan (1950) is less clear but appears to recognise three. For the purposes of the study radiate hairs are defined as those that have single celled branches radiating from a central point that is sessile on the leaf surface or supported by a short stalk that is only one cell wide. Seithe's developmental evidence suggests that these trichomes have not undergone the initial anticlinal cell division or the primary series of oblique periclinal divisions and elongations but gone straight to a second round of distal cell division. (Figure 12; I).

3) Stalked-Radiate hairs: This hair type differs from rosulate in the that although the single celled branches of the hair arise from a central point, that point is raised above the leaf surface by a stalk that is more than one cell thick. Developmentally these can be thought of as having gone through the initial series of oblique periclinal divisions and elongations but having rapidly moved on to the second round of distal cell division. (Figure 12; G).

4**) Dendroid**: This hair type is similar to the stalked-radiate type but differs in having a longer stalk. The stalk must be more than 3 times longer than broad. Developmentally this hair type is similar to the stalked-radiate type but differs in the degree of development of the stalk. It could therefore be thought of as homologous with the former type, but was initially scored separately as it was easy to spot. (Figure 12; J.).

5) Ramiform: All the hair types mentioned so far have a single branching point, although in some species this may be quite broad. The ramiform type consists of a short stalk similar to the that found in the stalked-radiate type but the arms that leave the apex of this stalk may be multicellular and are branched. Developmentally these can be thought of as having gone through the initial series of oblique periclinal divisions and elongations but for these to have become less discriminate. Another explanation may be that traits for both proximil and distal cell division are present. *R strigullosum* appears to have hairs that are both branched, forming a tangled indumentum, and somewhat glandular this is treated as and ambiguity here but may point to further developmental conclusions in the future. (Figure 12; H).

6**) Folioliferous**: This is an aberrant hair type as regards the developmental evidence discussed so far. They resemble dendroid type in that they consist of a distinct stalk and a radiate branches but the cells that make up both the stalk and branches are short and wide and although fused for the greater part of their length are reflexed at the tip. They are easy to spot when scoring specimens and so were scored separately but may be homologous with dendroid and stalked-radiate hairs at some level. (Figure 12; F).

7) **Cup-shaped**: These hairs have a short multicelled stalk and then expand into a large cup or funnel shaped structure. No differentiation is made here as to the exact shape of this structure as is done in Cowan (1950). The pattern of cells appears to be superficially similar to those in ramiform trichomes, the walls of the cells often being made up of somewhat elongate cells. It is unclear how this structure would be produced by the development traits suggested thus far and it is likely that it is the result of a novel trait. As with the ramiform type it may be that traits for both proximal and distal cell division are present. (Figure 12; D).

8) **Filament hairs**: A number of species have single celled hairs adorning the lower portion of the filaments. In some species these hairs also occur on the inner, lower surface of the corolla. They are not discussed by either Cowan (1950) or Seithe (1980) but are mentioned by Leppik (1974) who speculates that they serve to prevent droplets of nectar from leaving the flower or rain water from entering and diluting the nectar. Seithe (1980) refers to simple, single celled hairs (occurring on the leaves of lepidote species) as being derived from more complex structures. She does not put forward evidence in support of this and it is not the view held here. These filament hairs are assumed to be of independent origin.

9) **Papillae:** There is some dispute as to whether papillae should be treated as trichomes or not. For the purposes of this study they are included within the trichome classification. They occur on the lower surface of the leaves in a number of species and are defined as out growths from single cells that are less than twice as long as wide. It could be that these papillae are the early stages of full blown trichomes however they occur in presence of other, more complex, structures and so are treated as if of independent origin. (Figure 12; E).

Topographical Rules: The location and density of the different trichome types on the plant is often of importance for identification of species. These characters are deemed to be of little use from the point of view of comparative studies however as they are likely to be the result of minor genetic changes being influenced by the development of the particular organs. For this reason, in scoring the occurrence of hair types, the primary concern was given to whether the plant had the ability to produce that structure and, although the location of the structure was recorded, it was considered to be of secondary importance and excluded from the initial analysis.

Agglutination: The hairs of the indumenta of some species of subgenus *Hymenanthes* are fused into an agglutinate mass and this is commonly used as a character in identification of species as well as making the study of these indumenta problematic. The cause of this aglutination is obscure but may be the result of the presence of glands on branching hairs. Ascertaining whether this is correct is beyond the scope of this study but may shed light of the development of the different hair types. Agglutinate indumenta are distinct and were scored as a character.

Secondary Metabolites

Much work has been carried out on the chemotaxonomy of *Rhododendron* for example Harborne (1962, 1980 & 1986), Harborne & Williams (1971), Spethmann (1980 & 1987), Arisumi *et al* (1985), Reynolds *et al* (1969), De Loose (1970), King (1975), King (1977) Evans *et al* (1975a and b) and Hu & Xiao (1992) and references cited there in. The two works of most significance to this study are Spethmann (1980), which has been discussed above, and Harborne & Williams (1971) which will be dealt with in more detail here. Other works are either summarised in these papers or make minor additions to them.

Harborne & Williams (1971) surveyed the leaves of 206 species from across the genus for ten secondary metabolites. They believed this to represent one third of the total species. The 10 compounds surveyed were quercetin, gossypetin, kaempferol, myricetin, azaleatin, caryatin, dihydromyricetin, dihydroquercetin, dihydrokaempferol and coumarins. Quercetin was found to be ubiquitous and the occurrence of the other compounds was presented in tabular form. The paper makes a number of conclusions. It is noted that *Rhododendron* retains the whole gamut of primitive flavonoid characters and that this points toward the genus being an ancient relict taxon. Variation of chemical markers within the genus is so varied it may only be of use at the species level (in identifying specific, closely allied groups) although there are some larger trends such as the absence of gossypetin from the whole of subgenus *Pentanthera* and (as confirmed later by Harborne 1986) the whole of section *Vireya*.

The use of secondary metabolites as taxonomic markers is problematic. The presence of the marker is the result of a long chain of events and the loss of that marker may be the result of a break in that chain at any number of different points. Absence of a marker cannot therefore be used as an indication of relationship. Chemical data has additional disadvantages in that it may be possible to produce the same chemical marker via variations in the same (thus weakening the degree of relationship that can be inferred from the shared presence of the compound) and the markers may only be produced in certain organs or tissues of the plant or only at set times during the life cycle or under set environmental stress. Markers may also be derived form each other or by different branches in the same metabolic pathway. Thus for the purposes of phylogenetic reconstruction chemical markers can only be used at a very crude level. Although they are Available, Selective and may be Verifiable (via the use of a number of different detection systems) they do no meet the all important criteria of Homology or Independence.

As the primary interest of this study is the detection of phylogenetic groups it was decided not to dedicate any laboratory time to the detection of secondary metabolite markers. Because the data in Harborne & Williams (1971) was presented in tabular form it was added to the data matrix so that it could be mapped to any results obtained using other data.

Cytology.

The majority of Rhododendrons have the chromosome number 2n=26. (Janaki Ammal & Bridgewater 1950, Jones & Brighton 1972, Li 1957, Nakamura 1931, Sax 1930,) The exceptions to this rule mainly occur in subgenus *Rhododendron* and in the isolated case of *R. canadense* (L.) Torr. (subgenus *Pentanthera*) in western North America. (Nakamura 1931, Li 1957). Within subgenus *Rhododendron* there are several polyploid series known especially around subsection *Maddenia* (Hutch.) Sleumer which reaches dodecaploidy in *R. maddenii* ssp. *crassum* (Franch) Cullen (Janaki Ammal & Bridgewater 1950).

All counts made in subgenus *Hymenanthes* to date (numbering nearly 200) have been 2n=26. (Janaki Ammal & Bridgewater 1950, Jones & Brighton 1972, Li 1957, Nakamura 1931, Sax 1930). It was not felt, therefore, that it would be worthwhile carrying out a cytological study of the subgenus as part of this project as it does not appear likely that a significant amount of variability would be encountered.

Chapter Summary

- The potential sources of conventional taxonomic data were considered both in relation to the biology of *Rhododendron* and in terms of what has been used by other authors.
- Conclusions were reached as to which sources of data would be applicable to the current study.

CHAPTER 5: MORPHOLOGICAL STUDY

Introduction

The previous chapter gave an overview of the sources of conventional data available to the study. This chapter will deal with the sampling procedures used and subsequent analysis and re-scoring of the data. Only brief conclusions will be drawn here. More in depth conclusions will be drawn in the final chapter where different data sets can be compared.

Designing a sampling procedure has been a major concern for the study. A conventional taxonomic approach involves the construction of a data matrix of taxa by characters and the use a series of clustering techniques to make an assessment of the relationships of these taxa. To carry out this procedure it is necessary to have some way of delimiting the taxa and most studies use predifined species as OTUs (Operational Taxonomic Units). As has been illustrated in Chapter 2, this subject is somewhat problematic in subgenus *Hymenanthes* with few of the taxa, whether species or subsections, being easily defined. It is the aim of the study as outlined at the end of Chapter 1, however, to carry out a phylogenetic analysis of the group in order to establish a hierarchy. At the least to look at the relationships of the currently established sections. The difficulties in delimiting study taxa, though, suggests that an alternative, population genetics approach could be taken leading to an analysis of variance between populations. There are a number of reasons why such an analysis is impractical here:

- Sampling within the populations must be random, with each individual standing an equal chance of being sampled. Most of the samples available were collected in association with botanical exploration for horticultural purposes with only a few collections made especially for the study.
- The region in which the majority of the variation occurs is insufficiently known and it is difficult to define the separate populations.
- Information on the study taxa must be collected from diverse data sources (including taxonomic literature) and measures of variance are unlikely to be available for all taxa.
- The analysis presumes that the individuals are of the same species and therefore have the same variance.
- It is not clear how such an analysis would behave between species in such a complex case.

It was concluded that a conventional taxonomic approach should be taken as the ANOVA approach appeared impractical and the phylogenetic approach meets the aims of the project more closely but is was decided that some attempt should be made to estimate the variation that may occur within a species so that at least an intuitive assessment of the value of different characters could be made. It must be stressed that this would necessitate making a large assumption in extrapolating the variation found within a single taxon across a whole study group and so no attempt was made to do this formally.

Materials

The herbarium at Edinburgh (E) contains around 20,000 *Rhododendron* specimens, approximately half of which are from subgenus *Hymenanthes*. During the course of the study the living collections of RBGE contained around 4,000 accessions of *Rhododendron* of which 2,100 were from *Hymenanthes*, 1,800 were of names wild taxa and 1,550 of known wild provenance. In May 1994 field work was carried out in China during which numerous personal observations were made and specimens collected and added to Edinburgh. These materials, combined with the data from the literature, were the resources used in this study.

METHODS

On the basis of Chamberlain (1982), Stevenson (1947), Davidian (1989 & 1992), field observations and personal communication with *Rhododendron* growers a sample of 52 "Exemplar" species were selected. These species were chosen so as to represent the majority of the variation within the subgenus. In addition to these exemplars two species were chosen to act as outgroup species. These were *R. luteum* from subgenus *Pentanthera* and *R. ferrugineum* from subgenus *Rhododendron.*

Selection of Exemplar species

Distance measures and parsimony techniques analyses are highly affected by missing data. A high priority was therefore give to producing complete sets of data for all taxa and all characters, this restricted the number of taxa that could be sampled from the study group to 54; although an initial attempt was made to score a set of one hundred taxa. One species was chosen from each of the smaller subsections and more than one from the larger, more complex subsections. Species for which little material was available (e.g. *R. excelsum*) were excluded along with those that were suspected of being of hybrid origin (e.g. *R. fulvastrum*). Species selected are listed as part of Appendix F.

Delimitation of Exemplar species

Some of the species are quite distinct (e.g. *R. griffithianum*) but many others species hybridise and intergress for at least part of their range and these are more problematic (e.g. *R. decorum*). The only practical approach that could be taken was to use a typological definition to delimiting these species. Using a combination of type specimens, the original descriptions of the species, the monographic descriptions (Chamberlain 1982) and herbarium sheets bearing *determinavit* labels an image of each species was produced. Specimens were then included in the study under this taxon name only if they fell within this description in the majority of their characters. Specimens with aberrant traits were excluded as being of potentially hybrid origin. This is a far from adequate means of delimiting OTUs (see Chapter 2) but was the only one available to the study. A similar approach was taken by Hedegaard (1980a & 1980b) who sampled specimens that closely resembled the type.

Characters Used

The results section gives a catalogue of the characters used and how they were scored. The division of continuously variable characters is also dealt with there. The characters were divided into two groups. Those that were thought to be informative and those that were thought to be of secondary importance and therefore potentially misleading in the analysis. Thus the possession of the different hair types were classed as primary characters but the location of those hair types on the plant were treated as secondary characters. Estimates of flower colour were also treated as secondary characters (see chapter 4).

Scoring of characters

Data was collected directly onto a computer spread sheet. At the beginning of the project this was a Microsoft Works 3 spread sheet but data was transferred to a Microsoft Excel 5 workbook near the end of the study. At the beginning each specimen was given a row on the sheet and each character a column. Descriptions from the literature were included as rows as if they were specimens. As scoring proceeded the columns on the sheet were adapted as definitions of characters were refined. This was particularly so with the hair type characters. When sufficient specimens had been examined for a species the rows were averaged so as to produce a single row of data for each species.

Care was taken that the same structures were measured from specimen to specimen and that these

structures were at the same developmental stage. Mature, fully expanded leaves on the previous years growth were selected. Depending on the specimen, a number of leaves, up to a maximum of five, were measured and the figures averaged for that specimen. Likewise, in fertile specimens a number of fully expanded flowers or fruits on the point of dehiscence were scored. All measures were taken to the nearest millimetre apart from estimates of pollen tetrad diameter and plant height. All specimens at E (approximately 10,000) were examined and between five and fifteen (that met the criteria outlined above) selected to be formally scored for each species. Specimens scored are listed in Appendix C. Where possible living specimens were examined in the field or in cultivation. Measurements were not taken directly from living material but pressed specimens prepared and measurements taken from them so as to be comparable with those observations made in the herbarium.

Observations were made with the naked eye, using a 10X hand lens, at up to 50X with a binocular microscope and up to 400X times with a light microscope. A scanning electron microscope was used to clarify some issues; specimens being taken from suitable herbarium material and directly sputter coated.

The result of the scoring exercise was a 54 x 54 data matrix suitable for analysis. This matrix is given as part of the matrix in Appendix F.

Variation within a taxon

An attempt was made to give some estimate of the variation within a taxon so as to facilitate the division of continuous characters and give some indication of the significance of the inter-taxon variation. In order to accomplish this, a single plant of *R. ponticum* (the same plant used to provide the DNA sample for this species) was selected and the variation for the majority of non-binary characters within this individual recorded. A sample of 50 leaves, 50 flowers, 30 inflorescences and 30 infructescences was made. Statistics of this variation are given in the catalogue of characters below.

Analysis of data

On completion of the date set each of the non-binary characters was analysed to see if it could be divided into a binary or multistate character and to check for any anomalies. The mean, median, mode, standard deviation, standard variance, kurtosis, skewness and range were all calculated. Kurtosis characterises the relative peakedness or flatness of a distribution compared to the normal distribution. Positive kurtosis indicates a relatively peaked distribution. Negative kurtosis indicates a relatively flat distribution. Skewness characterises the degree of asymmetry of a distribution around its mean. Positive skewness indicates a distribution with an asymmetric tail extending towards more positive values. Negative skewness indicates a distribution with an asymmetric tail extending towards more negative values.

The original date set was treated in two ways. It was standardised, (by subtracting the mean and dividing by the standard deviation for each variable) so as to remove the effects of different scales, and it was converted into binary matrices so as to make it suitable for parsimony analysis. Two different methods were used in converting the matrix into a binary form; the first was simply to remove all non-binary characters so as to leave in a much smaller matrix; the second was to convert the continuously variable and meristic characters into binary characters. Because initial analysis of these characters had not provided good reasons for dividing them into ranges (see results and discussion below) they were simply split about their means. It should be noted that there is no strong theoretical justification for doing this and the operation was carried out purely as part of the empirical exploration of the data. The large and small binary matrices were then subjected to parsimony analysis using the PAUP computer program, Swofford 1985. Standardisation and splitting of characters was carried out using the NTSYS package, (Rohlf 1994).

Phenetic analysis: Similarity matrices were generated from the standardised matrix using the

average taxonomic distance coefficient (Sneath and Sokal 1973, page 124 also Rohlf 1994, page 7-12) and the Manhattan distances of (Sneath and Sokal 1973, page 125 also Rohlf 1994, page 7-12) to give the matrices DIST and MAN respectively. Cluster analyses were then done using the Neighbour Joining (NJ) method of Saitou & Nie (1987) and the UPGMA cluster algorithm. The NJ tree was rooted using *R. luteum* as an outgroup. Principle co-ordinates analyses (PCOORDA) of both these matrices were also carried out (Gower 1966) and minimum spanning trees (MST) calculated (Rohlf 1975). The cophenetic correlations between the matrices and the trees were calculated as a measure of goodness of fit (Rohlf & Fisher 1968). This involved producing a cophenetic matrix from the tree and then a Mantel Test (Mantel 1967) between the cophenetic matrix and the original similarity matrix. The cophenetic matrix is an idealised ultrametric matrix representative of the tree. The Mantel test is a statistic for comparison of matrices; values nearer to one representing closer agreement between the matrices and values nearer zero representing less similar matrices. The two similarity matrices were also compared using this statistic. All these calculations were done using the NTSYS package.

Parsimony Analyses: The number of possible rooted trees that may be produced for 54 taxa is very large, certainly well in excess of 10^{40} (Felsenstein 1978). Heuristic searches were carried out on both the large and small binary matrices using the general option on the PAUP program with the maximum number of trees stored at any one time set to one thousand. Branch and bound searches were also attempted but exhaustive searches were not. Resulting trees were rooted using *R. luteum* as an outgroup.

Results of character measurements.

Catalogue of characters

Histograms presented in this section represent the distribution of the continuous characters within the sample group. The range of each character is divided into eight equal bins and the frequence of taxa in any one of these bins is plotted on the vertical axis. The colours used in the histograms are intend to make them easier to read and are arbitrary.

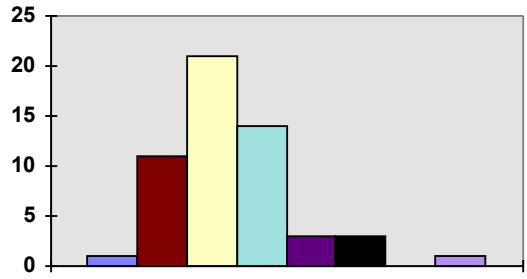
Habit (0 = never a tree 1 = capable of becoming arborescent). This character can not be scored from herbarium specimens, apart from the few where detailed field notes had been added, and so most of the species were scored from the literature and observations of living material. 33 out of the 54 taxa had state 1.

Height, average (metres). As above, this character can not be scored from herbarium specimens and so most of the species were scored from the literature and observations of living material. Statistics are given in Table 7 and Figure 13.

Table 7: *Statistics of height character for the 54 exemplar taxa.*

Statistic	Value
Mean	4.28
Median	3.83
Mode	5
Minimum	0.3
Maximum	15
Range	14.7
Standard Deviation	2.63
Sample Variance	6.90
Kurtosis	4.36
Skewness	1.61

Figure 13: *Histogram of height character data for 54 exemplar taxa.*



Bin	Frequency
0.3	1
2.4	11
4.5	21
6.6	14
8.7	3
10.8	3
12.9	0
More	1

Cataphylls (0 = absent 1 = present). This character was scored from the literature, herbarium specimens and living material. It was present in only three of the species scored; *R. sanguineum, R. forrestii* and *R. roxieanum* .

Leaf apex shape. (0 = obtuse 1 = acute). This character was scored from the literature, herbarium specimens and living material by comparison with a right angle cut in a piece of card. 33 out of 54 taxa had state 1.

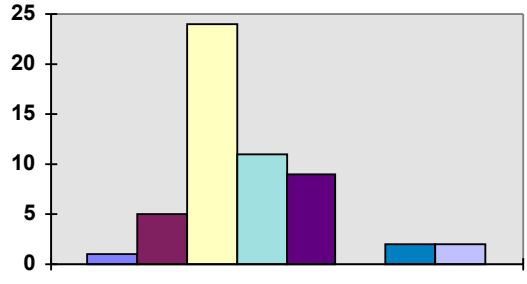
Leaf base shape. (0 = obtuse 1 = acute). As above, this character was scored from the literature, herbarium specimens and living material. 23 out of 54 taxa had state 1.

Leaf size (arbitrary measure). The measures of the length and width of the leaves were combined so as to avoid the bias that would be generated should they be included as separate characters. The square root of the product of the average of length and width for each taxon was taken as this measure. Statistics are given in Table 8 and Figure 14.

Table 8: *Statistics of leaf size character for the 54 exemplar taxa and R. ponticum.*

Statistic	Exemplars	*R. ponticum*
Mean	70.75	166.22
Median	61.86	170.00
Mode	57.97	180.00
Standard Deviation	34.95	24.40
Sample Variance	1221.38	595.28
Kurtosis	2.70	-0.95
Skewness	1.47	0.09
Range	171.97	95.00
Minimum	16.02	125.00
Maximum	187.99	220.00

Figure 14: *Histogram of leaf size character data for 54 exemplar taxa.*



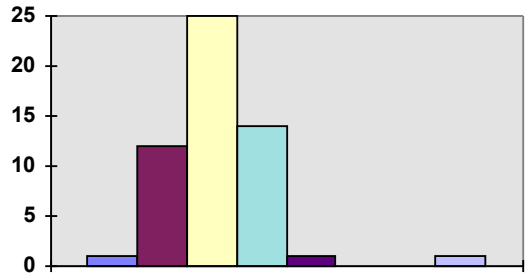
Bin	Frequency
16.02	1
40.58714	5
65.15429	24
89.72143	11
114.2886	9
138.8557	0
163.4229	2
More	2

Leaf length/width ratio. This character is a measure of how narrow the leaves are. It was measured from herbarium material in all taxa. Statistics are in Table 9 and Figure 15.

Table 9: *Statistics of leaf length/width character for the 54 exemplar taxa and R. ponticum.*

Statistic	Exemplars	*R. ponticum*
Mean	2.73	3.26
Median	2.55	3.33
Mode	2.50	3.00
Standard Deviation	0.95	0.34
Sample Variance	0.90	0.12
Kurtosis	12.34	5.30
Skewness	2.67	-1.71
Range	6.40	1.98
Minimum	1.20	1.82
Maximum	7.6	3.80

Figure 15: *Histogram of leaf length/width character data for 54 exemplar taxa*



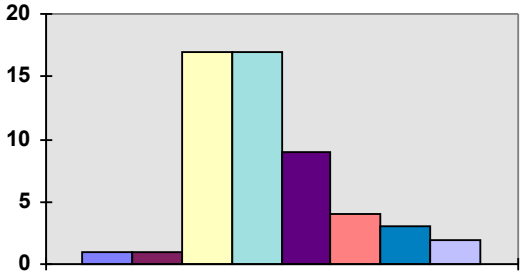
Bin	Frequency
1.2	1
2.114286	12
3.028571	25
3.942857	14
4.857143	1
5.771429	0
6.685714	0
More	1

Widest point of leaf. (Proportion). This character was scored entirely from herbarium material. The distance from the base to the widest point of the leaf was divided by the leaf length. Obovate leaves giving values greater than 0.5 and ovate leaves giving values less than 0.5. Statistics are Table 10 and Figure 16.

Table 10: *Statistics of widest point of leaf character for the 54 exemplar taxa and R. ponticum.*

Statistic	Exemplars	*R. ponticum*
Mean	0.56	0.60
Median	0.55	0.60
Mode	0.50	0.60
Standard Deviation	0.06	0.12
Sample Variance	0.00	0.02
Kurtosis	-0.04	21.96
Skewness	0.65	2.77
Range	0.27	1.11
Minimum	0.45	0.18
Maximum	0.72	1.29

Figure 16: *Histogram of widest point of leaf character for 54 exemplar taxa*



Bin	Frequency
0.45	1
0.49	1
0.53	17
0.57	17
0.60	9
0.64	4
0.68	3
More	2

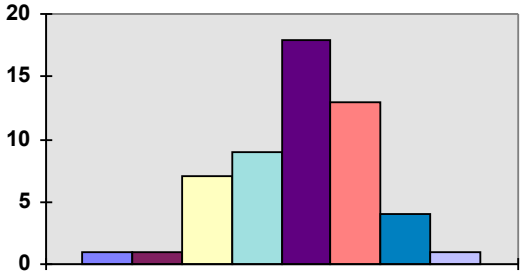
Leaf margins revolute(1 = revolute, 0 = flat). This character was initially recorded from herbarium material but species that scored 1 were cross checked with the literature and living specimens because of the danger of leaf curl being a drying artefact. 16 of the 54 taxa had state 1.

Petiole length (proportion). Average petiole lengths were measured in millimetres and later divided by the leaf size measures for the species. Some care was required in judging the lengths of petioles in leaves in which they were winged or flattened. Statistics for this character are given in Table 11 and Figure 17.

Table 11: *Statistics of petiole length character for the 54 exemplar taxa and R. ponticum.*

Statistic	Exemplars	*R. ponticum*
Mean	0.24	0.34
Median	0.25	0.34
Mode	0.13	0.36
Standard Deviation	0.09	0.04
Sample Variance	0.01	0.00
Kurtosis	0.63	-0.17
Skewness	-0.10	-0.19
Range	0.49	0.17
Minimum	0.00	0.25
Maximum	0.49	0.42

Figure 17: *Histogram of petiole length character for 54 exemplar taxa.*



Bin	Frequency
0.00	1
0.07	1
0.14	7
0.21	9
0.28	18
0.35	13
0.42	4
More	1

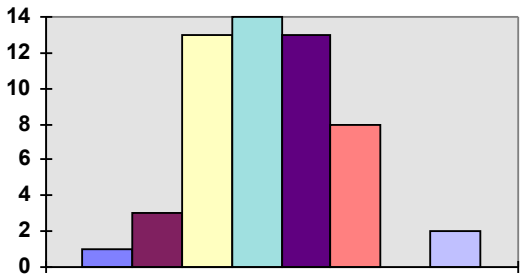
Petioles flattened or winged. (1 = flattened or winged, 0 = terete). This character was scored from herbarium material and cross checked with living material. 13 of the 54 taxa scored 1.

Number of flowers per inflorescence. (Integer). This character was scored from herbarium and living material. Statistics are given in Table 12 and Figure 18.

Table 12: *Statistics of flower number character for the 54 exemplar taxa and R. ponticum.*

Statistic	Value	*R. ponticum*
Mean	10.34	14.5
Median	10.00	14
Mode	12.00	14
Standard Deviation	4.92	2.89
Sample Variance	24.17	18
Kurtosis	0.44	0.4
Skewness	0.61	0.7
Range	24.00	12
Minimum	1.00	20
Maximum	25.00	8

Figure 18: *Histogram of the flower number character for 54 exemplar taxa.*



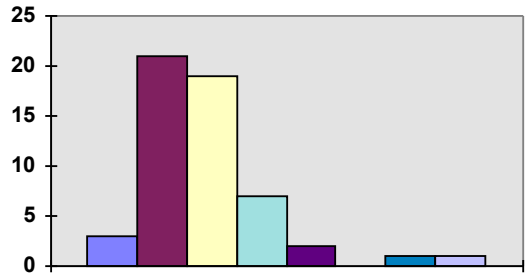
Bin	Frequency
1.00	1
4.43	3
7.86	13
11.29	14
14.71	13
18.14	8
21.57	0
More	2

Inflorescence rachis length (millimetres). This character was scored from flowering herbarium material only. Statistics are given Table 13 and Figure 19.

Table 13: *Statistics of inflorescence rachis length character for the 54 exemplar taxa and R. ponticum.*

Statistic	Exemplars	*R. ponticum*
Mean	11.71	52.60
Median	10.00	54.00
Mode	2.50	55.00
Standard Deviation	11.07	6.51
Sample Variance	122.62	42.37
Kurtosis	6.81	0.57
Skewness	2.22	-0.76
Range	60.00	30.00
Minimum	0.00	35.00
Maximum	60.00	65.00

Figure 19: *Histogram of the inflorescence rachis length character for 54 exemplar taxa.*



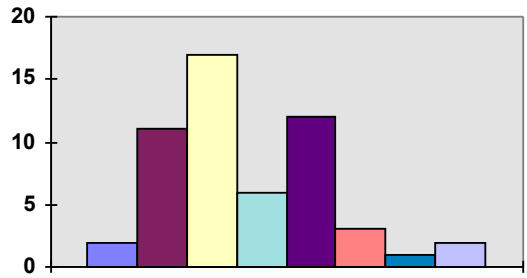
Bin	Frequency
0.00	3
8.57	21
17.14	19
25.71	7
34.29	2
42.86	0
51.43	1
More	1

Pedicel length in flower (millimetres). This character was scored from flowering material. Care was take to measure only the pedicels of fully open flowers as those of immature flowers were observed to be shorter. Statistics for this character are given in Table 14 and Figure 20.

Table 14: *Statistics of pedicel length in flower character for the 54 exemplar taxa and R. ponticum.*

Statistic	Value	*R, ponticum*
Mean	20.12	32.91
Median	17.50	32.00
Mode	17.50	32.00
Standard Deviation	8.84	4.25
Sample Variance	78.23	18.02
Kurtosis	0.80	-0.53
Skewness	0.93	-0.01
Range	39.50	16.00
Minimum	7.50	24.00
Maximum	47.00	40.00

Figure 20: *Histogram of the pedicel length in flower character for 54 exemplar taxa.*



Bin	Frequency
7.50	2
13.14	11
18.79	17
24.43	6
30.07	12
35.71	3
41.36	1
More	2

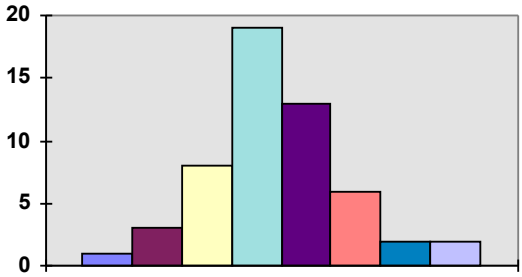
Pedicel length in fruit as a proportion of that in flower (Proportion). The average pedicel lengths of fruiting herbarium specimens was recorded and the divided by the average flowering pedicel length for the species. Statistics are given in Table 15 and Figure 21.

Table 15: *Statistics of pedicel length in fruit character for the 54 exemplar taxa and R. ponticum.*

Statistic	Value	*R. ponticum*
Mean	1.07	1.21
Median	1.00	1.20
Mode	1.00	1.20
Standard Deviation	0.19	0.17

Sample Variance	0.04	0.03
Kurtosis	0.36	2.79
Skewness	0.43	0.87
Range	0.92	0.88
Minimum	0.68	0.87
Maximum	1.60	1.75

Figure 21: *Histogram of the pedicel length in fruit character for 54 exemplar taxa.*



Bin	Frequency
0.68	1
0.81	3
0.94	8
1.07	19
1.21	13
1.34	6
1.47	2
More	2

Flower fragrance (1 = fragrant, 0 = not fragrant). The ability to detect a fragrance is a skill which is not possessed equally by everyone and may be genetically determined. The ideal method to standardise the detection of fragrance in a study would either be to restrict the scoring to a single individual or to a team of individuals, each of whom smells all the flowers. Unfortunately it was not possible for one person to smell several plants from of all the species in the study in the time available and so a different approach was taken. Four growers with field collecting experience of much of the subgenus were ask to consider each of the species in the exemplar group and whether the flowers had a distinct scent or not. Between them these growers and the author had experience of all the species with more than one person having an opinion on most of them. It was determined that six of the 54 species had some form of scent to the flowers, there was no conflict between the evidence supplied by any of the individuals surveyed.

Calyx size (0 = reduced, 1 = large and petaloid). This character was scored from herbarium material and cross checked with observations of living material. As mentioned in Chapter 4 the two states were clearly distinguishable. 17 of the 54 taxa scored state 1.

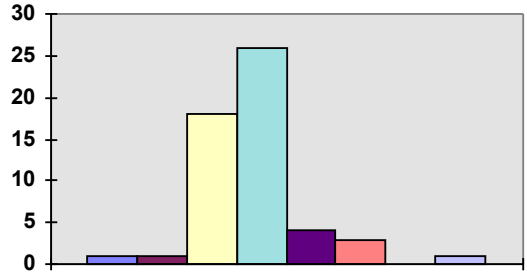
Number of Corolla lobes (0 = 5, 1 = greater than 5). This character was scored from herbarium material and cross checked with observations of living material. 10 of the 54 taxa scored state 1.

Corolla length (millimetres). This character was scored exclusively from herbarium material. The distance was measured from the base of the corolla to the tip of the longest lobe. This was always the upper (proximal) lobe. Statistics are given in Table 16 and Figure 22.

Table 16: *Statistics of corolla length character for the 54 exemplar taxa and R. ponticum.*

Statistic	Exemplars	*R. ponticum*
Mean	40.24	46.69
Median	38.75	47.00
Mode	40.00	50.00
Standard Deviation	11.82	4.19
Sample Variance	139.80	17.58
Kurtosis	6.03	-1.29
Skewness	1.62	-0.10
Range	76.50	14.00
Minimum	13.50	39.00
Maximum	90.00	53.00

Figure 22: *Histogram of the corolla length character for 54 exemplar taxa.*



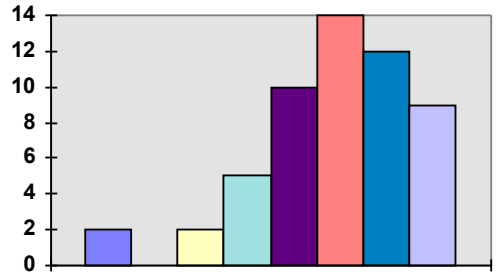
Bin	Frequency
13.50	1.00
24.43	1.00
35.36	18.00
46.29	26.00
57.21	4.00
68.14	3.00
79.07	0.00
More	1.00

Corolla tube length (proportion). As above, this character was only measured from herbarium material. The shortest distance from the base of the corolla to the bottom of cleft between two lobes was measured. This was always one of the clefts next to the lower (distal) lobe. The average distance for the specimen was divided by the average of the previous character to give a proportion. Statistics are given in Table 17 and Figure 23.

Table 17: *Statistics of corolla tube length character for the 54 exemplar taxa and R. ponticum.*

Statistic	Exemplars	*R. ponticum*
Mean	0.65	0.37
Median	0.65	0.36
Mode	0.70	0.36
Standard Deviation	0.09	0.06
Sample Variance	0.01	0.00
Kurtosis	0.97	3.45
Skewness	-0.78	1.85
Range	0.40	0.28
Minimum	0.40	0.30
Maximum	0.80	0.58

Figure 23: *Histogram of the corolla tube length character for 54 exemplar taxa.*



Bin	Frequency
0.40	2.00
0.46	0.00
0.51	2.00
0.57	5.00
0.63	10.00
0.69	14.00
0.74	12.00
More	9.00

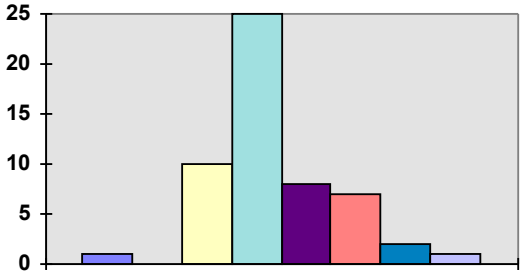
Corolla indumentum (0 = absent, 1 = present). Four of the 54 taxa showed some form of multicellular indumentum on the outside of the corolla. (It should be noted that this is distinct from the hair found to spread from the base of the stamens on to the inside of the corolla).

Stamen length (millimetres). The length of the longest stamen was measured. This character was only scored from herbarium material where it was frequently difficult to locate which position the longest stamen occupied as they often came loose during dissection. Observations of living material showed that the longest stamen was always the lower most. Statistics for this character are given in Table 18 and Figure 24.

Table 18: *Statistics of stamen length character for the 54 exemplar taxa and R. ponticum.*

Statistic	Exemplars	*R. ponticum*
Mean	29.34	40.59
Median	28.50	41.50
Mode	30.00	42.00
Standard Deviation	9.07	3.37
Sample Variance	82.22	11.35
Kurtosis	2.04	0.93
Skewness	0.91	-0.66
Range	52.00	16.00
Minimum	8.00	31.00
Maximum	60.00	47.00

Figure 24: *Histogram of the stamen length character for 54 exemplar taxa.*



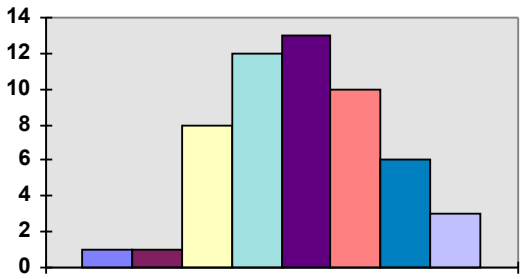
Bin	Frequency
8.00	1.00
15.43	0.00
22.86	10.00
30.29	25.00
37.71	8.00
45.14	7.00
52.57	2.00
More	1.00

Relative length of shortest stamen. (proportion). As above, this character was measured in herbarium material and complimented with observations made in living material. The shortest stamens were always those near the top of the flower. The length of the shortest stamen was divided by the length of the longest stamen on a flower by flower basis. Statistics for the character are given in Table 19 and Figure 25.

Table 19: *Statistics of shortest stamen character for the 54 exemplar taxa and R. ponticum.*

Statistic	Exemplars	*R. ponticum*
Mean	0.66	0.61
Median	0.65	0.59
Mode	0.67	0.76
Standard Deviation	0.13	0.07
Sample Variance	0.02	0.00
Kurtosis	-0.34	1.50
Skewness	0.07	1.52
Range	0.58	0.25
Minimum	0.38	0.51
Maximum	0.95	0.76

Figure 25: *Histogram of the shortest stamen character for 54 exemplar taxa.*



Bin	Frequency
0.38	1.00
0.46	1.00
0.54	8.00
0.62	12.00
0.70	13.00
0.79	10.00
0.87	6.00
More	3.00

Hairs at base of stamen (0 = absent 1 = present). As discussed in Chapter 4 single celled hairs were observed on the base of the filaments in some species. 28 of the 54 taxa scored 1 for this character.

Stamen hairs on corolla (0 = absent, 1 = present). In some species the hairs in the previous character were observed to have spread onto the inside of the corolla tube. 8 of the 28 taxa scoring 1 for the previous character also score 1 for this one. It is noted that these two characters are acting in an additive manner and so may produce misleading results. No specimens were observed in which these hairs were present on the inside of the corolla but not on the base on the filaments.

Approximation of pollen volume (μm^3). In the light of the work outlined in Chapter 4, on the importance of pollen volume in incompatibility mechanisms, an attempt was made to estimate the volume of the pollen grains. Tetrads of pollen grains were removed from herbarium specimens and soaked in a 10% glycerol solution for 20 minutes before being stained with iodine and observed at 400x magnification. The diameter of tetrads that were orientated as shown in Figure 26 (so that one pollen grain was obscured from view) were measured using a eye piece graticule (x).

From Figure 26 it can be seen, using simple trigonometry, that:

$$a = \sqrt{4r^2 - r^2}$$

or

$$a = \sqrt{3}r$$

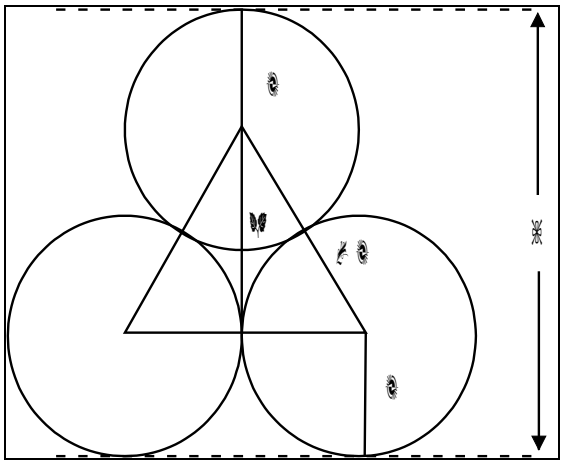
and so that:

$$x = \sqrt{3}r + 2r$$

$$x = 3.732r$$

The volume of the pollen grains can then be estimated as Πr^3.

Figure 26: *Estimation of pollen volume from tetrad diameter. The fourth grain, which would be above or below the plain of focus, has been omitted from this diagram.*

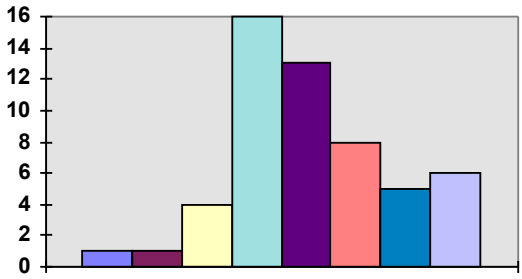


Given in Table 20 and Figure 27 are the statistics for this character in the sample group.

Table 20: *Statistics for the pollen volume character for the 54 exemplar taxa.*

Statistic	Value
Mean	11604
Standard Error	518
Median	11550
Mode	11550
Standard Deviation	3808
Sample Variance	14497847
Kurtosis	0
Skewness	0
Range	18095
Minimum	2636
Maximum	20731

Figure 27: *Histogram of the pollen volume character for 54 exemplar taxa.*



Bin	Frequency
2636	1
5221	1
7806	4
10391	16
12976	13
15561	8
18146	5
More	6

The distribution of the sizes of this character are very close to the normal distribution. The accuracy with which the distance x could be measured was very low (single eye piece units being 3.2µm apart). The diameter of an average size tetrad (of around 57.6µm across) could be estimated to ± 6.25% but this converts into an accuracy of +20% or -18.6% in the final pollen volume estimate. This inaccuracy was compounded by possible variations in exine thickness not having been taken into account. The pollen volume character was therefore treated with extreme caution. It was not measured for the *R. ponticum* study.

During the course of measuring tetrad diameters a number of deformed pollen tetrads were

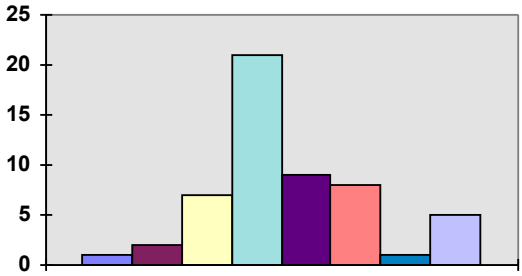
observed. It was common for one, two or even three of the grains to be either empty or markedly deformed. At the beginning of the study these deformities were scored as possible taxonomic characters and as possible signs of incapability of chromosomes at meiosis and therefore an indication of hybrid taxa. It became apparent, however, that the deformities were not consistent within species and so it was felt that the deformities were more likely to be the result physiological factors affecting pollen production and the recording was abandoned.

Ovary length (millimetres). This character was measured exclusively from herbarium material. Statistics are given Table 21 and Figure 28.

Table 21: *Statistics of ovary length character for the 54 exemplar taxa and R. ponticum.*

Statistic	Exemplars	*R. ponticum*
Mean	5.65	5.03
Median	5.00	5.00
Mode	5.00	5.00
Standard Deviation	1.66	0.54
Sample Variance	2.74	0.29
Kurtosis	0.43	0.86
Skewness	0.63	0.03
Range	8.00	2.00
Minimum	2.00	4.00
Maximum	10.00	6.00

Figure 28: *Histogram of the ovary length character for 54 exemplar taxa.*



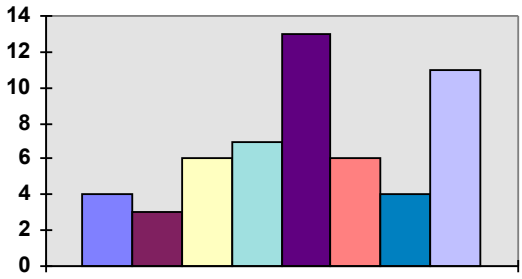
Bin	Frequency
2.00	1
3.14	2
4.29	7
5.43	21
6.57	9
7.71	8
8.86	1
More	5

Ovary width (proportion). The ovary width was measured in millimetres and the average for each specimen was divided by the average ovary length for that specimen in an attempt to give an unbiased measure of how narrow the ovary was. Statistics are given in Table 22 and Figure 29.

Table 22: *Statistics of ovary width character for the 54 exemplar taxa and R. ponticum.*

Statistic	Exemplars	*R. ponticum*
Mean	0.62	0.63
Median	0.60	0.60
Mode	0.60	0.60
Standard Deviation	0.13	0.08
Sample Variance	0.02	0.01
Kurtosis	-0.92	-0.01
Skewness	0.03	0.78
Range	0.43	0.30
Minimum	0.40	0.50
Maximum	0.83	0.80

Figure 29: *Histogram of the ovary width character for 54 exemplar taxa.*



Bin	Frequency
0.40	4
0.46	3
0.52	6
0.59	7
0.65	13
0.71	6
0.77	4
More	11

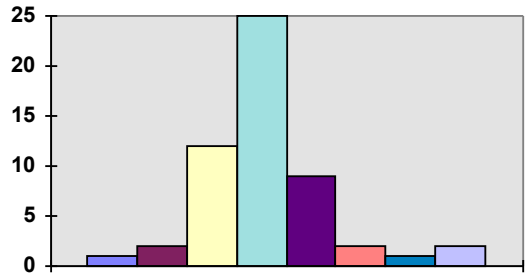
Style length (millimetres). This character was measured exclusively from herbarium material. Statistics are given in Table 23 and Figure 30. From the point of view of the incompatibility mechanisms discussed in Chapter 4 these statistics are somewhat misleading. It was established that the male/female style length ratio had to fall between 0.2 and 6 for crosses to be fertile in the species of *Vireya* studied. Examining the ratio of the ends of the range of style lengths found here gives ratios of 0.04 and 23.3. This range includes the two out group taxa (*R. luteum*, 4.5mm and *R. ferrugineum*, 3mm) however which are known to be incompatible with the other taxa. If these are ignored then the range of style lengths is 10mm to 70mm giving maximum ratios of 0.143 and 7; only just outside the range of compatibility suggested from the *Vireya* study. If either the lower most value (*R. barbatum*, 10mm) or the upper two values (*R. auriculatum*, 70mm and *R. griersonianum*, 62mm) or the upper most and lower most values of the range are excluded then the ratios fall within the 0.2 to 6 range. As in excess of 95% of all the sampled ingroup taxa fell within the style length compatibility range it was thought unlikely that the same mechanism was effective in this group.

A comparison was made between the style length and the pollen volume measure. No significant correlation (r = 0.196 for n = 54 taxa) was found between the two. This may have been due to the noise in the pollen volume data.

Table 23: *Statistics of style length character for the 54 exemplar taxa and R. ponticum.*

Statistic	Exemplars	*R. ponticum*
Mean	28.47	37.28
Median	28.00	37.00
Mode	30.00	42.00
Standard Deviation	11.45	4.16
Sample Variance	131.04	17.31
Kurtosis	3.81	-1.21
Skewness	1.16	-0.25
Range	67.00	13.00
Minimum	3.00	30.00
Maximum	70.00	43.00

Figure 30: *Histogram of the style length character for 54 exemplar taxa.*



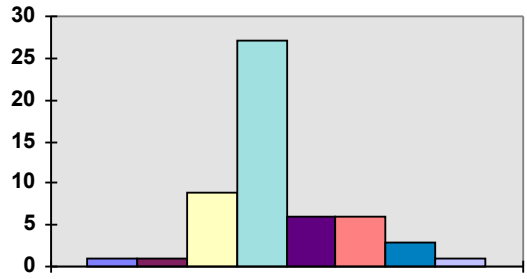
Stigma size (0 = small, 1 = large). As mentioned in Chapter 4 there is a clear distinction between species with a large stigma (over 4mm in diameter) and those with a small stigma, less that 2mm in diameter. Only 3 taxa were recorded as having state 1 for this character.

Capsule length (millimetres). This was measured entirely from herbarium material. Statistics are given in Table 24 and Figure 31.

Table 24: *Statistics of capsule length character for the 54 exemplar taxa and R. ponticum.*

Statistic	Exemplars	*R. ponticum*
Mean	21.66	18.94
Median	20.00	18.83
Mode	20.00	18.83
Standard Deviation	7.21	2.03
Sample Variance	52.01	4.10
Kurtosis	1.50	0.86
Skewness	0.98	0.03
Range	39.00	7.53
Minimum	6.00	15.06
Maximum	45.00	22.59

Figure 31: *Histogram of the capsule length character for 54 exemplar taxa.*



Bin	Frequency
6.00	1
11.57	1
17.14	9
22.71	27
28.29	6
33.86	6
39.43	3
More	1

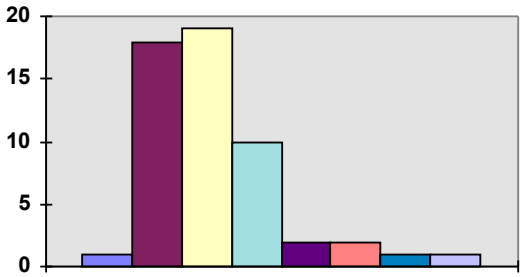
Bin	Frequency
3.00	1
12.57	2
22.14	12
31.71	25
41.29	9
50.86	2
60.43	1
More	2

Capsule width (proportion). The capsule width was measured in millimetres and the average for each specimen was divided by the average capsule length in millimetres to give an unbiased measure of how narrow the capsule was. Statistics are given in Table 25 and Figure 32.

Table 25: *Statistics of capsule width character for the 54 exemplar taxa and R. ponticum.*

Statistic	Exemplars	*R. ponticum*
Mean	3.61	3.38
Median	3.26	3.51
Mode	5.00	3.51
Standard Deviation	1.63	0.42
Sample Variance	2.64	0.18
Kurtosis	3.99	0.19
Skewness	1.73	-0.15
Range	8.50	1.58
Minimum	1.50	2.63
Maximum	10.00	4.21

Figure 32: *Histogram of the capsule width character for 54 exemplar taxa.*



Bin	Frequency
1.50	1
2.71	18
3.93	19
5.14	10
6.36	2
7.57	2
8.79	1
More	1

Hair type characters: The eight different hair types, described in Chapter 4, (excluding the filament hairs dealt with above) were scored as either present (1) or absent (0). The number of times each occurred in the 54 sampled taxa is given in Table 26.

Table 26: *Frequency of occurrence of the different hair types in the 54 exemplar taxa.*

Hair Type	Occurrence
Glands	34
Radiate hairs	5
Stalked radiate hairs	5
Dendroid hairs	11
Foiloliferous hairs	2
Ramiform hairs	9
Cup-shaped hairs	2
Papillae	22

Agglutination of indumentum (0 = absent 1 = present). Agglutination of longer indumenta was either clearly present or absent. It occurred in 10 out of the 54 taxa.

Secondary characters: Fifteen characters were scored as secondary character. These were all present/absent characters of some form and are present in the table below. No distinction was made between the different complex hair type in these characters. Flower colour was reduced to whether three main colours were present in the markings. Details of the occurrence of these characters is given in Table 27.

Table 27: *Frequency of occurrence of the secondary characters in the 54 exemplar taxa.*

Character	Occurrence
Young shoots hairy	36
Young shoots glandular	22

Character	Occurrence
Petiole hairy	27
Petiole glandular	24
Pedicel hairy	23
Pedicel glandular	34
Ovary hairy	20
Ovary glandular	31
Style hairy	5
Style glandular	14
Flower Red	49
Flower Blue	22
Flower Yellow	20
Basal blotch	20

RESULTS OF DATA ANALYSIS

Division of continuous and meristic characters into states.

It was concluded from the statistical evidence given above that it was not possible to divide any of the continuously variable meristic characters into separate states suitable for use in the cladistic analysis. Although some of the characters appear to have disjuctions, closer examination of these showed this to be the result of somewhat skewed but more or less normal distributions. The decision was taken, however, to arbitrarily divide these characters about their means as an exercise in exploring the data.

Phenetic Analysis

The PCOORDA of both similarity matrices produced very similar results (see page 68). Figure 33 is the plot of the first two components of the DIST matrix analysis with the MST superimposed on it. The data does not appear to resolve itself into clear clusters. The branches of the MST are long, joining points that are widely dispersed on the projection. One group of taxa do however appear to be somewhat separated on the plot and these have been highlighted with stars. The same taxa have been highlighted on the UPGMA, NJ and parsimony trees given below. Similar patterns were produced with plots of the second and third and the first and third co-ordinates. As can be seen from Table 28 the variance is spread widely across a large number of components and so it is unlikely that a two or three dimensional plot will be able to accurately reflect any structure that may be present in the data.

Table 28: *Eigenvalues and the percentage of variance accounted for by the first 40 components of the PCOORDA analysis for the DIST matrix.*

i	Eigenvalue	Percent	Cumulative	i	Eigenvalue	Percent	Cumulative
1	8.897227	16.7872	16.7872	21	0.696185	1.3136	90.6718
2	5.18619	9.7853	26.5725	22	0.596312	1.1251	91.7969
3	3.713906	7.0074	33.5799	23	0.57238	1.08	92.8769
4	3.373455	6.365	39.9449	24	0.501851	0.9469	93.8238
5	2.828454	5.3367	45.2816	25	0.442399	0.8347	94.6585
6	2.682644	5.0616	50.3432	26	0.408031	0.7699	95.4284
7	2.595241	4.8967	55.2399	27	0.365448	0.6895	96.1179
8	2.378013	4.4868	59.7267	28	0.32283	0.6091	96.727
9	2.003869	3.7809	63.5076	29	0.289844	0.5469	97.2739
10	1.96446	3.7065	67.2141	30	0.246647	0.4654	97.7393

i	Eigenvalue	Percent	Cumulative	i	Eigenvalue	Percent	Cumulative
11	1.676685	3.1636	70.3777	31	0.230527	0.435	98.1742
12	1.586238	2.9929	73.3706	32	0.176727	0.3334	98.5077
13	1.539712	2.9051	76.2757	33	0.156137	0.2946	98.8023
14	1.268621	2.3936	78.6693	34	0.149277	0.2817	99.0839
15	1.129949	2.132	80.8013	35	0.113242	0.2137	99.2976
16	1.043498	1.9689	82.7701	36	0.105819	0.1997	99.4972
17	1.014099	1.9134	84.6835	37	0.093925	0.1772	99.6745
18	0.904051	1.7058	86.3893	38	0.064627	0.1219	99.7964
19	0.809013	1.5264	87.9157	39	0.059047	0.1114	99.9078
20	0.76454	1.4425	89.3583	40	0.037021	0.0699	99.9777

Table 29 is a summary of the results obtained via the clustering algorithms. It shows the cross comparison of all the matrices produced by the two different similarity coefficients, DIST and MAN as well as the cophenetic matrices (DIST.Co. and MAN.Co.) derived via the clustering techniques, the UPGMA results are shown above the diagonal and the NJ below. All values in the diagonal are, of course, one and a single comparison is repeated above and below the diagonal (that of MAN x DIST the only combination in which no clustering technique was used.)

Table 29: *Mantel Statistics for comparison of four data matrices by using two clustering techniques. UPGMA trees represented above the diagonal NJ trees below. See text for full explanation of abbreviations.*

	DIST.	DIST. Co.	MAN.	MAN. Co.
DIST.	1.00	0.82	0.96	0.73
DIST.Co.	0.41	1.00	0.74	0.80
MAN..	0.96	0.40	1.00	0.74
MAN.Co	0.38	0.15	0.42	1.00

Rohlf (1994 page 10-7) suggests that when interpreting Mantel statistics the following subjective scale could be used: 0.0 to 0.7 is a very poor fit between the matrices, 0.7 to 0.8 is a poor fit, 0.8 to 0.9 is a good fit and 0.9 to 1.0 is a very good fit. On this basis it can be seen from Table 29 that there is a very good fit between the matrices produced by the two different similarity coefficients, as would be expected. It can also be seen that the results obtained by the UPGMA clustering are generally higher than those produced by the NJ method the cophenetic correlations (i.e. comparisons between real and cophenetic matrices) all scoring as poor or good fits for the UPGMA but as very poor fits for all the NJ trees.

Figure 34 is the UPGMA dendrogram produced from the DIST coefficient, the tree that had the highest cophenetic correlation (0.82) and should therefore most closely resemble the data. It should be noted that the NJ clustering of this matrix only produced a cophenetic correlation of 0.41. This tree is given in Figure 35. It can be seen that there is some similarity between the two trees but not a great deal. It should be noted that the lower branches of the NJ tree are very short suggesting weak clustering.

Cladistic Analysis

The heuristic search of the large binary data set produced a single tree of length 331 but with a very low consistency index of 0.124 and a rescaled consistency index of only 0.068 indicating that there is a very high level of homoplasy in the tree; many characters changing state more than once. The tree is given in Figure 37.

The search of the small binary data resulted in 1000 most parsimonious trees (the maximum permitted by the search criteria) of length 114. Consensus trees of this data produced little resolution. The 50% majority rule consensus tree is shown in Figure 36.

Conclusions

Brief conclusions will be made here concerning the interpretation of this data. A fuller discussion of the results is left until chapter nine where the comparisons can be made with the molecular results.

The UPGMA clustering method assumes the ultrametric condition, i.e. that a rooted tree can be drawn from the data in which all pairwise distances are equal to the sum of the lengths of the branches that join them and the distance from the root to the tip of any branch is the same. This can be referred to as assuming clock-like evolutionary change. The NJ method assumes that an additive unrooted tree can be drawn from the data in which all pairwise distances are equal to the sum of the lengths of the branches that join them. The additive trees are therefore a subset of ultrametric trees; all utrametric trees are additive. The calculation of the cophenetic distance assumes the ultrametric condition and so close similarity of the cophenetic matrix with the original similarity matrix indicates that the original matrix has a strong ultrametric component. This is the case with the UPGMA clustering of the MAN and DIST similarity matrices but especially with the DIST matrix. One would expect that if there is a strong ultrametric component to the matrix the NJ method would produce a similar result as to the UPGMA method. This is not supported by the results above where both the MAN and DIST matrices produce very low cophenetic correlations with the NJ algorithm. This raises doubts concerning the validity of the clusters produced by the UPGMA method. Examination of Figure 34 and Figure 35 show the topologies to be very different but to bear some similarities. This suggests that there is some hierarchical signal in the data but that it is weak.

The PCOORDA analysis emphasises the difficulty of dividing the data into clusters. These results (Figure 33 and Table 28) indicate that there is either no structure in the data or that the structure is so complex that it can not be represented well in a two or three dimensional projections. The evidence from the parsimony analyses tends to support the hypothesis that there is only a low level of structure within the data. The small binary matrix producing little resolution and the large binary data set producing a fully resolved but badly supported tree.

The taxa that have been highlighted in the PCOORDA projection and the trees in this chapter indicate that all the methods may be detecting the same basic structure in the data as they are often clustered near each other or in two distinct groups. The analysis that groups these taxa most distinctly is that of the large binary data set. If the clustering patterns are compared with the current classification there is little, if any , likeness. In none of the subsections for which there are more than one representative species do the species cluster together and the large binary data set fails to separate out the outgroup taxa. The similarity between the analyses and the disimilarity with previous taxonomies suggests that either an entirely new phylogeny is being revealed or the taxa are being clustered on the basis of some other feature.

As the marked taxa identified in the PCOORD analysis are generally larger in size than other taxa it is concluded that, despite all efforts to the contrary, the common variable influencing the clustering is size. This is confirmed by the very strong clustering found in the analyses of the larger binary data set. For this reason the decision was taken not to take the continuously variable data forward to further analysis either in its current form or arbitrarily converted into a binary matrix as it is only likely to mislead to subsequent analyses. The trees in this chapter will, however, be refered back to by the final chapter.

Chapter Summary

- Sampling and data collection techniques were described.
- The taxonomic characters used were described and results for each character presented separately.
- Continuously variable characters were divided into states.
- Phenetic and cladistic analyses of the data was carried out.

- It was concluded that the continuously variable characters were misleading and should not be taken forward to secondary analyses.

Figure 33: *PCOORDA of the first two components from the analysis of the DIST similarity matrix. Highlighted taxa are the same as those highlighted in the other trees presented in Chapter 5.*

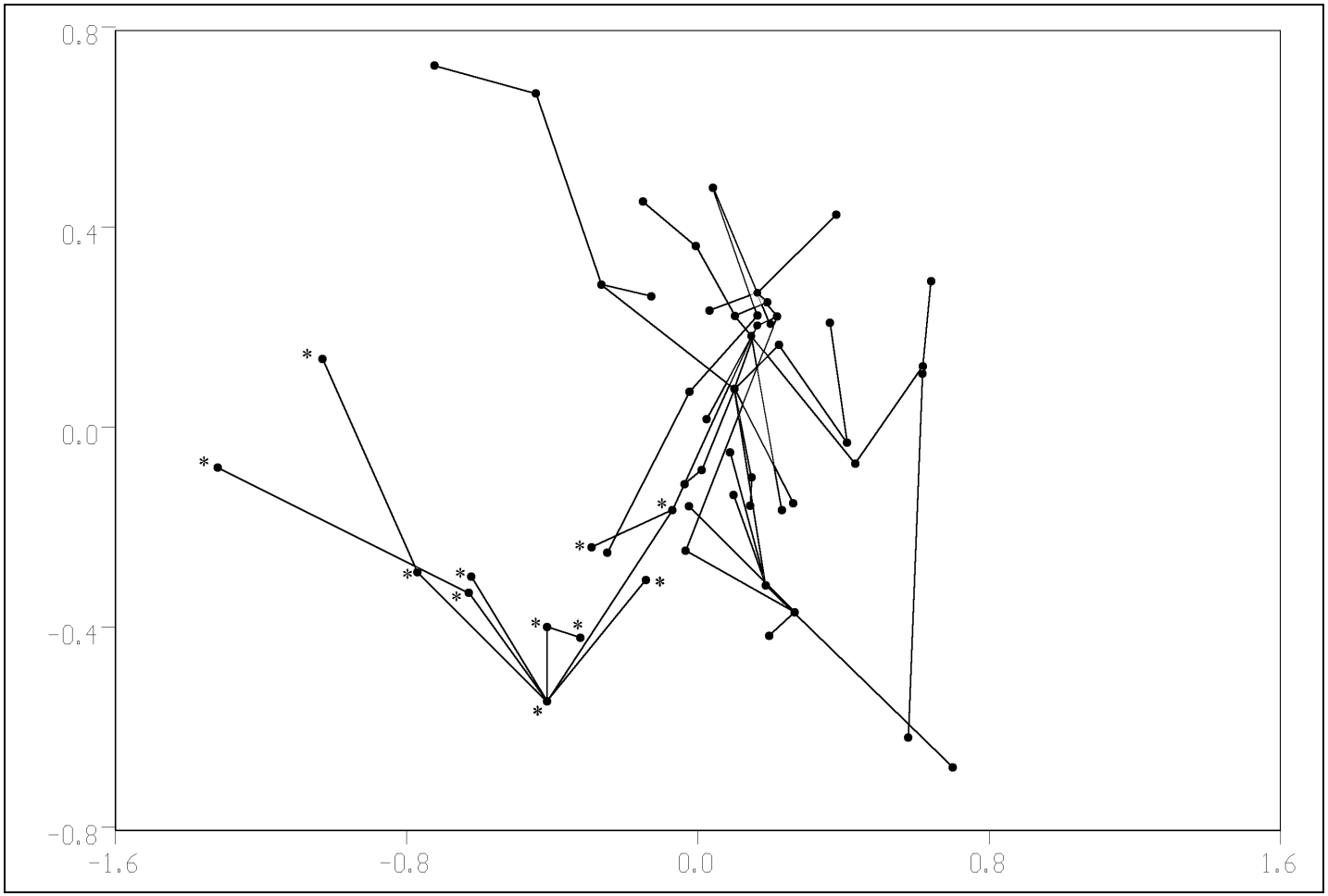


Figure 34: *UPGMA clustering of DIST similarity matrix. Highlighted taxa are the same as those highlighted in the other trees and the PCOORDA analysis presented in Chapter 5.*

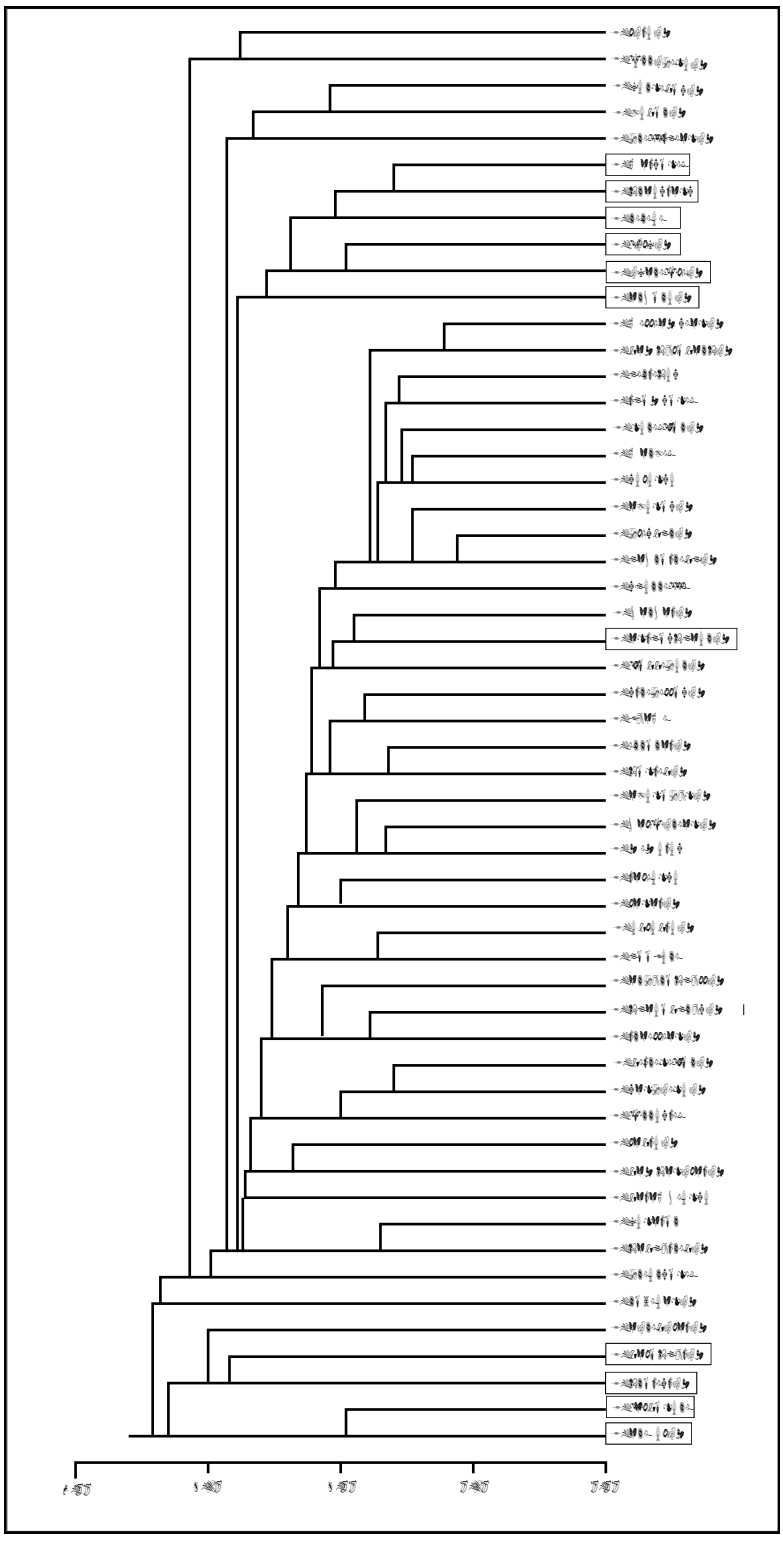


Figure 35: *Neighbour Joining tree of DIST similarity matrix. Highlighted taxa are the same as those highlighted in the other trees and the PCOORDA analysis presented in Chapter 5.*

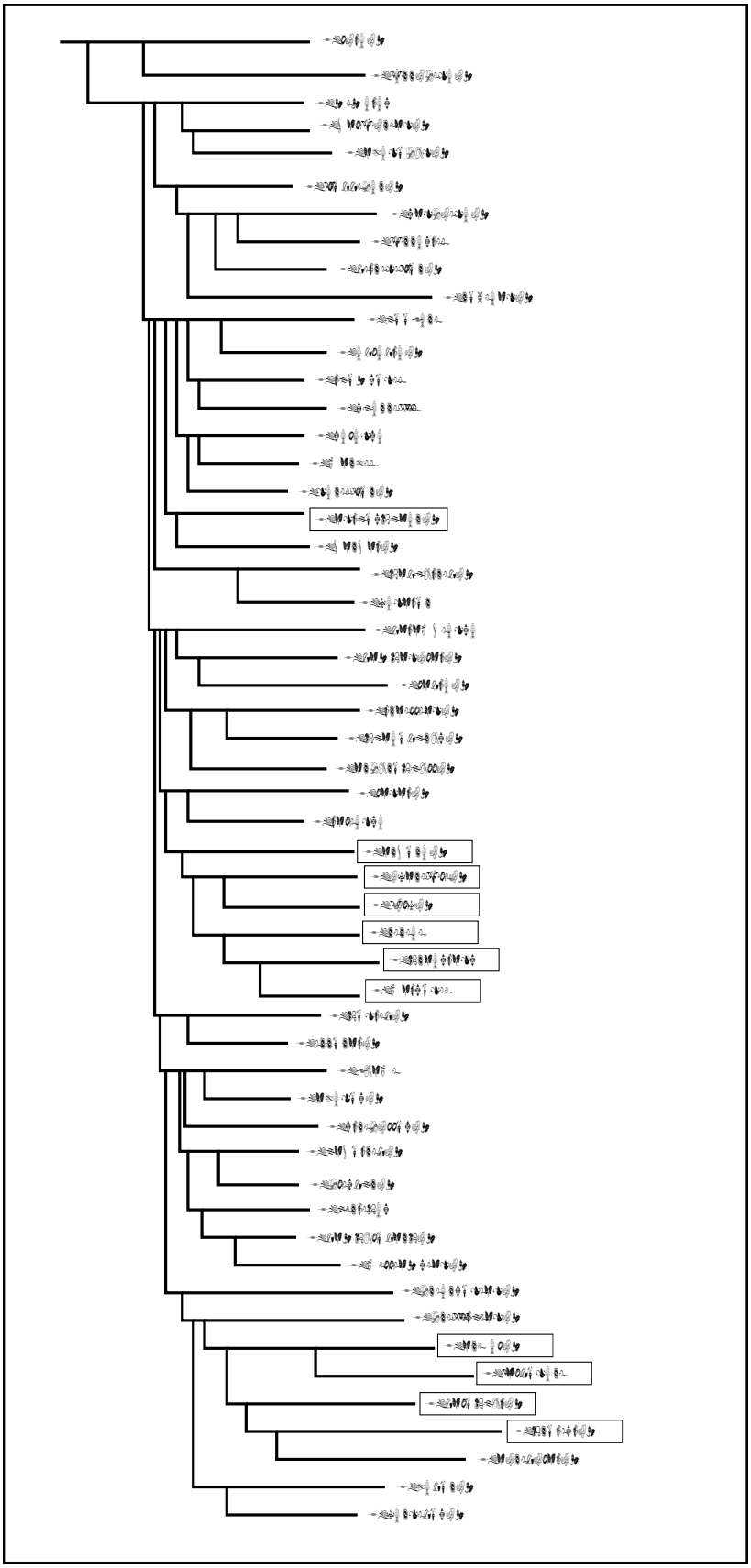


Figure 36: *50% majority rule consensus tree from the 100 trees produced in the parsimony analysis of the small binary matrix. Figures above the branches are the percentage of trees that support that branch. Highlighted taxa are the same as those highlighted in the other trees and the PCOORDA analysis presented in Chapter 5.*

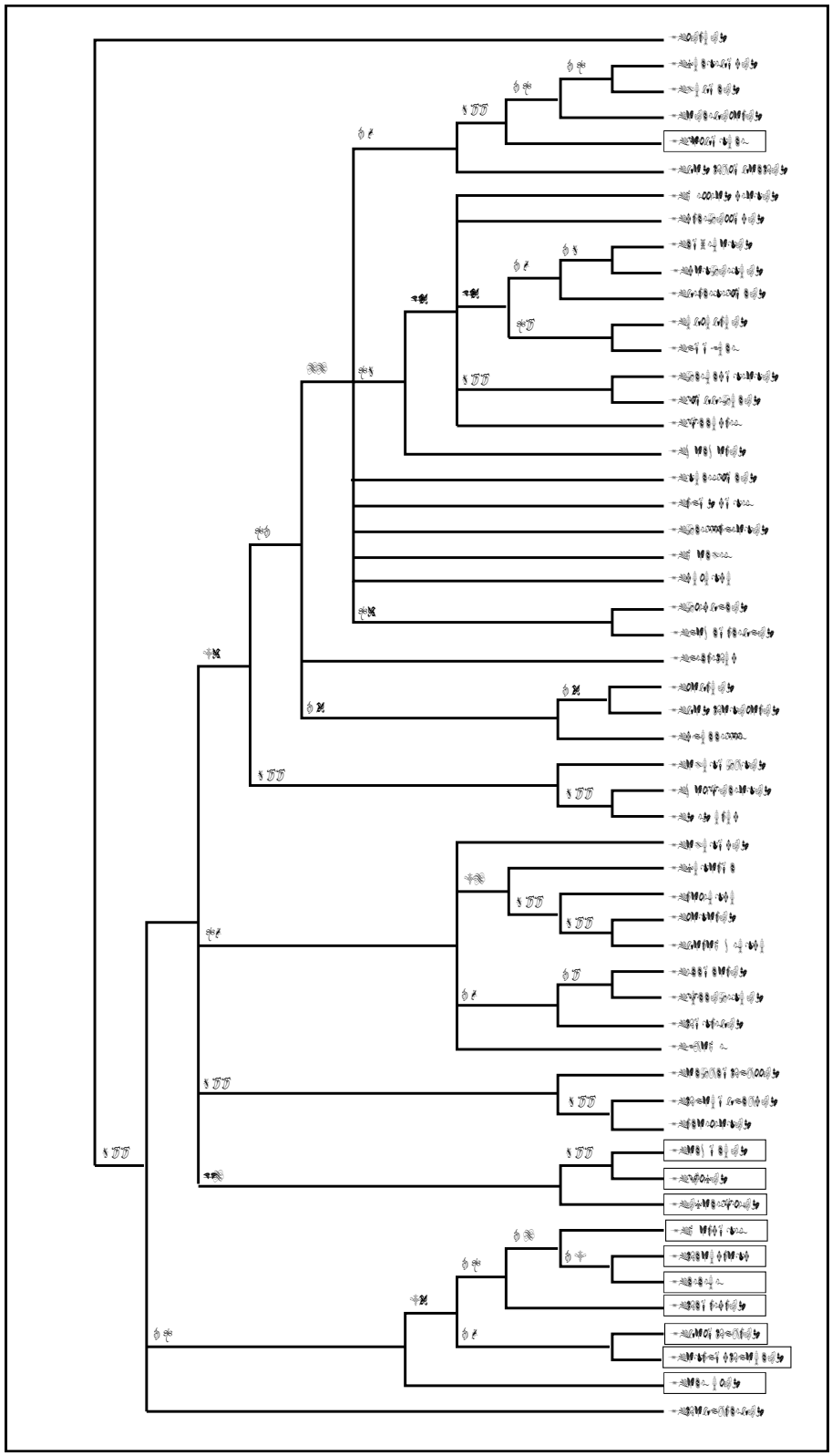
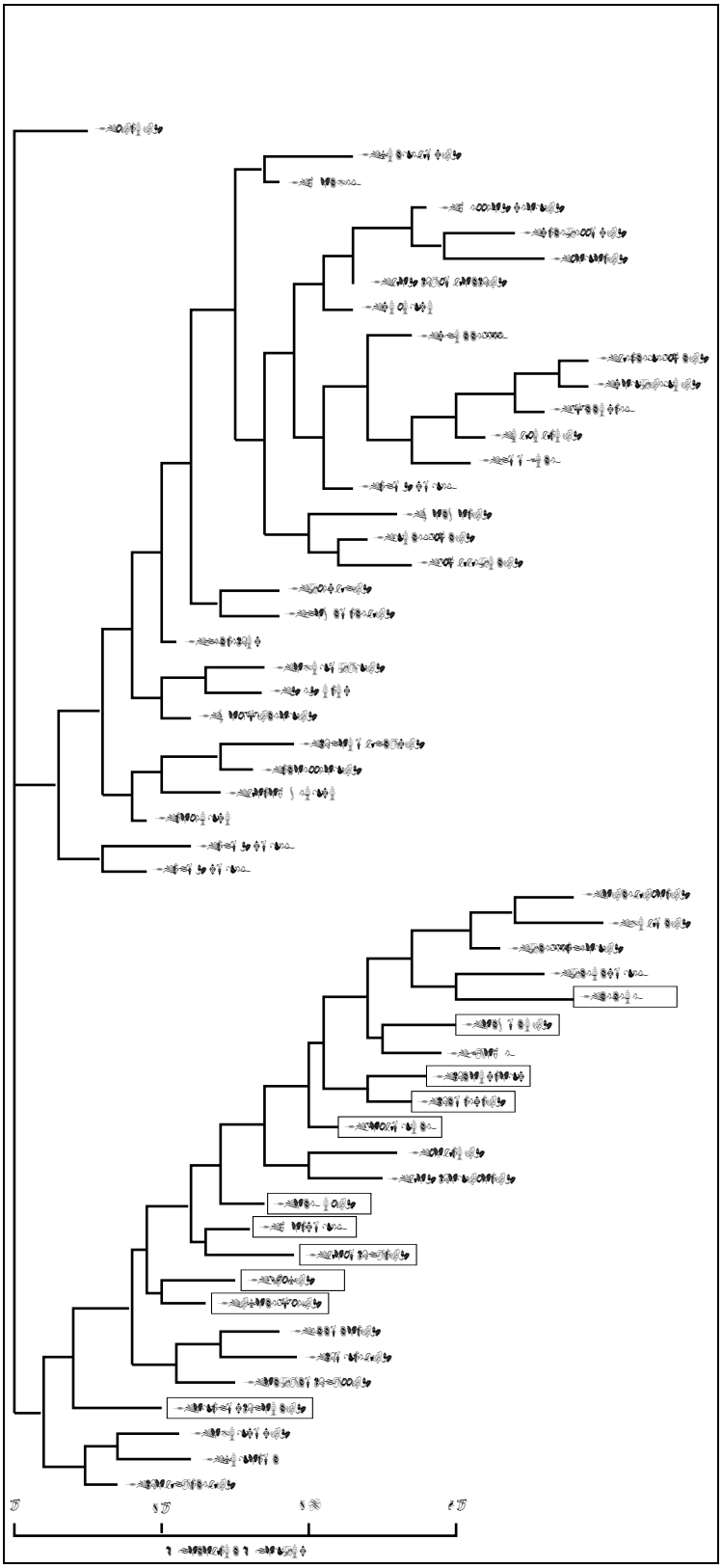


Figure 37: *Single tree produced from the parsimony analysis of the large binary data set. Branch lengths are shown proportionate to the number of character changes. Highlighted taxa are the same as those highlighted in the other trees and the PCOORD analysis presented in Chapter 5.*



CHAPTER 6: RAPD STUDY

Introduction

Despite the limitations of the RAPD technique, with respect to reproducibility and homology of amplified products, RAPDs are an attractive method for obtaining information on diversity because they are cheap and easy to use, do not require probes or prior sequence information for specific primer design, are highly polymorphic and can be detected on agarose gels without the need for florescent or radioactive labelling. For these reasons they have been extremely widely applied and have expanded the number of species in which molecular data could be obtained in an unprecedented way. (e.g. Castiglione *et al* 1993; Oxelman 1996; Smith et al 1994) At the time of initiating the *Rhododendron* study very little molecular work had been carried out in the genus, no microsatellites of RFLP probes were available and few sequences had been tested for variation. The *Rhododendron* genus is very large and *Hymenanthes* itself is huge, which makes the more intensive techniques, such as sequencing, daunting. A study was therefore undertaken to test out the usefulness of RAPDs in understanding the classification of *Hymenanthes*.

The RAPD study initially envisaged was ambitious. It was believed that production of RAPD fingerprints would be relatively straight forward and that a fingerprint data base would be established for 100 exemplar species from which taxonomic data could be extracted. The project was split into three phases which, to a degree, ran concurrently. These phases were; DNA extraction; survey of suitable primers; fingerprinting of taxa.

Materials

Plant material

One hundred exemplar species were selected from subgenus *Hymenanthes* following the procedure outlined at the beginning of Chapter 5 but with the additional criterion that they should occur as living specimens in the collections of RBGE, preferably as wild origin material. A single outgroup species (*R. ciliatum*) was selected. A programme of collection and extraction of this material was undertaken. Species were collected in batches of ten at a time, the DNA extracted, cleaned and run on a gel for each batch before the next batch was selected. The species, with the accession number of plants used and a reference to their subsection are given in Table 30.

Table 30: *Table of specimens used in the RAPD study. Accession numbers are those used in the living collections at Royal Botanic Garden Edinburgh. Subsection numbers are those used by Chamberlain (1983).*

Species	Accession	Subsection	Species	Accession	Subsection
R. aberconwayi	19370338A	11	*R. hyperythrum*	19710096A	12
R. adenogynum	19381023A	15	*R. irroratum*	19812433A	11
R. adenosum	19300433A	9	*R. japonicum*	19761903B	12
R. aganniphum	19698581A	15	*R. kyawii*	19772748B	20
R. alutaceum	19614571A	15	*R. lacteum*	19490491A	15
R. anthosphaerum	19231005A	11	*R. lanatum*	19810957A	17
R. arboreum	19790276A	14	*R. macrophyllum*	19690512A	12
R. argyrophyllum	19090009A	13	*R. makinoi*	19721124A	12
R. arizelum	19320082B	4	*R. mallotum*	19201013A	22
R. aureum	19450053A	12	*R. maximum*	19800047A	12
R. auriculatum	19160027A	2	*R. mimetes*	19825082B	15
R. balfourianum	19191004A	15	*R. neriiflorum*	19200019A	22
R. barbatum	19751635A	21	*R. niveum*	19701316A	14

Species	Accession	Subsection	Species	Accession	Subsection
R. beesianum	19698411B	15	*R. oreodoxa*	19150027A	1
R. brachycarpum	19731145C	12	*R. phaeochrysum*	19698780A	15
R. bureavii	19181009B	15	*R. ponticum*	19773079A	12
R. calophytum	19724038A	1	*R. principis*	19568065A	15
R. campanulatum	19720857D	18	*R. pronum*	19731826A	15
R. campylocarpum	19832543A	6	*R. proteoides*	19491025A	15
R. catawbiense	19340114A	12	*R. protistum*	19832100A	3
R. caucasicum	19521068A	12	*R. recurvoides*	19754048A	9
R. ciliatum	19831843A	Outgroup	*R. roxieanum*	19734042A	15
R. citriniflorum	19241043A	22	*R. rufum*	19501047A	15
R. clementinae	19331023A	15	*R. sanguineum*	19794033A	22
R. crinigerum	19491014A	9	*R. selense*	19241044A	8
R. cyanocarpum	19812501A	24	*R. sherriffii*	19560423A	23
R. dignabile	19710052A	15	*R. smirnowii*	19623832A	12
R. eclecteum	19231008A	24	*R. sphaeroblastum*	19730396A	15
R. faberi	19698801C	15	*R. strigillosum*	19754050A	7
R. falconeri	19790170A	4	*R. taliense*	19568652B	15
R. floccigerum	19491018A	22	*R. thomsonii*	19803353A	24
R. floribundum	19840941A	13	*R. traillianum*	19301045A	15
R. fulgens	19371010A	23	*R. ungernii*	19623836A	12
R. fulvum	19180010D	16	*R. uvariifolium*	19381079A	16
R. galactinum	19110045A	4	*R. vernicosum*	19141012A	1
R. griersonianum	19320271B	19	*R. wardii*	19481014B	6
R. habrotrichum	19180014A	9	*R. watsonii*	19500299A	3
R. haematodes	19730148A	22	*R. wightii*	19813601A	15
R. hirtipes	19251063A	8	*R. williamsianum*	19320138A	5
R. hookeri	19291007A	24	*R. yakushimanum*	19411005A	12

Buffers used

SDS buffer (Used in extraction method 1) 10mM Tris-HCl (pH8.0); 10mM EDTA; 1% SDS.

Prelysis buffer (Used in the extraction method 2): 250 mM Sorbitol; 25 mM Tris-HCl (pH 8.0); 10 mM EDTA (pH8.0).

CTAB buffer (Used in the extraction method 2): 1% CTAB; 10 mM Tris-HCl (pH 8.0); 20 mM EDTA (pH 8.0); 1.4 M NaCl; 5% PVP (4000); 350 mM DTT (DL-Dithiothreitol).

Urea buffer (Used in the extraction method 2): 8 M Urea; 350 mM NaCl; 2% SLS; 10mM Tris-HCl (pH 8.0); 10mM EDTA (pH 8.0).

2xCTAB buffer (Used in the extraction method 3): 2xCTAB Buffer; 2% CTAB; 100mM Tris-HCl pH 8; 20mM EDTA pH 8; 1.4M NaCl; 1% PVP (4000).

P.C.I. = phenol:chloroform:isoamylalcohol, 25:24:1 by volume.

TBE = 89mM Tris-HCl; 89mM Boric Acid; 11mM EDTA (pH8.3).

TE = 10mM Tris; 1mM EDTA (pH 7.5).

Primers Used.

Primers for use in the RAPD survey were kindly supplied by the laboratory of Francesco Sala, Pavia and were the same as those used in Castigilione *et al* (1993). They are listed in Table 31.

Table 31: *Sequences of primers used in the RAPD survey.*

Name	Sequence	Name	Sequence
10MER-01	5′-GATGGCCGGC-3′	20MER-01	5′-GAGGCCTACGCCCCATAGAA-3′
10MER-02	5′-GGGATCCGGC-3′	20MER-02	5′-AATGCGTTGAGGCGCAGCAG-3′
10MER-03	5′-GGGCCATGGC-3′	20MER-03	5′-TTCCCGTGTCTTCCGGCTTAC-3′
10MER-04	5′-CGTTGGCCCG-3′	20MER-04	5′-TTCTTCTCCTACCAGTATCG-3′
10MER-05	5′-CCAAGGGGGC-3′	20MER-05	5′-CAAGCGCTCATGAGCCCGAA-3′
10MER-06	5′-TAGGGGCCCC-3′	20MER-06	5′-CAGGAGTCGCATAAGGGAGA-3′
10MER-07	5′-CCGCCCGGAT-3′	20MER-07	5′-CTGTGAGAAAGATGAAAGAT-3′

METHODS

DNA extraction.

Three different methods were used to extract DNA. The first used was based on an SDS extraction buffer and Phenol Chloroform extractions, the second on CTAB extraction buffer with Phenol Chloroform extractions and the third on a CTAB buffer and Dichloromethane extractions. The second two methods were carried out as minipreps in 1.5ml eppendorf tubes.

Extraction Method 1 - SDS + Phenol Chloroform.

Six grams of fresh leaf material were ground in liquid Nitrogen in a pre-cooled pestle and mortar with a little sterile sand until the appearance of a fine powder was obtained. Before the powder could thaw it was divided between two 15ml, screw top, polypropylene centrifuge tubes each containing 5ml of extraction buffer and 2.5ml of P.C.I. The tubes were shaken firmly once or twice and then agitated gently for 30-40 minutes at room temperate during which time they were opened twice to release any pressure that had built up. Following incubation tubes were centrifuged at 10,000 rpm for 10 minutes at 4°C to remove cellular debris and the supernatant decanted from both tubes into a conical flask. The lower fraction was discarded. The volume was estimated and 0.1 x the volume of 3M Na acetate and 2.5 x the volume of 90% etOH added and gently mixed. The mixture was then incubated at -20°C for 2 hours before being transferred to glass centrifuge tubes and centrifuged as above. This time the supernatant was discarded and the pellet air dried for 15-30 minutes at room temperature. Sufficient 1 x TE to re-dissolve pellet was added to the tube and the suspension was incubated at 4°C for 16 hours. The resulting solution was transferred to 1.5ml eppendorf tubes, (500µl in each tube) and a further P.C.I. extraction carried out by adding 250µl of PCI to each tube, mixing well, centrifuging, in a microcentrifuge at 13,000 rpm for 3 minutes at room temperature and transferring the supernatant to a fresh tube. RNA was digested by incubating the supernatant for one hour at 37°C with 25µl RNase A (10mg/ml, DNase free). Protein was digested by incubating for half an hour at 37°C with 25 µl proteinaseK. (10mg/ml). A final P.C.I. extraction was carried out exactly as above. The DNA was precipitated by adding 0.1x the volume 3M Na acetate and 2.5 x the volume 90% etOH, mixing gently, incubating at -20°C for 2 hours and a single pellet was produced for each sample by centrifuging one of the tubes used for that sample at 13,000 rpm for 3 minutes, discarding the supernatant and replacing it with the suspension from another tube for that sample and centrifuging in the same way; this procedure being repeated until the DNA in suspension in all the tubes for any one sample had been pelletted out in a single tube. The resulting pellet was air dried for around 20 minutes and resuspended in 1 x TE.

Extraction Method 2 - CTAB + Phenol Chloroform.

Two grams of fresh leaf material were ground in liquid Nitrogen in a pre-cooled pestle and mortar with a little sterile sand until the appearance of fine powder was achieved. Enough powder was transferred to a 1.5ml eppendorf tube to just reach the 0.25ml mark on the side of the tube. Care was taken that the powder did not thaw when it was placed in the tube. To this end the eppendorf tube and spatula were both cooled in liquid Nitrogen prior to use. Using this technique it was possible to

grind samples place the powder in the cooled eppendorf tubes and place the closed tubes in a dewar of liquid Nitrogen so that a number of samples could subsequently be processed in parallel. (It even proved possible to process samples the following day by holding them at -80°C overnight.) Extraction was continued by adding 410µl of prelysis buffer, that had been cooled on ice, to the tube and suspending the sample using the tip of a disposable pipette and by gentle vortexing the tube. 260µl of lysis buffer, 5µl of RNase A (10mg/ml, DNase free) and 78µl of 15% Sarkosyl were then added to the tube and the suspension mixed thoroughly by inverting and flicking before incubating at 65°C for 30 minutes. 5µl Protinase K (10mg/ml) were then added to the mix and incubation continued for a further 20 minutes at 65°C. The tube was allowed to cool to room temperature and then a chloroform extraction carried out by adding 750µl chloroform:isoamyl alcohol (24:1) to the tube and centrifuging it at 10,000 rpm for 20 minutes in a bench top centrifuge. The upper, aqueous phase was transferred to a fresh tube and a P.C.I. extraction performed by adding 750 µl of P.C.I. mixing and centifuging at 10,000 rpm for 10 minutes. The upper, aqueous phase was transferred to a fresh tube and the DNA precipitated by the addition of 1ml of ice cold absolute ethanol and storing at -20°C for around 1 hour. The precipitated DNA was pelletted out of suspension by centrifuging the tube at 4,000 rpm for 15 minutes at room temperature and then discarding the supernatant. The pellet was gently washed with 500µl of 70% alcohol, centrifuged again, as in the previous step, the alcohol discard and the pellet briefly air dried before being resuspended in 500 µl of urea buffer. A P.C.I. extraction was carried out (as above) followed by a chloroform extraction (as above) and the DNA precipitated and pelletted out with absolute ethanol (as above). Finally the pellet was resuspended in 100 µl of TE buffer.

Extraction Method 3 - CTAB + Dichloromethane

Two grams of tissue were ground and a portion placed in an eppendorf tube, as described in the previous method. The powder was suspended in 750 µl of 2xCTAB buffer, that had been warmed to 65°C, by mixing with the tip of a disposable pipette and gentle vortexing. The tube was incubated for 30 minutes at 65°C without any agitation. After cooling to 35-40°C 750 µl of dichloromethane/isoamylalcohol (24:1) were added and mixed with the suspension by gently shaking and flicking of the tube. The tube was centrifuged for 5 minutes at 8,000 rpm and the upper, aqueous phase (of approximately 600ul) was divided between two fresh 1.5ml tubes into which 250 µl of 2xCTAB had already been placed. These tubes were mixed well, but not vortexed, and centrifuged for 5 minutes at 8,000 rpm. The aqueous phases were moved to fresh tubes and this procedure repeated (250 µl 2xCTAB, mixing, centrifuging). The upper aqueous phases were combined in a single tube (giving a total volume of around 500µl) and the RNA digested by the adding 10 µl of RNase A (10mg/ml) and incubating at 37°C for 15 minutes. If there was sufficient space in the tube 600 µl (approximately 0.6 volumes) of isopropanol (room temperature) was added and the DNA that precipitated was pelletted out by centrifuging at 6000 rpm for 5 minutes. If there was insufficient room in the tube to add enough isopropanol then the solution was divided between two tubes and the DNA precipitated by adding 0.6 volumes of room temperature isopropanol to each tube. A single pellet was formed in a single tube by centrifuging one tube as above, removing the supernatant and adding the contents of the second tube and centrifuging again, as above. The DNA pellet was washed for 20 minutes in 1ml of room temperature, 70% ethanol with continuous agitation, then centrifuged for 2 minutes at 6,000 rpm and the ethanol discarded. The pellet were then washed a second time, in exactly the same manner, before being air dried and resuspended in 100 µl of TE.

Extracted DNA was mixed with bromophenol blue loading buffer (Sigma G 2526) run in a 0.8% agarose gel (Sigma A 9539) with 1 x TBE buffer in a Biorad Mini Sub and Wide Mini Sub electrophoresis tanks at 80 volts for 90 minutes then visualised using ethidium bromide. The finished gel was photographed using Polaroid Type 667 film.

Results of Extractions

The first extraction technique was based on one that had been successfully used in wheat and tomato but appeared to be inappropriate for use in *Rhododendron*. Extractions were very high in polysaccharides, so much so in fact that it was difficult to load the extract in the well of submarine gel without it floating out. The second method had been developed and used on *Populus,* which was deemed to be high in polysaccharides. This method appeared to be somewhat more selective, producing less polysaccharide in the final extract but still producing a significant amount. RAPD reactions were attempted with these extracts but with limited success. A commercial DNA purification kit was therefore used as a subsequent clean up stage. The Elu-Quick DNA Purification Kit (Schkeucher & Schuekk, Dassel, German) made use of the affinity of DNA for glass in sodium perchlorate; the DNA being bound to glass beads using a proprietary binding buffer, washed with series of proprietary wash buffers and then eluted from the beads with distilled, deionised water. DNA produced in this manner appeared to be somewhat degraded but would produce RAPD fingerprints. The procedure was, however, exceedingly time consuming and complex with many opportunities for errors to occur. Thirty five samples were extracted successfully using this method. When the third, dichloromethane base, technique was attempted it was found to provide DNA of a superior quality with less polysaccharide present. Because of the perceived dependence of RAPD fingerprints on extraction quality of DNA the first 35 samples were therefore discarded and all samples extracted from scratch using the CTAB-dichloromethane method. This method was also used for all subsequent extractions in the study.

Survey of suitable primers.

The conditions outlined by Castigilione *et al* (1993) were taken as a starting point for the development of a RAPD protocol. Reactions were carried out in a total volume of 25 μl containing 10mM Tris-HCl pH 8.3; 50mM KCl; 5mM $MgCl_2$; 200mM of each dNTP; 0.5μg of primer; approximately 0.2mg/ml template DNA and 0.5 units of Taq Polymerase (Perkin Elmer Ampli Taq). A master mix of ingredients (minus DNA) was made up and aliquoted between tubes containing the template DNA. One tube was always included that contained no template DNA as a negative control. The thermal cycle consisted of: 92°C for 3 minutes followed by a cycle of 92°C for 30 seconds, 45°C for 60 seconds and 72°C for 2 minutes. This cycle was repeated 45 times and the mixture was chilled to 4°C for storage. Thermal cycling was performed with a Perkin Elmer Cetus 9600 machine. 10μl of the reaction products were visualised on a 2% 1:3 standard/wide range agarose gel (Sigma A7431) under the same conditions as were used for visualising genomic DNA, given above. A size standard consisting of a 123 base pair ladder (Sigma D5042) was run alongside the reactions for estimating the sizes of fragments.

Only a few, very feint, bands were produced using these conditions and so the reaction conditions were re-assessed. It was noted that Castigilione *et al* (1993) had used a different thermal cycler (an MJ Research machine) and that this machine had longer ramping times than the Cetus 9600 machine. A profile of the ramping times used by the MJ Research machine was obtained and the Cetus programmed to copy these times. The cycle now ramped took 1 minute 28 seconds to reach 94°C, 3 minutes 48 seconds to reach 37°C and 2 minutes 20 seconds to reach 72°C rather than as quickly as possible. These reaction conditions produced clearer and apparently more repeatable banding patterns. As some samples still produced indistinct patterns another form of *Taq* polymerase (Dynazyme by Finzyme) was substituted for the Perkin Elmer Ampli *Taq* and the reaction ingredients changed to 10mM Tris-HCl pH 8.3; 50mM KCl; 1.5mM $MgCl_2$; 200mM of each dNTP; 0.1% Triton x100; 0.5μg of primer; approximately 0.2mg/ml template DNA and 0.5units of Taq this was found produce more robust reactions.

A survey of the 14 primers listed in Table 31 was carried out using the above reaction conditions and species chosen from across the study group. The species selected for use in the primer study were: *R. vernicosum*, *R. falconeri*, *R. selense*, *R. anthosphearum*, *R. argyrphyllum*, *R. taliense* and *R. sherriffii*. Four of the primers were then selected for use in a survey of all the species, two

10mers (numbers 5 and 7) and two 20mers (numbers 3 and 7). Primers were selected on the basis of producing a number of bright clear bands, some of which were shared with others in the pilot sample and some of which were not. Primers that produced bands that were different in every individual or were the same in all individuals were excluded.

Fingerprinting of taxa

An attempt was made to work through all the DNAs that had been extracted to produce a fingerprint with each of the primers for each of them. Two major problems were run into in carrying out this survey.

Fingerprints were not produced from all primer/DNA combinations, even after a number of attempts at amplification had been made. Some combinations produced no bands at all, others produced only a small number of very faint bands (Figure 40). Once the survey had started it was not possible to adjust the reaction conditions as this may have had an effect on the fingerprints already produced and would entail starting the survey again for that primer.It was also not known whether the new conditions would work for the primer/DNA combinations that worked under the original conditions. The only variable that was altered, therefore, was the concentration of the DNA. This was only sometimes effective in producing a fingerprint. For a few species DNA was extracted for a second or third time in case errors had been made at this stage. Making decisions concerning the homology of bands on different gels became very problematic and a system needed to be devised to overcome this.

Scoring of banding patterns

Banding patterns produced by both the primer survey and the OTU survey proved to be highly polymorphic. A maximum of 26 fingerprinting reactions could be run on any one gel and so it was not possible to directly compare fingerprints produced by more than a small proportion of the taxa being examined, especially as not all the RAPD reactions were successful. It was decided that, because of the erratic nature of the results obtained, fingerprints would only be accepted, and bands scored, if the same DNA/primer combination produced a similar banding pattern on two separate occasions.

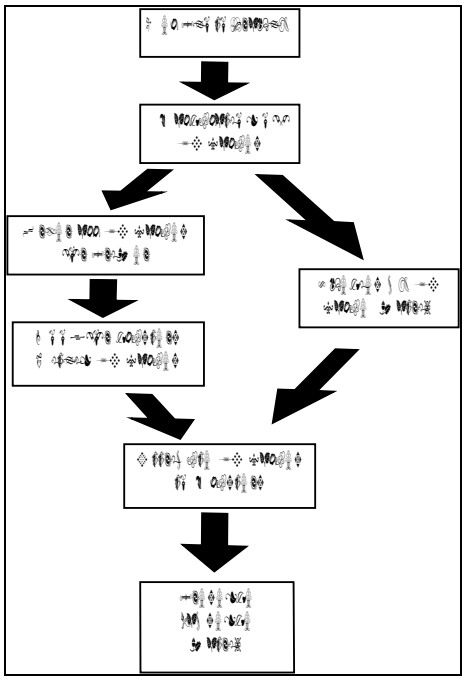
In making decisions about homology it is desirable to take into account all the bands produced by the study. To convert the gel photographs into a presence/absence matrix in the most explicit way possible a sampling strategy and computer program were devised that took all bands on all photographs into account.

Care was taken to ensure that there was a 123 bp size marker run on the outer lanes of each gel run or each portion of gel that was photographed. In order to score a gel a straight edge was fastened across the base of the photograph between the two smallest (and brightest) bands on the size ladder. This line was deemed to be equivalent to the solvent front on a thin layer chromatography plate. Each lane on the gel was then taken in turn and the distance from the well to this ‘solvent front’ measured along with the distance travelled by each of the clearly visible bands in the lane. The RF value for each of these bands was then calculated as a percentage (i.e. the distance migrated by the band divided by the distance migrated by the 123 base pair band multiplied by 100) This method was preferred to making estimates of band size because it was computationally simple and did not imply levels of precision that were not possible using this gel system. Band sizes were not necessary for the study as bands could be identified using relative migration distance.

Once RF values had been recorded for all the reactions made with all the DNAs for any one primer decisions had to be taken as to which bands were homologous with each other and therefore which bands were present in which species. To do this a computer program was written using the Microsoft QBASIC language. The code for this program is given in Appendix D and the operation of the program is summarised in Figure 38. The program took all the RF values for the primer, pooled them, ordered and then looked for clusters within distribution of RF values. Clusters were

defined as collections of RF values that could not be separated from other clusters by more than a set percentage from the averaged centre of that cluster. This percentage was always set at 5% but could be varied to explore the data. Once clusters had been identified the program returned to the RF values listed by species and scored each band as present or absent in the clusters defined, thus producing a presence/absence matrix for the primer. Appendix D contains an example of the output from the program.

Figure 38: *Flow chart illustrating the way in which the RAPD scoring computer program works.*



Analysis of data

The presence/absence matrix produced by the scoring program was examined using a spreadsheet and those bands that only occurred in a single taxon or occurred in all the taxa were deleted. The remaining matrix was then analysed using the NTSYS package mentioned in the Chapter 5. A similarity matrix was constructed using Jaccard's coefficient (Jaccard 1908). This being the most appropriate coefficient for RAPD data as it only assumes similarity due to shared bands. (see discussion in Chapter 3). UPGMA and Neighbour Joining (Saitou & Nei 1987) trees were then constructed from the similarity matrix and the cophenetic correlations of the trees to the data calculated (see Chapter 5 for further details of this procedure.)

RESULTS

Figure 39 shows two of the gels produced during the primer survey; one is an example of a primer that was chosen for use in the taxon survey and the other a primer that was not. Figure 40 is an example of one of the gels used in the taxon survey. It can be seen from both these figures that there is a very high level of polymorphism between the different fingerprints.

Figure 39: *Primer Survey. Gel 1 is an example of one of the primers (20mer-03) that was subsequently used in the taxon survey. Gel 2 is an example of one that was not used (20mer-05). Control lanes are not shown.*

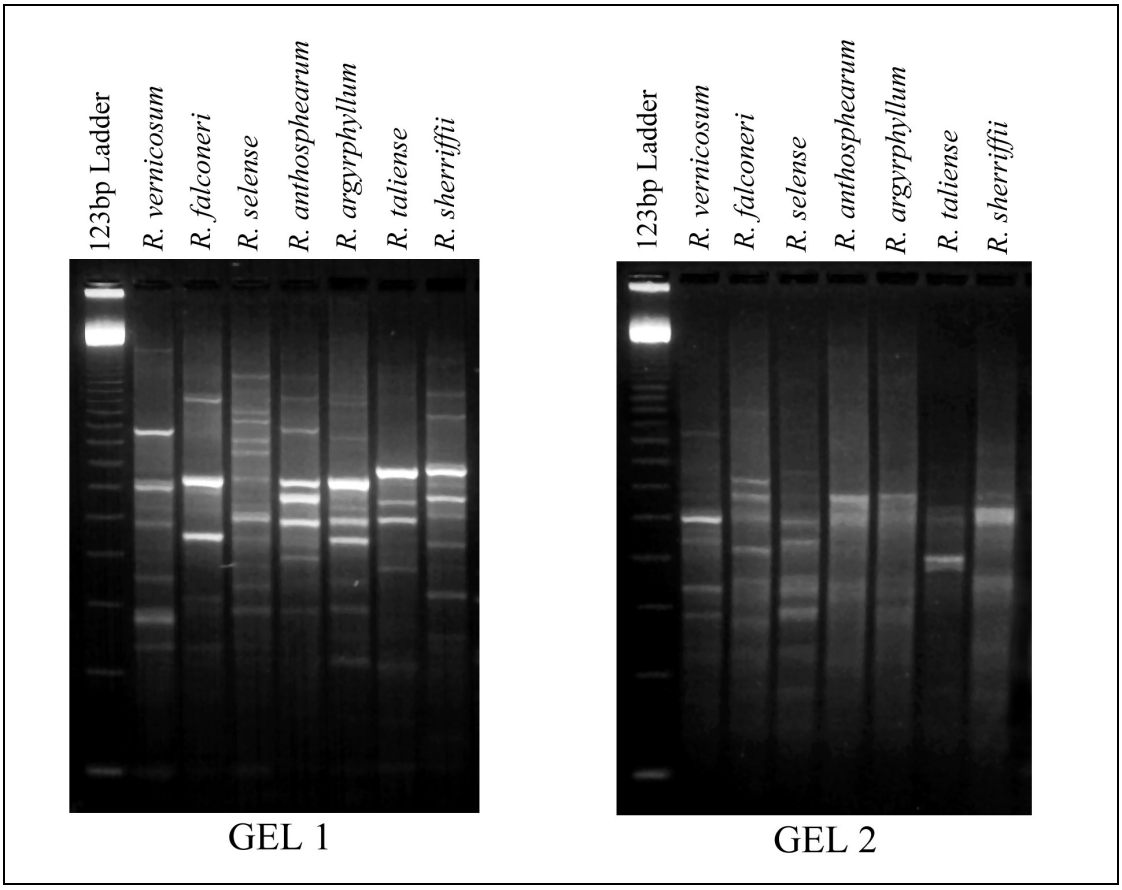
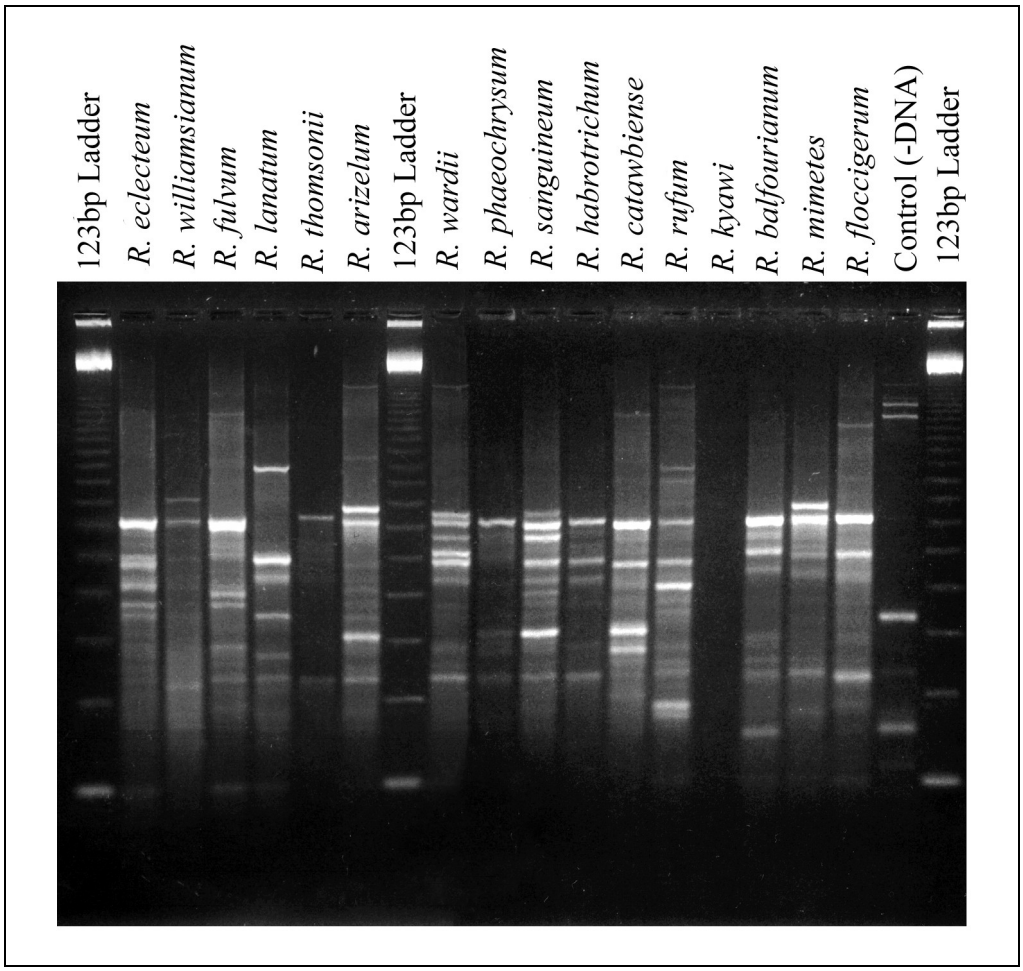


Figure 40 shows three other features that were symptomatic of the survey. The DNA free control reaction appears have produced a number of bands. This is thought to be due to contamination in one of the reaction ingredients, most likely the Taq polymerase, or to be caused by secondary structures being produced by the primers themselves. They are a common feature of RAPD reactions and are rarely consistent. The second feature of note is the completely blank lane produced by the *R. kyawii* reaction. It is remarkable that this reaction did not even produce the bands that are present in the control lane. This was a common occurrence throughout the study and was initially thought to be due to a Taq inhibitor present in the extraction. Specific PCR control reactions did not confirm this hypothesis (see Chapter 7) but personal communications with other workers using RAPD have confirmed that it is a common, if erratically occurring, aspect of the assay. A description of this phenomenon was not encountered in the literature. The third feature of note from this figure can be seen in the lane for *R. thomsonii.* This reaction appears to have 'fired' in that the Taq polymerase has amplified something but the reaction products do not appear to be compatible with those produced in the other samples. This was another common feature of the study.

Figure 40: *An example of an RAPD fingerprint gel from the taxon survey (using the primer 10mer-05).*



After several attempts had been made at fingerprinting all the extracted DNAs with the four chosen primers it was decided to carry out an assessment of the results obtained so far and to analyse those banding patterns that had been produced. Reproducible banding patterns had only been secured for 17 of the 80 extracted DNAs for the 4 primers and not all DNA/primer combinations had worked reliably leaving blank areas in the resulting matrix (The matrix is given in Appendix D).

Scoring of the gel images was problematic as it was only possible to measure band positions to the nearest 0.5mm. Analysis of the patterns gave a very weak hierarchical structure with cophenetic correlations of 0.12 for the NJ tree of the 17 taxa and 0.65 for the UPGMA tree. The seventeen taxa that were successfully scored did not represent an adequate cross section of morphological variation.

Conclusions

As was outlined in Chapter 3 RAPD does not produce markers that are ideally suited to phylogenetic analysis. It was assumed, however, that from the point of view of this study the advantages the technique had (in being quick and easy to set up) out-weighed these disadvantages. It became clear in January 1995 that RAPD was not proving easy to implement and that only relatively small amounts of data were being produced. In addition, it had proved possible to carry out specific PCR reactions on DNAs that were not proving suitable for RAPD reactions and this opened up the possibility of using other techniques, such as PCR-RFLP and sequencing (details of these techniques are given in the following chapters). In the light of these results and the theoretical questions raised by papers such as Clark & Lanigan (1993) it was decided that no more resources should be devoted to this technique.

With the benefit of hindsight there are a number of aspects of this study that should have been done differently. The aspects are listed below and, as they are of general nature and could be applied other RAPD studies; they take the form of a series of practical recommendations.

- RAPD is neither a simple nor and easy technique and should not be undertaken lightly by inexperienced molecular biologists as there are large number of variables that must be optimised and pipetting accuracy and good laboratory techniques is vital.
- Studies should be carried out on a small scale, examining only the number of samples that will fit on a single electrophoresis gel. This would typically be in the region of 26 samples on a 30 lane gel (allowing for a DNA free control and three size standards).
- DNA should be as clean as possible. Caesium Chloride gradient centrifugation would be an advantage.
- All DNAs should be extracted in the same way and preferably at the same time as fingerprints and optimisation of the RAPD reaction may be effected by contaminants.
- It is preferable that primers are purchased from a supplier who supplies them specifically for RAPD such as Operon Technologies or Pharmcia Biotech. This allows for some comparison with other studies though not the pooling of results.
- Conditions should be optimised for each primer used separately.
- Primers should be selected that give relatively few bands; there should be no danger of bands being confused on the gel.
- As large a number of primers as possible should be used.
- Reactions should be carried out in large enough volumes that they can be loaded onto a gel several times. It is rare for all the reactions on a gel to work perfectly first time and this allows for model gels to be run for scoring purposes. It is also useful if it becomes necessary to look at more samples than will fit on a single gel as different combinations reactions may be loaded for comparison.
- It appears that RAPD studies are most useful within species boundaries typically comparing populations or cultivars, often with a narrow genetic base. (e.g. Castiglione, *et al* 1993 and Smith *et al* 1994.)
- Even if reproducible banding patterns are obtained and scored, care should be taken that the correct analysis technique is employed. This is still an area of some debate. (see Chapter 3).

CHAPTER 7:RESTRICTION SITE ANALYSIS OF PCR AMPLIFIED FRAGMENTS

INTRODUCTION

One of the problems encountered in the course of the RAPD study outlined in the previous chapter was the failure of some of the DNAs to produce fingerprints with some or all of the primers. One hypothesis was that there was a substance present in some of the extractions that could inhibit the action of *Taq* polymerase. As a control specific PCR amplifications of the internal transcribed spacer regions of the nuclear ribosomal gene were attempted. These amplifications were successful and it was concluded that an inhibitor was not present in the extractions and that the problem lay elsewhere in protocol. A by-product of this process was the production of amplified portions of a highly variable region of the genome. It was thought that it would be of interest to examine these PCR generated fragments as a possible source of data. As sequencing facilities were not at the time available but restriction enzyme digestion could be easilly applied the fragments were, digested and polymorphisms were identified. This was the beginning of the PCR-RFLP study which is described in this chapter and which lead on to the sequencing study outlined in the following chapter.

Regions

As described in Chapter 3, restriction site data has advantages over data produced by anonymous, random markers such as RAPDs and AFLPs in that it is more demonstrably homologous and potentially verifiable. It is important that the data is restriction **site** rather than restriction **fragment** data for these benefits to accrue. Restriction analysis of PCR fragments (PCR-RFLP) is useful in that it is more straight forward to analyse the fragment patterns produced (so as to hypothesise gain and loss of restriction sites) than it is with total genomic or organellar digests, where maps must be devised. Decisions must be made, however, as to which regions will be studied. A study region must contain a sequence which varies at a rate suitable for detecting changes at the taxonomic level being addressed and PCR primers must be available. Ideally more than one region should be studied (Doyle 1992).

The first decision to be taken in selection of a region is whether it will come from an organellar or the nuclear genome. The chloroplast is the more well known of the plant cytoplasmic genomes (complete sequences now being available for several species) whilst the mitochondria is more complex in structure and somewhat less studied in plants. In animals the mitochondrial genome is widely used in phylogenetic studies. Cytoplasm is usually maternally inherited in plants, with the notable exception of the gymnosperms in which it is predominantly paternally inherited. Kron (1993) showed that, in subgenus *Pentanthera* at least, the cytoplasm is maternally inherited within the genus *Rhododendron*. Cytolplasmic genomes are not subjected to the recombination events associated with meiosis and fertilisation and so never undergo the reticulation process associated with hybridisation and speciation in the nuclear genome. Any structure that exists in their organellar nucleotide sequence data is therefore more likely to resemble a nested hierarchy of changes reflecting evolutionary history than that found in the nuclear genome. A combination of these factors has lead to the widespread use of chloroplast restriction and sequence data for reconstruction of phylogenies culminating in recent years with an analysis of the relationships between the majority of seed plant families using the rbcL gene. (Chase *et al* 1993).

Organellar molecular data is not the panacea for problems encountered in the reconstruction of species phylogenies however. The histories reconstructed from organellar data are the histories of the cytoplasm not those of the species-lineage itself. It is possible, through processes such as lineage sorting and hybrid speciation, for organelles to have different histories to the nucleus (Rieseberg & Soltis 1991, Doyle 1992). The mechanisms that give the organellar DNA the ability to record history so effectively are contrary to the mechanisms that maintain diversity in the nucleus. It

is therefore not surprising that diversity in the chloroplast genome is generally lower than that in the nucleus, reducing the resolution of data from this source. (A possible exception to this rule and a possible future source of data are chloroplast specific microsatellites. Powell *et al* 1995a & 1995b). Possible regions for investigation within the chloroplast have been outlined by Olmstead and Palmer (1994) and by Demesure *et al* (1995) (who also includes details of some mitochondrial regions).

As has already been mentioned, subgenus *Hymenanthes* consists of a large number of rapidly evolving and hybridising entities. Both these factors suggest that cytoplasm based markers may not be appropriate in analysing diversity in the group. The hybridisation events are likely to lead to populations, or even proto-species that either contain two chloroplast types (leading to sampling problems) or that contain the cytotype of only one of the parents. This has been demonstrated to occur in hybrid swarms between *R. flammeum* and *R. canescens*; many individuals in sampled populations being morphologically indistinguishable from *R. flammeum* but possessing the chloroplast genome of *R. canescens* (Kron 1993). Ctyoplasm based markers were therefore ruled out as a first choice of markers.

The nuclear genome is far more complex than the chloroplast genome, is less well known and has been used less as a source of data for constructing phylogenies. Much of what is known of the nuclear genome is based on sequencing, via reverse transcriptase, of functional genes in cDNA libraries. This technique does not generate information about the potentially more phylogenetically useful introns and intergenic spacers and has not generated a large number of useful regions.

The ITS regions of nrDNA

One region that has been used extensively is the internal transcribed spacer unit of nuclear ribosomal DNA. The nuclear ribosomal gene (nrDNA) consists of several parts. There are three coding regions that code for the 18S, 5.8S and 25S ribosomal proteins, there are two transcribed spacer regions that separate these coding regions, known as the Internal Transcribes Spacers (ITS1 and ITS 2) and there is an Intergenic Spacer (IGS) that contains controlling elements for transcription. The nrDNA gene is a multi copy gene with numerous copies being arranged as tandem repeats. Active nrDNA genes arranged at a single locus are known as nucleolus organiser regions (NORs) because the nucleolus, the site of nrDNA transcription and ribosome assembly, is organised around the nrDNA genes during interphase. These regions may be detected cytologically with silver staining at mitosis. The coding regions of this gene are highly conserved yet the ITS regions vary greatly. The two ITS regions plus the 5.8S region total around 1000 base pairs in length in most species. This provides an almost ideal system for phylogenetic analysis. PCR primers may be designed in the 18S and the 25S regions that will work over an extremely broad range of taxa (from fungi all the way to *Rhododendron*) and yet the ITS1 and ITS2 regions that lie between these priming sites are highly variable and thus may provide a large amount of information, either through use of a restriction site study or a sequencing study. In the latter case the length of the ITS/5.8S region is sufficient for long run sequencing reactions to span the whole region, or if reactions are problematic internal sequencing primers may be designed for the highly conserved 5.8S region thus allowing the entire region to be sequenced using either two or four sequencing reactions.

The potential weakness of the ITS system lies in the fact that the genes are present in the genome in many copies. As there appears to be little variation in gene sequence within a NOR region and even between NOR regions on different chromosomes within an individual or species it appears there must be a homogenisation mechanism that ensures the mutations occurring in one gene copy are either spread to all other copies or removed. Recent evidence suggests that mutations are homogenised rapidly within any NOR region but spread more slowly to others. A review of this process was given by Schlotterer and Tautz (1994). Genes evolving in this manner provide more complex models or analysis of evolutionary history than single copy or plastid genes, although the

ITS region has been treated as if it were a single copy gene in many studies (e.g. Downie & Katz-Downie 1996). Base pairs within the region are also typically treated as independent although by their presence the ITS regions indicate that they may well have a function and conserved sequences have been found within the sequences (Liu & Schardl 1994) The strength of the system is that if a taxon under examination was of recent hybrid origin, (i.e. two distinct ITS types have just come together within a single individual) it would be apparent from the restriction site or sequence data. This has been demonstrated in *Paeonia* by Sang *et al* (1995) where a reticulate evolutionary pattern was derived from ITS sequence data.

The ITS region of nrDNA therefore appears to have a number of characteristics that make it an ideal gene to examine in subgenus *Hymenanthes*. It is one of the most variable genes that have been used in phylogenetic studies, it is capable of detecting reticulate evolution (and possibly even modelling it) and primers that appear to work universally are readily available. It is fortuitous, then, that a pair of ITS primers were available to use in the control reactions during the RAPD study.

Baldwin *et al* (1995) gives a more in detailed review of the use of ITS of nrDNA in phylogenetic reconstruction.

*mat*K - A chloroplast region.

Although chloroplast data is likely to be less sensitive to detection of reticulate evolution its ability to retain a higher level of hierarchical information means that it may still be valuable, especially in combination with data from a nuclear gene. In their review of chloroplast DNA methods Olmstead and Palmer (1994) catalogued a series of regions for potential study (their table 1, page 1213). The most variable of these genes, based on a comparison of tobacco and rice, is the *mat*K gene. The *mat*K gene occurs as a 1.5 kilobase region embedded within a 2.5 kilobase intron that interrupts the two *trn*K exons. It was formerly termed ORFK and ORF509 and encodes for a maturase involved in splicing type II introns from RNA transcripts (Wolfe *et al* 1992). It has been successfully used in a number of phylogenetic investigations, notably in Saxifragaceae (Johnson & Soltis 1994) and Polemoniaceae (Steele & Vilgalys 1994). On the grounds of its apparent variability and utility this gene was chosen for the present study; to provide a complementary data set to that produced by the work on ITS. Primers were selected in the two exons of the trnK gene (after Johnson & Soltis 1994) so that the whole trnK intron could be amplified and cut, including the potentially more variable non-coding regions that flank the matK gene itself. This entire region will be referred to as matK for the rest of the study.

Materials

Plant material

Having learnt from the over ambitious approach taken in the RAPD study a subset of 27 species was selected from the set of exemplar species that had been used in the morphological study (Chapter 5) with the additional criterion that they should occur as wild origin, living specimens in the collections of RBGE. No outgroup species were included initially in this group as it had been established that an ITS sequencing program (that was being carried out as part of the 'Azalea' project) would provide the relevant data to root the trees produced. Material was collected from cultivation at the Royal Botanic Garden Edinburgh and DNA extracted using the third, dichloromethane technique outlined in Chapter 6. The species, with the accession number of plants used and a reference to their subsection are given in Table 32.

Table 32: *Table of specimens used in the PCR-RFLP study. Accession numbers are those used in the living collections at Royal Botanic Garden Edinburgh. Subsection numbers are those used by Chamberlain (1983).*

Species	Accession	Subsection	Species	Accession	Subsection
R. adenosum	19300433A	9	*R. lanatum*	19810957A	17
R. arboreum	19790276A	14	*R. neriiflorum*	19200019A	22
R. argyrophyllum	19090009A	13	*R. phaeochrysum*	19698780A	15
R. auriculatum	19160027A	2	*R. ponticum*	19773079A	12
R. barbatum	19751635A	21	*R. roxieanum*	19734042A	15
R. campanulatum	19720857D	18	*R. sherriffii*	19560423A	23
R. campylocarpum	19832543A	6	*R. strigillosum*	19754050A	7
R. falconeri	19790170A	4	*R. taliense*	19568652B	15
R. fulvum	19180010D	16	*R. thomsonii*	19803353A	24
R. griersonianum	19320271B	19	*R. venator*	19754062A	16
R. hirtipes	19251063A	8	*R. vernicosum*	19141012A	1
R. irroratum	19812433A	11	*R. watsonii*	19500299A	3
R. kyawii	19772748B	20	*R. williamsianum*	19320138A	5
R. lacteum	19490491A	15			

Primers

The primers used to amplify the ITS region of the nrDNA were those originally designed for use in fungi (White *et al* 1990) but subsequently used in higher plants notably Compositae (Baldwin 1992). The primers used to amplify matK were taken directly from Johnson & Soltis (1994). All primers were synthesised by Oswell Limited, Southampton (formerly Edinburgh) and their sequences are given in Table 33.

Table 33: *Sequences of primers used in the PCR-RFLP survey.*

Name	Location	Direction	Sequence
ITS5	18S nrDNA	Forward	5'-GGAAGTAAAAGTCGTAACAAGG-3'
ITS4	26S nrDNA	Reverse	5'-TCCTCCGCTTATTGATATGC-3'
trnK-3914F	5'trnK exon	Forward	5′-TGGGTTGCTAACTCAATGG-3′
trnK-2R	3'trnK exon	Reverse	5′-AACTAGTCGGATGGAGTAG-3′

Enzymes

A total of 16 restriction enzymes were used in the course of the study. All were supplies with manufactures buffer at 10x concentration. Table 34 summarises the enzymes used in the study, their recognition sites, working temperature and commercial supplier.

Methods

PCR Amplification.

All PCR reactions were carried out in a total reaction volumes of 50µl using Dynazyme taq polymerase on a Perkin Elmer Cetus 9600 thermal cycler. Each reaction contained: 10mM Tris-HCl pH 8.3; 50mM KCl; 1.5mM $MgCl_2$; 200 µM of each dNTP; 0.1% Triton x100; 1.0µM of each primer; approximately 0.2mg/ml template DNA and 0.5units of Dynazyme polymerase (Finnzymes Oy, Finland). For amplification of ITS the following thermal cycle was used: 94°C for 3 minutes followed by 30 cycles of 94.0°C for 1 minute, 55°C for 2 minutes and 72°C for 1 minute. The reaction was finally cooled to 4°C for storage. For matK a different cycle was used: 94°C for 1 minute followed by 20 cycles of 94°C for 1 minute 30 seconds, 48°C for 2 minutes and 72°C for 3 minutes there was then a final annealing period of 15 minutes at 72°C before the reactions were cooled to soak to 4°C for storage.

Table 34: *Restriction enzymes used in the PCR-RFLP study. The actual cut sites are marked by a "/" in the recognition sequence.*

Enzyme	Recognition sequence	Commercial Supplier	Temp.
Ase I	AT/TAAT	New England Biolabs 104S	37°C
Ava I	C/PyCGpuG	Sigma R-3379	37°C
*Bam*H I	G/GATCC	Sigma R 0260	37°C
Bgl I	GCCNNNNN/NGGC	Sigma R-6753	37°C
*Bst*N I	CC/(AT)GG	Sigma R-2759	60°C
Cfo I	GCG/C	Sigma R-1761	37°C
Cla I	AT/CGAT	Sigma R-7763	37°C
*Eco*R I	G/AATTC	Sigma R-2677	37°C
*Eco*R V	GAT/ATC	Sigma R-2756	37°C
HAE III	GG/CC	Sigma R-5628	37°C
Hind III	A/AGCTT	New England Biolabs 104S	37°C
Hinf I	G/ANTC	Sigma R-6760	37°C
Msp I	C/CGG	Sigma R-4506	37°C
Rsa I	GT/AC	Sigma R-4756	37°C
Sal I	G/TCGAC	Sigma R-0754	37°C
Taq I	T/CGA	NBL Genescience011106	65°C

All PCR products were visualised on a 2% 1:3 standard/wide range agarose gel (Sigma A7431) under the same conditions as were used for visualising genomic DNA, outlined in the previous chapter. Estimates were made of the concentration of DNA in the products by on gel comparisons with known standards (Gibco BRL Phage λDNA 14420-012). Successful reactions contained greater than 2ng/µl of DNA. If DNA concentrations were slightly below this level the reaction mixture was concentrated using a rotary evaporator (Savant DNA Speed 110) to reduce the volume. If the concentrations were judged to be a great deal below this level (<1ng/µl) the tube was discarded and another PCR reaction carried out. A check was made at this stage that there were no apparent size differences between the different fragments.

Restriction enzyme digestion

All restriction enzyme digests were conducted using the same conditions except for a slight modification for those that had optimum working temperatures of over 37°C. PCR reaction products with at least 2ng/µl DNA were digested directly; without further treatment. 5µl of reaction mixture were incubated with approximately 1.5-2 units restriction enzyme, 1.5µl manufacture's enzyme buffer (10x) and 1.5µl loading buffer (10x) in a total volume of 15µl water. The loading buffer was a 0.5% Orange G with 60% sucrose (at 10x) in water. The bromophenol blue based marker used during DNA extraction and in the RAPD study (Sigma G 2526) was found to inhibit the action of the restriction enzymes and so an Orange G (Sigma O-3756) based buffer was used. Orange G was chosen as a tracking dye because it migrated significantly faster than bromophenol blue. Tests were carried out to confirm that the loading buffer did not inhibit the action of the enzymes.

The majority of the enzymes have optimum working temperatures of 37°C. The reactions for these enzymes were carried out in 96 well microtitre plates. Because the plates are designed to allow the samples to 'breath' (even with a lid in place) and the reactions are being carried out in very small volumes a special assembly was devised. This employed a piece of high density foam rubber (of the type used in camping bed rolls) cut to fit just inside the lid of the plate. Reactions were set up in the microtitre plate on a bed of ice, and a sheet of cling film stretched over the top. The foam rubber and lid were then pushed down onto the plate and secured with two rubber bands. The plates could be incubated and stored in the refrigerator or freezer without loss until they were run on a gel. Care was taken to ensure the plates remained flat whenever the contents of the wells were liquid. If the

plate was ever opened and resealed the cling film would be replaced. Incubation was carried out in a hybridisation oven (Hybaid).

Some of the enzymes required working temperatures of over 60°C. Unfortunately the polystyrene microtitre plates used for the other enzymes would buckle and the lids leak at these temperatures and so reactions with these enzymes were carried out in 0.75ml ependorf tubes placed in dry heating blocks (Techne DriBlock DB2A).

The ITS region was digested with all 16 enzymes the *mat*K region with only 8.

Visualisation and estimation of fragment sizes

Restriction digests were visualised on 1:3 standard/wide range agarose gel (Sigma A7431) electrophoresis gels under the same conditions outlined in Chapter 6. Following the manufacturers recommendations 3% gels were used for the ITS fragments (which were all less than 1000 base pairs in length) and 2% gels were used for the *mat*K fragments (which were likely to be over 500 base pairs in length).

A 123 base pair ladder (Sigma D5042) was run along side the digests as a size standard and the distances of migration of the ladder fragments and the digest fragments were measured to the nearest half millimetre from the photograph of the gel. The least squared method was then used to calculate the approximate sizes of the fragments. To do this an implementation of a program written by Schaffer (1981) in FORTRAN (but converted into BASIC by Russel (pers. com.) and into MICROSOFT QBASIC by the author) was used.

Reactions were loaded in two different ways. For the main screening of the 27 exemplar taxa the reactions were loaded along side each other so as to make it easier to spot any size polymorphisms; an example is given in Figure 41. If clarification of the scoring of any individual was necessary, typically due to weak PCR reactions, then a PCR-RFLP fingerprint was produced, and an example is shown in Figure 43.

For each enzyme that gave polymorphic restriction patterns the cut sites were identified by hand. The level of polymorphism was so low that it was possible to do this for all but one enzyme and be sure that, from the point of view of this part of the study, the sites were homologous. Towards the end of the study ITS sequences became available from within *Rhododendron* and this greatly facilitated mapping cut sites as well as identifying certain anomalies. A cladogram of the groups formed by the polymorphic restriction sites was calculated by hand.

Results

Very low levels of polymorphism were detected in the ITS study and no polymorphism was detected in the matK study.

Figure 41 is an example of part of the survey of the ITS fragments and illustrates a number of features that were observed throughout the study. Where polymorphisms were found between taxa they were clearly visible, for example the differences between *R. fulvum* (which lacks the cut site termed Msp I-540) and *R. neriiflorum* which has that cut site. In *R. neriiflorum* the 540 base pair fragment is missing and is replaced by one of around 490 base pairs in length and one around 50-60 base pairs long. The smaller fragment is only just visible. With a number of the enzymes the occurrence of small fragments could be deduced by the effect of their loss on the larger fragments but were often not visible if they were less than around 70 base pairs in length. The lanes for *R. adenosum*, *R. venator*, *R. irroratum* and *R. lacteum* all show additive banding. Here all the bands found in the previous two examples are present, although the 540 base pair band is much fainter than in the previous examples. There are two main explanations for this. Either the samples have internal polymorphisms or the DNA has not been digested completely. The latter explanation is unlikely as in this case one would expect a small amount of DNA to be present at one or more of the

possible intermediate fragment sizes (e.g. 270 or 710) or as undigested DNA. *R. lacteum*, which also shows additive banding, does show some evidence of partial digestion as un-cut DNA is present as a fragment around 750 base pairs long but none of these symptoms are shown by the first three lanes. The most extreme example of additive banding and loss of small bands was found with the enzyme Taq I. It gave a large number of bands that could not be summed to make a fragment the same size as the PCR amplification product. Some of the bands were of varying intensity and so, although it was likely that polymorphism of some kind was present, fragments were not scored because clear statements of homology could not be made. Where ever additive banding occurred in other digests one set of bands was always much fainter than the other. The brighter bands were taken as being representative of the individual from the point of view of scorng the characters.

Figure 43 shows the PCR-RFLP fingerprint for the matK region of *R. ponticum*. This differs from the ITS region (Figure 41) in that bands are much larger and clearer and there are no signs of additive banding.

Table 35 summarises the results of study for the different enzymes. Only three scorable polymorphic restriction sites were found. The presence/absence matrix for these sites is given in Table 36.

It can be seen from Table 36 that there are five different combinations of restriction site found. Seven species (group A) have the sites BstN I and Cfo I, sixteen species (group B) have all the restriction sites, two species (group C) have just Msp I, *R. ponticum* has Cfo I and Msp I and *R. campanulatum* has none. It is relatively straight forward to carry out a cladistic analysis of so few taxa by so few characters and the resulting most parsimonious tree is shown in Figure 42. Three of the species that were highlighted in the trees produced by the morphological study are represented in this smaller set of exemplars. These species are *R. fulvum*, *R. falconeri* and *R. arboreum* and were found distributed across the groups A, B and C.

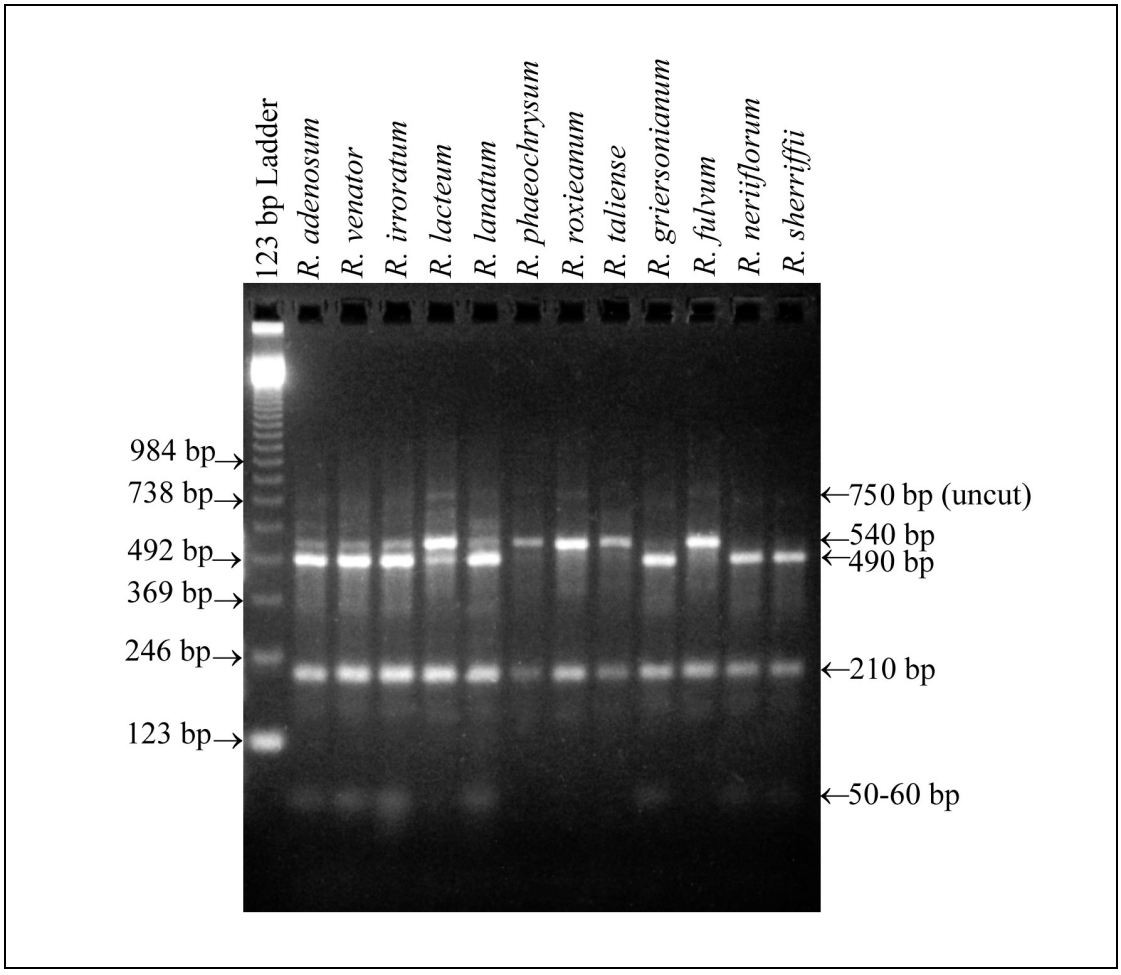
Table 35: *Summary of results for the different enzymes used in the PCR-RFLP study.*

Enzyme	ITS of nrDNA	matK intron of trnK
Ase I	No cut sites, No polymorphism	No cut sites, No polymorphism
Ava I	No cut sites, No polymorphism	No cut sites, No polymorphism
*Bam*H I	No cut sites, No polymorphism	Not Used
Bgl I	No cut sites, No polymorphism	Not Used
*Bst*N I	2 cut sites, 1 polymorphic	Not Used
Cfo I	3 cut sites, 1 polymorphic	No cut sites, No polymorphism
Cla I	1 cut site, No polymorphism	Not Used
*Eco*R I	No cut sites, No polymorphism	1 cut site, No polymorphism
*Eco*R V	1 cut site, No polymorphism	No cut sites, No polymorphism
HAE III	2 cut sites, No polymorphism	Not Used
Hind III	No cut sites, No polymorphism	1 cut site, No polymorphism
Hinf I	2 cuts sites, No polymorphism	Not Used
Msp I	2 cut sites, 1 polymorphic	1 cut site, No polymorphism
Rsa I	1 cut site, No polymorphism	3 cut sites, No polymorphism
Sal I	No cut sites, No polymorphism	Not Used
Taq I	Complex pattern not interpret	Not Used

Table 36: *Occurrence of three polymorphic restriction sites in the 27 exemplar taxa examined. Taxa are listed in subsection order. (1 = site present, 0 = site absent).*

Species	*Bst*N I	*Cfo* I	*Msp* I	Species	*Bst*N I	*Cfo* I	*Msp* I
R. vernicosum	1	1	0	*R. lacteum*	1	1	0
R. auriculatum	1	1	1	*R. phaeochrysum*	1	1	0
R. watsonii	1	1	1	*R. roxieanum*	1	1	0
R. falconeri	1	1	1	*R. taliense*	1	1	0
R. williamsianum	1	1	1	*R. fulvum*	1	1	0
R. campylocarpum	1	1	1	*R. lanatum*	0	0	1
R. strigillosum	1	1	1	*R. campanulatum*	0	0	0
R. hirtipes	1	1	0	*R. griersonianum*	1	1	1
R. adenosum	1	1	1	*R. kyawii*	1	1	1
R. venator	1	1	1	*R. barbatum*	1	1	1
R. irroratum	1	1	1	*R. neriiflorum*	1	1	1
R. ponticum	0	1	1	*R. sherriffii*	1	1	1
R. argyrophyllum	1	1	1	*R. thomsonii*	1	1	1
R. arboreum	0	0	1				

Figure 41*: Msp I digest of ITS of nrDNA for 12 species. Figures on the right of the gel are approximate fragment sizes in base pairs, those on the left are actual sizes of ladder fragments.*

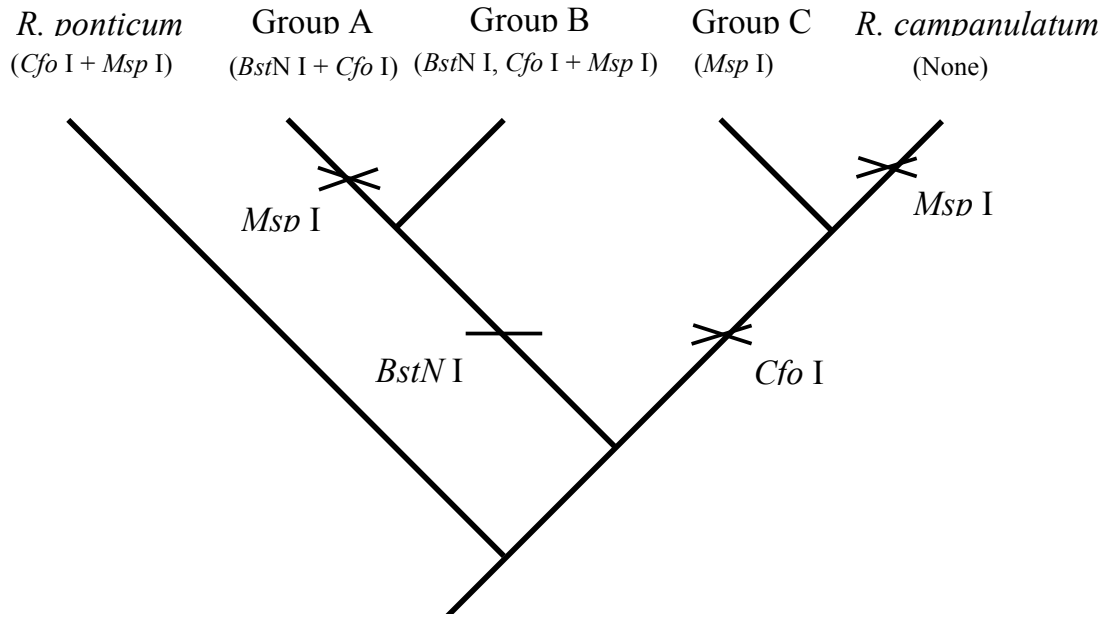


Conclusions

PCR-RFLP has been used successfully in a number of studies, examples include Arnold *et al* (1991), Laguerre, G.; Rigottier-Gois, L. & Lemanceau, P. (1994), Liston (1992), Liston *et al* (1992),

Quinn, W. (1992), Reiseberg, Hanson & Philbrick (1992) and Slade *et al* (1993). It has proved that it is capable of producing clear, scorable taxonomic characters in this study. Unfortunately the number of characters produced is very small. A pilot study that was carried out in parallel, (using the same protocol and eight of the same enzymes to examine the ITS region for twelve species) on *Matthiola* (Cruciferae) produced six scorable characters in only three working days. This, combined with the success of the studies listed above, suggests that the low level of variability in the markers is due to a low level of variation within the regions examined, rather than the low resolution of the technique.

Figure 42: *The most parsimonious tree for the five groups and three characters produced by the study. Length = 4. Rooting on R. ponticum is arbitrary.*



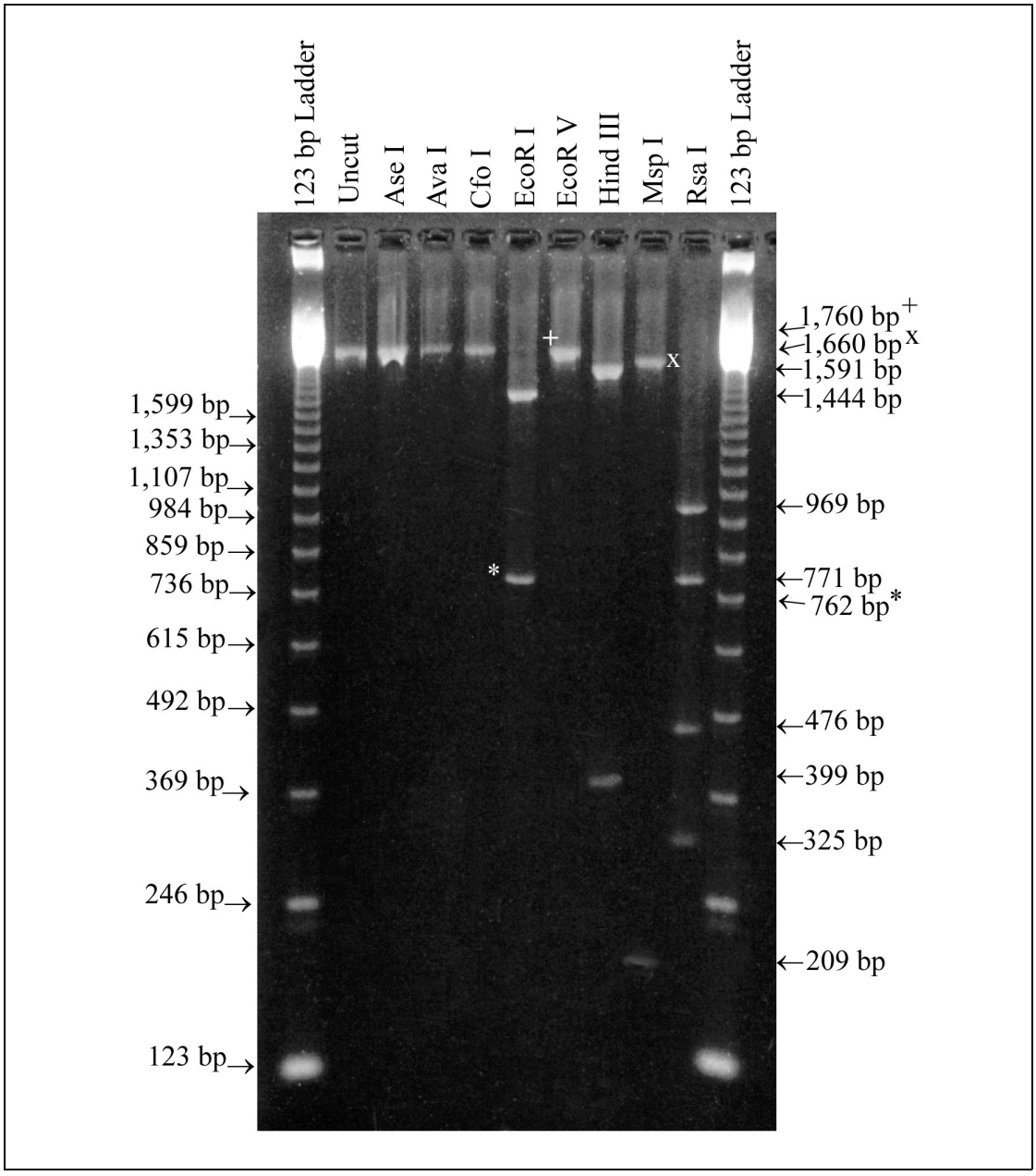
Towards the end of this part of the study sequences of the ITS region of *Rhododendron* as a whole became available as well as some from within subgenus *Hymenanthes*. This had two effects; it became apparent that some of the restriction site data that had been obtained may be misleading and it appeared that, because of the low level of variation within the region, it would be more efficient to gather data by sequencing than by continuing to digest the ITS region with different enzymes. The PCR-RFLP study was therefore halted and the ITS regions for the 27 exemplar taxa were sequenced. This is the topic of the next chapter. The data from the ITS restriction site survey and the sequencing study are obviously intertwined and as a comparison of the results will be given in the final chapter no further discussion will be made of it here.

The occurrence of additive banding is indicative that at least some of the taxa are either of hybrid origin or are rapidly speciating. The fact that the additional bands were typically very faint in comparison to the main bands indicates that there is one major sequence type present in the ITS populations in each individual or that one type is favoured over another by the PCR reactions. The faint additional bands may even be the result of pseudo genes that are no longer functional but are still picked out by the PCR conditions. These results indicate that care should be taken when interpreting the sequencing reactions in Chapter 8.

The matK region was chosen as being the most variable chloroplast region for which primers were available (Olmstead & Palmer 1994). As digestion with eight enzyme had yielded no polymorphisms it was concluded that the region was not very variable within the study group and that it would be more practical to take a sequencing approach here as has been taken in other studies (e.g. Johnson & Soltis 1994 and Steele & Vilgalys 1994). If this region failed to produce significant data then another possibility may be to examine the chloroplast specific microsatellites described by Powell *et al* (1995a and 1995b) although this would entail overcoming the problems associated with

construction of phylogenies from this kind of marker that were outlined in Chapter 3 although the authors have stated that they do not believe these restriction hold for chloroplast SSRs. As had been established at the beginning of this chapter the chloroplast was given a lower priority than ITS region.

Figure 43: *Digest of matK region of R. ponticum by eight different enzymes. Figures on the right of the gel are approximate fragment sizes in base pairs, those on the left are actual sizes of ladder fragments.*



Chapter Summary

- A PCR-RFLP study was undertaken looking at two regions, ITS and *mat*K.
- A procedure based on microtitre plates was devised to screen samples rapidly.
- A very low level variation was encountered in the ITS region.

- No variation was encountered in the *mat*K region.
- Data from the ITS region was analysed and a tree produced.

CHAPTER 8: SEQUENCING STUDY

Introduction

Towards the end of the PCR-RFLP study the opportunity arose to spend several weeks at Professor Guido Volkaert's laboratory at the Catholic University of Leuven, Belgium. As part of the 'Azalea' project this laboratory was sequencing the ITS of nrDNA of a number of species of *Rhododendron* from across the genus (details in Appendix G). During this training period, sequencing techniques and allied protocols involving the use of a ABI 373 sequencing machine were attempted, these include dye terminator and dye primer chemistries as a well as the cloning of PCR fragments. (see Chapter 3). Although a complete data set was not produced at this time, enough partial sequences were produced to enable conclusions to be drawn, regarding the nature of the restriction sites, that lead to the cessation of that part of the project (see Chapter 9) and the decision to sequence these fragments instead. Two months were therefore devoted to producing a complete sequence data set for the 27 exemplar species chosen at the outset of the PCR-RFLP study as outlined below. Two of these species were subsequently included within the 'Azalea' project and so acted as controls between two different sequencing methods. Sequences from the 'Azalea' project were also used as outgroups in the analysis.

Materials

Plant Material and DNA

The same plant material was used during the sequencing study as in the PCR-RFLP study and total genomic DNA was extracted in exactly the same way using the third, CTAB/phenolchloroform method outlined in Chapter 6. Table 32 gives details of the taxa used.

Primers

PCR amplification and two of the sequencing reactions for each fragment were carried out using the same primers as described in Chapter 7. In addition to these, two internal primers were used to sequence out from the 5.8s region. These primers were designed as part of the 'Azalea' project and differ somewhat from the fungal primers advocated by White *et al* (1990). The sequences of all these primers are given in Table 37.

Table 37: *PCR and sequencing primers. (ITS2R and ITS3R were specifically designed for use within Rhododendron during the 'Azalea' study.)*

Name	Location	Direction	Sequence
ITS2	5.8S nrDNA	Reverse	5'-GCTGCGTTCTTCATCGATGC-3'
ITS2R	5.8S nrDNA	Reverse	5'-CCGAGATATCCGTTGCCGAG-3'
ITS3	5.8S nrDNA	Forward	5'-GCATCGATGAAGAACGCAGC-3'
ITS3R	5.8S nrDNA	Forward	5'-AACGGATATCTCGGCTCTT-3'
ITS4	26S nrDNA	Reverse	5'-TCCTCCGCTTATTGATATGC-3'
ITS5	18S nrDNA	Forward	5'-GGAAGTAAAAGTCGTAACAAGG-3'

Methods

Direct Sequencing of PCR fragments.

The ITS regions of nrDNA were amplified in exactly the same way as described in the PCR-RFLP study (outlined in the previous chapter) using primers ITS4 and ITS5. The results of the PCR reactions were cleaned by running the entire contents of each reaction on a 1% low melting point agarose gel containing ethidium bromide (as described for visualisation of genomic DNA and restriction digests in previous chapters). Under ultraviolet illumination the band containing the PCR

fragment was excised from the gel and placed in 1ml of TE buffer in a 1.5ml eppendorf tube (leaving many of the reaction by-products in the remains of the gel). The TE was discarded and the agarose melted and cooled by placing the eppendorf first in a heating block at 70˚C for 10 minutes and then in a water bath at 43˚C. After at least 10 minutes in the water bath 3µl of agarase was added to the tube and the mixture incubated for two hours. The tube was briefly cooled on ice, to check that the agarose had been entirely digested, before its contents were brought to 400µl with water. A phenol extraction was carried out by adding 400µl of phenol, mixing and centrifuging at 13,000 rpm for 2 minutes. The aqueous phase was removed and placed in a fresh tube where a chloroform extraction was carried out by adding 400µl of chloroform, mixing and centrifuging as before; placing the aqueous phase in a fresh 1.5ml eppendorf tube. The DNA was precipitated out of solution by adding 1ml of absolute alcohol and 8µl of 5M NaCl mixing well and placing on ice for 10 minutes before centrifuging at 13,000 rpm for 20 minutes. The supernatant was discarded and the pellet resuspended in 25µl of water.

Sequencing reactions were carried out on the cleaned PCR fragments using Perkin Elmer AmpliCycle™ sequencing kit (N808-0175), following the suppliers instructions and using [α-^{33}P]-dATP as an internal label. An additional 20µM dATP/dTTP mix was added for A+T rich templates (as had been recommended by other users of the kit under the same conditions) and 5µl of template were used, spread between the 4 reactions. The thermal cycle for the reactions was 95˚C for 2 minutes followed by 25 cycles of 1 minute at 95˚C, 1 minute at 55˚C and 1 minute at 72˚C. The tubes were then cooled to 45˚C before the commercial suppliers stop solution/loading buffer was added.

Four sequencing reactions were carried out for each template; one with each of the primers ITS2R, ITS3R, ITS4 and ITS5. The reactions were visualised by running on Long Ranger polyacrylamide gels at 55 watts using 1x TBE as a running buffer and exposing on Kodak Biomax MR (8715187) autoradiography film for three days. Each reaction was run three times. The first two times the reactions were loaded in the order ACGT and the gel either run until bromophenol blue tracking dye had just reached the end of the gel (to read the first 100-200 base pairs) or until the xylene cyanole had reached the end (to read as far beyond the 150 base pair mark as possible.) The reactions for one primer from 10 different templates were generally run on a single gel. The third time the reactions were loaded they were run as dideoxy fingerprints all the 'A' reactions from all the different samples being run on one gel, the 'C' reactions on another and likewise for the 'G' and 'T' reactions. The dideoxy fingerprint gels were run only once; until 20 minutes after the bromophenol blue dye had hit the bottom buffer tank. A complete sequencing reactions of ACGT was run on each dideoxy fingerprint gel to ascertain the position of any polymorphisms observed.

Scoring and Alignment

All the autoradiographs were scored by hand directly into the text editor of a computer. Gels from the reverse primers were inverted before being read which meant that no sequence manipulation software was required. Base pair positions that could not be read on more than one gel were scored as N. Particular attention was paid to the cross checking of possible additive banding - banding that indicate termination events for more than one base at a single locus. The sequences proved so similar that they could easily be aligned by eye, along with two outgroup sequences from the 'Azalea' project. The outgroup species selected were the same as those used in the morphological study; *R. luteum* and *R. ferrugineum*. Once sequences had been aligned it proved convenient to convert the variable sites into a presence/absence matrix that could be combined with the morphological matrix later.

Analysis

The presence/absence matrix of variable sites was reduced so as to only represent those sites that were non autapomorphic (i.e. potentially informative) and species that had exactly the same scores

for all sites were combined into groups. A UPGMA and a Neighbour Joining clustering analysis was carried out on the basis of the simple matching coefficient of similarity using the NTSYS computer package and cophenetic correlations calculated (as described in Chapter 5). A parsimony analysis was also carried out using the PAUP package; as in Chapter 5.

Results

Sequencing

It proved straight forward to obtain sequences for both the ITS1 and ITS2 regions using this manual sequencing technique, although the read lengths of gels only made it possible to read into the 5.8s exon with a few primer/template combinations. As no polymorphisms were observed in those sequences that were obtained for the 5.8s regions (and none were found in the 'Azalea' study) it was decided not to attempt to obtain sequences for the exon.

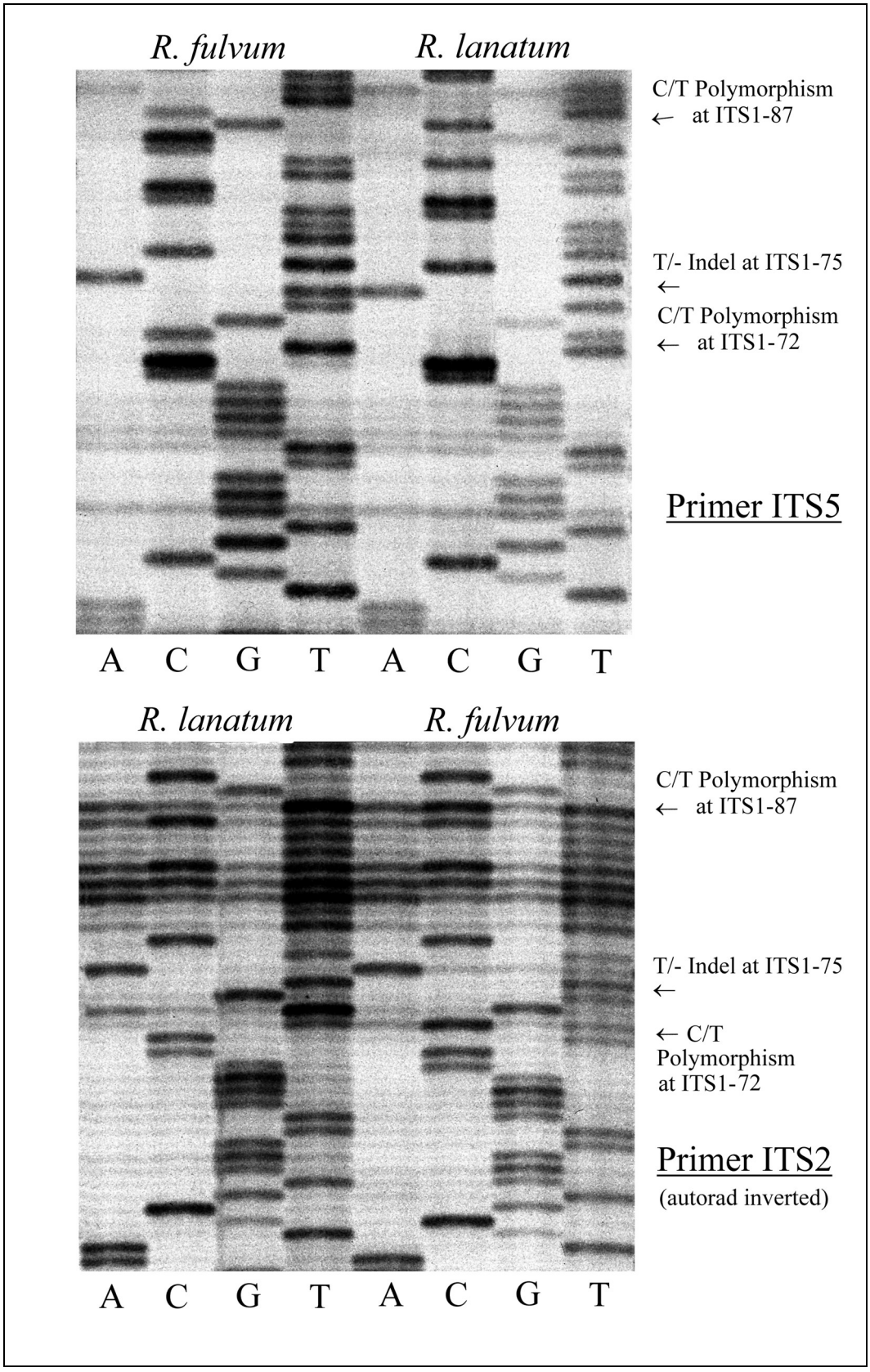
The consensus length of ITS1 was 243 base pairs and of ITS2 368 base pairs. The sequences were found to be 54.5% G/C rich.

Figure 44 illustrates some of the observations made during the sequencing study. It shows two small portions of two sequencing autoradiographs; the results of the sequencing reactions for two primers for the same part of ITS1 (Primer ITS5 and Primer ITS2). The lower image has been inverted so that it reads in the same sense as the upper image. Visible on both autorads are horizontal lines running across the gel and obscuring some of the bands. This was considered to be the product of secondary structures being formed during the sequencing reactions and found, to a degree, on all the gels that were run. Where a sequence was entirely obscured by such banding it could usually be read in the reaction with the reverse primer for that region. In the few cases where this was not possible the base pairs were scored as N. There was one insertion/deletion event encountered in the study at ITS1 base pair position 75 (ITS1-75). This can be seen clearly on both the gels in the figure, along with a C/T point mutation ITS1-72. A second C/T point mutation at ITS1-87 is less visible in the lower gel image but is clearly visible in the upper picture.

The full sequences obtained are given in Appendix E. These represent the results of the four different primers and have been cross checked with the partial sequences obtained during the training period in Leuven as well as the complete sequences that were later produced for *R. ponticum* and *R. argyrophyllum* by the 'Azalea' project. No conflicts were observed between the machine-read sequences and those produced manually. The area of sequence visible in Figure 44 and all the polymorphic sites are highlighted in the Appendix E. A number of restriction sites have also been marked on the sequences. These will be discussed in the next chapter.

Examination of the sequencing gels revealed a number of base pair positions that appeared to show additive banding. Careful checking against the complementary primer reactions did not, however, confirm any of these observations. The additive banding was either absent from the second primer reaction or secondary structures obscured that position. No bands were therefore scored as being additive although the data does not demonstrate conclusively that additive banding was not present. This matter is discussed further in the next chapter where restriction site data is compared with the sequence data.

Figure 44: *Examples of two portions of sequencing gel for the same region of ITS1. See text for full explanation.*



Analysis

The level of polymorphism in the sequences was low. When aligned with the outgroup there were a total of 39 polymorphic sites, two of which were point deletions the rest being single base substitution mutations. Of these sites 13 were autapomorphic for taxa within the ingroup (i.e. only occurring in single ingroup taxa), 9 were autapomorphic for one or other of the outgroup taxa and 5 were synapomorphies for the ingroup. This left just 14 characters that could be considered informative (i.e. occurring in more than one but not all of the ingroup taxa).Two groups, one of fourteen taxa (*R. vernicosum, R. auriculatum, R. watsonii, R. falconeri, R. williamsianum, R. campylocarpum, R. adenosum, R. venator, R. irroratum, R. griersonianum, R. kyawii, R. barbatum, R. neriiflorum* & *R. sherriffii.*) and one of three (*R. lacteum, R. phaeochrysum* & *R. taliense*) have the same combination of non autapomorphic characters. From the point of view of the analysis in this chapter these species were merged into two groups (Groups A and B respectively). The distribution of the non autapomorphic characters between the taxa is given in Table 38.

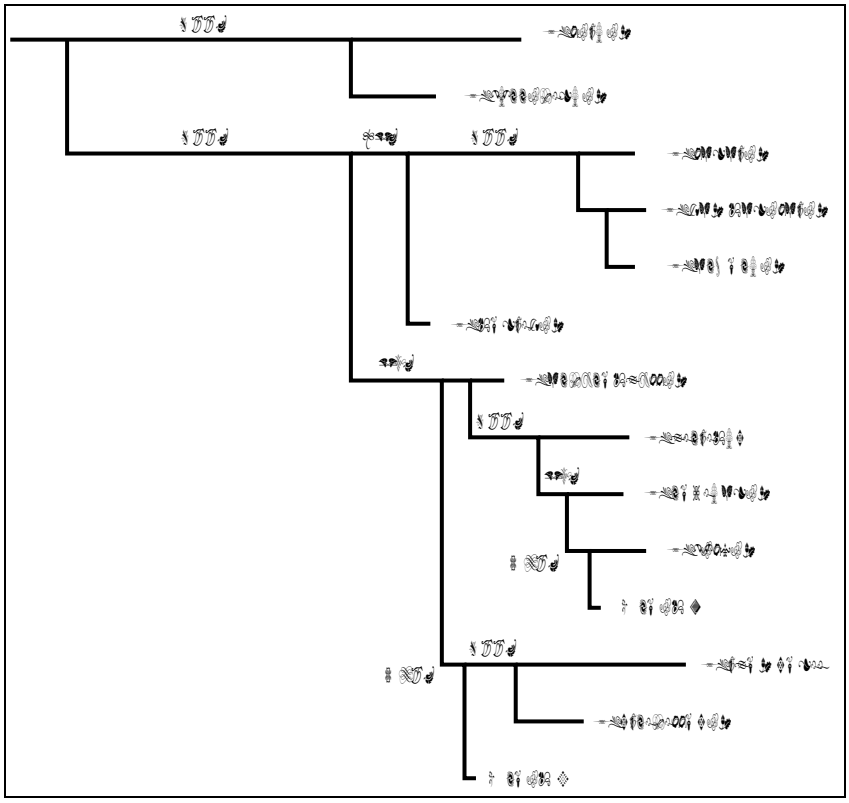
Table 38: *Distribution of non autapomorphic characters in the sequencing study. (c.f. Appendices E and F for location of sites).*

Species	**Non Autapomorphic Characters**																		
Group-A	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Group-B	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
R. strigillosum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
R. hirtipes	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0
R. ponticum	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
R. argyrophyllum	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
R. arboreum	0	1	1	0	0	1	0	0	0	1	0	0	1	1	0	0	0	0	0
R. roxieanum	0	0	0	1	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0
R. fulvum	0	0	0	1	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0
R. lanatum	0	1	1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0
R. campanulatum	0	1	1	0	0	1	0	0	1	1	0	0	1	1	0	0	0	0	0
R. thomsonii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	1
R. ferrugineum	1	0	0	0	1	1	0	1	1	0	1	1	0	0	0	1	0	0	0
R. luteum	1	1	0	0	1	1	1	1	1	0	1	1	0	0	0	0	0	1	0

The results of the different clustering analyses of these data are shown in Figure 45 and Figure 46. The parsimony analysis gave the same nine trees of length 29, consistency index 0.66 and rescaled consistency index 0.49 using both the heuristic and branch and bound search options. The majority rule consensus of these nine trees had almost exactly the same topology as the NJ tree shown in Figure 45. The percentage of trees that supported each branch have therefore been marked on the nodes in this figure. Those with values of less than 50% were collapsed in the consensus tree.

The cophenetic value of the UPGMA tree was high (0.92) whilst that of the NJ tree was low (0.04) suggestive of the same contradiction (as was encountered in Chapter 6) of an the data being ultrametric but not additive (see comments made in Chapter 6).

Figure 45: *NJ tree of taxa shown in Table 38. Branch lengths are drawn proportionately. Numbers on branches are the support for these branches in the majority rule consensus tree. (see text for full explanation)*



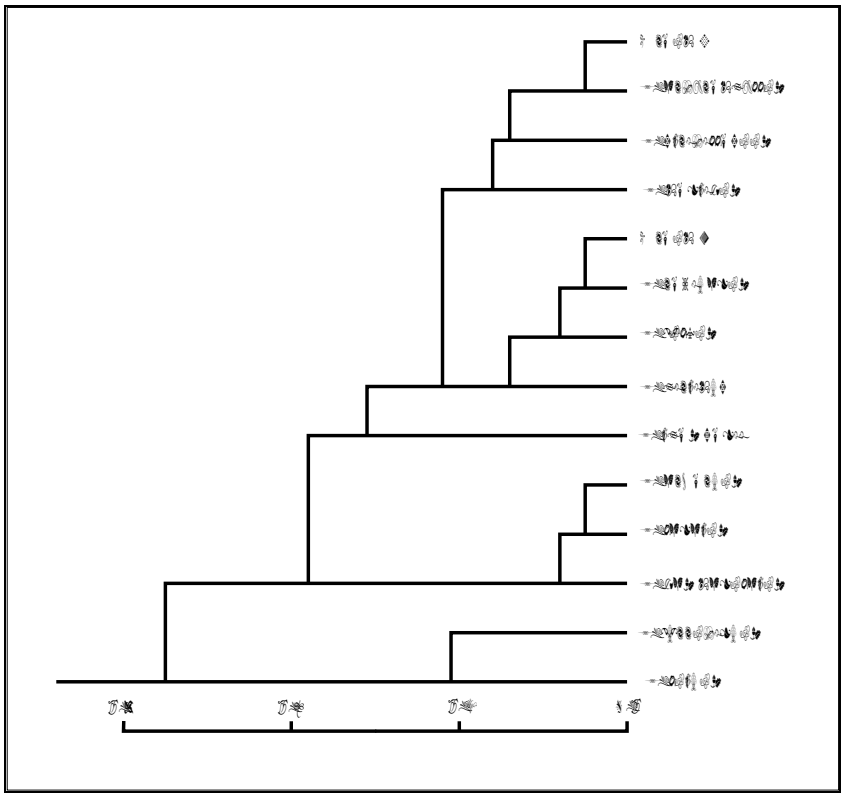
Several groupings are supported in both the UPGMA and the NJ/Parsimony trees. The outgroup, of *R. ferrugineum* and *R. luteum*, are supported in all trees with six characters separating them from the other taxa. Group-B along with *R. hirtipes*, *R. roxieanum* and *R. fulvum* is supported as a single grouping by all trees and the cluster of *R. lanatum, R. campanulatum* and *R. arboreum* is supported by all trees. Where the NJ/Parsimony trees differ from the UPGMA tree is in the positioning of the other taxa. The UPGMA analysis has clustered all of the taxa but *R. thomsonii* into a single grouping (possibly on the basis of their dissimilarity to the other taxa) the NJ/Parsimony analyses, on the other hand, have attempted to place them basally to the undisputed groupings. There is little evidence as to where these taxa should be placed, as is reflected in the short branch length on the NJ tree and the lack of support in the consensus parsimony tree. The only additional grouping that is supported by both the NJ and all the most parsimonious trees is that of *R. strigillosum* and *R. thomsonii.*

To summarise; there are two groups whose members have the same scorings of informative characters and therefore are placed together by default. (Group-A and Group-B) and there are three groupings that are suggested by the analysis of shared characters, these are;

- Group-B (*R. lacteum, R. phaeochrysum* & *R. taliense*) along with *R. hirtipes*, *R. roxieanum* and *R. fulvum*
- *R. lanatum, R. campanulatum* and *R. arboreum*
- *R. strigillosum* and *R. thomsonii*

Other taxa (including the large Group-A) appear to be basal to these groupings but there is insufficient evidence to place them absolutely.

Figure 46: *UPGMA clustering of taxa in Table 38*



Conclusions

Relationships within subgenus *Hymenanthes*.

The results obtained by the sequencing study are an improvement on those obtained in the PCR-RFLP study in that enough data was generated to draw some conclusions regarding the possible relationships of the taxa however the level of variability is very low and this means that there is little resolution in the trees produced. There is some evidence that the groupings suggested by the analysis have some biological bases as is indicated by the fact that three of the four representatives of subsection *Taliensia* included in the molecular exemplar sample all come within Group-B whilst the fourth member, *R. roxieanum*, comes within the same, undisputed grouping. Speculation as the importance of the other groupings will be dealt with in the next chapter where a comparison of the different data sets is made.

A sample of twenty seven species represents less than 10% of the classical species suggested to exist within the subgenus. The question must be asked whether this is an appropriate size of sample to represent the variation present. If the samples had been randomly chosen this may well be the case but as an attempt was made to choose species from across the range of diversity the effective sample size is much larger and possibly sufficient. The fact that of the twenty five positions that were variable within the ingroup eleven (44%) were autapomorphic suggests that if further sampling were carried out homologues would be found for these characters and they would become informative. This would provide greater resolution moving up the tree, sub-groupings would be found within Group-A for example. Working on the principle that major groups have already been included in the study, however, further sampling of the ITS region could never strengthen the weakly supported lower branches of the trees. If new apomorphies (variable sites) were discovered they can, by definition, only define new groups and not strengthen of clarify existing relationships. It is safe to say that, in this respect, the ITS region of nrDNA has been exhausted for the grosser level differentiation of relationships and another source of data must be used. For finer levels of differentiation the region is still a potential source of data.

Low levels of variability in ITS sequences.

Although the data does not contain a large number of useful markers for phylogenetic reconstruction conclusions can be drawn from the fact that such a low level of variation is present. Perhaps the most obvious hypothesis would be that the species sampled have undergone extremely rapid speciation with insufficient time for mutations to occur and leave a phylogenetic pattern. (Examples include: Clark *et al* 1995, Lang *et al* 1994, Oxelman *et al* 1996, Ritland *et al* 1993, Soltis *et al* 1993 and Zechman *et al* 1994) There are, however, other possible hypotheses.

As has already been discussed, in Chapter 7, ITS is part of a tandemly repeated gene that is homogeneous within individuals. Although the mechanism of homogenisation is not fully understood certain conclusions may be drawn from what evidence is available. It has been demonstrated that homogenisation occurs within NOR regions faster that between NOR regions and that a slower rate of homogenisation occurs between homologous chromosomes (Schlötterer & Tautz 1994). These homogenisation events would appear to occur on mitotic and meiotic time scales. At a grosser level homogenisation between individuals is likely to occur at a generational level, assortment events bring NOR regions containing different sequence types into contact within individuals thus maintaining a uniform sequence type within populations. Immigration between populations would likewise maintain homogeneity within biological species. Should interspecific hybridisation events occur however this would lead to homogenisation between species. (These comments are summarised in Table 39.) From the point of view of reconstructing phylogenies from ITS sequence data, homogenisation below the species level is a good thing; it makes direct sequencing possible and it ensures that any one individual may be taken as representing the species as a whole. Between species homogenisation on the other hand is likely to destroy phylogenetic signal and any loss of signal will be efficiently spread to all copies of the gene in all members of the species.

It has been demonstrated that species within subgenus *Hymenanthes* are promiscuous, that horticulturists have no difficulty in crossing most combinations of species, that species hybrids often occur in the wild (see page 24) and that the one known incompatibility mechanism, from section *Vireya* (see 54) is unlikely to occur in the subgenus. It therefore seems highly likely that there will be a degree of interspecific homogenisation of the ITS region in this group.

Table 39: *Rates of homogenisation of ITS regions at different levels of diversity.*

Level of Homogenisation	**Time Scale**
Within NOR region.	Mitotic
Between NOR regions (within chromosomes)	Mitotic (Meiotic?)
Between chromosomes.	Meiotic, through cross over events (possibly also mitotic?)
Between individuals	Generational through assortment of different NOR regions.
Between Populations	Multigenerational (dependent of degree on isolation of populations)
Between 'Species'	Impossible in 100% biological species. (multigenerational if interspecific hybridisation events occur).

Summary

Sequencing of the ITS regions did not prove problematic but only a low level of variation was encountered. The variation present does suggest some groupings that may be informative but there is little resolution within these groupings and the relationships between the groupings are unclear. Further sequencing would lead to greater resolution within groupings but not to a greater understanding of the their relationships. There are two potential explanations for the lack of

variability in the ITS region and both may play a part in maintaining low levels of variation. Further resolution of relationships can only be achieved by combining this data with other data sets. This will be the subject of the next chapter.

CHAPTER 9: FINAL ANALYSIS AND CONCLUSIONS

Introduction

The project has produced four data sets, as well as observations concerning the nature of diversity and the utility of different molecular techniques. This final chapter draws together these different aspects of the study into a single analysis; comes to a single set of conclusions and makes recommendations for further study.

For reasons outlined in Chapter 6 the RAPD data set will not be used as part of the combined analysis, although further conclusions will be made at the end concerning the suitability of the different techniques. Likewise the continuously variable characters of the morphological study were not carried forward for reasons outlined in Chapter 5. The most directly comparable data sets produced are those from the PCR-RFLP and Sequencing studies and so these will be dealt with first and combined into a single molecular data set that will then be compared with the morphological data.

Comparison of Sequencing and PCR-RFLP results

The sequencing, in Chapter 8, of the same PCR fragments that were analysed by PCR-RFLP in Chapter 7 allow direct comparison of the two sets of results.

Method

The sequences produced in Chapter 8 were edited so that they all included the 5.8s exon, that had only been sequenced from a few of them. This gave the complete sequences for the fragments that had been digested. (The assumption was made here that all individuals would have the same sequence for the exon, which appears likely as no variation was found in either this study or the 'Azalea' study). A word processor was then used to identify the cut sites for all the enzymes used in the study and these sites were compared with those found in the PCR-RLFP study.

Results

None of the enzymes that failed to cut the PCR fragments were subsequently found to have cut sites in the sequences but of the eight enzymes that gave scorable patterns in the digestions half were found to have been misinterpreted when compared with the sequencing results. The restriction sites found with the two different approaches are shown in Table 40. Those enzymes which gave conflicting results have been highlighted. Selected restriction sites have also been highlighted in Appendix E. The results obtained for a series of enzymes is discussed below.

Table 40: *Comparison of restriction sites observed in the PCR-RFLP study with those found by analysis of the sequences.*

Enzyme	Observed in PCR-RFLP	Visible from Sequence results
Ase I	No cut sites, No polymorphism	No cut sites, No polymorphism
Ava I	No cut sites, No polymorphism	No cut sites, No polymorphism
*Bam*H I	No cut sites, No polymorphism	No cut sites, No polymorphism
Bgl I	No cut sites, No polymorphism	No cut sites, No polymorphism
Bst NI	2 cut sites, 1 polymorphic	2 cut sites, 1 polymorphic
***Cfo* I**	**3 cut sites, 1 polymorphic**	**4 cut sites, 1 polymorphic**
Cla I	1 cut site, No polymorphism	1 cut site, No polymorphism
*Eco*R I	No cut sites, No polymorphism	No cut sites, No polymorphism
*Eco*R V	1 cut site, No polymorphism	1 cut site, No polymorphism
***HAE* III**	**2 cut sites, No polymorphism**	**3 cut sites, No polymorphism**

Enzyme	Observed in PCR-RFLP	Visible from Sequence results
Hind III	No cut sites, No polymorphism	No cut sites, No polymorphism
***Hinf* I**	**2 cuts sites, No polymorphism**	**3 cut sites, 2 polymorphic**
Msp I	2 cut sites, 1 polymorphic	2 cut sites, 1 polymorphic
***Rsa* I**	**1 cut site, No polymorphism**	**2 cut sites, No polymorphism**
Sal I	No cut sites, No polymorphism	No cut sites, No polymorphism
***Taq* I**	**Complex pattern not interpreted**	**4 cut sites, 1 polymorphic**

***Cfo* I.** There are two Cfo I sites very close together at ITS1-163 and ITS1-73. These are far too close to be detected using the PCR-RFLP method used here. Fortunately neither of the sites is variable but if they had both been polymorphic it would not have been possible to detect which of the two was present leading to problems with homology. In addition to this the Cfo I site at ITS1-163 overlaps with an MSP I site that starts at ITS1-160. These two sites will not, therefore, behave as independent characters as regards at least one base position.

***Hae* III.** As with the close sites in *Cfo* I there are two *Hae* III sites that were not detected in the PCR-RFLP study; these are at ITS2-177 and ITS2-205. Neither are polymorphic but if both were it would be difficult to tell them apart with PCR-RFLP and it certainly would not have been possible under the electrophoresis conditions used here.

***Hinf* I.** The danger of two spatially close restriction sites both being polymorphic and being misinterpreted that has been described above is illustrated well by the two *Hinf* I sites at ITS2-37 and ITS2-54. Although the first site is obscured by secondary structure in many of the species the site is definitely missing from *R. kyawii.* Within fourteen base pairs the second restriction site is highly polymorphic between the species but is present in *R. kyawii.* As these sites were only visible as a single site they were scored as a single, non-polymorphic site rather than two polymorphic ones.

***Rsa* I.** A similar situation exists in *Rsa* I as with the enzymes above. The two sites at ITS2-319 and ITS2-334 were scored as one uniform site even though one of them is absent in *R. strigillosum*.

Taq I. The distribution of Taq I sites is illustrative of how difficult restriction fragment patterns can be to interpret, even in PCR-RFLP. The four restriction sites present, in the sequence data, produce five fragments. Two of these fragments are close to 300 base pairs long, two are on the limits of detectable size at around 60 bp and one is probably not detectable at around 40 bp. All the bands therefore tend to mask each other and the one polymorphic site can not be detected. The patterning encountered in the PCR-RFLP study was more complex than this indicating that some of these sites may have been polymorphic within individuals. No evidence of this was produced by the sequencing reactions.

***Msp* I.** Although the PCR-RFLP and sequencing results scored for *Msp* I did not conflict there were, however, faint additive bands present in a number of individuals in the PCR-RFLP study that were not encountered in the sequencing project. As was discussed in Chapter 8 no additive banding could be confirmed to have occurred in any of the sequencing reactions.

Conclusions

From the theoretical stand point PCR-RFLP appears to be a very useful technique, as is outlined in Chapter 3, but in this study it does not appear to have met the five criteria of good taxonomic data. The markers were not **selective** enough within the ingroup to answer the questions posed and sequencing has shown that some the restriction sites recorded were not **homologous** or **independent**. The methods of internal **verification** do not seem to have worked (parts of fragments were thought to have summed to the total PCR fragment when in fact they didn't) and, although the markers appeared readily **available** at the outset it proved just as easy and quick to produce a more detailed data set by sequencing the same fragments.

One area that remains unresolved is the additive banding that occurs in the PCR-RFLP study but is

not supported by the sequencing results. There are three explanations for the contradiction:

- There are no internal polymorphisms within the taxa and the extra bands are caused by partial digestion of the fragments. This was ruled out by the ancillary evidence discussed in Chapter 7.
- There are internal polymorphisms and the lack of bands in the sequences are due to dynamics of the sequencing reaction. This is possible but unexpected as the same annealing temperature was used for the sequencing reaction as for the PCR reactions.
- There are internal polymorphisms but they are masked in the sequencing reactions by secondary structures. This is mitigated against by the fact that the sequence appears clear at some of the restriction sites that show additive banding.

Without further study (as suggested at the end of this chapter) this matter will not be resolved.

Combining the two data sets is straight forward. There is no occasion, other than for the faint additive bands, where the sequences do not provide a full explanation for the banding encountered during the PCR-RFLP study. As the correct data obtained during the restriction site study is only a subset of that obtained during the sequencing study the sequence data set may be taken as representing them both and will be carried forward into the rest of the comparative analysis as the molecular data set.

Combined Analysis of Morphological and Molecular Data Sets

Method

The morphological data is available for 54 taxa whilst molecular data is only available for 29 taxa (a subset of the taxa for which the morphological data is available). A combined data set was therefore made up for the 29 molecular exemplar species that included both the molecular characters and the binary morphological characters. This data set was analysed and interpreted in detail before an attempt was made to extrapolate the findings to the larger morphological data set.

Both phenetic and cladistic analyses were carried out on the data. The matrix first being converted into two similarity matrices using the average taxonomic distance coefficient (DIST, see Chapter 5) and the simple matching coefficient (SM). The simple matching coefficient is the number of matching states divided by the total number of characters and so weights presence or absence of a state equally. This is appropriate here as designation of presence or absence of character state was arbitrary. (c.f. use of Jaccard's coefficient in Chapter 6). The two similarity matrices were then clustered using the UPGMA and Neighbour Joining algorithms (see Chapter 5). Cophenetic correlations were calculated for the clusterings produced. A parsimony analysis was carried out using the PAUP computer program, as described previously.

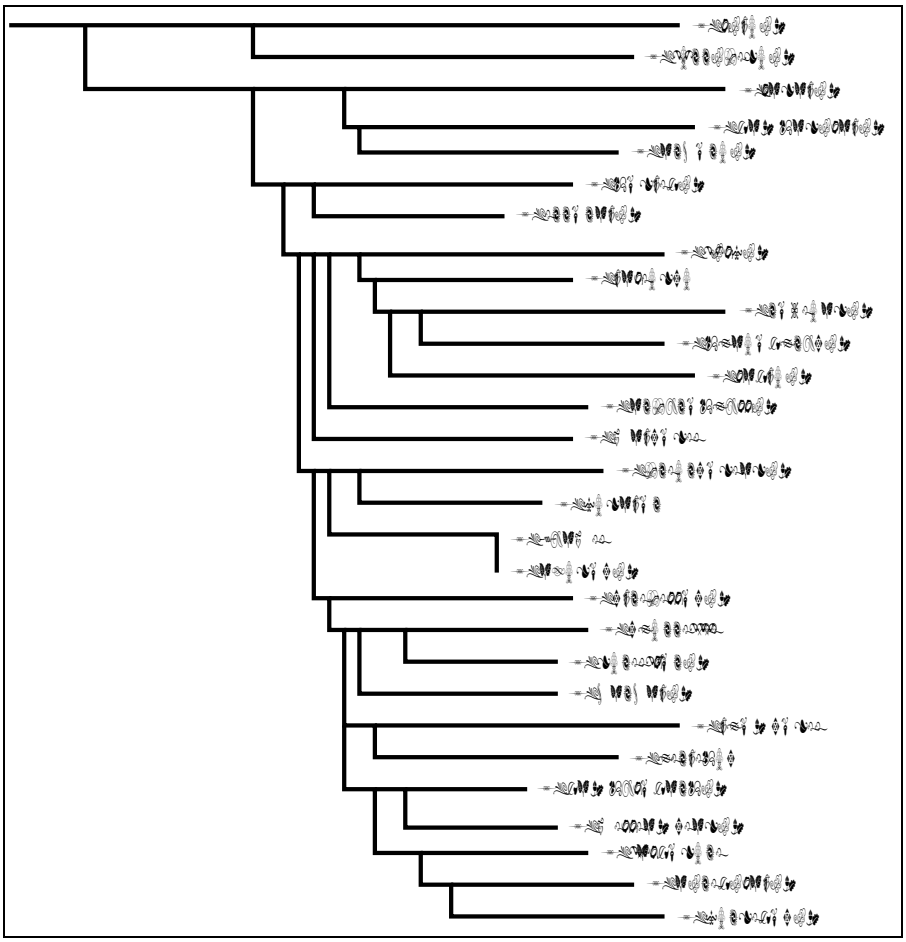
In order to assess the relationship between the two different types of data present in the matrix a third analysis was carried out. This took the form of a clustering analysis of the characters on the basis of the taxa, effectively turning the data matrix on its side. All autapomorphic characters were removed and a similarity matrix produced using the simple matching coefficient. The characters were then clustered using the UPGMA clustering algorithm.

Results

Figure 48 is the UPGMA clustering of the characters by the taxa. It can be seen from this figure that molecular characters are clustered into four groups. Cross comparison between this figure and the complete data matrix presented in Appendix F reveals the reasons for these clusters. The top group clusters together because all characters are synapomorphic for the ingroup; only scoring 1 in the outgroup. The next group are clustered because they occur in two taxa each, one in the outgroup and another in the ingroup (exception to this are ITS2-294T and ITS3-310A which occur in two

ingroup members, one of which also scores 1 for another member of this group.) The third group of molecular characters are those that define the *R. lanatum* group of taxa described in the last chapter and the fourth group define the *R. taliense* group.

Figure 47: *Neighbour Joining tree from DIST matrix of combined data set.*



None of the morphological characters appear to be closely linked with any of the molecular characters. The character 'Corolla Indumentum' is nested between the first two molecular character clusters on the basis that it occurs in the outgroup and only one ingroup member. The other morphological characters that separate the groups of molecular characters have no close affinities and appear to cluster there by chance. 'Flowers Fragrant', for example, clusters with 'Corolla Lobes not Five' because they are shared by two individuals, both are placed near the molecular characters because 'Flowers Fragrant' occurs in the outgroup.

The NJ and UPGMA trees produced from the DIST and SM similarity matrices produced trees that differed somewhat in their lower branches. The cophenetic values of these trees were DIST-NJ 0.75, DIST-UPGMA 0.91, SM-NJ 0.29 and SM-UPGMA 0.91. Figure 47 is the DIST-NJ tree and illustrates why the other trees were different, most of the lower branches on the tree are very short with one of zero length.

The parsimony analysis found six equally most parsimonious trees of length 129, Consistency Index 0.473 and Rescaled Consistency Index 0.253. Figure 49 shows the strict consensus tree of these six trees. The majority rule tree had the same topology as this tree because branches were either supported by all the most parsimonious trees or by less that three of them. Marked on the branches of the tree are the number of character changes that were hypothesised to occur on each branch (using the AccTran option in PAUP). These figures were the same for all trees except where marked. Also shown in Figure 47 is the data matrix from which the trees were generated (with the autapomorphic characters removed). Looking at this matrix it is not possible to pick out any

relationships between morphological and molecular characters that may have been missed in examining the UPGMA clustering given in Figure 48.

Conclusions

There does not appear to be a correlation between the morphological and the molecular data and although the level of resolution obtained here is higher than that produced with molecular data alone (in Chapter 8) there seems to be little agreement between the clustering techniques as to the correct topology of the tree. This indicates that more noise may have been introduced into the data. *R. fulvum*, for example, was closely allied with Group-B by the molecular data alone (see Figure 45 & Figure 46) no matter which analysis was used but in the combined data set it is placed either with *R. argyrophyllum* and *R. watsonii* or with the *R. taliense*-like species of Group-B. Two out of three of the groupings produced by the molecular analysis (see page 120) are supported in the combined analysis but none are visible in the morphological analysis. Some groupings that appear in the combined analysis are not present in molecular analysis but are weakly present in the morphological analysis. It can only be concluded that the major groupings present in the combined analysis are the result of a signal coming from the molecular data or from the morphological data set and exist despite the other data being present rather than with its support. In the light of the above conclusions it is not feasible to extrapolate the results of the combined analysis to the larger exemplar group. Had the distribution of any of the individual morphological characters, or groups of characters, coincided with any of the molecular characters then it would have been possible to hypothesise that other individuals showing these morphological characters also carried the molecular character. This does not appear possible here.

Despite the lack of concordance between the data sets some groupings of taxa remain more or less clear, clustering together in analysis of either molecular or morphological data sets and not being lost in analyses of the combined data sets. These are:

- The four representatives of subsection *Taliensia*, (often in association with *R. fulvum* and/or *R. hirtipes*.) - from molecular data
- *R. lanatum*, *R. campanulatum* and *R. arboreum*. -from molecular data.
- *R. falconeri*, *R. auriculatum* and *R. vernicosum* - from morphological data.
- *R. neriiflorum*, *R. sherriffii* and *R. thomsonii* (and possibly *R. barbatum*) - from morphological data.

Comparison with previous classifications

The groupings listed above allow some conclusions to be drawn regarding specific proposals made by previous taxonomic treatments.

Spethmann (1980-1987) split the subgenus into two sections, section *Hymenanthes* and section *Lactanthes*, section *Lactanthes* containing two subsections; a broadly delimited *Taliensia* (that included series Lactea) and a broadly delimited *Falconera* including series *Grandia* (represented by *R. watsonii* in this analysis)(see page 18). Section *Lactanthes* is not supported by the analysis, the constituent species being separated by a number of characters in every analyses although, as with Chamberlain (1982), the broad delimitation of subsection *Taliensia* is. Within section *Hymenanthes* Spethmann recognises three groups, the division between groups two and three is not supported, (*Campanulata* and *Arborea* coming in separate groups despite being very closely related in the analysis) but group three is somewhat supported by the clustering of *R. neriiflorum*, *R. sherriffii, R. thomsonii* and *R. barbatum* in the analysis. In summary, Spethmann's classification is not supported by the data but its most ambitious element (section Lactanthes) is not 'unsupported' by the molecular data, only the traditional data.

Figure 48: *UPGMA clustering of a simple matching matrix of characters by taxa.*

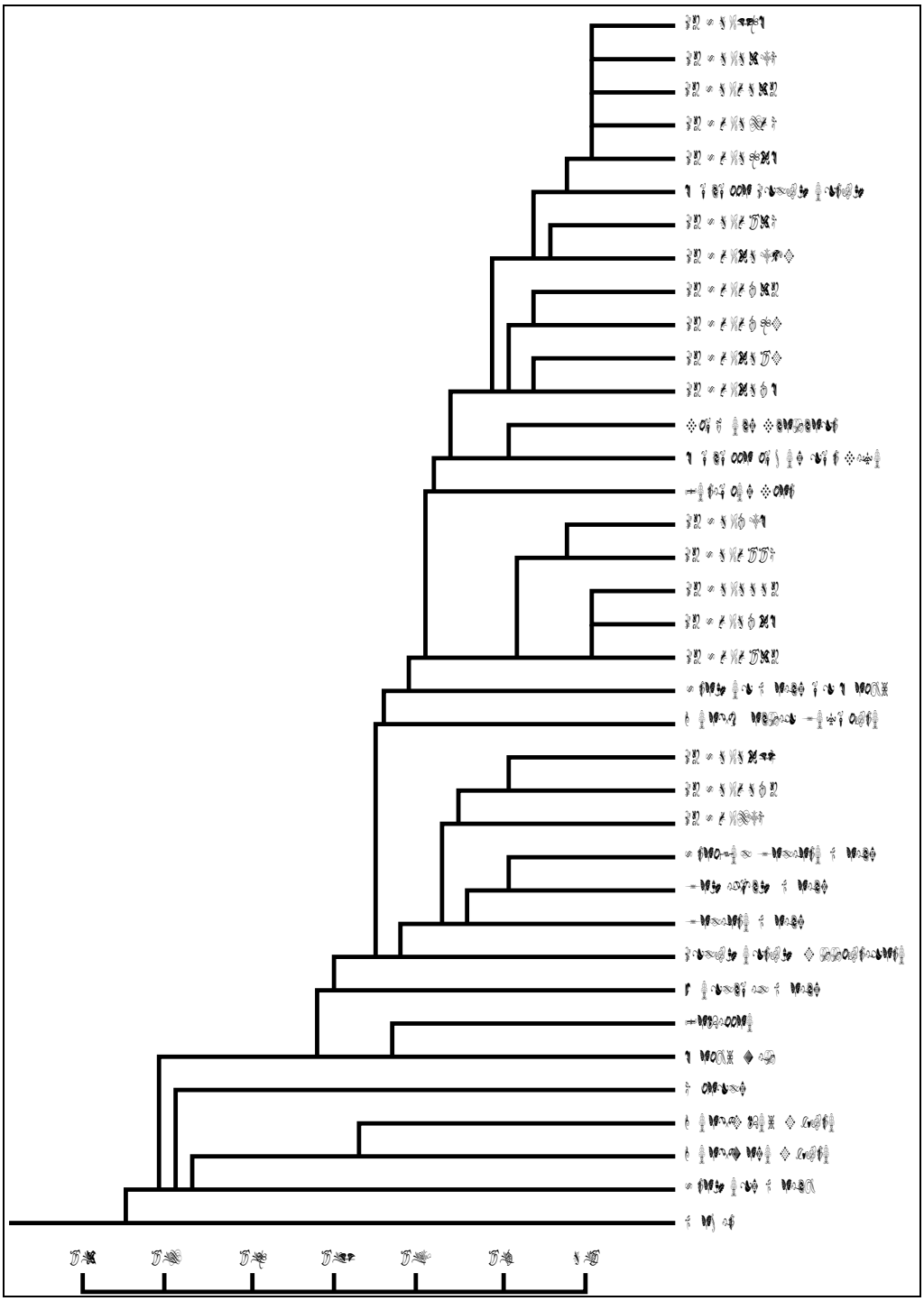
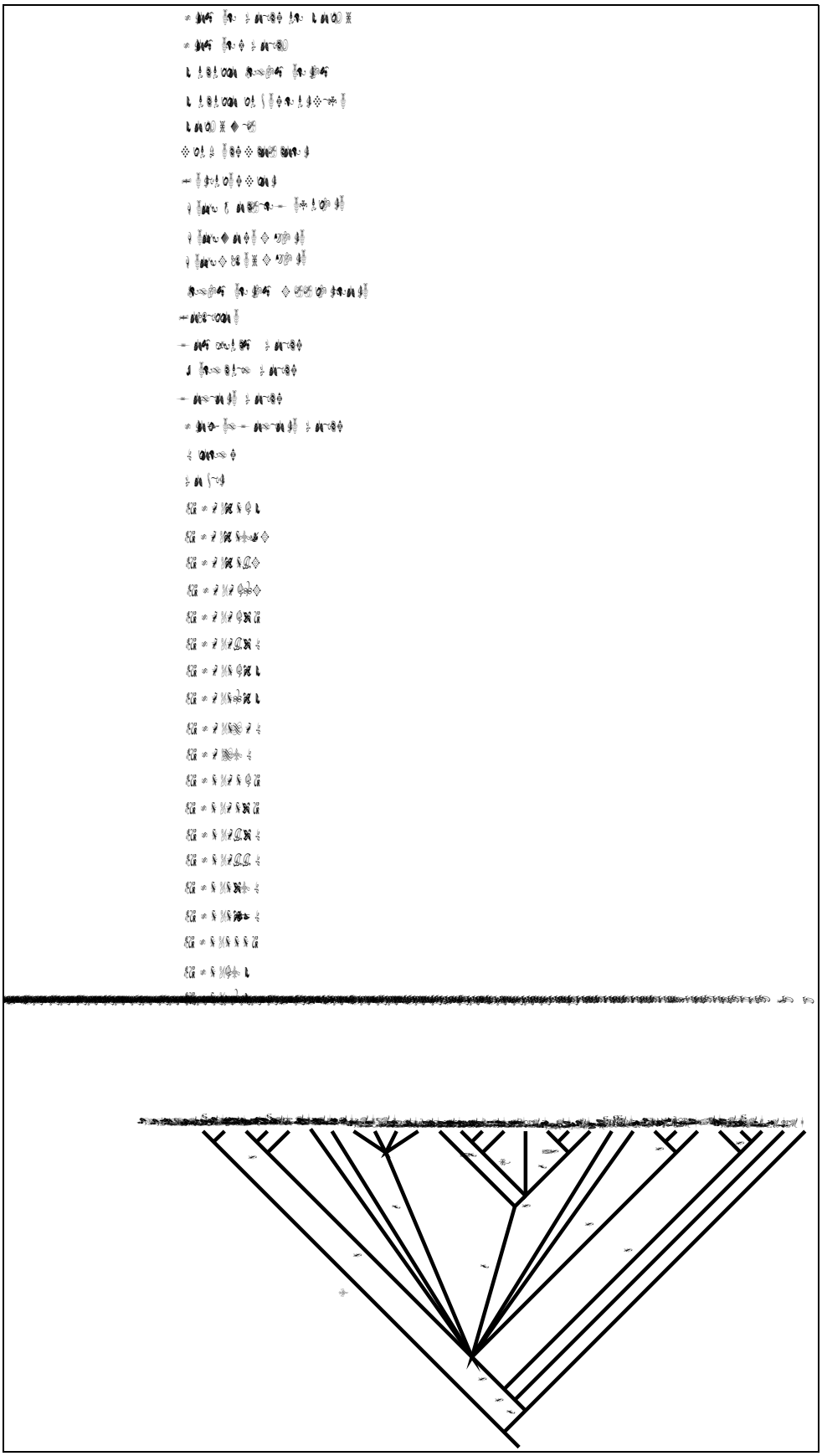


Figure 49: *Summary diagram of combined analysis of morphological and molecular data. (see text for full explanation).*



Chamberlain (1982) (see page 15) did not believe the subgenus warranted being split into sections and this appears to have been supported by the molecular data, with the possible exception of the *R. lanatum* group discussed below. As with Spethmann the wider delimitation of subsection *Taliensia* to include series *Lacteum* is supported by molecular evidence. Chamberlain suggests a possible affinity between subsections *Fulva* and either *Taliensia* or *Argyrophylla*. The molecular evidence suggests that the affinity is closer with *Taliensia* than with *Argyrophylla* but that *Argyrophylla* is still fairly closely related although this breaks down when the morphological evidence is considered. A possible relationship with *R. hirtipes* (subsection *Selensia*) has not be suggested before.

From the molecular point of view the best supported group is that of subsections *Lanatum*, *Campanulatum* and *Arboreum* (represented by their type species). Chamberlain predicts the grouping of *Lanatum* and *Campanulatum* but not the link with *Arboreum* (page 371). He also suggests a link with *Taliensia*, which is supported in the cladistic analysis of the combined data set but not by the molecular data alone. He suggests a link between *Auriculata* and *Fortunea* and this is supported by the morphological data but he does not mention the link the with *Falconera* (instead suggesting *Grandia*, an affinity which is not supported). The erection of subsection *Lanata* out of *Campanulata* is neither supported nor refuted by these data but even if they remain separate taxa on phenetic grounds they are clearly sister taxa. Chamberlain also erected the subsection *Fulgensia* as a link between subsections *Nerriiflora*, *Thomsonia* and *Barabata* (page 415). This is supported by the morphological analysis. When **Sleumer (1949)** erected the subsections he split subseries *Argyrophyllum* from *Arboreum* to make two separate sections, this is supported by the molecular evidence.

Final Conclusions

Concerning the aims of the study.

Four specific proposals were made at the end of Chapter 1, these are dealt with in turn here.

To examine whether either conventional of molecular techniques will produce evidence of an underlying phylogenetic pattern to the variation in the subgenus.

Both molecular and morphological approaches have produced evidence of some form of structure within the group. The two sources of data have been complementary but not additive in as much as no groupings were supported by both techniques. Whether other sources of data will reveal more structure can not be established.

To establish whether the subsections proposed by Sleumer (1980) and Chamberlain (1982) can be arranged into a hierarchy.

Because of the low level of support for the lower branches (in all the analyses) there is little evidence of a strong, truly hierarchical structure, only of a single level loose confederations of species.

To ascertain whether the sectional treatment proposed by Spethmann (1987) is valid and so should be more widely applied.

The morphological evidence rejects the sectional treatment proposed by Spethmann. The molecular evidence provides no evidence to support such a treatment. It is not recommended that this classification is followed.

To suggest subsets of taxa that could be studied in more detail.

The most well defined grouping encountered in the study is that of *R. lanatum*, *R. campanulatum* and *R. arboreum*. As this group has a number of well defined apomorphies it could form the nucleus

of a more detailed study. Species could be screened for these apomorphies prior to inclusion in the study and more sensitive molecular markers used to examine relationships within the group.

Concerning subgenus *Hymenanthes*.

At the beginning of the study subgenus *Hymenanthes* was a larger complex group of species with little or no hierarchical structure. At the conclusion of the study the group appears to be equally impenetrable, however a great deal has been clarified concerning the nature of variation in the group. It appears likely that there is no strong phylogenetic pattern within the subgenus although it is not possible to prove this absolutely. There are two main hypotheses to account for this lack of structure. One is that the group has undergone exceedingly rapid, perhaps explosive, evolution from a single ancestral stock resulting in a large number of very closely related taxa. Under such a model each taxon is equally distantly related to each other taxon and to the ancestral taxon and so there is no structure. The second hypothesis is that the group as a whole acts as a single evolutionary unit. Breeding barriers are so permeable that gene exchange is relatively common between morphologically distinct entities and that single populations may contain a large proportion of the genes present in the whole subgenus. In this way the group is able to respond to the rapidly changing environmental patterns that occur in the eastern Himalaya and to exploit the vast number of diverse niches that occur. (The anthropomorphism here is intended. The biology of the plants clearly has not evolved so as to respond to the changing environment but rather the environment causes them to have this biology.) It is likely that a combination of the two hypotheses is true. The group has evolved in an explosive manner and the diversity is maintained by the breeding systems of the plants.

Speculation as the origin of the subgenus is out of place here but is rather a matter for the study of the genus as a whole. The timings of events can only be calculated relative to other groups and so linked into geological events. The fact the *R. ponticum* frequently comes out basally may be of relevance.

Concerning the nature of biodiversity

Chapter 2 highlighted the theoretical problems associated with the orthodox approach to complex groups. If the combined species concept, that was proposed there, is applied to the subgenus (in the light of the results of the study) then it is clear that either the entire group is a single species or the group falls into that area of biodiversity for which the species is an inadequate paradigm. Despite our increasing knowledge of subgenus *Hymenanthes* we still have problems in describing the diversity present using the current models. Just as a molecular marker may be deemed inappropriate in a certain situation, so a theoretical model may not be appropriate. It may be concluded then that a different theoretical approach is needed to the group. Other models for describing diversity could be specimen or character based rather than taxon base, making use of relational data structures without imposing an absolute hierarchy. Such systems should be considered legitimate areas for further study. The variation in subgenus *Hymenanthes* will not be adequately described until significant advances have been made in the theoretical aspects of the study of biodiversity.

Concerning Molecular Markers.

Chapter 3 considered the major types of molecular marker and three of these markers were used in Chapters 6, 7 and 8. RAPD were found to be theoretically problematic and technically more difficult than envisaged. PCR-RFLP was theoretically sound but found to be misleading in this group. Sequencing was found to be the most productive technique but gave low levels of variation.

During the course of the study the availability of sequencing technology has increased dramatically. Both institutions involved acquiring automated sequencers at the end of the practical study period. There is no doubt that if these facilities had been readily available from the first day of the project the PCR-RFLP study would not have been carried out and, possibly, the RAPD study would not

have been attempted, it being more likely that an automated AFLP approach would have been taken. In addition to the automation of sequencing and other molecular techniques there have been advances in DNA extraction made by a number of companies and there are now several off-the-shelf kits for extracting plant genomic DNA, one being developed in collaboration with the 'Azalea' project.

It is likely that if the same project was begun today it would take a very different course. The same amount of molecular data presented here could be generated in a fraction of the time taken during this study. This is not true of the morphological data though. No significant change in amassing morphological data has occurred in the last four years and none is currently foreseen. Approximately half the time allotted to this study was dedicated to morphology and half to molecular work. If a similar approach were taken today the data obtained from the molecular approach would far exceed that produced from the morphological methods.

The main conclusion that can be drawn is that when examining a morphologically distinct group the first technique that should be attempted is sequencing. This produces the most theoretically robust data for phylogentic reconstruction and is relatively easy to produce using current technologies. If the most variable gene available does not provide enough data then a more sensitive approach should be considered. The most theoretically desirable is SSRs. If time permits an SSR library should be generated and microsatellites selected that vary at an appropriate rate. If time is not available an RAPD or AFLP approach could be adopted but great care should be taken in analysing any banding patterns obtained from such studies.

Recommendations for further study.

Theory: As has been outlined above, one of the major problems associated with the describing the biodiversity in subgenus *Hymenanthes* may be in trying to impose a rigid hierarchical system on non hierarchical diversity. Further study could take the form of constructing a large, specimen-based data base to included morphological and molecular data and then to produce *ad hoc* classifications as and when required. The subgenus is a useful group in which to try and develop such as system that may then be applicable to other complex groups.

Further Exploration of ITS of nrDNA. It has already been stated, in Chapter 8, that the further sequencing of ITS for other species of the subgenus would not lead to a resolution of the basal polytomy. It would, however, lead to clustering of individuals to the those lineages that are already present and thus produce a non-hierarchical but potentially useful classification. This may help to resolve the boundaries of some of the subsections currently proposed.

There is potentially a large amount of information in the degree to which the ITS region is homogenised in any one individual that is not made use of when direct sequencing of PCR fragments is undertaken. This is shown by the additive banding encountered in the PCR-RLFP study (but not in the sequencing study) and warrants further investigation. Two approaches could be taken. One would be the use of SSCP technology to ascertain the number of fragment types produced from any one individual under specific PCR conditions, perhaps sequencing individual bands produced by this method. The danger of this approach is that so many different versions of the fragment may be present under all PCR conditions that a smear would be produced on the SSCP gel. Another more easily implemented approach would be to clone the PCR fragments from several individuals and sequence as many clones as possible. The weakness in this latter approach is that it does not allow for specificity in the cloning procedure and so some fragments may be missed.

Sequencing other genes: There are other regions of the genome that may be more variable than the ITS of nrDNA. One candidate region is 5S rDNA gene which is also arranged in large tandemly repeated units. (Sastri *et al* 1992). It consists of transcribed and non-transcribed portions, the non transcribed portions being highly length variable and appearing to contain a microsatellite in some species (Baum & Johnson 1994). Direct sequencing of this region is likely to be difficult because of

the length variations but a cloning/sequencing approach may be a productive source of data.

Microsatellites: Perhaps the most powerful marker to use below the species level, microsatellites would be the best source of data for further analysis of the subgenus. It has already been shown, in the 'Azalea' project (see Appendix G), that it is possible to generate microsatellites for other subgenera of *Rhododendron* and should a large scale project be considered on *Hymenanthes* the best way forward would be to produce such a library and select microsatellites that vary at a low enough rate to be informative across the broadest possible range of morphological variation. Edwards *et al* (1996) outline a viable method of producing such libraries. Powell *et al* (1995a & 1995b) has described the occurrence of microsatellites with highly conserved primer sites in the chloroplast genome that could be a source of information concerning chloroplast inheritance in the group.

End Word

One of the major problems in suggesting further studies in *Rhododendron* subgenus *Hymenanthes* is its large size and lack of internal subdivision. Unlike more easily subdivided groups, where each section may be completely revised by a single worker, the only way that progress can be made here is by individuals contributing what they can and accepting that the group may not be fully understood for many years to come. I hope that this study has contributed something to the growing body of knowledge on the subgenus.

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Appendix B: List of recognised species within *Rhododendron* subgenus *Hymenanthes* by Biological Recording Unit. (Hollis & Brummit 1992).

ALA-OO (United States - Alabama)
- ***Rhododendron catawbiense*** Michx.

ALT-OO (Commonwealth of Independent States - Altay)
- ***Rhododendron aureum*** Georgi
  - **—var. *aureum***

AMU-OO (Commonwealth of Independent States - Amur)
- ***Rhododendron aureum*** Georgi
  - **—var. *aureum***
  - **—var. *hypopytis*** (Pojark.) D.F.Chamb.

ASS-AP (India - Arunachal Pradesh)
- ***Rhododendron arboreum*** Sm.
  - **—ssp. *delavayi*** (Franch.) D.F.Chamb. **var. *delavayi***
  - **—ssp. *cinnamomeum*** (Lindl.) Tagg **var. *roseum*** Lindl.
- ***argipeplum*** Balf.f. & R.E.Cooper
- ***barbatum*** Wall. ex G.Don
- ***beanianum*** Cowan
- ***campylocarpum*** Hook.f.
  - **—ssp. *campylocarpum***
- ***exasperatum*** Tagg
- ***falconeri*** Hook.f.
  - **—ssp. *eximium*** (Nutt.) D.F.Chamb.
  - **—ssp. *falconeri***
- ***fulgens*** Hook.f.
- ***grande*** Wight
- ***griffithianum*** Wight
- ***hodgsonii*** Hook.f.
- ***hookeri*** Nutt.
- ***kendrickii*** Nutt.
- ***lanatum*** Hook.f.
- ***lanigerum*** Tagg
- ***neriiflorum*** Franch.
  - **—ssp. *phaedropum*** (Balf.f. & Farrer) Tagg
- ***pocophorum*** Balf.f. ex Tagg
  - **—var. *hemidartum*** (Tagg) D.F.Chamb.
  - **—var. *pocophorum***
- ***subansiriense*** D.F.Chamb.
- ***succothii*** Davidian
- ***thomsonii*** Hook.f.
  - **—ssp. *thomsonii***
- ***tsariense*** Cowan
- ***wightii*** Hook.f.
- ***arboreum*** Sm. **ssp. *delavayi*** (Franch.) D.F.Chamb. **var. *peramoenum*** (Balf.f. & Forrest) D.F.Chamb.
- ***tanastylum*** Balf.f. & Kingdon-Ward
  - **—var. *tanastylum***
- ***papillatum*** Balf.f. & Cooper
- ***arboreum*** Sm. **ssp. *cinnamomeum*** (Lindl.) Tagg
  - **—ssp. *delavayi*** (Franch.) D.F.Chamb.
- ***blumei*** Nutt.
- ***tsariense*** Cowan **var. *tsariense***

ASS-AS (India - Assam)
- ***Rhododendron arboreum*** Sm.
  - **—ssp. *delavayi*** (Franch.) D.F.Chamb. **var. *delavayi***
  - **—ssp. *delavayi*** (Franch.) D.F.Chamb.

ASS-MA (India - Manipur)
- ***Rhododendron arboreum*** Sm.
  - **—ssp. *delavayi*** (Franch.) D.F.Chamb. **var. *delavayi***
- ***elliottii*** Watt ex Brandis
- ***macabeanum*** Watt ex Balf.f.
- ***wattii*** Cowan
- ***arboreum*** Sm. **ssp. *delavayi*** (Franch.) D.F.Chamb.

ASS-ME (India - Meghalaya)
- ***Rhododendron arboreum*** Sm.
  - **—ssp. *delavayi*** (Franch.) D.F.Chamb. **var. *delavayi***
  - **—ssp. *delavayi*** (Franch.) D.F.Chamb.

BHU-BH (Bhutan)
- ***Rhododendron arboreum*** Sm.
  - **—ssp. *arboreum***
  - **—ssp. *cinnamomeum*** (Lindl.) Tagg **var. *roseum*** Lindl.
- ***argipeplum*** Balf.f. & R.E.Cooper
- ***barbatum*** Wall. ex G.Don
- ***campanulatum*** D.Don
  - **—ssp. *aeruginosum*** (Hook.f.) D.F.Chamb.
  - **—ssp. *campanulatum***
- ***campylocarpum*** Hook.f.
  - **—ssp. *campylocarpum***
- ***falconeri*** Hook.f.
  - **—ssp. *falconeri***
- ***fulgens*** Hook.f.
- ***grande*** Wight
- ***griffithianum*** Wight
- ***hodgsonii*** Hook.f.
- ***kendrickii*** Nutt.
- ***kesangiae*** D.G.Long & Rushforth
- ***lanatum*** Hook.f.
- ***neriiflorum*** Franch.
  - **—ssp. *phaedropum*** (Balf.f. & Farrer) Tagg
- ***niveum*** Hook.f.
- ***succothii*** Davidian
- ***thomsonii*** Hook.f.
  - **—ssp. *thomsonii***
- ***tsariense*** Cowan
- ***wallichii*** Hook.f.
- ***wightii*** Hook.f.
- ***flinckii*** Davidian
- ***bhutanense*** D.G.Long & Bowes Lyon
- ***papillatum*** Balf.f. & Cooper
- ***poluninii*** Davidian
- ***tsariense*** Cowan **var. *magnum*** Davidian
- ***kesangiae*** D.G.Long & Rushforth **var. *album*** Namgyel & D.G.Long
  - **—var. *kesangiae***
- ***tsariense*** Cowan **var. *tsariense***

BHU-SI (India - Sikkim)
- ***Rhododendron arboreum*** Sm.
  - **—ssp. *arboreum***
  - **—ssp. *cinnamomeum*** (Lindl.) Tagg **var. *cinnamomeum*** (Wall. ex G.Don) Lindl.
  - **—ssp. *cinnamomeum*** (Lindl.) Tagg **var. *roseum*** Lindl.

argipeplum Balf.f. & R.E.Cooper
barbatum Wall. ex G.Don
campanulatum D.Don
—**ssp.** ***aeruginosum*** (Hook.f.) D.F.Chamb.
—**ssp.** ***campanulatum***
campylocarpum Hook.f.
—**ssp.** ***campylocarpum***
falconeri Hook.f.
—**ssp.** ***falconeri***
fulgens Hook.f.
grande Wight
griffithianum Wight
hodgsonii Hook.f.
lanatum Hook.f.
niveum Hook.f.
thomsonii Hook.f.
—**ssp.** ***thomsonii***
wallichii Hook.f.
wightii Hook.f.
$x$sikkimense Pradhan & Lachumgpa
arboreum Sm. **ssp.** ***cinnamomeum*** (Lindl.) Tagg

BMA-OO (India - Sikkim)
Rhododendron annae Franch.
anthosphaerum Diels
aperantum Balf.f. & Kingdon-Ward
araiophyllum Balf.f. & W.W.Sm.
arboreum Sm.
—**ssp.** ***delavayi*** (Franch.) D.F.Chamb. **var.** ***delavayi***
bainbridgeanum Tagg & Forrest
basilicum Balf.f. & W.W.Sm.
beanianum Cowan
beesianum Diels
campylocarpum Hook.f.
—**ssp.** ***caloxanthum*** (Balf.f. & Farrer) D.F.Chamb.
cerasinum Tagg
coelicum Balf.f. & Farrer
decorum Franch.
—**ssp.** ***diaprepes*** (Balf.f. & W.W.Sm.) T.L.Ming
dichroanthum Diels **ssp.** ***apodectum*** (Balf.f. & W.W.Sm.) Cowan
—**ssp.** ***septentrionale*** Cowan
—**ssp.** ***scyphocalyx*** (Balf.f. & Forrest) Cowan
eclecteum Balf.f. & Forrest **var.** ***eclecteum***
exasperatum Tagg
forrestii Balf.f. ex Diels **ssp.** ***forrestii***
glischrum Balf.f. & W.W.Sm. **ssp.** ***glischrum***
griersonianum Balf.f. & Forrest
habrotrichum Balf.f. & W.W.Sm.
haematodes Franch.
—**ssp.** ***chaetomallum*** (Balf.f. & Forrest) D.F.Chamb.
kyawii Lace & W.W.Sm.
mallotum Balf.f. & Kingdon-Ward
martinianum Balf.f. & Forrest
meddianum Forrest **var.** ***meddianum***
—**var.** ***atrokermesinum*** Tagg
montroseanum Davidian
neriiflorum Franch.
—**ssp.** ***neriiflorum***
—**ssp.** ***phaedropum*** (Balf.f. & Farrer) Tagg
preptum Balf.f. & Forrest
recurvoides Tagg & Kingdon-Ward
rex H.L,v.
—**ssp.** ***fictolacteum*** (Balf.f.) D.F.Chamb.
sidereum Balf.f.
sinogrande Balf.f. & W.W.Sm.
spilotum Balf.f. & Farrer
stewartianum Diels
dichroanthum Diels
protistum Balf.f. & Forrest **var.** ***protistum***
facetum Balf.f. & Kingdon-Ward
glischrum Balf.f. & W.W.Sm.
hylaeum Balf.f. & Farrer
protistum Balf.f. & Forrest **var.** ***giganteum*** (Forrest ex Tagg) D.F.Chamb.
eclecteum Balf.f. & Forrest
meddianum Forrest
forrestii Balf.f. ex Diels
tanastylum Balf.f. & Kingdon-Ward
protistum Balf.f. & Forrest
parishii C.B.Clarke
chionanthum Tagg & Forrest
sperabile Balf.f. & Farrer
euchroum Balf.f. & Kingdon-Ward
magnificum Kingdon-Ward
arizelum Balf.f. & Forrest
vesiculiferum Tagg
tanastylum Balf.f. & Kingdon-Ward **var.** ***tanastylum***
arboreum Sm. **ssp.** ***delavayi*** (Franch.) D.F.Chamb.
sperabile Balf.f. & Farrer **var.** ***sperabile***
delavayi Franch. **var.** ***albomentosum*** Davidian
araiophyllum Balf.f. & W.W.Sm. **ssp.** ***araiophyllum***
decorum Franch. **ssp.** ***decorum***

BRC-OO (Canada - British Columbia)
Rhododendron macrophyllum D.Don ex G.Don

BUL-OO (Bulgaria)
Rhododendron ponticum L.

CAL-OO (United States - California)
Rhododendron macrophyllum D.Don ex G.Don

CHC-GU (China - Guizhou)
Rhododendron aberconwayi Cowan
$x$agastum Balf.f. & W.W.Sm.
annae Franch.
arboreum Sm.
—**ssp.** ***delavayi*** (Franch.) D.F.Chamb. **var.** ***delavayi***
argyrophyllum Franch. **ssp.** ***nankingense*** (Cowan) D.F.Chamb.
auriculatum Hemsl.
calophytum Franch.
—**var.** ***calophytum***
decorum Franch.
floribundum Franch.
fortunei Lindl.
—**ssp.** ***discolor*** (Franch.) D.F.Chamb.
irroratum Franch. **ssp.** ***irroratum***
longesquamatum C.K.Schneid.
maculiferum Franch.
ririei Hemsl. & E.H.Wilson
simiarum Hance **var.** ***simiarum***
vernicosum Franch.
williamsianum Rehder & E.H.Wilson
wiltonii Hemsl. & E.H.Wilson

denudatum H.L,v.
coeloneuron Diels
simiarum Hance
davidii Franch.
huianum W.P.Fang
glanduliferum Franch.
faithae Chun
fortunei Lindl. **ssp.** ***fortunei***
leishanicum W.P.Fang & S.S.Chang
brevinerve Chun & W.P.Fang
spanotrichum Balf.f. & W.W.Sm.
longipes Rehder & E.H.Wilson **var.** ***longipes***
haofui Chun & W.P.Fang
arboreum Sm. **ssp.** ***delavayi*** (Franch.) D.F.Chamb.
oligocarpum W.P.Fang & S.S.Chang
magniflorum W.K.Hu
oblancifolium M.Y.Fang
sutchuenense Franch.
longipes Rehder & E.H.Wilson
guizhouense M.Y.Fang

CHC-HU (China - Hubei)

Rhododendron adenopodum Franch.
argyrophyllum Franch. **ssp.** ***hypoglaucum*** (Hemsl.) D.F.Chamb.
auriculatum Hemsl.
fortunei Lindl.
—ssp. ***discolor*** (Franch.) D.F.Chamb.
maculiferum Franch.
oreodoxa Franch.
—var. ***fargesii*** (Franch.) D.F.Chamb.
praeteritum Hutch.
praevernum Hutch.
praeteritum Hutch. **var.** ***hirsutum*** W.K.Hu
sutchuenense Franch.
praeteritum Hutch. **var.** ***praeteritum***

CHC-SI (China - Sichuan)

Rhododendron adenogynum Diels
adenopodum Franch.
adenosum Davidian
alutaceum Balf.f. & W.W.Sm. **var.** ***alutaceum***
argyrophyllum Franch.
—ssp. ***hypoglaucum*** (Hemsl.) D.F.Chamb.
—ssp. ***argyrophyllum***
—ssp. ***omeiense*** (Rehder & E.H.Wilson) D.F.Chamb.
auriculatum Hemsl.
balfourianum Diels
—var. ***aganniphoides*** Tagg & Forrest
beesianum Diels
calophytum Franch.
—var. ***openshawianum*** (Rehder & E.H.Wilson) D.F.Chamb.
—var. ***calophytum***
decorum Franch.
eclecteum Balf.f. & Forrest **var.** ***eclecteum***
elegantulum Tagg & Forrest
floribundum Franch.
fortunei Lindl.
—ssp. ***discolor*** (Franch.) D.F.Chamb.
galactinum Balf.f. ex Tagg
hemsleyanum E.H.Wilson
hunnewellianum Rehder & E.H.Wilson **ssp.** ***hunnewellianum***
insigne Hemsl. & E.H.Wilson
irroratum Franch.
—ssp. ***irroratum***
longesquamatum C.K.Schneid.
lukiangense Franch.
maculiferum Franch.
mimetes Tagg & Forrest **var.** ***mimetes***
—var. ***simulans*** Tagg & Forrest
orbiculare Decne.
—ssp. ***orbiculare***
oreodoxa Franch.
—var. ***fargesii*** (Franch.) D.F.Chamb.
—var. ***oreodoxa***
pachytrichum Franch.
peregrinum Tagg
phaeochrysum Balf.f. & W.W.Sm.
—var. ***levistratum*** (Balf.f. & Forrest) D.F.Chamb.
—var. ***phaeochrysum***
—var. ***agglutinatum*** (Balf.f. & Forrest) D.F.Chamb.
praevernum Hutch.
proteoides Balf.f. & W.W.Sm.
rex H.L,v.
—ssp. ***rex***
ririei Hemsl. & E.H.Wilson
roxieanum Forrest
—var. ***roxieanum***
—var. ***oreonastes*** (Balf.f. & Forrest) T.L.Ming
—var. ***cucullatum*** (Hand.-Mazz.) D.F.Chamb.
rufum Batalin
selense Franch. **ssp.** ***dasycladum*** (Balf.f. & W.W.Sm.) D.F.Chamb.
sikangense W.P.Fang
souliei Franch.
sphaeroblastum Balf.f. & Forrest
strigillosum Franch.
thayerianum Rehder & E.H.Wilson
traillianum Forrest & W.W.Sm. **var.** ***traillianum***
uvariifolium Diels
vernicosum Franch.
wardii W.W.Sm. **var.** ***puralbum*** (Balf.f. & W.W.Sm.) D.F.Chamb.
—var. ***wardii***
wasonii Hemsl. & E.H.Wilson
watsonii Hemsl. & E.H.Wilson
williamsianum Rehder & E.H.Wilson
wiltonii Hemsl. & E.H.Wilson
przewalskii Maxim.
pingianum W.P.Fang
irroratum Franch. **ssp.** ***pogonostylum*** (Balf.f. & W.W.Sm.) D.F.Chamb.
denudatum H.L,v.
traillianum Forrest & W.W.Sm.
bureavioides Balf.f.
wardii W.W.Sm.
balangense W.P.Fang
faberi Hemsl.
eclecteum Balf.f. & Forrest
selense Franch.
coeloneuron Diels
detersile Franch.
nigroglandulosum Nitz.
roxieoides D.F.Chamb.

prattii Franch.
barkamense D.F.Chamb.
huidongense T.L.Ming
bonvalotii Bureau & Franch.
asterochnoum Diels
davidii Franch.
huianum W.P.Fang
fortunei Lindl. **ssp.** ***fortunei***
platypodum Diels
ochraceum Rehder & E.H.Wilson
dasycladoides Hand.-Mazz.
longipes Rehder & E.H.Wilson **var.** ***longipes***
—**var.** ***chienianum*** (W.P.Fang) D.F.Chamb.
hunnewellianum Rehder & E.H.Wilson **ssp.** ***rockii*** (E.H.Wilson) D.F.Chamb.
brevipetiolatum M.Y.Fang
sphaeroblastum Balf.f. & Forrest **var.** ***wumengense*** K.M.Feng
urophyllum W.P.Fang
miyiense W.K.Hu
nymphaeoides W.K.Hu
gonggashanense W.K.Hu
ochraceum Rehder & E.H.Wilson **var.** ***brevicarpum*** W.K.Hu
asterochnoum Diels **var.** ***brevipedicellatum*** W.K.Hu
decorum Franch. **ssp.** ***parvistigmatis*** W.K.Hu
orbiculare Decne. **ssp.** ***oblongum*** W.K.Hu
danbaense L.C.Hu
wasonii Hemsl. & E.H.Wilson **var.** ***wenchuanense*** L.C.Hu
ebianense M.Y.Fang
wolongense W.K.Hu
longicalyx M.Y.Fang
verruciferum W.K.Hu
oreodoxa Franch. **var.** ***adenostylosum*** M.Y.Fang & H.K.Hu
calophytum Franch. **var.** ***pauciflorum*** W.K.Hu
pachytrichum Franch. **var.** ***tenuistylosum*** W.K.Hu
sutchuenense Franch.
insigne Hemsl. & E.H.Wilson **var.** ***hejiangense*** (W.P.Fang) M.Y.Fang
clementinae Forrest **ssp.** ***clementinae***
asterochnoum Diels **var.** ***asterochnoum***
balfourianum Diels **var.** ***balfourianum***
decorum Franch. **ssp.** ***decorum***
insigne Hemsl. & E.H.Wilson **var.** ***insigne***
ochraceum Rehder & E.H.Wilson **var.** ***ochraceum***
pachytrichum Franch. **var.** ***pachytrichum***
sikangense W.P.Fang **var.** ***sikangense***
sphaeroblastum Balf.f. & Forrest **var.** ***sphaeroblastum***
uvariifolium Diels **var.** ***uvariifolium***
wasonii Hemsl. & E.H.Wilson **var.** ***wasonii***
alutaceum Balf.f. & W.W.Sm.
hunnewellianum Rehder & E.H.Wilson
longipes Rehder & E.H.Wilson
mimetes Tagg & Forrest
trichogynum L.C.Hu
pugenense L.C.Hu
przewalskii Maxim. **ssp.** ***przewalskii***

CHC-YU (China - Yunnan)
Rhododendron aberconwayi Cowan
adenogynum Diels
aganniphum Balf.f. & Kingdon-Ward **var.** ***aganniphum***
—**var.** ***flavorufum*** (Balf.f. & Forrest) D.F.Chamb.
$x$agastum Balf.f. & W.W.Sm.
albertsenianum Forrest
alutaceum Balf.f. & W.W.Sm. **var.** ***alutaceum***
—**var.** ***russotinctum*** (Balf.f. & Forrest) D.F.Chamb.
—**var.** ***iodes*** (Balf.f. & Forrest) D.F.Chamb.
annae Franch.
anthosphaerum Diels
aperantum Balf.f. & Kingdon-Ward
araiophyllum Balf.f. & W.W.Sm.
arboreum Sm.
—**ssp.** ***delavayi*** (Franch.) D.F.Chamb. **var.** ***delavayi***
argyrophyllum Franch.
—**ssp.** ***argyrophyllum***
bainbridgeanum Tagg & Forrest
balfourianum Diels
—**var.** ***aganniphoides*** Tagg & Forrest
basilicum Balf.f. & W.W.Sm.
bathyphyllum Balf.f. & Forrest
beesianum Diels
bureavii Franch.
callimorphum Balf.f. & W.W.Sm. **var.** ***myiagrum*** (Balf.f. & Forrest) D.F.Chamb.
—**var.** ***callimorphum***
calophytum Franch.
—**var.** ***openshawianum*** (Rehder & E.H.Wilson) D.F.Chamb.
—**var.** ***calophytum***
campylocarpum Hook.f.
—**ssp.** ***caloxanthum*** (Balf.f. & Farrer) D.F.Chamb.
catacosmum Balf.f. ex Tagg
chamaethomsonii (Tagg & Forrest) Cowan & Davidian
—**var.** ***chamaethomsonii***
citriniflorum Balf.f. & Forrest
—**var.** ***citriniflorum***
—**var.** ***horaeum*** (Balf.f. & Forrest) D.F.Chamb.
clementinae Forrest
coelicum Balf.f. & Farrer
coriaceum Franch.
coryanum Tagg & Forrest
crinigerum Franch. **var.** ***crinigerum***
—**var.** ***euadenium*** Tagg & Forrest
cyanocarpum (Franch.) W.W.Sm.
decorum Franch.
—**ssp.** ***diaprepes*** (Balf.f. & W.W.Sm.) T.L.Ming
dichroanthum Diels **ssp.** ***apodectum*** (Balf.f. & W.W.Sm.) Cowan
—**ssp.** ***septentrionale*** Cowan
—**ssp.** ***scyphocalyx*** (Balf.f. & Forrest) Cowan
—**ssp.** ***dichroanthum***
diphrocalyx Balf.f.
eclecteum Balf.f. & Forrest **var.** ***eclecteum***
—**var.** ***bellatulum*** Balf.f. ex Tagg
elegantulum Tagg & Forrest
eudoxum Balf.f. & Forrest **var.** ***mesopolium*** (Balf.f. & Forrest) D.F.Chamb.
—**var.** ***eudoxum***

floccigerum Franch.
forrestii Balf.f. ex Diels **ssp. *forrestii***
fulvum Balf.f. & W.W.Sm.
glischrum Balf.f. & W.W.Sm. **ssp. *glischrum***
—**ssp. *rude*** (Tagg & Forrest) D.F.Chamb.
griersonianum Balf.f. & Forrest
habrotrichum Balf.f. & W.W.Sm.
haematodes Franch.
—**ssp. *haematodes***
—**ssp. *chaetomallum*** (Balf.f. & Forrest) D.F.Chamb.
irroratum Franch.
—**ssp. *irroratum***
kyawii Lace & W.W.Sm.
lacteum Franch.
lukiangense Franch.
mallotum Balf.f. & Kingdon-Ward
martinianum Balf.f. & Forrest
meddianum Forrest **var. *meddianum***
microgynum Balf.f. & Forrest
neriiflorum Franch.
—**ssp. *neriiflorum***
—**ssp. *phaedropum*** (Balf.f. & Farrer) Tagg
oreodoxa Franch.
—**var. *oreodoxa***
pachytrichum Franch.
phaeochrysum Balf.f. & W.W.Sm.
—**var. *levistratum*** (Balf.f. & Forrest) D.F.Chamb.
—**var. *phaeochrysum***
—**var. *agglutinatum*** (Balf.f. & Forrest) D.F.Chamb.
pocophorum Balf.f. ex Tagg
—**var. *hemidartum*** (Tagg) D.F.Chamb.
—**var. *pocophorum***
praestans Balf.f. & W.W.Sm.
pronum Tagg & Forrest
proteoides Balf.f. & W.W.Sm.
rex H.L,v.
—**ssp. *fictolacteum*** (Balf.f.) D.F.Chamb.
—**ssp. *rex***
rothschildii Davidian
roxieanum Forrest
—**var. *roxieanum***
—**var. *oreonastes*** (Balf.f. & Forrest) T.L.Ming
—**var. *cucullatum*** (Hand.-Mazz.) D.F.Chamb.
sanguineum Franch.
—**ssp. *didymum*** (Balf.f. & Forrest) Cowan
—**ssp. *sanguineum* var. *haemaleum*** (Balf.f. & Forrest) D.F.Chamb.
—**ssp. *sanguineum* var. *sanguineum***
—**ssp. *sanguineum* var. *himertum*** (Balf.f. & Forrest) D.F.Chamb.
—**ssp. *sanguineum* var. *didymoides*** Tagg & Forrest
—**ssp. *sanguineum* var. *cloiophorum*** (Balf.f. & Forrest) D.F.Chamb.
selense Franch. **ssp. *setiferum*** (Balf.f. & Forrest) D.F.Chamb.
—**ssp. *dasycladum*** (Balf.f. & W.W.Sm.) D.F.Chamb.
—**ssp. *jucundum*** (Balf.f. & W.W.Sm.) D.F.Chamb.
—**ssp. *selense***
semnoides Tagg & Forrest
sidereum Balf.f.
sikangense W.P.Fang
sinogrande Balf.f. & W.W.Sm.
sperabile Balf.f. & Farrer **var. *weihsiense*** Tagg & Forrest
sphaeroblastum Balf.f. & Forrest
stewartianum Diels
strigillosum Franch.
taliense Franch.
temenium Balf.f. & Forrest **var. *temenium***
traillianum Forrest & W.W.Sm. **var. *traillianum***
—**var. *dictyotum*** (Balf.f. ex Tagg) D.F.Chamb.
uvariifolium Diels
vernicosum Franch.
wardii W.W.Sm. **var. *puralbum*** (Balf.f. & W.W.Sm.) D.F.Chamb.
—**var. *wardii***
temenium Balf.f. & Forrest
irroratum Franch. **ssp. *pogonostylum*** (Balf.f. & W.W.Sm.) D.F.Chamb.
denudatum H.L,v.
dichroanthum Diels
traillianum Forrest & W.W.Sm.
protistum Balf.f. & Forrest **var. *protistum***
crinigerum Franch.
facetum Balf.f. & Kingdon-Ward
glischrum Balf.f. & W.W.Sm.
wardii W.W.Sm.
dimitrium Balf.f. & Forrest
aganniphum Balf.f. & Kingdon-Ward
eclecteum Balf.f. & Forrest
meddianum Forrest
selense Franch.
forrestii Balf.f. ex Diels
schistocalyx Balf.f. & Forrest
arboreum Sm. **ssp. *delavayi*** (Franch.) D.F.Chamb. **var. *peramoenum*** (Balf.f. & Forrest) D.F.Chamb.
tanastylum Balf.f. & Kingdon-Ward
sinofalconeri Balf.f.
protistum Balf.f. & Forrest
codonanthum Balf.f. & Forrest
pubicostatum T.L.Ming
dumicola Tagg & Forrest
comisteum Balf.f. & Forrest
nakotiltum Balf.f. & Forrest
chionanthum Tagg & Forrest
chamaethomsonii (Tagg & Forrest) Cowan & Davidian **var. *chamaedoron*** (Tagg & Forrest) D.F.Chamb.
neriiflorum Franch. **ssp. *agetum*** (Balf.f. & Forrest) Tagg
bijiangense T.L.Ming
sperabile Balf.f. & Farrer
davidii Franch.
huianum W.P.Fang
glanduliferum Franch.
magnificum Kingdon-Ward
arizelum Balf.f. & Forrest
calvescens Balf.f. & Forrest **var. *calvescens***
—**var. *duseimatum*** (Balf.f. & Forrest) D.F.Chamb.
dasycladoides Hand.-Mazz.

esetulosum Balf.f. & Forrest
vesiculiferum Tagg
mengtszense Balf.f. & W.W.Sm.
spanotrichum Balf.f. & W.W.Sm.
fulvoides Balf.f. & Forrest
tanastylum Balf.f. & Kingdon-Ward **var.** ***tanastylum***
—**var.** ***pennivenium*** (Balf.f. & Forrest) D.F.Chamb.
leptopeplum Balf.f. & Forrest
farinosum H.L,v.
arboreum Sm. **ssp.** ***delavayi*** (Franch.) D.F.Chamb.
serotinum Hutch.
erastum Balf.f. & Forrest
sanguineum Franch. **ssp.** ***sanguineum***
laojunense T.L.Ming
gongshanense T.L.Ming
flavoflorum T.L.Ming
montiganum T.L.Ming
sikangense W.P.Fang **var.** ***exquisitum*** (T.L.Ming) T.L.Ming
pingbianense M.Y.Fang
delavayi Franch. **var.** ***pilostylum*** K.M.Feng
sphaeroblastum Balf.f. & Forrest **var.** ***wumengense*** K.M.Feng
punctifolium L.C.Hu
zhongdianense L.C.Hu
fictolacteum Balf.f. **var.** ***miniforme*** Davidian
roxieanum Forrest **var.** ***parvum*** Davidian
pilostylum W.K.Hu
decorum Franch. **ssp.** ***cordatum*** W.K.Hu
rex H.L,v. **ssp.** ***gratum*** (T.L.Ming) M.Y.Fang
araiophyllum Balf.f. & W.W.Sm. **ssp.** ***lapidosum*** (T.L.Ming) M.Y.Fang
clementinae Forrest **ssp.** ***clementinae***
araiophyllum Balf.f. & W.W.Sm. **ssp.** ***araiophyllum***
balfourianum Diels **var.** ***balfourianum***
decorum Franch. **ssp.** ***decorum***
pachytrichum Franch. **var.** ***pachytrichum***
sphaeroblastum Balf.f. & Forrest **var.** ***sphaeroblastum***
uvariifolium Diels **var.** ***uvariifolium***
alutaceum Balf.f. & W.W.Sm.
callimorphum Balf.f. & W.W.Sm.
calvescens Balf.f. & Forrest
eudoxum Balf.f. & Forrest

CHH-OO (China - Hainan)
Rhododendron simiarum Hance
—**var.** ***deltoideum*** P.C.Tam

CHM-JI (China - Jilin)
Rhododendron aureum Georgi
—**var.** ***aureum***

CHN-GA (China - Gansu)
Rhododendron hunnewellianum Rehder & E.H.Wilson **ssp.** ***hunnewellianum***
maculiferum Franch.
oreodoxa Franch.
—**var.** ***fargesii*** (Franch.) D.F.Chamb.
—**var.** ***oreodoxa***
rufum Batalin
watsonii Hemsl. & E.H.Wilson
przewalskii Maxim.
potaninii Batalin
hunnewellianum Rehder & E.H.Wilson **ssp.** ***rockii*** (E.H.Wilson) D.F.Chamb.
kansuense Millais
gannanense Z.C.Feng & X.G.Sun
przewalskii Maxim. **ssp.** ***przewalskii***

CHN-SA (China - Shaanxi)
Rhododendron argyrophyllum Franch.
—**ssp.** ***argyrophyllum***
clementinae Forrest
maculiferum Franch.
oreodoxa Franch.
purdomii Rehder & E.H.Wilson
oreodoxa Franch. **var.** ***shensiense*** D.F.Chamb.
clementinae Forrest **ssp.** ***aureodorsale*** W.P.Fang ex J.Q.Fu
sutchuenense Franch.

CHS-AN (China - Anhui)
Rhododendron fortunei Lindl.
—**ssp.** ***discolor*** (Franch.) D.F.Chamb.
simiarum Hance **var.** ***simiarum***
—
fortunei Lindl. **ssp.** ***fortunei***
anwheiense E.H.Wilson
shanii W.P.Fang

CHS-FU (China - Fujian)
Rhododendron fortunei Lindl.
simiarum Hance **var.** ***simiarum***
—
fortunei Lindl. **ssp.** ***fortunei***

CHS-GD (China - Guangdong)
Rhododendron fortunei Lindl.
simiarum Hance **var.** ***simiarum***
—
faithae Chun
fortunei Lindl. **ssp.** ***fortunei***
brevinerve Chun & W.P.Fang
haofui Chun & W.P.Fang

CHS-GX (China - Guangxi)
Rhododendron annae Franch.
arboreum Sm.
—**ssp.** ***delavayi*** (Franch.) D.F.Chamb. **var.** ***delavayi***
fortunei Lindl.
—**ssp.** ***discolor*** (Franch.) D.F.Chamb.
maculiferum Franch.
orbiculare Decne.
—**ssp.** ***orbiculare***
simiarum Hance **var.** ***simiarum***
faithae Chun
fortunei Lindl. **ssp.** ***fortunei***
platypodum Diels
orbiculare Decne. **ssp.** ***cardiobasis*** (Sleumer) D.F.Chamb.
chihsinianum Chun & W.P.Fang
brevinerve Chun & W.P.Fang
haofui Chun & W.P.Fang
arboreum Sm. **ssp.** ***delavayi*** (Franch.) D.F.Chamb.
maoerense W.P.Fang & G.Z.Li
ziyuanense P.C.Tam
fangchengense P.C.Tam
polytrichum W.P.Fang
oligocarpum W.P.Fang & S.S.Chang
orbiculare Decne. **ssp.** ***oblongum*** W.K.Hu

simiarum Hance **var. *versicolor*** (Chun & W.P.Fang) M.Y.Fang
ziyuanense P.C.Tam **var. *ziyuanense***
—var. *pachyphyllum* (W.P.Fang) G.Z.Li

CHS-HA (China - Hunan)
Rhododendron argyrophyllum Franch.
auriculatum Hemsl.
fortunei Lindl.
—ssp. *discolor* (Franch.) D.F.Chamb.
orbiculare Decne.
—ssp. *orbiculare*
simiarum Hance **var. *simiarum***
—
fortunei Lindl. **ssp. *fortunei***
anwheiense E.H.Wilson
brevinerve Chun & W.P.Fang
haofui Chun & W.P.Fang
ziyuanense P.C.Tam
polytrichum W.P.Fang
shimenense Q.X.Liu & C.M.Zhang
ziyuanense P.C.Tam **var. *pachyphyllum*** (W.P.Fang) G.Z.Li

CHS-HK (China - Hunan)
Rhododendron simiarum Hance **var. *simiarum***
—

CHS-HN (China - Henan)
Rhododendron henanense W.P.Fang **ssp. *henanense***
—ssp. *lingbaoense* W.P.Fang
—

CHS-JS (China - Jiangsu)
Rhododendron anwheiense E.H.Wilson

CHS-JX (China - Jiangxi)
Rhododendron fortunei Lindl.
simiarum Hance **var. *simiarum***
—
fortunei Lindl. **ssp. *fortunei***
anwheiense E.H.Wilson
jingangshanicum P.C.Tam
xiaoxidongense W.K.Hu

CHS-ZH (China - Zhejiang)
Rhododendron fortunei Lindl.
—ssp. *discolor* (Franch.) D.F.Chamb.
simiarum Hance **var. *simiarum***
—
fortunei Lindl. **ssp. *fortunei***

CHT-QI (China - Qinghai)
Rhododendron przewalskii Maxim.
—ssp. *chrysophyllum* W.P.Fang & S.X.Wang
—ssp. *huzhuense* W.P.Fang & S.X.Wang
—ssp. *yushuense* W.P.Fang & S.X.Wang
—ssp. *dabanshanense* (W.P.Fang & S.X.Wang) W.P.Fang & S.X.Wang
—ssp. *przewalskii*

CHT-XI (China - Xizang)
Rhododendron adenogynum Diels
aganniphum Balf.f. & Kingdon-Ward **var. *aganniphum***
—var. *flavorufum* (Balf.f. & Forrest) D.F.Chamb.
alutaceum Balf.f. & W.W.Sm. **var. *alutaceum***
anthosphaerum Diels
arboreum Sm.
—ssp. *cinnamomeum* (Lindl.) Tagg **var. *roseum*** Lindl.
argipeplum Balf.f. & R.E.Cooper
bainbridgeanum Tagg & Forrest
barbatum Wall. ex G.Don
bathyphyllum Balf.f. & Forrest
beesianum Diels
campylocarpum Hook.f.
—ssp. *campylocarpum*
—ssp. *caloxanthum* (Balf.f. & Farrer) D.F.Chamb.
catacosmum Balf.f. ex Tagg
cerasinum Tagg
chamaethomsonii (Tagg & Forrest) Cowan & Davidian
—var. *chamaethauma* (Tagg) Cowan & Davidian
—var. *chamaethomsonii*
citriniflorum Balf.f. & Forrest
—var. *citriniflorum*
—var. *horaeum* (Balf.f. & Forrest) D.F.Chamb.
coriaceum Franch.
coryanum Tagg & Forrest
crinigerum Franch. **var. *crinigerum***
dignabile Cowan
eclecteum Balf.f. & Forrest **var. *eclecteum***
—var. *bellatulum* Balf.f. ex Tagg
eudoxum Balf.f. & Forrest **var. *mesopolium*** (Balf.f. & Forrest) D.F.Chamb.
—var. *eudoxum*
exasperatum Tagg
faucium D.F.Chamb.
floccigerum Franch.
forrestii Balf.f. ex Diels **ssp. *forrestii***
—ssp. *papillatum* D.F.Chamb.
fulgens Hook.f.
glischrum Balf.f. & W.W.Sm. **ssp. *glischrum***
grande Wight
haematodes Franch.
—ssp. *chaetomallum* (Balf.f. & Forrest) D.F.Chamb.
hirtipes Tagg
hodgsonii Hook.f.
kendrickii Nutt.
lanatum Hook.f.
lanigerum Tagg
lukiangense Franch.
martinianum Balf.f. & Forrest
microgynum Balf.f. & Forrest
montroseanum Davidian
neriiflorum Franch.
—ssp. *neriiflorum*
—ssp. *phaedropum* (Balf.f. & Farrer) Tagg
oreodoxa Franch.
pachytrichum Franch.
parmulatum Cowan
phaeochrysum Balf.f. & W.W.Sm.
—var. *levistratum* (Balf.f. & Forrest) D.F.Chamb.
—var. *phaeochrysum*
—var. *agglutinatum* (Balf.f. & Forrest) D.F.Chamb.
piercei Davidian
pocophorum Balf.f. ex Tagg
—var. *hemidartum* (Tagg) D.F.Chamb.
—var. *pocophorum*

praestans Balf.f. & W.W.Sm.
principis Bureau & Franch.
proteoides Balf.f. & W.W.Sm.
pudorosum Cowan
ramsdenianum Cowan
rex H.L,v.
—**ssp.** ***fictolacteum*** (Balf.f.) D.F.Chamb.
roxieanum Forrest
—**var.** ***oreonastes*** (Balf.f. & Forrest) T.L.Ming
—**var.** ***cucullatum*** (Hand.-Mazz.) D.F.Chamb.
sanguineum Franch.
—**ssp.** ***didymum*** (Balf.f. & Forrest) Cowan
—**ssp.** ***sanguineum*** **var.** ***haemaleum*** (Balf.f. & Forrest) D.F.Chamb.
—**ssp.** ***sanguineum*** **var.** ***sanguineum***
—**ssp.** ***sanguineum*** **var.** ***himertum*** (Balf.f. & Forrest) D.F.Chamb.
—**ssp.** ***sanguineum*** **var.** ***didymoides*** Tagg & Forrest
—**ssp.** ***sanguineum*** **var.** ***cloiophorum*** (Balf.f. & Forrest) D.F.Chamb.
selense Franch. **ssp.** ***setiferum*** (Balf.f. & Forrest) D.F.Chamb.
—**ssp.** ***selense***
semnoides Tagg & Forrest
sherriffii Cowan
sinogrande Balf.f. & W.W.Sm.
sperabiloides Tagg & Forrest
stewartianum Diels
temenium Balf.f. & Forrest **var.** ***dealbatum*** (Cowan) D.F.Chamb.
—**var.** ***temenium***
—**var.** ***gilvum*** (Cowan) D.F.Chamb.
thomsonii Hook.f.
—**ssp.** ***lopsangianum*** (Cowan) D.F.Chamb.
traillianum Forrest & W.W.Sm. **var.** ***dictyotum*** (Balf.f. ex Tagg) D.F.Chamb.
tsariense Cowan
uvariifolium Diels
—**var.** ***griseum*** Cowan
venator Tagg
wallichii Hook.f.
wardii W.W.Sm. **var.** ***puralbum*** (Balf.f. & W.W.Sm.) D.F.Chamb.
—**var.** ***wardii***
wightii Hook.f.
temenium Balf.f. & Forrest
traillianum Forrest & W.W.Sm.
crinigerum Franch.
glischrum Balf.f. & W.W.Sm.
hylaeum Balf.f. & Farrer
wardii W.W.Sm.
aganniphum Balf.f. & Kingdon-Ward
viscidifolium Davidian
eclecteum Balf.f. & Forrest
selense Franch.
forrestii Balf.f. ex Diels
tanastylum Balf.f. & Kingdon-Ward
comisteum Balf.f. & Forrest
pomense Cowan & Davidian
circinnatum Cowan & Kingdon-Ward
lanatoides D.F.Chamb.
trilectorum Cowan
eudoxum Balf.f. & Forrest **var.** ***brunneifolium*** (Balf.f. & Forrest) D.F.Chamb.
chamaethomsonii (Tagg & Forrest) Cowan & Davidian **var.** ***chamaedoron*** (Tagg & Forrest) D.F.Chamb.
miniatum Cowan
populare Cowan
calvescens Balf.f. & Forrest **var.** ***calvescens***
—**var.** ***duseimatum*** (Balf.f. & Forrest) D.F.Chamb.
esetulosum Balf.f. & Forrest
vesiculiferum Tagg
fulvoides Balf.f. & Forrest
arboreum Sm. **ssp.** ***cinnamomeum*** (Lindl.) Tagg
erastum Balf.f. & Forrest
erosum Cowan
eurysiphom Tagg & Forrest
sanguineum Franch. **ssp.** ***sanguineum***
ramipilosum T.L.Ming
tsariense Cowan **var.** ***trimoense*** Davidian
fictolacteum Balf.f. **var.** ***miniforme*** Davidian
megalanthum M.Y.Fang
oreodoxa Franch. **var.** ***adenostylosum*** M.Y.Fang & H.K.Hu
xizangense (W.P.Fang & W.K.Hu) Q.Z.Yu
tanastylum Balf.f. & Kingdon-Ward **var.** ***lingzhiense*** M.Y.Fang
tsariense Cowan **var.** ***tsariense***
uvariifolium Diels **var.** ***uvariifolium***
alutaceum Balf.f. & W.W.Sm.
calvescens Balf.f. & Forrest
eudoxum Balf.f. & Forrest
oreogenum L.C.Hu
lulangense L.C.Hu & Y.Tateishi

CTA-OO (Commonwealth of Independent States - Chita)
Rhododendron aureum Georgi
—**var.** ***aureum***

DEL-OO (United States - Delaware)
Rhododendron maximum L.

GEO-OO (United States - Georgia)
Rhododendron catawbiense Michx.
maximum L.

IND-HP (India - Himachal Pradesh)
Rhododendron arboreum Sm.
—**ssp.** ***arboreum***
campanulatum D.Don
—**ssp.** ***campanulatum***

IND-TN (India - Tamil Nadu)
Rhododendron arboreum Sm.
—**ssp.** ***nilagiricum*** (Zenker) Tagg

IND-UP (India - Uttar Pradesh)
Rhododendron arboreum Sm.
—**ssp.** ***arboreum***
barbatum Wall. ex G.Don
campanulatum D.Don
—**ssp.** ***campanulatum***

IND-WB (India - West Bengal)
Rhododendron arboreum Sm.
—**ssp.** ***arboreum***
—**ssp.** ***cinnamomeum*** (Lindl.) Tagg **var.** ***cinnamomeum*** (Wall. ex G.Don) Lindl.
—**ssp.** ***cinnamomeum*** (Lindl.) Tagg **var.** ***roseum***

Lindl.
barbatum Wall. ex G.Don
campanulatum D.Don
—**ssp.** ***campanulatum***
falconeri Hook.f.
—**ssp.** ***falconeri***
fulgens Hook.f.
grande Wight
griffithianum Wight
hodgsonii Hook.f.
wallichii Hook.f.
arboreum Sm. **ssp.** ***cinnamomeum*** (Lindl.) Tagg

JAP-OO (Japan)
Rhododendron aureum Georgi
—**var.** ***aureum***
brachycarpum D.Don ex G.Don
—**ssp.** ***brachycarpum***
—**ssp.** ***fauriei*** (Franch.) D.F.Chamb. **forma** ***nematoanum*** (Makino) Murata
—**ssp.** ***fauriei*** (Franch.) D.F.Chamb.
degronianum CarriŠre **ssp.** ***degronianum***
—**ssp.** ***heptamerum*** (Maxim.) H.Hara **var.** ***heptamerum*** (Maxim.) Sealy
makinoi Tagg
degronianum CarriŠre **ssp.** ***heptamerum*** (Maxim.) H.Hara **var.** ***kyomaruense*** (T.Yamaz.) H.Hara
—
—**ssp.** ***yakushimanum*** (Nakai) H.Hara
—**ssp.** ***heptamerum*** (Maxim.) H.Hara **var.** ***hondoense*** (Nakai) H.Hara
—**ssp.** ***heptamerum*** (Maxim.) H.Hara **var.** ***kyomaruense*** (T.Yamaz.) H.Hara **forma** ***amagianum*** (T.Yamaz.) H.Hara
—**ssp.** ***yakushimanum*** (Nakai) Kitam. **var.** ***yakushimanum***
—**ssp.** ***yakushimanum*** (Nakai) H.Hara **var.** ***intermedium*** (Sugim.) H.Hara
—**ssp.** ***heptamerum*** (Maxim.) H.Hara

JMK-OO (India - Jammu-Kashmir)
Rhododendron arboreum Sm.
—**ssp.** ***arboreum***
campanulatum D.Don
—**ssp.** ***campanulatum***

KAM-OO (Commonwealth of Independent States - Kamchatka)
Rhododendron aureum Georgi
—**var.** ***aureum***

KHA-OO (Commonwealth of Independent States - Khabarovsk)
Rhododendron aureum Georgi
—**var.** ***aureum***

KOR-NK (Commonwealth of Independent States - Khabarovsk)
Rhododendron aureum Georgi
—**var.** ***aureum***

KOR-SK (Commonwealth of Independent States - Khabarovsk)
Rhododendron brachycarpum D.Don ex G.Don
—**ssp.** ***brachycarpum***
—**ssp.** ***fauriei*** (Franch.) D.F.Chamb.

KRA-OO (Commonwealth of Independent States - Krasnoyarsk)
Rhododendron aureum Georgi
—**var.** ***aureum***

KTY-OO (United States - Kentucky)
Rhododendron catawbiense Michx.

KUR-OO (Commonwealth of Independent States - Kuril Is)
Rhododendron aureum Georgi
—**var.** ***aureum***

LAO-OO (Commonwealth of Independent States - Kuril Is)
Rhododendron serotinum Hutch.

LBS-LB (Lebanon)
Rhododendron ponticum L.

MAI-OO (United States - Maine)
Rhododendron maximum L.

MAS-OO (United States - Massachusetts)
Rhododendron maximum L.

MLY-PM (Malaysia - Peninsular Malaysia)
Rhododendron wrayi King & Gamble

NBR-OO (Canada - New Brunswick)
Rhododendron maximum L.

NCA-OO (United States - North Carolina)
Rhododendron catawbiense Michx.
maximum L.

NCS-SO (Commonwealth of Independent States - Severo-Osetiya)
Rhododendron caucasicum Pall.

NEP-OO (Nepal)
Rhododendron arboreum Sm.
—**ssp.** ***cinnamomeum*** (Lindl.) Tagg **var.** ***roseum*** Lindl. **forma** ***album*** Wall.
—**ssp.** ***cinnamomeum*** (Lindl.) Tagg **var.** ***cinnamomeum*** (Wall. ex G.Don) Lindl.
—**ssp.** ***cinnamomeum*** (Lindl.) Tagg **var.** ***roseum*** Lindl.
barbatum Wall. ex G.Don
campanulatum D.Don
—**ssp.** ***aeruginosum*** (Hook.f.) D.F.Chamb.
—**ssp.** ***campanulatum***
campylocarpum Hook.f.
—**ssp.** ***campylocarpum***
falconeri Hook.f.
—**ssp.** ***falconeri***
fulgens Hook.f.
grande Wight
griffithianum Wight
hodgsonii Hook.f.
thomsonii Hook.f.
—**ssp.** ***thomsonii***
wallichii Hook.f.
wightii Hook.f.
arboreum Sm. **ssp.** ***cinnamomeum*** (Lindl.) Tagg

NSC-OO (Canada - Nova Scotia)
Rhododendron maximum L.

NWH-OO (United States - New Hampshire)
Rhododendron maximum L.

NWY-OO (United States - New York)
Rhododendron maximum L.

ORE-OO (United States - Oregon)
Rhododendron macrophyllum D.Don ex G.Don

PEN-OO (United States - Pennsylvania)
Rhododendron maximum L.

POR-OO (Portugal)
Rhododendron ponticum L.

PRM-OO (Commonwealth of Independent States - Primorye)
Rhododendron aureum Georgi
—**var.** ***aureum***
SAK-OO (Commonwealth of Independent States - Sakhalin)
Rhododendron aureum Georgi
—**var.** ***aureum***
SCA-OO (United States - South Carolina)
Rhododendron maximum L.
SPA-SP (Spain)
Rhododendron ponticum L.
SRL-OO (Sri Lanka)
Rhododendron arboreum Sm.
—**ssp.** *zeylanicum* (Booth) Tagg
SUM-OO (Indonesia - Sumatera)
Rhododendron irroratum Franch.
korthalsii Miq.
irroratum Franch. **ssp.** ***kontumense*** (Sleumer) D.F.Chamb.
TAI-OO (Taiwan)
Rhododendron formosanum Hemsl.
hyperythrum Hayata
morii Hayata
pseudochrysanthum Hayata
pachysanthum Hayata
pseudochrysanthum Hayata **forma** ***rufovelutinum*** T.Yamaz.
—**ssp.** ***morii*** (Hayata) T.Yamaz. **var.** ***taitunense*** T.Yamaz.
—**ssp.** ***morii*** (Hayata) T.Yamaz. **var.** ***nankotaisanense*** (Hayata) T.Yamaz.
TCS-AB (Commonwealth of Independent States - Abkhasiya)
Rhododendron caucasicum Pall.
ponticum L.
TCS-AR (Commonwealth of Independent States - Abkhasiya)
Rhododendron caucasicum Pall.
ponticum L.
TCS-GR (Commonwealth of Independent States - Gruziya)
Rhododendron caucasicum Pall.
ponticum L.
smirnowii Trautv.
ungernii Trautv.
TEN-OO (United States - Tennessee)
Rhododendron maximum L.
THA-OO (Thailand)
Rhododendron arboreum Sm.
—**ssp.** ***delavayi*** (Franch.) D.F.Chamb. **var.** ***delavayi***
—**ssp.** ***delavayi*** (Franch.) D.F.Chamb.
TUR-OO (Turkey)
Rhododendron caucasicum Pall.
ponticum L.
smirnowii Trautv.
ungernii Trautv.
VER-OO (United States - Vermont)
Rhododendron maximum L.
VIE-OO (Vietnam)
Rhododendron arboreum Sm.
irroratum Franch.
protistum Balf.f. & Forrest **var.** ***giganteum*** (Forrest ex Tagg) D.F.Chamb.
tanastylum Balf.f. & Kingdon-Ward
sinofalconeri Balf.f.
protistum Balf.f. & Forrest
nhatrangense Dop
mengtszense Balf.f. & W.W.Sm.
spanotrichum Balf.f. & W.W.Sm.
irroratum Franch. **ssp.** ***kontumense*** (Sleumer) D.F.Chamb.
tanastylum Balf.f. & Kingdon-Ward **var.** ***pennivenium*** (Balf.f. & Forrest) D.F.Chamb.
serotinum Hutch.
excelsum A.Chev.
VRG-OO (United States - Virginia)
Rhododendron catawbiense Michx.
maximum L.
WAS-OO (United States - Washington)
Rhododendron macrophyllum D.Don ex G.Don

Appendix C: Specimens Scored.

This is where the list of scored specimens goes..

Balakrishnan (1971) D-601 *R. arboreum*.

Ball, J. (1884) *R. catawbiense*.

Bartholomew, B. (1974) 150 *R. barbatum*.

Bigger (1987) 2892 *R. thomsonii*.

Biltmore Herbarium (1897) 6566 *R. catawbiense*.

Bowes Lyon, S. (1994) 10012 *R. lanatum*; — (1994) 10016a *R. thomsonii*.

Bozeman, J.R., Ramseur, G.S.& Radford, A.E. (1966) 45204 *R. catawbiense*.

c6143, cultivated at Edinburgh (1958) *R. williamsianum*.

Cave, G.H. 6712 *R. falconeri;* — 6813 *R. falconeri;* — 6923 *R. falconeri*.

Chamberalin (1975) 158a *R. campanulatum;* — (1975) 138 *R. barbatum*.

Chamberlain, Cox and Hutchison (1989) 4026 *R. watsonii*.

Chengdu Edinburgh Expedition (1991) (1991) 209 *R. calophytum;* — (1991) 133 *R. strigillosum;* — (1991) 171 *R. pachytrichum;* — (1991) 191 *R. strigillosum;* — (1991) 285 *R. ririei*.

Chu, K.L. (1936) 2524 *R. argyrophyllum*.

Cooper, R.E. (1915) 3257 *R. barbatum*; — (1914) 3507 *R. barbatum*; — (1915) 3818 *R. barbatum*; — (1915) 4830 *R. barbatum*; — (1916) 5736 *R. campanulatum*; — (1916) 5737 *R. campanulatum*; — (1916) 5926 *R. campanulatum*; — (1914) 3484 *R. lanatum*.

Cox & Hutchinson (1965) 580 *R. barbatum*.

Cramer (1976) 4735 *R. arboreum*.

Dassanayake (1970) *R. arboreum*.

Davis, P. and Hedge, I.C. (1957) D32899 *R. ponticum*.

Drummond, J.R. (1884) 2761 *R. campanulatum;* — (1885) 22254 *R. campanulatum;* — (1888) 22498 *R. campanulatum;* — (1904) 22707 *R. campanulatum;* —. (1904) 22759 *R. campanulatum*.

Duzenli (1978) 1188 *R. ponticum*.

EN Yunnan Expedition. (1964) 1438 *R. argyrophyllum*.

Fang, W.P. (1928) 874 *R. argyrophyllum;* — (1928) 1018 *R. argyrophyllum;* — (1928) 1141 *R. argyrophyllum;* — (1928) 1188 *R. argyrophyllum;* — (1928) 3653 *R. watsonii;* — (1928) 2971 *R. strigillosum;* — (1928) 2971 *R. strigillosum*.

Farges 1508 *R. auriculatum*.

Farrer, R. (1920) 1753 *R. kyawii*.

Feng, K.M. (1940) 8270 *R. sanguineum;* — (1959) 23319 *R. balfourianum*.

Forrest, G. (1917) 14190 *R. wardii*; — (1906) 4136 *R. taliense*; — (1906) 4160 *R. lacteum*; — (1906) 4167 *R. taliense*; — (1910) 6772 *R. taliense*; — (1910) 6778 *R. lacteum*; — (1912) 8939 *R. neriiflorum*; — (1919) 10293 *R. sanguineum*; — (1919) 10315 *R. sanguineum*; — (1923) 11056 *R. eclecteum*; — (1924) 11503 *R. anthospaerum*; — (1913) 11575 *R. lacteum*;

— (1913) 11579 *R. taliense*; — (1913) 11583 *R. taliense*; — (1913) 11911 *R. neriiflorum*; — (1914) 13259 *R. forrestii*; — (1914) 13304 *R. sanguineum*; — (1914) 13442 *R. forrestii*; — (1914) 13448 *R. taliense*; — (1914) 13542 *R. sanguineum*; — (1917) 14011 *R. forrestii*; — (1917) 14188 *R. forrestii*; — (1917) 14357 *R. wardii*; — (1917) 14534 *R. forrestii*; — (1917) 14971 *R. sanguineum*; — (1917) 15272 *R. forrestii*; — (1917) 15412 *R. wardii*; — (1917) 15568 *R. lacteum*; — (1921) 16320 *R. mimetes*; — (1918) 16531 *R. uvariifolium*; — (1918) 16535 *R. forrestii*; — (1918) 16565 *R. praestans*; — (1918) 16678 *R. forrestii*; — (1918) 17088 *R. mimetes*; — (1918) 17089 *R. balfourianum*; — (1918) 17096 *R. uvariifolium*; — (1918) 17291 *R. mimetes*; — (1918) 17291 *R. mimetes*; — (1918) 17319 *R. sanguineum*; — (1919) 17324 *R. selense*; — (1921) 17368 *R. mimetes*; — (1921) 17377 *R. mimetes*; — (1918) 17408 *R. uvariifolium*; — (1919) 17413 *R. protistum*; — (1919) 17420 *R. protistum*; — (1918) 17626 *R. barbatum*; — (1918) 17696 *R. griesonianum*; — (1918) 17705 *R. neriiflorum*; — (1918) 17749 *R. neriiflorum*; — (1919) 17905 *R. protistum*; — (1919) 18049 *R. griesonianum*; — (1919) 18168 *R. anthospaerum*; — (1919) 18220 *R. neriiflorum*; — (1925) 18395 *R. kyawii*; — (1925) 18395 *R. kyawii*; — (1925) 18395 *R. kyawii*; — (1919) 18458 *R. protistum*; — (1919) 18458 *R. protistum*; — (1919) 18624 *R. protistum*; — (1919) 18669 *R. roxieanum*; — (1919) 18742 *R. neriiflorum*; — (1918) 18748 *R. neriiflorum*; — (1919) 18750 *R. kyawii*; — (1919) 18811 *R. protistum*; — (1919) 18814 *R. protistum*; — (1918) 18829 *R. griesonianum*; — (1919) 19204 *R. sanguineum*; — (1919) 19212 *R. wardii*; — (1921) 19411 *R. selense*; — (1919) 19512 *R. wardii*; — (1921) 19565 *R. wardii*; — (1921) 19628 *R. roxieanum*; — (1921) 20053 *R. eclecteum*; — (1921) 20053 *R. eclecteum*; — (1921) 20213 *R. phaeochrysum*; — (1922) 20305 *R. floccigerum*; — (1921) 20419 *R. mimetes*; — (1921) 20428 *R. mimetes*; — (1921) 20455 *R. balfourianum*; — (1921) 20836 *R. eclecteum*; — (1921) 21044 *R. balfourianum*; — (1921) 21055 *R. balfourianum*; — (1921) 21380 *R. balfourianum*; — (1921) 21417 *R. mimetes*; — (1922) 21769 *R. eclecteum*; — (1922) 21840 *R. eclecteum*; — (1922) 21842 *R. eclecteum*; — (1922) 21881 *R. eclecteum*; — (1922) 21883 *R. eclecteum*; — (1922) 21949 *R. lacteum*; — (1922) 22021 *R. lacteum*; — (1922) 22325 *R. lacteum*; — (1921) 22541 *R. balfourianum*; — (1922) 22710 *R. eclecteum*; — (1922) 22711 *R. selense*; — (1922) 22805 *R. floccigerum*; — (1922) 22806 *R. floccigerum*; — (1922) 22807 *R. floccigerum*; — (1922) 22809 *R. floccigerum*; — (1922) 22810 *R. floccigerum*; — (1923) 23309 *R. uvariifolium*; — (1923) 23335 *R. traillianum*; — (1923) 23336 *R. traillianum*; — (1923) 23338 *R. traillianum*; — (1923) 23339 *R. traillianum*; — (1918) 24064 *R. neriiflorum*; — (1924) 24092 *R. habrotrichum*; — (1918) 24116 *R. griesonianum*; — (1924) 24121 *R. habrotrichum*; — (1924) 24193 *R. arizelum*; — (1924) 24236 *R. arizelum*; — (1918) 24280 *R. griesonianum*; — (1924) 24315 *R. habrotrichum*; — (1924) 24542 *R. kyawii*; — (1924) 24600 *R. anthospaerum*; — (1924) 24687 *R. kyawii*; — (1924) 24740 *R. arizelum*; — (1924) 25020 *R. fulvum*; — (1924) 25064 *R. arizelum*; — (1924) 25158 *R. griesonianum*; — (1918) 25251 *R. neriiflorum*; — (1924) 25483 *R. fulvum*; — (1924) 25573 *R. campylocarpum*; — (1924) 25583 *R. lacteum*; — (1924) 25608 *R. arizelum*; — (1924) 25616 *R. glischrum*; — (1924) 25627 *R. arizelum*; — (1924) 25725 *R. glischrum*; — (1924) 25747 *R. glischrum*; — (1924) 25752 *R. campylocarpum*; — (1924) 25782 *R. arizelum*; — (1924) 25785 *R. glischrum*; — (1924) 25792 *R. glischrum*; — (1925) 25998 *R. kyawii*; — (1924) 26037 *R. kyawii*; — (1924) 26038 *R. arizelum*; — (1918) 26048 *R. griesonianum*; — (1918) 26487 *R. neriiflorum*; — (1925) 26734 *R. griesonianum*; — (1925) 26746 *R. griesonianum*; — (1925) 26935 *R. arizelum*; — (1925) 27108 *R. arizelum*; — (1925) 27128 *R. kyawii*; — (1925) 27245 *R. kyawii*; — (1925) 27616 *R. arizelum*; — (1925) 27624 *R. arizelum*; — (1925) 27624 *R. arizelum*; — (1925) 27792 *R. arizelum*; — (1929) 28235 *R. taliense*; — (1929) 28237 *R. taliense*; — (1929) 28239 *R. taliense*; — (1929) 28248 *R. lacteum*; — (1922) 28248 *R. lacteum*; — (1929) 28251 *R. neriiflorum*; — (1929) 28255 *R. lacteum*; — (1929) 28273 *R. taliense*; — (1929) 28286 *R. taliense*; — (1921) 29263 *R. balfourianum*; — (193?) 29264 *R. balfourianum*; — (193?) 29265 *R. balfourianum*; — (193?) 29280 *R. mimetes*; —

(1918) 29281 *R. mimetes*; — (19??) 29282 *R. balfourianum*; — (1932) 29306 *R. uvariifolium*; — (1932) 29308 *R. floccigerum*; — (1931) 29322 *R. wardii*; — (1931) 29345 *R. griesonianum*; — (1931) 29621 *R. arizelum*; — (1931) 29626 *R. arizelum*; — (1931) 29679 *R. griesonianum*; — (1918) 29762 *R. griesonianum*; — (1931) 29785 *R. arizelum*; — (??) 30392 *R. griesonianum*; — (1931) 30890 *R. wardii*.

Gale, U, Mg (1962) 9124 *R. arboreum*.

Gray, A. & Carey, J. (1841) *R. catawbiense*.

Grierson & Long, D. (1979) 1047 *R. falconeri*; — (1979) 1089 *R. thomsonii*; — (1979) 1226 *R. griffithianum*.

Heller, A. (1893) 1079 *R. catawbiense*.

Hu, W.K. (1946) 8207 *R. pachytrichum*; — (1946) 8208 *R. pachytrichum*; — (1946) 8209 *R. pachytrichum*; — (1946) 8211 *R. pachytrichum*; — (1946) 8222 *R. calophytum*; — (1946) 8230 *R. calophytum*; — (1946) 8232 *R. calophytum*; — (1946) 8235 *R. calophytum*; — (1946) 8244 *R. pachytrichum*; — (1946) 8246 *R. pachytrichum*; — (1946) 8247 *R. strigillosum*; — (1946) 8251 *R. calophytum*; — (1946) 8269 *R. strigillosum*; — (1946) 8286 *R. calophytum*; — (1946) 8294 *R. strigillosum*; — (1946) 8339 *R. calophytum*; — (1946) 8367 *R. strigillosum*; — (1946) 8432 *R. ririei*; — (1946) 8436 *R. ririei*; — (1946) 8442 *R. ririei*; — (1946) 8701 *R. ririei*; — (1946) 8712 *R. ririei*; — (1946) 8716 *R. ririei*; — (1946) 8720 *R. ririei*; — (1946) 8737 *R. ririei*.

Hupeh (1885) 5029 *R. auriculatum*.

Kanai, Murata, Ohashi, Tanaka & Yamazaki (1967) 8837 *R. falconeri*.

Kingdon-Ward, F. (1931) 1676 *R. neriiflorum*; — (1919) 3042 *R. glischrum*; — (1922) 5040 *R. adenogynum*; — (1922) 5105 *R. adenogynum*; — (1928) 6281 *R. glischrum*; — (1928) 6285 *R. venator*; — (1928) 6285 *R. venator*; — (1947) 8238 *R. hookeri*; — (1931) 9321 *R. neriiflorum*; — (1931) 9506 *R. neriiflorum*; — (1930) 9629 *R. forrestii*; — (1947) 13650 *R. hookeri*; — (1938) 14288 *R. lanatum*; — (1938) 14314 *R. lanatum*.

Kirkpatrick, G. (1990) 30 *R. barbatum*; — (1990) 38 *R. campanulatum*; — (1990) 47 *R. campanulatum*; — (1990) 52 *R. campylocarpum*.

Koelz, W. (1937) 11251 *R. arboreum*.

Kunming Edinburgh Gothenburg Expedition (1993) 1403 *R. balfourianum*; — 1716 *R. glischrum*; — 1291 *R. uvariifolium*.

Kunming-Edinburgh Yulon-Shan Expedition (1985) *R. vernicosum*.

Lee, T.C. (1940) 4451 *R. ririei*.

Ludlow, F. & Sherriff, G. (1934) 595 *R. thomsonii*; — (1936) 1352 *R. neriiflorum*; — (1936) 1381 *R. fulvum*; — (1936) 1389 *R. lanatum*; — (1936) 1390 *R. sherriffii*; — (1936) 1557 *R. lanatum*; — (1936) 1558 *R. lanatum*; — (1936) 1610 *R. lanatum*; — (1936) 1893 *R. campylocarpum*; — (1936) 2085 *R. wardii*; — (1937) 2983 *R. falconeri*; — (1937) 2988 *R. barbatum*; — (1937) 3041 *R. falconeri*; — (1937) 3063 *R. lanatum*; — (1937) 3096 *R. barbatum*.

Ludlow, Sherriff & Elliot (1938) 3994 *R. campylocarpum*; — (1947) 12484 *R. campylocarpum*; — (1950) 12526 *R. campylocarpum*; — (1947) 13783 *R. forrestii*; — (1947) 13969 *R. forrestii*; — (1947) 15070 *R. forrestii*; — (1947) 15098 *R. forrestii*; — (1947) 15285 *R. forrestii*; — (1947) 15326 *R. phaeochrysum*.

Ludlow, Sherriff & Hicks (1930) 30 *R. thomsonii*; — (1949) 16068 *R. griffithianum*; — (1949) 16116 *R. thomsonii*; — (1949) 16371 *R. falconeri*; — (1949) 18719 *R. falconeri*; — (1949)

18850 *R. thomsonii*; — (1949) 18890 *R. lanatum*; — (1949) 18945 *R. lanatum*; — (1949) 19046 *R. thomsonii*; — (1949) 20648 *R. lanatum*; — (1949) 21483 *R. griffithianum*.

Ludlow, Sherriff & Taylor (1938) 3849 *R. wardii*; — (1938) 3942 *R. forrestii*; — (1938) 4396 *R. wardii*; — (1938) 4620 *R. wardii*; — (1938) 4773 *R. wardii*; — (1938) 5679 *R. wardii*; — (1947) 13619 *R. wardii*; — (1947) 13668 *R. wardii*; — (1947) 13733 *R. wardii*; — (1947) 15009 *R. wardii*; — (1947) 15040 *R. wardii*.

MacLaren (?) 295 *R. watsonii*.

Maire, E. E. (1914) 35 *R. decorum*; — (1914) 47 *R. decorum*.

Mao, P.I. (1952) 761 *R. decorum*; — (1952) 1017 *R. lacteum*; — (1952) 1130 *R. decorum*.

Matthew, K.M. (1975) 14791 *R. arboreum*; — (1975) 14917 *R. arboreum*.

McLaren (1935) 101a *R. irroratum*; — (1935) AD170 *R. pachytrichum*; — (1935) AF457 *R. pachytrichum*; — (1935) AH277 *R. pachytrichum*; — (1935) AH284 *R. pachytrichum*; — (1935) AH293 *R. pachytrichum*; — (1935) AH431 *R. pachytrichum*; — (1935) C44 *R. neriiflorum*; — (1935) C5 *R. neriiflorum*; — (1935) D18 *R. uvariifolium*; — (1935) D217 *R. forrestii*; — (1935) Z31 *R. selense*; — (1935) Z31 *R. selense*; — (1935) Z33 *R. pachytrichum*; — (1935) 42 *R. uvariifolium*; — (1935) 71 *R. irroratum*; — (1935) L 108A *R. lacteum*; — (1935) L 126A *R. lacteum*.

Milciladze, Gagnidze, Davlianidzia *R. ponticum*.

Mombeig, P. (1907) 165 *R. floccigerum*; — (1907) 171 *R. uvariifolium*.

Mueller-Dombois & Cooray (1967) 67070811 *R. arboreum*.

Murata & Togashi (1963) 6304903 *R. falconeri*.

Noshiro, Akiyama & Acharya (1992) 9261186 *R. thomsonii*; — (1992) 9261209 *R. thomsonii*; — (1992) 9261211 *R. campylocarpum*; — (1992) 9261225 *R. thomsonii*; — (1992) 9261229 *R. campylocarpum*; — (1992) 9261247 *R. thomsonii*; — (1992) 9261324 *R. campylocarpum*.

RBGE (1989) 1976.0891 *R. fulvum*.

Robyns, A.G. (1969) 6953 *R. arboreum*.

Rock (1932) 22096a *R. eclecteum*; — (1949) 32 *R. floccigerum*; — (1948) 45 *R. sanguineum*; — (1948) 62 *R. sanguineum*; — (1932) 103 *R. praestans*; — (1949) 112 *R. sanguineum*; — (1932) 118 *R. praestans*; — (1949) 126 *R. sanguineum*; — (1949) 136 *R. sanguineum*; — (1949) 138 *R. roxieanum*; — (1949) 149 *R. sanguineum*; — (1949) 150 *R. sanguineum*; — (1948) 180 *R. fulvum*; — (1932) 2301 *R. fulvum*; — (1923) 3941 *R. adenogynum*; — (1923) 3969 *R. adenogynum*; — (1923) 3976 *R. adenogynum*; — (1923) 3980 *R. adenogynum*; — (1922) 4098 *R. decorum*; — (1922) 4214 *R. decorum*; — (1922) 5191 *R. decorum*; — (1922) 5394 *R. traillianum*; — (1922) 6253 *R. taliense*; — (1922) 6346 *R. lacteum*; — (1922) 6681 *R. decorum*; — (1922) 6832 *R. traillianum*; — (1922) 7796 *R. traillianum*; — (1923) 8090 *R. decorum*; — (1923) 8151 *R. anthospaerum*; — (1923) 8153 *R. anthospaerum*; — (1922) 8202 *R. traillianum*; — (1923) 8210 *R. anthospaerum*; — (1923) 8211 *R. anthospaerum*; — (1923) 8212 *R. anthospaerum*; — (1923) 8218 *R. uvariifolium*; — (1923) 8219 *R. uvariifolium*; — (1922) 8224 *R. traillianum*; — (1923) 8350 *R. uvariifolium*; — (1923) 8360 *R. uvariifolium*; — (1922) 8382 *R. traillianum*; — (1923) 8424 *R. uvariifolium*; — (1923) 8450 *R. uvariifolium*; — (1923) 8506 *R. anthospaerum*; — (1923) 8530 *R. decorum*; — (1923) 8531 *R. decorum*; — (1923) 8701 *R. anthospaerum*; — (1923) 8836 *R. selense*; — (1923) 8846 *R. selense*; — (1923) 8850 *R. selense*; — (1923) 8859 *R. selense*; — (1923) 8882 *R. anthospaerum*; — (1923) 8922 *R. roxeanum*; — (1923) 9065 *R. selense*; — (1923) 9083 *R. selense*; — (1923) 9159 *R. anthospaerum*; — (1923) 9175 *R. anthospaerum*; — (1923) 9177 *R. anthospaerum*; — (1923) 9183 *R. anthospaerum*;

— (1923) 9238 *R. eclecteum*; — (1923) 9542 *R. anthospaerum*; — (1923) 9591 *R. decorum*; — (1924) 9820 *R. anthospaerum*; — (1923) 10929 *R. selense*; — (1923) 10961 *R. selense*; — (1923) 10979 *R. roxieanum*; — (1932) 10999 *R. floccigerum*; — (1923) 11028 *R. selense*; — (1923) 11055 *R. eclecteum*; — (1923) 11062 *R. selense*; — (1923) 11134 *R. adenogynum*; — (1923) 11134 *R. adenogynum*; — (1923) 11367 *R. adenogynum*; — (?) 11404 *R. decorum*; — (1923) 11471 *R. adenogynum*; — (1923) 11634 *R. praestans*; — (1923) 16034 *R. adenogynum*; — (1979) 16089 *R. mimetes*; — (1923) 16310 *R. adenogynum*; — (1928) 16366 *R. phaeochrysum*; — (1928) 17095 *R. eclecteum*; — (1929) 17127 *R. glischrum*; — (1928) 17147 *R. floccigerum*; — (1928) 17151 *R. floccigerum*; — (1928) 17235 *R. decorum*; — (1928) 17564 *R. phaeochrysum*; — (1928) 17655 *R. vernicosum*; — (1929) 18021 *R. decorum*; — (1929) 18139 *R. vernicosum*; — (1929) 18153 *R. balfourianum*; — (1929) 18153 *R. balfourianum*; — (1929) 18160 *R. balfourianum*; — (1929) 18164 *R. balfourianum*; — (cult. 1952) 18228 *R. adenosum*; — (1929) 18386 *R. glischrum*; — (1929) 18387 *R. glischrum*; — (1929) 18420 *R. glischrum*; — (1929) 18449 *R. glischrum*; — (1932) 22069 *R. forrestii*; — (1932) 22109 *R. campylocarpum*; — (1932) 22269 *R. eclecteum*; — (1932) 22332 *R. praestans*; — (1932) 22445 *R. eclecteum*; — (1932) 22500 *R. forrestii*; — (1932) 22592 *R. eclecteum*; — (1932) 22594 *R. eclecteum*; — (1932) 22603 *R. fulvum*; — (1932) 22636 *R. floccigerum*; — (1932) 22662 *R. eclecteum*; — (1932) 22693 *R. praestans*; — (1932) 22814 *R. phaeochrysum*; — (1923) 22908 *R. praestans*; — (1932) 22909 *R. fulvum*; — (1923) 22911 *R. praestans*; — (1932) 23007 *R. fulvum*; — (1923) 23019 *R. praestans*; — (1932) 23027 *R. fulvum*; — (1932) 23285 *R. praestans*; — (1932) 23481 *R. eclecteum*; — (1932) 23496 *R. praestans*; — (1932) 23520 *R. praestans*; — (1932) 23587 *R. praestans*; — (1932) 23818 phaeochrysum var *R. agglutinatum*; — (1932) 24017 phaeochrysum var *R. agglutinatum*; — (1932) 25042 *R. irroratum*; — (1932) 25102 *R. fulvum*; — (1932) 25110 *R. fulvum*; — (1932) 25114 *R. uvariifolium*; — (1932) 25124 *R. fulvum*; — (1932) 25148 *R. fulvum*; — (1932) 25149 *R. fulvum*; — (1932) 25151 *R. fulvum*; — (1932) 25155 *R. roxieanum*; — (1932) 25251 *R. uvariifolium*; — (1932) 25349 *R. adenogynum*; — (1932) 25352 *R. uvariifolium*; — (1932) 25375 *R. adenogynum*; — (1932) 25419 *R. uvariifolium*; — (1932) 25421 *R. uvariifolium*; — (1932) 25431 *R. fulvum*; — (1932) 25468 *R. fulvum*; — (1929) 17201 *R. vernicosum*.

Rushforth (1987) 1234 *R. lanatum*.

Ruth, A. (1890) 164 *R. catawbiense*.

Shriver (1879) 164 *R. catawbiense*.

Sinclair (1945) 4165 *R. griffithianum*.

Sino-American Botanical Expedition (1984) 545 *R. lacteum*.

Sino-American Expedition (1980) 1322 *R. argyrophyllum*; — 1758 *R. argyrophyllum*.

Sino-British Expedition to Cangshan (1981) 235 *R. lacteum*; — 345 *R. lacteum*.

Sino-British Expedition to Lijiang (1987) 204 *R. traillianum*; — 237 *R. traillianum*.

Small, J.K. & Heller, A.A. (1891) 237 *R. catawbiense*.

Stainton, J.D.A. (1956) 379 *R. thomsonii*; — (1956) 749 *R. campylocarpum*.

Staiton, Sykes & Williams (1954) 235 *R. campanulatum*; — (1954) 845 *R. campanulatum*; — (1954) 928 *R. campanulatum*; — (1954) 2601 *R. campanulatum*; — (1954) 2607 *R. campanulatum*; — (1954) 5096 *R. campanulatum*; — (1954) 8293 *R. campanulatum*; — (1954) 9106 *R. campanulatum*; — (1954) 9107 *R. campanulatum*.

Steward, Chiao, Cheo (1931) 332 *R. auriculatum*; — (1931) 421 *R. auriculatum*.

Stonor, C.R. (1954) 26 *R. campylocarpum*; —. (1954) 26 *R. campylocarpum*; — (1954) 28 *R. campylocarpum*.

Taylor (1932) *R. arboreum*.

Tokyo University 3rd Botanical Expedition to E Himalaya(1967) 4302 *R. barbatum*; — 12345 *R. thomsonii*; — 12669 *R. barbatum*.

Tsai, H.T. (1932) 50832 *R. calophytum*; — (1932) 50907 *R. argyrophyllum*; — (1932) 50927 *R. argyrophyllum*.

Tsiang (1930) 7707 *R. auriculatum*; — (1930) 7740 *R. auriculatum*.

Vos & Corbett (1965) 32 *R. barbatum*; — (1965) 50 *R. falconeri*; — (1965) 81 *R. barbatum*.

Walker 36 *R. arboreum*.

Wang, F.T. (1930) 20857 *R. pachytrichum*; — (1930) 21025 *R. pachytrichum*.

Ward (1922) 5432 *R. sanguineum*; — (1922) 5432 *R. sanguineum*.

Watt, G. (1881) 2257 *R. falconeri*; — (1881) 5382 *R. falconeri*; — (1881) 7003 *R. barbatum*; — (1881) 7007 *R. falconeri*.

Wilson, E.H. (1909) 1203 *R. pachytrichum*; — (1909) 1349 *R. pachytrichum*; — (1908) 1367 *R. calophytum*; — (1911) 1467 *R. auriculatum*; — (cult 1926) 1467 *R. auriculatum*; — (1907) 3442 *R. argyrophyllum*; — (1907) 3443 *R. argyrophyllum*.

Yang, Z.H. (1981) 81-0178 *R. calophytum*; — (1981) 81-0182 *R. strigillosum*; — (1981) 810182 *R. strigillosum*.

Yu, S.W. (1962) 100268 *R. traillianum*.

Yu, T.T. (1932) 359 *R. ririei*; — (1932) 381 *R. ririei*; — (1932) 473 *R. calophytum*; — (1932) 640 *R. calophytum*; — (1937) 10684 *R. traillianum*; — (1937) 10953 *R. traillianum*; — (1937) 13927 *R. adenogynum*; — (1937) 13928 *R. adenogynum*; — (1937) 13995 *R. traillianum*; — (1937) 14955 *R. adenogynum*; — (1937) 15157 *R. traillianum*; — (1937) 15300 *R. adenogynum*; — (1938) 19047 *R. fulvum*; — (1938) 19677 *R. fulvum*; — (1938) 20623 *R. sanguineum*; — (1938) 20665 *R. fulvum*.

Zhongdian expodition (1963) 4122 *R. lacteum*.

Appendix D: RAPD SURVEY

Code for Microsoft QBASIC program used in scoring RAPD bands.

```
' Smooths data
' This version takes a file in which the data is present as follow.
' Each RF value appeares on a separate line
' Each OTU begins with a blank line then a name on its own line
' followed by each rf value on its own line.
' there are no bands valued at zero.

CLS
INPUT "Name of File containing RF Values by OTU please"; df$
INPUT "Name of File for output please"; rslt$
INPUT "What tolerance do you want"; tol

' Count the number of records
count = 0
OPEN df$ FOR INPUT AS #1
DO UNTIL EOF(1)
 LINE INPUT #1, rec$
 count = count + 1
LOOP
PRINT "total lines ="; count
CLOSE #1

' Dimension Arrays and open input file
DIM array(count)
DIM bands(INT(count / 4), INT(count / 4))
band = 1
position = 1
OPEN df$ FOR INPUT AS #1

' Read in all rf values into array and sorts them
FOR a = 1 TO count
   INPUT #1, b
   IF b = 0 THEN
      no.otus = no.otus + 1
      INPUT #1, name$
      a = a + 1
   ELSE
      no.rf.values = no.rf.values + 1
      array(no.rf.values) = b
   END IF
NEXT a

' Report on the number of RF values and OTUs
PRINT "Number of RF Values ="; no.rf.values
PRINT "Number of OTUs ="; no.otus

' Sort the RF Values into numerical order.
PRINT "Sorting: Please wait....."
```

```
   FOR sort1 = 1 TO no.rf.values
      FOR sort2 = sort1 TO no.rf.values
         IF array(sort1) >= array(sort2) THEN
            SWAP array(sort1), array(sort2)
         END IF
      NEXT sort2
   NEXT sort1

' Close the input file
CLOSE 1#
BEEP

' initialise clustering array by
' putting the first data point in the first
' position for the first band.
bands(1, 1) = array(1)

' Start the clustering loop
FOR a = 2 TO no.rf.values

' Calculate the av. for the band currently being built
   total = 0
   FOR b = 1 TO position
      total = total + bands(band, b)
   NEXT b
   band.av = total / position

' does data.point fall within limits of question
' If it does then add it to that row
   IF array(a) - band.av < tol THEN
      position = position + 1
      bands(band, position) = array(a)
' If it does not then move down a row and put it in the first position.
   ELSE
      band = band + 1
      position = 1
      bands(band, position) = array(a)
   END IF
   no.bands = band
NEXT a

' Report on number of bands found and
PRINT "Number of Bands Identified ="; no.bands

' Work through OTUs and produce matrix
PRINT "Scoring OTUs for bands: Please wait...."
DIM matrix(no.otus, no.bands)
DIM name$(no.otus)
OPEN df$ FOR INPUT AS #1
otu = 0
PRINT "Scoring ";
FOR a = 1 TO (count - no.otus)
```

```
    INPUT #1, b
    IF b = 0 THEN
      otu = otu + 1
      INPUT #1, name$(otu)
      PRINT name$(otu); ": ";
    ELSE
      FOR band = 1 TO no.bands
        FOR position = 1 TO INT(count / 4)
          IF bands(band, position) = b THEN
            matrix(otu, band) = 1
          END IF
        NEXT position
      NEXT band
    END IF
  NEXT a
  PRINT
  BEEP

  ' Write the arrays file.
  OPEN rslt$ FOR OUTPUT AS #2
  PRINT #2, "RF Values ordered by size"
  PRINT #2,
  FOR a = 1 TO no.rf.values
    PRINT #2, array(a); CHR$(9);
  NEXT a
  PRINT #2,
  PRINT #2,
  PRINT #2, "Attributed list of RF Values by Bands"
  FOR band = 1 TO no.bands
    PRINT #2,
    PRINT #2, "Band "; band; CHR$(9);
    FOR position = 1 TO INT(count / 4)
      IF bands(band, position) > 0 THEN
        PRINT #2, bands(band, position); CHR$(9);
      END IF
    NEXT position
  NEXT band
  PRINT #2,
  PRINT #2,

  PRINT #2, "Resulting Data Matrix is...."
  PRINT #2,
  PRINT #2, "Band"; CHR$(9);
  FOR a = 1 TO no.bands
    PRINT #2, a; CHR$(9);
    NEXT a
    PRINT #2,
    FOR a = 1 TO no.otus
      PRINT #2, name$(a); CHR$(9);
      FOR b = 1 TO no.bands
        PRINT #2, matrix(a, b); CHR$(9);
      NEXT b
```

```
    PRINT #2,
  NEXT a

CLOSE #2
PRINT "Results written to: "; rslt$
BEEP
BEEP
END
```

Example of Output file.

RF Values ordered by size

13.67521 14.03509 15.51724 15.9292 16.66667 17.3913 18.42105 20.68966 21.73913 22.22222 22.80702 23.07692 23.07692 23.72881 24.34783 24.78633 25 25.86207 26.31579 26.95652 27.11864 27.58621 29 29.31034 29.66102 29.66102 30.0885 30.43478 30.70175 31.35593 31.57895 32.47863 33.8983 34.48276 36.2069 37.60684 38.98305 39.65517 39.65517 39.65517 43.10345 43.22034 43.58974 44.06779 45.61404 45.76271 46.55172 46.55172 46.61017 47.00855 47.36842 47.82609 48.27586 48.27586 50.87719 51.30435 51.69492 51.72414 51.75439 52.17391 52.54237 52.58621 52.58621 53.09734 53.44828 55.17241 56.52174 58.11966 58.40708 59.29203 60.34483 60.86956 61.01695 61.9469 62.6087 63.55932 64.34782 64.95727 65.25423 65.48672 66.37931 68.42105 70.08547 71.30434 72.4138 72.56637

Attributed list of RF Values by Bands

Band 1 13.67521 14.03509
Band 2 15.51724 15.9292 16.66667
Band 3 17.3913
Band 4 18.42105
Band 5 20.68966
Band 6 21.73913 22.22222 22.80702 23.07692 23.07692
Band 7 23.72881 24.34783 24.78633 25
Band 8 25.86207 26.31579 26.95652 27.11864
Band 9 27.58621
Band 10 29 29.31034 29.66102 29.66102 30.0885 30.43478
Band 11 30.70175 31.35593 31.57895
Band 12 32.47863
Band 13 33.8983 34.48276
Band 14 36.2069
Band 15 37.60684
Band 16 38.98305 39.65517 39.65517 39.65517
Band 17 43.10345 43.22034 43.58974 44.06779
Band 18 45.61404 45.76271 46.55172 46.55172 46.61017 47.00855
Band 19 47.36842 47.82609 48.27586 48.27586
Band 20 50.87719 51.30435 51.69492 51.72414 51.75439 52.17391 52.54237 52.58621 52.58621
Band 21 53.09734 53.44828
Band 22 55.17241
Band 23 56.52174
Band 24 58.11966 58.40708
Band 25 59.29203
Band 26 60.34483 60.86956 61.01695
Band 27 61.9469 62.6087
Band 28 63.55932 64.34782
Band 29 64.95727 65.25423 65.48672

Band 30 66.37931
Band 31 68.42105
Band 32 70.08547
Band 33 71.30434
Band 34 72.4138 72.56637

Resulting Data Matrix is....

```
Band 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
32 33 34
OTU01 0 0 0 0 0 0 0 1 0 1 1 0 0 0 0 1 1 0 0 1 0 0 0 0 0 0 0 1 1 0 0 0 0 0
OTU02 0 0 0 0 0 1 1 0 0 0 0 1 0 0 1 0 1 1 0 0 0 0 0 1 0 0 0 0 1 0 0 1 0 0
OTU06 0 0 0 0 0 0 1 0 0 1 0 0 1 0 0 0 1 1 0 1 0 0 0 0 0 1 0 0 0 0 0 0 0 0
OTU08 0 0 0 0 0 0 0 0 1 1 1 0 0 1 1 0 0 0 0 0 1 0 0 0 0 0 0 1 0 0 0 1 0 0 0 1
OTU10 1 0 0 0 0 0 1 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0
OTU11 0 1 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 1 0 0 1 0 0 1 0 0 0 0 0 0 0
OTU12 0 0 0 0 0 1 0 1 0 0 0 0 0 0 0 0 1 0 1 1 1 0 1 0 0 1 0 0 0 1 0 0 0 0 1
OTU31 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0
OTU33 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0
OTU35 0 0 1 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 0 1 0 0 1 0 1 0 0 0 0 1 0
OTU36 0 0 0 0 0 0 1 0 0 0 1 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 1 0 0 0 0 0 0
OTU37 0 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 0 0 1 0 0 0
OTU40 1 1 0 0 0 0 0 0 1 0 0 1 0 0 0 0 0 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0
```

RAPD DATA SET USED IN ANALYSIS.

```
anthospaerum
0 0 0 0 0 1 1 1 0 1 1 0 0 1 0 0 0 0 0 1 1 0 0 1 1 1 0 1 1 1 0 0 0 1 0 1 1 0 0 0 0 0 1
0 0 1 0 0 0 0 0 0 0 0 0 0 1 0 0 0 1 0 1 0 0 0 0 1 0 0 0 0 0 1 1 0 0 1 0 0 0 0 0 0 0
1 0
argyrophyllum
0 0 1 1 0 0 0 0 0 0 1 1 0 0 0 1 0 0 0 0 1 0 0 1 1 1 0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 0
0 0 1 1 0 0 1 1 0 1 0 1 1 1 0 0 0 0 0 0 0 0 0 1 1 0 1 0 1 0 0 1 0 0 0 1 0 0 0 0 0 0
0 0
falconeri
0 0 0 1 0 1 0 1 0 1 1 0 1 0 0 1 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0
0 0 1 0 0 0 0 1 0 0 0 0 0 0 0 0 1 1 0 1 0 0 0 1 1 0 1 0 1 0 0 1 0 0 0 0 1 0 0 0 0
0 0
selense
0 0 0 0 1 1 0 1 0 0 0 0 1 0 0 1 0 0 0 1 1 0 1 0 0 1 0 1 0 0 0 1 0 0 0 1 0 1 1 0
0 0 0 1 0 1 0 1 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 1 1 0 1 0 0 0 0 0 1 0 0 0 1 0 0 0 0
0 0
sherriffii
1 0 1 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 0 0 0 1 1 1 0 0 0 0 0 0 0 0 0 0
0 0 0 1 0 0 0 1 0 0 1 1 0 0 0 0 0 0 0 0 0 1 0 1 1 0 1 0 1 0 1 0 1 0 0 1 0 0 0 0 0
0 0
taliense
0 1 0 0 0 1 0 0 0 0 0 0 0 1 1 0 1 0 0 0 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 1 0 0 1
0 1 0 1 0 0 0 1 0 0 1 0 0 0 1 1 0 0 0 1 1 0 1 1 0 1 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0
0 0
vernicosum
0 0 0 1 0 0 0 0 1 0 1 1 1 0 0 0 0 0 1 1 0 1 0 1 0 1 0 0 0 0 0 1 0 0 0 0 1 0 0 0
0 0 1 1 0 1 0 1 0 1 0 0 0 0 0 0 1 0 0 1 0 1 0 0 0 1 0 0 0 0 0 1 0 0 0 0 1 1 0 1 1
0 0
aureum
0 1 0 0 0 0 0 0 1 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1 0 1 0 0 0 0 0 0 0
0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 1 0 0 0 1
0 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
yakushimanum
? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 0 0 0 0
0 0 1 1 1 1 1 1 1 1 0 0 0 0 1 1 0 0 1 1
0 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
macrophyllum
0 0 0 0 0 0 0 0 1 1 1 0 1 0 0 0 0 0 0 0 1 0 0 1 0 0 0 0 0 0 1 0 0 1 0 0 0 1 0 0
0 0 1 1 0 0 1 0 1 1 0 1 0 0 0 1 0 0 1 1 0 1 1 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0
0 0
brachycarpum
? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 0 0 1 1 1 0 1 0 0 1 0 0 0 0 0 0 0 1 0 0
0 0 1 1 0 0 1 1 0 1 1 0 0 0 1 1 1 1 0 0 0 0 0 1 1 0 1 0 0 0 0 0 1 0 0 0 0 0 1 0
1 0
japonicum
```

0 0 0 1 1 0 0 0 0 0 0 0 1 0 0 1 0 1 0 0 1 0 1 0 1 0 0 0 0 0 1 0 0 0 0 0 1 0 1 0
1 0 1 0 0 0 1 0 0 1 1 1 0 1 1 1 0 0 0 0 0 0 1 0 0 0 1 0 0 1 0 0 1 0 0 0 0 0 0 0
0 0
ungernii
0 0 1 0 0 1 0 0 0 0 0 0 1 1 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 0 0 0 0 0 0 0 0 0
0 1 0 0 1 0 0 0 0 0 1 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 0 0 0 1 1 0 0 0 0 1 0
0 0
maximum
0 0 1 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 0 0 0 0 ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? 0 0 0 0
0 0 0 1 0 1 1 0 1 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 1 0 0 1 0 0 0 0 0 0 1 0 0 0
1 1
makinoi
? 0 0 0 0
0 1 1 0 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 0 1 0 1 1 0 0
0 0
catawbiense
? 0 0 0 0
1 0 1 1 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 1 0 1 0 0 0 0 0 0 1 1 0 0 0 1 0 0 0 0 1 0 1
0 1
hyperythrum
1 1 0 0 1 0 1 0 0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 1 0 0 0 0 1 0 0 1 0 0 1 1
1 ? 0 1 0 1 0 1 1 0 0 0 0 1 0 0
0 0 0 1 0 0 0

Appendix E: Sequences from ITS1 and ITS2.

Base pair positions are numbered from one for ITS1 and ITS2. The regions of sequence illustrated in Figure 44 are in bold. Poly morphic positions have been marked with arrows and selected restriction sites have been underlined (see Chapter 9).

	1111111111122222222233333333334444444444
ITS 1	1234567890123456789012345678901234567890123456789
R. vernicosum	TCGAAACCTGCCAACAAGCAGAAAACTTGCGAACTTGTCTAATACAGTG
R. auriculatum	TCGAAACCTGCCAACAAGCAGAAAACTTGCGAACTTGTCTAATACAGTG
R. watsonii	TCGAAACCTGCCAACAAGCAGAAAACTTGCGAACTTGTCTAATACAGTG
R. falconeri	TCGNAACCTGCCAACAAGCAGAAAACTTGCGAACTTGTCTAATACAGTG
R. williamsianum	TCGAAACCTGCCAACAAGCAGAAAACTTGCGAACTTGTCTAATACAGTG
R. campylocarpum	TCGAAACCTGCCAACAAGCAGAAAACTTGCNAACTTGTCTAATACAGTG
R. strigillosum	TCGNAACCTGCCAACAAGCAGAAAACTTGCNAACTTGTCTAATACAGTG
R. hirtipes	TCGAAACCTGCCAACAAGCAGAAAACTTGCGAACTTGTCTAATACAGTG
R. adenosum	TCGAAACCTGCCAACAAGCAGAAAACTTGCGAACTTGTCTAATACAGTG
R. venator	TCGAAACCTGCCAACAAGCAGAAAACTTGCGAACTTGTCTAATACAGTG
R. irroratum	TCGAAACCTGCCAACAAGCAGAAAACTTGCGAACTTGTCTAATACAGTG
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R. kyawii	TCGAAACCTGCCAACAAGCAGAAAACTTGCGAACTTGTCTAATACAGTG
R. barbatum	TCGAAACCTGCCAACAAGCAGAAAACTTGCGAACTTGTCTAATACAGTG
R. neriiflorum	TCGAAACCTGCCAACAAGCAGAAAACTTGCGAACTTGTCTAATACAGTG
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Restriction Sites	Taq I
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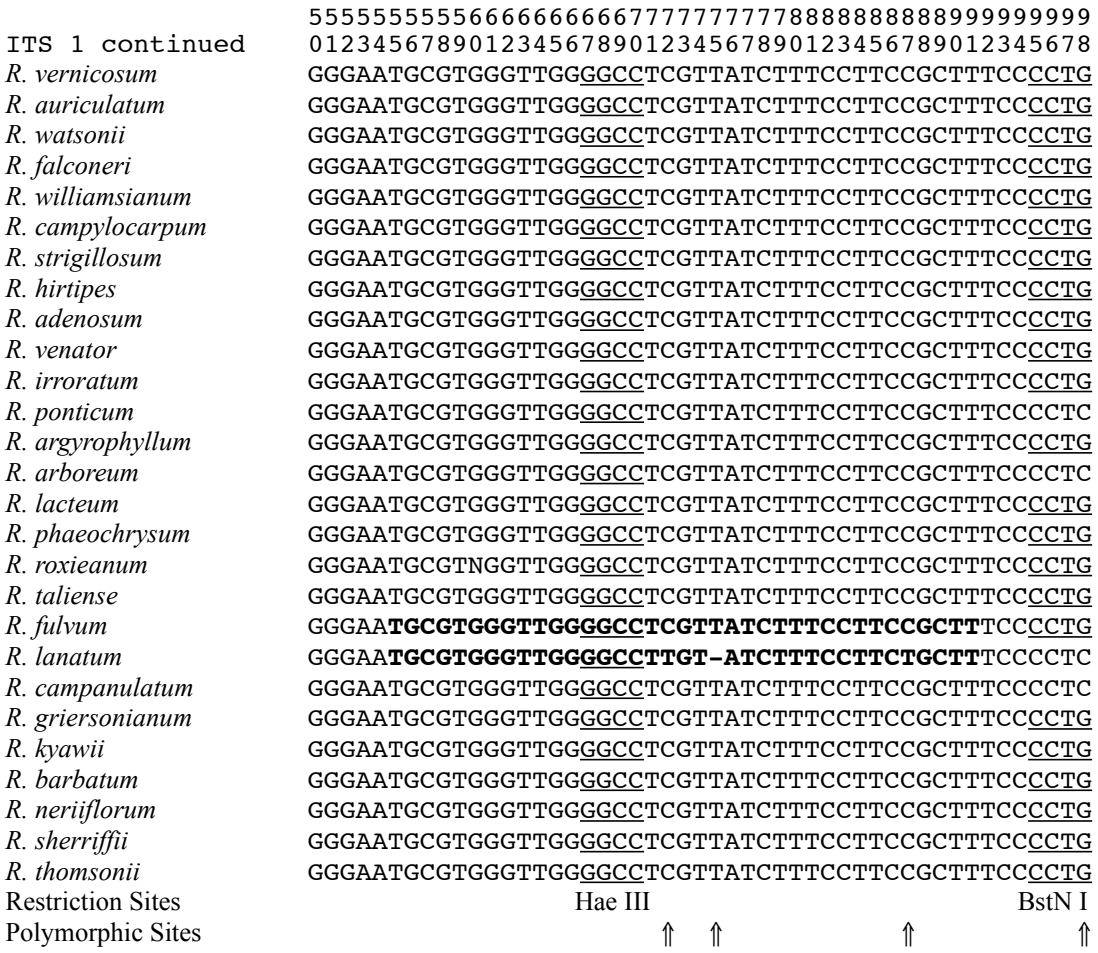
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R. thomsonii GGGAATGCGTGGGTTGGGGCCTCGTTATCTTTCCTTCCGCTTTCCCCTG
Restriction Sites Hae III BstN I
Polymorphic Sites

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R. falconeri        GCGAGTAGATGTGCGCGGAGCTTTCGGGCAACGTGTTCATTTACTTGTC
R. williamsianum    GCGAGTAGATGTGCGCGGAGCTTTCGGGCAACGTGTTCATTTACTTGTC
R. campylocarpum    GCGAGTAGATGTGCGCGGAGCTTTCGGGCAACGTGTTCATTTACTTGTC
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R. taliense         GCGAGTAGATGTGCGCGGAGCTTTCGGGCAACGTGTTCGTTTACTTGTC
R. fulvum           GCGAGTAGATGTGCGCGGAGCTTTCGGGCAACGTGTTCGTTTACTTGTC
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R. campanulatum     GCGAGTAGATGCTCGCGGAGCTTTCGGGCAACGTGTTCATTTACTTGTC
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R. kyawii           GCGAGTAGATGTGCGCGGAGCTTTCGGGCAACGTGTTCATTTACTTGTC
R. barbatum         GCGAGTAGATGTGCGCGGAGCTTTCGGGCAACGTGTTCATTTACTTGTC
R. neriiflorum      GCGAGTAGATGTGCGCGGAGCTTTCGGGCAACGTGTTCATTTACTTGTC
R. sherriffii       GCGAGTAGATGTGCGCGGAGCTTTCGGGCAACGTGTTCATTTACTTGTC
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Restriction sites                 Cfo I
Polymorphic sites                 ⇑⇑                          ⇑
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R. falconeri	AAACAACGAACCCCGGCGCAAAACGCGCCAAGGATAATTGAACAAAGTT
R. williamsianum	AAACAACGAACCCCGGCGCAAAACGCGCCAAGGATAATTGAACAAAGTT
R. campylocarpum	AAACAACGAACCCCGGCGCAAAACGCGCCAAGGATAATTGAACAAAGTT
R. strigillosum	AAACAACGAACCCCGGCGCAAAACGCGCCAAGGATAATTGAACAAAGTT
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R. kyawii	AAACAACGAACCCCGGCGCAAAACGCGCCAAGGATAATTGAACAAAGTT
R. barbatum	AAACAACGAACCCCGGCGCAAAACGCGCCAAGGATAATTGAACAAAGTT
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R. sherriffii	AAACAACGAACCCCGGCGCAAAACGCGCCAAGGATAATTGAACAAAGTT
R. thomsonii	AAACAACGAACCCCGGCGCAAAACGCGCCAAGGATAATTGAACAAAGTT
Restriction Sites	MspI/Cfo I Cfo I

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R. falconeri	TGTTNACGTCCCCTGCCCGTTTCCGGGTGGTGTTGGCGTGCACATCT
R. williamsianum	TGTTCACGTCCCCTGCCCGTTTCCGGGTGGTGTTGGCGTGCACATCT
R. campylocarpum	TGTTNACGTCCCCTGCCCGTTTCCGGGTGGTGTTGGCGTGCACATCT
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R. hirtipes	TGTTCACGTCCCCTGCCCGTTTTCGGGTGGTGTTGGCGTGCACATCT
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R. arboreum	TGTGCACGTCCCCTGCCCGTTTCCGGGTGGTGTTGGCGTGCACATCT
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R. phaeochrysum	TGTTCACGTCCCCTGCCCGTTTTCGGGTGGTGTTGGCGTGCACATCT
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R. fulvum	TGTTCACGTCCCCTGCCCGTTTTCGGGTGGTGTTGGCGTGCACATCT
R. lanatum	TGTGCACGTCCCCTGCCCGTTTCCGGGTGGTGTTGGCGTGCACATCT
R. campanulatum	TGTGCACGTCCCCTGCCCGTTTTCGGGTGGTGTTGGCGTGCACATCT
R. griersonianum	TGTTCACGTCCCCTGCCCGTTTCCGGGTGGTGTTGGCGTGCACATCT
R. kyawii	TGTTCACGTCCCCTGCCCGTTTCCGGGTGGTGTTGGCGTGCACATCT
R. barbatum	TGTTCACGTCCCCTGCCCGTTTCCGGGTGGTGTTGGCGTGCACATCT
R. neriiflorum	TGTTCACGTCCCCTGCCCGTTTCCGGGTGGTGTTGGCGTGCACATCT
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R. thomsonii	TGTTCACGTCCCCTGCCCGTTTCCGGGTGGTGTTGGCGTGCACATCT
Restriction Sites	Msp I
Restrictions Sites	⇑ ⇑ ⇑ ⇑

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Restriction Sites                                                    Hinf I
Polymorphic Sites                                                    ⇑
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R. neriiflorum      CATCGAGTCTTTGAACGCAAGTTGCGCCTNAAGCCATTAGGTTGAAGGC
R. sherriffii       CATCGAGTCTTTGAACGCAAGTTGCGCCTGAAGCCATTAGGTTGAAGGC
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Restriction Sites     Taq I/Hinf I              Cfo I
Polymorphic Sites               ⇑
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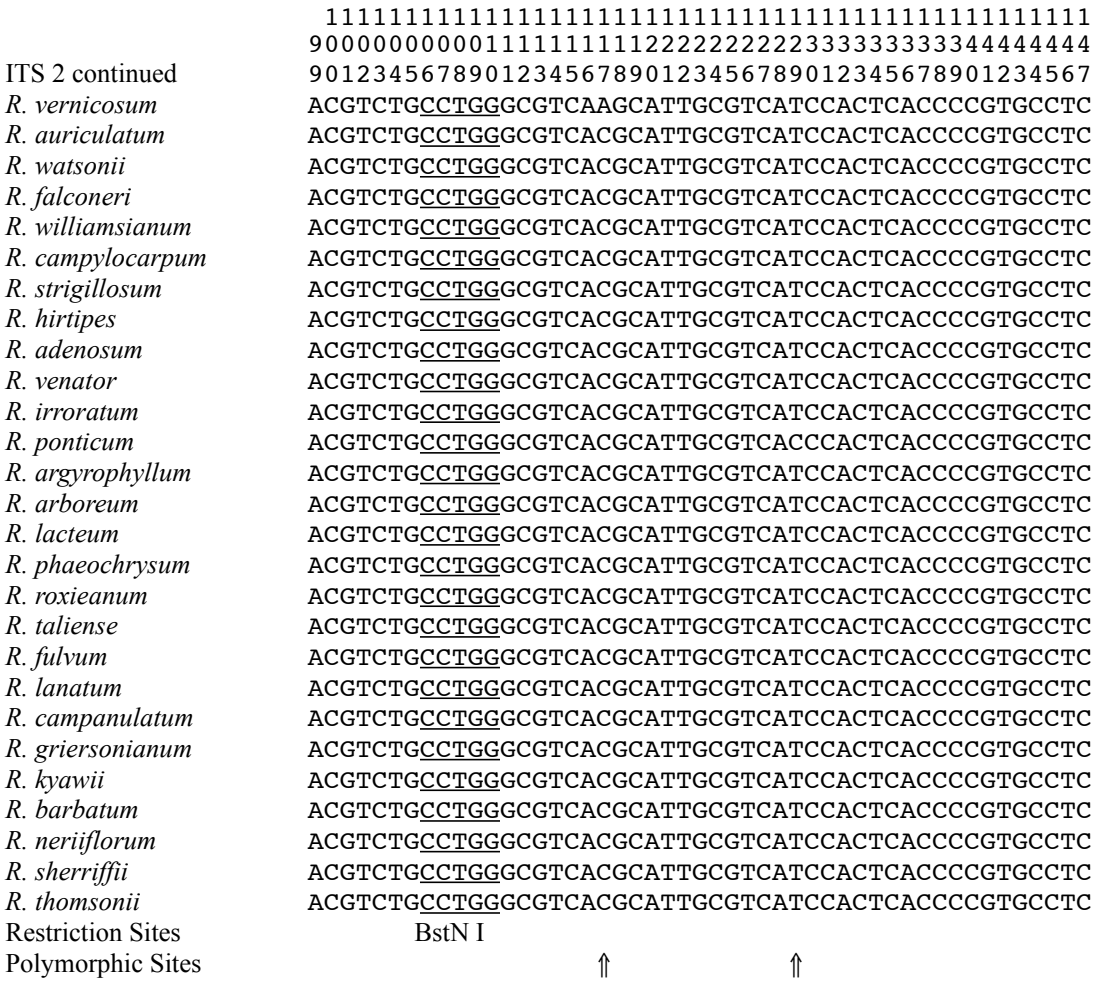
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Restriction Sites BstN I
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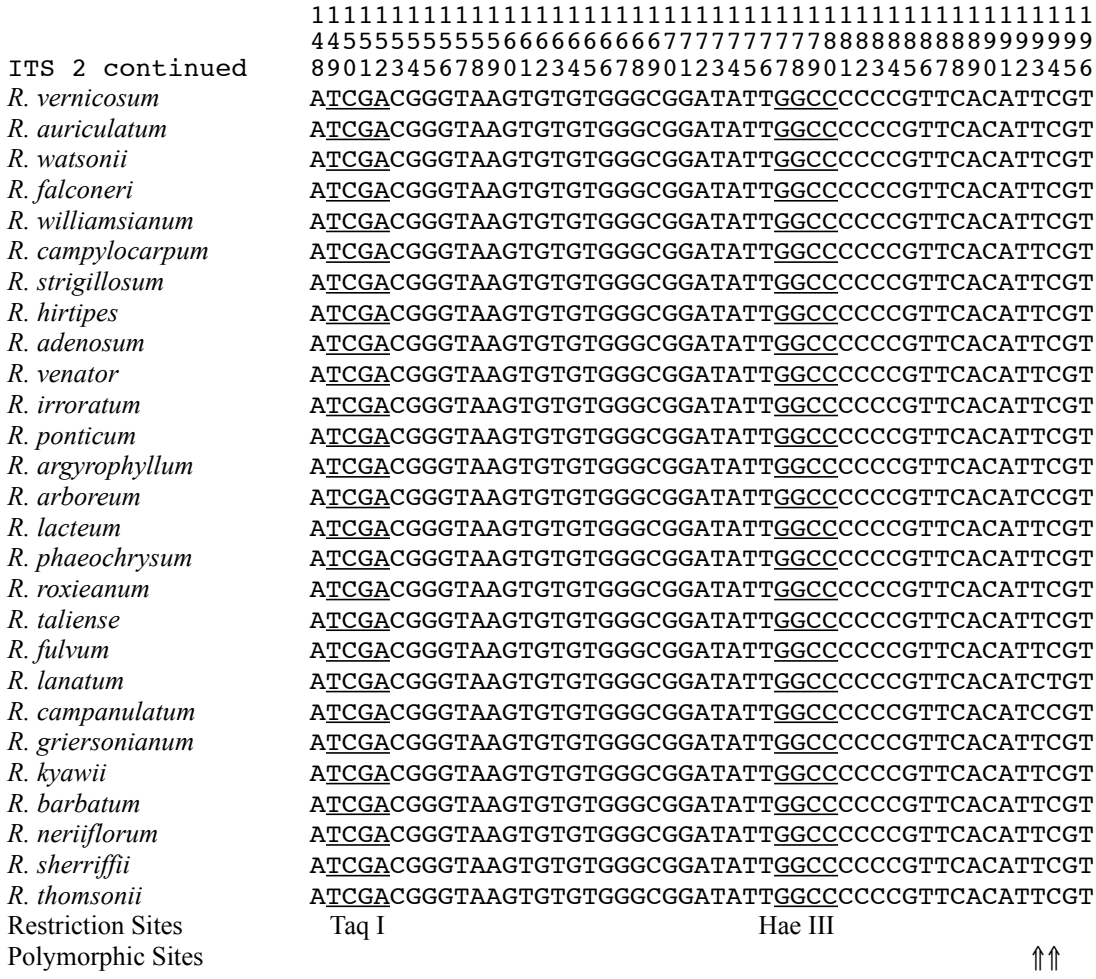
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R. watsonii ATCGACGGGTAAGTGTGTGGGCGGATATTGGCCCCCCGTTCACATTCGT
R. falconeri ATCGACGGGTAAGTGTGTGGGCGGATATTGGCCCCCCGTTCACATTCGT
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R. campylocarpum ATCGACGGGTAAGTGTGTGGGCGGATATTGGCCCCCCGTTCACATTCGT
R. strigillosum ATCGACGGGTAAGTGTGTGGGCGGATATTGGCCCCCCGTTCACATTCGT
R. hirtipes ATCGACGGGTAAGTGTGTGGGCGGATATTGGCCCCCCGTTCACATTCGT
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Restriction Sites Taq I Hae III
Polymorphic Sites ⇑⇑

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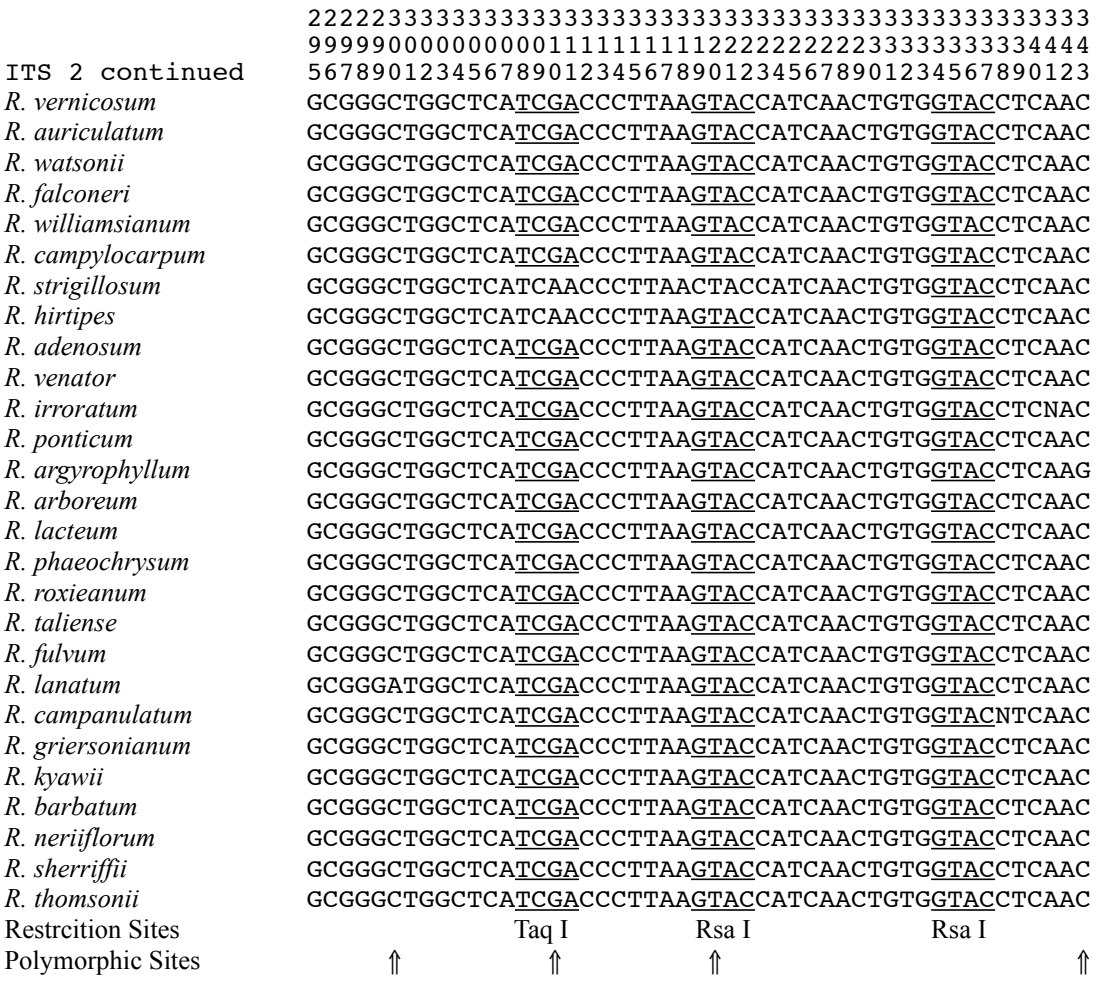
ITS 2 continued	7890123456789012345678901234567890123456789012345
R. vernicosum	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. auriculatum	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. watsonii	GCTCGGTCGGCCNNNNAATGACGGTCCCCGATGATGGACATCACGGCAA
R. falconeri	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. williamsianum	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. campylocarpum	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. strigillosum	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. hirtipes	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. adenosum	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. venator	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. irroratum	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. ponticum	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. argyrophyllum	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. arboreum	GCTCGGTTGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. lacteum	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. phaeochrysum	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. roxieanum	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. taliense	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. fulvum	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. lanatum	GCTCGGTTGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. campanulatum	GCTCGGTTGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. griersonianum	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. kyawii	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. barbatum	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. neriiflorum	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. sherriffii	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
R. thomsonii	GCTCGGTCGGCCTAAAAATGACGGTCCCCGATGATGGACATCACGGCAA
Restriction Sites	Hae III
Polymorphic Sites	⇑

	222222222222222222222222222222222222222222222222
	444455555555556666666666777777777788888888899999
ITS 2 continued	678901234567890123456789012345678901234567890123 4
R. vernicosum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. auriculatum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. watsonii	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. falconeri	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. williamsianum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. campylocarpum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. strigillosum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. hirtipes	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. adenosum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. venator	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. irroratum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. ponticum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. argyrophyllum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. arboreum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. lacteum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. phaeochrysum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. roxieanum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. taliense	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. fulvum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTT
R. lanatum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. campanulatum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. griersonianum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. kyawii	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. barbatum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. neriiflorum	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. sherriffii	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
R. thomsonii	GTGGTGGTTGCCAAACCGTCGCGTCATGTCGTGCATGCCATTCTTTGTC
Polymorphic Sites	⇑


2222233333333333333333333333333333333333333333333
9999900000000001111111111222222222233333333334444
ITS 2 continued 5678901234567890123456789012345678901234567890123
R. vernicosum GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. auriculatum GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. watsonii GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. falconeri GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. williamsianum GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. campylocarpum GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. strigillosum GCGGGCTGGCTCATCAACCCTTAACTACCATCAACTGTGGTACCTCAAC
R. hirtipes GCGGGCTGGCTCATCAACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. adenosum GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. venator GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. irroratum GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCNAC
R. ponticum GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. argyrophyllum GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAG
R. arboreum GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. lacteum GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. phaeochrysum GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. roxieanum GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. taliense GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. fulvum GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. lanatum GCGGGATGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. campanulatum GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACNTCAAC
R. griersonianum GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. kyawii GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. barbatum GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. neriiflorum GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. sherriffii GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
R. thomsonii GCGGGCTGGCTCATCGACCCTTAAGTACCATCAACTGTGGTACCTCAAC
Restrcition Sites Taq I Rsa I Rsa I
Polymorphic Sites

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	444444555555555666666666
ITS 2 continued	456789012345678901234567 8
R. vernicosum	TGCGACCCCAGGTCAGGCGGGATTA
R. auriculatum	TGCGACCCCAGGTCAGGCGGGATTA
R. watsonii	TGCGACCCCAGGTCAGGCGGGATTA
R. falconeri	TGCGACCCCAGGTCAGGCGGGATTA
R. williamsianum	TGCGACCCCAGGTCAGGCGGGATTA
R. campylocarpum	TGCGACCCCAGGTCAGGCGGGATTA
R. strigillosum	TGCGACCCCAGGTCAGGCGGGATTA
R. hirtipes	TGCGACCCCAGGTCAGGCGGGATTA
R. adenosum	TGCGACCCCAGGTCAGGCGGGATTA
R. venator	TGCGACCCCAGGTCAGGCGGGATTA
R. irroratum	TGCGACCCCAGGTCAGGCGGGATTA
R. ponticum	TGCGACCCCAGGTCAGGCGGGATTA
R. argyrophyllum	TGCGACCCCAGGTCAGGCGGGATTA
R. arboreum	TGCGACCCCAGGTCAGGCGGGATTA
R. lacteum	TGCGACCCCAGGTCAGGCGGGATTA
R. phaeochrysum	TGCGACCCCAGGTCAGGCGGGATTA
R. roxieanum	TGCGACCCCAGGTCAGGCGGGATTA
R. taliense	TGCGACCCCAGGTCAGGCGGGATTA
R. fulvum	TGCGACCCCAGGTCAGGCGGGATTA
R. lanatum	TGCGACCCCAGGTCAGGCGGGATTA
R. campanulatum	TGCGACCCCAGGTCAGGCGGGATTA
R. griersonianum	TGCGACCCCAGGTCAGGCGGGATTA
R. kyawii	TGCGACCCCAGGTCAGGCGGGATTA
R. barbatum	TGCGACCCCAGGTCAGGCGGGATTA
R. neriiflorum	TGCGACCCCAGGTCAGGCGGGATTA
R. sherriffii	TGCGACCCCAGGTCAGGCGGGATTA
R. thomsonii	TGCGACCCCAGGTCAGGCGGGATTA
ITS 2 continued	BstN I

Appendix F: The complete Data Set.

The completed molecular and morphological data matrix is given over the next twelve pages. It has been split into two parts. The first part deals with the species that were sampled for both molecular and morphological characters and the second part with those species that were sampled for just morphological data.

Appendix G: Summary of the 'Azalea' Project.

This appendix gives and outline of the 'Azalea' project that ran in parellel with the present study. The text is largely taken from Chamberlain & Hyam (1997).

The genus *Rhododendron* - a case study to test the value of various molecular techniques as measures of biodiversity

The Genus *Rhododendron* comprises around 1000 species that are variously arranged in subgenera, sections, subsections and series within the genus. The classification of *Rhododendron* has been studied by a number of authors and several, often conflicting, schemes of classification are available.

The Royal Botanic Garden Edinburgh has played a leading role in these studies for almost the whole of this century and has accumulated unparalleled knowledge in the taxonomy of the genus. For this reason *Rhododendron* was selected as a representative group of naturally occurring plants, against which the value of various molecular tools in the study of biosystematics could be tested. The classification followed was that proposed by Chamberlain (1982), Cullen (1980), Judd & Kron (1995), Kron, (1993) and Philipson & Philipson (1986). While it was assumed that this classification, which was largely derived from classical, morphological and anatomical studies, reflected the probable ‘natural’ groupings in the genus fairly accurately, it was expected from the start that there would be mismatches when compared with the data derived from even the best of the molecular data. Indeed, the ultimate success of a method was to be partially judged by the discrepancies indicated as compared with the interpretations derived on morphology and anatomy alone.

Two essentially different problems were selected. The first was a collaborative project involving several laboratories in which a subset of specimens from across the whole of *Rhododendron* (but with particular emphasis on the ‘Azaleas’ subgenera) were investigated using different techniques, this is discussed in here. The second, largely carried out at Edinburgh, involved a survey of selected species from Subgenus *Hyemenanthes.*

Materials and methods.

Leaf material for this project was supplied from authentically named plants in the collections at Edinburgh and its satellite gardens. The plants were selected so that different levels of the hierarchical classification were represented in the sample. They included; multiple samples from several accessions; multiple samples from several species; representatives of all the subgenera and most of the sections; outgroup taxa and one known hybrid taxon. Identical batches of 42 samples representing 33 species were despatched to separate laboratories where they were analysed using a series of different techniques. These techniques included RAPD, AFLP, oligonucleotide fingerprinting and ITS nrDNA sequencing. Presented here is a comparison of an RAPD, an AFLP and a sequence data set for all 42 samples. (The oligonucleotide sequencing proved so sensitive that only the most similar taxa could be scored and so is excluded here.) Table 41 gives a summary of the samples used in the study arranged according to the classification used at Edinburgh at the beginning of the study.

Table 41: *Species sampled during the course of the study, in taxonomic order. Numbers in bracts represent the number of replicates where more than one individual was sampled from each individual.*

Subgenus (Genus)	Section	Species Sampled
Rhododendron	*Vireya*	*R. leptanthum, R. jarvanicum*
	Pogonanthum	*R. anthopogon*

	Rhododendron	*R. ferrugineum*
Hymenanthes	*Ponticum*	*R. ponticum, R. argyrophyllum*
Tsutsusi	*Tsutsusi*	*R. yedoense, R. kaempferi,R. kiusianum, R. tschonoskii, R. tsusiophyllum*
	Brachycalyx	*R. wadanum,R. reticulatum*
Pentanthera	*Pentanthera*	*R. arborescens, R. luteum* (x6)*, R. occidentale*(x6)*, R. molle, 'Summer Fragrance'*
	Rhodora	*R. canadense, R. vaseyi*(x2)*,*
	Sciadorhodion	*R. albrechtii, R. schlippenbachii*
Azaleastrum	*Azaleastrum*	*R. honkongense*
	Choniastrum	*R. moulmainense*
Therorhodion		*R. camtschaticum*
Mumeazalea		*R. semibarbatum*
Candidastrum		*R. albiflorum*
(Ledum)		*L. groenlandicum*
(Bejaria)		*B. laevis*
(Menziesia)		*M. lasiophylla*
(Daboecia)		*D. cantabrica*

The RAPD data set was produced using commercially available primers to generate a total of 240 polymorphic bands across all 42 taxa. The AFLP data set used three different primer combinations to produce 180 different bands. ITS sequence data was gathered using direct sequencing on an ABI373 machine.

Assessment criteria

As *Rhododendron* was acting as a model system here it was necessary, from the outset, to establish criteria by which the results obtained could be assessed. Two approaches were taken. The first involved the calculation of the cophenetic correlations (Rohlf & Fisher 1968) between the trees produced by the data and the actual data as a measure of the amount of hierarchical structure present. This is the kind of control that could be carried out during the course of a study in which there is no *a priori* knowledge of the group. The second method relied on the fact that the genus has been well studied in the past. A set of ten *a priory* precepts were established based on facts about the sampled species that were not contradicted by any of the classifications already in existence. Examples of the ten precepts are; that all the representative of *R. luteum* should cluster together; that individuals from the two accessions of *R. luteum* should come out separately; that members of subgenus *Rhododendron* (a very well defined, natural group) should come together.

Half the precepts concerned groupings that could be considered to occur below the species level and half above the species level. Each of the clusterings produced were scored against the ten precepts, scoring 0.10 for a correct clustering, 0.05 for a partially correct clustering (i.e. one aberrant individual) or zero for an incorrect clustering. In this way a scoring of between zero and one was produced for each analysis.

Analysis

The sequencing approach and random dominant marker methodologies produce two distinctly different types of data. It is possible to make strong statements of homology concerning sequence data (and therefore use it in parsimony analyses) but assumptions of homology with RAPD and AFLP studies are more problematic. In order to compare the results obtained all the three matrices were initially analysed phenetically (on the basis of overall similarity). The sequence data was later analysed using parsimony.

Two similarity matrices were produced for the two binary data sets, one using Nei's coefficient of

similarity (Nei 1972) and one with Jaccard's coefficient of similarity (Jaccard 1908). A single similarity matrix was produced from the sequence data using the Jukes Cantor model (Jukes & Cantor 1969) after they had been aligned using CLUSTALW (Thompson *et al* 1994). The similarity matrices were clustered using the UPGMA and the Neighbour Joining (Saitou & Nei 1987) methods. Cophenetic matrices were then produced from these trees (Rohlf & Sokal 1981) and compared with the original similarity matrices using the Mantel test (Mantel 1967) to give the cophenetic correlations (Rohlf & Fisher 1968) of the trees to the data. All these analyses were carried out using the NTSYS computer package (Rohlf 1994).

The UPGMA clustering method assumes the ultrametric condition, i.e. that a rooted tree can be drawn from the data in which all pairwise distances are equal to the sum of the lengths of the branches that join them and the distance from the root to the tip of any branch is the same. This can be referred to as assuming clock-like evolutionary change. The NJ method assumes that an additive unrooted tree can be drawn from the data in which all pairwise distances are equal to the sum of the lengths of the branches that join them, (although the resulting tree tends towards the ultrametric). The additive trees are a subset of ultrametric trees; all utrametric trees are additive. The calculation of the cophenetic distance matrices assumes the ultrametric condition and so close similarity of the cophenetic matrix with the original similarity matrix indicates that the original matrix has a strong ultrametric component.

A further round of analysis was carried out on the sequence data in which the alignments were manually adjusted, the indels scored as separate characters and a heuristic parsimony analysis done using the PAUP computer package on the default settings.

Results and discussion.

The histogram in figure 1 shows the combination of the cophenetic values for each of the techniques used for each of the data sets and the highest scores on the *a priori* criteria. Rohlf (1994 page 10-7) suggests that when interpreting Mantel statistics the following subjective scale could be used: 0.0 to 0.7 is a very poor fit between the matrices, 0.7 to 0.8 is a poor fit, 0.8 to 0.9 is a good fit and 0.9 to 1.0 is a very good fit. On this basis it can be seen from the histogram that the two analyses that made use of the UPGMA clustering gave trees that were either a good fit or a very good fit with the data, suggesting that there is a strong ultrametric element in the matrices. The analyses that made use of the NJ method scored very differently, the most consistent result being that the sequence data just about reaches the level of a good fit with both clustering methods. This appears to be a contradiction as a strong ultrametric data set should give a strong additive tree with NJ. If the *a priori* scorings are considered then it can be seen that the RAPD and AFLP data scores just over half whilst the sequence data scores 0.85.

It is possible to imagine a scenario in which the RAPD and AFLP data are taken in isolation and an analysis carried out on them using either of the measures of similarity plus the UPGMA clustering algorithm. In this case it would be concluded that the data is highly structured, that the tree closely resembles this structure and that the tree represents the relationships of the taxa. It can be seen from the results of this experiment, however, that this is not true as the trees produced by this method only get around half of the clusterings correct. The only combination of techniques that tend to give an indication of the true value of the trees is that of the Jaccard similarity coefficient and the NJ clustering, although this technique tends to under estimate the value of the RAPD and ALFP data sets. In summary, use of Nei and UPGMA on AFLP or RAPD data sets appears to produced a robust hierarchy but when compared with *a priori* knowledge the trees are misleading. Additive methods such as NJ give a more pessimistic picture of these data.

Considering the data sets themselves the RAPD and AFLP scored almost equally well in defining the groups below the species level but generally failed to cluster the above species level groups. What is surprising is the fact that the sequencing approach scored as well below the species level as the other techniques. (The precepts on which it failed were also failed by the RAPD and AFLP

techniques.)

In conclusion the results suggest that, in similar groups of plants, sequencing approaches should be exhausted before other techniques are employed and that when forming hierarchies from RAPD and AFLP like data both additive and ultrametric approaches should be taken and the results compared.

The parsimony analysis of the sequence data found a total of 243 trees with a consistency index of 0.76. The topology of the majority rule consensus tree from this analysis is very similar to that of the NJ trees produced using the similarity analysis and is presented in figure 2. The taxonomic conclusions discussed below are based on this tree.

Taxonomic conclusions.

From the three out-groups chosen, the ITS Sequencing clearly indicates that *Befaria* and *Daboecia* are distinct from *Rhododendron*, but that the third, *Menziesia*, is not. This is the only totally unexpected and unexplained result in the analysis as there is no morphological evidence to suggest that it is in any way related to section *Pentanthera*, as suggested by the ITS sequences. *R. camtschaticum* (subgenus *Therorhodion*) appears to be basal to the remaining species of *Rhododendron*; the results strongly support to the suggestion made by some authors that it should be excluded from *Rhododendron* as *Therorhodion camschaticum*. Subgenus *Hymenanthes*, subgenus *Rhododendron* and subgenus *Tsutsusi* are clearly defined monophyletic groups; the monotypic subgenus *Candidastrum* (*R. albiflorum*) is also confirmed. While the sampling is not comprehensive enough to make firm conclusions, the recognition of two sections within subgenus *Tsutsusi* is supported, while the results are compatible with the three sections recognised in subgenus *Rhododendron*. The inclusion of the genus *Ledum* in subgenus *Rhododendron*, as proposed by Kron & Judd (1990), is confirmed. While both section *Azaleastrum* (*R. hongkongense*) and section *Choniastrum* (*R. moulmainense*) are distinguished by the analysis, they do not appear to be related to one another. This implies that the morphological characters used to group them together should be reappraised. The affinity proposed between *R. hongkongense* and *R. semibarbatum* (subgenus *Mumeazalea*) questions the significance of the morphological characters, especially the strongly dimorphic stamens of the latter, that have been used to delimit the monotypic subgenus *Mumeazalea*. The results differ most radically from the classification proposed on morphological grounds in respect to subgenus *Pentanthera*. Section *Pentanthera* is clearly demarcated but section *Rhodora* (*R. canadense* & *R. vaseyi*) and section *Sciadorhodion* (*R. schippenbachii* and *R. albrechtii*) are apparently artificial groupings and the species are not apparently related to section *Pentanthera*.

In summary it may be seen that ITS sequencing accurately confirms the higher level infrageneric classification proposed on morphological grounds to which more than 98% of the species of *Rhododendron* belong. Therefore, reappraisal of the morphological evidence for the classification of the remaining 2% is necessary. This, however, was not the purpose of the project and is not pursued further here.

Acknowledgements.

This study was only made possible by the cooperation, over a three year period, of thirteen laboratories spread across Europe. We would like to thank all at these laboratories would contributed but particularly those at the laboratory of Dr Ana Vazquez in Madrid (who supplied the RAPD data set used here), Dr Michaela Matthes at Zeneca Seeds, UK (who supplied the AFLP data set used here) and Dr Rita Aert and Professor Guido Volckaert at the Catholic University of Leuven, Belgium (who supplied the sequences data used here).

Figure 50: *Histogram of cophenetic correlations for the four different combinations of similarity and clustering techniques used and the highest scorings obtained against the a priori precepts for the RAPD, AFLP and ITS sequencing data sets.*

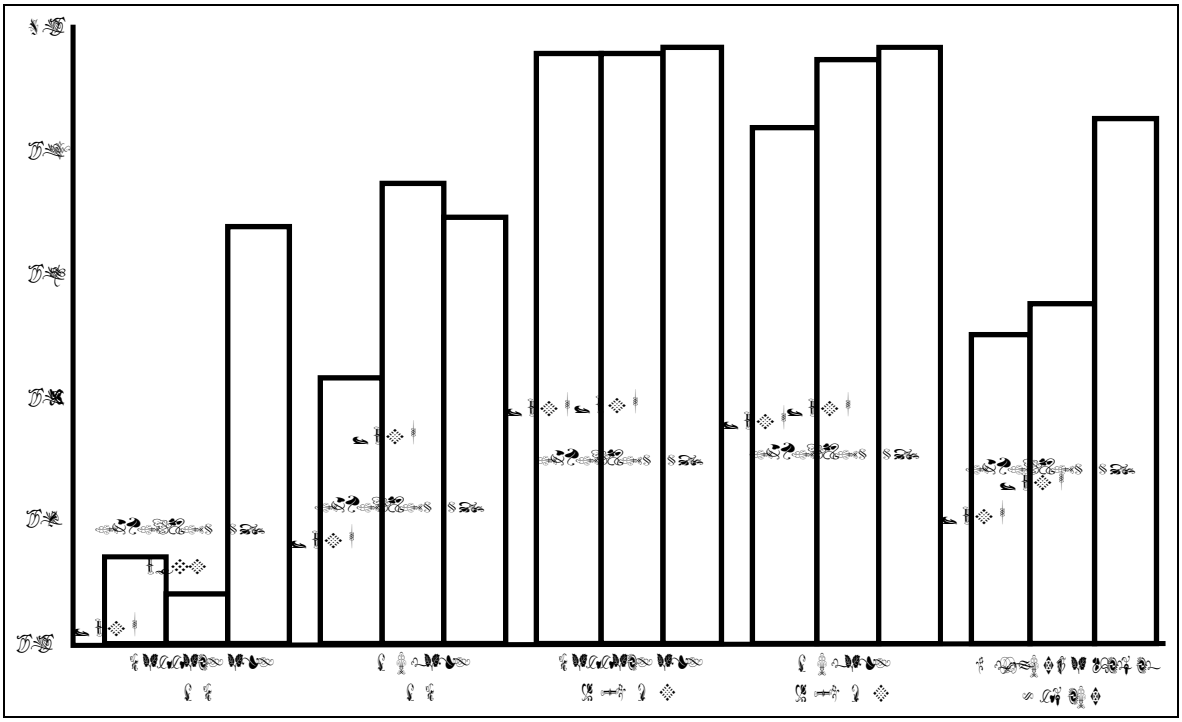
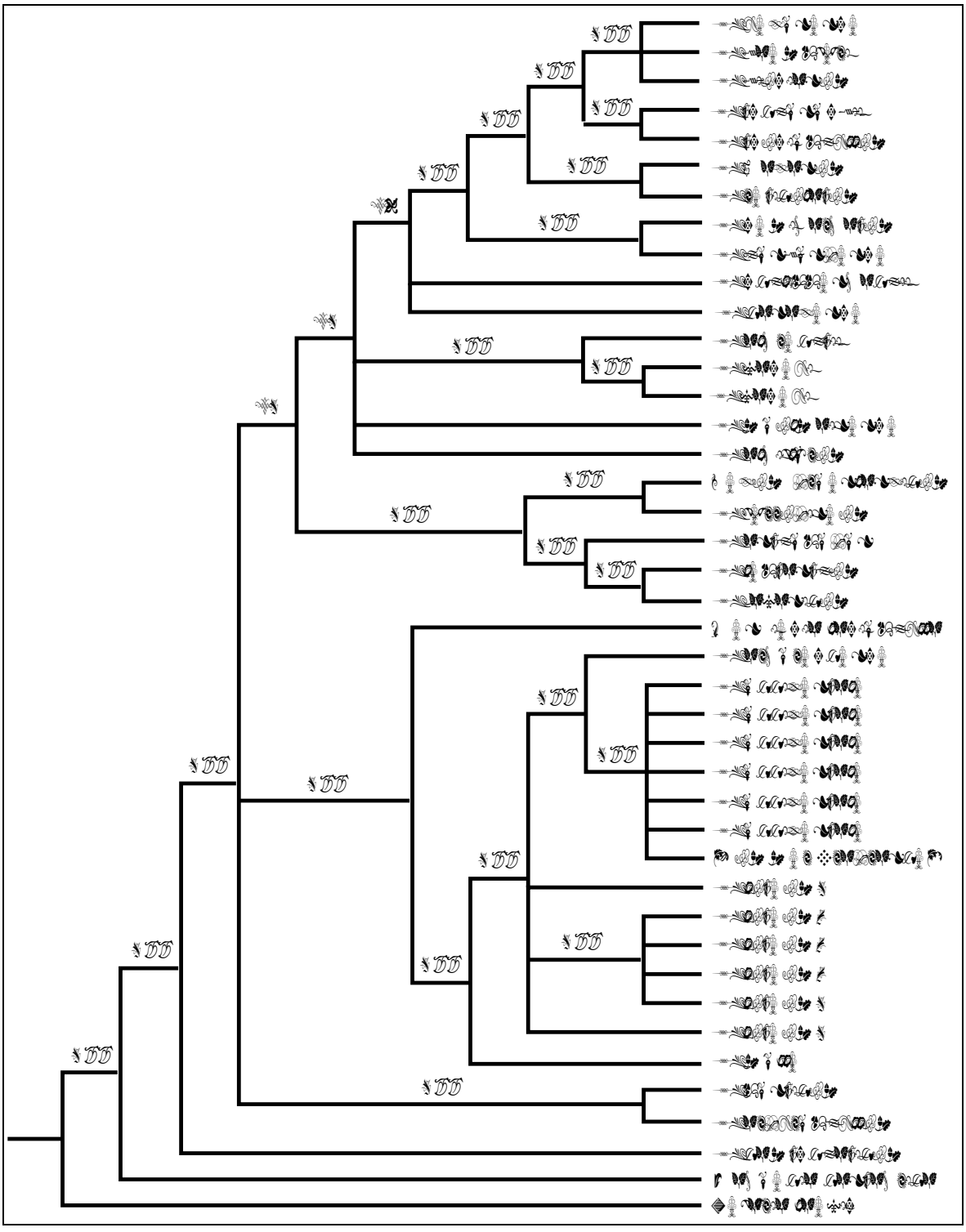


Figure 51: *The 50% majority rule consensus tree of the 167 most parsimonious trees from the parsimony analysis of the ITS sequence data. Numbers on branches are the percentage .*



Appendix A: The Synonomy of *Rhodododendron* subgenus *Hymenanthes*. (After Chamberlain *et al* 1996)

Subgenus *Hymenanthes* (Blume) K.Koch (1872)
Section *Ponticum* G.Don (1834)
Subsection *Arborea* Sleumer (1949)
arboreum Sm. [ASS-AP, ASS-AS, ASS-MA, ASS-ME, BHU-BH, BHU-SI, BMA-OO, CHC-YU, CHC-GU, CHS-GX, CHT-XI, IND-HP, IND-TN, IND-UP, IND-WB, JMK-OO, NEP-OO, SRL-OO, THA-OO, VIE-OO] syn: *Rhododendron nepalense* hort., *R. purpureum* Buch.-Ham. ex D.Don, *R. rollisonii* Lindl., *R. undulatum* Sweet ex Steudel
— ssp. ***arboreum*** [JMK-OO, IND-HP, IND-UP, IND-WB, BHU-SI, BHU-BH] syn: *Rhododendron arboreum* Sm. ssp. *windsori* (Nutt.) Tagg, *R. puniceum* Roxb., *R. windsorii* Nutt.
— ssp. ***cinnamomeum*** (Lindl.) Tagg [NEP-OO, IND-WB, ASS-AP, BHU-SI, CHT-XI]
— — var. ***cinnamomeum*** (Wall. ex G.Don) Lindl. [NEP-OO, IND-WB, BHU-SI] syn: *Rhododendron arboreum* Sm. ssp. *campbelliae* (Hook.f.) Tagg, *R. campbelliae* Hook.f., *R. cinnamomeum* Wall. ex G.Don
— — var. ***roseum*** Lindl. [NEP-OO, IND-WB, ASS-AP, BHU-SI, BHU-BH, CHT-XI]
— — — forma ***album*** Wall. [NEP-OO] syn: *Rhododendron album* Buch.-Ham. ex D.Don, *R. gloxinaeflorum* hort.
— ssp. ***delavayi*** (Franch.) D.F.Chamb. [ASS-ME, ASS-MA, ASS-AS, ASS-AP, BMA-OO, THA-OO, CHC-YU, CHC-GU CHC-GU]
— — var. ***delavayi*** [ASS-ME, ASS-MA, ASS-AS, ASS-AP, BMA-OO, THA-OO, CHC-YU, CHC-GU, CHS-GX] syn: *Rhododendron delavayi* Franch., *R. pilovittatum* Balf.f. & W.W.Sm.
— — var. ***peramoenum*** (Balf.f. & Forrest) D.F.Chamb. [ASS-AP, CHC-YU] syn: *Rhododendron delavayi* Franch. var. *peramoenum* (Balf.f. & Forrest) T.L.Ming, *R. peramoenum* Balf.f. & Forrest
— ssp. ***nilagiricum*** (Zenker) Tagg [IND-TN] syn: *Rhododendron nilagiricum* Zenker
— ssp. ***zeylanicum*** (Booth) Tagg [SRL-OO] syn: ***hunnewellianum*** Rehder & E.H.Wilson [CHC-SI, CHC-GA]
— ssp. ***hunnewellianum*** [CHC-SI] syn: *Rhododendron leucolasium* Diels
— ssp. ***rockii*** (E.H.Wilson) D.F.Chamb. [CHN-GA, CHC-SI] syn: *Rhododendron rockii* E.H.Wilson
insigne Hemsl. & E.H.Wilson [CHC-SI]
— var. ***hejiangense*** (W.P.Fang) M.Y.Fang [CHC-SI] syn: *Rhododendron argyrophyllum* Franch. ssp. *hejiangense* W.P.Fang
— var. ***insigne*** [CHC-SI]
longipes Rehder & E.H.Wilson [CHC-SI, CHC-GU]
— var. ***chienianum*** (W.P.Fang) D.F.Chamb. [CHC-SI] syn: *Rhododendron chienianum* W.P.Fang
— var. ***longipes*** [CHC-SI, CHC-GU]
oblancifolium M.Y.Fang [CHC-GU]
pingianum W.P.Fang [CHC-SI]
ririei Hemsl. & E.H.Wilson [CHC-SI, CHC-GU]
shimenense Q.X.Liu & C.M.Zhang [CHS-HA]
simiarum Hance [CHS-GX, CHS-GD, CHS-HA, CHS-JX, CHS-AN, CHS-ZH, CHS-HK, CHS-FU, CHH-OO, CHC-GU] syn: *Rhododendron fokienense* Franch., *R. fordii* Hemsl.
— var. ***deltoideum*** P.C.Tam [CHH-OO]
— var. ***simiarum*** [CHS-GX, CHS-GD, CHS-JX, CHS-HA, CHS-AN, CHS-ZH, CHS-HK, CHS-FU, CHC-GU]
— var. ***versicolor*** (Chun & W.P.Fang) M.Y.Fang [CHS-GX] syn: *Rhododendron versicolor* Chun & W.P.Fang
thayerianum Rehder & E.H.Wilson [CHC-SI]
Subsection *Auriculata* Hutch. (1922)
auriculatum Hemsl. [CHC-SI, CHC-HU, CHC-GU, CHS-HA]
chihsinianum Chun & W.P.Fang [CHS-GX]
Rhododendron arboreum Sm. ssp. *kingianum* (Watt ex Hook.f.) Tagg, *R. arboreum* Sm. var. *kingianum* Watt ex Hook.f., *R. zeylanicum* Booth
delavayi Franch. var. ***albomentosum*** Davidian [BMA-OO] (See introduction)
— var. ***pilostylum*** K.M.Feng [CHC-YU]
lanigerum Tagg [ASS-AP, CHT-XI] syn: *Rhododendron lanigerum* Tagg var. *silvaticum* (Cowan) Davidian, *R. silvaticum* Cowan
niveum Hook.f. [BHU-SI, BHU-BH]
Subsection *Argyrophylla* Sleumer (1949)
adenopodum Franch. [CHC-SI, CHC-HU] syn: *Rhododendron simiarum* Hance ssp. *youngae* (W.P.Fang) D.F.Chamb., *R. youngae* W.P.Fang
argyrophyllum Franch. [CHN-SA, CHC-YU, CHC-SI, CHS-HU]
— ssp. ***argyrophyllum*** [CHN-SA, CHC-YU, CHC-SI] syn: *Rhododendron argyrophyllum* Franch. var. *cupulare* Rehder & E.H.Wilson, *R. chionophyllum* Diels
— ssp. ***hypoglaucum*** (Hemsl.) D.F.Chamb. [CHC-SI, CHC-HU] syn: *Rhododendron gracilipes* Franch., *R. hypoglaucum* Hemsl.
— ssp. ***nankingense*** (Cowan) D.F.Chamb. [CHC-GU] syn: *Rhododendron argyrophyllum* Franch. var. *leiandrum* Hutch., *R. argyrophyllum* Franch. var. *nankingense* Cowan
— ssp. ***omeiense*** (Rehder & E.H.Wilson) D.F.Chamb. [CHC-SI] syn: *Rhododendron argyrophyllum* Franch. var. *omeiense* Rehder & E.H.Wilson
brevipetiolatum M.Y.Fang [CHC-SI]
coryanum Tagg & Forrest [CHC-YU, CHT-XI]
denudatum H.Lév. [CHC-YU, CHC-SI, CHC-GU] syn: *Rhododendron xanthoneuron* H.Lév.
ebianense M.Y.Fang [CHC-SI]
fangchengense P.C.Tam [CHS-GX]
farinosum H.Lév. [CHC-YU]
floribundum Franch. [CHC-SI, CHC-GU]
formosanum Hemsl. [TAI-OO]
haofui Chun & W.P.Fang [CHC-GU, CHS-GX, CHS-GD, CHS-HA]
Subsection *Barbata* Sleumer (1949)
argipeplum Balf.f. & R.E.Cooper [BHU-SI, BHU-BH, ASS-AP, CHT-XI] syn: *Rhododendron macrosmithii* Davidian, *R. smithii* Nutt. ex Hook.f.
barbatum Wall. ex G.Don [IND-UP, IND-WB, NEP-OO, BHU-SI, BHU-BH, ASS-AP, CHT-XI] syn: *Rhododendron aristatum* Royle, *R. lancifolium* Hook.f., *R. nobile* Wall.
erosum Cowan [CHT-XI]
exasperatum Tagg [ASS-AP, BMA-OO, CHT-XI]
x *imberbe* Hutch. syn: *Rhododendron barbatum* Wall. ex G.Don forma *imberbe* (Hutch.) H.Hara
succothii Davidian [BHU-BH, ASS-AP] syn: *Rhododendron nishiokae* H.Hara
Subsection *Campanulata* Sleumer (1949)
campanulatum D.Don [JMK-OO, IND-HP, IND-UP, IND-WB, NEP-OO, BHU-SI, BHU-BH] syn: *Rhododendron edgarii* Gamble, *R. planifolium* Nutt.
— ssp. ***aeruginosum*** (Hook.f.) D.F.Chamb. [BHU-SI, BHU-BH, NEP-OO] syn: *Rhododendron aeruginosum* Hook.f., *R. campanulatum* D.Don var. *aeruginosum* Hook.f. ex Cowan & Davidian
— ssp. ***campanulatum*** [JMK-OO, IND-HP, IND-UP, IND-WB, NEP-OO, BHU-SI, BHU-BH]
gannanense Z.C.Feng & X.G.Sun [CHN-GA]
wallichii Hook.f. [NEP-OO, IND-WB, BHU-SI, BHU-BH, CHT-XI] syn: *Rhododendron heftii* Davidian
Subsection *Campylocarpa* Sleumer (1949)
callimorphum Balf.f. & W.W.Sm. [CHC-YU]
— var. ***callimorphum*** [CHC-YU] syn: *Rhododendron cyclium* Balf.f. & Forrest, *R. hedythamnum* Balf.f. & Forrest
— var. ***myiagrum*** (Balf.f. & Forrest) D.F.Chamb. [CHC-YU] syn: *Rhododendron myiagrum* Balf.f. &

Forrest
campylocarpum Hook.f. [NEP-OO, BHU-SI, BHU-BH, ASS-AP, CHT-XI, CHC-YU, BMA-OO]
— ssp. ***caloxanthum*** (Balf.f. & Farrer) D.F.Chamb. [BMA-OO, CHT-XI, CHC-YU] syn: *Rhododendron caloxanthum* Balf.f. & Farrer, *R. campylocarpum* Hook.f. ssp. *telopeum* (Balf.f. & Forrest) D.F.Chamb., *R. telopeum* Balf.f. & Forrest
— ssp. ***campylocarpum*** [NEP-OO, BHU-SI, BHU-BH, ASS-AP, CHT-XI]
henanense W.P.Fang [CHS-HN]
— ssp. ***henanense*** [CHS-HN]
— ssp. ***lingbaoense*** W.P.Fang [CHS-HN]
longicalyx M.Y.Fang [CHC-SI]
souliei Franch. [CHC-SI] syn: *Rhododendron cordatum* H.Lév.
wardii W.W.Sm. [CHT-XI, CHC-YU, CHC-SI]
— var. ***puralbum*** (Balf.f. & W.W.Sm.) D.F.Chamb. [CHT-XI, CHC-YU, CHC-SI] syn: *Rhododendron puralbum* Balf.f. & W.W.Sm.
— var. ***wardii*** [CHT-XI, CHC-YU, CHC-SI] syn: *Rhododendron astrocalyx* Balf.f. & Forrest, *R. croceum* Balf.f. & W.W.Sm., *R. gloeoblastum* Balf.f. & Forrest, *R. litiense* Balf.f. & Forrest, *R. mussoti* Franch., *R. oresterum* Balf.f. & Forrest, *R. prasinocalyx* Balf.f. & Forrest

Subsection *Falconera* Sleumer (1949)
arizelum Balf.f. & Forrest syn: *Rhododendron arizelum* Balf.f & Forrest var. *rubicosum* Cowan & Davidian, *R. rex* H.Lév. ssp. *arizelum* (Balf.f. & Forrest) D.F.Chamb.
basilicum Balf.f. & W.W.Sm. [BMA-OO, CHC-YU] syn: *Rhododendron megaphyllum* Balf.f. & Forrest, *R. regale* Balf.f. & Kingdon-Ward
coriaceum Franch. [CHT-XI, CHC-YU] syn: *Rhododendron foveolatum* Rehder & E.H.Wilson
x *decipiens* Lacaita
falconeri Hook.f. [NEP-OO, IND-WB, BHU-SI, BHU-BH, ASS-AP]
— ssp. ***eximium*** (Nutt.) D.F.Chamb. [ASS-AP] syn: *Rhododendron eximium* Nutt.
— ssp. ***falconeri*** [NEP-OO, IND-WB, BHU-SI, BHU-BH, ASS-AP]
fictolacteum Balf.f. var. ***miniforme*** Davidian [CHT-XI, CHC-YU] (See introduction)
galactinum Balf.f. ex Tagg [CHC-SI]
hodgsonii Hook.f. [NEP-OO, IND-WB, BHU-SI, BHU-BH, ASS-AP, CHT-XI]
preptum Balf.f. & Forrest [BMA-OO]
rex H.Lév. [CHC-SI, CHC-YU, CHT-XI, BMA-OO]
— ssp. ***fictolacteum*** (Balf.f.) D.F.Chamb. [BMA-OO, CHT-XI, CHC-YU] syn: *Rhododendron fictolacteum* Balf.f., *R. lacteum* Franch. var. *macrophyllum* Franch.
— ssp. ***gratum*** (T.L.Ming) M.Y.Fang [CHC-YU] syn: *Rhododendron gratum* T.L.Ming
— ssp. ***rex*** [CHC-SI, CHC-YU]
rothschildii Davidian [CHC-YU]
semnoides Tagg & Forrest [CHT-XI, CHC-YU]
sinofalconeri Balf.f. [CHC-YU, VIE-OO]

Subsection *Fortunea* Sleumer (1949)
asterochnoum Diels [CHC-SI]
— var. ***asterochnoum*** [CHC-SI]
— var. ***brevipedicellatum*** W.K.Hu [CHC-SI]
calophytum Franch. [CHC-YU, CHC-SI]
— ssp. ***jinfuense*** M.Y.Fang [CHC-SI] syn: *Rhododendron calophytum* Franch. var. *jinfuense* M.Y.Fang & W.K.Hu
— var. ***calophytum*** [CHC-YU, CHC-SI, CHC-GU]
— var. ***openshawianum*** (Rehder & E.H.Wilson) D.F.Chamb. [CHC-SI, CHC-YU] syn: *Rhododendron openshawianum* Rehder & E.H.Wilson
— var. ***pauciflorum*** W.K.Hu [CHC-SI]
davidii Franch. [CHC-YU, CHC-SI, CHC-GU]
decorum Franch. [BMA-OO, CHC-YU, CHC-SI, CHC-GU]
— ssp. ***decorum*** [BMA-OO, CHC-YU, CHC-SI] syn: *Rhododendron franchetianum* H.Lév., *R. giraudissii* H.Lév., *R. spooneri* Hemsl. & E.H.Wilson
— ssp. ***diaprepes*** (Balf.f. & W.W.Sm.) T.L.Ming [BMA-OO, CHC-YU] syn: *Rhododendron diaprepes* Balf.f. & W.W.Sm., *R. rasile* Balf.f. & W.W.Sm.
— var. ***parvistigmatis*** W.K.Hu [CHC-SI]
— var. ***cordatum*** W.K.Hu [CHC-YU]
faithae Chun [CHS-GX, CHS-GD, CHC-GU]
fortunei Lindl. [CHC-SI, CHC-HU, CHC-GU, CHS-GX, CHS-GD, CHS-HA, CHS-JX, CHS-FU, CHS-AN, CHS-ZH]
— ssp. ***discolor*** (Franch.) D.F.Chamb. [CHC-SI, CHC-HU, CHC-GU, CHS-GX, CHS-HA, CHS-AN, CHS-ZH] syn: *Rhododendron discolor* Franch., *R. houlstonii* Hemsl. & E.H.Wilson, *R. kirkii* Millais, *R. kwangfuense* Chun & W.P.Fang, *R. mandarinorum* Diels
— ssp. ***fortunei*** [CHC-SI, CHS-GX, CHS-GD, CHS-HA, CHS-JX, CHS-FU, CHS-AN, CHS-ZH, CHC-GU] syn: *Rhododendron albicaule* H.Lév.
x *geraldii* Ivens syn: *Rhododendron sutchuenense* Franch. var. *geraldii* Hutch.
glanduliferum Franch. [CHC-YU, CHC-GU]
gonggashanense W.K.Hu [CHC-SI]
griffithianum Wight [NEP-OO, IND-WB, BHU-SI, BHU-BH, ASS-AP] syn: *Rhododendron aucklandii* Hook.f., *R. griffithianum* Wight var. *aucklandii* (Hook.f.) Hook.f., *R. oblongum* Griff.
hemsleyanum E.H.Wilson [CHC-SI] syn: *Rhododendron chengianum* W.P.Fang
huianum W.P.Fang [CHC-YU, CHC-SI, CHC-GU]
jingangshanicum P.C.Tam [CHS-JX]
magniflorum W.K.Hu [CHC-GU]
maoerense W.P.Fang & G.Z.Li [CHS-GX]
miyiense W.K.Hu [CHC-SI]
nymphaeoides W.K.Hu [CHC-SI]
orbiculare Decne. [CHC-SI, CHS-GX, CHS-HA]
— ssp. ***cardiobasis*** (Sleumer) D.F.Chamb. [CHS-GX] syn: *Rhododendron cardiobasis* Sleumer
— ssp. ***oblongum*** W.K.Hu [CHC-SI, CHS-GX]
— ssp. ***orbiculare*** [CHC-SI, CHS-GX, CHS-HA] syn: *Rhododendron rotundifolium* David
oreodoxa Franch. [CHC-YU, CHC-SI, CHC-HU, CHN-SA, CHN-GA, CHT-XI]
— var. ***adenostylosum*** M.Y.Fang & H.K.Hu [CHT-XI, CHC-SI]
— var. ***fargesii*** (Franch.) D.F.Chamb. [CHC-SI, CHC-HU, CHN-GA] syn: *Rhododendron erubescens* Hutch., *R. fargesii* Franch.
— var. ***oreodoxa*** [CHC-YU, CHC-SI, CHN-GA] syn: *Rhododendron haematocheilum* Craib, *R. limprichtii* Diels, *R. reginaldii* Balf.f.
— var. ***shensiense*** D.F.Chamb. [CHN-SA] syn: *Rhododendron shensiense* Ching
platypodum Diels [CHC-SI, CHS-GX]
praeteritum Hutch. [CHC-HU]
— var. ***hirsutum*** W.K.Hu [CHC-HU]
— var. ***praeteritum*** [CHC-HU]
praevernum Hutch. [CHC-SI, CHC-HU]
serotinum Hutch. [CHC-YU, VIE-OO, LAO-OO]
sutchuenense Franch. [CHC-SI, CHC-HU, CHN-SA, CHC-GU]
vernicosum Franch. [CHC-YU, CHC-SI, CHC-GU] syn: *Rhododendron adoxum* Balf.f. & Forrest, *R. araliiforme* Balf.f. & Forrest, *R. euanthum* Balf.f. & W.W.Sm., *R. hexamerum* Hand.-Mazz., *R. lucidum* Franch., non Nutt., *R. rhantum* Balf.f. & W.W.Sm., *R. sheltonii* Hemsl. & E.H.Wilson
verruciferum W.K.Hu [CHC-SI]
wolongense W.K.Hu [CHC-SI]

Subsection *Fulgensia* D.F.Chamb. (1979)
fulgens Hook.f. [NEP-OO, IND-WB, BHU-SI, BHU-BH, ASS-AP, CHT-XI]
miniatum Cowan [CHT-XI]

Subsection *Fulva* Sleumer (1949)
fulvoides Balf.f. & Forrest [CHT-XI, CHC-YU]
fulvum Balf.f. & W.W.Sm. [CHC-YU]
uvariifolium Diels [CHT-XI, CHC-YU, CHC-SI]
— var. ***griseum*** Cowan [CHT-XI]
— var. ***uvariifolium*** [CHT-XI, CHC-YU, CHC-SI] syn: *Rhododendron dendritrichum* Balf.f. & Forrest, *R. mombeigii* Rehder & E.H.Wilson, *R. niphargum*

Balf.f. & Kingdon-Ward

Subsection *Glischra* (Tagg) D.F.Chamb.

adenosum Davidian [CHC-SI] syn: *Rhododendron glischrum* Balf.f. & W.W.Sm. var. *adenosum* Cowan & Davidian, *R. kuluense* D.F.Chamb.

crinigerum Franch. [CHT-XI, CHC-YU]

— var. ***crinigerum*** [CHT-XI, CHC-YU] syn: *Rhododendron ixeunticum* Balf.f. & W.W.Sm.

— var. ***euadenium*** Tagg & Forrest [CHC-YU]

diphrocalyx Balf.f. [CHC-YU] syn: *Rhododendron burrifolium* Balf.f. & Forrest

glischrum Balf.f. & W.W.Sm. [BMA-OO, CHT-XI, CHC-YU]

— ssp. ***glischroides*** (Tagg & Forrest) D.F.Chamb. [BMA-OO] syn: *Rhododendron glischroides* Tagg & Forrest var. *arachnoideum* Tagg, *R. glischroides* Tagg & Forrest

— ssp. ***glischrum*** [BMA-OO, CHT-XI, CHC-YU]

— ssp. ***rude*** (Tagg & Forrest) D.F.Chamb. [CHC-YU] syn: *Rhododendron rude* Tagg & Forrest

habrotrichum Balf.f. & W.W.Sm. [BMA-OO, CHC-YU]

recurvoides Tagg & Kingdon-Ward [BMA-OO]

spilotum Balf.f. & Farrer [BMA-OO]

vesiculiferum Tagg [BMA-OO, CHT-XI, CHC-YU]

Subsection *Grandia* Sleumer (1949)

balangense W.P.Fang [CHC-SI]

grande Wight [NEP-OO, IND-WB, BHU-SI, BHU-BH, ASS-AP, CHT-XI] syn: *Rhododendron argenteum* Hook.f., *R. longifolium* Nutt.

kesangiae D.G.Long & Rushforth [BHU-BH]

— var. ***album*** Namgyel & D.G.Long [BHU-BH]

— var. ***kesangiae*** [BHU-BH]

macabeanum Watt ex Balf.f. [ASS-MA]

magnificum Kingdon-Ward [BMA-OO, CHC-YU]

montroseanum Davidian [BMA-OO, CHT-XI] syn: *Rhododendron mollyanum* Cowan & Davidian

praestans Balf.f. & W.W.Sm. [CHT-XI, CHC-YU] syn: *Rhododendron coryphaeum* Balf.f. & Forrest, *R. semnum* Balf.f. & Forrest

protistum Balf.f. & Forrest [BMA-OO, CHC-YU, VIE-OO]

— var. ***giganteum*** (Forrest ex Tagg) D.F.Chamb. [BMA-OO, VIE-OO] syn: *Rhododendron giganteum* Forrest ex Tagg

— var. ***protistum*** [BMA-OO, CHC-YU] syn: *Rhododendron giganteum* Tagg var. *seminudum* Tagg & Forrest

pudorosum Cowan [CHT-XI]

sidereum Balf.f. [BMA-OO, CHC-YU]

sinogrande Balf.f. & W.W.Sm. [BMA-OO, CHT-XI, CHC-YU] syn: *Rhododendron sinogrande* Balf.f. & W.W.Sm. var. *boreale* Tagg & Forrest

watsonii Hemsl. & E.H.Wilson [CHC-SI, CHN-GA]

wattii Cowan [ASS-MA]

Subsection *Griersoniana* Davidian ex D.F.Chamb. (1979)

griersonianum Balf.f. & Forrest [BMA-OO, CHC-YU]

Subsection *Irrorata* Sleumer (1949)

aberconwayi Cowan [CHC-YU, CHC-GU]

annae Franch. [BMA-OO, CHC-YU, CHC-GU, CHS-GX] syn: *Rhododendron annae* Franch. ssp. *laxiflorum* (Balf.f. & Forrest) T.L.Ming, *R. hardingii* Tagg, *R. laxiflorum* Balf.f. & Forrest

anthosphaerum Diels [BMA-OO, CHT-XI, CHC-YU] syn: *Rhododendron anthosphaerum* Diels var. *eritimum* (Balf.f. & W.W.Sm.) Davidian, *R. chawchiense* Balf.f. & Farrer, *R. eritimum* Balf.f. & W.W.Sm., *R. gymnogynum* Balf.f. & Forrest, *R. heptamerum* Balf.f., *R. hylothreptum* Balf.f. & W.W.Sm., *R. persicinum* Hand.-Mazz.

araiophyllum Balf.f. & W.W.Sm. [BMA-OO, CHC-YU]

— ssp. ***araiophyllum*** [BMA-OO, CHC-YU]

— ssp. ***lapidosum*** (T.L.Ming) M.Y.Fang [CHC-YU] syn: *Rhododendron lapidosum* T.L.Ming

brevinerve Chun & W.P.Fang [CHC-GU, CHS-GX, CHS-GD, CHS-HA]

excelsum A.Chev. [VIE-OO]

gongshanense T.L.Ming [CHC-YU]

irroratum Franch. [CHC-YU, CHC-SI, VIE-OO, SUM-OO]

— ssp. ***irroratum*** [CHC-YU, CHC-SI, CHC-GU] syn: *Rhododendron ninguenense* Hand.-Mazz.

— ssp. ***kontumense*** (Sleumer) D.F.Chamb. [VIE-OO, SUM-OO] syn: *Rhododendron atjehense* Sleumer, *R. kontumense* Sleumer, *R. langbianense* A.Chev. ex Dop, *R. ninguenense* sensu Sleumer, non Hand.-Mazz.

— ssp. ***pogonostylum*** (Balf.f. & W.W.Sm.) D.F.Chamb. [CHC-YU, CHC-SI] syn: *Rhododendron adenostemonum* Balf.f. & W.W.Sm., *R. pogonostylum* Balf.f. & W.W.Sm.

kendrickii Nutt. [BHU-BH, ASS-AP, CHT-XI] syn: *Rhododendron pankimense* Cowan & Kingdon-Ward, *R. shepherdii* Nutt.

korthalsii Miq. [SUM-OO]

laojunense T.L.Ming [CHC-YU]

leptopeplum Balf.f. & Forrest [CHC-YU]

lukiangense Franch. [CHT-XI, CHC-YU, CHC-SI] syn: *Rhododendron admirabile* Balf.f. & Forrest, *R. adroserum* Balf.f. & Forrest, *R. ceraceum* Balf.f. & W.W.Sm., *R. gymnanthum* Diels

mengtszense Balf.f. & W.W.Sm. [CHC-YU]

papillatum Balf.f. & Cooper [BHU-BH, ASS-AP] syn: *Rhododendron epapillatum* Balf.f. & Cooper

pingbianense M.Y.Fang [CHC-YU]

ramsdenianum Cowan [CHT-XI]

spanotrichum Balf.f. & W.W.Sm. [CHC-YU, CHC-GU]

tanastylum Balf.f. & Kingdon-Ward [ASS-AP, BMA-OO, CHC-YU, CHT-XI, VIE-OO]

— var. ***lingzhiense*** M.Y.Fang [CHT-XI] syn: *Rhododendron rubro-punctata* T.L.Ming

— var. ***pennivenium*** (Balf.f. & Forrest) D.F.Chamb. [CHC-YU, VIE-OO] syn: *Rhododendron* x *agastum* Balf.f. & W.W.Sm. var. *pennivenium* (Balf.f. & Forrest) T.L.Ming, *R. pennivenium* Balf.f. & Forrest, *R. petelotii* Dop

— var. ***tanastylum*** [ASS-AP, BMA-OO, CHC-YU] syn: *Rhododendron cerochitum* Balf.f. & Forrest, *R. ombrachares* Balf.f. & Kingdon-Ward

wrayi King & Gamble [MLY-PM] syn: *Rhododendron corruscum* Ridl., *R. dubium* King & Gamble, *R. wrayi* King & Gamble var. *ellipticum* Ridl., *R. wrayi* King & Gamble var. *minor* Ridl.

Subsection *Lanata* Hook.f.

circinnatum Cowan & Kingdon-Ward [CHT-XI]

flinckii Davidian [BHU-BH]

lanatoides D.F.Chamb. [CHT-XI]

lanatum Hook.f. [BHU-BH, BHU-SI, ASS-AP, CHT-XI] syn: *Rhododendron lanatum* Hook.f. var. *luciferum* Cowan, *R. luciferum* (Cowan) Cowan

poluninii Davidian [BHU-BH]

tsariense Cowan [BHU-BH, ASS-AP, CHT-XI]

— var. ***magnum*** Davidian [BHU-BH]

— var. ***trimoense*** Davidian [CHT-XI]

— var. ***tsariense*** [BHU-BH, ASS-AP, CHT-XI]

Subsection *Maculifera* Sleumer (1949)

anwheiense E.H.Wilson [CHS-AN, CHS-HA, CHS-JX, CHS-JS] syn: *Rhododendron maculiferum* Franch. ssp. *anwheiense* (E.H.Wilson) D.F.Chamb.

longesquamatum C.K.Schneid. [CHC-SI, CHC-GU] syn: *Rhododendron brettii* Hemsl. & E.H.Wilson

maculiferum Franch. [CHN-GA, CHN-SA, CHC-SI, CHC-HU, CHC-GU, CHS-GX]

monosematum Hutch. syn: *Rhododendron strigillosum* Franch. var. *monosematum* (Hutch.) T.L.Ming (See introduction)

morii Hayata [TAI-OO]

ochraceum Rehder & E.H.Wilson [CHC-SI]

— var. ***brevicarpum*** W.K.Hu [CHC-SI]

— var. ***ochraceum*** [CHC-SI]

oligocarpum W.P.Fang & S.S.Chang [CHC-GU, CHS-GX]

pachysanthum Hayata [TAI-OO] syn: *Rhododendron rufum* Batalin var. *pachysanthum* (Hayata) S.S.Ying

pachytrichum Franch. [CHC-YU, CHC-SI]

— var. ***pachytrichum*** [CHC-YU, CHC-SI]

— var. ***tenuistylosum*** W.K.Hu [CHC-SI]

pilostylum W.K.Hu [CHC-YU]

polytrichum W.P.Fang [CHS-HA, CHS-GX]

pseudochrysanthum Hayata [TAI-OO]
— ssp. ***morii*** (Hayata) T.Yamaz. var. ***nankotaisanense*** (Hayata) T.Yamaz. [TAI-OO] syn: *Rhododendron nankotaisanense* Hayata (See introduction)
— — var. ***taitunense*** T.Yamaz. [TAI-OO]
— forma ***rufovelutinum*** T.Yamaz. [TAI-OO]
sikangense W.P.Fang [CHC-SI, CHC-YU]
— var. ***exquisitum*** (T.L.Ming) T.L.Ming [CHC-YU] syn: *Rhododendron exquisitum* T.L.Ming
— var. ***sikangense*** [CHC-SI] syn: *Rhododendron cookeanum* Davidian
strigillosum Franch. [CHC-SI, CHC-YU]
ziyuanense P.C.Tam [CHS-GX, CHS-HA]
— var. ***pachyphyllum*** (W.P.Fang) G.Z.Li [CHS-HA, CHS-GX] syn: *Rhododendron pachyphyllum* W.P.Fang
— var. ***ziyuanense*** [CHS-GX]

Subsection *Neriiflora* Sleumer (1949)
albertsenianum Forrest [CHC-YU]
aperantum Balf.f. & Kingdon-Ward [BMA-OO, CHC-YU] syn: *Rhododendron aperantum* Balf.f. & Kingdon-Ward var. *subpilosum* Cowan
beanianum Cowan [BMA-OO, ASS-AP]
bijiangense T.L.Ming [CHC-YU]
catacosmum Balf.f. ex Tagg [CHT-XI, CHC-YU]
chamaethomsonii (Tagg & Forrest) Cowan & Davidian [CHT-XI, CHC-YU]
— var. ***chamaedoron*** (Tagg & Forrest) D.F.Chamb. [CHT-XI, CHC-YU] syn: *Rhododendron repens* Balf.f. & Forrest var. *chamaedoron* Tagg & Forrest
— var. ***chamaethauma*** (Tagg) Cowan & Davidian [CHT-XI] syn: *Rhododendron repens* Balf.f. & Forrest var. *chamaethauma* Tagg
— var. ***chamaethomsonii*** [CHT-XI, CHC-YU] syn: *Rhododendron repens* Balf.f. & Forrest var. *chamaethomsonii* Tagg & Forrest
chionanthum Tagg & Forrest [BMA-OO, CHC-YU]
citriniflorum Balf.f. & Forrest [CHT-XI, CHC-YU]
— var. ***citriniflorum*** [CHT-XI, CHC-YU] syn: *Rhododendron chlanidotum* Balf.f. & Forrest
— var. ***horaeum*** (Balf.f. & Forrest) D.F.Chamb. [CHT-XI, CHC-YU] syn: *Rhododendron citriniflorum* Balf.f. & Forrest ssp. *aureolum* Cowan, *R. citriniflorum* Balf.f. & Forrest ssp. *horaeum* (Balf.f. & Forrest) Cowan, *R. citriniflorum* Balf.f. & Forrest ssp. *rubens* Cowan, *R. horaeum* Balf.f. & Forrest
coelicum Balf.f. & Farrer [BMA-OO, CHC-YU]
dichroanthum Diels [BMA-OO, CHC-YU]
— ssp. ***apodectum*** (Balf.f. & W.W.Sm.) Cowan [BMA-OO, CHC-YU] syn: *Rhododendron apodectum* Balf.f. & W.W.Sm., *R. dichroanthum* Diels var. *apodectum* (Balf.f. & W.W.Sm.) T.L.Ming, *R. jangtzowense* Balf.f. & Forrest, *R. liratum* Balf.f. & Forrest
— ssp. ***dichroanthum*** [CHC-YU]
— ssp. ***scyphocalyx*** (Balf.f. & Forrest) Cowan [BMA-OO, CHC-YU] syn: *Rhododendron dichroanthum* Diels ssp. *herpesticum* (Balf.f. & Kingdon-Ward) Cowan, *R. herpesticum* Balf.f. & Kingdon-Ward, *R. scyphocalyx* Balf.f. & Forrest, *R. torquatum* Balf.f. & Farrer
— ssp. ***septentrionale*** Cowan [BMA-OO, CHC-YU] syn: *Rhododendron dichroanthum* Diels var. *septentrionale* (Cowan) T.L.Ming, *R. scyphocalyx* Balf.f. & Forrest var. *septentrionale* Tagg ex Davidian
erastum Balf.f. & Forrest [CHT-XI, CHC-YU] syn: *Rhododendron porphyrophyllum* Balf.f. & Forrest, *R. serpens* Balf.f. & Forrest
euchroum Balf.f. & Kingdon-Ward [BMA-OO]
eudoxum Balf.f. & Forrest [CHT-XI, CHC-YU]
— var. ***brunneifolium*** (Balf.f. & Forrest) D.F.Chamb. [CHT-XI] syn: *Rhododendron brunneifolium* Balf.f. & Forrest, *R. eudoxum* Balf.f. & Forrest ssp. *brunneifolium* (Balf.f. & Forrest) Tagg
— var. ***eudoxum*** [CHT-XI, CHC-YU] syn: *Rhododendron eudoxum* Balf.f. & Forrest ssp. *trichomiscum* (Balf.f. & Forrest) Tagg, *R.* x *fulvastrum* Balf.f. & Forrest ssp. *trichomiscum* (Balf.f. & Forrest) Cowan, *R.* x *fulvastrum* Balf.f. & Forrest ssp. *trichophlebium* (Balf.f. & Forrest) Cowan, *R. temenium* Balf.f. & Forrest ssp. *albipetalum* Cowan, *R. temenium* Balf.f. & Forrest ssp. *rhodanthum* Cowan, *R. trichomiscum* Balf.f. & Forrest, *R. trichophlebium* Balf.f. & Forrest
— var. ***mesopolium*** (Balf.f. & Forrest) D.F.Chamb. [CHT-XI, CHC-YU] syn: *Rhododendron asteium* Balf.f. & Forrest, *R. epipastum* Balf.f. & Forrest, *R. eudoxum* Balf.f. & Forrest ssp. *asteium* (Balf.f. & Forrest) Tagg, *R. eudoxum* Balf.f. & Forrest ssp. *epipastum* (Balf.f. & Forrest) Tagg, *R. eudoxum* Balf.f. & Forrest ssp. *mesopolium* (Balf.f. & Forrest) Tagg, *R.* x *fulvastrum* Balf.f. & Forrest ssp. *epipastum* (Balf.f. & Forrest) Cowan, *R.* x *fulvastrum* Balf.f. & Forrest ssp. *mesopolium* (Balf.f. & Forrest) Cowan, *R. mesopolium* Balf.f. & Forrest
floccigerum Franch. [CHT-XI, CHC-YU]
forrestii Balf.f. ex Diels [BMA-OO, CHT-XI, CHC-YU]
— ssp. ***forrestii*** [BMA-OO, CHT-XI, CHC-YU] syn: *Rhododendron forrestii* Diels var. *repens* (Balf.f. & Forrest) Cowan & Davidian, *R. repens* Balf.f. & Forrest
— ssp. ***papillatum*** D.F.Chamb. [CHT-XI]
x *fulvastrum* Balf.f & Forrest
x *fulvastrum* Balf.f. & Forrest var. ***fulvastrum***
haematodes Franch. [BMA-OO, CHT-XI, CHC-YU]
— ssp. ***chaetomallum*** (Balf.f. & Forrest) D.F.Chamb. [BMA-OO, CHT-XI, CHC-YU] syn: *Rhododendron chaetomallum* Balf.f. & Forrest, *R. chaetomallum* Balf.f. & Forrest var. *glaucescens* Tagg & Forrest
— ssp. ***haematodes*** [CHC-YU] syn: *Rhododendron haematodes* Franch. var. *calycinum* Franch., *R. haematodes* Franch. var. *hypoleucum* Franch.
x *hillieri* Davidian
mallotum Balf.f. & Kingdon-Ward [BMA-OO, CHC-YU] syn: *Rhododendron aemulorum* Balf.f.
microgynum Balf.f. & Forrest [CHT-XI, CHC-YU] syn: *Rhododendron gymnocarpum* Balf.f. ex Tagg, *R. perulatum* Balf.f. & Forrest
neriiflorum Franch. [ASS-AP, BMA-OO, BHU-BH, CHT-XI, CHC-YU]
— ssp. ***agetum*** (Balf.f. & Forrest) Tagg [CHC-YU] syn: *Rhododendron agetum* Balf.f. & Forrest, *R. neriiflorum* Franch. var. *agetum* (Balf.f. & Forrest) T.L.Ming
— ssp. ***neriiflorum*** [BMA-OO, CHT-XI, CHC-YU] syn: *Rhododendron euchaites* Balf.f. & Forrest, *R. neriiflorum* Franch. ssp. *euchaites* (Balf.f. & Forrest) Tagg, *R. neriiflorum* Franch. ssp. *phoenicodum* (Balf.f. & Farrer) Tagg, *R. phoenicodum* Balf.f. & Farrer
— ssp. ***phaedropum*** (Balf.f. & Farrer) Tagg [BHU-BH, ASS-AP, BMA-OO, CHT-XI, CHC-YU] syn: *Rhododendron floccigerum* Franch. ssp. *appropinquans* (Tagg & Forrest) D.F.Chamb., *R. floccigerum* Franch. var. *appropinquans* Tagg & Forrest, *R. neriiflorum* Franch. var. *phaedropum* (Balf.f. & Forrest) T.L.Ming, *R. phaedropum* Balf.f. & Farrer, *R. tawangense* K.C.Sahni & H.B.Naithani
parmulatum Cowan [CHT-XI]
piercei Davidian [CHT-XI] syn: *Rhododendron beanianum* Cowan var. *compactum* Cowan
pocophorum Balf.f. ex Tagg [ASS-AP, CHT-XI, CHC-YU]
— var. ***hemidartum*** (Tagg) D.F.Chamb. [ASS-AP, CHT-XI, CHC-YU] syn: *Rhododendron hemidartum* Balf.f. ex Tagg
— var. ***pocophorum*** [ASS-AP, CHT-XI, CHC-YU]
sanguineum Franch. [CHT-XI, CHC-YU]
— ssp. ***didymum*** (Balf.f. & Forrest) Cowan [CHT-XI, CHC-YU] syn: *Rhododendron didymum* Balf.f. & Forrest, *R. sanguineum* Franch. var. *didymum* (Balf.f. & Forrest) T.L.Ming
— ssp. ***sanguineum*** [CHT-XI, CHC-YU]
— — var. ***cloiophorum*** (Balf.f. & Forrest) D.F.Chamb. [CHT-XI, CHC-YU] syn: *Rhododendron asmenistum* Balf.f. & Forrest, *R. cloiophorum* Balf.f. & Forrest ssp. *asmenistum* (Balf.f. & Forrest) Tagg, *R. cloiophorum* Balf.f. & Forrest, *R. cloiophorum*

(Balf.f. & Forrest) Tagg ssp. *leucopetalum* (Balf.f. & Forrest) Tagg, *R. leucopetalum* Balf.f. & Forrest, *R. sanguineum* Franch. ssp. *cloiophorum* (Balf.f. & Forrest) Cowan, *R. sanguineum* Franch. ssp. *leucopetalum* (Balf.f. & Forrest) Cowan

— — var. ***didymoides*** Tagg & Forrest [CHT-XI, CHC-YU] syn: *Rhododendron cloiophorum* Balf.f. & Forrest ssp. *mannophorum* (Balf.f & Forrest) Tagg, *R. cloiophorum* Balf.f. & Forrest ssp. *roseotinctum* (Balf.f & Forrest) Tagg, *R. mannophorum* Balf.f. & Forrest, *R. roseotinctum* Balf.f. & Forrest, *R. sanguineum* Franch. ssp. *consanguineum* Cowan, *R. sanguineum* Franch. ssp. *didymoides* (Tagg & Forrest) Cowan, *R. sanguineum* Franch. ssp. *roseotinctum* (Tagg & Forrest) Cowan

— — var. ***haemaleum*** (Balf.f. & Forrest) D.F.Chamb. [CHT-XI, CHC-YU] syn: *Rhododendron haemaleum* Balf.f. & Forrest, *R. sanguineum* Franch. ssp. *haemaleum* (Balf.f. & Forrest) Cowan, *R. sanguineum* Franch. ssp. *mesaeum* Balf.f. ex Cowan

— — var. ***himertum*** (Balf.f. & Forrest) D.F.Chamb. [CHT-XI, CHC-YU] syn: *Rhododendron himertum* Balf.f. & Forrest, *R. nebrites* Balf.f. & Forrest, *R. poliopeplum* Balf.f. & Forrest, *R. sanguineum* Franch. ssp. *aizoides* Cowan, *R. sanguineum* Franch. ssp. *himertum* (Balf.f. & Forrest) Cowan, *R. sanguineum* Franch. ssp. *melleum* Cowan

— — var. ***sanguineum*** [CHT-XI, CHC-YU] syn: *Rhododendron sanguineum* Franch. ssp. *sanguineoides* Cowan

sperabile Balf.f. & Farrer [BMA-OO, CHC-YU]

— var. ***sperabile*** [BMA-OO]

— var. ***weihsiense*** Tagg & Forrest [CHC-YU]

sperabiloides Tagg & Forrest [CHT-XI]

Subsection *Pontica* Sleumer (1949)

aureum Georgi [ALT-OO, KRA-OO, CTA-OO, AMU-OO, KHA-OO, PRM-OO, KAM-OO, KUR-OO, SAK-OO, KOR-NK, JAP-OO, CHM-JI]

— var. ***aureum*** [ALT-OO, KRA-OO, CTA-OO, AMU-OO, KHA-OO, PRM-OO, KAM-OO, KUR-OO, SAK-OO, KOR-NK, JAP-OO, CHM-JI] syn: *Rhododendron chrysanthum* Pall., *R. flavum* Pall., *R. officinale* Salisb.

— var. ***hypopytis*** (Pojark.) D.F.Chamb. [AMU-OO] syn: *Rhododendron hypopytis* Pojark.

brachycarpum D.Don ex G.Don [KOR-SK, JAP-OO]

— ssp. ***brachycarpum*** [KOR-SK, JAP-OO] syn: *Rhododendron brachycarpum* G.Don forma *normale* Kitam., *R. brachycarpum* D.Don ex G.Don var. *roseum* Koidz., *R. brachycarpum* G.Don ssp. *tigerstedtii* Nitz., *R. fauriei* Franch. var. *rufescens* Nakai

— ssp. ***fauriei*** (Franch.) D.F.Chamb. [KOR-SK, JAP-OO] syn: *Rhododendron brachycarpum* D.Don ex G.Don var. *roseiflorum* Miyoshi, *R. fauriei* Franch.

— — forma ***nematoanum*** (Makino) Murata [JAP-OO] syn: *Rhododendron brachycarpum* D.Don ex G.Don var. *nematoanum* Makino, *R. brachycarpum* D.Don ex G.Don var. *nematoanum* Makino forma *fauriei* Makino (Franch.) Murata, *R. brachycarpum* D.Don ex G.Don var. *nematoanum* Makino forma *nematoanum* Makino

catawbiense Michx. [NCA-OO, VRG-OO, ALA-OO, GEO-OO, KTY-OO]

caucasicum Pall. [TUR-OO, TCS-AR, TCS-AB, NCS-SO, TCS-GR] syn: *Rhododendron caucaseum* Sims, *R. caucasicum* Pall. var. *stramineum* Hook.

x *charadzeae* A.P.Khokhr. & Mazurenko

degronianum Carrière [JAP-OO]

— ssp. ***degronianum*** [JAP-OO] syn: *Rhododendron degronianum* Carrière var. *nakaii* (Komatsu) Nakai, *R. degronianum* Carrière forma *spomtaneum* Nakai, *R. degronianum* Carrière forma *variegatum* Nakai, *R. hymenanthes* (Blume) Makino var. *pentamerum* Makino, *R. japonicum* (Blume) C.K.Schneid. var. *pentamerum* (Maxim.) Hutch., *R. metternichii* Siebold & Zucc. ssp. *pentamerum* (Maxim.) Sugim., *R. metternichii* Siebold & Zucc. var. *pentamerum* Maxim., *R. nakaii* Komatsu, *R. pentamerum* (Maxim.) Matsum.

— ssp. ***heptamerum*** (Maxim.) H.Hara [JAP-OO]

— — var. ***heptamerum*** [JAP-OO] syn: *Hymenanthes japonica* Blume, *Rhododendron hymenanthes* (Blume) Makino, *R. japonicum* (Blume) C.K.Schneid., *R. japonoheptamerum* Kitam., *R. maximum* Thunb., non L., *R. metternichii* Siebold & Zucc. var. *heptamerum* Maxim., *R. metternichii* Siebold & Zucc. forma *latifolium* Sugim., *R. metternichii* Siebold & Zucc. var. *micranthum* Nakai, *R. metternichii* Siebold & Zucc.

— — var. ***hondoense*** (Nakai) H.Hara [JAP-OO] syn: *Rhododendron degronianum* Carrière ssp. *hondoense* Nakai, *R. japonoheptamerum* Kitam. var. *hondoense* (Nakai) Kitam., *R. metternichii* Siebold & Zucc. var. *hondoense* Nakai

— — var. ***kyomaruense*** (T.Yamaz.) H.Hara [JAP-OO] syn: *Rhododendron metternichii* Siebold & Zucc. var. *kyomaruense* T.Yamaz.

— — — forma ***amagianum*** (T.Yamaz.) H.Hara [JAP-OO] syn: *Rhododendron metternichii* Siebold & Zucc. forma *amagianum* T.Yamaz.

— ssp. ***yakushimanum*** (Nakai) H.Hara [JAP-OO] syn: *Rhododendron degronianum* Carrière ssp. *yakushimanum* (Nakai) Kitam. var. *yakushimanum* (Nakai) Kitam.

— — var. ***intermedium*** (Sugim.) H.Hara [JAP-OO] syn: *Rhododendron metternichii* Siebold & Zucc. var. *intermedium* Sugim.

— — var. ***yakushimanum*** [JAP-OO] syn: *Rhododendron degronianum* Carrière ssp. *yakushimanum* (Nakai) H.Hara, *R. degronianum* Carrière var. *yakushimanum* (Nakai) Kitam., *R. metternichii* Siebold & Zucc. var. *yakushimanum* (Nakai) Ohwi, *R. metternichii* Siebold & Zucc. ssp. *yakushimanum* (Nakai) Sugim., *R. yakushimanum* Nakai

hyperythrum Hayata [TAI-OO] syn: *Rhododendron rubropunctatum* Hayata

intermedium Wender

x *kesselringii* E.Wolf

x *kurokimense* Arakawa

macrophyllum D.Don ex G.Don [WAS-OO, ORE-OO,

temenium Balf.f. & Forrest [CHT-XI, CHC-YU]

— var. ***dealbatum*** (Cowan) D.F.Chamb. [CHT-XI] syn: *Rhododendron eudoxum* Balf.f. & Forrest ssp. *glaphyrum* (Balf.f. & Forrest) Tagg, *R. glaphyrum* Balf.f. & Forrest, *R. temenium* Balf.f. & Forrest ssp. *dealbatum* Cowan, *R. temenium* Balf.f. & Forrest ssp. *glaphyrum* (Balf.f. & Forrest) Cowan

— var. ***gilvum*** (Cowan) D.F.Chamb. [CHT-XI] syn: *Rhododendron temenium* Balf.f. & Forrest ssp. *chrysanthum* Cowan, *R. temenium* Balf.f. & Forrest ssp. *gilvum* Cowan

— var. ***temenium*** [CHC-YU, CHT-XI] syn: *Rhododendron eudoxum* Balf.f. & Forrest ssp. *pothinum* (Balf.f. & Forrest) Tagg, *R. eudoxum* Balf.f. & Forrest ssp. *temenium* (Balf.f. & Forrest) Tagg, *R. pothinum* Balf.f. & Forrest, *R. temenium* Balf.f. & Forrest ssp. *pothinum* (Balf.f. & Forrest) Cowan

trilectorum Cowan [CHT-XI]

x *xanthanthum* (Tagg & Forrest) D.F.Chamb. syn: *Rhododendron chaetomallum* Balf.f. & Forrest var. *xanthanthum* Tagg & Forrest

Subsection *Parishia* Sleumer (1949)

elliottii Watt ex Brandis [ASS-MA]

facetum Balf.f. & Kingdon-Ward [BMA-OO, CHC-YU] syn: *Rhododendron eriogynum* Balf.f. & W.W.Sm.

flavoflorum T.L.Ming [CHC-YU]

huidongense T.L.Ming [CHC-SI]

kyawii Lace & W.W.Sm. [BMA-OO, CHC-YU] syn: *Rhododendron agapetum* Balf.f. & Kingdon-Ward, *R. prophantum* Balf.f. & Forrest

parishii C.B.Clarke [BMA-OO]

schistocalyx Balf.f. & Forrest [CHC-YU]

urophyllum W.P.Fang [CHC-SI]

CAL-OO, BRC-OO] syn: *Rhododendron californicum* Hook.f., *R. maximum* Hook., *R. washingtonianum* hort.

makinoi Tagg [JAP-OO] syn: *Azalea makinoi* (Tagg) Makino, *A. makinoi* (Tagg) Makino var. *muranoana* Makino, *Rhododendron metternichii* Siebold & Zucc. forma *angustifolium* Makino, *R. stenophyllum* Makino, *R. yakushimanum* Nakai ssp. *makinoi* (Tagg) D.F.Chamb.

maximum L. [NSC-OO, MAI-OO, NWH-OO, VER-OO, NWY-OO, MAS-OO, PEN-OO, DEL-OO, VRG-OO, WVA-OO, NCA-OO, TEN-OO, SCA-OO, GEO-OO, NBR-OO] syn: *Rhododendron ashleyii* Coker, *R. fragrans* hort., *R. latifolium* Hoffmanns., *R. maximum* L. var. *album* Pursh, *R. maximum* L. var. *purpureum* Pursh, *R. procerum* Salisb., *R. purpureum* (Pursh) G.Don, *R. purpureum* (Pursh) G.Don var. *tigrinum* Steudel, *R. purshii* G.Don

x *nikomontanum* (Komatsu) Nakai syn: *Rhododendron brachycarpum* G.Don var. *lutescens* Koidz., *R. chrysanthum* Pall. var. *nikomontanum* Komatsu

ponticum L. [SPA-SP, POR-OO, BUL-OO, TUR-OO, TCS-GR, TCS-AR, TCS-AB, LBS-LB] syn: *Rhododendron adansonii* Pépin, *R. algarvense* Page, *R. azaleoides* Desf., *R. baeticum* Boiss. & Reut., *R. hyacinthiflorum* hort., *R. lancifolium* Moench, *R. lowei* hort., *R. obtusum* hort., *R. odoratum* Lodd. ex Steudel, *R. parviflorum* Dum.Cours., *R. ponticum* L. ssp. *baeticum* (Boiss. & Reuter) Hand.-Mazz., *R. ponticum* L. var. *brachycarpum* Boiss., *R. speciosum* Salisb.

smirnowii Trautv. [TUR-OO, TCS-GR]

x *sochadzeae* Char & Davidian. syn: *Rhododendron ponticum* L. x *Rhododendron caucasicum* Pall.

ungernii Trautv. [TUR-OO, TCS-GR]

Subsection *Selensia* Hayata (1913)

bainbridgeanum Tagg & Forrest [BMA-OO, CHT-XI, CHC-YU]

calvescens Balf.f. & Forrest [CHT-XI, CHC-YU]

— var. ***calvescens*** [CHT-XI, CHC-YU]

— var. ***duseimatum*** (Balf.f. & Forrest) D.F.Chamb. [CHT-XI, CHC-YU] syn: *Rhododendron duseimatum* Balf.f. & Forrest, *R. selense* Franch. var. *duseimatum* (Balf.f. & Forrest) Cowan & Davidian, *R. selense* Franch. ssp. *duseimatum* (Balf.f. & Forrest) Tagg

dasycladoides Hand.-Mazz. [CHC-YU, CHC-SI]

esetulosum Balf.f. & Forrest [CHT-XI, CHC-YU] syn: *Rhododendron manopeplum* Balf.f. & Forrest

hirtipes Tagg [CHT-XI]

martinianum Balf.f. & Forrest [BMA-OO, CHT-XI, CHC-YU]

selense Franch. [CHT-XI, CHC-YU, CHC-SI]

— ssp. ***dasycladum*** (Balf.f. & W.W.Sm.) D.F.Chamb. [CHC-YU, CHC-SI] syn: *Rhododendron dasycladum* Balf.f. & W.W.Sm., *R. dolerum* Balf.f. & Forrest, *R. rhaibocarpum* Balf.f. & W.W.Sm., *R. selense* Franch. var. *dasycladum* (Balf.f. & Forrest) T.L.Ming, *R. selense* Franch. ssp. *dolerum* (Balf.f. & Forrest) Tagg

— ssp. ***jucundum*** (Balf.f. & W.W.Sm.) D.F.Chamb. [CHC-YU] syn: *Rhododendron blandulum* Balf.f. & W.W.Sm., *R. jucundum* Balf.f. & W.W.Sm., *R. selense* Franch. var. *jucundum* (Balf.f. & W.W.Sm.) T.L.Ming

— ssp. ***selense*** [CHT-XI, CHC-YU] syn: *Rhododendron axium* Balf.f. & Forrest, *R. chalarocladum* Balf.f. & Forrest, *R. metrium* Balf.f. & Forrest, *R. nanothamnum* Balf.f. & Forrest, *R. pagophilum* Balf.f. & Kingdon-Ward, *R. probum* Balf.f. & Forrest, *R. selense* Franch. ssp. *axium* (Balf.f. & Forrest) Tagg, *R. selense* Franch. ssp. *chalarocladum* (Balf.f. & Forrest) Tagg, *R. selense* Franch. ssp. *metrium* (Balf.f. & Forrest) Tagg, *R. selense* Franch. ssp. *nanothamnum* (Balf.f. & Forrest) Tagg, *R. selense* Franch. var. *pagophilum* (Balf.f. & Forrest) Cowan & Davidian, *R. selense* Franch. ssp. *pagophilum* (Balf.f. & Kingdon-Ward) Tagg, *R. selense* Franch. var. *probum* (Balf.f. & Forrest) Cowan & Davidian, *R. selense* Franch. ssp. *probum* (Balf.f. & Forrest) Tagg

— ssp. ***setiferum*** (Balf.f. & Forrest) D.F.Chamb. [CHT-XI, CHC-YU] syn: *Rhododendron setiferum* Balf.f. & Forrest, *R. vestitum* Tagg & Forrest

xizangense (W.P.Fang & W.K.Hu) Q.Z.Yu [CHT-XI]

Subsection *Taliensia* Sleumer (1949)

adenogynum Diels [CHT-XI, CHC-YU, CHC-SI] syn: *Rhododendron adenophorum* Balf.f. & W.W.Sm.

aganniphum Balf.f. & Kingdon-Ward [CHT-XI, CHC-YU]

— var. ***aganniphum*** [CHT-XI, CHC-YU] syn: *Rhododendron aganniphum* Balf.f. & Kingdon-Ward var. *glaucopeplum* (Balf.f. & Forrest) T.L.Ming, *R. aganniphum* Balf.f. & Kingdon-Ward var. *schizopeplum* (Balf.f. & Forrest) T.L.Ming, *R. doshongense* Tagg, *R. fissotectum* Balf.f. & Forrest, *R. glaucopeplum* Balf.f. & Forrest, *R. schizopeplum* Balf.f. & Forrest

— var. ***flavorufum*** (Balf.f. & Forrest) D.F.Chamb. [CHT-XI, CHC-YU] syn: *Rhododendron flavorufum* Balf.f. & Forrest

alutaceum Balf.f. & W.W.Sm. [CHT-XI, CHC-YU, CHC-SI]

— var. ***alutaceum*** [CHT-XI, CHC-YU, CHC-SI] syn: *Rhododendron globigerum* Balf.f. & Forrest, *R. roxieanum* Forrest var. *globigerum* (Balf.f. & Forrest) D.F.Chamb.

— var. ***iodes*** (Balf.f. & Forrest) D.F.Chamb. [CHC-YU] syn: *Rhododendron iodes* Balf.f. & Forrest

— var. ***russotinctum*** (Balf.f. & Forrest) D.F.Chamb. [CHC-YU] syn: *Rhododendron russotinctum* Balf.f. & Forrest, *R. triplonaevium* Balf.f. & Forrest, *R. tritifolium* Balf.f. & Forrest

balfourianum Diels [CHC-YU, CHC-SI]

— var. ***aganniphoides*** Tagg & Forrest [CHC-YU, CHC-SI]

— var. ***balfourianum*** [CHC-YU, CHC-SI]

barkamense D.F.Chamb. [CHC-SI]

bathyphyllum Balf.f. & Forrest [CHT-XI, CHC-YU]

beesianum Diels [BMA-OO, CHT-XI, CHC-YU, CHC-SI] syn: *Rhododendron colletum* Balf.f. & Forrest, *R. emaculatum* Balf.f. & Forrest

bhutanense D.G.Long & Bowes Lyon [BHU-BH]

bureavii Franch. [CHC-YU] syn: *Rhododendron cruentum* H.Lév.

bureavioides Balf.f. [CHC-SI]

clementinae Forrest [CHC-YU, CHN-SA]

— ssp. ***aureodorsale*** W.P.Fang ex J.Q.Fu [CHN-SA]

— ssp. ***clementinae*** [CHC-YU, CHC-SI]

codonanthum Balf.f. & Forrest [CHC-YU]

coeloneuron Diels [CHC-SI, CHC-GU]

comisteum Balf.f. & Forrest [CHT-XI, CHC-YU]

danbaense L.C.Hu [CHC-SI]

detersile Franch. [CHC-SI]

dignabile Cowan [CHT-XI]

dumicola Tagg & Forrest [CHC-YU]

elegantulum Tagg & Forrest [CHC-YU, CHC-SI]

faberi Hemsl. [CHC-SI] syn: *Rhododendron faberi* Hemsl. ssp. *faberi*, *R. faberioides* Balf.f., *R. wuense* Balf.f.

lacteum Franch. [CHC-YU] syn: *Rhododendron mairei* H.Lév.

mimetes Tagg & Forrest [CHC-SI]

— var. ***mimetes*** [CHC-SI]

— var. ***simulans*** Tagg & Forrest [CHC-SI] syn: *Rhododendron simulans* (Tagg & Forrest) D.F.Chamb.

montiganum T.L.Ming [CHC-YU]

nakotiltum Balf.f. & Forrest [CHC-YU]

nhatrangense Dop [VIE-OO]

nigroglandulosum Nitz. [CHC-SI]

phaeochrysum Balf.f. & W.W.Sm. [CHT-XI, CHC-YU, CHC-SI]

— var. ***agglutinatum*** (Balf.f. & Forrest) D.F.Chamb. [CHT-XI, CHC-YU, CHC-SI] syn: *Rhododendron agglutinatum* Balf.f. & Forrest, *R. dumosulum* Balf.f. & Forrest, *R. lophophorum* Balf.f. & Forrest, *R. syncollum* Balf.f. & Forrest

— var. ***levistratum*** (Balf.f. & Forrest) D.F.Chamb. [CHT-XI, CHC-YU, CHC-SI] syn: *Rhododendron*

aiolopeplum Balf.f. & Forrest, *R. dichropeplum* Balf.f. & Forrest, *R. helvolum* Balf.f. & Forrest, *R. intortum* Balf.f. & Forrest, *R. levistratum* Balf.f. & Forrest, *R. sigillatum* Balf.f. & Forrest, *R. theiophyllum* Balf.f. & Forrest, *R. vicinum* Balf.f. & Forrest

— var. ***phaeochrysum*** [CHT-XI, CHC-YU, CHC-SI] syn: *Rhododendron cupressens* Nitz., *R. dryophyllum* Balf.f. & Forrest

pomense Cowan & Davidian [CHT-XI]

prattii Franch. [CHC-SI] syn: *Rhododendron faberi* Hemsl. ssp. *prattii* (Franch.) D.F.Chamb., *R. leei* W.P.Fang

principis Bureau & Franch. [CHT-XI] syn: *Rhododendron principis* Bureau & Franch. var. *vellereum* (Hutch. ex Tagg) T.L.Ming, *R. vellereum* Hutch. ex Tagg

pronum Tagg & Forrest [CHC-YU]

proteoides Balf.f. & W.W.Sm. [CHT-XI, CHC-YU, CHC-SI] syn: *Rhododendron lampropeplum* Balf.f. & Forrest

przewalskii Maxim. [CHT-QI, CHN-GA, CHC-SI] syn: *Rhododendron dabanshanense* W.P.Fang & S.X.Wang, *R. kialense* Franch.

pubicostatum T.L.Ming [CHC-YU]

punctifolium L.C.Hu [CHC-YU]

roxieanum Forrest [CHT-XI, CHC-YU, CHC-SI]

— var. ***cucullatum*** (Hand.-Mazz.) D.F.Chamb. [CHT-XI, CHC-YU, CHC-SI] syn: *Rhododendron coccinopeplum* Balf.f. & Forrest, *R. cucullatum* Hand.-Mazz., *R. porphyroblastum* Balf.f. & Forrest

— var. ***oreonastes*** (Balf.f.) T.L.Ming [CHT-XI, CHC-YU, CHC-SI] syn: *Rhododendron recurvum* Balf.f. & Forrest var. *oreonastes* Balf.f. & Forrest

— var. ***parvum*** Davidian [CHC-YU]

— var. ***roxieanum*** [CHC-YU, CHC-SI] syn: *Rhododendron aishropeplum* Balf.f. & Forrest, *R. poecilodermum* Balf.f. & Forrest, *R. recurvum* Balf.f. & Forrest

roxieoides D.F.Chamb. [CHC-SI]

rufum Batalin [CHC-SI, CHN-GA] syn: *Rhododendron weldianum* Rehder & E.H.Wilson

shanii W.P.Fang [CHS-AN]

sphaeroblastum Balf.f. & Forrest [CHC-YU, CHC-SI]

— var. ***sphaeroblastum*** [CHC-YU, CHC-SI]

— var. ***wumengense*** K.M.Feng [CHC-YU, CHC-SI]

taliense Franch. [CHC-YU]

traillianum Forrest & W.W.Sm. [CHT-XI, CHC-YU, CHC-SI]

— var. ***dictyotum*** (Balf.f. ex Tagg) D.F.Chamb. [CHT-XI, CHC-YU] syn: *Rhododendron dictyotum* Balf.f. ex Tagg

— var. ***traillianum*** [CHC-YU, CHC-SI] syn: *Rhododendron aberrans* Tagg & Forrest

wasonii Hemsl. & E.H.Wilson [CHC-SI] syn: *Rhododendron rhododactylum* Millais

— var. ***wasonii*** [CHC-SI]

— var. ***wenchuanense*** L.C.Hu [CHC-SI]

wightii Hook.f. [NEP-OO, BHU-BH, BHU-SI, ASS-AP, CHT-XI]

wiltonii Hemsl. & E.H.Wilson [CHC-SI, CHC-GU]

zhongdianense L.C.Hu [CHC-YU]

Subsection *Thomsonia* Sleumer (1949)

bonvalotii Bureau & Franch. [CHC-SI]

cerasinum Tagg [BMA-OO, CHT-XI]

cyanocarpum (Franch.) W.W.Sm. [CHC-YU] syn: *Rhododendron cyanocarpum* (Franch.) W.W.Sm. var. *eriphyllum* Balf.f. & W.W.Sm. ex Tagg, *R. hedythamnum* Balf.f. & Forrest var. *eglandulosum* Hand.-Mazz., *R. thomsonii* Hook.f. var. *cyanocarpum* Franch.

eclecteum Balf.f. & Forrest [BMA-OO, CHT-XI, CHC-YU, CHC-SI]

— var. ***bellatulum*** Balf.f. ex Tagg [CHC-YU, CHT-XI]

— var. ***eclecteum*** [BMA-OO, CHT-XI, CHC-YU, CHC-SI] syn: *Rhododendron brachyandrum* Balf.f. & Forrest, *R. eclecteum* Balf.f. & Forrest var. *brachyandrum* (Balf.f. & Forrest) Cowan & Davidian

eurysiphom Tagg & Forrest [CHT-XI]

faucium D.F.Chamb. [CHT-XI]

hookeri Nutt. [ASS-AP]

hylaeum Balf.f. & Farrer [BMA-OO, CHT-XI]

meddianum Forrest [BMA-OO, CHC-YU]

— var. ***atrokermesinum*** Tagg [BMA-OO]

— var. ***meddianum*** [BMA-OO, CHC-YU]

megalanthum M.Y.Fang [CHT-XI]

populare Cowan [CHT-XI]

ramipilosum T.L.Ming [CHT-XI]

sherriffii Cowan [CHT-XI]

x *sikkimense* Pradhan & Lachumgpa [BHU-SI]

stewartianum Diels [BMA-OO, CHT-XI, CHC-YU] syn: *Rhododendron aiolosalpinx* Balf.f. & Farrer, *R. nipholobum* Balf.f. & Farrer, *R. stewartianum* Diels var. *aiolosalpinx* (Balf.f. & Farrer) Cowan & Davidian, *R. stewartianum* Diels var. *tantulum* Cowan & Davidian

subansiriense D.F.Chamb. [ASS-AP]

thomsonii Hook.f. [NEP-OO, BHU-SI, BHU-BH, ASS-AP, CHT-XI]

— ssp. ***lopsangianum*** (Cowan) D.F.Chamb. [CHT-XI] syn: *Rhododendron lopsangianum* Cowan, *R. thomsonii* Hook.f. var. *lopsangianum* (Cowan) T.L.Ming

— ssp. ***thomsonii*** [NEP-OO, BHU-SI, BHU-BH, ASS-AP]

viscidifolium Davidian [CHT-XI]

Subsection *Venatora* D.F.Chamb. (1979)

venator Tagg [CHT-XI]

Subsection *Williamsiana* Balf.f. (1916)

leishanicum W.P.Fang & S.S.Chang [CHC-GU]

williamsianum Rehder & E.H.Wilson [CHC-SI, CHC-GU]

Subsection Unplaced

x *agastum* Balf.f. & W.W.Sm. [CHC-YU, CHC-GU]

x *batemannii* Hook.f. syn: *Rhododendron wallichii* Hook.f. x *arboreum* Sm.

blumei Nutt. [ASS-AP]

x *candelabrum* Hook.f. syn: *Rhododendron thomsonii* Hook.f. var. *candelabrum* (Hook.f.) C.B.Clarke, *R. thomsonii* Hook.f. ssp. *candelabrum* (Hook.f.) D.F.Chamb., *R. thomsonii* Hook.f. var. *pallidum* Cowan

x *chlorops* Cowan

x *detonsum* Balf.f. & Forrest syn: *Rhododendron ochrocalyx* hort.

dimidiatum Balf.f.

dimitrium Balf.f. & Forrest [CHC-YU]

x *erythrocalyx* Balf.f. & Forrest syn: *Rhododendron beimaense* Balf.f. & Forrest, *R. cymbomorphum* Balf.f. & Forrest, *R.* x *erythrocalyx* Balf.f. & Forrest ssp. *beimaense* (Balf.f. & Forrest) Tagg, *R.* x *erythrocalyx* Balf.f. & Forrest ssp. *docimum* Balf.f. ex Tagg, *R.* x *erythrocalyx* Balf.f. & Forrest ssp. *eucallum* (Balf.f. & Forrest) Tagg, *R.* x *erythrocalyx* Balf.f. & Forrest ssp. *truncatulum* (Balf.f. & Forrest) Tagg, *R. eucallum* Balf.f. & Forrest, *R. panteumorphum* Balf.f. & W.W.Sm., *R. truncatulatum* Balf.f. & Forrest, *R. truncatulum* Balf.f. & Forrest

x *hemigymnum* (Tagg & Forrest) D.F.Chamb. syn: *Rhododendron chaetomallum* Balf.f. & Forrest var. *hemigymnum* Tagg & Forrest

x *holmbaense* Rehder

x *inopinum* Balf.f.

kansuense Millais [CHN-GA]

x *magorianum* Millais

maximowiczianum H.Lév.

x *paradoxum* Balf.f.

peregrinum Tagg [CHC-SI]

x *peregrinum* Tagg

planetum Balf.f.

potaninii Batalin [CHN-GA]

purdomii Rehder & E.H.Wilson [CHN-SA]

x *pyrrhoanthum* Balf.f.

x *salmoneum* Vilmorin

x *smithii* Sweet

venosum Nutt.

wallaceanum Millais

x *welsianum* hort.