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A CHEMOSYSTEMATIC AND CLADISTIC STUDY OF THE SOUTHERN AFRICAN ENDEMIC FAMILY BRUNIACEAE DC

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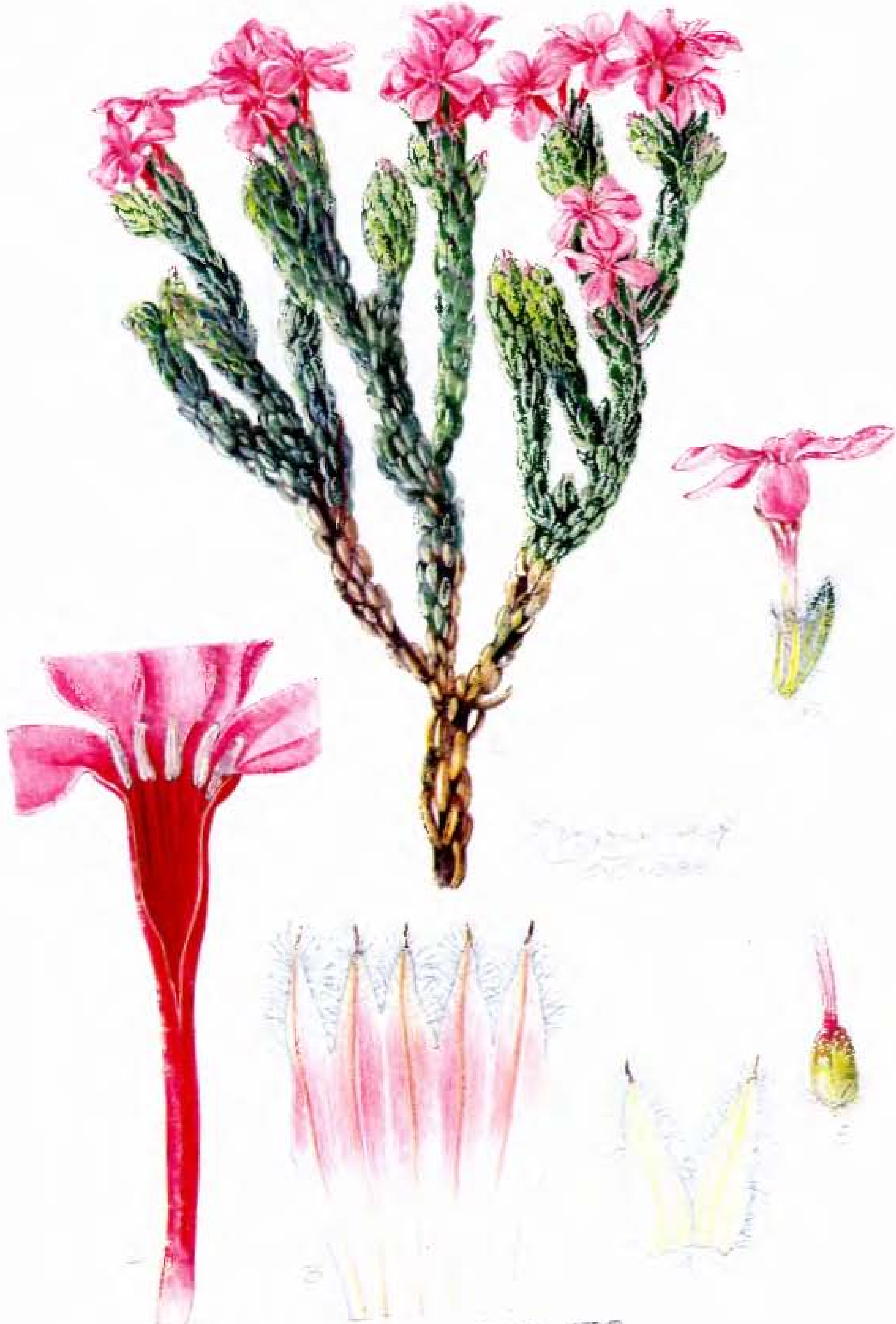

LONCHOSTOMA ESTERHUYSENIAE STRID

COLL. G. SCOTT 23. DEC. 1988. RIVERSONDEREND MTS.

1. LIFE SIZE 2, 3, 4, 5 x 8 6 x 2

"There is a need to begin with biosystematic, evolutionary and population studies in southern Africa. Little is known about the pattern of evolution and speciation in woody, herbaceous or annual taxa. There are many questions regarding the origin of endemic and characteristic southern African plants that should be investigated in detail. Only with a good understanding of the history of the flora can its present status be properly appreciated."

Goldblatt, 1978

This work is respectfully dedicated to Elsie E. Esterhuysen, whose contribution to botany has been an inspiration.

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ABSTRACT

A CHEMOSYSTEMATIC AND CLADISTIC STUDY OF THE SOUTHERN AFRICAN ENDEMIC FAMILY BRUNIACEAE DC

Bruniaceae, one of the southern African endemic plant families, comprises 76 species in 12 genera. The most recent revision of the family is that of Pillans (1947). No phylogenetic analysis of within-family relationships has been carried out to date and the results of recent cladistic studies have been equivocal as regards the affinities of the family. The present study was undertaken in order to identify sister group(s) of Bruniaceae and using this information, to explore the phylogeny of the family.

Analyses were based on non-molecular data obtained from the published literature, mainly from morphology, anatomy and palynology. An additional data set was generated from analysis, in the present study, of foliar flavonoid profiles of 58 species representing all genera in Bruniaceae. Eight species representing Grubbiaceae, Diapensiaceae, Ericaceae, Retziaceae and Geissolomaceae, families with which Bruniaceae has been allied in recent classifications, were included in the phytochemical survey.

Investigation of flavonoid patterns in leaf extract hydrolysates of species in Bruniaceae revealed the presence of the flavonol aglycones myricetin in 57,3%, kaempferol in 54%, isorhamnetin in 70%, fisetin in 13.8% and quercetin in 100% of species examined. Quercetin 3-O-methyl ether was recorded from 12.1% of species. The flavone aglycones luteolin and chrysoeriol were detected in 17.24% and 13.8% respectively of species examined. None of the common flavonols were recorded from Geissolomataceae but the rarer flavonol gossypetin was detected in *Erica arborea* and the two species of Diapensiaceae investigated in this study. Luteolin was detected in all three species of Grubbiaceae while the common flavones apigenin and diosmetin were not recorded from any species examined.

In Bruniaceae the proanthocyanidin prodelphinidin was recorded from 63.8% and procyanidin from 96.6% of species. In Grubbiaceae and Diapensiaceae only procyanidin was recorded as present, while neither Retziaceae nor Geissolomaceae appeared to synthesise condensed tannins. The phenolic acids ellagic and gallic acid were not detected in any species in Bruniaceae, in *Erica arborea* or *Retzia capensis,* but were recorded from all species in Grubbiaceae and Diapensiaceae examined in this study. Ellagic but not gallic acid was detected in *Geissoloma marginatum.*

A preliminary cladistic analysis, aimed at identifying sister group(s) to Bruniaceae, utilised 24 taxa and 22 characters, of which 4 were generated by phytochemical analysis. The results suggested that the families Ericaceae and Epacridaceae were appropriate out-groups for inclusion in a second analysis, aimed at exploring within-family relationships. For this purpose, a total of 52 species of Bruniaceae, 6 genera in Epacridaceae and 7 in Ericaceae were scored for 32 characters; 5 of these were chemical characters generated by the present study.

The results of the second analysis showed that 7 out of 12 currently recognised genera in Bruniaceae comprise monophyletic lineages. *Lonchostoma* was shown to be closest to the ancestors of the family. The most significant result was the identification of *Dracophyllum* (Epacridaceae) as sister to Bruniaceae. This finding is supported by the microfossil record, which indicates the presence of Epacridaceae at three sites in southern Africa at the Cretaceous/Tertiary boundary. Bruniaceae may be regarded as an ancient Gondwanan relict family, the closest relatives of which are to be found in Australia.

10 January 2000

CHAPTER 1

INTRODUCTION

Bruniaceae is one of the families endemic to southern Africa. The family was revised by Pillans (1947), whose nomenclature and taxonomy are followed here. At present, 76 species in 12 genera (Table 1) are recognised.

Table 1. SPECIES IN BRUNIACEAE

Audouinia
A. capitata (L.) Brongn.

Berzelia
B. abrotanoides (L.) Brongn., *B. burchellii* Dümmer, *B. commutata* Sonder,, *B. cordifolia* Schldl., *B. dregeana* Colozza, *B. ecklonii* Pill., *B. galpinii* Pill., *B intermedia* Schldl., *B. lanuginosa* (L.) Brongn., *B. rubra* (Willd.) Schldl.

Brunia
B. albiflora E. Phillips, *B. alopecuroides* Thunb., *B. macrocephala* Willd., *B. laevis* Thunb., *B. neglecta* Schldl., *B. nodiflora* L., *B. stokoei* E. Phillips.

Linconia
L. alopecuroides L., *L. cuspidata* (Thunb.) Swartz, *L. ericoides* E.G.H.Oliv.

Lonchostoma
L. esterhuyseniae Strid, *L. monogynum* (Vahl) Pill., *L. myrtoides* (Vahl) Pill., *L. pentandrum* (Thunb.) Druce, *L. purpureum* Pill.

Mniothamnea
M. bullata Schltr., *M. callunoides* (Oliver) Niedenzu

Nebelia
N. fragarioides (Willd.) Kuntze, *N. laevis* (E. Meyer) Kuntze, *N. paleacea* (Bergius) Sweet, *N. sphaerocephala* (Sonder) Kuntze, *N. stokoei* Pill., *N. tulbaghensis* Schltr. ex Dümmer

Pseudobaeckia
P. africana (Burm. f.) Pill., *P. cordata* (Burm. f.) Niedenzu, *P. stokoei* Pill., *P. teres* (Oliver) Dümmer

Raspalia
R. angulata (Sonder) Niedenzu, *R. bernardii* Pill., *R. dregeana* (Sonder) Niedenzu, *R. globosa* (Lam.) Pill., *R. microphylla* (Thunb.) Brongn., *R. oblongifolia* Pill., *R. palustris* (Schltr. ex Kirchner) Pill., *R. phylicoides* (Thunb.) Arnott, *R. sacculata* (Bolus ex Kirchner) Pill., *R. schlechteri* Dümmer, *R. staavioides* (Sonder) Pill., *R. stokoei* Pill., *R. variabilis* Pill., *R. villosa* Presl,. *R. virgata* (Brongn.) Pill.

Staavia
S. brownii Dümmer, *S. capillella* (Thunb.) Sonder, *S. dodii* Bolus, *S. dregeana* Presl., *S. glutinosa* (L.) Dahl, *S. phylicoides* Pill., *S. radiata* (L.) Dahl, *S. verticillata* (L. f.) Pill,. *S. zeyheri* Sonder

Thamnea
T. depressa Oliver, *T. diosmoides* Oliver, *T. gracilis* Oliver, *T. hirtella* Oliver, *T. massoniana* Dümmer, *T. thesioides* Dümmer, *T. uniflora* Solander ex Brongn.

Tittmannia
T. esterhuyseniae E. Powrie, *T. hispida* Pill., *T. laevis* Pill., *T. laxa* (Thunb.) Presl.

1. DESCRIPTION

Species in Bruniaceae comprise woody shrubs varying in stature from prostrate (e.g. *Thamnea massoniana)* to almost arborescent (e.g. *Brunia albiflora)* species. The family is morphologically, anatomically and palynologically extremely diverse (Pillans, 1947; Carlquist, 1978 and 1991; Hall, 1988).

1. 1. LEAVES

The foliage may be soft or highly sclerophyllous, glabrous, pilose or densely hirsute. Individual leaves may be minute and scale-like, linear, lanceolate or ovate, isolateral or bifacial or intermediate between these conditions, but not ericoid. They may be closely adpressed to the stem or laxly borne, and all contain either rhomboids or druses of calcium oxalate. Juvenile leaf forms occur in two species, *Staavia dodii* and *Berzelia cordifolia,* and dimorphic leaves are seen in adult plants of *Pseudobaeckia cordata.*

The leaves are alternate and entire and bear a characteristic black tip or apiculum that persists throughout the life of the leaf except in *Audouinia capitata*, in which species it is lost early in leaf development. The origin, composition and function of this tissue is uncertain; Kirchner (in Dümmer, 1912) considered it to be composed of cork-like cells produced by meristematic tissue and to possess a secretory or protective function. Carlquist (1991) however, noted that the bulk of this tissue was not produced by the phellogen and that it might function as a repository for large amounts of terpenoids.

Carlquist (1991), in a study of the leaf anatomy of the family, found the leaves of Bruniaceae to be "extraordinarily xeromorphic by comparison with those of the angiosperms at large", a feature which he considered to reflect the windy,

summer-dry conditions prevailing in much of the distribution range of the family. However, he found leaf mesomorphy values to be correlated with habitat, species with higher values characteristically occupying moist habitats and vice versa.

1.2. FLOWERS

Flowers may be axillary or terminal, borne singly on short shoots (e.g. *Audouinia, Tittmannia and Thamnea*;), in terminal spikes or panicles (e.g. *Pseudobaeckia, Raspalia*) or in globose (*Brunia, Berzelia, Nebelia*) or capitate (*Staavia*) heads. The flowers are actinomorphic, pentamerous and generally very small (petal length 1-2 mm), exceptions being those of *Audouinia, Lonchostoma* and individual *Thamnea, Linconia* and *Tittmannia* species. Corolla colour is usually white or cream but may be red (*Audouinia capitata, Brunia stokoei*), pink (*Lonchostoma esterhuyseniae, Linconia alopecuroidea*), or purple (*Lonchostoma purpureum, Staavia dodii*). Individual flowers, whether borne singly or in an inflorescence, are subtended by one or several bracts. Up to twelve such bracts are present in *Audouinia, Thamnea* and *Tittmannia*, while those of *Nebelia* are conspicuously lengthened and protrude beyond the exserted stamens. In *Staavia*, the bracts are enlarged and ray-like, forming an involucre surrounding the inflorescence. This arrangement, similar to that of a composite flower, prompted Dümmer (1912) to compare *S. dodii* to a "shrubby chrysanthemum".

The petals may be free, connate at the base or, in the case of *Lonchostoma* species, united to form a short tube. Clawed petals characterise *Audouinia, Thamnea* and some species of *Linconia, Lonchostoma* and *Tittmannia*; in the remaining genera the petals are lanceolate, linear or oblong. Aestivation is imbricate and insertion usually epi- or perigynous, occasionally hypogynous (*Lonchostoma*). The calyx, often adnate to the ovary, may have the lobes free or

connate at the base; the latter are frequently clothed with trichomes, particularly on the abaxial surface. The ovary is most often half to completely inferior, rarely superior, and may be unilocular (*Berzelia*, *Mniothamnea*), trilocular (*Audouinia*) or bilocular (the remaining genera). De Lange (1991) recorded the occasional occurrence of up to six loculi in ovaries of *Audouinia*. Loculi generally house one or two pendulous ovules, but may have up to 12 per loculus in *Lonchostoma* (Rebelo, 1980). *Thamnea* differs from the other genera in having up to 10 ovules suspended from a free central column.

Styles are equal in number to the locules in the ovary and may be free or united along part or all of their length. Stamens alternate with the petals, to which they may adhere laterally, and are prominently exserted in the genera *Brunia*, *Berzelia* and *Nebelia*. In *Lonchostoma* the stamens are epipetalous, inserted in the throat of the corolla or adnate to the base of the petals. The remaining genera have included stamens, an exception being *Raspalia dregeana*. Anthers are introrse, dorsifixed and usually longtitudinally dehiscent, except in *Linconia*, where only the lower half of the thecae is sporogenous (Dahlgren & van Wyk, 1988). Sagittate to ovate anthers characterise most genera; in *Audouinia*, *Thamnea* and *Tittmannia* they are linear-oblong.

1.3. FRUITS

Pillans (1947) noted that the fruits of Bruniaceae were imperfectly known in most genera and unknown in two; however, he recorded the presence of dehiscent fruits in *Brunia* and *Nebelia*. According to Niedenzu (1891) and Dyer (1975) dry, indehiscent nutlets characterise *Audouinia*, *Tittmannia*, *Thamnea*, *Pseudobaeckia*, *Mniothamnea* and *Berzelia*, while the remaining genera have either loculicidal capsules or a schizocarp with two cocci. De Lange (1992)

1. Berzelia abrotanoides

2. Brunia nodiflora

3. Nebelia sphaerocephala

4. Thamnea diosmoides

5. Audouinia capitata

6. Linconia alopecuroides

7. Lonchostoma esterhuyseniae

8. Staavia glutinosa

9. Raspalia globosa

10. Tittmannia esterhuyseniae

Representative species of 10 (out of 12) genera in Bruniaceae, showing within-family diversity in inflorescence structure

Nos. 1, 2, 5 and 9 are from the slide collection of the National Botanical Institute at Kirstenbosch,
No 6 was kindly loaned by Dr C. Paterson-Jones and the remainder are from the author's own collection

confirmed the presence of indehiscent fruits in *Audouinia*. An arillus appears to be present in some genera, e.g. *Linconia* and *Staavia*,

1.4. SUBTERRANEAN ORGANS

Lignotubers are known to be present in at least 21 species, representing nine out of the 12 genera of the family (Carlquist, 1978; Pillans, 1947; Rebelo, 1980; Broll, 1980). All *Audouinia*, *Linconia*, *Tittmannia* and *Lonchostoma* species appear to possess these organs; *Raspalia*, *Pseudobaeckia* and *Mniothamnea* species apparently lack them altogether. Lignotubers enable the species that possess them to survive the fires which are considered to be a feature of fynbos ecology (Deacon *et al.*, 1992), but may also function as storage organs for water or nutrients (James, 1984; Carlquist, 1978). The latter author noted that species without lignotubers tended to occur in moist sites and concluded that lignotubers might be adaptive to the hot, dry summers, high wind incidence and porous sandstone soils that characterise much of the CFR. In the same study, an investigation of the wood anatomy of Bruniaceae, Carlquist commmented that this feature of the family was exemplary for adaptation to xeromorphy. Allsopp (1993) noted the general occurrence of vesicular-arbuscular mycorrhizas (VAM) in Bruniaceae, which de Lange (1992) confirmed for *Audouinia*.

Carlquist (1978) drew attention to the great diversity in wood anatomy seen in Bruniaceae as well as to its extraordinarily primitive character, phenomena which in his opinion suggested a long evolutionary history, permitting "exploitation of a wide variety of growth forms and distinctive habitats". Hall (1987) attributed the diversity seen in Bruniaceae and the limited geographical range of many of its species to "long-term isolation by climatic and oceanic barriers in a cul de sac at the southern tip of Africa". The morphological diversity of Bruniaceae prompted

Dümmer (1912) to comment that "the polymorphism and the varied cases of mimicry which prevail in the order form one of its most striking and interesting features". The superficial though striking resemblance of some genera in Bruniaceae to other plant groups was a source of confusion to early systematists and has contributed to the taxonomic shuffling of the family between various plant orders. Linnaeus (1767) and Thunberg (1803) placed *Audouinia capitata* and *Linconia cuspidata* in *Diosma* (Rutaceae), while *Staavia radiata* and *S. trichotoma* were included in *Phylica* (Rhamnaceae) by Linnaeus (1755) and Thunberg (1804). *Lonchostoma pentandrum* was assigned to *Passerina* (Thymelaeaceae) and then to *Gnidia* (Thymelaeaceae) by Thunberg (1794, 1801) and to *Stilbe* (Stilbaceae) by Lamarck (1817). *Raspalia globosa* was placed in *Diosma* by Meyer (1844) and in *Passerina* by Lamarck (1797). To this day *Audouinia capitata* is known by the folk name of "false heath" on account of its superficial resemblance to some *Erica* species (Ericaceae), while Hooker (1865) referred to the same species as "an epacridaceous subshrub".

2. OCCURRENCE AND DISTRIBUTION

All but one of the species in the family are confined to the Cape Floristic Region (CFR) as defined by Kruger (1978), Taylor (1979) and Bond & Goldblatt (1984), with a single outlier in southern Kwazulu-Natal (Fig.1). As can be seen from Fig. 2 (from Oliver *et al.*, 1983) the greatest concentration of species is found in the Caledon grid square (Edwards and Leistner, 1971) 3418 BB, with a gradual decline in species numbers to the north and east of the CFR. The occurrence of single outlier (*Raspalia trigyna*) on the southern coast of Kwazulu-Natal some 450 km distant from its closest relative, is noteworthy and has been explained in terms

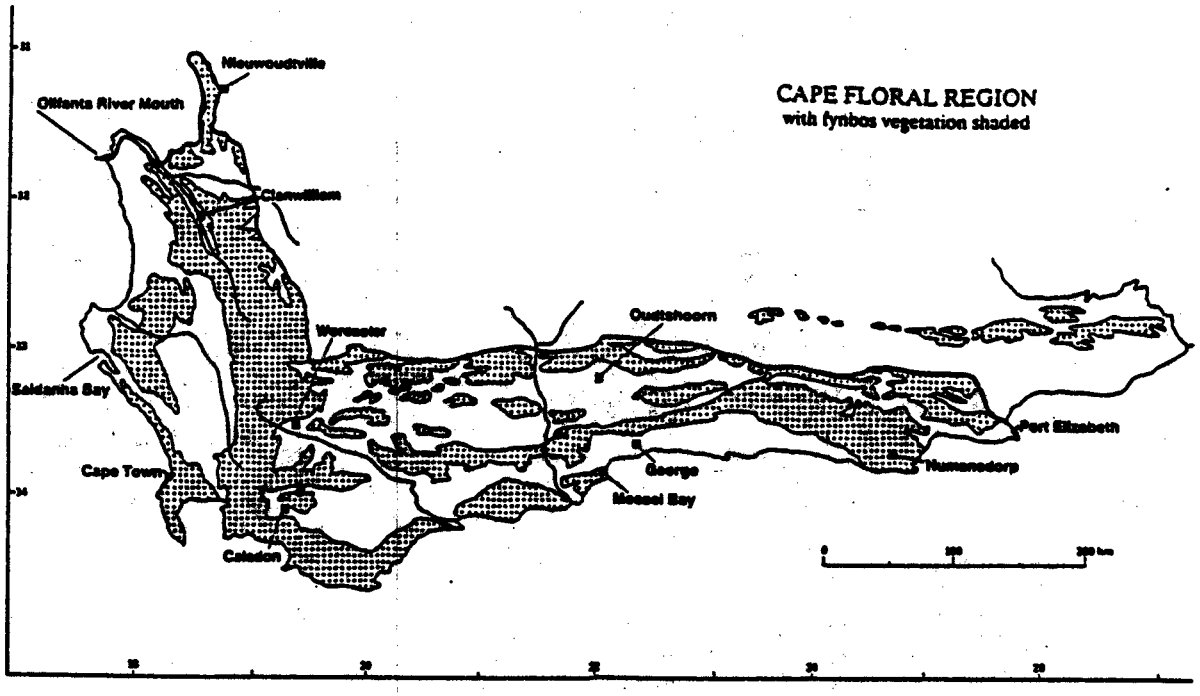


FIGURE 1. Cape Floristic Region, with fynbos vegetation shaded (from Bond and Goldblatt, 1984).

of geology by Dümmer (1912) and Pillans (1947) who noted the preference of members of Bruniaceae for substrates derived from Table Mountain sandstone (TMS). This formation traverses the whole of the CFR, entering the sea in the vicinity of Port Elizabeth, to reappear at Port St Johns, on the Pondoland coast of Kwazulu-Natal.

Within the CFR, the family occurs mainly as a component of fynbos (Taylor, 1978; Kruger, 1978) with little representation in other vegetation types, e.g. forest, thicket or karroid shrubland. Species in Bruniaceae are particularly common in ericaceous fynbos (Campbell, 1985), a community encountered on wet, south-

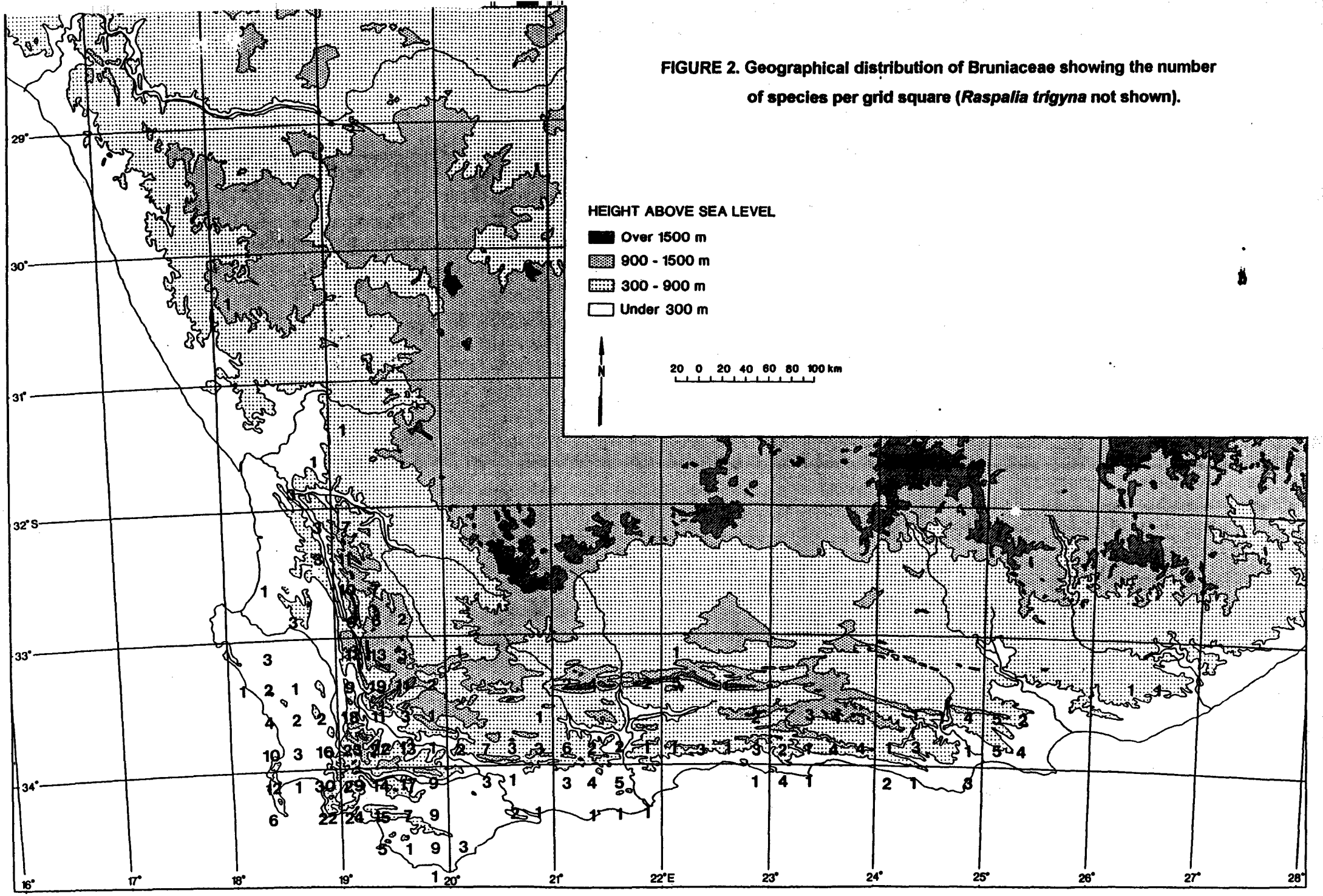


FIGURE 2. Geographical distribution of Bruniaceae showing the number of species per grid square (*Raspalia trigyna* not shown).

facing slopes of the Cape coastal mountains. A preference for sites offering some moisture, e.g. river banks, high-altitude swamps, seep areas or rock crevices is a characteristic feature of the family and dense communities of individual species, e.g. *Berzelia lanuginosa*, may become established in such habitats. There are occasional exceptions to this general observation, e.g. *Berzelia cordifolia*, a native of dry lowland hills and coastal plains of the Swellendam and Bredasdorp divisions and *Thamnea thesioides*, found on the lower slopes of the Ceres mountains, in the Karroo/fynbos ecotone. Bruniaceae, in common with the six CFR endemic families (Penaeaceae, Grubbiaceae, Stilbaceae, Retziaceae, Roridulaceae and Geissolomataceae), can thus be described as largely confined to fynbos of moist montane habitats (Goldblatt, 1978).

A noteworthy feature of the family is the extremely restricted distribution of many of its members. Only about 25% of the species in Bruniaceae are locally frequent (Hall, 1987) the remainder being rare and often confined to the upper slopes of the coastal mountains where they grow as isolated patches of a few individuals. A few species e.g. *Staavia radiata*, *Berzelia lanuginosa* and *Brunia nodiflora* have wide geographical ranges and tolerate large variations in altitude. The rarity and restricted distribution of many species in Bruniaceae is reflected in their conservation status (Hilton-Taylor, 1995). About one-third have been accorded Red Data category status; *Staavia* stands out in having 8 out of 9 currently recognised species in this category.

3. SYSTEMATICS

Despite the early controversy over placement of some of its species, the infrafamilial taxonomy of Bruniaceae since its establishment by Brown (1818), has

been fairly consistent. Brongniart (1826) was responsible for delimitation of 9 out of 12 of the currently recognised genera and subsequent authors differed mainly as regards whether these should be further split or some merged. *Brunia* was divided into subgeneric groupings by Brongniart (1826), Sonder (1861-62) and Hooker (1865), while Niedenzu (1891) established *Pseudobaeckia* and *Mniothamnea* as separate from *Brunia* and *Berzelia* respectively. *Lonchostoma*, included in the family by Brown, was excluded by several later authors on account of its tubular corolla, but all classifications subsequent to 1853 regarded this genus as correctly placed in Bruniaceae. The major classifications are presented below in table form.

TABLE 2. BRUNIACEAE ACCORDING TO DE CANDOLLE (1825)

Genera	Brunia	Staavia	Linconia
a) Stamens	arising from base of petals	opposite petals	alternating with petals
b) Fruits	indehiscent	bilocular capsule	bicoccus
c) Ovary	bi- to unilocular	—	Bilocular
d) Inflorescence	dense head	capitate	flowers clustered
e) Styles	free, subconnate at the base	fused at base free at apex	Subdivergent
f) Species	19	2	3

TABLE 3. BRUNIACEAE ACCORDING TO BRONGNIART (1826)

GENERA	DISTINGUISHING FEATURES					
	Fruit	Styles	Ovary position	no. of locules in ovary	No. of ovules per loculus	No. of species
a) *Brunia*	indehiscent	2	semi-inferior	2	1-2	5
	Section 1:	Calyx hirsute, stamens exserted				
	Section 2:	Calyx glabrous, stamens included				
b) *Staavia*	dehiscent	1	semi-inferior	2	1	4
c) *Berardia*	dehiscent	2	semi-inferior	2	1	3
d) *Linconia*	dehiscent	2	semi-inferior	2	2	2
e) *Raspalia*	—	2	inferior	2	1	1
f) *Thamnea*	—	1	inferior	1	many	1
g) *Audouinia*	—	1	semi-inferior	3	2	1
h) *Tittmannia*	—	1	inferior	2	2	1
i) *Berzelia*	indehiscent	1	inferior	1	1	2

TABLE 4. BRUNIACEAE ACCORDING TO SONDER (1861-62)

GENERA	DISTINGUISHING FEATURES					
	Fruit	Styles	Ovary position	No. of locules in ovary	No. of ovules per locule	Flowers
a) *Berzelia*	indehiscent	1	half inferior	1	1	dense heads
b) *Tittmannia*	indehiscent	1	inferior	2	2	axillary
c) *Brunia*	indehiscent	2	half inferior	2	1-2	heads or panicles
d) *Lonchostoma*	dehiscent	2 or 1	—	2	2	terminal spikes
e) *Linconia*	dehiscent	2	half inferior	2	2	axillary spikes
f) *Berardia*	dehiscent	2		2	1	dense heads
g) *Staavia*	dehiscent	1	half inferior	2	1	heads
h) *Audouinia*	—	3	half inferior	3	2	terminal spikes
i) *Thamnea*	—	1	inferior & disc	1(5?)	±10 (2?)	solitary, terminal

TABLE 5. BRUNIACEAE ACCORDING TO HOOKER (1865)

GENERAL		DISTINGUISHING FEATURES				
	Fruit	**Styles**	**No. of locules in ovary**	**No. of ovules per locule**	**Anthers**	**Flowers**
a) *Berzelia*	indehiscent	1	1	1	oblong or linear	globose, capitate
b) *Thamnea*	—	1	1	6-8	oblong - linear	solitary
c) *Tittmannia*	indehiscent	1	2	1-2	oblong	solitary, axillary
d) *Brunia*	indehiscent	2	2	1-2	oblong or didymous	capitate or paniculate
e) *Raspalia*	dehiscent	2	2	1-2	oblong	small globose capitate heads
f) *Berardia*	dehiscent	2	2	1-2	oblong	congested capitate heads
g) *Staavia*	dehiscent	1	2	1-2	Oblong	involucrate capitulum
h) *Linconia*	dehiscent	2	2	1-2	triangular - hastate	solitary axillary
i) *Audouinia*	—	1	3	2	Linear	congested oblong capitula
j) *Lonchostoma*	dehiscent	2	2	2	oblong - hastate	terminal capitula

TABLE 6. BRUNIACEAE ACCORDING TO NIEDENZU (1891)

GENERA	DISTINGUISHING FEATURES					
A. AUDOUINEAE	**Anthers**	**Fruit**	**Locules in ovary**	**Inflores-cence**	**Ovules/ locule**	**Stamen length**
Audouinia	Linear or		3			
Titmannia	elongate-	indehiscent	2			
Thamnea	rectang-ular		2			
B. BRUNIEAE						
Lonchostoma	hastate	dehiscent capsule	2	raceme	2	
Linconia		capsule				
Raspalia	ovate	dehiscent		dense		
Diberara		capsule	2	head		
Staavia					1	
Pseudobaeckia						shorter than petals
Brunia		indehiscent				longer than petals
Mniothamnea		nutlet	1			shorter than petals
Berzelia			1			longer than petals

TABLE 7. BRUNIACEAE ACCORDING TO DYER (1975)

GENERA	DIAGNOSTIC CHARACTERS						
	Flowers	**Bracts**	**Flower colour**	**Locules in ovary**	**Sepal shape**	**Sepal fusion**	**Styles**
Thamnea	solitary terminal						
Audouinia		4-12 conspic-	red	3			
Linconia		uous, forming	not red	2			
Titmannia		an involucre					
Pseudobaeckia	axillary or				rounded	fused	
Lonchostoma	in dense	flowers in					
Staavia	compound	axil of bract					
Mniothamnea	heads	of foliage leaf			lanceolate		1
Raspalia		with 1 - 2					2
Berzelia		bracteoles				free	1
Brunia							2

Subsequent to the publication of Pillans' revision of the family (1947), Powrie (unpublished MS) suggested merging *Brunia/Nebelia*, *Raspalia/Pseudobaeckia*, *Berzelia/Mniothamnea* and *Thamnea/Tittmannia*, while Tahktajan (1987), recognised four lineages within Bruniaceae, as follows:

1. Audouinioideae: *Audouinia, Thamnea, Tittmannia*
2. Brunioideae: *Linconia, Raspalia, Nebelia, Staavia, Pseudobaeckia, Brunia*
3. Lonchostomoideae: *Lonchostoma*
4. Berzelioideae: *Berzelia, Mniothamnea*

SUMMARY STATEMENT: INVESTIGATIONS UNDERTAKEN IN THIS RESEARCH

Cladistic method, while ideally suited to exploring infrafamilial relationships such as those proposed by Powrie and Takhtajan, had not been applied to Bruniaceae. The central goal of the present study was to undertake, for the first time, a phylogenetic analysis of the family. Such a study required as a first step the assembling of all available evidence relevant to the taxon under investigation. As can be seen from Tables 2-7, traditional classifications of Bruniaceae were all based on a limited number of morphological characters: number of ovary chambers, number of ovules per ovary loculus, number of styles, fruit type and inflorescence construction. Since 1975, the results of several important studies of Bruniaceae had however become available. These studies included investigations of wood and leaf anatomy (Carlquist, 1978, 1991), palynology

(Hall, 1988) and chromosome cytology of some genera in the family (Goldblatt, 1981), all of which offered a number of characters for cladistic analysis.

Several phylogenetic studies (Hufford, 1992; Anderberg, 1992, 1993) have included information from secondary chemistry, in addition to that available from morphology, anatomy and palynology, on the grounds that this represented an independent data set. Major phylogenetic studies based on total evidence from molecular and non-molecular data (Doyle *et al.*, 1994; Chase *et al.*, 1995; Nandi *et al.*, 1998) have also included information from secondary chemistry. In the latter study 87 out of 252 non-molecular characters were from this source.

The secondary chemistry of Bruniaceae was not well known (Hegnauer 1962-1992). Apart from a preliminary study of leaf flavonoids (Jay, 1968) and a statement that the family lacked iridoids (Jensen *et al.* 1975), few phytochemical data were available. The work of Jay, together with the results of a pilot study, suggested that an investigation of flavonoid patterns in Bruniaceae would provide informative characters for incorporation in a cladistic study of within-family relationships. No molecular data were available for Bruniaceae and facilities for undertaking molecular studies were at the time very limited; an analysis of non-molecular characters was therefore the focus of the study.

Critical to an investigation of within-family relationships was the identification of sister group(s) of the family. Bruniaceae had always been regarded as taxonomically isolated (Goldblatt, 1978, Linder *et al.*, 1992). The placement of the family in traditional classifications had been controversial (see Table 8), alliances having been suggested with taxa representing at least three of Cronquist's (1981) subclasses, namely Dilleniidae, Rosidae and Hamamelidae

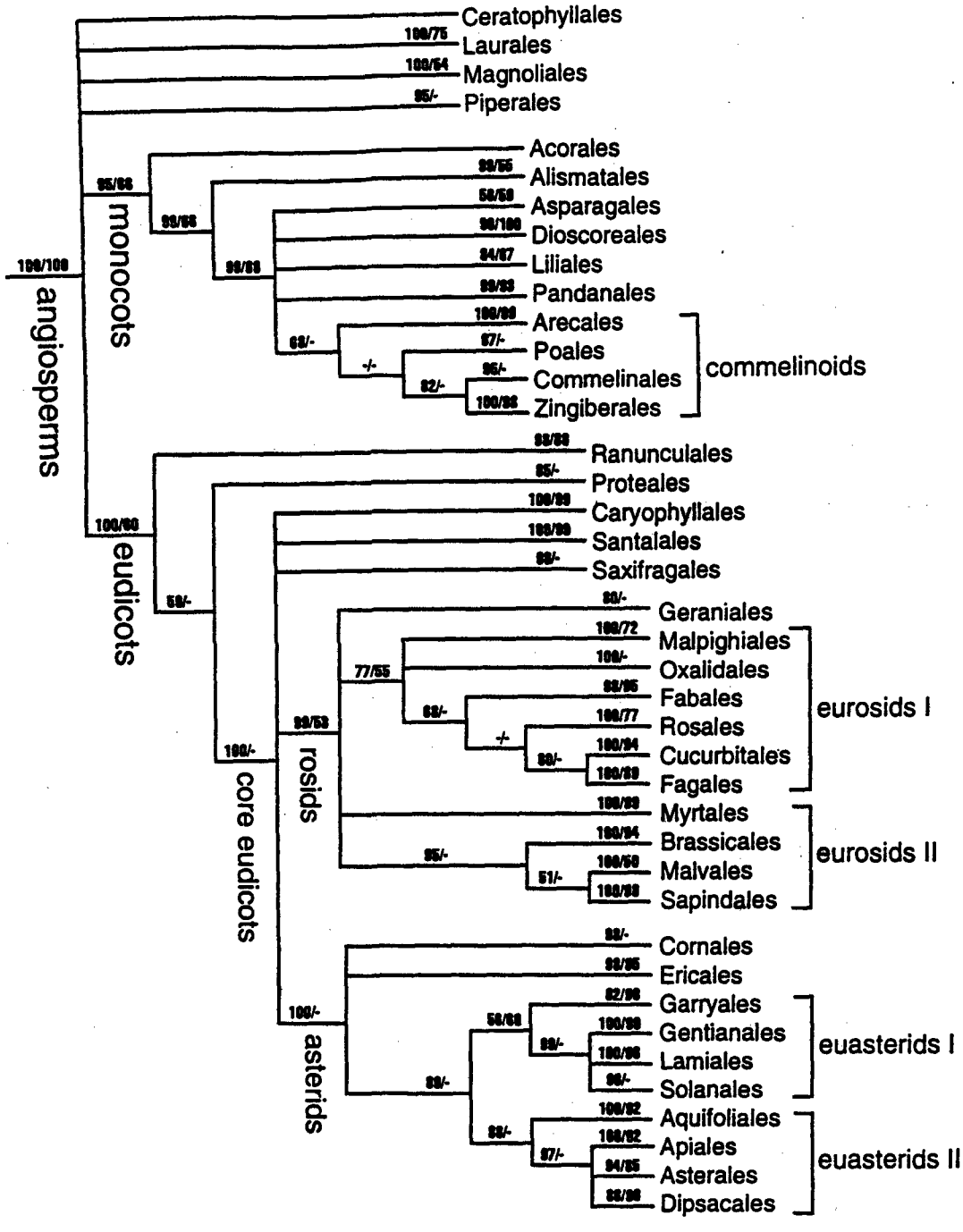
TABLE 8 AFFINITIES OF BRUNIACEAE ACCORDING TO VARIOUS AUTHORS

Date	Author	Allied taxa	Order or clade
1818	Brown	Corneae, Hamamelideae	
1825	De Candolle	Rhamnaceae	
1826	Brongniart	Cornaceae (Myrtes), Hamamelidaceae	
		Haloragaceae, Araliaceae	
1836-40	Endlicher		Hamamelideae, Corneae
1853	Lindley	Hamamelideae , Grubbia	
1861-2	Sonder	Saxifragaceae Hamamelidaceae	
1865	Hooker	Saxifragaceae	
1868	Harvey		Hamamelideae
1872	Baillon	Saxifragaceae	Hamamelideae
1891	Niedenzu	Grubbiaceae	
1897	Van Tieghem	Cornaceae	"Umbelliferae"(Rosalean)
1912	Hallier		Rosales
1915	Bessey		Rosales
1930	Niedenzu & Harms		Rosales (isolated position)
1935	Wettstein		Rosales
1953	Soó		Hamamelidales
1964	Schulze-Menz		Rosales
1969	Hutchinson		Hamamelidales
1969 1987 1997	Tahktajan	Pittosporaceae, Escalloniaceae, Roridulaceae Geissolomaceae Grubbiaceae	Saxifragales Bruniales, superorder Ericanae
1976 1992	Thorne	Grubbiaceae, Roridulaceae, Geissolomaceae, Myrothamnaceae, Hydrostachyaceae	Pittosporales, suborder Brunineae Bruniales, superorder Rosanae
1981 1988	Cronquist	Cunoniaceae, Pittosporaceae, Roridulaceae	Rosales Ericales
1980 1983	Dahlgren	Grubbiaceae Cunoniaceae, some non-South African families	Cunoniales
1988	Dahlgren & van Wyk	Grubbiaceae	Bruniales, near Ericales
1989	Dahlgren, G.		Bruniales, part of Ericales
1991	Carlquist	Grubbiaceae	Rosales Hamamelidales
1992	Hufford, L.		Corniflorae-asterid group of Rosidae
1993	Chase *et al.*	Apiaceae, Pittosporaceae, Valerianaceae, Dipsacaceae	Asteridae *s.l.*
1993	Olmstead *et al.*	Escalloniaceae, Cornaceae	Asteridae *s.l.*
1997	Soltis *et al.*	Lamiaceae, Solanaceae, Boraginaceae, Apocynaceae	Asteridae *s.l.*
1998	Nandi *et al.*	Eucommiales, Icacinaceae	Asteridae *s.l.*
1998	APG	Apiales, Asterales, Aquifoliales	Euasterids 2
1999	Hoot *et al.*	Hydrangeaceae, Araliaceae	Asterids

(Brongniart, 1826; van Tieghem, 1897; Harvey, 1868; Baillon, 1872; Hallier, 1912; Bessey, 1912; Niedenzu and Harms, 1930; Wettstein, 1935; Soo, 1953; Schulze-Menz, 1964; Thorne, 1976, 1983, 1992; Cronquist, 1981; Carlquist, 1991). Recent opinion (Dahlgren and van Wyk, 1988; Cronquist, 1988; G. Dahlgren, 1989; Tahktajan, 1997) had however favoured affinities with the order Ericales (subclass Dilleniidae).

The development of techniques in phylogenetic systematics and their application to the analysis of both molecular and non-molecular data has greatly influenced current thinking regarding relationships among angiosperm families. Support has been forthcoming for the monophyly of many major groups above the family level, now formalized as an ordinal classification (APG, 1998). In this system, Bruniaceae has been placed in "euasterid II" (see diagram, from APG, 1998).

While all major cladistic studies (Hufford, 1992; Chase *et al.*, 1993, Olmstead *et al.*, 1993; Soltis *et al.*, 1997; Nandi *et al.*, 1998; Hoot *et al.*, 1999) have agreed as to the association of Bruniaceae with an asterid lineage, there has been no agreement as regards sister relationships of the family (see table 8). The results obtained by Chase *et al.* (1993) were used as a guide to the identification of potential outgroup(s) for the family, permitting re-examination in the present study of both its position in existing classifications and its phylogeny.



Phylogenetic interpretation of the orders of flowering plants, compiled from recent cladistic analyses (from APG, 1998). Jackknife support is given on the branches (a dash for values <50%); first jackknife values from analysis of 545 sequences of the *rbc*L, *atp*B and 18S rDNA genes (Soltis, D.E., Chase, M.W., Soltis, P.S., Albach, D., Mort, M.E., Savolainen, V., Zanis, M. and Farris, J.S., unpublished, in prep.) Second jackknife values from analysis of 2538 *rbc*L sequences (Källersjö *et al.*, 1998).

The specific objectives of the present study were:

1. To carry out a survey of foliar flavonoid patterns of 56 species representing all genera in Bruniaceae, as well as of 8 species representing the following families, with which Bruniaceae had been allied in traditional classifications: Grubbiaceae, Ericaceae, Diapensiaceae, Retziaceae and Geissolomaceae. Owing to the unavailability, at the time, of guidelines from molecular studies as to the identity of sister taxa of Bruniaceae, assessment of flavonoid patterns was confined to those taxa associated with Bruniaceae in the classifications of Cronquist (1981) and G. Dahlgren (1989) as well as the monograph of Dahlgren and van Wyk, (1988). A preliminary survey of infraspecific variation in foliar flavonoid patterns of three selected species would be conducted in order to establish adequate sampling strategies.

2. Using the data generated in 1, together with information from the published literature, to carry out a cladistic analysis of Bruniaceae and putative allies (as suggested by the results of Chase et al., 1993), in order to establish the identity of sister group(s) of the family.

3. Incorporating the sister-group(s) identified in 2 above, to carry out a cladistic study of within-family relationships in Bruniaceae, based on data generated in 1 as well as information from morphology, anatomy, cytology and palynology available in the published literature. The focus of the present study was accumulation of evidence from secondary chemistry and therefore the evidence in the literature was not re-examined for the purpose of cladistic analysis.

CHAPTER 2

THE USE OF FLAVONOID DATA IN SYSTEMATICS

1. INTRODUCTION

The foundations of biochemical systematics (chemotaxonomy) can be traced back to early pharmacological studies (see Bell, 1979) in which plants with similar medicinal properties were classed together (Petiver, 1699), but the possibility of using chemical characters in formal plant systematics was first raised by Abbott (1886). It was not until the early 1950's that improved analytical methods, principally chromatographic techniques, ushered in the true beginning of the biochemical era of plant systematics. Developments in this field have been rapid and phytochemical data have come to be utilised in both phenetic and phylogenetic work at various taxonomic levels. Heywood (1973) noted that "it is clear ... that chemical information may be of value in most aspects of plant systematics, from the population to the family level and above".

2. SECONDARY METABOLITES IN SYSTEMATICS

Until recently almost all the biochemical information used in systematic work came from studies of variation in plant secondary metabolism and the distribution of products of the shikimic acid, acetate and isoprenoid pathways. In the last 15-20 years, variation in plant primary metabolism has become increasingly apparent and the sequencing of plant proteins e.g. plastocyanin, ferredoxin and cytochrome c, as well as of chloroplast and nuclear genomes, has provided much new and useful information particularly suited to cladistic studies (Soltis and Soltis, 1995). The decision to use micro- or macromolecular information is largely a matter of cost, access to appropriate technology or expertise, and

availability of plant material. Cronquist (1980) noted that secondary metabolites had at the time proved most useful in systematics and these compounds have continued to provide valuable information at all taxonomic levels (Harborne and Turner, 1984; Giannasi and Crawford, 1986; Hegnauer, 1986).

Reviews of micromolecular systematics have been presented by Alston and Turner (1963), Swain (1966), Harborne (1970a), Bendz and Santesson (1973), Smith (1976), Bisby *et al.* (1980), Young and Siegler (1981), Harborne and Turner (1984) and Giannasi and Crawford (1986). Other significant contributors in this field include Swain (1974), Fairbrothers *et al.* (1975) and Hegnauer (1986), while Gottlieb (1982) added an ecological dimension, relating phytochemistry and phytogeography. The most detailed texts to date are Gibbs' (1974) and Hegnauer's (1962-1992) descriptions of the phytochemical profiles of all known plant families. Three recent systems of plant classification, namely those of Hutchinson (1980), G. Dahlgren (1989), and Thorne (1992a) have incorporated microchemical characters, as have some large-scale phylogenetic studies (Doyle *et al.* 1994; Chase *et al.*, 1995; Nandi *et al.*, 1998).

Of the many classes of secondary plant metabolites, flavonoids have been the most widely utilised in systematic work because of their ubiquitous occurrence in most aerial parts of land plants, structural diversity and stability, such that valuable information may be gleaned from the analysis of old herbarium material as well as fresh tissue. Standard methods of flavonoid investigation are simple, inexpensive and do not require large amounts of vegetable material, the compounds concerned being easily detected in low concentration (Markham, 1989). The distribution of flavonoids in the plant kingdom is well documented and the biosynthetic pathways of all major flavonoid classes elucidated (see Koes *et al.*, 1994 and references therein).

3. THE FLAVONOIDS

3.1. Description

The flavonoids comprise a group of mostly low molecular mass biosynthetically related phenolic compounds whose presence in plant tissues was first demonstrated in the middle of the 19th century, following the search for natural dyestuffs. Of more than 4 000 known structures (Harborne, 1994), all possess a basic C_6-C_3-C_6 skeleton, that is, two aromatic rings linked by a third cyclic (pyrone) system. According to the level of oxidation of the central pyrone ring, some twelve classes of flavonoid compound may be recognised, each having characteristic biochemical and biological properties. Table 9 lists the properties and Figure 3 shows the structures of the nine major flavonoid classes.

TABLE 9. Properties of the major flavonoid classes (from Harborne and Turner, 1984)

Flavonoid class	Distribution	Characteristic properties
1. Anthocyanins	scarlet, red, mauve and blue flower pigments; also in leaf and other tissues	water-soluble, visible max. 515-545 nm, mobile in BAW on paper
2. Proanthocyanidins	mainly colourless, in heartwoods and in leaves of woody plants; astringent, tannin properties	yield anthocyanidins (colour extractable into amyl alcohol) when tissue is heated for 0-5 h in 2M HCl
3. Flavonols	mainly colourless co-pigments in both cyanic and acyanic flowers; widespread in leaves. UV protectants in leaves.	after acid hydrolysis, bright yellow spots in UV light on Forestal chromatograms; spectral max. 350-386nm.
4. Flavones	as flavonols	after acid hydrolysis, dull absorbing brown spots on Forestal chromatograms; spectral max. 330-350 nm
5. C-glycosyl flavonoids	as flavonols	contain C-C linked sugar; mobile in water unlike normal flavones
6. Biflavonoids	colourless; almost entirely confined to the gymnosperms	On BAW chromatograms dull absorbing spots of very high Rf
7. Chalcones and aurones	yellow flower pigments; occasionally present in other tissues	give red colours with ammonia (colour change can be observed *in situ*), visible max. 370-410 nm
8. Flavanones and dihydrochalcones	colourless; in leaf and fruit (especially in *Citrus*)	give intense red colours with Mg/HCl; occasionally an intense bitter taste
9. Isoflavonoids	colourless; often in root; only common in one family, the Leguminosae; oestrogenic or fungitoxic	mobile on paper in water; no specific colour tests available

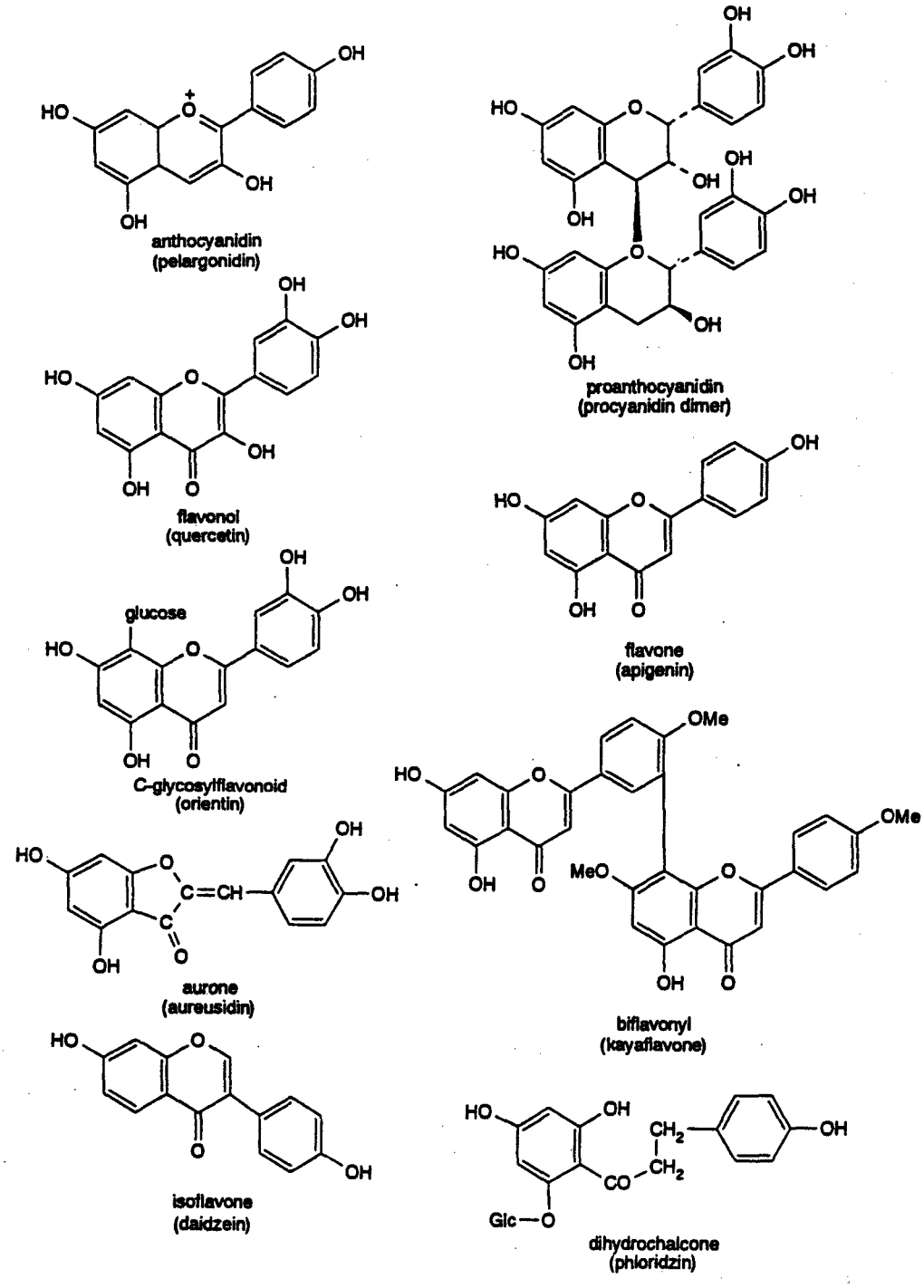
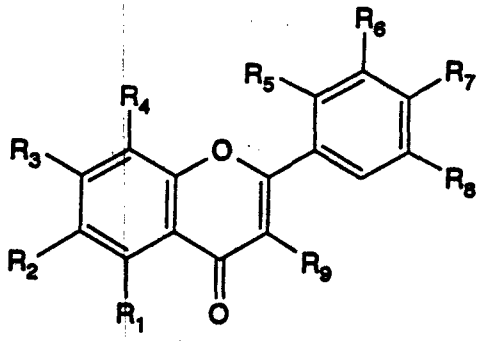


Figure 3. Structures of the major flavonoid classes

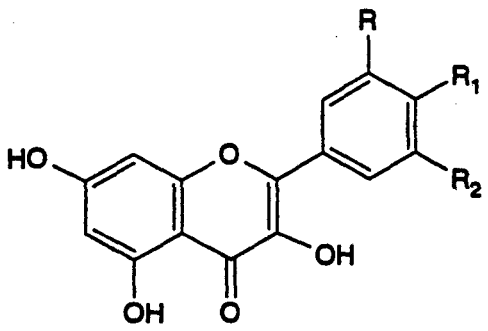
The pattern of hydroxylation in the flavonoids can vary considerably, although nine main types are recognisable (Table 10).

TABLE 10. Hydroxylation patterns observed in flavonoids (from Goodwin and Mercer, 1983)

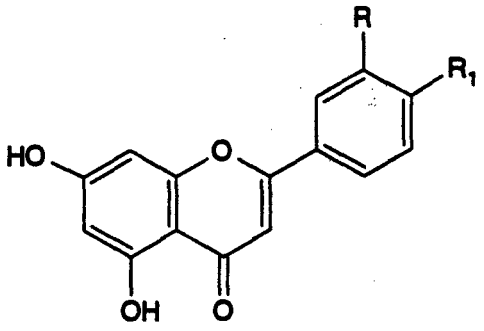


Flavonoid	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9
5-Hydroxyflavone	OH	H	H	H	H	H	H	H	H
5,8-Dihydroxyflavone	OH	H	H	OH	H	H	H	H	H
Digicitrin	OH	OCH_3	OCH_3	OCH_3	H	OH_3	CH_3	OH	OCH_3
Morin	OH	H	OH	H	OH	H	OH	H	OH
Isoetin	OH	H	OH	H	OH	H	OH	OH	H
Gossypetin	OH	H	OH	OH	H	OH	OH	H	OH
Quercetagetin	OH	OH	OH	H	H	OH	OH	H	OH
6-Methoxyluteolin	OH	OCH_3	OH	H	H	OH	OH	H	H
Diosmetin	OH	H	OH	H	H	OH	OCH_3	H	H

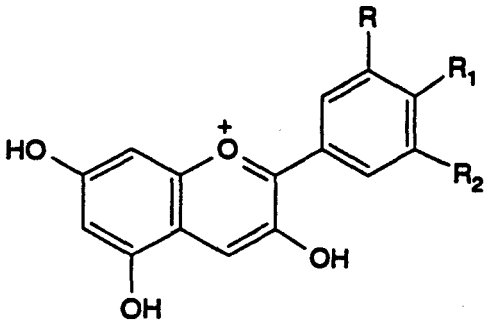
A frequently encountered pattern is 5,7 hydroxylation of the A ring together with varying degrees of 2', 3' and 4' hydroxylation of the B ring. This is seen in the eight most common flavonoids (Fig. 4) i.e. the flavonols myricetin (1), quercetin (2) and kaempferol (3), the flavones apigenin (4) and luteolin (5) and the anthocyanins pelargonidin, (6), cyanidin (7) and delphinidin (8).



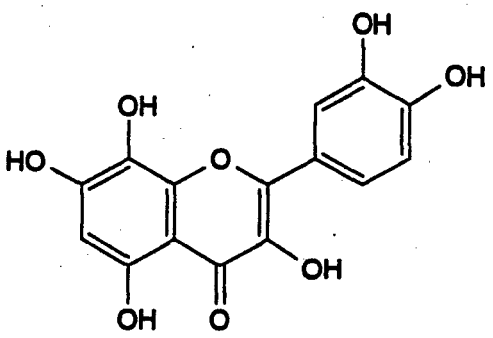
1. myricetin : R = R_1 = R_2 = OH
2. quercetin : R = R_1 = OH; R_2 = H
3. kaempferol : R = R_2 = H; R_1 = OH



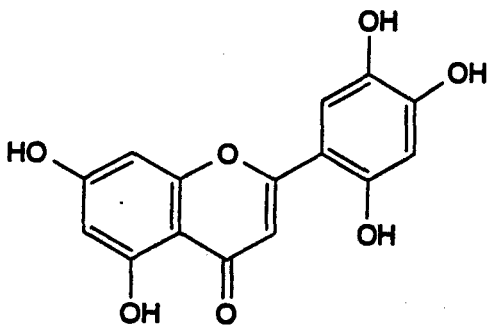
4. apigenin : R = H; R_1 = OH
5. luteolin : R = R_1 = OH



6. pelargonidin : R = R_2 = H; R_1 = OH
7. cyanidin : R = R_1 = OH; R_2 = H
8. delphinidin : R = R_1 = R_2 = OH



9. gossypetin



10. isoetin

Figure 4. Common flavonoid hydroxylation patterns

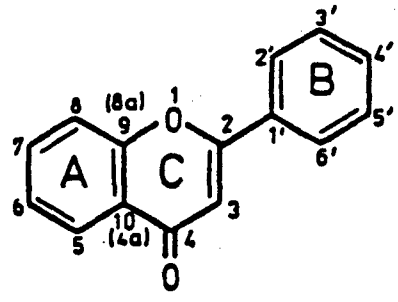
Introduction of additional OH groups to ring A, or less commonly B, may cause a significant change in colour, e.g. gossypetin (9) (8-OH quercetin) and isoetin (10) (2'-OH luteolin) are yellow compared with quercetin and luteolin (both pale cream).

As a group, flavonoids have the following common properties, many of which are utilised in their detection and identification (see Markham, 1982).

(a) Intense absorption in the ultra-violet (UV) region of the spectrum, a property of the aromatic nuclei. A minority absorb also in the visible region e.g. all anthocyanins and some flavones. The different flavonoid classes have characteristic spectral properties, the wavelengths (λ) of principal and subsidiary maxima being one of the most useful means of distinguishing between these groups. Absorption band I (principal maximum) occurs in the range 300 - 400 nm and is considered to originate from the B-ring cinnamoyl system and band II (subsidiary maximum) which occurs in the range 240-285 nm and is due to the A-ring benzoyl system (Table 11).

TABLE 11. Ultra-violet/visible absorption ranges for flavonoids (from Markham, 1982)

Band II (nm)	Band I (nm)	Flavonoid type
250-280	310-350	Flavone
250-280	330-360	Flavonols (3-OH substituted)
250-280	350-385	Flavonols (3-OH free)
245-275	310-330 shoulder	Isoflavones
	c. 320 peak	Isoflavones (5-deoxy-6,7-dioxygenated
275-295	300-330 shoulder	Flavanones and dihydroflavonols
230-270 (low intensity)	380-390	Chalcones
230-270 (low intensity)	380-430	Aurones
270-280	465-560	Anthocyanidins and anthocyanins



Hydroxylation of the molecule produces a bathochromic shift (i.e. towards a longer λ) in band II. This effect may be seen by comparing the spectral maxima of myricetin, quercetin and kaempferol (Table 12).

TABLE 12. Effect of hydroxylation pattern on flavonoid UV spectra.

	BAND I	BAND II
Myricetin	378	256
Quercetin	374	255
Kaempferol	368	268

In the case of flavanones, dihydroflavonols and isoflavones, conjugation between the B ring and the CO group of the pyrone ring cannot occur and absorption band 1 is reduced to a shoulder.

(b) A weakly acidic character (a property of the phenolic OH group) which renders all flavonoids soluble in alkali, a reaction accompanied usually by a change in colour and a bathochromic shift in the spectrum. This underpins the use of sodium hydroxide (NaOH), a strong alkali, and sodium acetate (NaOAc), a weak alkali, as "shift" reagents in the routine identification of flavonoids. Ionisation of the phenolic hydroxyls is complete in the former and a bathochromic shift in both absorption bands of the order of 40-60 nm is observed, the shift varying with the individual compound. In NaOAc, ionisation of only the most acidic of the phenolic groups occurs, with the 7-OH group being affected but not

the 5-OH group. Since absorption band 2 is due to the A ring, a bathochromic shift of 10-20 nm is observed in this band on the addition of NaOAc, provided the 7-OH group is free. Should this group be glycosidically bound, no shift is observed (Ribéreau Gayon, 1973).

(c) The capacity to form hydrogen bonds (a property of all phenols, unless sterically hindered), which in the case of flavonoids may be intramolecular, e.g. between the 5-OH and 4-CO of many flavonoids such as quercetin and taxifolin which have o-OH acetophenone nuclei.

(d) In the case of flavonoids with catechol nuclei (o-dihydroxy groups), the ability to form complexes with metals, iron and aluminium in particular. This property is both biologically important, being concerned with the natural colouration of plants and analytically useful in revealing otherwise invisible spots on chromatograms, since the complexes are usually coloured. In the spectophotometric analysis of flavonoids, aluminium chloride ($AlCl_3$) is used as a 'shift' reagent to detect the presence of o-dihydroxy groups and also of free OH groups in positions 3 or 5 on which complexation can take place in conjunction with the CO group of the pyrone ring. The addition of dilute hydrochloric acid (HCl) to the complex will result in decomposition of the relatively unstable o-dihydroxy complex but not of the CO-OH complex. These reactions are observed as characteristic changes in the neutral spectra, a strong bathochromic shift of the entire spectrum accompanying complexation.
The addition of borate (H_3B0_3) to the solution of a flavonoid + NaOAc bridges the two hydroxyls in an o-dihydroxy group and is used to detect the presence of such a group.

(e) Although flavonoids may occur in the free state in plants, particularly on external surfaces of leaves or fronds, they are more frequently encountered in

glycosidic combination. Although glycosylation is theoretically possible at any of the substituent OH groups, sugars are most often attached at B-ring hydroxyls, commonly also in the 3- position of flavonols and the 7-position of flavones, less commonly at the 5- position, except in anthocyanidins. The glycosides so formed are generally soluble in water, methanol and ethanol but insoluble in other organic solvents unless highly methylated, while the free aglycones are usually sparingly soluble in water/ethanol but soluble in ether and less polar solvents. The sugars involved are almost exclusively aldoses, with glucose, rhamnose, galactose and arabinose commonly encountered in the pyranose form, and attached by a β-linkage between C_1 of the sugar and a hydroxyl oxygen. Besides monosaccharides, di- and trisaccharides - e.g. rutinose (glucose-rhamnose) are quite common. Diglycosides, where monosaccharides are attached to different OH groups, are not unusual e.g. the 3,5 diglucosides of *Campanula* and *Dahlia* spp.

Glycoside formation is not limited to hydroxyl oxygen, but in many flavonoid classes extends also to the carbon nucleus of the aromatic ring at the 6- and/or 8- position. These C-glycosylflavonoids may have additional sugars bound to a phenolic OH or to a C-bound sugar and are much more resistant to hydrolysis than are O-glycosides, a characteristic which is usually applied in the identification of the former on paper chromatograms of hydrolysed plant extracts run in water. C-glycoflavonoids are mobile in water compared with most flavonoid aglycones, which remain at the origin. Acylated and sulphated sugars are frequently encountered and uronic acids may replace sugars as substituent groups.

(f) The flavonoids frequently form ethers, whereby phenolic hydroxyls are methylated. The methoxyl group is common in nature and both chemically and

biologically stable, unlike the glycosidic bond which is easily ruptured on treatment with mineral acid. Methylation masks the phenolic function and introduces a greater degree of lipid solubility such that highly methoxylated flavonoids may occur in leaf waxes.

The great variety of flavonoid oxygenation, methoxylation and glycosylation patterns seen in nature provides the characters used in biochemical systematics.

3.2. Classification

The following flavonoid classes are of particular importance in systematics:

(a) ANTHOCYANINS

These are sap-soluble pigments responsible for the pink, mauve, violet, blue, scarlet, and red colouration of flowers, leaves and fruits, and for red autumn hues (Harborne, 1967). The colour of a particular plant organ is dependent on the pH of the cell sap and the presence/absence of metal ions as well as on the proportions of the various anthocyanin pigments present. The effects of hydroxylation are particularly striking in the flavylium system of anthocyanins, with small differences in B-ring hydroxylation profoundly affecting the resultant colours (Strack and Wray, 1989 and 1994). Pelargonidin for example (4'-OH) is scarlet, cyanidin (3', 4'-dihydroxy) is crimson and delphinidin (3',4',5'-trihydroxy) mauve. These natural pigments, together with their methyl ethers peonidin, petunidin and malvidin, constitute three of the eight most commonly occurring flavonoids. They are ubiquitous within the angiosperms except in the order Centrospermales where they are replaced by purple betalain pigments. Anthocyanins have also been detected in mosses, young fern fronds and gymnosperms (Markham, 1988; Niemann, 1988) but appear to have their greatest ecological significance in flowering plants in providing floral colour for

the attraction of pollinating vectors (Harborne, 1993). It has been suggested that natural selection has operated from cyanidin as the more primitive pigment, via the loss of an OH group from the B ring, to yield pelargonidin which provides bright scarlet colouration that has been correlated with natural selection by animal pollinators such as hummingbirds (Harborne, 1993). Delphinidin on the other hand, via gain of a B ring OH, provides the deep blue colours correlated with selection by bees. These two pigments are particularly frequent in flowers but rare in vegetative tissues, which usually contain cyanidin. Cyanidin is the most common of the anthocyanin pigments, occurring in 80% of pigmented leaves, 69% of fruits and 50% of flowers (Brouillard, 1988). While methylation of anthocyanin B ring OH groups is common, A ring methoxyl groups are rare and confined to specific taxa; the 5-methyl ethers of malvidin and petunidin for instance occur exclusively in the family Plumbaginaceae e.g. in the light blue corollas of the indigenous *Plumbago auriculata* (Harborne and Grayer, 1988).

Glucose, rhamnose and galactose are commonly encountered in glycosidal combination with anthocyanin aglycones, attachment being frequently at the 3 or 5 position. Sugars may be acylated by a molecule of organic acid esterified to an OH group (Harborne, 1986). Both aromatic acids (e.g. caffeic, ferulic, sinapic, p-coumaric) and aliphatic dicarboxylic acids (e.g. malonic, malic, succinic) occur as sugar esters; acylation renders the flavylium cation zwitterionic and makes possible the distinguishing of these pigments from other anthocyanins by means of paper electrophoresis in a weakly acid buffer. Zwitterionic cations are present in a number of angiosperm families e.g. Asteraceae, Lamiaceae, Scrophulariaceae and Ranunculaceae and may provide useful systematic information (Harborne and Grayer, 1988).

(b) FLAVONOLS

These generally colourless or pale cream compounds are, like the anthocyanins, very widespread in nature and include a further three of the eight most commonly occurring flavonoids i.e. myricetin, quercetin and kaempferol, which correspond in B ring hydroxylation patterns to the anthocyanins delphinidin, cyanidin and pelargonidin respectively. While flavonols often accompany anthocyanins as floral co-pigments, they are almost universally present in angiosperm leaves. Over 60% of 1 000 species sampled contained one or other of the three common flavonols, with quercetin being present in 50%, myricetin in 10% and kaempferol in 48% (Harborne and Williams, 1988). Myricetin appears to have a more restricted distribution in the foliage of mainly woody plants, while kaempferol and quercetin are encountered throughout the angiosperms. The distribution of flavonols is not confined to flowering plants however; they have also been isolated from the former fern allies, true ferns and gymnosperms (Williams and Harborne, 1994).

Flavonols differ structurally from the closely related flavones in possessing a 3-OH group and can be distinguished from them on the basis of spectral properties, mobility in PC and TLC and appearance in UV light, with and without the presence of alkali (NH_3 vapour). Unlike the anthocyanins, whose aglycones are few in number, over 200 flavonol aglycones are known (Harborne, 1998), varying in their pattern of methylation and hydroxylation . Glycosylation patterns likewise vary widely. The introduction of additional A ring OH groups at the 6 or 8 positions gives quercetagetin or gossypetin, examples of a few flavonols that are pigments in their own right. Methylation is common; the three flavonol methyl ethers isorhamnetin, larycitrin and syringetin are fairly widespread and

correspond in structure to the anthocyanidins peonidin, petunidin and malvidin respectively.

Apart from their role as floral co-pigments, flavonols have been implicated as insect feeding repellents or as UV screens in foliage, particularly in plants of arid habitats which may contain flavonol (or flavone) aglycones in external secretions e.g. leaf waxes (Mc Clure, 1975; Harborne, 1985).

(c) FLAVONES

Like the related flavonols, flavones are extremely common in angiosperms where they appear to replace the former in more specialised herbaceous plant families. Two of the eight most common flavonoids are the flavones apigenin and luteolin, corresponding in B ring hydroxylation patterns to kaempferol and quercetin respectively. The flavone equivalent of myricitin is tricetin (3',4',5'-trihydroxyflavone) which is known, but has a limited distribution in nature (Wollenweber, 1994).

Flavones readily form C-glycosides (see Jay, 1994) e.g. vitexin and orientin (the 8 C-glycosides of apigenin and luteolin respectively), although they occur widely in the usual O-glycoside form (Williams and Harborne, 1994). Dimeric biflavones are commonly encountered, particularly in gymnosperms, but are by no means confined to this group and are increasingly being isolated from angiosperms as well. An example is amentoflavone (3', 8"-biapigenin).

Flavones, like flavonols, are generally cream or colourless and may act as floral co-pigments, although they are occasionally present alone in petal tissue.

(d) FLAVANONES/DIHYDROFLAVONOLS

These are reduction products of flavones and flavonols respectively (see Bohm, 1994), being formed first in the biosynthetic pathway and undergoing oxidation in the 2,3 position to give more highly oxidised forms (Grayer, 1989). Although fairly widespread, they do not generally accumulate in any quantity in plant organs, exceptions being naringenin and eriodictyol which correspond structurally to apigenin and luteolin. Members of this flavonoid class frequently occur in the free state in the heartwoods of trees or as glycosides in leaves. Reduction of the 4 CO group of dihydroflavonols to OH and H gives rise respectively to the flavan - 3,4 diols and flavan -3 - ols.

Flavanones are widespread in Fabaceae and Asteraceae but accumulate in a number of other angiosperms e.g. Rutaceae (*Citrus* spp.), Rosaceae (*Prunus* spp.) and gymnosperm families e.g. Pinaceae (*Pinus* spp.). Dihydroflavonols occur in over 50 plant families and are characteristic of Anacardiaceae, Asteraceae, Ericaceae, Fabaceae as well as several gymnosperm genera e.g. *Pinus, Abies, Podocarpus* (Grayer, 1989).

(e) ISOFLAVONOIDS

These are most commonly flavone isomers in which the B ring is attached at C_3 instead of C_2 of the pyrone nucleus (see Dewick, 1994). Examples of this group include daidzein (7,4'-dihydroxyisoflavone) formononetin (7-hydroxy-4'-methoxyisoflavone) and genistein (5,7,4'-trihydroxyisoflavone). The remaining isoflavones have isoprenyl substitution which leads in many cases to extra heterocyclic rings and/or allyl side chains (Williams and Harborne, 1989) e.g. osajin and pomiferin. These compounds differ from the general flavonoid substitution pattern in their frequent lack of 5-hydroxylation and presence of 6' and 2' and 6' hydroxylation. C-glycosidation of isoflavones is rare.

Rarer isoflavonoids include the pterocarpans, rotenoids and coumestans, which are of biological interest as phytoalexins, insecticides and anti-estrogenics respectively.

Their distribution in nature is fairly restricted; they characterise the fabaceous subfamily *Papilionoideae* and have also been isolated from the heartwood of *Pinus* species, from *Iris* species and from a further twelve dicot, monocot and gymnosperm families, where isoflavonoid occurrence appears to be limited to single species or genera (Williams and Harborne, 1989).

(f) CHALCONES/AURONES

These arise early in the flavonoid biosynthetic pathway, chalcones being converted via ring closure to the corresponding flavanones by the catalytic action of the enzyme chalcone-flavanone isomerase (see Bohm, 1994). The central 3 C chain that unites rings A and B in most flavonoids is not therefore the usual O heterocycle of six atoms but is in the form of a linear chain in the case of chalcones or a pentacyclic ring (aurones). A limited number of these yellow pigments are known; however they contribute to the floral colour of a number of angiosperm species, particular members of the family Asteraceae in which they may constitute the sole source of petal colour (Mabry and Markham, 1975).

They differ from yellow carotenoid pigments, which they often accompany, in their lack of lipid solubility and their colour change in the presence of alkali to a bright red-orange, a reaction which has given rise to the collective term anthochlor pigments for chalcones and aurones. It has been suggested that they function not only as floral pigments but as UV absorbing nectar guides to the centre of the flower, although their occurrence extends to other plant tissues e.g.

the heartwoods and seeds of some legumes (Bohm, 1988). Reduction of chalcones gives rise to dihydrochalcones e.g. phloridzin.

(g) PROANTHOCYANIDINS (condensed tannins)

These are colourless oligomers and polymers of flavan-3-ols but, unlike hydrolysable tannins as well as other flavonoid classes, do not occur in association with sugars. Their molecular mass is in the general range of 1 000 to 5 000. The most common class of proanthocyanidin comprises the procyanidins which consist of chains of catechin and/or epicatechin linked 4→6, or 4→8 (Porter, 1989 and 1994; de Bruyne *et al.*, 1999).

Their detection in plant tissues is based upon their oxidation by hot mineral acid to the corresponding coloured anthocyanidin pigment, most commonly cyanidin but also delphinidin. A similar oxidation process accompanies the preparation of beverage plants such as ceylon tea, cocoa and cola and is responsible for the red colour of the "fermented" product and that of some drugs and spices e.g. cinnamon and *Cinchona* barks (Evans, 1996).

Their polyphenolic character permits ready complexation with proteins, reducing the enzyme function and nutritive value of these compounds. This accounts also for their use as tanning materials, converting raw hides to leather, and for the astringency and unpalatability of plant tissues in which they occur in quantity.

(h) BIFLAVONOIDS

These dimeric compounds comprise two flavonoid units, of similar or different classes, joined by means of a C-C bond or less commonly, by a C-O-C bond (see Geiger, 1994). The constituent monomers are usually flavones or

flavanones, but isoflavones, aurones, chalcones and dihydrochalcones are not uncommon. Monomer units are most frequently linked by a carbon-carbon 3',8"-bond e.g.amentoflavone, a 6, 8"-bond e.g. agathisflavone, a 6,3"-bond e.g. robustaflavone or a carbon-oxygen-carbon 6',4"-bond as in hinokiflavone. A 5,7,4'-or 5,7,3',4'-oxygenation pattern is the general rule amongst biflavonoids and methylation of one or more OH groups is common (Geiger and Quinn, 1988).

Apart from their widespread distribution in the lower plant orders, biflavonoids have been reported from 32 genera in 15 angiosperm families, assigned to eight of Dahlgren's superorders (see Harborne and Williams, 1989). They are known to accumulate in quantity in some species, in a variety of different tissues, including leaf, bark, root, heartwood, fruit and seed (Williams and Harborne, 1989).

(i) FLAVAN -3 - OLS (catechins)

Unlike the related anthocyanins and flavones, catechins do not normally exist as glycosides nor do they occur as methylated forms. The most common members of this class differ only in the number of B ring hydroxyl groups and all have two chiral centres (at C_2 and C_3) giving rise to four optical isomers. On treatment with hot dilute mineral acid they give rise to yellow-brown insoluble products of high molecular mass (phlobaphenes) whereas the flavan-3,4-diols produce some coloured anthocyanin in addition to phlobaphene (see Porter, 1994).

3.3. Biosynthesis

The biosynthetic origin of flavonoids was established by Neish and co-workers (Underhill *et al.*, 1957; Watkin *et al.*, 1957) following feeding experiments with buckwheat and red cabbage in the early 1950's, and the essential steps of

flavonoid biosynthesis were known by 1965. Since then much further study has been undertaken and the topic reviewed by Hahlbrock and Grisebach (1975), Harborne (1977), Herbert (1981), Ebel and Hahlbrock (1982), Heller and Forkmann (1988 and 1994) and Koes *et al.* (1994). The main outline of the biosynthetic pathway, the inter-relationships of different flavonoid classes and the enzymes catalysing the various steps are now well known, although the genetic control of the various processes still requires some clarification.

A characteristic of flavonoid biosynthesis is the production, via different pathways, of the two component aromatic rings of the flavan nucleus. The phenylpropane residue (Ring B and carbon atoms 2,3 and 4) is derived from a p-coumaroyl fragment via the shikimic acid pathway. Ring A on the other hand is formed from three acetate units, joined head to tail and represents a special case of polyketide synthesis. Polyketides are all assembled from acetate derived C_2 units and include not only the aromatic A rings of flavonoids but also the fatty acids and certain phenols.

A key intermediate in flavonoid biosynthesis is 4,2',4',6'-tetrahydroxychalcone, formed by the condensation of p-coumaroyl-coenzyme A with three molecules of malonyl-CoA, and from which all the major flavonoid classes are derived. The flavanone naringenin is the first true flavonoid to arise, via chalcone isomerase - catalysed closure of the pyran ring (C). The various stages in flavonoid biosynthesis are shown in Fig. 5, together with the enzymes responsible for each transformation (Table 13).

TABLE 13. List of enzymes leading to various flavonoid classes (From Heller and Forkmann, 1994). EC number=enzyme commission number

Enzyme	*Acronym*	*EC number*
NON-FLAVONOID PRECURSORS		
I Acetyl-CoA carboxylase	ACC	6.4.1.2
II Phenylalanine ammonia-lyase	PAL	4.3.1.5
III Cinnamate 4-hydroxylase	C4H	1.14.13.11
IV 4-Coumarate:CoA ligase	4CL	6.2.1.12
V 4-Coumaroyl-CoA 3-hydroxylase	CC3H	
FLAVONOID CLASSES		
1. Chalcone synthase	CHS	2.3.1.74
2. Polyketide reductase	PKR	
3. Chalcone isomerase	CHI	5.5.1.6
4. 2-Hydroxyisoflavanone synthase	IFS	
5. 2-Hydroxyisoflavanone dehydratase	IFD	
6. Flavone synthase I	FNS 1	

7. Flavanone 4-reductase	FNR	1.1.1.234
8. Flavanone 3-hydroxylase	FHT	1.14.11.9
9. Flavonol synthase	FLS	
10. Dihydroflavonol 4-reductase	DFR	1.1.1.219
11. Leucoanthocyanidin 4-reductase	LAR	
(flavan-3,4-*cis*-diol 4-reductase)		
12. Anthocyanidin synthase	ANS	
13. Flavonoid (anthocyanidin/flavonol)	FGT	2.4.1.91
3-*O* glucosyl-transferase		

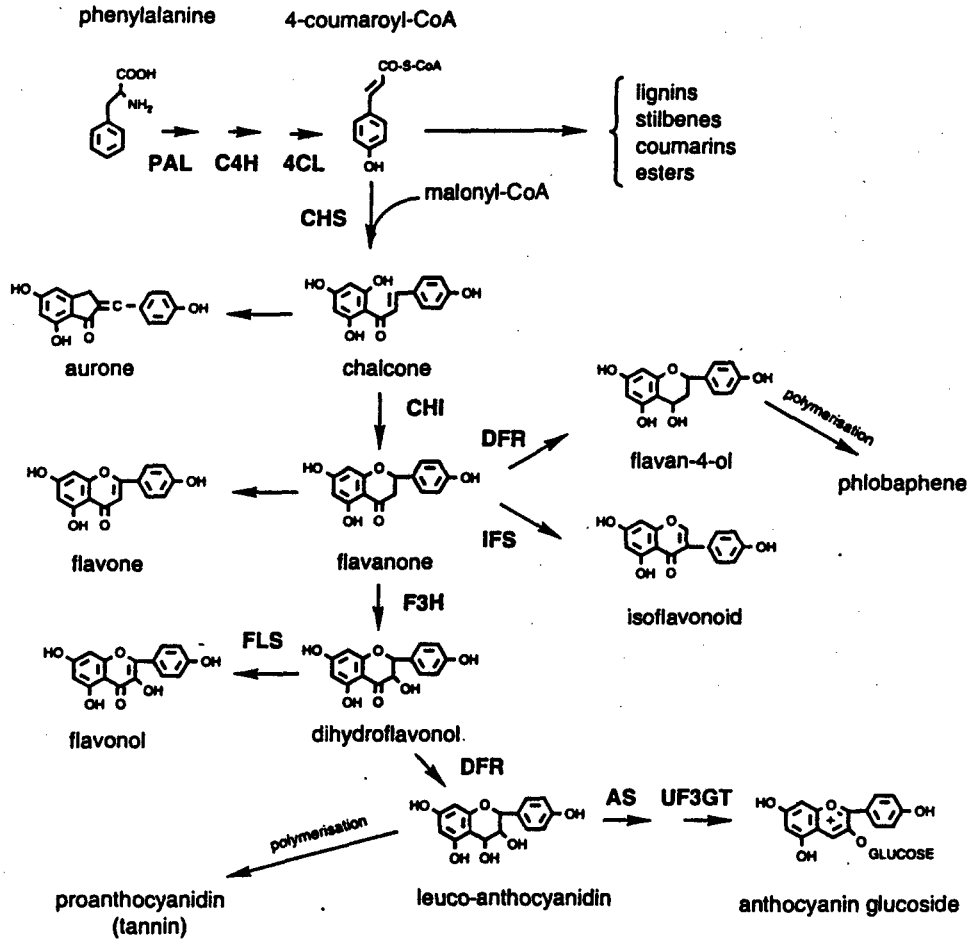


FIGURE 5. Simplified diagram of the flavonoid biosynthetic pathway (from Koes *et al.*,1994).

The main types of flavonoids are represented and the enzymes catalysing some key reactions are indicated by the following abbreviations:

PAL= phenylalanine ammonia-lyase; C4H= cinnamate 4-hydroxylase; 4CL=4-coumaroyl-coenzymeA ligase; CHS= chalcone synthase; CHI= chalcone flavanone isomerase; F3H= flavanone 3β-hydroxylase; DFR= dihydroflavonol 4-reductase; FLS= flavonol synthase; IFS= isoflavonoid synthase; AS= anthocyanin synthase; UF3GT, UDP-glucose= flavonoid 3-O-glucosyltransferase.

3.4. Isolation and identification

During the years 1945-65 the development of chromatography, particularly paper chromatography (PC), and UV spectroscopy made possible the separation and identification of flavonoids in plant material (Mabry *et al* 1970; Markham, 1982). More recently, improved techniques of separation e.g. high pressure liquid chromatography (HPLC), droplet counter current chromatography and centrifugal thin layer chromatography (TLC) have, together with mass spectrometry and nuclear magnetic resonance (NMR), permitted the separation, quantitation and identification of flavonoids present in plant extracts. The choice of appropriate techniques is a question of scale, economics and class of compound to be investigated; current approaches to flavonoid analysis are discussed by Harborne (1998).

3.5. Flavonoid distribution

Overviews of the occurrence of flavonoids in the various plant orders have been presented by Harborne (1972, 1975) and Harborne and Mabry (1982). More recently, the distribution of flavonoids in bryophytes and pteridophytes has been reviewed by Markham (1988), in gymnosperms (Niemann, 1988), in dicots (Giannasi, 1988) and monocots (Williams and Harborne, 1988). Flavonoids appear to be absent from the algae but are widespread in liverworts and mosses. Of the liverwort species studied 41% were found to contain flavonoids of several classes but excluding isoflavones, chalcones, biflavonyls and proanthocyanidins. Amongst the mosses, 48% of species examined were shown to possess a variety of flavonoids but flavonols, dihydroflavonols and 3-OH anthocyanins were not detected (Markham, 1988). Amongst the Bryophytes flavonoid synthesis appears to be limited to one or a few types, e.g. Isoetales possess only flavones,

Lycopodiales only flavones and Equisetales flavonols, proanthocyanidins and a few flavones. In both Psilotales and Selaginales biflavonoids appear to predominate, while the ferns, by contrast, synthesise most flavonoid types, except aurones, isoflavones and dihydroflavonols (Markham, 1988).

The Ginkgoales, Gnetales and Cycadales synthesise either biflavones or C-glycoflavones, together with proanthocyanidins and some flavones/flavonols, Biflavones, generally widespread in most coniferous families, are absent from Pinaceae which otherwise synthesises a complex mixture of flavones, flavonols, dihydroflavonols and C-glycosylflavones (Niemann, 1988).

The angiosperms have received most attention as regards flavonoid surveys and dicots appear to have the capacity for synthesis of all known flavonoid classes. Asteraceae, in particular, are notable for flavonoid complexity, while Scrophulariales, Lamiales and Rubiaceae produce a diverse although less striking array of flavonoids (Giannasi, 1988). Monocots by comparison appear to have less complex flavonoid profiles: chalcones, aurones, dihydrochalcones and isoflavonoids have very restricted distributions in monocot orders, biflavonoids were until recently thought to be entirely absent and flavanones/flavonols appear sporadically. Proanthocyanidins on the other hand are surprisingly common, despite their correlation with the woody habit, which is very rare in monocots (Williams and Harborne, 1988).

The distribution of different classes of flavonoids throughout the plant kingdom suggests that they have appeared sequentially during evolution (Koes *et al.*, 1994). This is schematically depicted in Figure 6.

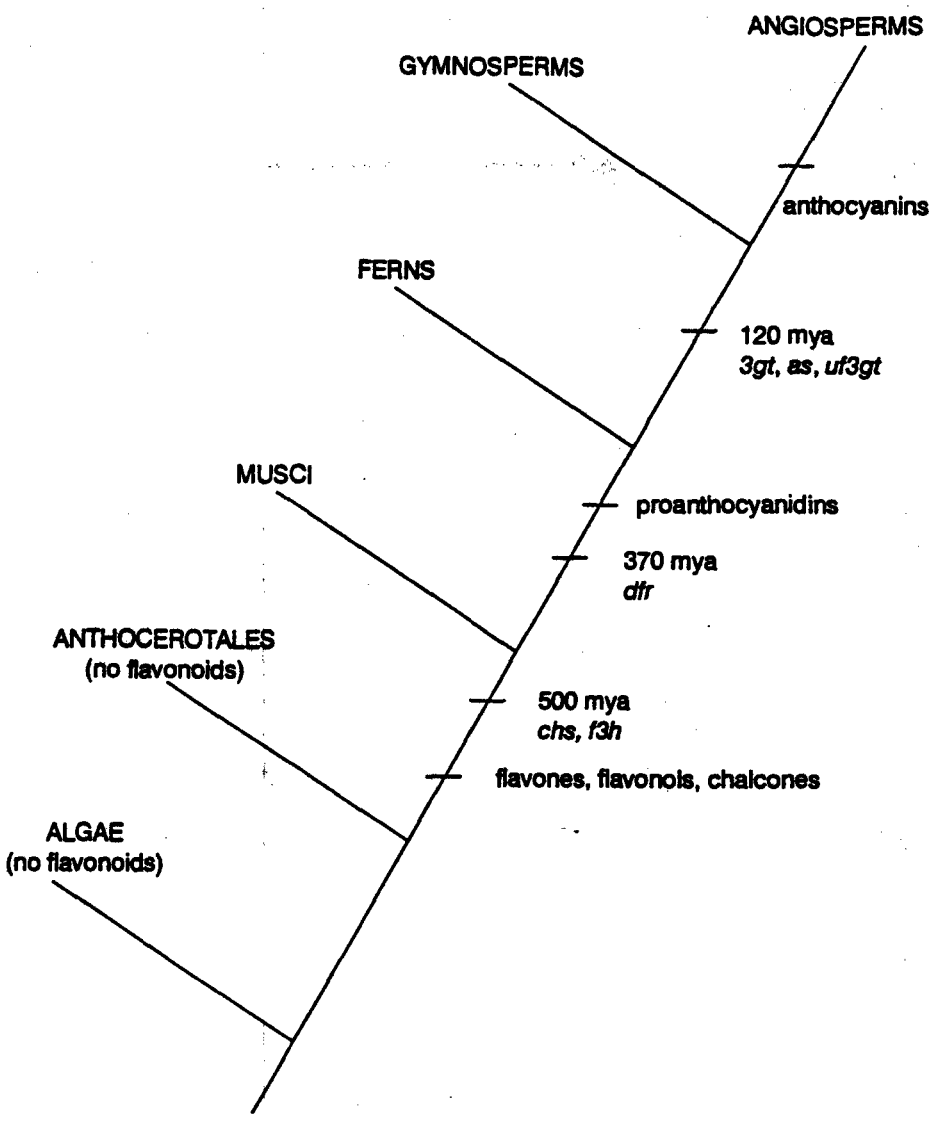


Figure 6. Appearance of different flavonoid classes (see Figure 5) during plant evolution (from Koes *et al.*, 1994).
The enzymes involved in the synthesis of each group of compounds and an estimation of the moment of appearance of such enzymes are also indicated.
(mya = millions of years ago)

3.6 Flavonoid function

The possible biological function of plant secondary metabolites has attracted a good deal of attention in the last twenty years. In the opinion of Markham (1988), flavonoid accumulation is likely to have developed originally in early green plants as a protection against the intense ultra-violet radiation that reached earth before the build-up of oxygen and ozone layers. The hypothesis that

successful colonisation of the land would have been dependent, amongst other things, on the ability to synthesise UV screens, appears to be supported by the absence of flavonoids from the algae, their ubiquitous presence in almost all other plant orders and their presence in the chloroplasts of all angiosperms. Support for a UV-shield function is also suggested by the transcriptional activation of flavonoid biosysnthetic genes that has been observed as a generalised reaction in plant seedlings exposed to UV light. Further evidence is provided by the accumulation of flavonoids, after induction, mainly in epidermal cells (Koes *et al.*, 1994).

Much of the current interest in flavonoid function has come from ecologists seeking reasons for herbivore food selection or exploring plant-pollinator interactions, from plant pathologists seeking the causes of disease resistance and from physiologists investigating allelopathy. Anthocyanins, in addition to their role as plant pigments (see Brouillard and Dangles, 1994), are thought to be involved in the inhibition of larval growth in insects. *Citrus* flavanones e.g. hesperidin and naringenin are of interest because of their extremely bitter taste to humans and higher mammals, a property that has been implicated in herbivore deterrence. Biflavonoids may act as natural fungitoxins or insect antifeedants (see Harborne and Grayer, 1994) and isoflavonoid phytoalexins as agents in disease resistance (Harborne, 1993).

This suggests a role for flavonoids in plant survival or fitness for life, rather than in essential growth processes. Flavonoids have however been implicated in plant growth and development e.g. as promotors or inhibitors of growth hormones and as plant growth regulators. A role in respiration, photosynthesis, morphogenesis, sex determination (McClure, 1975) and in pollen tube development (Mo *et al.*, 1992) has also been suggested. Isoflavonoids may act as deterrents to insect predators but also appear to play a role in host recognition

systems. In particular, the common isoflavones daidzein and genistein have been identified in the root exudates of legumes, where they have the ability to inhibit the nodulating ability of *Rhizobium* in the nitrogen-fixing symbiosis (Harborne, 1988). Other isoflavonoids have however been implicated in promotion of nodulation (Dakora *et al.* 1993; Kosslak *et al.*, 1987).

Swain and Cooper-Driver (1981), in a comprehensive review of biochemical evolution in early land plants, suggested that the capacity to synthesise secondary metabolites of various kinds was an important factor in determining the ultimate success of land plants, in that it enabled them to withstand the physical stresses of a land-based existence (dehydration or UV radiation) as well as the challenge of predators and pathogens. An elaboration of the flavonoid biosynthetic pathway would have extended the adaptive utility of these secondary compounds to include not only UV protection but also pathogen/predator defence (proanthocyanidins, isoflavonoid phytoalexins) and the attraction of pollinating or seed dispersal agents (anthocyanins, flavones, chalcones, aurones). The capacity of flavonoids to fulfil these various functions has been documented by Harborne (1977, 1982, 1993), Swain (1977), Rozenthal & Jansen (1979) and others.

A biosynthetic flexibility, permitting diversity in chemical structure and hence in bioactivity, may well account for the persistence of flavonoids in most extant plant taxa. The discovery of yet more functions for these compounds will contribute to a better understanding of ecological and co-evolutionary processes.

3.7 The use of flavonoid data in systematics

The foundations for the possible use of flavonoids in plant systematics were laid in the 1930's when variations in floral anthocyanin patterns were shown to be

related to the taxonomy of the families examined. Since that time a steadily growing volume of flavonoid distributional data has been applied to the solution of problems at every taxonomic level. Classic studies were those of Alston and Turner (1967) at the specific level, Erdtman (1956) at the generic level, Harborne and Williams (1973) at familial level, Bate Smith (1962) at ordinal level and Williams *et al.* (1988) at supraordinal level.

In the early period of chemosystematics, most flavonoid data were treated phenetically, but a number of important studies attempted to assess their phylogenetic content. Bate Smith's observations (1962) concerning the distribution of proanthocyanidins in ferns, gymnosperms and mainly woody angiosperms convinced him of the primitive nature of these tannins and led to his devising an "advancement index" based on the distribution of proanthocyanidins, ellagic acid and simple phenols in various plant orders. Bate Smith introduced the concept of a flavonoid scoring system in his studies of the systematics of *Geranium* (Bate Smith, 1973) and *Ulmus* (Bate Smith and Richens, 1973). Rezende and Gottlieb (1973) likewise used a points system, in order to determine the phylogenetic significance of xanthone oxygenation patterns. Sporne (1982) showed the presence of leaf proanthocyanidins to be positively correlated with some 13 anatomical features deemed primitive on fossil evidence, using this information to assign an advancement index to several angiosperm families.

Harborne (1966, 1967) demonstrated that evolutionary advancement in the angiosperms, as seen in a change from woody to herbaceous habit, was accompanied by changes in leaf flavonoid patterns whereby:

(i) Flavones replaced flavonols in more advanced herbaceous plant families,

(ii) the ability to synthesise proanthocyanidins and tri-hydroxylated B-ring flavonoids, e.g. myricetin, was lost.

In a review of flavonoids in relation to the evolution of the angiosperms, Harborne (1977) assessed primitiveness/advancement of certain of these compounds (Table 14) on the basis of three criteria, namely their distribution patterns, correlation with other biological characters and biosynthetic complexity.

TABLE 14. Evolution of some flavonoid characters in the Angiosperms (from Harborne, 1975).

Character	Primitive state	Advanced state
Anthocyanin in petal	Based on cyanidin; simple glucosylation	Based on delphinidin or pelargonidin (or rarely apigeninidin/luteolinidin); often methylated; complex glycosylation and acylation
Proanthocyanidin in the leaf	Present	Absent
Flavonols/flavones in leaf	Flavonols present, especially myricetin; flavones absent; *O*-methylation absent	Flavonoles based on quercetin or kaempferol, but being replaced by flavones; *O*-methylation frequent
Extra A-ring hydroxylation of Flavonols/flavones	in 8-position	in 6-position
Glycosylflavone in leaf	Present sometimes accompanied by mangiferin	Absent
Biflavonoids in leaf	Present	Absent
Yellow anthochlors in flower	Chalcones alone	Aurones, often accompanied by chalcones

Harborne expressed evolutionary advancement in terms of flavonoid scores and advancement indices, using the technique to clarify phylogeny in Apiaceae (tribe Caucalideae) and Polemoniaceae (Harborne, 1977).

Gornall and Bohm (1978), Crawford (1978) and Richardson (1982) questioned the validity of earlier concepts of primitiveness or advancement. In particular they noted that:

1. Complexity of flavonoid structure was not necessarily indicative of evolutionary advancement. Although in a theoretical biosynthetic pathway A → B → C → D, compound D might be considered most advanced in terms of the number of enzyme mediated steps required to produce its greater structural complexity, in fact the dominant trend in angiosperms was one of reduction (Gornall and Bohm, 1978). The most advanced members of a particular taxon might therefore have the simplest compounds, and in the pathway A → B → C → D, compound C might be primitive or advanced with respect to D. Gornall and Bohm suggested that flavonoid compounds had three character states: primitive, advanced and highly advanced, and that the primitive/highly advanced states might be phenotypically similar. Thus the absence of a particular flavonoid might be interpreted as primitive (an inability to synthesise) or highly advanced (secondary loss of the ability to synthesise). In drawing phylogenetic conclusions it was therefore important that not only the presence of a particular compound be ascertained, but also its phylogenetic polarity.

2. Flavonoid classes formerly deemed primitive by virtue of their widespread occurrence in lower plant orders or families considered primitive on morphological grounds, were, as more distributional data became available, increasingly being isolated from 'advanced' taxa. Biflavonoids, for example, were at one time thought to be confined to pteridophytes and gymnosperms but were later shown to occur also in several angiosperm families. A further example was the widespread occurrence of chalcones and flavanones in Asteraceae. However, as Harborne (1977) argued, this might merely represent the retention of a primitive character on the part of certain taxa.

3. Flavonoid structural complexity (a putatively advanced feature) was not only evident in advanced plant taxa, as shown by the occurrence of several

advanced features (3-deoxygenation, methylation, acetylation and complex glycosylation) in Ranunculaceae floral pigments.

Richardson and Young (1982) criticised the use of flavonoid point scores and advancement indices, noting that average or total scores might conceal valuable phylogenetic information. For example, two species with similar flavonoid scores might contain the same or different compounds, necessitating some means of adequately displaying the phylogenetic content of point scores. Richardson and Young (1982) advocated the application of either Hennigian phylogenetic methods (Hennig, 1966) or the Wagner-ground plan divergence method (Wagner, 1980) to the analysis of flavonoid data, in order to overcome this difficulty.

The suggestion that Hennigian methods be applied to phytochemical data had been put forward earlier by Humphries and Richardson (1980) who argued that previous studies, aimed at evolutionary interpretation of chemical data, were narrative rather than analytical. They claimed that Hennigian methods would raise flavonoid presence/absence information from a merely supportive role in systematics to the status of raw data that might be used in its own right to erect falsifiable hypotheses and remove subjectivity, as shown by their study of flavonoid patterns in Fabaceae (Humphries and Richardson, 1980).

Richardson (1982) applied Hennigian analytical methods to flavonoid data from Lemnaceae and *Solanum* section *Androceras* (Solanaceae), demonstrating that it was possible to use both presence of particular flavonoid classes and variation occurring at particular carbon atoms of the flavonoid skeleton as phylogenetic characters. Since that time there have been relatively few studies in which cladistic method has been applied to the analysis of flavonoid data *per se*. This is a pity in view of the greatly improved computer capability, ideally suited to

the analysis of flavonoid data, that has become available to the systematist over the past 15 years.

The paucity of flavonoid cladistic studies is partly due to the increased focus on molecular (DNA restriction sites and sequences) rather than secondary chemical data in recent years. Molecular data, in addition to providing large numbers of characters for phylogenetic analysis, reflect the entire genotype and are thought to give a clearer view of relationships than do secondary chemical characters, which represent only part of the genotype and may in addition be influenced by the environment.

3.8. Problems associated with the use of flavonoid data in systematics.

The preceding review of the application of flavonoid data to the solution of problems in systematics sets in context the research to be undertaken in the present study. In particular, an assessment of the proposed methodology can be made. There are two central questions to be addressed:

1. How should infraspecific variation in flavonoid patterns be taken into account?

Studies using flavonoid data as systematic characters have been criticised for failing to take into account the effects of season, geography and other extrinsic or intrinsic factors on plant secondary chemistry (Bohm, 1987). The existence of chemical variation at the population level underscores the need for adequate sampling, both within and between populations of a given species, as is routinely done in morphological work (Harborne and Turner, 1984).

In the present study, it was proposed in the first instance that a minimum of two collections be made of each species, from different geographical localities. Within- and between-population variation in foliar flavonoid patterns could not be

investigated for all 66 species included in the study, but an assessment of this variation could be made by selecting three representative species having different distribution ranges (wide vs. narrow) and reproductive strategies (obligate reseeding vs. resprouting capacity). Within- and between-population variation in foliar flavonoid patterns would be statistically treated, as recommended by Bohm (1987), in order to establish whether more than two collections per species would be necessary to adequately represent its flavonoid profile.

2. Is the use of flavonoid data appropriate to the taxonomic level of the present study?

In the present study, phylogenetic information was sought at the level of both family and genus. Giannasi and Crawford (1986) argued that the debate over the most effective taxonomic level of flavonoid utility was circular in that the assignment of taxonomic level to two or more taxa was a subjective decision taken by systematists. Flavonoid data could in their view be most profitably used when and where they were most useful, rather than forcing them into some possibly artificial taxonomic constraint.

Grayer *et al.*, (1999), more recently, noted that flavonoid data had proved to be more taxonomically useful within families, genera and species rather than at or above family level. This has to do with problems of chemical convergence and/or parallelism. Unlike molecular sequence data, very few secondary chemical characters are shared by all major plant groups, so that the value of these characters in addressing phylogenetic questions above the family level is very limited. Flavonoids are an exception in this respect in that they are almost ubiquitous in the plant kingdom, but suffer from the disadvantage that similar

flavonoid classes or individual flavonoids may occur in taxa that do not appear on other evidence to be closely related. Since the flavonoid biosynthetic pathway appears to operate in a similar manner in all plant groups studied, this is more likely to be the result of parallel evolution than of chemical convergence as typified by the occurrence of tropane alkaloids in the apparently unrelated families Solanaceae and Erythroxylaceae or of ergot-type alkaloids in *Claviceps purpurea* as well as Convolvulaceae.

The phylogenetic significance of the presence/absence of flavonoid classes or compounds may therefore difficult to assess at family level and above (Grayer *et al.*, 1999). This does not render flavonoids unsuitable for phylogenetic analysis at or above family level, but does suggest that a reliable phylogeny will not be retrieved using flavonoid data alone (Giannasi & Crawford (1978); Harborne & Turner (1984). These authors explicitly warned against placing a too-emphatic reliance on single flavonoids, flavonoid classes or patterns of substitution and/or distribution as key systematic markers of a given taxon.

Parallelism is evident not only in respect of flavonoids but also of many morphological, anatomical and cytological characters (see Soltis and Soltis, 1995). This has not precluded their use in phylogenetic studies; rather the approach has been to combine all available information in a single analysis. This was the approach followed in the present study.

CHAPTER 3

INVESTIGATION OF INFRASPECIFIC VARIATION IN FOLIAR FLAVONOID PATTERNS OF SELECTED MEMBERS OF BRUNIACEAE

INTRODUCTION

Studies using flavonoid data as systematic characters have been criticised for failing to take into account the effects of season, geography and other extrinsic or intrinsic factors on plant secondary chemistry (Bohm, 1987). The existence of chemical variation at the population level underscores the need for adequate sampling, both within and between populations of a given species, as is routinely done in morphological work (Harborne and Turner, 1984). Studies at generic level e.g. Nicholls and Bohm (1982), Bohm *et al.* (1984) or specific level e.g. Hillis (1966), Saleh and Towers (1972) have more frequently addressed infraspecific flavonoid variation than have studies at family level or above. In the latter it has been common practice to analyse 1-2 specimens of each species e.g. Williams *et al.* (1986) in a tribal classification for the Iridaceae and Williams *et al.* (1988) in a chemical appraisal of leaf flavonoids of Dahlgren's Liliiflorae.

The present study, at family level, did not permit much allowance to be made for the effects of external factors such as seasonality on foliar secondary chemistry. However, it has been noted (Harborne, pers.comm.) that these effects are usually more quantitative than qualitative and, since the aim of this study was not to exhaustively analyse each species, they can be discounted. Some attempt was made to allow for genetic variability however, by sampling a minimum of two populations per species, from different geographical localities. The need for an assessment of whether this approach was adequate (in terms of representing a

particular species' flavonoid profile) provided the rationale for the present analysis of infraspecific variation. The information generated could, in addition, be applied to the systematics of Bruniaceae e.g. in deciding whether Cedarberg populations of *Berzelia lanuginosa* merit separate status, a matter of debate owing to their morphological distinctness.

The following specific questions were addressed:

1) Is a greater degree of variation in foliar flavonoid profile to be encountered within or between populations of selected species in Bruniaceae?

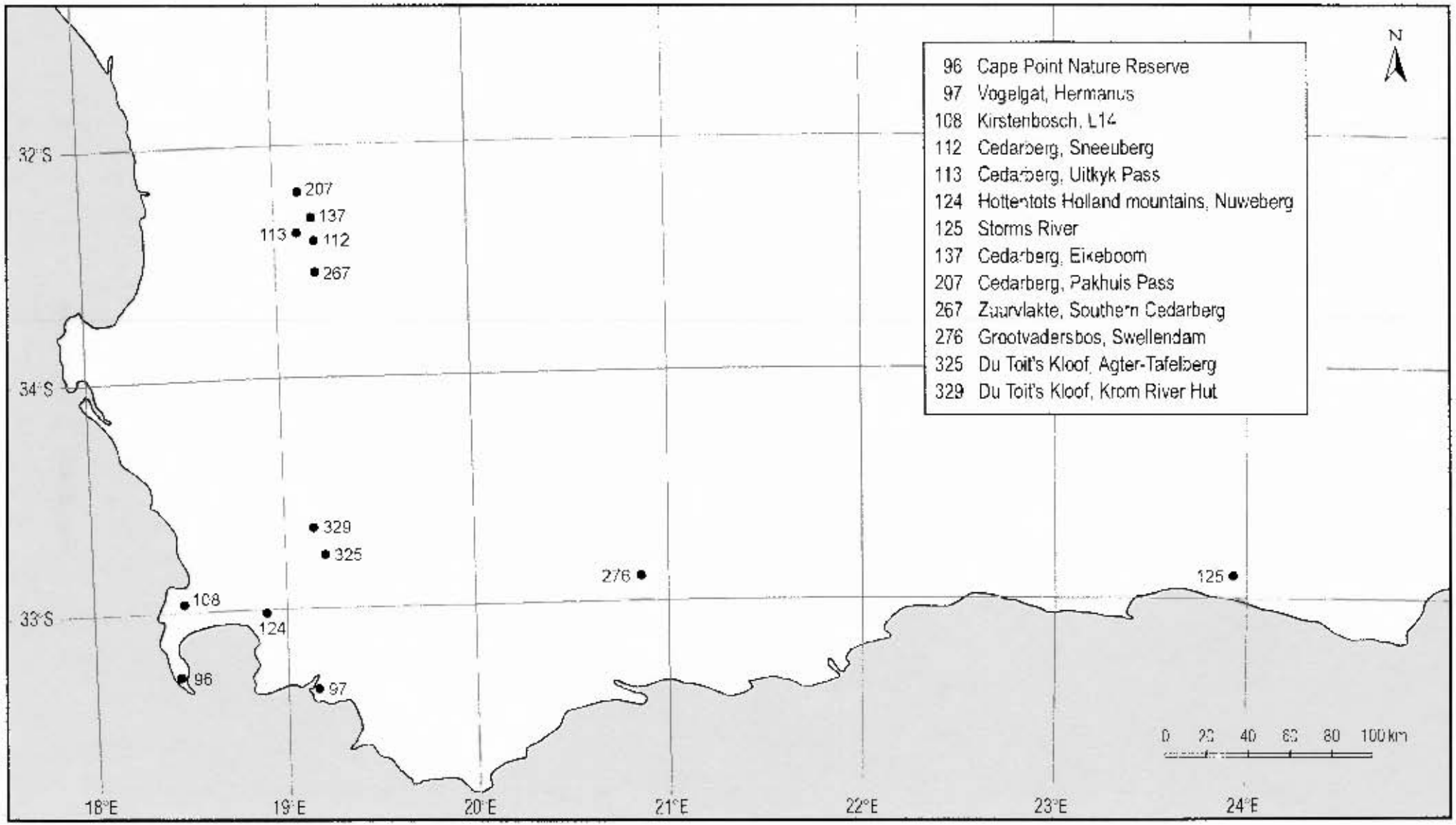
2) Is a similar pattern of variation to be encountered in two species with wide distributional ranges, one of which is killed by fire i.e. is an obligate reseeder (*Berzelia lanuginosa*) and the other able to survive fire by resprouting from a subterranean rootstock (*Brunia nodiflora*) and a putatively primitive lignotuberous species (*Audouinia capitata*) with a narrow distribution range and represented in nature by only a few small populations.

3) Is there evidence of the emergence of flavonoid chemotypes among groups of populations situated in close geographical proximity?

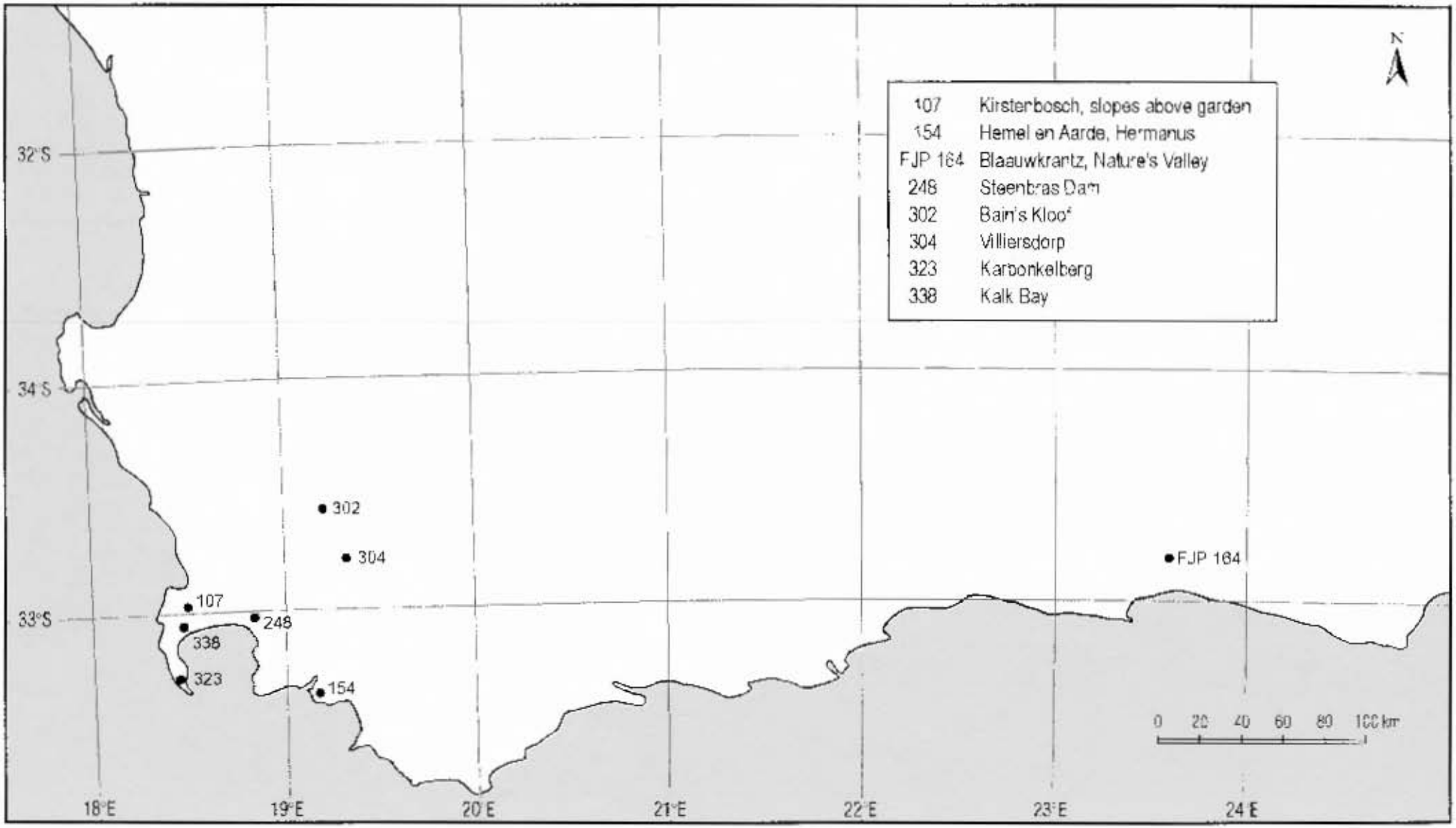
A. METHODS

1. SAMPLE COLLECTION, EXTRACTION AND ANALYSIS

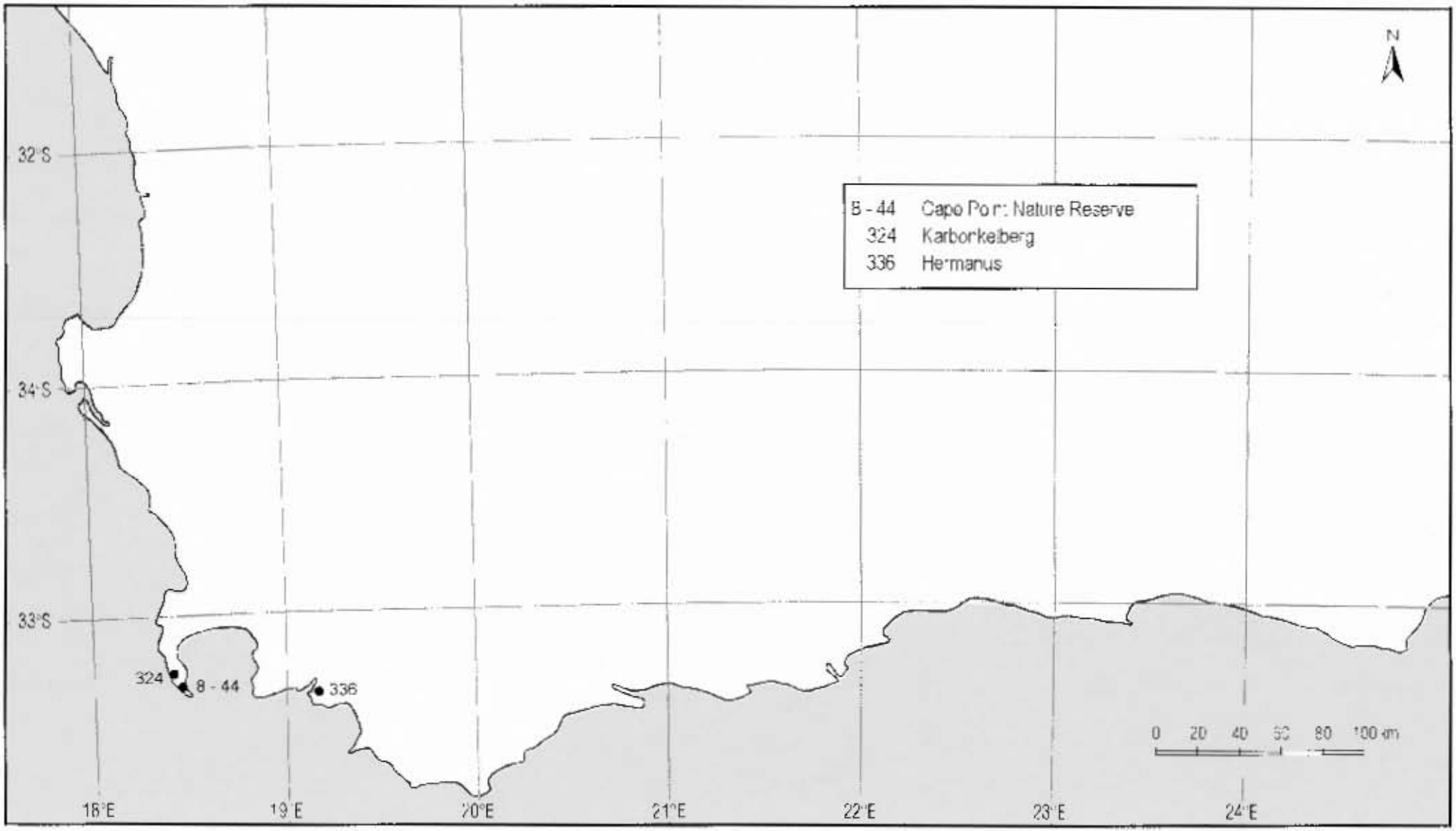
In order to explore the question of within-population variation in leaf secondary chemistry of species used in this study, fifteen plants each of *Berzelia lanuginosa*, *Brunia nodiflora* and *Audouinia capitata* were sampled from a single



Geographical distribution of 13 populations of Berzelia lanuginosa.



Geographical distribution of 8 populations of *Brunia nodiflora*.



Geographical distribution of 3 populations of *Audouinia capitata*.

population of the respective species. The first two species were sampled from populations occurring at Kalk Bay (Cape Peninsula) and *Audouinia* from one of the populations found in the Cape Point Nature Reserve. For the study of geographical variation, one plant of each species was sampled from several different populations with as wide as possible a distribution within the species' geographical ranges. In the case of *Audouinia capitata*, estimates of geographical variation were limited to the three known localities (see distribution maps of the three species).

Dried leaf material (500 mg) was extracted with 3x5 ml quantities of 90% methanol (MeOH) followed by 3x5 ml quantities of 50% MeOH. The combined solutions were evaporated to near dryness on a watchglass and the residue adjusted to a volume of 4 ml with 70% ethanol (EtOH). After centrifuging, 200 µl aliquots of the clear supernatant were applied to Whatman No.1 chromatography paper and the chromatograms developed two dimensionally in BAW and 15% AcH using rutin as a marker. The dried chromatograms were viewed in UV light, before and after fuming with NH_3 vapour. The corresponding spots on each set of chromatograms were numbered and the presence/absence of each compound recorded in a matrix (Tables 15-19).

2. DATA ANALYSIS

2.1. WITHIN AND BETWEEN POPULATION VARIATION IN FOLIAR FLAVONOID PROFILES

In order to address questions 1 and 2 above, regard was had to the qualitative nature of the chemical data generated in this study; these possess the properties of a binomial distribution i.e. fall into one of two mutually exclusive categories, in this instance presence/absence. This situation is defined by the

TABLE 15: Flavonoids detected by two-dimensional paper chromatography of leaf extracts from 16 plants of *Berzelia lanuginosa* growing at Kalk Bay.

	SAMPLE NUMBER																
NO 339	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**XI**	**XII**	**XIV**	**XV**	**XVI**	**XVII**	**UV/NH_3**
1	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	invisible / pale yellow
2	+	+	+	+	+	+	+	+	+	+	-	+		+	+	+	invisible / egg yellow
3	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	taupe / lemon
4	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	taupe / yellow-green
5	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	taupe / egg
6	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	taupe / green-yellow
7	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	sky / aqua
8	+	+	+	-	+	+	+	+	+	+	+	-		-	+	+	faint blue / no colour change
9	+	-	+	-	-	+	+	+	+	+	+	+		+	+	+	invisible / aqua
10	+	+	+	+	+	+	+	+	+	+	+	+		+	+	+	mustard / no colour change
11	-	+	+	+	+	+	+	+	+	+	+	+		+	+	-	blue / bright blue
12	-	+	-	+	-	-	+	-	+	+	+	+		-	+	-	pale yellow-white / no colour change
13	-	+	-	-	+	+	+	-	-	-	-	-		-	-	-	faint yellow-white / bright

No number 14 recorded

Major compounds 3,4,5,6 present in all samples

TABLE 16: Flavonoids detected by two-dimensional paper chromatography of leaf extracts from 13 populations of *Berzelia lanuginosa.*

	SAMPLE NUMBER													
Coll. No	96	97	108	112	113	124	125	137	207	267	276	325	329	UV/NH_3
1	+	+	+	+	+	+	+	+	+	+	+	+	+	invisible / pale yellow
2	+	-	-	+	+	+	+	+	+	+	+	+	-	invisible / egg yellow
3	+	+	+	+	-	+	+	+	+	+	+	+	+	taupe / lemon
4	+	+	+	+	+	+	+	+	+	+	+	+	+	taupe / yellow-green
5	+	+	+	+	+	+	+	+	+	+	+	+	+	taupe / egg
6	+	+	+	-	+	-	-	-	-	-	-	-	+	taupe / green-yellow
7	+	+	+	+	+	+	+	+	+	+	+	+	+	sky / aqua
8	+	+	+	+	+	+	+	+	+	+	+	+	+	faint blue / no colour change
9	+	+	+	+	+	+	+	+	+	+	+	+	+	invisible / aqua
10	+	+	+	-	-	+	-	+	+	+	-	-	-	mustard / no colour change
11	+	+	+	-	+	+	+	+	+	+	-	-	-	blue / bright blue
12	-	+	+	+	-	+	-	+	-	-	-	-	+	pale yellow-white / no colour change
13	+	-	-	+	-	-	-	-	-	-	+	+	+	faint yellow-white / bright
14	-	-	-	-	+	-	+	-	-	-	+	-	-	taupe / mustard

96 Cape Point Nature Reserve

97 Vogelgat, Hermanus

108 Kirstenbosch, L14

112 Cedarberg, Sneeuberg

113 Cedarberg, Uitkyk Pass

124 Hottentots Holland mountains, Nuweberg

125 Storms River

137 Cedarberg, Eikeboom

207 Cedarberg, Pakhuis Pass

267 Zuurvlakte, Southern Cedarberg

276 Grootvadersbos, Swellendam

325 Du Toits Kloof, Agter - Tafelberg

329 Du Toit's Kloof, Krom River Hut

TABLE 17: Flavonoids detected by two-dimensional paper chromatography of leaf extracts from 14 plants of *Brunia nodiflora* growing at Kalk Bay.

	SAMPLE NUMBER														
NO	**ai**	**aii**	**aiii**	**aiv**	**av**	**avi**	**avii**	**bi**	**bii**	**biii**	**biv**	**bv**	**bvi**	**bvii**	**UV/NH$_3$**
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	invisible / light egg
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	invisible / lemon
3	-	+	+	+	+	+	+	+	+	+	+	+	+	+	invisible / faint yellow
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	invisible / pale yellow
5	+	+	-	+	+	+	-	-	-	-	-	+	-	-	invisible / uv blue
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	taupe / yellow-green
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	sky / aqua
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	invisible / powder blue
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	yellow / brighter
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	faint taupe / yellow
11	+	+	+	-	+	+	-	+	+	+	+	+	+	+	bright blue
12	-	+	-	-	+	-	-	+	-	+	-	+	+	-	faint taupe
13	+	+	-	+	-	+	+	+	+	+	+	+	+	+	invisible / egg
14	+	+	+	+	-	+	-	+	+	+	+	+	+	+	bright blue / no colour change
15	-	+	+	-	-	-	-	+	+	+	+	+	+	+	pale yellow / brighter

TABLE 18: Flavonoids detected by two-dimensional paper chromatography of leaf extracts from 7 populations of *Brunia nodiflora*.

	SAMPLE NUMBER								
NO	107	154	FJP 164	248	302	304	323	338	UV/NH_3
1	+	+	+	+	+	+	+	+	invisible / light egg
2	+	+	+	+	-	+	+	+	invisible / lemon
3	-	+	-	+	+	+	+	+	invisible / faint yellow
4	+	+	+	+	+	+	+	+	invisible / pale yellow
5	-	-	-	-	-	-	-	+	invisible / uv blue
6	+	+	+	+	+	+	+	+	taupe / yellow-green
7	+	+	-	+	+	+	+	+	sky / aqua
8	+	+	+	+	+	+	+	+	invisible / powder blue
9	-	+	-	-	+	+	+	+	yellow / brighter
10	-	-	-	-	+	-	+	+	faint taupe / yellow
11	+	+	+	+	+	+	+	+	bright blue
12	-	+	-	-	+	+	-	+	faint taupe
13	-	+	-	+	+	+	+	-	invisible / egg
14	-	+	+	-	-	-	-	-	bright blue / no colour change
15	-	-	-	+	+	-	-	-	pale yellow / brighter

107 Kirstenbosch, slopes above garden

154 Hemel en Aarde, Hermanus

FJP 164 Blaauwkrantz, Nature's Valley

248 Steenbras Dam

302 Bain's Kloof

304 Villiersdorp

323 Karbonkelberg

338 Kalk Bay

TABLE 21. Between population variation in foliar flavonoid profiles of *Berzelia lanuginosa, Brunia nodiflora* and *Audouinia capitata,* expressed as percentage probability (p).

	p (%)	variance	std.dev.
Berzelia lanuginosa	74.2	0.00146	0.038
Brunia nodiflora	68.3	0.00180	0.042
Audouinia capitata	77.8	0.0030	0.056

2.2. SIMILARITY COEFFICIENTS

In order to address questions 2 and 3 above, Sørensen coefficients of similarity were calculated for all populations of *Berzelia lanuginosa* and *Brunia nodiflora* and *Audouinia capitata* surveyed, using the formula: $S = \frac{2a}{2a+b+c}$

where S=Sφrensen coefficient

a=number of compounds common to both samples

b=number of compounds in sample 1

c=number of compounds in sample 2

The results were expressed as half-matrices of similarity coefficients (Tables 22-24).

i	100	82	95	80	86	90	87	95	91	91	86	86	90	91	100	
ii		100	87	91	96	92	96	87	92	92	87	87	82	92	82	
iii			100	86	91	96	92	100	96	96	91	91	95	95	95	
iv				100	86	82	78	86	91	91	86	95	90	91	80	
v					100	96	83	91	87	87	82	82	86	87	86	
vi						100	96	96	92	92	87	87	91	92	92	
vii							100	92	88	88	92	92	87	88	87	
viii								100	96	96	91	91	95	95	95	
ix									100	100	96	96	91	100	91	
x										100	96	96	91	100	91	
xi											100	91	86	96	78	
xii												100	95	96	86	
xiii													100	91	90	
xiv														100	91	
xv															100	
xvi																100
	i	ii	iii	iv	v	vi	vii	viii	ix	x	xi	xii	xiii	xiv	xv	xvi

Table 22a. Half-matrix of Sørensen coefficients of similarity (as per cent) for 16 plants of *Berzelia lanuginosa* growing at Kalk Bay, based on foliar flavonoid data.

	96	97	108	112	113	124	125	137	207	267	276	325	329
96	100	87	87	82	82	87	82	87	91	91	82	86	82
97		100	100	76	76	91	76	91	86	86	67	70	86
108			100	76	76	82	76	91	86	86	67	70	86
112				100	70	86	80	86	80	80	90	95	90
113					100	76	90	76	80	80	80	74	70
124						100	86	100	95	95	76	80	76
125							100	86	90	90	90	84	70
137								100	95	95	76	80	76
207									100	100	80	84	70
267										100	80	84	70
276											100	94	80
325												100	84
329													100

Table 22b. Half-matrix of Sørensen coefficients of similarity (as per cent) for 13 populations of *Berzelia lanuginosa*, based on foliar flavonoid data.

	a.i.	a.ii	a.iii	a.iv	a.v.	a.vi	a.vii	b.i	b.ii	b.iii	b.iv	b.v.	b.vi	b.vii
a.i.	100	89	83	83	83	96	82	85	88	85	88	89	88	88
a.ii		100	89	89	89	93	80	97	93	97	96	100	97	93
a.iii			100	83	83	88	82	92	96	92	96	89	92	96
a.iv				100	83	96	91	85	88	85	88	89	85	88
a.v.					100	88	82	85	80	88	80	89	88	72
a.vi						100	87	89	92	74	92	93	89	92
a.vii							100	83	87	83	87	80	83	87
b.i.								100	96	100	96	97	100	96
b.ii.									100	96	100	93	96	100
b.iii										100	96	97	100	93
b.iv											100	97	96	100
b.v.												100	97	93
b.vi													100	96
b.vii														100

Table 23a. Half-matrix of Sørensen coefficients of similarity (as per cent) for 14 plants of *Brunia nodiflora* growing at Kalk Bay, based on foliar flavonoid data.

		107	154	FJP 164	248	302	304	323	338
	107	100	74	86	82	63	67	78	74
	154		100	63	82	83	96	87	83
FJP	164			100	71	53	67	67	63
	248				100	82	76	86	73
	302					100	87	87	83
	304						100	91	87
	323							100	87
	338								100

Table 23b. Half-matrix of Sørensen coefficients of similarity (as per cent) for 8 populations of *Brunia nodiflora*, based on foliar flavonoid data.

	8	15	16	19	24	25	26	28	29	30	41	42	43	44
8	100	90	90	86	87	87	90	93	90	93	90	90	87	96
15		100	94	90	91	97	94	97	91	90	94	94	91	87
16			100	97	90	90	100	97	90	90	88	93	90	86
19				100	93	87	97	93	93	86	90	90	93	89
24					100	94	90	94	94	87	97	90	94	90
25						100	90	94	88	93	91	97	88	83
26							100	97	90	90	88	93	90	86
28								100	94	93	91	97	94	90
29									100	87	97	90	100	90
30										100	84	97	87	89
41											100	90	94	87
42												100	90	86
43													100	87
44														100

Table 24a. Half-matrix of Sørensen coefficients of similarity (as per cent) for 14 plants of *Audouinia capitata* growing at Cape Point, based on foliar flavonoid data.

	44	324	336
44	100	85	69
324	—	100	85
336	—	—	100

Table 24b. Half-matrix of Sørensen coefficients of similarity (as per cent) for 3 populations of *Audouinia capitata*, based on foliar flavonoid data.

The clustering algorithm UPGMA (Kovach, 1993) was used to generate dendrograms (Figures 8 and 9) based on the raw data for *Berzelia lanuginosa* and *Brunia nodiflora*.

Examination of a plant species throughout its geographical range for the possible emergence of distinct chemotypes is an essentially phytosociological process, although in the present analysis it is individuals and populations of the same species rather than vegetation samples that are the subjects of comparison. For assessment of similarity between vegetation samples or quadrats, the Sorensen coefficient is often used since it can accommodate qualitative data and in addition, gives weight to characters common to both populations rather than to those occurring only in either sample. The use of UPGMA (average-linkage method) as a sorting strategy for the production of a hierarchical classification was a matter of convenience, although it does offer the advantage of producing clearer dendrograms than do single-linkage clustering methods.

B. RESULTS

1. WITHIN AND BETWEEN POPULATION VARIATION IN FOLIAR FLAVONOID PROFILES.

The probability (expressed here as a percentage) of encountering a particular flavonoid in leaf extracts of a plant collected anywhere within the distribution ranges of the three species examined is 68.3% in the case of *Brunia nodiflora*, 74.2% in the case of *Berzelia lanuginosa* and 77.8% for *Audouinia capitata* (Table 21). This suggests an increasing degree of between-population variability

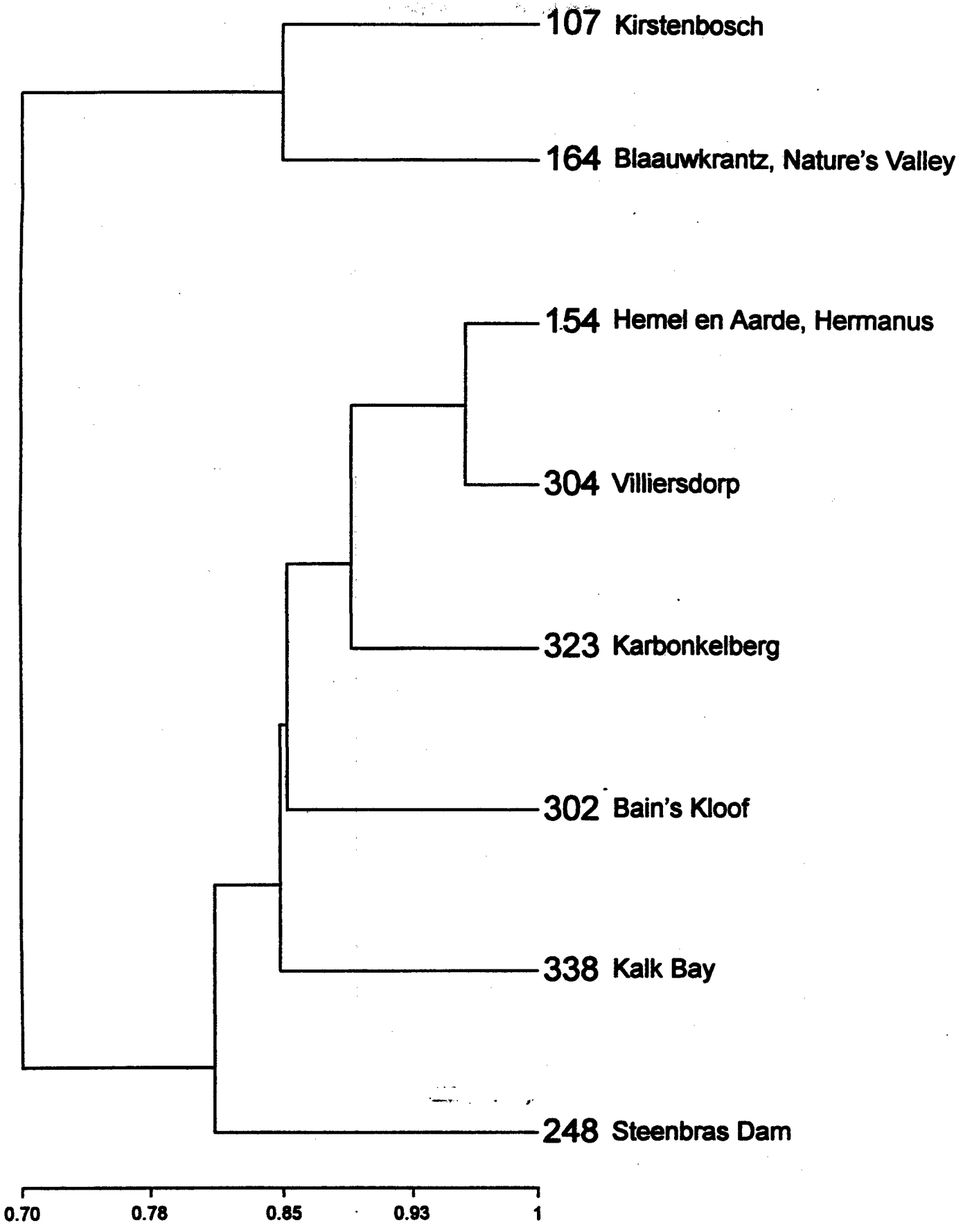


FIGURE 8:
Dendrogram generated by UPGMA analysis of foliar flavonoid data from two-dimensional paper chromatograms of leaf extracts from 8 populations of *Brunia nodiflora*.

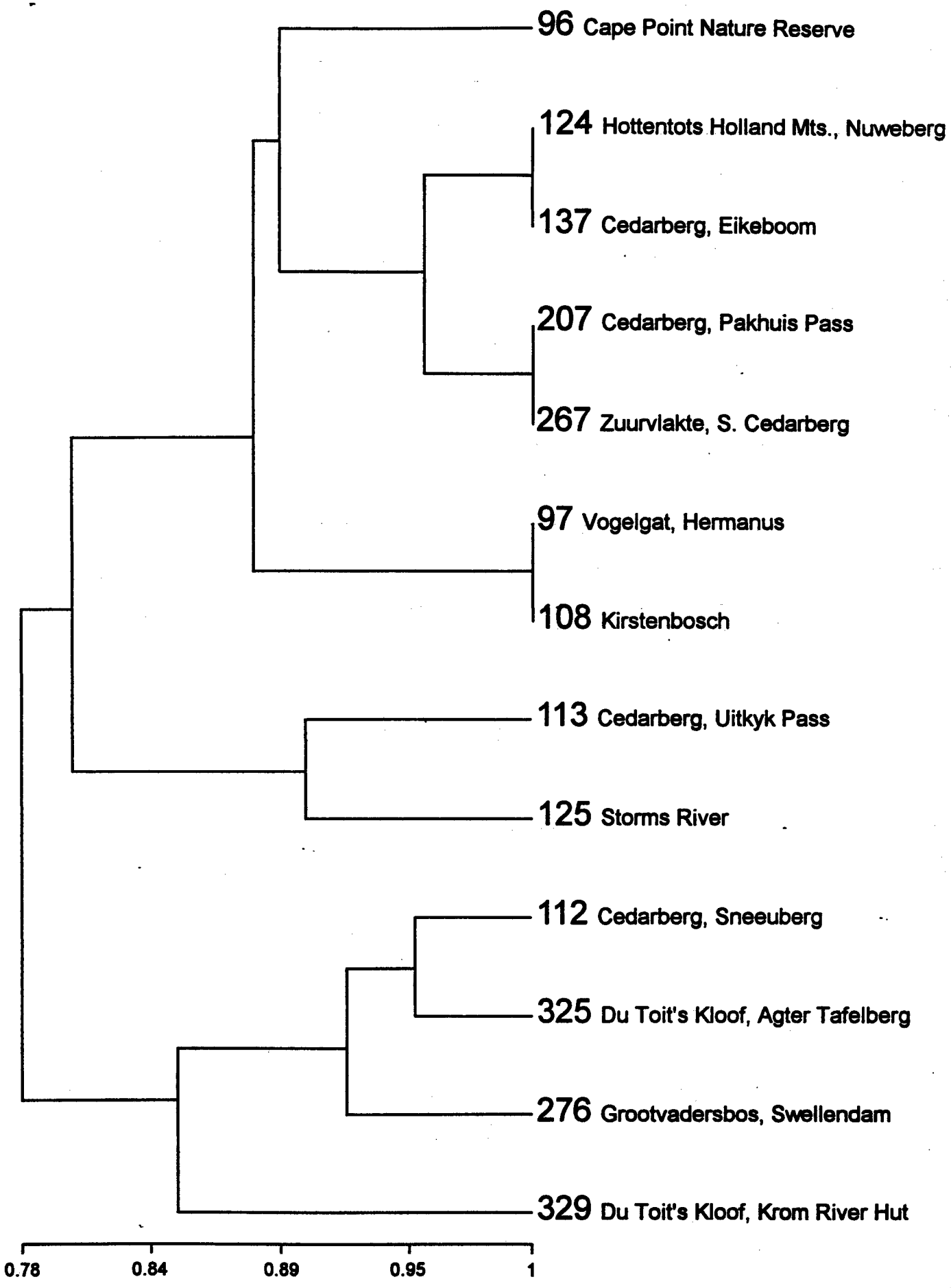


FIGURE 9:
Dendrogram generated by UPGMA analysis of foliar flavonoid data from two-dimensional paper chromatograms of leaf extracts from 13 populations of *Berzelia lanuginosa*.

in foliar flavonoid profile in the order *Audouinia capitata* → *Berzelia lanuginosa* → *Brunia nodiflora.*

Within-population variability in foliar flavonoid profile appears however to increase in the order *Brunia nodiflora* (86.7%) → *Berzelia lanuginosa* (86.2%) → *Audouinia capitata* (72.6%), as based on percentage probability (Table 20). The trend for within-population variability is thus the reverse of that for between-population variability, although little difference was evident between *Brunia nodiflora* and *Berzelia lanuginosa* in terms of within-population variation. The degree of variability between populations appeared higher than that within populations in the case of *Berzelia lanuginosa* and *Brunia nodiflora*; this was most noteworthy in the latter (18.4% difference in percentage probability for between and within population variability) as compared with *Berzelia lanuginosa* (12%). *Audouinia* on the other hand appeared to have more variability within individual populations, although differing by only 5.2% from that recorded between populations.

2. SIMILARITY COEFFICIENTS

2.1. *BERZELIA LANUGINOSA*

Despite the fact that this species has the widest distribution range of any member of Bruniaceae and exhibits a great deal of morphological variation, there did not appear to be as great a degree of chemical variability as might be expected. Within-population similarity coefficients ranged between 78% and 100%, with more than half exceeding 90% (Table 22a)

Geographical variation was expressed in terms of similarity coefficients within a range of 70-100% (Table 22b). Of the five Cedarberg collections, 113 and 137 (from the Uitkyk Pass and Eikeboom) were only 70% similar despite their geographical proximity, while 207/267, from the Zuurvlakte and Pakhuis Pass, were 100% similar although the two sites are situated at opposite ends of the Cedarberg range. Populations at the extremes of the geographical range of *Berzelia lanuginosa*, from Pakhuis Pass (207) and Storms River (125), were 90% similar while those toward the centre, in the S.W. Cape at Grootvadersbos (276) and Nuweberg (124), only 76%.

From the dendrogram (Figure 9) it can be seen that populations 124/137/207/267 formed a cluster at the 95% similarity level. The four collections were from the northern, central and southern Cedarberg and the foot of the Hottentots Holland mountains. A second cluster of populations comprised 112/325/276 - from the southern Cedarberg, Du Toit's Kloof and the Swellendam mountains respectively. A second collection from the Du Toit's Kloof mountains was linked to this cluster but at a lower level of similarity. A fifth population from the Cedarberg, at Uitkyk Pass (113) was most closely to linked to a Storms River collection (125). It does not appear from these results that the five Cedarberg collections consitute a distinct chemotype or that flavonoid races of *Berzelia lanuginosa* are emerging in any region of the species' distribution range.

2.2. *BRUNIA NODIFLORA*

At a within-population level (Table 23a), there appeared to be a greater degree of variation in chemical profile in this species than in *Berzelia lanuginosa*, although no one individual sampled was more than 30% dissimilar from any other. The greatest source of variation seemed to be between burnt and unburnt plants (samples a and b respectively), although mature foliage was used in both

cases. In only two instances however, was the similarity coefficient less than 80%.

The percentage similarity in chemical profile between the eight populations sampled varied between 53% and 96% (Table 23b); the Villiersdorp (304) and Hermanus (154) populations were most similar while the Bain's Kloof (302) and Blaauwkrantz (FJP 164) were least similar. The latter two sites approximate the extremes of the distribution range of this species while the former two are from near its centre and the findings suggest that *Brunia nodiflora* may have extended its region in a northwards and eastwards direction from a possible centre of origin in the south-western Cape. The samples used for further analysis in this study came from Gysmanshoek Pass and Kalk Bay Mountain, both sites fairly close to the postulated centre of origin.

The dendrogram (Figure 8) linked populations 154/304/323 at the 90% similarity level. The collections, from Hermanus, Villiersdorp and Karbonkelberg respectively, are from sites too geographically separated to suggest the emergence of a distinct chemotype. Collections 107/164, from Table Mountain and Blaauwkrantz Pass, were linked at the 85% similarity level but the remaining populations did not form recognisable clusters.

2.3. *AUDOUINIA CAPITATA*

As can be seen from Tables 19 and 24a, the 14 plants examined did not appear to vary greatly in profile, with no individual being less than 80% similar to any other. Compounds 1-11, 14 and 16 were almost universally present while the remainder had a more sporadic distribution. This suggested that it would not be necessary to sample more than one plant from any population in order to obtain a representative flavonoid profile. By contrast, the three different populations of *Audouinia* (Table 24b) did indicate a degree of infraspecific variation in

secondary chemistry, with collections from Cape Point and Hermanus being only 69% similar, while those from Cape Point and Karbonkelberg were 85% similar. Both the Cape Point and Hermanus populations were therefore sampled for the purposes of chemical analysis, in order to establish whether there were any differences in the flavonoids features utilised for systematic purposes. According to de Lange (1992 and pers. comm.) the Karbonkelberg population differs from the other two known populations in terms of a greater occurrence of putatively primitive features, e.g. four to five locular ovaries, three free styles.

C. DISCUSSION

A number of factors affect the distribution of genetic variability within a single species. According to Hamrick (1983) and others, the major determinants include seed dispersal mechanism, mating system, geographical distribution range and primary mode of reproduction. Others include effective population size, successional stage at which the species occurs and favoured community.

In a study of the effects of four of these traits on the distribution of genetic variability, Hamrick found mating system and seed dispersal mechanism to directly affect the degree of between and within population variation. Plants with the greatest potential for gene movement i.e. sexually-reproducing, wind pollinated species with winged or plumose seeds, were found to have little difference between and relatively more within populations. Inasmuch as none of the three species examined in the present analysis appears to be wind-pollinated and two out of three have indehiscent fruits, a greater proportion of genetic variability would be expected between than within their respective populations. The reproductive biology of neither *Berzelia lanuginosa* nor *Brunia nodiflora* has

been much studied however and it is not known to what extent outcrossing as opposed to selfing is the rule in these species.

1. WITHIN AND BETWEEN POPULATION VARIATION IN FOLIAR FLAVONOID PROFILES

As regards variation in foliar flavonoid profile between different populations of the three species examined, *Audouinia* should be regarded as a special case because of the small number of known extant populations. The fact that this species exhibited the lowest degree of between-population variability was unexpected in view of the small likelihood of gene flow between populations and the possibility that these have been separated for a long time by significant geographical barriers. Evidence of some genetic drift would not have been surprising. It was to be expected that this species exhibited the greatest degree of within-population variability of the species examined, in view of the apparent longevity of individual plants, fruit indehiscence, very short seed dispersal distance (max. 2,5m) and tendency to reproduce vegetatively. However, this species when reproducing sexually is almost 100% outcrossing (De Lange, pers.comm.)

The fact that *Berzelia lanuginosa* and *Brunia nodiflora* appeared similar in having fairly uniform within-population foliar flavonoid patterns suggests that there is free gene flow between individual plants at a particular site, despite the indehiscent fruits of the former and resprouting capacity of the latter. One would therefore probably be justified in sampling only one or two plants from a population for chemical analysis. As regards between-population variation, the relatively high degree shown by *Brunia nodiflora* suggests that more intensive sampling would be necessary. Whether this holds true for other resprouting

species in Bruniaceae is not known and it is difficult to explain, without an understanding of other traits affecting gene flow between populations, why this species should have a particularly high between:within population ratio of genetic variability.

2. SIMILARITY COEFFICIENTS

2.1. *BERZELIA LANUGINOSA*

The general impression gained from inspection of both the Sorensen half-matrix (Table 22b) and the dendrogram (Figure 9) is that the populations of *Berzelia lanuginosa* examined do not differ sufficiently in foliar flavonoid profiles to warrant sampling of more than two within the distribution range of the species. Free gene flow appears to be possible between populations that are geographically quite widely separated e.g. those of the Hottentots Holland (Collection 124) and central Cedarberg (Collection 137) mountains, resulting in identical chemical profiles. While there is evidence of some clustering of the Cedarberg populations e.g. collections 137, 207 and 267, yet others e.g. 112 and 113 appear to be more similar to distant than to neighbouring relatives. The morphological distinctiveness of the Cedarberg form of *Berzelia lanuginosa* e.g. having bright yellow flowers compared with pale cream elsewhere, does not appear to be accompanied by similarly unusual foliar flavonoid patterns and no emergence of a local chemical race could be detected in this species.

2.2. BRUNIA NODIFLORA

The dendrogram (Figure 8) and Sorensen matrix (Table 23b) suggest that *Brunia nodiflora* populations do not have as free an exchange of genes as do

those of *Berzelia lanuginosa*. No tight clustering of populations was evident from the dendrogram and geographically close localities yielded collections that appeared quite dissimilar in foliar flavonoid profile e.g. the three populations from the Cape Peninsula, at Kalk Bay, Karbonkelberg and the mountain slopes above Kirstenbosch (338, 323, 107). This finding confirms the results of analysis of within and between population variability.

CHAPTER 4

SURVEY OF FOLIAR FLAVONOID DISTRIBUTION PATTERNS IN BRUNIACEAE

A. METHODS

1. COLLECTION AND DRYING OF LEAF MATERIAL

The aim of this study was to examine fresh leaf material of all currently recognized species in Bruniaceae. This proved to be impossible; three species are could not be found despite careful searching and a further four appear to have gone extinct due to habitat destruction or too-frequent fires (Hilton-Taylor, 1996). Six are known from a single poorly-described locality or remote mountain habitat and a new species, *Linconia ericoides* E.G.H. Oliv. (Oliver, 1999), was discovered too recently for inclusion the present study. Nevertheless, a total of 56 out of 76 species, representing all genera in Bruniaceae, was collected from natural stands of vegetation. Two separate collections were made of 31 species, from different localities within their distribution ranges. The three species in Grubbiaceae, together with the monotypic *Geissoloma marginatum* and *Retzia capensis*, were also sampled from natural vegetation, in duplicate where possible. Voucher specimens were lodged at NBG (see APPENDIX 1). Dried leaf material of two species belonging to Diapensiaceae was obtained from the Royal Botanic Garden, Edinburgh and of *Erica arborea* from Chelsea Physic Garden, London. Voucher specimens for these species are lodged at the respective institutions. All material was dried at 40°C in a ventilated oven for 72 hours, ground in a Moulinex grinder and passed through a sieve (212 µm mesh) prior to extraction.

2. PHYTOCHEMICAL METHODS

The choice of a fractionation technique appropriate to this study took into account considerations of cost, laboratory capability, numbers of samples requiring analysis, the (in many cases) small amounts of leaf material available for each species and general convenience. An attempt was made to separate aglycone mixtures using preparative centrifugal thin layer chromatography (Hostettmann, 1985) on 4 mm cellulose layers, but this was found to be time-consuming and therefore unsuitable for dealing efficiently with large numbers of samples. Paper chromatography, although slow, appeared to be the best choice in this instance and was used throughout the study, supplemented by TLC on cellulose (Schleicher and Schull pre-coated plates) where necessary. High performance liquid chromatography (HPLC) would also have been an appropriate technique for flavonoid analysis but was not available to me at the time (1993-4).

2.1. ANALYSIS OF GLYCOSIDES

Direct extracts were prepared (see Figure 10) of a total of 60 species, representing all genera in Bruniaceae, all members of Grubbiaceae, two of Diapensiaceae and one species each from the putatively allied families Geissolomataceae, Ericaceae and Retziaceae. The extracts were applied to Whatman No. 3 chromatography paper (4x ½ sheets per sample) and developed by 2D PC in BAW and AcH 15%. The separated compounds were eluted by overnight soaking in 70% EtOH and purified by running as a band in either BAW or 15% AcH or both. Rf values in each solvent were noted, as was the position of

LEAF MATERIAL (500mg dry powder)

i. extract with 90% and 50% methanol
ii. combine extracts and remove bulk of solvent in rotary evaporator (40°C)

DIRECT EXTRACT:GLYCOSIDES, TANNINS, COUMARINS, SIMPLE PHENOLIC ACIDS

i. evaporate to dryness in fume cupboard
ii. take up residue in 90% ethanol (1 ml)
iii. divide into 2 x 0,5ml aliquots

ALIQUOT1

i. spot on to Whatman 3MM paper (4x1/4 sheets)
ii. develop in BAW rutin marker
iii. dry and develop in 15% AcH

ALIQUOT2

i. Hydrolyse: 2NHCl/45 mins/100°C (waterbath)
ii. Extract with 3x10ml portions diethyl ether
iii. Extract with 3x10ml portions of amyl alcohol

2-D CHROMATOGRAMS (4)

i. Measure Rf values
ii. Note behaviour in UV light
iii. cut out corresponding spots
iv. Elute overnight in 70% ethanol
v. Evaporate eluate to dryness

ISOLATED GLYCOSIDES (impure)

i. Run as a band in BAW
ii. Check Rf value and behaviour in UV light
iii. Elute and run in 15% AcH

PURIFIED GLYCOSIDES

i. Measure UV spectra (shift reagents)
ii. Attempt provisional ID (compare published spectra)
iii. compare with aglycone pattern
iv. If sufficient material, attempt confirmation of ID (^{1}H NMR,MS)

AGLYCONES + PHENOLIC ACIDS

ETHER EXTRACT

i. wash with water to remove acid
ii. Evaporate to dryness
iii. Take up residue and run on Whatman N°1 paper or TLC cellulose plates
Solvents: BAW
Forestal
PhOH
Water
CAW } TLC
Markers: flavones
flavonols
C-glycosides
isoflavone
phenolic acids
iv. Note Rf vlaues behaviour in UV light and colour rxn with NA
v. Attempt aglycone ID

AMYL ALCOHOL EXTRACT

i. Evaporate to dryness
ii. Take up in 1% HCl in MeOH
iii. Run on Whatman N°1 paper using cyanidin, delphinidin and pelargonidin markers
Solvents: BAW
Roux
Forestal
50% AcH
iv. Measure Rf values of separated compounds, note behaviour in UV light
v. Attempt aglycone ID

FIGURE 10. Flow chart depicting the isolation and identification of leaf flavonoid glycosides, aglycones and phenolic acids.

TABLE 25. Flavonols, anthocyanidins and phenolic acids detected in a pilot study of leaf hydrolysates of genera of Bruniaceae and allied families.

FAMILY	GENUS	FLAVONOLS				PHENOLIC ACIDS		ANTHOCYANINS	
		M	Q	K	Iso	E.A	G.A	C	D
BRUNIACEAE	*Linconia*	—	+	—	+	—	—	—	—
	Audouinia	tr.	+	tr	—	—	—	+	—
	Tittmannia	—	+	tr.	+	—	—	+	—
	Nebelia	tr.	+	—	+	—	—	+	—
	Thamnea	—	+	+	+	—	—	+	—
	Berzelia	+	+	+	+	—	—	+	+
	Brunia	+	+	+	+	—	—	+	+
	Pseudobaeckia	+	+	+	+	—	—	+	+
	Mniothamnea	tr.	+	tr.	+	—	—	+	+
	Raspalia	+	+	—	+	—	—	+	+
	Lonchostoma	+	+	—	—	—	—	+	+
	Staavia	+	+	—	+	—	—	tr.	+
PENAEACEAE	*Penaea*	—	+	—	—	+	+	tr.	+
GRUBBIACEAE	*Grubbia*	—	+	—	—	+	+	tr.	—
RETZIACEAE	*Retzia*	—	—	—	—	—	—	—	—
GEISSOLOMACEAE	*Geissoloma*	—	tr.	—	—	+	—	—	—
DIAPENSIACEAE	*Galax*	—	+	—	tr.	+	+	—	—
CUNONIACEAE	*Cunonia* *	—	+	—	—	+	+	—	+

* from Jay (1968)

M=myricetin
Q=quercetin
K=kaempferol
Iso=isohamnetin
tr.=trace

EA=ellagic acid
GA=gallic acid
C=cyanidin
D=delphinidin

the compound on the finished PC and its behaviour in UV light before and after fuming with NH_3 vapour.

The purified glycosides were redissolved in spectral grade methanol and analysed on a Beckman 35 UV-visible spectrophotometer according to the method of Mabry *et al.* (1970), using shift reagents as an aid to identification. A discussion of the principles of this method appears in Chapter 2 (Section 3.1, page 25). The results appear in Appendix 5.

As an additional aid to diagnosis of compounds separated by PC, 50µl aliquots of direct extract were applied to each of 4 x 1/4 sheets of Whatman N° 1 paper and the chromatograms developed two-dimensionally in BAW and 15% AcH. Each of the 4 dried chromatograms was sprayed with one of the following reagents (see APPENDIX 2):

1) Naturstoffreagenz A
2) $AlCl_3$ reagent
3) Pauly's reagent
4) Vanillin/HCl reagent

The chromatograms were viewed in natural and UV light (365nm) and the colour reactions of separated compounds with the four reagents noted.

2.2. ANALYSIS OF AGLYCONES

Residues of extracts prepared in 2.1. were hydrolysed on a boiling water bath for 30 minutes with 2N HCl and the cooled hydrolysate extracted first with diethyl ether (3 × 15 ml), then with amyl alcohol (3 × 15ml), each extract being evaporated to dryness on a watch glass and the residue taken up in 90% ethanol (EtOH). Aliquots of 50 µl were applied to 1 M Whatman paper for one-

dimensional chromatography and the resultant chromatograms developed by the descending method in the following solvents, the composition of which appears in Appendix 2.

a) Ether fraction: BAW, CAW, Forestal, 50% AcH, PhOH, H_2O.

b) Amyl alcohol fraction: BAW, Forestal, Roux solvent.

In the case of ether fractions run in CAW, Schleicher and Schull pre-coated cellulose TLC plates were used and developed by ascending chromatography. The following markers were used:

Flavonols: myricetin; quercetin, kaempferol, isorhamnetin, gossypetin, fisetin.

Flavones: apigenin, luteolin, diosmetin, chrysoeriol

C-glycosides: vitexin, orientin

Anthocyanidins: delphinidin, cyanidin, pelargonidin

Phenols: ellagic acid, gallic acid

Isoflavones: daidzein

The developed chromatograms were dried, viewed in ultra-violet (UV) light of wave-length (λ) 365 nm, before and after fuming with ammonia vapour (NH_3), and finally sprayed with Naturstoffreagenz A (2-amino-ethyl diphenylborinate 1% in MeOH). Compounds were identified (see APPENDIX 3) from their Rf values in the solvent systems used, co-chromatography with markers and behaviour in UV light as well as with the visualising reagent. The results appear in APPENDIX 6. A flow chart (Figure 10) shows the procedure followed in the isolation and identification of leaf flavonoid glycosides, aglycones and phenolic acids.

2.3. ANALYSIS OF FLAVONOID SULPHATES AND GLUCURONIDES

Direct extracts were prepared of the following species: *Audouinia capitata*, *Lonchostoma esterhuyseniae*, *Brunia stokoei*, and *Berzelia cordifolia*. Aliquots were applied to Whatman No.1 chromatography paper and the presence/absence of flavonoid sulphates/glucuronides ascertained using paper electrophoresis in buffer solutions of pH 2,2 (formate-acetate) and pH 4,4 (acetate) respectively. The composition of the buffer solutions appears in APPENDIX 2. Quercetin-3-sulphate and apigenin-7-0-glucuronide were used as markers in the respective systems.

B. RESULTS

1. ANALYSIS OF GLYCOSIDES

Extraction of dried leaf material with successive portions of 90% and 50% methanol yielded a mixture containing flavonoid glycosides, phenolic acids, coumarins, tannins and chlorophylls, together with other polar to moderately polar compounds. The chlorophylls were deposited on the sides of the rotary evaporator flask during solvent removal, leaving a more or less clear yellow solution which was taken to dryness in a fume cupboard.

The method used for fractionation of the direct extract i.e. preparative paper chromatography, although slow, permitted several extracts to be processed simultaneously in the large chromatanks available. This was an advantage in view of the high number of samples to be processed, as was the low cost of the technique. The two dimensional chromatograms provided preliminary diagnostic information as to the:

i) classes and degree of glycosylation of flavonoid compounds present, inferred from their position on the chromatogram (see APPENDIX 4A).

ii) structure of compounds present, inferred from their behaviour in UV-light (365nm), with or without the presence of NH_3 vapour. (see APPENDIX 4B)

iii) Identity of compounds present, inferred from their Rf values in the two solvents used (BAW and 15% AcH) when compared with published values (Mabry *et al*, 1970; Harborne 1967 and 1984; Markham; 1982).

Elution of the compounds separated by 2D-PC, followed by purification by running as bands on 3M paper in at least two different solvents, appeared satisfactory in that single bands were usually obtained after the second run. In retrospect however, it would have been better to carry out a final clean-up of each compound on Sephadex to remove traces of impurities which contaminated some of the samples and rendered interpretation of their spectra more difficult.

The purpose of direct extract analysis was to obtain a flavonoid glycoside profile for each genus included in the study. Rather than attempt to identify each compound present on the chromatogram, only the major flavonoids were selected, according to spot size, position and colour reactions in UV light, for further investigation. Since the procedure followed in each case was identical, a detailed account of chromatographic and spectral interpretations is given for only one species. Corresponding data for the remainder appear in APPENDIX 5.

The procedure followed in this study for the analysis of flavonoid glycoside patterns, is exemplified by the identification, in *Berzelia cordifolia* leaf extracts, of quercetin-3-0-rhamnoside (quercitrin)=*B. cordifolia* fraction 6.

1.1 Preliminary diagnosis

Fraction 6 appeared, when viewed in UV light (365nm), as a deep puce/purple compound at Rf 0,79 (BAW) and 0.54 (AcH 15%). A colour change to bright mustard yellow was noted on fuming with NH_3 vapour and the compound was rendered visible peach-orange in natural light, on spraying with NA reagent.

Its behaviour in UV light suggested that the compound could be a 5-OH flavone or flavonol (3-0-substituted, with a 4'-OH group) or possibly a 5-OH flavanone or 4' OH chalcone with no B-ring hydroxyl groups. The position of the compound on a 2D PC suggested a flavonol 3-0-mono or diglycoside and its colour reaction with NA reagent the presence of a 3',4'-di-OH pattern.

1.2 Ultra-violet spectroscopy

UV spectroscopy is a standard tool for the determination of flavonoid functional groups. Neutral spectral maxima provide information as to flavonoid class, while the use of "shift" reagents assists with establishing the presence, absence or possible substitution of A and B ring OH groups. Spectral shape and maxima can be compared with published data, allowing an approximate identification to be made. The spectra obtained for the compound under investigation appear in Figure 11 and spectral shift data in Table 26.

Table 26. UV/visible spectral shifts for *Berzelia cordifolia* Fraction 6.

	spectral maxima (nm)			
solution in MeOH	Band I	Band II	Band III	Spectral effect
i) Alone	258		348	27 nm hypsochromic shift compared to quercetin (Band II 375 nm)
ii)+2 drops 5% $AlCl_3$	295		434	86 nm bathochromic shift in Band III
iii) Soln (ii)+2 drops HCl	298	351	396	48 nm bathochromic shift in Band III
iv)+NaOAc	267	327	373	9 nm bathochromic shift in Band I
v) Soln (iv)+H_3BO_3	260		367	19 nm bathochromic shift in Band III
vi) +2 drops 2M NaOH	271	330	394	46 nm bathochromic shift in Band III

The methanol spectrum (Table 26 i) suggested either a flavone (Band I 250-270 nm; Band III 330-350 nm) or a flavonol (Band I 250-270 nm; Band III 350-390 nm). The hypsochromic shift in Band III as compared with quercetin suggested that the 3-OH was substituted; this would be consistent with a flavonol 3-O-glycoside as indicated by the position of the compound on a 2D PC.

A large bathochromic shift in Band III seen on addition of 5% $AlCl_3$ (Table 26 ii) to the methanolic solution (i) was indicative of a free OH group at C_3 or C_5 and/or an o-dihydroxy system. Reduction in the magnitude of the shift on addition of HCl (iii) suggested the latter arrangement was present (acid-labile complexation with $AlCl_3$), while a bathochromic shift in Band III of 48 nm relative to the MeOH spectrum remaining after the addition of HCl (Table 26 iii) suggested the presence of a free 3- or 5-hydroxyl group. The size of the shift was consistent with either a 5-OH flavone or 5-OH, 3 substituted flavonol (bathochromic shift of 35-55 nm in Band III with $AlCl_3$/HCl). Flavonols with a free 3-OH group characteristically give acid-stable bathochromic shifts of more than 60 nm with

$AlCl_3$/HCl. A complete regeneration of the methanol spectrum on addition of HCl to the $AlCl_3$ complexed flavonoid would have indicated either absence or substitution of both 3-and 5-OH groups.

Addition of powdered sodium acetate (NaOAc) to the methanolic solution produced a 9nm bathochromic shift in Band I (Table 26 iv). This was indicative of the presence of a 7-OH group, ionisation of which by NaOAc characteristically produces a Band I shift. Ionisation of a 3- and/or 4'-OH group is also seen with this reagent, but does not affect Band I.

Addition of powdered boric acid (H_3BO_3) to (iv) produced a bathochromic shift of 19nm in Band III (Table 26 v); this suggested the presence of B ring o-diOH groups, which chelate with H_3BO_3 in the presence of NaOAc, giving bathochromic Band III shifts of 12-30nm. Other o-diOH groups such as 6,7 or 7,8 (A ring) characteristically exhibit a smaller (5-10nm) shift.

Addition of 2M NaOH to (i) produced a stable bathochromic shift of 46nm (Table 26 vi) in Band III. This is diagnostic for the presence of a free 4' OH group (40-65 nm bathochromic shift in Band III, with no loss of intensity). An unstable NaOH spectrum would have suggested one of the following hydroxylation patterns: 3, 4'; 3, 3', 4'; 5, 6, 7; 5, 7, 8; 3', 4', 5'. Flavonoids with this arrangement are unstable in NaOMe.

The combined information provided by the UV spectra suggested that compound 6 was likely to be a flavonol with a 3-0 linked sugar and a 3',4',5,7 hydroxylation pattern. The fairly high Rf value in BAW indicated a single sugar (more than one would tend to lower the Rf value in BAW and raise that in AcH 15%), probably with a methyl group, as in rhamnose. From tables of Rf values and MeOH spectral maxima (Band III), quercetin 3-0-rhamnoside (quercetrin, Band III maximum 350 nm) was selected as being closest to the unknown as regards spectral characteristics, although the Rf values in BAW/AcH 15% were

(iv) A sharp singlet at δ 5,26, consistent with the presence of an 0-rhamnoside, the C-1 proton of which has an equatorial - equatorial coupling with the C-2 proton and gives a resonance signal in the region δ 5,0-5,3. This is a property of the α-linkage common to most natural flavonoid rhamnosides and contrasts with the β-linkage of most natural glucosides. The latter gives rise to diaxial coupling between the C-1 and C-2 protons of both 3-0- and 7-0-glucosides, the signals appearing as a doublet and multiplet respectively.

(v) Two doublets, at δ 6,19 (J = 1,6Hz) and δ 6,39 (J = 1,6Hz), representing the meta-related protons of C-6 and C-8 respectively. These chemical shifts are characteristic of flavones, flavonols and isoflavones having a 5,7 di-OH substitution pattern (H-6: δ 6,0-6,2 and H-8: δ 6,3-6,5) and no 7-0-linked sugar. The latter, if present, causes a downfield shift of both signals as does the absence of a 5-OH group which gives rise, in addition, to a doublet at ± δ 8 due to o-coupling between the C-5 and C-6 protons.

(vi) A sharp doublet at δ 6,87 (J = 8,0 Hz), characteristic of the H-5' proton of 3',4' di-OH-flavonoids, which is o-coupled to that of C-6'. In 4'-OH-flavonoids, a C-3' doublet would be superimposed on its identical C-5' counterpart and a larger signal recorded.

(vii) One doublet, at δ 7,30 (J = 1,2 Hz), representing the C-2' proton and a double doublet at δ 7,25 (J = 8,0; 1,2 Hz) representing C-6'. The C-6' and C-2' resonances are characteristic for 3',4', di-OH-flavonols (range δ 7,2,-7,9) as opposed to 4'-OH-flavonols (range δ 7,9-8,1)

The ^{1}H NMR spectrum supported the identification of Fraction 6 as quercitrin, although the diagnosis was based on data relating to flavonoid trimethylsilyl (TMS) ethers dissolved in carbon tetrachloride (CCl_4), rather than underivatised flavonoids in DMSO-d_6. However, the DMSO-d_6 spectrum of Fraction 6 compared well with that of quercitrin TMS-ether/CCl_4 (Mabry *et al*, 1970) as did

chemical shift data for a range of common flavonoids in DMSO-d_6 with that of their TMS-ether counterparts in CCl_4 (Batterham and Highet, 1964; Hillis and Horn, 1965). This suggested that ^{1}H NMR data for flavonoid TMS-ethers/CCl_4 could be applied to the interpretation of DMSO-d_6 spectra.

1.4 Identification of aglycone

The material recovered from ^{1}H NMR spectroscopy after removal of DMSO-d_6 (cold air draught, fume hood) was treated with 2N HCl for 30 minutes on a boiling water bath and analysed for the presence of common flavonoid aglycones. The identity of Fraction 6 aglycone was confirmed as quercetin, further supporting diagnosis of the parent glycoside as quercitrin.

This procedure was useful in that it permitted the tentative identification of quercitrin in other *Berzelia* species from observation of their 2D paper chromatograms. Quercitrin, because of its relatively high Rf value in BAW as compared with the majority of glycosides occurring in the genus, is well separated from these and visible as a deep puce-purple spot in the far left hand segment of a 2D PC, becoming mustard-yellow on fuming with ammonia and giving a bright yellow to peach colour with NA reagent. Its presence was tentatively identified in all *Berzelia* species examined except *Berzelia lanuginosa* and *B. abrotanoides*, and confirmed by later UV-spectral analysis. Quercitrin was also identified in *Brunia albiflora* and *Erica arborea*, but not in any other species examined in the present study.

2. ANALYSIS OF AGLYCONES

The determination of an aglycone profile for the family provided baseline information that could be directly applied to systematics as well as used for comparison with data obtained from glycoside analysis. The results of aglycone analysis (APPENDIX 6) are expressed only in terms of those compounds for which markers were available for co-chromatography. The sugars released during hydrolysis were not identified.

2.1. PHENOLS

The presence of the simple phenols gallic and ellagic acid was checked in chromatograms of leaf extract hydrolysates. Neither was detected in any species of Bruniaceae, Retziaceae or in *Erica arborea*, sole representative of Ericaceae. Both compounds were found in all species of Grubbiaceae and in the two representatives of Diapensiaceae included in this study. Ellagic but not gallic acid was detected in Geissolomaceae. The two tannin hydrolysates were distinguishable from flavonoids on paper chromatograms on the basis of their pale lilac (yellow in NH_3) and bright blue fluorescence (ellagic and gallic acid respectively) in UV light. The absence of hydrolysable tannins from Bruniaceae does not suggest a close relationship between this family and either Grubbiaceae, Diapensiaceae or Geissolomaceae.

The presence of the phenolic glycoside arbutin, reported from subfamilies Vaccinioideae and Pyroloideae of Ericaceae (Stevens, 1971) by Harborne and Williams (1973) was checked in 2D chromatograms sprayed with Folin reagent. Neither arbutin nor its aglycone hydroquinone were identified in any of the species examined.

2.2. PROANTHOCYANIDINS

Procyanidin was detected in all members of Bruniaceae except the two *Linconia* spp., in *Erica arborea*, *Shortia soldanelloides*, *Galax urceolata* and in trace amounts in the three *Grubbia* species, but not in Geissolomaceae or Retziaceae. Prodelphinidin had a more limited distribution, not being recorded from Geissolomaceae, Retziaceae, Diapensiaceae, Grubbiaceae or from *Tittmannia*, *Thamnea*, *Audouinia*, *Nebelia* and *Linconia* (Bruniaceae). Those genera in Bruniaceae that did have prodelphinidin were almost uniformly positive for this compound. Grubbiaceae, Geissolomataceae and Diapensiaceae thus appear to synthesise mainly hydrolysable tannins, Bruniaceae and *Erica arborea* only condensed tannins and Retziaceae neither.

2.3. FLAVONOLS

Quercetin was present in all species examined except *Geissoloma marginatum*, myricetin in 35 species (57,3% of total) and kaempferol in 33 species (54% of total). In Bruniaceae, myricetin was detected in 7/12 genera: 9/9 *Berzelia*, 5/6 *Brunia*, 5/5 *Lonchostoma*, 1/1 *Mniothamnea*, 2/3 *Pseudobaeckia*, 4/9 *Raspalia* and 5/5 *Staavia* species; kaempferol occurred in 8/12 genera: 8/9 *Berzelia*, 6/6 *Brunia*, 2/2 *Linconia*, 2/3 *Pseudobaeckia*, 4/9 *Raspalia*, 2/5 *Staavia*, 3/4 *Thamnea* and 3/3 *Tittmannia* species. The quercetin methyl ether isorhamnetin was detected in 70% of species in all genera of Bruniaceae except *Audouinia* and in *Galax urceolata* (Diapensiaceae) but not in the other species investigated. Its occurrence in 0/1 *Audouinia*, 1/5 *Lonchostoma*, 4/9 *Berzelia*, 5/6 *Brunia*, 6/9 *Raspalia*, 4/5 *Thamnea* and all other species of the family examined in this study may be of phylogenetic significance. Although isorhamnetin has

rather similar Rf values to the common flavonols myricetin, quercetin and kaempferol in several solvents, it separated well in CAW and PhOH, running ahead of the other flavonols.

Of the families surveyed, Geissolomaceae was unusual in apparently lacking common flavonols altogether. Among the rarer flavonols, gossypetin was recorded as present in *Erica arborea, Galax urceolata* and possibly in *Shortia soldanelloides*, but not in any other species surveyed. The 5-deoxy flavonol fisetin was recorded as present in 8/9 *Raspalia* species, a finding that was consistent with the detection, in this genus, of glycosides lacking a 5-OH function.

Quercetin 3-0-methyl ether, distinguishable on paper chromatograms from most other flavonols by its deep purple colour in UV light changing to yellow on fuming with ammonia, was recorded as present in several genera of Bruniaceae, namely *Nebelia, Staavia, Berzelia* and *Brunia.* Although the Rf value of 3-O-methyl quercetin is higher than that of the common flavonols in most of the solvents used for chromatography, it may be confused with flavone aglycones such as chrysoeriol and the presence of this compound in Bruniaceae requires confirmation by co-chromatography.

A tentative identification, based on Rf values in the solvents used and colour reactions in UV light compared with published data, was made of compounds for which markers were not available. These included quercetin 5-methyl ether (azaleatin) and quercetin 3,5 dimethyl ether (caryatin).

2.3 FLAVONES

Of the flavones for which marker compounds were available, neither diosmetin nor apigenin was detected in any of the species examined. Luteolin was

recorded as present in 3/4 *Nebelia*, 7/9 *Raspalia and* 3/3 *Grubbia* species and chrysoeriol in 3/4 *Nebelia* and 5/9 *Raspalia* species.

The presence of the C-glycosyl flavone orientin was tentatively identified in some *Nebelia* species, although iso-orientin, usually produced by isomerisation during acid hydrolysis, was not detected on paper chromatograms run in water as solvent. The occurrence of orientin therefore requires confirmation. The apigenin analogue vitexin was not found.

3. OCCURRENCE OF FLAVONOID SULPHATES AND GLUCURONIDES

The four species of Bruniaceae investigated, namely *Audouinia capitata, Brunia stokoei, Berzelia cordifolia* and *Lonchostoma esterhuyseniae*, did not appear to contain either flavonoid sulphates or glucuronides, as evidenced by lack of mobility of their leaf direct extracts during paper electrophoresis. Although further examination may reveal the presence of these electrically charged flavonoids in species of Bruniaceae, the preliminary investigation does not suggest that they are a characteristic feature of the family.

C. DISCUSSION OF RESULTS

On the basis of the results obtained, it was possible to provide a flavonoid profile at family, generic and specific level. At family level Bruniaceae was characterised by the presence of proanthocyanidins in 54/56 species investigated and a total absence of hydrolysable tannins. Quercetin was recorded in almost all species examined, while the common flavonols myricetin and kaempferol had a more limited distribution and characterised only certain genera. Isorhamnetin was

present in 11/12 genera and quercetin-3-methyl ether in 4/12. Flavones were detected only in *Nebelia* and *Raspalia*. Flavonoids lacking a 5-OH function were found in *Raspalia*, *Pseudobaeckia*, *Staavia*, *Nebelia* and *Berzelia* species; this could be the effect of 5-deoxygenation (fisetin was identified in 7/9 *Raspalia* spp.), 5-O-glycosylation or substitution and is an aspect of the secondary chemistry of Bruniaceae that merits further investigation.

At generic level, the following profiles were established:

Audouinia

A simple profile, based on glycosides of quercetin and possibly kaempferol, characterises this genus. Prodelphinidin, isorhamnetin, fisetin and flavones appear to be absent.

Berzelia

A fairly uniform profile, based on myricetin in 9/9, quercetin in 9/9, kaempferol in 9/9, isorhamnetin in 5/9, procyanidin in 9/9, prodelphinidin in 8/9 and quercetin-3-methyl ether in 3/9 species, was identified. Fisetin and flavones were not detected. Glycosides appear to be based mainly on isorhamnetin and quercetin. Quercitrin was detected in 4/9 species, and glycosides lacking a 5-OH function in 2/9 species.

Brunia

This genus also has a uniform profile based on procyanidin in 6/6, prodelphinidin in 5/6, myricetin in 5/6, quercetin and kaempferol in 6/6, isorhamnetin in 5/6 and quercetin-3-methyl ether in 2/6 species. Flavones and fisetin were not detected. Glycosides of all the common flavonols were found, but none lacking a 5-OH function.

Linconia

The aglycones quercetin, kaempferol, isorhamnetin and glycosides based on these were detected in 2/2 species. Proanthocyanidins, flavones and fisetin appear to be absent.

Lonchostoma

A simple uniform profile, based on quercetin, myricetin, procyanidin and prodelphinidin in 5/5 species, characterises this genus. Isorhamnetin and glycosides of isorhamnetin were detected in *L. purpureum* only. Kaempferol, flavones and fisetin were not found.

Mniothamnea

The single species sampled from this small genus has a simple profile based on myricetin, quercetin, procyanidin, prodelphinidin and isorhamnetin. Flavones, kaempferol and fisetin were not detected. Glycosides appear to be based mainly on isorhamnetin.

Nebelia

A uniform profile, based on quercetin in 4/4, isorhamnetin in 4/4, luteolin in 3/4 and chrysoeriol in 3/4 species, is characteristic of this genus. Prodelphinidin (PD), kaempferol and myricetin were not found except for PD in *N. sphaerocephala.* Glycosides lacking a 5-OH function were detected in *N. laevis,* and quercetin-3-methyl ether in *N. paleacea.*

Pseudobaeckia

The three species analysed have a non-uniform profile with myricetin in 2/3, kaempferol in 2/3 and isorhamnetin in 2/3 species. Procyanidin and prodelphinidin were detected in 3/3 and flavones in 0/3 species. Glycosides appear to be based mainly on quercetin and isorhamnetin. Glycosides lacking a 5-OH function (possibly based on kaempferol-5-methyl ether or azaleatin) were detected in 2/3 species.

Raspalia

The largest genus in the family, *Raspalia* has the most complex profile of genera in Bruniaceae. Myricetin was detected in 3/9, kaempferol in 2/9, isorhamnetin in 6/9, fisetin in 8/9, luteolin in 7/9 and chrysoeriol in 5/9 species. Glycosides of fisetin (?), luteolin and quercetin were detected while glycosides lacking a 5-OH function were recorded from 4/9 species.

Staavia

Staavia species appear to possess a uniform profile based on myricetin, quercetin, isorhamnetin, procyanidin, prodelphinidin in 5/5 species, with kaempferol in 1/5. Neither fisetin nor flavones were detected, while glycosides lacking a 5-OH function were recorded from 1/5 species.

Thamnea

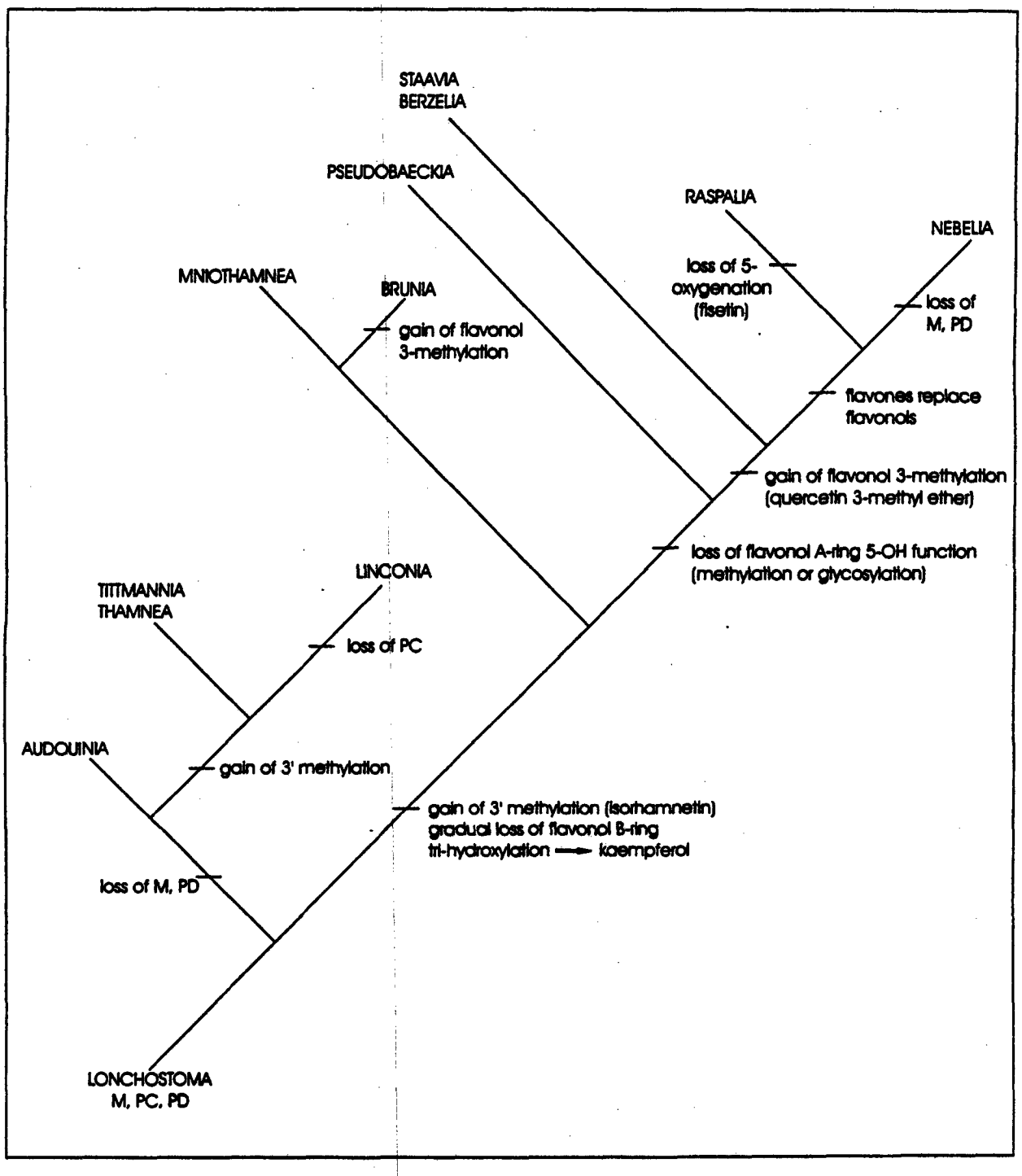
Quercetin was detected in 4/4, kaempferol in 3/4, isorhamnetin in 3/4 and procyanidin in 4/4 species. Myricetin, prodelphinidin, flavones and fisetin were not found.

Titmannia

This genus has a profile similar to that of *Thamnea*, based on quercetin, kaempferol, isorhamnetin, procyanidin in 3/3 species. Myricetin, prodelphinidin, flavones and fisetin were not found.

These findings are in accordance with the results of Jay's (1968) survey of common flavonoids and phenolic acids in 6 species of Bruniaceae. On the basis of flavonoid distribution patterns noted in the present study, it is possible to propose a phylogeny for Bruniaceae. If, as has been proposed by Harborne, development within a particular plant group is accompanied by loss of B-ring hydroxylation, replacement of flavonols by flavones and increasing 0-methylation, then evolution within Bruniaceae may have proceeded as depicted below.

In terms of this hypothesis, *Lonchostoma* is closest to the ancestral condition. The merging of *Thamnea* and *Tittmannia*, as proposed by Powrie (unpublished MS), is supported, but not of *Brunia* with *Nebelia*, *Raspalia* with *Pseudobaeckia* or *Berzelia* with *Mniothamnea*. However, as discussed in Chapter 2, a phylogeny based on a limited data set is unlikely to be reliable. The flavonoid data obtained in the present study, in combination with all other available evidence, should be assessed by cladistic method.



Hypothesis for the evolution of Bruniaceae, based on foliar flavonoid patterns.

M = myricetin, PC = procyanidin, PD = prodelphinidin

CHAPTER 5

CLADISTIC ANALYSES

A. METHODS

Two separate phylogenetic analyses were undertaken; the first had as its objective the identification of sister group(s) of Bruniaceae (analysis 1) and the second, based on the results of the first, examination of relationships within the family (analysis 2). Trees were rooted by designating outgroups (Nixon and Carpenter, 1993) and the character analysis programme CLADOS (Nixon, 1991) used to obtain diagnostic information on character distribution on the cladograms.

1. Selection of terminal taxa

1.1 Analysis 1: Identification of sister group(s) of Bruniaceae

The placement of Bruniaceae in classifications based on both traditional phyletic systematics and cladistic studies has been controversial (see Chapter 1, pp. 17-19). The present analysis attempted to identify with greater precision, using data from chemistry, morphology, anatomy, palynology, embryology and cytology, the sister group(s) of Bruniaceae, using the currently available molecular phylogenies as a guide to the inclusion of appropriate taxa. That of Chase *et al.* (1993), based on a large data set of some 500 species of seed plants, was the main point of reference rather than the smaller set (223 species) analysed by Soltis *et al.* (1997). The Asterid 2 and 3 clades (between which Bruniaceae was placed) retrieved in the Chase *et al.* analysis provided a guide to the choice of terminal taxa for use in the present analysis; the aim of this approach was to limit the number of possible sister group options in order to place Bruniaceae less equivocally within the Asteridae *s.l.* This was the approach adopted by Olmstead

et al. (1993) in their analysis of relationships in Asteridae *s.l.* These authors argued that "the coarse nature of the Chase *et al.* analysis weakens its ability to generate rigorous hypotheses, but serves a useful purpose by suggesting sets of taxa that merit inclusion in a more detailed analysis". Representatives were selected in the present study from all the major subclades in Asterid 2 and 3 as follows:

Asterid 2: Asteraceae, Valerianaceae, Dipsacaceae, Campanulaceae, Calyceraceae, Goodeniaceae, Escalloniaceae, Aquifoliaceae, Pittosporaceae, Apiaceae, Cornaceae.
Asterid 3: Diapensiaceae, Theaceae, Ericaceae, Epacridaceae, Actinidiaceae.

Terminal taxa were families in most cases despite the fact that Cornaceae appeared polyphyletic in the Chase *et al.* analysis. It has also been suggested that Ericaceae, unless merged with Epacridaceae and Empetraceae, is paraphyletic (Judd and Kron, 1993; Kron and Chase, 1993; Chase *et al.*, 1993; Anderberg, 1993). For the purpose of testing the hypothesis that Bruniaceae has ericalean affinities and in order to avoid problems of paraphyly, Ericaceae was represented by its three major subfamilies (recognised as separate families by Hutchinson, 1969) according to Stevens (1971) and Epacridaceae by the *Styphelia*, *Richea* and *Epacris* groups identified by Powell *et al.* (1996) in a cladistic analysis of relationships within that family. The latter authors also identified a *Cosmelia* lineage, which was found in the present analysis to be identical to the *Epacris* group (i.e. redundant) and was therefore omitted from the final matrix. Genera of Watson's tribe Epacrideae (1967) did not constitute a distinct lineage in the analysis of Powell *et al.* but were included as such on the grounds that most existing classifications of Epacridaceae (Drude, 1889; Bentham and Hooker, 1876; Watson, 1967) recognise a *Woollsia/Archeria/Epacris/Lysinema/Rupicola* grouping. Crayn *et al.* (1996), in a preliminary cladistic analysis of Epacridaceae based on molecular evidence, retrieved *Styphelia*, *Epacris* and *Richea* clades. Grubbiaceae was included

because of its recent close association with Bruniaceae by G. Dahlgren (1989) and Takhtajan (1987) as a separate order Bruniales, near Ericales.
Bruniaceae itself was subdivided into a *Lonchostoma* (the 5 currently recognised species) and a Brunioid grouping (the remaining species of the family) in recognition of the distinctiveness of *Lonchostoma*, as noted by Tahktajan (1987). Trees generated in the analysis were rooted by designating Dilleniaceae as outgroup; in the Chase *et al.* analysis this family was located in Rosid 3 clade i.e. well below Asterid clades 2 and 3.

1.2. Analysis 2: Examination of relationships within Bruniaceae

The sister groups of Bruniaceae having been identified in the first analysis as Epacridaceae and Ericaceae, a second analysis of relationships within the family was carried out in order to determine the existence of distinct lineages or groupings. A total of 51 of the currently recognised species in Bruniaceae was included together with 6 genera from Epacridaceae and 7 from Ericaceae, representing the major lineages in these two families (see 1.1), as follows:

Ericaceae: *Erica, Calluna, Scyphogyne, Arctostaphylos, Gaultheria, Pernettya, Daboecia*
Epacridaceae: *Dracophyllum, Epacris, Lebetanthus, Prionotes, Leucopogon, Monotoca*
Empetraceae, which has recently been shown to be part of a newly-circumscribed monophyletic Ericaceae (Anderberg, 1993; Judd and Kron, 1993), was represented by *Empetrum.* The tree was rooted by designating Clethraceae as outgroup on the grounds that this family appeared to be basal in the ericalean lineages identified in the Chase *et al.* (1993) and Soltis *et al.* (1997) molecular phylogenies, as well as in studies of relationships within ericalean lineages by Kron and Chase (1993), Anderberg (1993) and Kron (1996), based on non-molecular data and molecular data.

2. Selection of characters and character state delimitation

Fundamental to cladistic method is an assessment of variation within a group of organisms and its subsequent representation in a data matrix. The process of converting the observed variation into a matrix i.e. identification of informative characters and their partitioning into discrete states, is a critical step in cladistic analysis but may be interpreted differently by individual workers. Stevens (1991) noted that the subdivision of characters into states was not subject to formal rules, particularly in the case of quantitative characters. He called for explicit justification of character state delimitation as a matter of course in phylogenetic studies. Hawkins *et al.* (1997) discussed concepts of primary homology and recommended the application of conventional character coding, in terms of which individual characters are considered to be independent entities capable of existing in different states which are transformations of each other. These authors drew attention to the fact that assessment of primary homology, i.e that process whereby a proposition of homology is generated, is a two-stage process involving:

1. Definition of the characters that in turn define columns in a data matrix.
2. Partitioning of those characters into states which are coded and codings assigned to terminal taxa, each as a single column in the matrix.

The conventional view of cladistic characters embodies the concept that characters and their states are distinguishable and that the latter represent evolutionary transformations from an original (ancestral) condition. The characters used in analyses 1 and 2 were chosen mainly on the basis of the availability of a reasonably complete data set for the terminal taxa included. They represent for the most part non-overlapping qualitative entities capable of partition into discrete states (Stevens, 1991). Coding followed the guidelines of Hawkins *et al.* (1997). Of the five quantitative characters used in analysis 2 (11, 16, 20, 21 and 30), four were easily delimited: stamen number, number of

ovules/ovary loculus, number of ovary chambers and pollen colpus number are discrete meristic characters and cannot be said to exemplify continuous variation in state. The fourth quantitative character, namely corolla length (character 11) was not as readily divisible into states and the recognition of two size classes was based on the classification of *Erica* according to Guthrie and Bolus (1905), as discussed in Baker and Oliver (1967). Justification for the approach taken in coding of this character is set out in 6.2. below.

Although coding of the chemical characters used may appear not to conform to the requirement that character states are transformations of each other, the presence or absence of the various secondary metabolites implies either gain or loss of a step in the biosynthetic pathway concerned, and represents transformations along those paths. As discussed in Chapter 2 (see pp. 46-51), the presence of a particular flavonoid may represent either the plesiomorphic or apomorphic state, depending on whether the ability to synthesise the compound has been secondarily lost. For this reason, most recent phylogenetic analyses using data from secondary chemistry have treated these as presence/absence characters (see studies by Anderberg, 1993; Nandi *et al.*, 1998).

A further problem associated with the use of secondary chemical characters is the possibility that, depending on the analytical method used, one or more compounds may go undetected. At species or population level, where exploration of subtle variations in flavonoid pattern may be essential, failure to detect a particular flavonoid may lead to loss of potentially useful information. At generic or family level, it may be unnecessary to consider the variation at particular carbon atoms in the flavonoid and the presence or absence of classes of flavonoids may be used as taxonomic characters. In the latter case, failure to detect a minor compound is almost irrelevant. In practice, both kinds of character have been used in phylogenetic analyses (see Richardson, 1983; Hufford, 1992; Nandi *et al.*, 1998); the choice depends on which is most informative as regards the taxa under investigation.

3. Character coding

Potential sources of ambiguity can arise in respect of binary characters that are either polymorphic for the two character states designated, may be inapplicable (e.g. petal fusion in an apetalous taxon) or for which information is lacking. All three situations are often coded as ? (missing data) although a polymorphic taxon in fact has both character states (0 and 1), while in the case of an inapplicable character neither state is present and in that of unknown data either of two states (0 or 1) may be present (Platnick *et al.*, 1991).

3.1. Polymorphic characters

A common problem associated with the representation of organismal variation in a data matrix concerns characters which vary within terminal taxa included in the analysis, so that both character states may be present in a particular taxon. This becomes particularly troublesome when terminal taxa are large families e.g. Asteraceae or Ericaceae, in which very few characters are invariant throughout all members. Such polymorphic characters are usually dealt with either by coding as ? (missing data), by subdividing the taxon concerned into subunits that are monomorphic for the particular character (an approach recomended by Nixon and Davis, 1991) or by coding the taxon as having the plesiomorphous state. Adoption of the first approach (missing data) may lead to a range of errors, including:

1. incorrect calculation of cladogram length
2. incorrect character consistencies
3. selection of less than most parsimonious topologies
4. failure to recognise polyphyletic taxa

At best there will be a loss of information, in that character change within terminals will be hidden by a "missing" coding. The same applies to coding of polymorphic taxa as having the plesiomorphous state when in fact both

plesiomorphic and apomorphic states are present (Pimentel and Riggins, 1987). Nixon and Wheeler (1990) argued that "the basic criterion for delimiting terminal lineages is that each should bear only one state for each character utilised in the analysis". However, as Stevens (1991) pointed out, subdivision of polymorphic taxa into monomorphic subunits may lead to a situation where an attempt is made to analyse taxa at different hierarchical levels. This may in turn affect the pattern of variation of characters and hence the delimitation of their states. Evolution might not proceed uniformly in the different lineages and discrete patterns of variation in a character in one lineage might be blurred by variation in that same character in another lineage.

Despite the disadvantages of coding polymorphic characters as missing, many recent analyses of phylogenetic relationships based on morphological data have adopted this approach (Hufford, 1992; Anderberg, 1992, 1993; Judd and Kron, 1993). Although none of these authors discussed their reasons for doing so, it is assumed that, as no adequate procedure for analysing polymorphisms existed before the advent of PAUP 3.1, the large data sets involved may have rendered further subdivision of terminal taxa impractical.

Nixon and Davis (1991) argued that, provided the terminal taxa in an analysis could safely be assumed to be monophyletic and that all combinations of polymorphic characters did occur in terminal units that were scored as missing, the inclusion of a limited number of polymorphic characters was justified in terms of not affecting the topology of the most parsimonious trees. In analysis 1 both states do occur in all the variable taxa and monophyly could be assumed for Asteraceae, Campanulaceae, Goodeniaceae, Dipsacaceae and Valerianaceae but not Cornaceae.

The approach taken in analysis 1 was that, inasmuch as it represented a first-time exploration of the affinities of Bruniaceae, a coding compromise was reached in terms of which some of the families identified by molecular phylogenetic analysis as possible sister groups to Bruniaceae were subdivided into units monomorphic for the character states recognised in the data matrix. This was considered particularly necessary for Ericaceae and Epacridaceae, in

terms of exploring the hypothesis that Bruniaceae has ericalean affinities. It was acknowledged that the results of the analysis might not be optimal in terms of cladogram length, character consistency and parsimony and that those taxa scored in analysis 1 as variable for one of three characters (perianth merosity, leaf arrangement and anther dehiscence) might in future analyses require subdivision into units monomorphic for those characters. However this approach would mean addressing the question of the phylogeny of the monomorphic taxa as well as the affinities of Bruniaceae, which may render the analysis unwieldy (Maddison and Maddison, 1992).

Polymorphisms in analysis 2 are associated almost entirely with the outgroup taxa and represent infrageneric rather than infraspecific variation. An exception is character 30 (pollen colpus number) which was shown by Hall (1987) to vary within some species in Bruniaceae. These taxa were coded as ? in the analysis using Hennig 86, which does not offer a facility for handling polymorphic data. In PAUP 3.1.1., which can handle polymorphic characters, separate analyses were made; in the first taxa were coded as for the Hennig analysis (i.e. as ?) and in the second according to the two states known to occur for this character.

The availability of a fairly complete data set, particularly for the ingroup, was a major criterion for character selection but it proved difficult to assemble more than thirty characters that fell into this category. This is partly to do with the fact that several aspects of Bruniaceae e.g. reproductive biology, embryology and chromosome cytology, have not been the subject of detailed scientific study and resulted in an unfavourable character to taxon ratio in analysis 2. This being the case, the subdivision of polymorphic characters into units monomorphic for a particular character state was not considered viable in that such a step would have compounded the problem.

Wiens (1995), in a discussion of the effects of infraspecific variation on phylogeny inference, noted that while polymorphic characters were less reliable than their fixed counterparts, they should not on this account necessarily be excluded from cladistic analyses. A subsequent analysis (Wiens and Serviedo,

1997) showed that exclusion of polymorphic characters in fact resulted in decreased accuracy in phylogenetic analyses.

3.2. Inapplicable characters

Analysis 1 contained one inapplicable character i.e. corolla fusion in Grubbiaceae, in which the flowers are apetalous. Hawkins *et al.* (1997) recommended the division of such characters into two separate entities: in this case presence/absence of a corolla and free petals/fused petals. This approach would however have resulted in a coding of ? (missing data) for Grubbiaceae for the second of these two characters. None of the characters used in analysis 2 fall into the category "inapplicable"

3.3. Missing data

Missing data in analysis 1 was confined to nodal anatomy (character 22) in Bruniaceae, Aquifoliaceae and Richeoideae, nucelly in Richeoideae and occurrence of endosperm haustoria in Aquifoliaceae and Escalloniaceae. In analysis 2 missing data in respect of e.g. fire response strategy, occurrence of leaf calcium oxalate crystals, ploidy level and pollen colpus number characterise some outgroup taxa.

4. Character weighting

Although "no one supposes that characters in general all deserve the same weight and yield equally strong evidence" (Farris, 1983) in Carpenter (1988), the latter author rejected *a priori* character weighting as being too subjective. However, many analyses retrieve multiple most parsimonious cladograms from which one is selected as a phylogenetic hypothesis and *a posteriori* weighting of characters has been widely applied to the problem of choosing among a set of equally parsimonious solutions. Both Hennig and PAUP offer a successive

approximations weighting option (Farris, 1969) whereby character weights are set according to their fit on the most parsimonious trees, with highly consistent characters being most strongly weighted. In addition to facilitating a choice among many equally parsimonious cladograms, *a posteriori* weighting may be applied to improving the resolution of cladograms generated from unweighted data. In the present study, *a posteriori* weighting was applied in analysis 2 only, and was based on the rescaled consistency index, which is the product of the character consistency index and the character retention index (Farris,1969; Carpenter, 1988).

5. Cladogram generation

The chemical data obtained in the present study, together with morphological, anatomical, embryological and cytological information obtained from the literature, were analysed using the m*; bb* options of Hennig 86 (ver.1.5; Farris 1988) in the case of analysis1. The i.e. option was tried but found to be time-consuming. The first command (m* = mhennig) constructs several cladograms, each by a single pass through the data, adding the terminals in several different sequences and retaining the shortest trees found. Branch-swapping is then applied to each of the initial trees, retaining no more than one tree for each initial tree. The command bb* applies extended branch-swapping to the trees found by m* and retains all the trees that may be stored in the available space. This combination was found to perform best in heuristic analyses (Platnick, 1989).

In view of the large size of the data matrix used in analysis 2, an initial set of trees was generated from the unweighted data using the Hennig commands h* and bb*. A second set of 200 trees was calculated from a random input sequence in PAUP (ver.3.1.1, Swofford, 1990), with TBR (branch-swapping tree bisection and reconnection) and MULPARS off. Although this protocol meant that there was no guaranteee of discovering all multiple islands, the search times were shorter than would have been the case had MULPARS been activated.

The shortest trees from this initial search were kept and inputted to a TBR search with MULPARS on, to find the full set of trees of that length. This protocol, using a large number of random input sequences, was designed to locate all possible islands of parsimony (Maddison, 1991).

6. Characters used in the analyses

6.1. Analysis 1: Identification of sister group(s) of Bruniaceae

A combination of phytochemical, morphological, anatomical and embryological characters was used for the analysis. Main sources of information were as follows:

Phytochemical characters: Harborne and Williams, 1973; Bate-Smith, 1962; Gibbs, 1974; Hegnauer, 1962-73; Gornall *et al.* 1979; Jensen *et al.*,1975, the present study.

Anatomical characters: Metcalfe and Chalk, 1950; Allsopp and Stock, 1993; Watson, 1967.

Morphological characters: Pillans, 1947; Powell *et al.*, 1996; Stevens, 1973; Mabberley, 1989; Hutchinson, 1973; Ladd, 1994; Bentham, 1869; Smith-White, 1955, Ladd and Donaldson, 1993.

Embryological characters: Dahlgren and van Wyk, 1988; Johri *et al.*, 1992

1. Inulin

The polysaccharide fructan inulin appears to characterise Asteraceae and families of Cronquist's Campanulales as well as Boraginaceae and Menyanthaceae, in which it replaces starch as a storage carbohydrate in subterranean organs. Some taxa in Cornaceae (Cronquist, 1981) also accumulate inulin. The presence of inulin was used by Bremer (1987) as a

character supporting a hypothesized relationship between Asteraceae, Lobeliaceae and Campanulaceae.

2. Ellagic acid

Hydrolysable tannins, on treatment with hot mineral acid or esterases, yield sugar polyols (usually glucose) and phenolic acids e.g. gallic, hexahydroxydiphenic acids. The latter, on release from the parent tannin, forms its dilactone ellagic acid, easily detected on paper chromatograms by Rf value and violet fluorescence. The limited distribution of ellagitannins in only dicotyledonous families has been used as a taxonomic character, particularly by Dahlgren (1975, 1987), whose Rosiflorae, Myrtiflorae, Theiflorae and Dilleniflorae are notable for the presence of leaf ellagitannins. Of the families included in the present cladistic analysis, ellagitannins have been recorded as present in Grubbiaceae, Diapensiaceae, Theaceae, Clethraceae, Cornaceae, Asteraceae and subfamily Vaccinioideae of Ericaceae. The absence of ellagic acid, which is frequently accompanied by the presence of iridoids, has been used by Cronquist (1981) as a taxonomic character, but co-occurrence of these two classes of secondary metabolite is known is some taxa e.g. Fouquieriaceae (Bate Smith, 1984).

3. Proanthocyanidins

Precursors in the biosynthesis of condensed tannins, proanthocyanidins have a much wider distribution in the plant kingdom than do the hydrolysable tannins and consequently make less useful taxonomic characters. They tend nevertheless to predominate in woody dicot families (Bate Smith, 1962,1968); some herbaceous taxa e.g. Dipsacaceae, Campanulaceae lack these compounds entirely. The co-occurrence of proanthocyanidins and lignin has been the subject of discussion by Stafford (1988) and its phylogenetic significance by Ribereau-Gayon (1972), Bate Smith (1973) and Harborne (1977).

4. Gossypetin

The distribution of this rare yellow flavonol (8-OH-quercetin) is limited to a few dicot taxa, being commonly encountered in Primulaceae, Asteraceae, Empetraceae and subfamily Rhododendroideae of Ericaceae. Other ericalean taxa e.g. Epacridaceae, Clethraceae and Diapensiaceae (except *Galax aphylla*) do not appear to synthesise gossypetin (Harborne and Williams, 1973).

5. Myricetin

Flavonoids with tri-hydroxylated B-rings e.g. myricetin, delphinidin, have in the past been utilised both as indicators of lack of evolutionary advancement and as taxonomic markers (see Chapter 2). According to Gornall *et al.* (1979), myricetin is often found to co-occur with ellagic acid and both compounds tend to be absent from plant taxa possessing polyacetylenes, iridoids or benzylisoquinoline alkaloids. Of the families included in the present analysis, only Ericaceae, Epacridaceae, Theaceae, Dilleniaceae and Bruniaceae appear to synthesise myricetin.

6. Iridoids

These constitute a group of monoterpenoids the abundance of which in certain dicot taxa (13 orders of Dahlgren's Hamamelidae, Cornanae, Gentiananae, Loasanae and Lamiidae) and complete absence from others (Dahlgren's Asterales, Campanulales) has been considered taxonomically significant (Jensen *et al.*, 1975) The latter authors recognised two major groupings (carbocyclic iridoids and seco-iridoids), based mainly on chemical structure but also reflecting a bifurcation in the biosynthetic pathway. No differentiation was made in the present analysis between the two classes of iridoids, only presence/absence being recorded. Although only one species of Bruniaceae, *Staavia radiata*, has as far as is known ever been examined for the presence of iridoids (Jensen *et al.*, 1975) the family is coded as state 0 (iridoids absent) based on a negative finding for this species.

7. Polyacetylenes

Fatty acid derived polyacetylenic compounds are common in Asteraceae, Araliaceae and Apiaceae, well-documented in a further 15 dicot familes (of interest in this analysis are Pittosporaceae, Campanulaceae and Goodeniaceae) and recorded as present in Valerianaceae and Euphorbiaceae (Bohlmann *et al.*, 1973). The presence of polyacetylenes (and absence of iridoids, as well as morphological and anatomical congruence) has been used as an indicator of phylogenetic relationship between Asterales and Apiales by Dahlgren (1989).

8. Hydrocyanic acid (HCN)

The release of HCN as one of the products of hydrolysis is a property that unites an otherwise biosynthetically diverse group of glycosides. The use of cyanogenesis *per se* as a taxonomic character may criticised on the grounds that the associated aglycones may be of different biosynthetic origin i.e. not homologous. Nonetheless, the release of HCN as a probable defence against herbivory implies some evolutionary homology.

9. Black leaf tips (apiculae)

The presence of black apiculae on leaf tips of almost all members of Bruniaceae has been noted by many authors and their possible function discussed by Dummer (1912) and Carlquist (1991). The former author considered these structures to play a protective or secretory role but the latter suggested that they may serve as a repository for large amounts of terpenoid compounds. This remains to be confirmed. *Audouinia* has been recorded as lacking apiculae, but de Lange (1993) noted their presence in the young leaf, although not in more mature foliage.

10. Corolla fusion

Choripetaly characterises Theaceae, Dilleniaceae, Actinidiaceae, Apiaceae, Escalloniaceae, Cornaceae, Aquifoliaceae and "Brunioideae". Sympetaly is considered a characteristic feature of Cronquist's Asteridae, but is known outside the group e.g. in Ericaceae, Epacridaceae and several other families. The

occurrence of sympetaly in the Asteridae *sensu lato* identified in recent phylogenetic studies was discussed by Olmstead *et al.* (1992, 1993) who concluded that corolla fusion had either evolved several times within this lineage or reversed to polypetaly in some more recent taxa. Erbar (1991), in a study of corolla development in Asteridae *s.l.* according to Tahktajan (1987), Cronquist (1981,1988) and Dahlgren (1989), established that there were two developmental patterns leading to sympetaly in this lineage. In early sympetaly the corolla was initiated as a ring from which petal lobes later developed. In late sympetaly, the corolla was initiated as distinct petals which fused later in development. Early sympetaly was found to characterise Asteraceae, Goodeniaceae, Calyceraceae, Dipsacaceae, Valerianaceae and Campanulaceae. The other families included in the present study were not discussed by Erbar who did however note that the corolla in certain members of Apiales was initiated as a ring of which the development ceased on initiation of the petal lobes, giving rise to apparently free petals.

Occasional members of the otherwise sympetalous Ericaceae (*Leiophyllum, Elliottia, Befaria*) and Empetraceae (*Ceratiola*) are choripetalous. The placement of these taxa on cladograms resulting from molecular phylogenetic analysis of Ericaceae, Epacridaceae and Empetraceae led Kron and Chase (1993) to conclude that choripetaly may not represent the traditional ancestral condition within Ericaceae. It is not certain whether similar developmental patterns to those observed by Erbar (1991) for Asterales/Apiales account for the occurrence of polypetaly in the ericalean lineages and if the sympetalous corollas of Asterales and Ericales can be considered to be homologous structures. In the present analysis, pending elucidation of this question, it is accepted that they are.

What is interesting is the occurrence of epipetalous stamens in some epacrid lineages (see character 14 below) and in *Lonchostoma* (Bruniaceae), but not in Ericaceae. A study of corolla development in these families may shed light on the evolution of sympetaly in the Asteridae *s.l.* identified in recent phylogenetic analyses. Erbar's (1991) study focused specifically on sympetaly associated with epipetalous stamens and was based on an Asteridae *s.l.* as recognised in

existing systems of classification rather than on the redefined Asteridae *s.l.* identified in phylogenetic studies carried out subsequent to 1991.

The species of Grubbiaceae have only a single whorl of perianth parts and the family was coded as ? (inapplicable character). Both sympetaly (*Lonchostoma* species) and choripetaly (the remaining species in the family) occur in Bruniaceae, which was subdivided into units monomorphic for this character. The question of homology with sympetalous corollas of other taxa included in the study arises here as well and calls for investigation.

11. Leaf arrangement

Opposite leaves are found in Grubbiaceae, Dipsacaceae and Valerianaceae. The remaining families analysed have spiral/alternate leaves. Exceptions include Campanulaceae, Goodeniaceae and Cornaceae which are variable for this character and were coded here as ?, although leaves of Cornaceae are only exceptionally alternate and have therefore been coded as opposite in some cladistic analyses e.g. Anderberg (1992). Leaf arrangement has been utilised in several studies aimed at exploration of relationships within Ericales as well as Cronquist's Rosidae (Hufford, 1992; Anderberg, 1992, 1993) and was considered an important character in the present analysis in terms of examining the proposed close association of Bruniaceae and Grubbiaceae.

12. Perianth merosity

Pentamery is a feature of most taxa in this analysis, except Grubbiaceae, Aquifoliaceae and subfamily Ericoideae of Ericaceae. Dipsacaceae, Asteraceae, Valerianaceae and Cornaceae are variable for this character and were coded as ?

The occurrence of pentamery vs. tetramery may be a key issue in understanding evolution within Asteridae *s.l.* (Soltis *et al.*, 1997) and particularly within Cronquist's Ericales. Hufford (1992) however coded Ericaceae as uniformly pentamerous in his study of Cronquist's Rosidae, while Anderberg (1993) did not

take account of tetramery and pentamery in his analysis of relationships within Ericales.

13. Perianth insertion

Hypogyny is a feature of the "ericalean" taxa included here, with the exception of subfamily Vaccinioideae of Ericaceae. "Lonchostomoideae", Escalloniaceae and Aquifoliaceae also have a hypogynous perianth. In the remainder of taxa the perianth is epigynous. A detailed study regarding the evolution of epigyny (the putatively derived condition) in Asteridae *s.l.* has been advocated by Soltis and Soltis (1995) and is of particular interest in the present study owing to the occurrence of both epi- and hypogyny in Bruniaceae.

14. Stamen attachment

Epipetalous stamens are found in several of the taxa included in the analysis: the Styphelia and Epacris clades of Epacridaceae, Lonchostoma (Bruniaceae), Theaceae and Diapensiaceae well as families of Dahlgren's Asterales and Campanulales. This character has important implications for understanding relationships within Asteridae *s.l.*, Cronquist's Ericales and individual families e.g. Epacridaceae and Bruniaceae. In Epacridaceae for instance, epipetaly is common but reversals to the free state occur in a number of lineages (Powell *et al.*, 1996). This has not always been recognised in recent phylogenetic analyses, in which the family is coded as uniformly epipetalous (Anderberg, 1992). In Bruniaceae, *Lonchostoma* species generally have epipetalous stamens and are regarded as derived on these grounds as well as possession of a sympetalous corolla. This too has not been recognised in cladistic analyses (Hufford, 1992) which have coded the family as having uniformly free stamens.

15. Stamen number

Taxa having either true diplostemony or anthers greater in number than twice the perianth parts, include the three subfamilies of Ericaceae, Grubbiaceae, Theaceae, Dilleniaceae, Actinidiaceae and Escalloniaceae. The remainder of the

taxa included in this analysis either have the haplostemonous condition or anthers numbering less than the perianth parts. This character has been used in several recent phylogenetic studies of Ericales but interpretation has not been uniform e.g. Anderberg (1992) coded Epacridaceae as obdiplostemonous but later (1993) as haplostemonous.

16. Anther dehiscence

Anthers dehiscing by terminal pores are present in all the subfamilies of Ericaceae included here, as well as Actinidiaceae. The remaining families have anthers which dehisce by means of longitudinal slits. Taxa of Campanulaceae may have either condition and the family is coded as ?

17. Pollen presenters

In four of the families included here, namely Campanulaceae, Asteraceae, Calyceraceae and Goodeniaceae, specific structures for pollen distribution (other than anthers) are common. In Asteraceae, the style grows through a tube of more or less connate introrse anthers and extrudes the pollen by piston action. A similar condition, although modified in its details, is known in Goodeniaceae and Calyceraceae. This mechanism exemplifies active pollen presentation (Ladd, 1994), as opposed to the passive type which involves other floral modifications. In Campanulaceae stylar elongation occurs in some species, but the family is considered to have the passive type of pollen presentation, although Ladd (1994) noted that there seemed to be a number of nuances in pollen presentation mechanism in Campanulaceae. This author was of the opinion that pollen presentation in the Campanulales/Asterales was likely to have been derived from a common ancestor.

Anderberg (1992) considered the pollen presentation apparatuses of Asteraceae, Campanulaceae, Goodeniaceae and Calyceraceae to be homologous floral modifications. The occurrence of a (passive) pollen presenter has been recorded in only a single species of Epacridaceae, *Achrotriche serrulata*, and the family is coded as state 0 (pollen presenters absent). Bruniaceae (Ladd and Donaldson,

1993) does not appear to possess pollen presenters. Families in which pollen presenters are present in all species examined are coded as having State 1. Likely selective advantages in the development of pollen presenters have been hypothesised to include greater accuracy in pollen transfer and avoidance of interference between male and female organs in the flower.

18. Pollen release

Both Epacridaceae and Ericaceae shed pollen in tetrads. However, in genera of the *Styphelia* clade (Epacridaceae), only one (occasionally 2 or 3) out of four grains of the tetrad develops to maturity, giving rise to pseudomonads. True monadeny is coded here as state 0, tetradeny as state 1 and pseudomonadeny as state 2 for this character. Although the fossil record (Friis, 1985; Nixon and Crepet, 1993) suggests that monadeny represents the plesiomorphous condition, the evolutionary direction leading to tetradeny as opposed to pseudomonadeny is uncertain and this character was therefore coded as unordered multistate.

19. Number of integuments

Unitegmic ovules are characteristic of most taxa included in this analysis; bitegmy is encountered only in Dilleniaceae and Theaceae.

20. Endosperm haustoria

Endosperm haustoria are generally present in families of Dahlgren's Ericales, with the exception of Diapensiaceae. Dahlgren (1983) utilised this character in the delimitation of his superorder Corniflorae, although not all families in this group possess endosperm haustoria. Bruniaceae appears to have a micropylar haustorium, although this needs confirmation (Dahlgren and van Wyk, 1988). Theaceae, Pittosporaceae, Apiaceae and some of Dahlgren's Campanulales and Asterales lack haustoria.

21. Ovule nucelly

In Dilleniaceae, Cornaceae and Aquifoliaceae the ovule is crassinucellate; the remainder of taxa have tenuinucellate ovules. Bruniaceae, according to Dahlgren and van Wyk (1988), are either "almost tenuinucellate" or "weakly crassinucellate", depending on one's definition of tenui- vs. crassinucelly. Dahlgren (1988) noted that existing embryological studies of Bruniaceae show the megaspore mother cell and early megaspore tetrad separated from the nucellar epidermis by at least one cell layer. This state could not in his view be directly compared to the tenuinucelly of Ericales. Bruniaceae is coded as tenuinucellate in this analysis, with some reservation; the issue can be resolved only by detailed embryological study.

22. Nodal anatomy

This character is not well known for Bruniaceae, which is coded as ? Unilacunar nodes occur in Ericaceae, Epacridaceae and families generally regarded as having ericalean affinity, as well as in Campanulaceae, Escalloniaceae, Calyceraceae and Goodeniaceae. The remaining families used in the present analysis have tri/multilacunar nodes.

TABLE 27 CHARACTER STATES FOR ANALYSIS AIMED AT IDENTIFICATION OF SISTER GROUP(S) OF BRUNIACEAE

1. Inulin: not accumulated (0); accumulated (1)
2. Foliar ellagic acid: present (0); absent (1)
3. Foliar proanthocyanidins: present (0); absent (1)
4. Foliar gossypetin: absent (0); present (1)
5. Foliar myricetin: present (0); absent (1)
6. Iridoids: absent (0); present (1)
7. Polyacetylenes: absent (0); present (1)

8. Foliar HCN: absent (0); present (1)
9. Leaf apex: leaves not terminating in a black apiculus (0); leaves terminating in a black apiculus (1)
10. Corolla fusion: choripetaly (0); sympetaly (1)
11. Leaf arrangement: spiral/alternate (0); opposite (1)
12. Perianth merosity: pentamerous (0); tetramerous (1)
13. Perianth insertion: hypogynous (0); epigynous (1)
14. Stamen attachment: free from petals (0); adnate to petals (1)
15. Stamen number: ≥ 2×perianth segments (0); ≤ perianth segments (1)
16. Anther dehiscence: by longitudinal slits (0); by apical pores (1)
17. Pollen presentation mechanism: no floral modification for pollen presentation (0); flowers with specialised pollen presentation mechanism (1)
18. Pollen shed: monads (0); tetrads (1); pseudomonads (2)
19. Ovule integuments: bitegmy (0); unitegmy (1)
20. Endosperm: haustoria present (0); haustoria not present (1)
21. Ovules: crassinucellate (0); tenuinucellate (1)
22. Nodal anatomy: nodes unilacunar (0); nodes tri/multilacunar (1)

TABLE 28 CHARACTER STATE MATRIX FOR ANALYSIS AIMED AT IDENTIFICATION OF SISTER GROUP(S) OF BRUNIACEAE

Taxa	Characters																					
Dilleniaceae	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Theaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0
Diapensiacea	0	0	0	0	1	0	0	0	0	1	0	0	0	1	1	0	0	0	1	1	1	0
Actinidiaceae	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	1	0
Ericoideae	0	1	0	0	0	0	0	1	0	1	0	1	0	0	0	1	0	1	1	0	1	0
Rhododendroid	0	1	0	1	0	0	0	1	0	1	0	0	0	0	0	1	0	1	1	0	1	0
Vaccinioideae	0	0	0	0	0	1	0	1	0	1	0	0	1	0	0	1	0	1	1	0	1	0
Epacrideae	0	1	0	0	0	1	0	1	0	1	0	0	0	1	1	0	0	1	1	0	1	0

Richioideae	0	1	0	0	0	0	0	1	0	1	0	0	0	0	1	0	0	1	1	0	1	?
Styphelieae	0	1	0	0	0	0	0	1	0	1	0	0	0	1	1	0	0	2	1	0	1	0
Grubbiaceae	0	0	0	0	1	0	0	0	0	?	1	1	1	0	0	0	0	0	1	0	1	1
Pittosporaceae	0	1	0	0	1	0	1	0	0	1	0	0	0	0	1	0	0	0	1	1	1	1
Apiaceae	0	1	0	0	1	0	1	1	0	0	0	0	1	0	1	0	0	0	1	1	1	1
Lonchostoma	0	1	0	0	0	0	0	0	1	1	0	0	0	1	1	0	0	0	1	0	1	?
Brunioideae	0	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	1	0	1	?
Campanulacea	1	1	1	0	1	0	1	1	0	1	?	0	1	0	1	?	1	0	1	0	1	0
Dipsacaceae	0	1	1	0	1	1	0	0	0	1	1	?	1	1	1	0	0	0	0	1	1	1
Asteraceae	1	0	0	1	1	0	1	1	0	1	0	?	1	1	1	0	1	0	1	0	1	1
Valerianaceae	0	1	1	0	1	1	1	1	0	1	1	?	1	1	1	0	0	1	1	1	1	1
Escalloniaceae	0	1	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	?	1	0
Calyceraceae	1	1	1	0	?	1	0	0	0	1	0	0	1	1	1	0	1	0	1	0	1	0
Goodeniaceae	1	1	1	0	1	1	1	1	0	1	?	0	1	?	1	0	1	0	1	0	1	0
Aquifoliaceae	0	1	1	0	1	0	0	?	0	0	0	1	0	0	1	0	0	0	1	?	0	?
Cornaceae	1	0	1	0	1	1	0	0	0	0	?	?	1	0	1	0	0	0	1	0	0	1

6.2. Analysis 2: Examination of relationships within Bruniaceae

For Bruniaceae and the selected outgroup taxa, a combination of chemical, morphological, anatomical and embryological characters was used, together with one cytological and one palynological character. In addition to the sources given in 4.1, morphological data were from Powell *et al.* (1992, 1996), Dyer (1975), de Lange (1992) and Powrie (pers. comm.); anatomical data were obtained from Carlquist (1978, 1991), palynological data from Hall (1987) and cytological data from Goldblatt (1981).

1. Ellagic acid (see 6.1)

Not identified in any species of Bruniaceae or Epacridaceae, ellagic acid has been recorded as present in *Clethra*, *Empetrum* and *Arctostaphylos*.

2. Isorhamnetin

The methylated flavonol isorhamnetin (quercetin 3-methyl ether) is widespread in leaves of Bruniaceae, being absent only from *Lonchostoma* (except *L. purpureum*), *Audouinia*, some *Berzelia* and *Raspalia* species, *Brunia albiflora* and *Thamnea hirtella*. Although of common occurrence in angiosperm families, isorhamnetin has not been shown to be present in Epacridaceae, Empetraceae, Clethraceae or Ericaceae. According to Harborne (1977), O-methylation of the flavonoid B-ring is a derived character involving acquisition of additional steps in the flavonoid biosynthetic pathway.

3. Proanthocyanidins (see 6.1)

The loss of capacity to synthesise tri-hydroxylated flavonoid B-rings has been considered by Harborne (1977) to represent an evolutionary advancement and by Gornall and Bohm (1978) to be "highly advanced" i.e. representative of a two-stage process involving the gain and subsequent loss of biosynthetic capacity. The occurrence of prodelphinidin (PD) in leaves of Bruniaceae is fairly genus-specific according to Pillans' delimitation, in that it appears to be absent from *Audouinia*, *Linconia*, *Thamnea*, *Tittmannia* and *Nebelia* (except *N. macrocephala*) but present throughout all the other genera. The distribution of PD is not known for some genera in Epacridaceae; it appears to be present in *Dracophyllum*, *Leucopogon* and *Monotoca* but is absent from *Epacris*. In Ericaceae PD has been recorded as present in *Erica*, *Calluna*, *Daboecia* and *Arctostaphylos*, but no record could be found for *Pernettya*, *Gaultheria* or *Scyphogyne*. Procyanidins are universally present in all three families except in the genus *Linconia* (Bruniaceae).

4. Myricetin (see 6.1.)

The distribution of myricetin in Bruniaceae is rather similar to that of prodelphinidin in that genera either possess or lack both compounds. Exceptions to this rule are some *Raspalia* and *Staavia* species. Epacrid genera *Lebetanthus*

and *Prionotes* and amongst Ericaceae, *Erica* and *Daboecia*, synthesise myricetin Harborne and Williams, 1973).

5. Kaempferol

The last in a series of flavonols with decreasing B-ring hydroxylation is kaempferol, the distribution of which in Bruniaceae is also fairly genus-specific. It was not recorded in the present study in any *Lonchostoma*, *Nebelia*, *Staavia* or *Mniothamnea* nor in most *Raspalias* species. Of the outgroup taxa *Clethra*, *Dracophyllum*, *Erica* and *Pernettya* lack kaempferol.

6. Fire-response strategy

The occurrence of woody subterranean stems, from which regeneration is possible following a fire, does not seem to have been well studied in Ericaceae but burls (=lignotubers) are known from *Arctostaphylos* (Keeley and Keeley, 1988) and a few *Erica* species (Oliver, pers. comm.). Bell *et al.* (1996) noted that data for fire-response strategy in Epacridaceae was sparse, but recorded the presence of lignotubers in occasional members of Styphelieae and Cosmelieae. Only *Arctostaphylos* could be coded with certainty for this character and the remainder of the outgroups are recorded as unknown (?). In Bruniaceae, lignotubers are found in 21 species in all genera except *Raspalia*, *Pseudobaeckia* and *Mniothamnea* (Pillans, 1947; Carlquist, 1978; Rebelo, 1980; Broll, 1980). It is thought that, in addition to their role in fire survival, lignotubers may function as storage organs for water and/or nutrients or as a means of regeneration following frost (Gill, 1975; Carlquist, 1978; James, 1984; Zedler and Zammit, 1989; Boucher *et al.*, 1990).

7. Leaf margin

Serrate margins occur in many Ericaceae (Stevens, 1971) e.g. *Vaccinium* and *Gaultheria* and in occasional Epacridaceae (*Lebetanthus*, *Prionotes*). Leaves of the remaining taxa in the present analysis have entire margins. This character was utilised by Anderberg (1993) in a phylogenetic investigation of relationships

in Ericales and by Powell *et al.* (1996) in an analysis of relationships within Epacridaceae.

8. Leaf apiculae (see 6.1)

Black leaf apiculae are characteristic of all members of Bruniaceae, although in *Audouinia* these appear to be lost early in development (de Lange, 1992) and are not evident in the mature leaf. The function of these structures is not clear, but they do not appear to occur in epacrid or ericalean taxa. Some genera of Epacridaceae have leaves terminating in spines (Powell *et al.*, 1986; Powell, 1992), but the homology of pungent and apiculate leaf apices is doubtful.

9. Stipules

These are recorded as present in the bruniaceous genera *Linconia* and *Tittmannia* and in some *Berzelia*, *Brunia* and *Staavia* species (Pillans 1947); leaves of Ericaceae and Epacridaceae appear to be exstipulate (Stevens, 1971; Powell *et al.*,1992).

10. Perianth insertion

Epigyny is the rule in most genera of Bruniaceae, except *Lonchostoma* (Pillans, 1947). According to Dyer (1975) the ovary in *Raspalia* is "more or less superior" and species are coded as hypogynous in the present analysis. Superior ovaries occur throughout Epacridaceae (Powell *et al.*, 1992) and in most Ericaceae except for Vaccinioideae, some genera of which are epigynous (Stevens, 1971). Anderberg (1992, 1993), Judd and Kron (1993) and Powell *et al.* (1996) all considered perianth insertion to be informative on questions of circumscription of, as well as relationships within, Ericales.

11. Corolla fusion

Sympetaly, the rule in almost all taxa of Ericaceae and Epacridaceae, is a condition that has been considered indicative of close relationship between these groups (Copeland, 1954; Stevens, 1971). Varying degrees of corolla fusion are

evident in *Lonchostoma* species (Rebelo, 1980); this represents one of several features that set this genus apart from others in Bruniaceae, in which the petals free or occasionally very slightly connate at the base. Patterns of sympetaly and polypetaly in Asteridae *s.l.* have been discussed by Olmstead *et al.* (1993) and used by Anderberg (1993) in a cladistic examination of relationships within Ericales.

12. Corolla length

The majority of species in Bruniaceae have minute flowers (2-3 mm in length), often grouped in globose or capitate heads. Exceptions are *Lonchostoma*, *Audouinia* and some *Thamnea* species (corolla length exceeding 5mm). Within Bruniaceae the distinction is quite clear and almost genus-specific, with little overlap between these two size classes. The flowers of Epacridaceae are variable as regards size; *Leucopogon* and *Monotoca* generally have small inconspicuous flowers, while those of *Epacris* and *Dracophyllum* are usually larger and more showy (Powell *et al.*, 1986; Powell, 1992). Amongst the Ericaceae, there are some small-flowered *Erica* species, particularly in subgenus Euerica which is defined by corolla length of less than 8mm. Within Euerica, sections Ephebus, Ceramia, Chlorocodon and Arsace are defined by corolla lengths of less than 6, 5, 2 and 3mm respectively (Guthrie and Bolus, 1905, as discussed in Baker and Oliver, 1967) while in the remaining sections the corolla is longer than 5mm. In the South African endemic *Scyphogyne* and in *Daboecia* and *Calluna* the corolla is short; in *Pernettya* and *Gaultheria* both long and short-flowered species are found and in *Arctostaphylos* the corolla exceeds 5mm in length (Stevens, 1971). Coding of this character in the outgroup taxa is not exact in that exact measurements could not be made and *Pernettya* and *Gaultheria* were coded as variable. These two taxa were not divided into units monomorphic for corolla length in view of the already large data matrix, but this might be done in future analyses. Corolla length is not a character that has been included in any of the phylogenetic studies of relationships in Ericaceae/Ericales to date, but may well prove informative. Oliver stated (1991) that there appeared to have been an

evolutionary trend within *Erica* from small and inconspicuous to large-flowered forms, followed by a reversal to the basal condition in more derived groups. This represented in his opinion one aspect of development of different pollination syndromes in the group. The fossil record (Friis 1985; Nixon and Crepet, 1993) suggests that very small flowers (1,2-1,9 mm long) may be ancestral for ericalean lineages.

13. Corolla colour

White or cream-coloured flowers are the rule in Bruniaceae, but *Thamnea, Lonchostoma, Audouinia* and *Staavia* species are notable for their pink, purple, or red blooms. Ericaceae and Epacridaceae generally have brightly-coloured corollas, exceptions being *Scyphogyne, Arctostaphylos* and *Leucopogon* (Stevens, 1971; Powell *et al.*, 1996). This character could not be established with certainty for *Gaultheria* and *Pernettya* and these taxa were coded as ? *Daboecia* may have white, pink or red flowers and was also coded as ?

14. Flowers subtended by conspicuous white bracts

The flowers of Bruniaceae, whether borne singly or in an inflorescence, are subtended by one to several bracts. Up to twelve bracts are present in *Audouinia, Thamnea* and *Tittmannia*, while those of *Nebelia* are conspicuously lengthened and protrude beyond the exserted stamens. In *Staavia*, the bracts are enlarged and ray-like, forming an involucre surrounding the capitate inflorescence. The white bracts of *Staavia* may be very conspicuous e.g. in *S. glutinosa* and presumably play a role in the pollination syndrome of the group. They are not known to occur in other members of the family nor in the outgroup taxa.

15. Calyx lobes

In *Raspalia* the ovary appears to be more or less superior, in contrast to most other genera of the family. Calyx lobes in this taxon are adjacent at the base, forming a V-shaped angle, a character used by Pillans (1947) as diagnostic for

Raspalia in his key to Bruniaceae. This suggests that a residual calyx tube is present in *Raspalia* as it is in *Lonchostoma*, also hypogynous. In the remaining genera of Bruniaceae, which are epigynous, the sepals are free but adnate to the ovary for part of their length, forming a false tube at the base. Taxa of Ericaceae generally have the sepals fused into a tube or cup, while those of Epacridaceae have free sepals.

16. Fruit type

Capsular fruits (septicidal or loculicidal) characterise *Clethra*, *Lonchostoma*, *Lebetanthus*, *Prionotes*, *Dracophyllum*, *Gaultheria*, *Calluna* and *Erica* (Pillans, 1947; Stevens, 1971; Powell *et al.*, 1992). Non-capsular but dehiscent fruits occur in *Brunia*, *Linconia*, *Nebelia*, *Raspalia* and *Staavia* and dry indehiscent fruits in the remainder of genera in Bruniaceae (Pillans, 1947; Dyer, 1975) as well as in *Scyphogyne*. *Pernettya*, *Empetrum*, *Leucopogon*, *Monotoca*, and *Arctostaphylos* have fleshy indehiscent fruits. Fruit type was used as an important character both in the recognition of subfamilial groupings in Epacridaceae by Bentham and Hooker (1876) and Drude (1889) and in a phylogenetic analysis of relationships within the family by Powell *et al.* (1996). It has also been considered evolutionarily informative in recent cladistic studies in Ericales and Ericaceae (Judd and Kron, 1993; Anderberg, 1993). In the opinion of Oliver (1989) fruit dehiscence constitutes a key character for distinguishing Ericeae and Salaxideae, the former having dehiscent capsules and the latter a berry or drupe. He considered the Salaxideae to be a more advanced grade derived from a basic ericoid ground plan by reduction and fusion. The fossil record suggests that capsular fruits are ancestral within Ericales (Friis, 1985; Nixon and Crepet, 1992) but the evolutionary direction within the taxa used in the present analysis is uncertain and this character was coded as unordered multistate.

17. Stamen number (see 6.1)

In *Clethra* and Ericaceae there are generally two whorls of stamens, while in Epacridaceae and Bruniaceae only one is present. In some taxa of Ericoideae e.g. *Scyphogyne*, stamens may be reduced from 10 to 5 or less.

18. Stamen position

Some genera of Bruniaceae (*Brunia, Nebelia, Berzelia*) are notable for globose inflorescences in which the flowers have stamens much exserted; it has been generally assumed that this is an adaptation for insect pollination in Bruniaceae (Whitehead *et al.*, 1987) but wind pollination has been associated with prominently exserted stamens in other taxa of the Cape flora (Koutnik, 1987, Oliver, 1991). Some *Lonchostoma* species and *Raspalia dregeana* also have exserted stamens; *Erica* and *Scyphogyne* are variable for this character and are coded as ? on the grounds that further subdivision of these taxa would make the analysis too cumbersome. However, as in the case of character 12, this should be further explored in future cladistic studies. The remainder of the outgroups have the stamens included or at least not markedly exserted. This character is potentially problematic in that it represents what Stevens (1991) described as quantitative masquerading as qualitative information, but it was considered preferable not to exclude any information that would be likely to improve resolution, particularly in view of the low character to taxon ratio in this analysis. The pollination biology of Bruniaceae is not well understood and further study would contribute to a better understanding of family phylogeny.

19. Anther dehiscence

The anthers in *Clethra* and Ericaceae dehisce by apical pores or short apical slits, while in Epacridaceae and Bruniaceae dehiscence is uniformly by means of longitudinal slits over the full length of the anther.

20. Stamen insertion

Anthers in Ericaceae, Clethraceae and Bruniaceae are free from the corolla, except in *Lonchostoma*, where they are epipetalous in most species and inserted

in the throat of a tubular corolla. This is the condition for many taxa of Epacridaceae and has been used in the past (Bentham and Hooker, 1876; Drude, 1889) as an important character for delimitation of sub-familial groups. Of the genera included in this analysis, *Epacris, Leucopogon and Monotoca* have epipetalous stamens; in *Lebetanthus* and *Prionotes* these organs are free.

21. Ovule number/ovary loculus

In *Thamnea*, most *Lonchostoma* species, *Epacris, Erica, Calluna, Pernettya, Gaultheria, Dracophyllum, Lebetanthus* and *Prionotes*, there are numerous ovules in each ovary chamber. In *Monotoca* and *Leucopogon* (Styphelieae), as well as in *Scyphogyne, Arctostaphylos, Nebelia, Berzelia, Mniothamnea, Raspalia* and some *Brunia* species, ovule number is reduced to one per loculus (Pillans, 1947; Watson, 1967; Stevens, 1971; Oliver, 1991). The remaining genera of Bruniaceae generally have 2 ovules per chamber. This character has been used by various authors in generic or tribal delimitation in both Bruniaceae and Epacridaceae and is a feature of some of the "minor genera" of Ericoideae, where the reduction in the number of ovules/ovary chamber is considered to be part of an evolutionary trend involving decrease in flower size and loss of flower colour, together with development of fruit indehiscence, pollen monadeny and stamen exsertion within this lineage (Oliver, 1991). Evolutionary direction in development of this character in the taxa included in the present study is uncertain and it was therefore coded as unordered multistate.

22. Number of ovary chambers

In Bruniaceae there are normally 1-2 loculi in the ovary, exceptions to the rule being *Audouinia* (usually 3 but occasionally 4 or 5, according to de Lange (1992) and the geographic outlier *Raspalia trigyna* (2-3). In Epacridaceae there are 5 ovary chambers in Epacrideae but a variable number in Styphelieae (1-10): of the taxa included in the present analysis *Monotoca* has 1 and *Leucopogon* 2 or more (Powell *et al.*, 1992). Ericaceae also appear to be variable for this character; *Erica* species have 8, *Scyphogyne* 1, *Calluna, Pernettya, Daboecia*

and *Gaultheria* usually the same as the number of petals (Stevens, 1971). Although the fossil record (Friis, 1985; Nixon and Crepet, 1993) suggests that the ancestral condition in ericalean lineages may be a 3-4 chambered ovary, this character was coded as unordered multistate on the grounds that directionality is not known with certainty in the study group.

23. Placentation

All members of Epacridaceae except Styphelieae have axile placentation (Powell *et al.*, 1996) as do most Ericaceae except *Arctostaphylos* (Stevens, 1971) and Bruniaceae, except *Thamnea* and *Tittmannia* species, in which it is apical (Pillans, 1947).

24. Pollen release (see 6.1)

A feature held in common by Ericaceae and Epacridaceae is the maturing of pollen in tetrads, a character not known in Bruniaceae. In some members of Styphelieae, notably *Monotoca* and *Leucopogon*, not all of the four grains mature and the resultant grain is pseudomonadenous. In Ericoideae, anemophilous species have monadenous pollen which appears to be part of the overall pollination syndrome in these taxa (Oliver, 1991); in *Erica* and *Scyphogyne* it is tetradenous. This character was coded as unordered multistate as in the previous analysis.

25. Leaf calcium oxalate crystals

Oxalic acid is rarely found in the free state in the plant cell but its calcium salt is very common and occurs in crystalline form as the trihydrate or monohydrate, which belong to the tetragonal and monoclinic crystal systems respectively (Evans, 1996). The distribution of different crystalline forms of calcium oxalate (rhomboids and druses) in leaves of Bruniaceae formed part of a detailed anatomical study by Carlquist (1991), who used the results to define various infrafamilial lineages. According to Oliver (pers. comm.) both rhomboids (single rectangular crystals or prisms) and druses (cluster crystals or rosettes) occur in

Erica, which was coded as variable, on the grounds that insufficient information was available to permit recognition of units monomorphic for this character. The distribution of these two morphs is not well known for other taxa of Ericaceae included in the present study, nor for Epacridaceae. The evolutionary significance and directionality of this character is not clear. A case might be made for considering absence of calcium oxalate as the plesiomorphous state, the ability to synthesise simple crystals as a gain mutation followed by coalescence of single crystals into compound clusters, with the presence of both crystal types as intermediate between these two states. The function of the different crystalline forms of calcium oxalate, which include, in addition to druses and rhomboids, also single acicular crystals (raphides) and microsphenoids, is however not well understood and the character was coded as unordered multistate.

26. Stomatal distribution

Carlquist's study (1991) of leaf anatomy in Bruniaceae demonstrated the existence of great variation in stomatal distribution both between and within currently recognised genera of the family. In *Erica*, part of the ericoid leaf syndrome, which has in turn been interpreted, *inter alia*, as an adaptation to summer drought (Stock *et al.*, 1994), is the presence of rolled leaves with stomata on the abaxial surface only. In *Calluna*, stomata are not confined to the lower leaf surface while in *Daboecia*, *Pernettya*, *Scyphogyne* and most *Gaultheria* species they are; in *Arctostaphylos* both states occur (Stevens, 1971). In Epacridaceae, *Dracophyllum* species have stomata on both leaf surfaces while the remainder of taxa included in the present analysis have only abaxial stomata (Powell *et al.*, 1996).

27. Leaf hairs

A feature that has been used to differentiate between Ericaceae and Epacridaceae is the occurrence of multicellular leaf hairs in the former and their absence from the latter family, with the exception of *Lebetanthus* and *Prionotes*

(Stevens, 1971). Bruniaceae resembles Epacridaceae in having only simple unicellular clothing hairs, which are abundant in some taxa.

28. Anther appendages

As in the case of leaf hairs, the presence or absence of anther appendages is a feature that distinguishes Ericaceae from Epacridaceae, whose close relationship on grounds of both non-molecular (Copeland, 1954; Stevens, 1971; Anderberg, 1992 and 1993; Judd and Kron, 1993) and molecular (Kron and Chase, 1993; Kron, 1996) evidence is otherwise strongly indicated. Anthers in Ericaceae are generally adorned with awns, wings or other ornamentation, while those of Epacridaceae and Bruniaceae lack these appendages. Oliver (1991) noted that anther ornamentation was generally lacking in bird and wind-pollinated species of *Erica*, but since these constitute only an estimated 20% of the genus (Rebelo *et al.* 1985), this taxon was coded as having appendages commonly present.

29. Anther thecae

Pillans (1947) noted the occurrence, in *Linconia* species, of anther thecae which diverged at maturity, a feature that he regarded as sufficiently distinctive to be included in his key to the Bruniaceae. It appears that this character may be implicated in pollen dispersal since the upper connate portion of the anther thecae is abortive. Information as to the occurrence of divergent anthers was not available for the other taxa represented in the present analysis, which were coded as ? in the matrix.

30. Ploidy level

The chromosome cytology of Bruniaceae has been only partially studied (Goldblatt, 1981) but *Staavia radiata, Berzelia abrotanoides* and *B. ecklonii* appear to be polyploids. The ploidy level of other taxa in this study could not be established with certainty and these are coded as ? Smith-White (1955), in a study of chromosome numbers and pollen types in Epacridaceae, concluded that the family was exceptionally variable as regards chromosome number,

particularly in the tribe Styphelieae in which he recorded 9 different haploid numbers, derived from polyploid and aneuploid changes on base numbers of 4 and 6. In view of the complexity of epacrid cytology, coding for this character was taken as uncertain.

31. Pollen colpus number

In a detailed palynological study of Bruniaceae, Hall (1988) found tricolporate pollen grains to be most common in the family but recorded a range of aperture numbers of up to 10. Colpus number was not constant in currently recognised genera and varied in some cases within species. In the latter case the character was coded as ? in the Hennig analysis 86 and as both ? or as polymorphic in two separate PAUP analyses. Ericaceae appear to be fairly constant in having tricolporate pollen but the condition for Epacridaceae could not be established and these taxa are also coded as ?

32. Floral bracts extending beyond corolla

In *Nebelia* species the flowers are tightly clustered in head-like inflorescences, each flower being subtended by a prominent cream-coloured bract. The bract is stiff, sharply pointed and extends beyond the corolla, particularly in *N. paleacea* where it is at least twice as long as the petals. Its adaptive significance is not clear but a protective function in the developing flower may be suggested. Homology of this bract with that of *Staavia* species (character 13) is uncertain but unlikely, since in *Staavia* an involucre is present while in *Nebelia* all florets in the inflorescence bear a subtending bract. Prominent scarious bracts are not known to occur in other species in Bruniaceae nor in the outgroup taxa.

TABLE 29. CHARACTERS AND STATES USED IN ANALYSIS 2.

Leaf secondary chemistry

0. Ellagic acid: present (0); absent (1)
1. Isorhamnetin: absent (0); present (1)
2. Proanthocyanidins (PA): both procyanidins (PC) and prodelphinidins (PD) present (0); only procyanidins present (1)
3. Myricetin: present (0); absent (1)
4. Kaempferol: absent (0); present (1)

Morphology

5. Fire-response strategy: regeneration possible from a subterreanean stem (0); regeneration not possible from a subterranean stem (1)
6. Leaf margin: serrate (0); entire (1)
7. Leaf apex: leaves not terminating in a black apiculus(0); leaves terminating in a black apiculus (1)
8. Stipules: absent (0); present (1)
9. Corolla insertion: hypogynous (0); epigynous (1)
10. Corolla fusion: choripetaly (0); sympetaly (1)
11. Corolla length: >5mm (0); <5mm (1)
12. Corolla colour: corolla brightly coloured (0); corolla not brightly coloured (1)
13. Bracts: inflorescence not subtended by an involucre of white bracts (0); inflorescence subtended by an involucre of prominent white bracts (1)
14. Calyx lobes: not adjacent at the base to form a V-shaped angle (0); adjacent at the base and forming a V-shaped angle (1)
15. Fruit: loculicidal/septicidal capsule (0); dehiscent non-capsular fruits (1); indehiscent nutlet, drupe or pyrene (2)
16. Stamen number: = / > 2 × perianth segments (0); =/ < the number of perianth segments
17. Stamen position: stamens included (0); stamens prominently exserted (1)
18. Anther dehiscence: pores (0); apical slits (1)
19. Stamen attachment: epipetalous (0); free (1)
20. Number of ovules/ ovary locule: three or more (0); two (1); one (2)

21. Number of ovary chambers: >3 (0); 3 (1); 2 (2); 1 (3)

Anatomy

22. Placentation: axile (0); apical (1); basal (2)
23. Pollen release: monads (0); pseudomonads (1); tetrads (2)
24. Calcium oxalate crystals in leaf: rhomboids and druses (0); druses (1); rhomboids (2); neither (3)
25. Stomata: present on both leaf surfaces (0); one leaf surface only (1)
26. Leaf hairs: multicellular leaf hairs present (0); unicellular leaf hairs present (1)
27. Anther ornamentation: anthers usually adorned with appendages (0); anthers not usually ornamented (1)
28. Anther thecae: not diverging above the middle at maturity (0); diverging above the middle at maturity (1)

Cytology

29. Ploidy level: diploid (0); tetraploid (1)

Palynology

30. Pollen colpus number: 3 (0); 4 (1); 5 (2); 6 (3); 7 (4); 8 (5); 10 (6)

Gross morphology

31. Inflorescence structure: flowers not subtended by prominent, scarious bracts exceeding the corolla (0); flowers grouped in globose heads, each flower subtended by a prominent, scarious bract exceeding the corolla (1).

TABLE 30. CHARACTER STATE MATRIX USED IN ANALYSIS OF WITHIN-FAMILY PHYLOGENY.

Taxa	Characters						
clethra	00010	??000	0010?	00?01	00001	1????	00
empetr	00011	?1000	0100?	21011	20221	?????	00
monoto	10011	?1000	11?0?	21010	2311?	111??	?0
epacris	10111	?1000	1000?	01010	0002?	111??	?0
dracoph	10010	?1000	1000?	01011	0002?	011??	?0
lebetan	10?01	?0000	1??0?	01?11	0002?	101??	?0
prionot	10?01	?0000	1?00?	01011	0002?	101??	?0
leucop	100?1	?1000	1110?	21010	2001?	?11??	?0
erica	?0000	?1000	1000?	00?01	00020	100??	00
calluna	?0011	?1000	1100?	10001	0002?	010??	00
scypho	?????	?1000	1110?	21?01	2302?	100??	00
arctos	00011	01000	1010?	20001	00122	000??	00
pernet	?0?10	?1000	1??0?	20001	0002?	100??	00
gaulth	?0?11	?0000	1??0?	00001	00021	000??	00
daboec	10000	?1000	11?0?	00001	0002?	1????	00
audcap	10111	01101	00000	21011	11002	01100	00
berabr	11100	01111	01100	21111	23002	01101	00
bercor	11011	011?1	01100	21111	23002	0110?	00
bereck	11001	011?1	01100	21111	23002	01101	00
bergal	10001	111?1	01100	21111	2300?	?1100	00
berinc	10001	011?1	01100	21111	23002	0110?	00
berlan	11001	111?1	01100	21111	2300?	?1100	00
berrub	11001	111?1	01000	21111	23000	01100	00
bersqu	10001	11111	01100	21111	23002	0110?	00
brualb	11001	111?1	01100	11111	22002	01100	00
brualo	11001	111?1	01100	11111	22002	1110?	00
brulae	11001	011?1	01100	11111	1200?	?110?	30
brunod	11011	01101	01100	11111	1200?	?1100	20
brusto	11001	01111	01000	11111	2200?	?1100	00
brumac	11101	?11?1	01100	11111	12002	1110?	50
lonpur	11000	11100	10000	01111	12001	11100	?0
lonest	10000	11100	10000	01110	02001	1110?	20

lonmon	10100	11100	10100	01010	02001	11100	?0
lonmyr	10000	01100	10000	01111	02001	1110?	40
lonpen	10000	01100	10000	01010	02001	1110?	40
linalo	11111	11111	00000	11011	12001	1111?	00
lincus	11101	01111	01100	11011	12001	1111?	00
mniocal	11000	11101	01100	21011	23001	1110?	10
nebfrag	11110	11101	01100	11111	22000	11100	21
neblaev	11110	11101	01100	11111	22002	1110?	?1
nebpale	11110	11101	01100	11111	22002	01100	?1
nebsph	11110	11101	01100	11111	22002	1110?	?1
pseafr	11010	11101	01100	21001	12001	01100	?0
psecor	11011	11101	01100	21011	22001	0110?	10
rasbar	10001	11100	01101	11011	22001	1110?	?0
rasdre	10010	?1100	01101	11111	?2003	1110?	?0
rasglo	11000	?1100	01001	11011	22001	11100	?0
rasmic	11010	11100	01101	11011	22001	11100	10
rassta	11011	?1100	01101	11011	22001	0110?	?0
rasvar	10001	11100	01101	11011	22001	1110?	10
rastrig	11110	?1100	01101	11011	?2003	1110?	10
rasvillo	11011	?1100	01101	11011	22001	1110?	10
rasvir	11001	11100	01101	11011	22003	1110?	10
stadod	11000	11111	01010	11011	22001	01100	20
stacap	11000	01111	01010	11011	22001	01100	?0
stadreg	11001	?1111	01010	11011	22001	?110?	?0
staglut	11000	11111	01010	11011	22001	0110?	?0
starad	11000	01111	01010	11011	22001	01101	10
stazey	11001	?1101	01010	11011	22001	01100	20
thahir	10110	01101	00000	21011	02?02	1110?	20
thadio	11111	01101	00000	21011	02100	1110?	?0
thamas	11111	11101	00000	21011	02102	1110?	00
thathe	11111	?1101	01100	21011	03002	1110?	00
titlaev	11111	11111	01100	21011	12102	0110?	00
titlaxa	11111	01111	01100	21011	12102	0110?	00
titest	11111	01111	00100	21011	12102	0110?	00

7. Consensus trees

The use of consensus trees as a basis for formal classification has been recommended by Anderberg and Tehler (1990) who argue that, in the commonly encountered situation where multiple equally parsimonious cladograms are generated from the same data set, there is no way of knowing with certainty which is preferable as a phylogenetic hypothesis. In their view, a strict consensus tree, although not of itself an hypothesis, represents an unambiguous statement about the interrelationships of the taxa investigated and is therefore the most reliable basis for naming groups of terminal taxa for use in a working classification. In depicting only those components common to all most parsimonious cladograms i.e. unequivocally supported by the data, a strict consensus tree combines high explanatory power with minimal inconsistency. However, as West and Faith (1990) and Bremer (1988) point out, a consensus tree is always less resolved than any of the set of most parsimonious trees from which it is derived and therefore does not represent a parsimonious solution to character state change. It reveals little about process (Miyamoto, 1985) and cannot be used as an hypothesis of phylogeny. Carpenter (1988) argued that one of the most parsimonious cladograms should always be preferred over a consensus tree as a phylogenetic hypothesis and outlined a method for choosing among multiple most parsimonious trees. Most recent cladistic studies have adopted the approach that one of the most parsimonious cladograms be used as a basis for examining process e.g. character evolution and a consensus tree as a framework for classification. As Bremer (1988) noted: "with morphological data, consensus trees are not enough. Parsimonious cladograms are necessary as a reference scheme for character interpretation".

Consensus trees may be generated in a number of ways, which differ in the criteria used for retaining groups or subsets of taxa in the consensus. The most commonly used are strict, semi-strict (Bremer, 1988), Adams consensus (Adams, 1972) and majority rule (Margush and McMorris, 1981) trees. Strict consensus trees contain only those groups appearing in all of the equally parsimonious trees generated by analysis of the data set and represent the most conservative

approach to consensus. Semi-strict trees differ from strict counterparts in that they retain groups that are not supported in all parsimonious trees but are not specifically contradicted. Majority-rule consensus finds groups that appear in a pre-specified percentage of the total set of most parsimonious trees, while Adams consensus preserves more structure than does strict method.
In the present study strict consensus trees were generated in analysis 2 using the nelsen command in Hennig and the strict option in PAUP.

8. Assessment of confidence

Cladograms are currently being used as a basis for botanical classification and formal naming of taxa as well as a means of presenting phylogenetic hypotheses or exploring biogeographical pattern (Linder, 1991). The assessment of confidence in a particular cladogram or its nodes has become increasingly necessary. The simplest estimate of support for an individual branch relates to the number of character state changes associated with that branch (i.e. branch length) but also to the nature of change, greater support being provided by complex rather than simple morphological change and by non-homoplastic as opposed to homoplasious characters (Bremer, 1994). Homoplasy, which is according to the latter author common in morphological data at higher taxonomic levels, presents a particular problem when assessing branch support from branch length alone. More robust methods are now preferred for the testing of cladogram or clade stability. These fall into two broad categories, involving perturbation of the data set and of the analytical method respectively (Davis, 1993; Bremer, 1994).

8.1. Data perturbation

8.1.1. Bootstrapping

This is a method for the estimation of statistical error in situations where the underlying sample distribution is unknown or difficult to derive analytically (Felsenstein, 1985; Sanderson, 1989). The bootstrap offers a way of approximating this distribution by resampling from the original data set by simultaneous duplication and elimination of characters to give a set of bootstrap replicates of the same size as the original data set. These are used to construct a new set of trees of which a majority-rule consensus is generated. The proportion of replicates supporting a given clade is considered a measure of support i.e. a clade appearing in X% percent of bootstrap trees is associated with a confidence level of X%. This provides a quantitative value for the occurrence of specific clades, given the available data.

Sanderson, in a detailed discussion of the method, noted that its application was restricted to hypotheses regarding membership of terminal taxa i.e individual clades. He also drew attention to its limitations in respect of data sets with low character to taxon ratios and/or moderate to high levels of character conflict, a *caveat* echoed by Olmstead *et al.* (1992). Hillis and Bull (1993), in an assessment of the value of bootstrapping as a measure of repeatability and accuracy, concluded that the method was a biased but highly conservative estimate of the probability of correctly inferring clades. Nevertheless, estimates of bootstrap support are commonly included in phylogenetic studies.

In the present study bootstrap analyses were made of both data sets (analyses 1 and 2) as a first measure of confidence in the clades discovered by parsimony analysis. In the case of analysis 1, bootstrapping was carried out only on the unweighted data set, while in analysis 2 both unweighted and weighted data sets were analysed. In all cases, 200 replicates were specified and trees generated by heuristic search methods, using a simple addition sequence and the TBR branch-swapping algorithm with MULPARS option in effect.

8.1.2. Character removal/character deletion

This is a non-statistical method for assessing the stability of each clade resolved by a data set (Davis, 1993), in terms of which an estimate is made of the minimum number of characters that must be removed before resolution of the clade is lost i.e. it no longer appears in the consensus tree. The clade stability index (CSI), which is the ratio of the minimum number of required character removals to the number of informative characters in the data set, provides a direct quantitative measure of support for a given clade in terms of characters. Clade CSI cannot exceed a value of 1, while a CSI value of 0 indicates that a clade is not retrieved even by the complete data set. Davis argued that character removal provided an index of quality of cladistic data sets that was distinct from that which was measured by the bootstrap and noted that while bootstrap frequencies and minimum character removal scores appeared to be correlated, each was an imprecise predictor of the other. Davis' method, criticised by Bremer (1994), is similar to the technique of jack-knifing (Farris *et al.*, 1997) which involves successive deletion of characters from a data matrix.

8.2. Bremer support

Bremer (1988), in a examination of the use and limitations of amino acid data in inferring angiosperm phylogeny, drew attention to the fact that single changes in even large data matrices could result in retrieval of cladograms with topologies quite different from those obtained following analysis of the unchanged data set. This led him to the conclusion that when examining protein sequence data, decisions as to correct phylogeny should be based not only on the topologies of the most parsimonious trees, but also on that of cladograms several steps longer. Bremer noted the difference in his approach to branch support (based on the number of character state changes) from that of Davis (1993), based on number of character changes. He suggested a method for extending his estimate of branch support to estimates of support of an entire topology, by calculation of a total support index (the sum of all branch support values).

Estimates of "Bremer support" (Bremer, 1988, 1994) i.e. the loss of resolution in less than parsimonious trees, or the extra length needed to lose a branch in the consensus of near most parsimonious trees, were made in the present study. This approach, referred to as decay analysis by Donoghue *et al.* (1992) and Olmstead *et al.* (1993), represented an assessment, independent of bootstrap support, of confidence in the topologies discovered in analyses 1 and 2.

8.3. Constraint analysis

This constitutes a means of evaluating the cost of alternative topologies. Two constraint analyses, based respectively on the unweighted and weighted data sets used for analysis 2, were carried in order to test the hypotheses that:

1. *Brunia* species constitute a monophyletic group, as recognised by Pillans (1947) - unweighted data set.
2. *Audouinia* is the most basal taxon in the family, as suggested by Dahlgren and van Wyk (1988) -unweighted and weighted data set.

B. RESULTS OF ANALYSIS 1

IDENTIFICATION OF SISTER GROUP(S) OF BRUNIACEAE

The use of m*bb* in the present analysis retrieved a single tree, 67 steps in length, with a consistency index of 0,34 and a retention index of 0,61 (Figure 14). All of the ingroup taxa, with the exception of Theaceae comprised a clade (A) diagnosed by twin synapomorphies: absence of foliar myricetin (Character 4) and possession of unitegmic ovules (Character 18). Within this lineage, the following sub-clades were evident:

1. A Grubbiaceae/Aquifoliaceae/Cornaceae clade, supported by twin parallelisms of perianth tetramery and tri/multilacunar nodal anatomy (Characters 11 and 21)

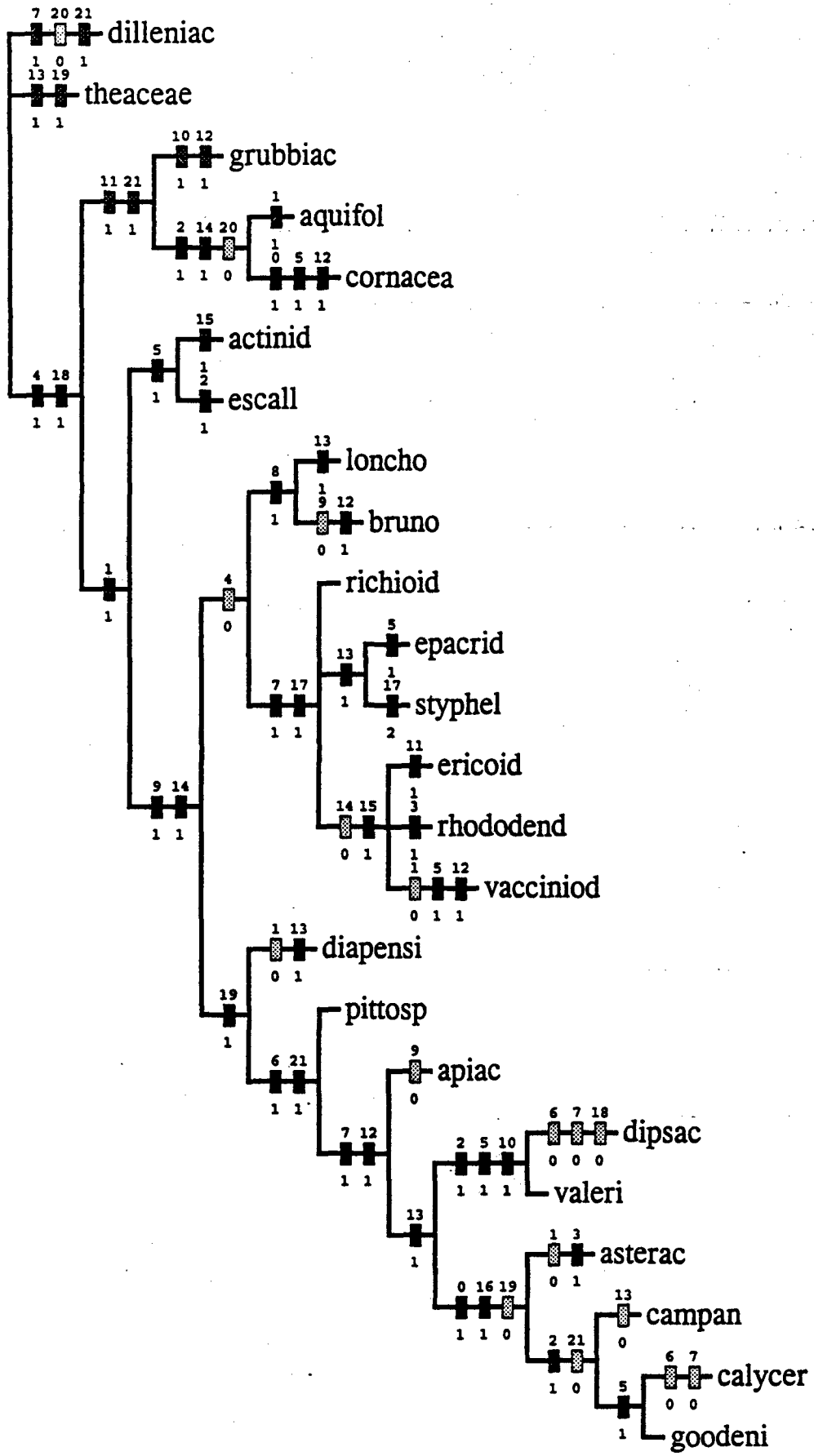


Figure 14. Single tree obtained in analysis 1 (identification of sister group of Bruniaceae), using Hennig 86.

Non-homoplasious forward changes=solid bars; homoplasious forward changes (parallelisms)=dark stippling; reversals=light stippling

and sister to the remaining ingroup taxa (Clade B). Within this clade, Grubbiaceae was sister to a Cornaceae/Aquifoliaceae lineage, diagnosed by two parallelisms {Characters 11 + 21 {perianth tetramery and tri/multilacunar nodal anatomy)} and one reversal (Character 20: crassinucellate ovules).

2. Within Clade B, which was diagnosed by a single parallelism (absence of foliar ellagic acid), an Escalloniaceae/Actinidiaceae lineage, supported by Character 5 (presence of iridoids), was sister to a grouping comprising the remaining ingroup taxa (Clade C), supported by one synapomorphy (Character 9: sympetaly) and one parallelism (Character 14: number of stamens equal to or less than the number of perianth segments).

3. Clade C contained two distinct lineages: a Bruniaceae + Ericaceae + Epacridaceae grouping (Clade D), diagnosed by a single reversal (presence of foliar myricetin; Character 4) and a clade comprising all of the remaining families included in the present analysis (Clade E).

4. Within Clade D Bruniaceae, comprising a monophyletic lineage supported by the synapomorphy of black leaf apiculae (Character 9), was sister to an Ericaceae/Epacridaceae grouping diagnosed by one synapomorphy (Character 17: pollen shed in tetrads) and one parallelism (Character 7: presence of leaf HCN). The epacridalean lineages did not form a distinct clade; rather the *Richea* and *Epacris/Styphelia* groupings were sisters to Ericaceae, which appeared to represent a monophyletic lineage diagnosed by one parallelism (Character 15: anther dehiscence by apical pores) and one reversal (Character 14: stamen number equal to or more than twice the number of perianth segments).

5. In Clade E, supported by the single parallelism of absence of an endosperm haustorium (Character 19), Diapensiaceae occupied a position as sister to a lineage in which Pittosporaceae and Apiaceae were placed at the base of a sub-clade diagnosed by one synapomorphy (Character 6: presence of polyacetylenes) and one parallelism (Character 21: tri/multilacunar nodal anatomy). Within this lineage two groupings were evident: a Dipsacaceae + Valerianaceae lineage (Clade F) that appeared well-supported by three parallelisms: absence of foliar proanthocyanidins, presence of iridoids and

opposite leaf arrangement (Characters 2, 5 and 10 respectively) and a Campanulaceae + Calyceraceae + Goodeniaceae + Asteraceae grouping (Clade G) diagnosed by one parallelism, one synapomorphy and one reversal: capacity to accumulate inulin, presence of pollen presenters and of endosperm haustoria (Characters 0, 16 and 19 respectively).

Apiaceae was sister to the lineage comprising Clades F and G, which was supported by a single parallelism: Character 13 (stamens adnate to petals).

6. Within Clade G, Asteraceae was sister to a grouping comprising Campanulaceae, Calyceraceae and Goodeniaceae, diagnosed by one parallelism (Character 2: absence of foliar proanthocyanidins) and one reversal (Character 21: unilacunar nodal anatomy). Campanulaceae was sister, in this lineage, to Calyceraceae/Goodeniaceae, supported by a single parallelism: presence of iridoids (Character 5).

In the bootstrap analysis, with the exception of the Ericaceae clade (55%) and Dipsacaceae/Valerianaceae lineage (61%), support for the groups shown on the cladogram was less than 50%. Estimates of "Bremer support" (decay index) gave similar results in that all nodes collapsed in the strict consensus tree of the 244 trees one step longer than the single most parsimonious tree, except for the nodes below the Ericaceae grouping and Clade F. All nodes collapsed in trees two steps longer than the strict consensus tree.

The current view that Ericaceae is paraphyletic unless merged with Epacridaceae and Empetraceae, based on the results of several phylogenetic studies (Judd and Kron, Kron and Chase, Anderberg, Chase *et al.*, all 1993; Crayn *et al.*, 1996 and Kron, 1996) and formalised by Kron (1997), is not however supported by the results of the present analysis.

C. DISCUSSION OF RESULTS OF ANALYSIS 1

IDENTIFICATION OF SISTER GROUP(S) OF BRUNIACEAE

1. Sister relationship of Bruniaceae and Ericaceae/Epacridaceae

The sister relationship of Bruniaceae and Ericaceae/Epacridaceae suggested by the results of the present analysis is significant for two reasons. Firstly, it may represent an advance on the findings of recent phylogenetic studies (see Sect. 1.1. below). Secondly, it is compatible with most traditional classifications (see Sect. 1.2. below), despite the fact that these have disagreed to the extent of placing Bruniaceae in three of Cronquist's (1981) superorders.

This result must nevertheless be viewed as tentative in that support for the Bruniaceae/Ericaceae/Epacridaceae clade is not strong either in terms of number of characters or in bootstrap and Bremer support. Inclusion of more characters in the analysis may result in better support and the need for detailed studies of reproductive biology, floral ontogeny and anatomy, fruit morphology and anatomy, embryology and chromosome cytology in Bruniaceae is highlighted. Apart from the flavonoid profile of the family, investigated in this study, its secondary chemistry is also not well understood. Only one species has been examined for the presence of iridoids, while the occurrence of polyacetylenes and sesquiterpene lactones, characteristic of Apiales and Asteraceae, has never been investigated in Bruniaceae.

Poor support for some internal nodes of a cladogram has been a problem in other phylogenetic analyses based on non-molecular data. In a study of relationships in Ericales (Anderberg; 1992), support for an Empetraceae + Ericaceae + Epacridaceae clade was limited to a single homoplasious character: diplostemony. This monophyletic lineage has been nevertheless identified in three major phylogenetic studies based on molecular data. In a second study of Ericales, Anderberg (1993) identified a Vaccinioid + Arbutoid + Epacridaceae clade, also based on a single homoplasious character: early anther inversion. A similar relationship was uncovered in the large-scale molecular phylogenetic analysis of Chase *et al.* (1993). Molecular studies of Bruniaceae are needed in

order to make an independent assessment of the relationships suggested by the present study.

1.1 Comparison of the results of the present study with those of recent phylogenetic analyses

Bruniaceae has been included in six large-scale recent phylogenetic studies, of which four (Olmstead *et al.*, 1993; Chase *et al.*, 1993; Soltis *et al.*, 1997; Hoot *et al.*, 1999) utilised molecular data, one (Nandi *et al.*, 1998) combined molecular and non-molecular data sets and one (Hufford, 1992) morphological and chemical information. These studies do not agree as to the sister relationship(s) of Bruniaceae and none agree with the results of the present study in its identification of Epacridaceae and Ericaceae as sister taxa to Bruniaceae. The areas of conflict are discussed below, dealing in chronological order with the relevant studies.

HUFFORD (1992)

Hufford's analysis of relationships among Cronquist's Rosidae and other non-magnoliid taxa placed Bruniaceae as sister to a large "corniflorae-asterid" clade which mainly comprised families of Cronquist's Asteridae but also of his Dilleniidae and Hamamelidae. Ericaceae was included in this lineage i.e. a similar position with respect to Bruniaceae to that indicated by the present study. The importance of Hufford's study lay in its identification of a more broadly-circumscribed Asteridae than had previously been recognised and in its association of Bruniaceae with this lineage. Internal support for the "corniflorae-asterid" clade was not however assessed in this study.

The results of Hufford's analysis were not based on accurate information for Bruniaceae, so cannot be directly compared with those of the present study. He coded the family as having pollen grains 20-30μ or larger with reticulate tectal

sculpture, crassinucellate ovules, marginal and/or axile placentation and no foliar proanthocyanidins. The correct states are: pollen grains mostly < 20μm with foveolate sculpture, tenuinucellate ovules (although this needs further study), axile-apical placentation and proanthocyanidins universally present.

Epacridaceae was not represented in Hufford's study, while Ericaceae was coded as having a pentamerous corolla and ellagic acid present. This does not reflect the polymorphism that exists within Ericaceae for these characters: members of subfamily Ericoideae have tetramerous flowers, while ellagic acid has been recorded from subfamily Vaccinioideae only. Subdivision of the family into units monomorphic for these characters may well have influenced the topology of trees discovered in the study.

CHASE *et al.* (1993)

This analysis, which addressed the question of spermatophyte phylogeny using DNA sequences of the chloroplast gene *rbc*L form 499 taxa, retrieved an monophyletic "asterid" group composed of Cronquist's Asteridae together with elements of his Dilleniidae, Rosidae and Hamamelidae. Within this clade five subclades, termed Asterid I-V, were distinguishable. Bruniaceae, represented by *Berzelia lanuginosa*, was placed in Asterid II, while Ericaceae and Epacridaceae (represented by 15 and 5 species respectively) were placed in Asterid III, with Epacridaceae nested within Ericaceae. Molecular evidence thus differed from non-molecular evidence (Hufford, 1992) in suggesting that Bruniaceae was part of, rather than sister to, an asterid lineage and also in its basal placement of Ericaceae with respect to Bruniaceae. Within Asterid II, Bruniaceae was found in the study of Chase *et al.* to be sister to a large clade comprising Asteraceae + Goodeniaceae + Campanulaceae and Cornaceae/Pittosporaceae/Apiaceae. Dipsacaceae/Valerianaceae was sister to this lineage (see Figure 15).
As Chase *et al.* noted, the importance of their study lay in its predictive value, rather than in the accuracy of the topologies discovered, internal support for

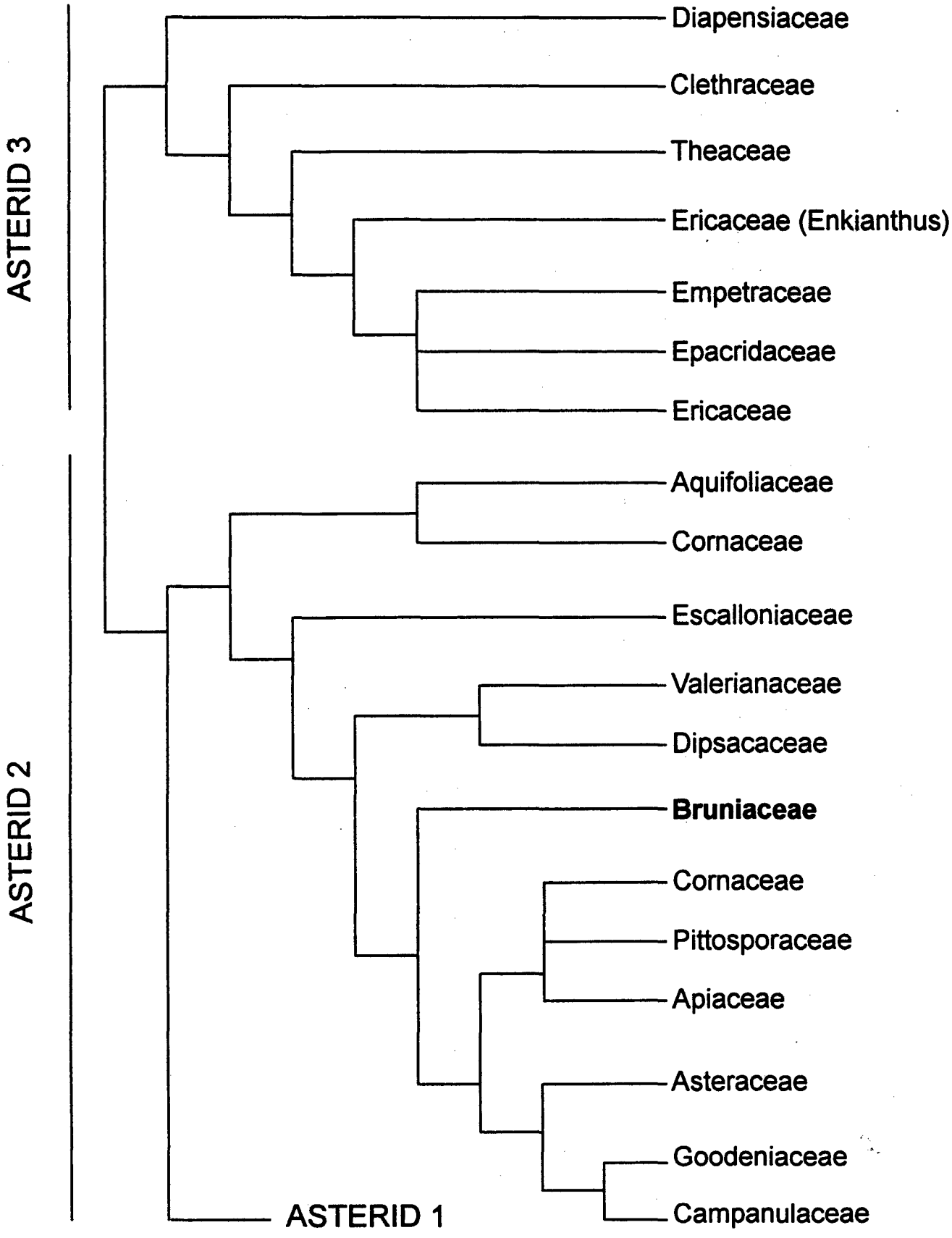


FIGURE 15:
Simplified strict consensus tree of Search I, from Chase *et al.* (1993) analysis of seed plant phylogeny.

which could not be adequately evaluated on account of the size of the data set. The results of the study were particularly valuable as a guide to the identification of potential sister-groups to Bruniaceae to be included in the present cladistic analysis. Terminal taxa were selected from the Asterid II and III clades retrieved in the Chase *et al.* analysis, thus representing families both above and below Bruniaceae in Asteridae *s.l.*

OLMSTEAD *et al.* (1993)

A position of Bruniaceae within (rather than as sister to) Asteridae *s.l.* was also supported by the results of an analysis by Olmstead *et al.* (1993), which examined relationships within Asteridae *s.l.*, based on *rbc*L sequence data. Bruniaceae (again represented by *Berzelia lanuginosa*) was not resolved to a particular clade in this study. This is surprising considering its more focused purpose in comparison with that of Chase *et al.* (1993). The initial analyses identified 11 clades that were congruent between all islands, with Bruniaceae not resolved to any of these. In a second analysis, of relationships between 3 of the 11 clades, namely Asterales, Dipsacales and Apiales, Bruniaceae was not resolved, either within a Dipsacales + Apiales clade (Island-80) or within the entire Asterales/Apiales/Dipsacales group (Island-196; see Figure 17).
No relationship was suggested in any of the analyses between Bruniaceae and an ericalean clade, which included representatives of both Ericaceae and Epacridaceae (5 and 1 species respectively) in the initial analyses but not in the second (1 representative of Ericaceae).

SOLTIS *et al.* (1997)

Soltis *et al.* (1997) addressed the question of angiosperm phylogeny as revealed by cladistic analysis of sequences from the nuclear gene 18S ribosomal DNA. Bruniaceae was represented in this study by *Berzelia lanuginosa* and Ericaceae by *Arctostaphylos uva-ursi* and *Vaccinium macrocarpa* - both members of

Simplified strict consensus tree of 5294 shortest trees resulting from the exploratory phylogenetic analysis of 223 species of angiosperms (from Soltis *et al.*, 1997)

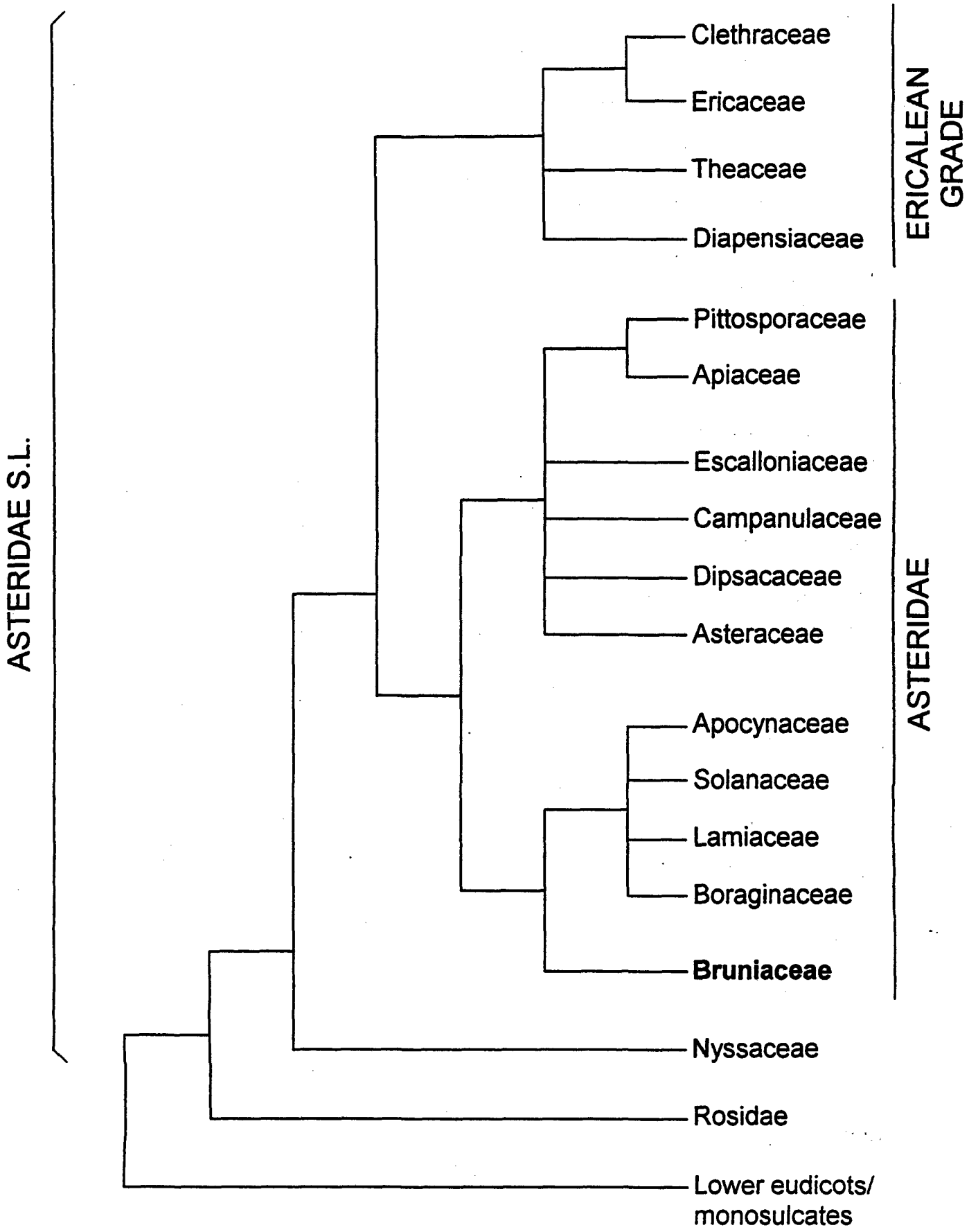

ASTERIDAE S.L.
Clethraceae
Ericaceae
Theaceae
Diapensiaceae
ERICALEAN GRADE
Pittosporaceae
Apiaceae
Escalloniaceae
Campanulaceae
Dipsacaceae
Asteraceae
Apocynaceae
Solanaceae
Lamiaceae
Boraginaceae
Bruniaceae
ASTERIDAE
Nyssaceae
Rosidae
Lower eudicots/ monosulcates

FIGURE 16

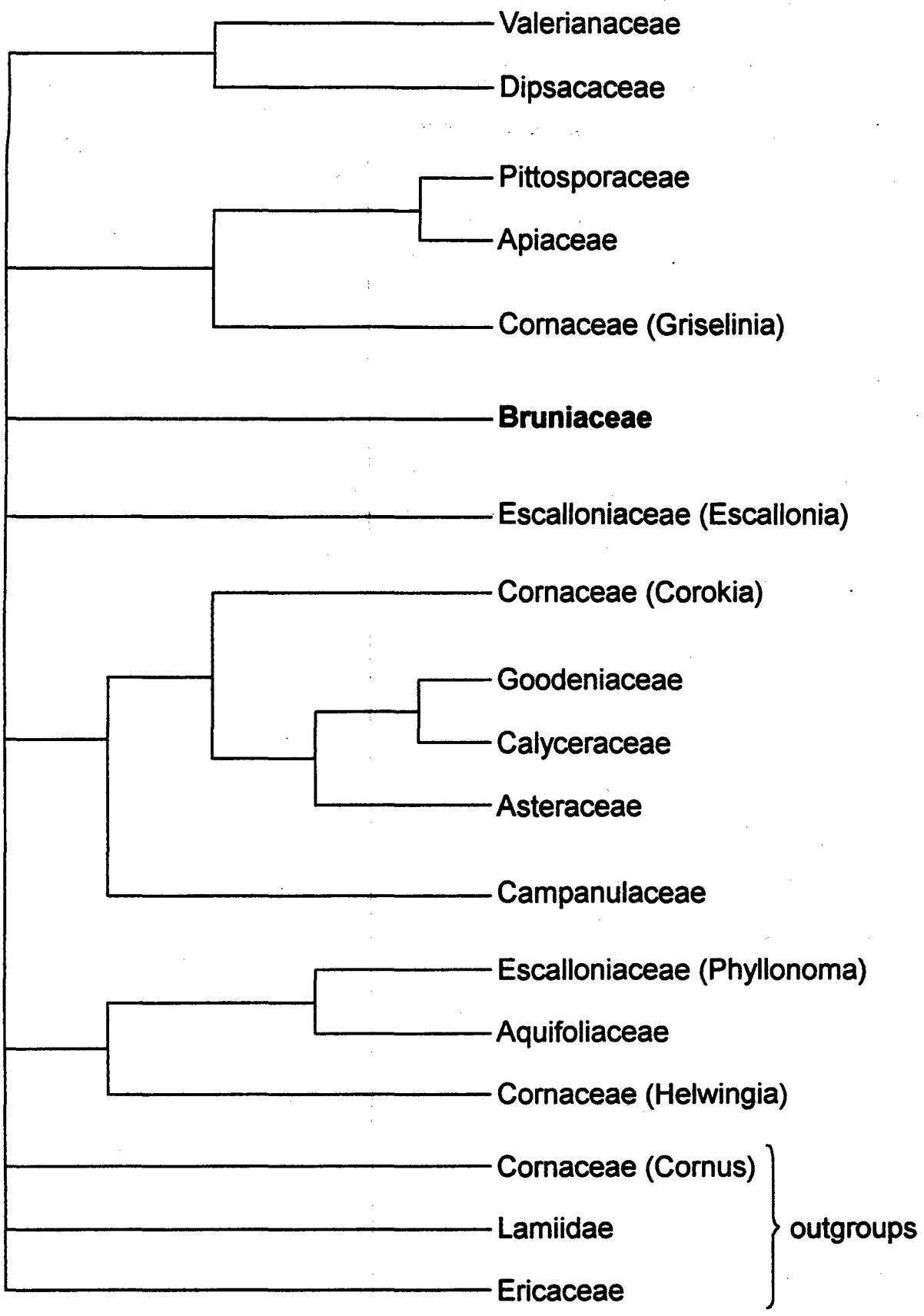


FIGURE 17:
Simplified strict consensus tree of 196 trees (Island 196) obtained in the phylogenetic analysis by Olmstead *et al.* (1993) of relationships in Asteridae *s.l.* This section of the analysis explored relationships between three of the clades identified in the primary analysis: namely Asterales *s.l.*, Apiales and Dipsacales.

subfamily Vaccinioideae according to Stevens (1973). Epacridaceae was not represented. This second large-scale study retrieved topologies that were largely congruent with those found by Chase *et al.*, although based on fewer taxa (223 species). In particular, all searches conducted in the analysis of Soltis *et al.* identified a large eudicot clade in which two subclades could be distinguished, largely corresponding to Rosidae and Asteridae *s.l.*

In the strict consensus of 5294 shortest trees resulting from phylogenetic analysis, Bruniaceae was placed in Asteridae s.l. as part of an Asteridae clade, at the base of a lineage comprising families of Cronquist's Lamiidae. An Ericalean grade, together with Caryophyllidae *s.l.* was sister to Asteridae. Parsimony jackknife analysis was used to assess support for the topologies obtained, but neither the Asteridae nor the Ericalean grade had values above 50.

The analyses of Chase *et al.* and Soltis *et al.*, based on chloroplast and nuclear gene sequences respectively, thus disagreed both in respect of the sister-relationships of Bruniaceae and of its position with respect to Ericaceae/Epacridaceae.

NANDI *et al.* (1998)

These workers conducted a cladistic study of 162 angiosperm taxa selected according to topologies obtained in various cladistic studies including those of Chase *et al.* (1993) and Hufford (1992). Both non-molecular and molecular (*rbc*L) data sets were analysed, separately and in combination. Bruniaceae was represented by *Berzelia lanuginosa* and Ericaceae by *Erica australis* only. Epacridaceae was not included. A total of 252 non-molecular characters was used in the first analysis, which retrieved a poorly-supported Asterid lineage. Within this clade three subclades were distinguishable: one comparable to Asterid III of Chase *et al.*, a second comprising families from Chase *et al.*'s Asterid clades I, II and IV and a third composed of taxa from Chase *et al.*'s Asterid I, II, III and V together with families of Cronquist's Rosidae and Dilleniidae that were not included in the analysis of Chase *et al.*

Within the first of these three subclades, an ericalean lineage was reasonably well-supported (59% bootstrap value) but none of the remaining clades had bootstrap values of greater than 50. Nandi *et al.* attributed the lack of internal support for tree topology obtained in the analysis of non-molecular data to a high number of empty cells i.e. missing information.

Analysis of the *rbcL* sequence data retrieved a moderately well-supported (bootstrap value 52%) Asterid III subclade *sensu* Chase *et al.*, including Ericaceae, together with poorly-supported Asterid I+II and IV subclades. Bruniaceae was placed as sister to Campanulales, in Asterid I+II.

Analysis of the combined data sets with successive weighting produced a single most parsimonious tree with no well-supported nodes (bootstrap values all less than 50%). As in the *rbc*L analysis, Bruniaceae was part of an Asterid I+II subclade, but in this instance as sister to Eucommiales and Icacinaceae (see Figure 19).

The different sister relationships for Bruniaceae discovered in the three analyses may have to do with the fact that, in the non-molecular data set, the family had 45.2% missing information (117 cells out of 242). In addition, at least ten of the characters were polymorphic for the family and could have been coded differently had a representative other than *Berzelia lanuginosa* been selected.

HOOT *et al.* (1999)

This study examined relationships among basal eudicots, using sequences of three genes: the chloroplast genes *atp*B and *rbc*L and nuclear ribosomal 18S DNA. Taxa (73) were selected on the basis of representivity of most of Tahktajan's subclasses and superorders (Tahktajan, 1997) with *Berzelia lanuginosa* the sole placeholder for his Ericanae. Asteridae according to Tahktajan were not represented in this study.

In three separate analyses based on individual gene sequences as well as two based on combined data sets (*atp*B/*rbc*L and *atp*B/*rbc*L/nr18S), *Berzelia* was placed as sister to *Hedera* (Araliaceae). This lineage was very well-supported

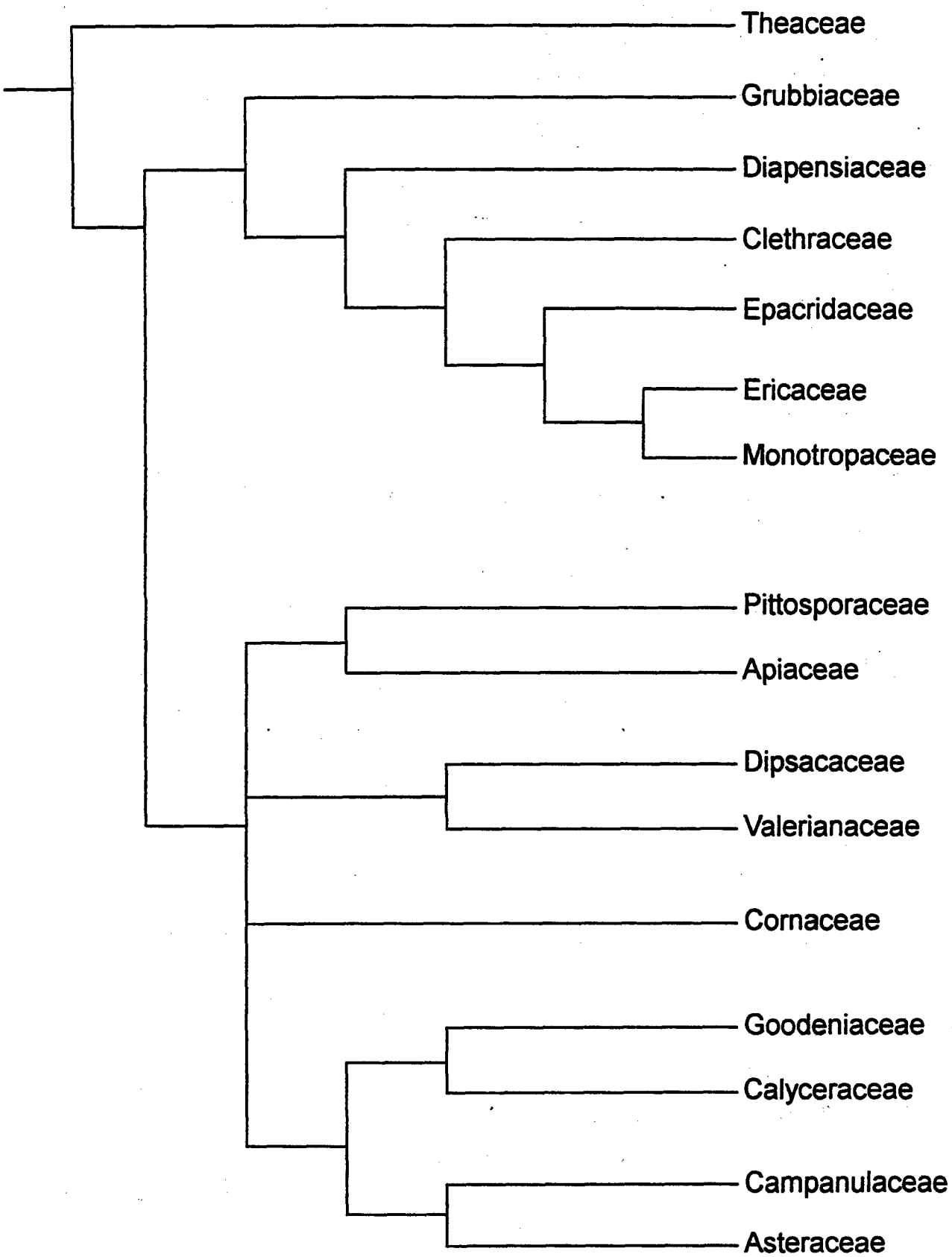


FIGURE 18:
Simplified strict consensus of 100 most parsimonious trees generated in an analysis of the circumscription of Ericales by Anderberg (1992), based on non-molecular data.

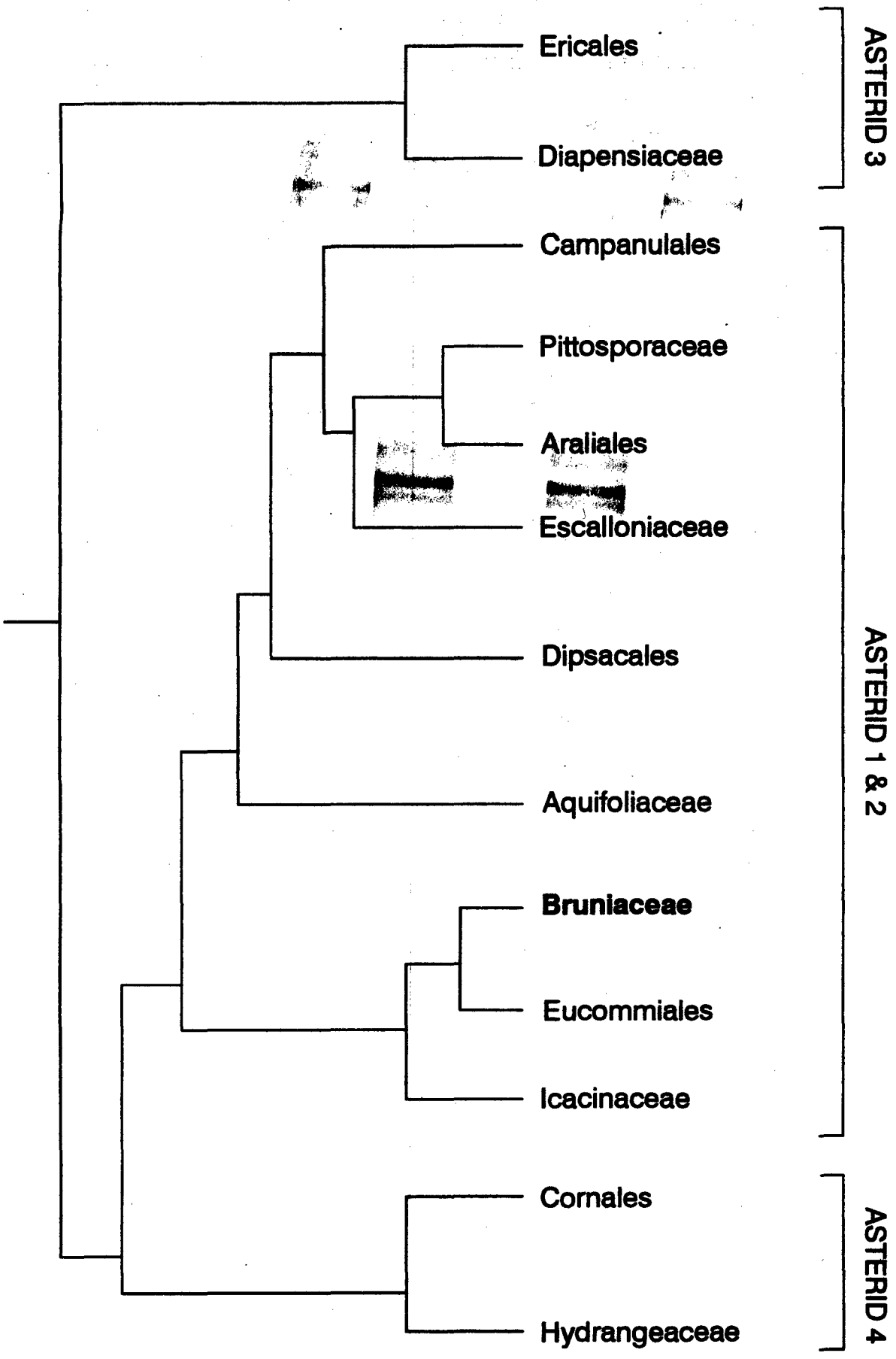


FIGURE 19:
Asterid clade (simplified) found in the single most-parsimonious tree generated in a combined cladistic analysis of angiosperms by Nandi *et al.* (1998), using *rbc*L and non-molecular data sets and successive weighting.

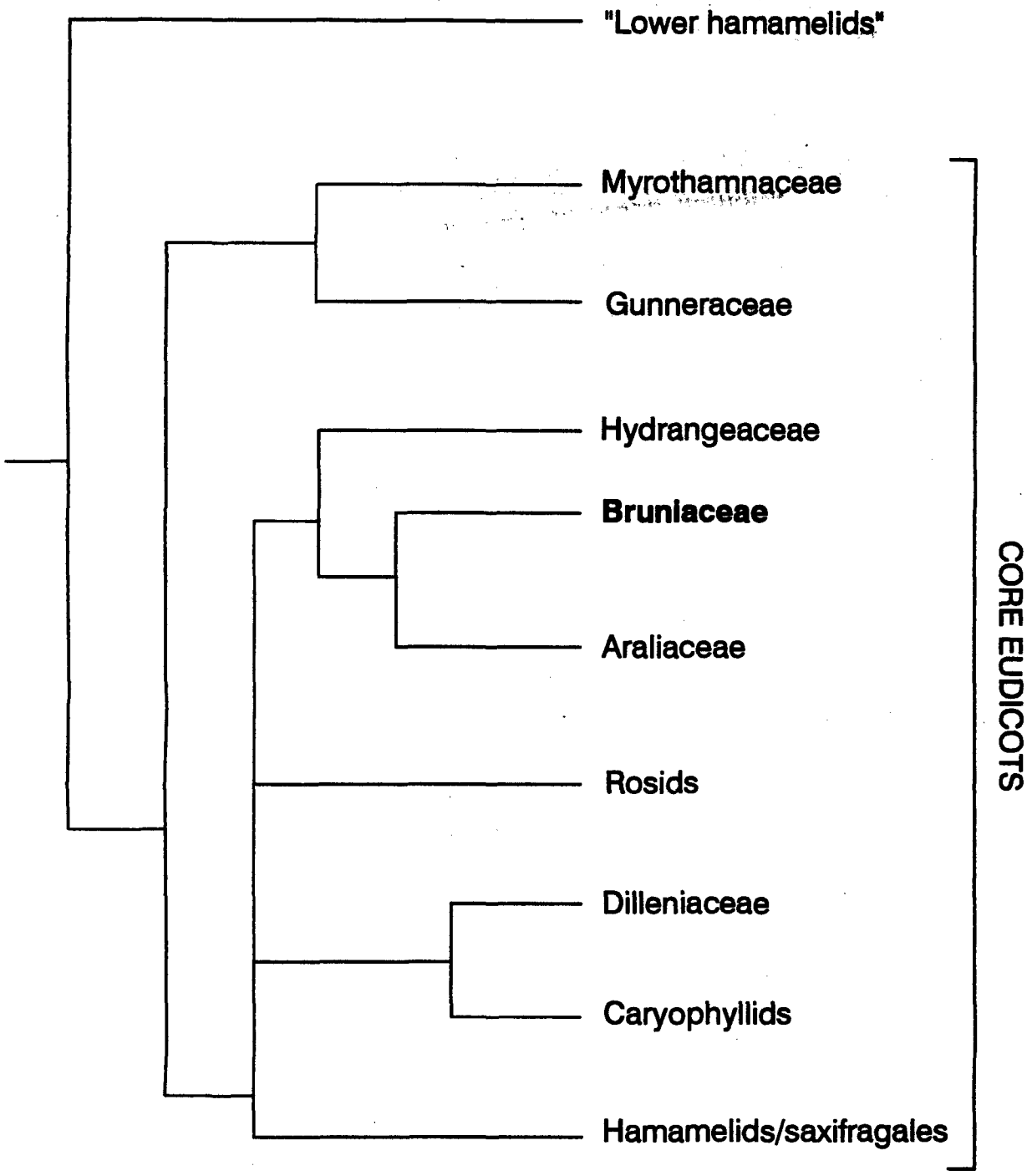


FIGURE 20:
Strict consensus tree (simplified to show the position of Bruniaceae) of the 15 shortest trees obtained from combined data (*atp*B, *rbc*L and 18S) used in a cladistic analysis of relationships among basal eudicots by Hoot *et al.* (1999).

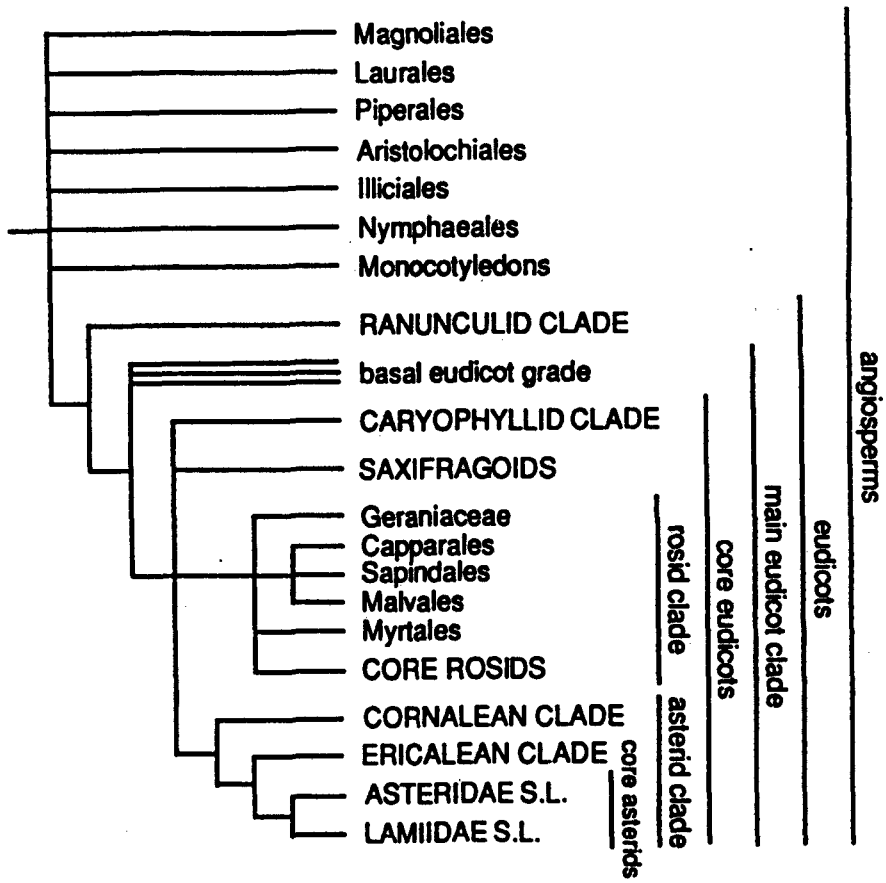
(85-100% of bootstrap replicates) in all except the analysis based on nr18S sequences. *Hydrangea* (Hydrangeaceae) was sister to this species-pair in all except the nr18S analysis (see Figure 20). The retrieval of this lineage is significant in that it unites representatives of two of Tahktajan's subclasses, not placed in his Asteridae but included in Asteridae *s.l. sensu* Cronquist by Chase *et al.* (1993), Soltis *et al.* (1997) and Nandi *et al.* (1998). However, in the analysis of Chase *et al.* Bruniaceae was part of Asterid II, while Hydrangeaceae and Araliaceae were included in Asterid IV.

ANDERBERG (1992)

A further study of interest is Anderberg's analysis of the circumscription of Ericales (1992), based, as in the case of the present analysis, on non-molecular data. Although Anderberg did not include Bruniaceae in his study, he focused on relationships that are relevant to the results of the present one. As can be seen from Figure 18, the topology of the strict consensus tree obtained by Anderberg closely resembles that of the tree shown in Figure 14, although very different sampling strategies and character sets were used in the two studies.

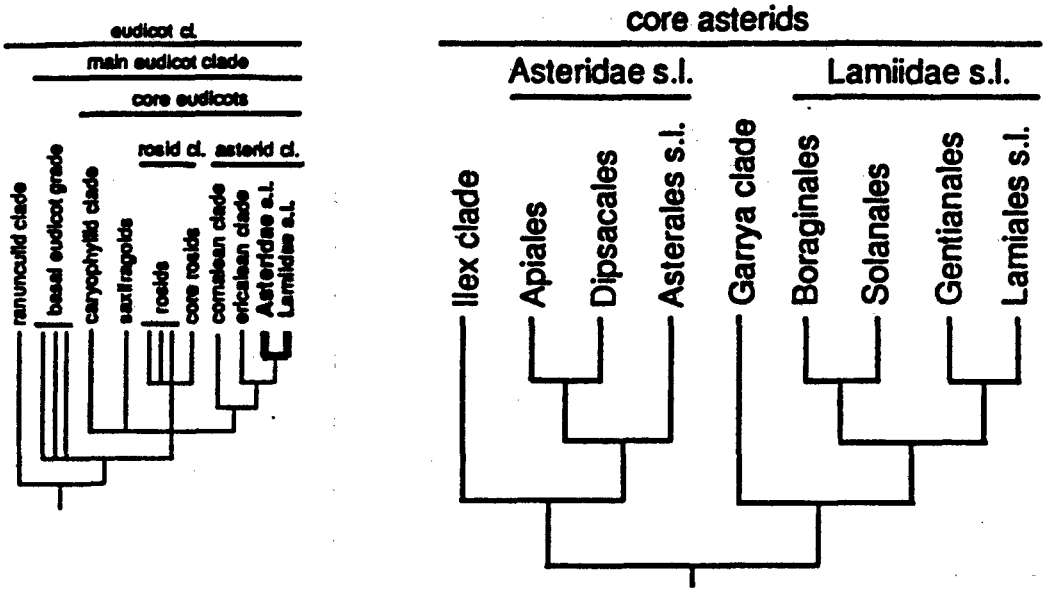
1.2. The position of Bruniaceae within the eudicots

All six studies discussed above agreed in associating Bruniaceae with a monophyletic asterid lineage (=asterids *sensu* Chase *et al.* 1993; Asterids *sensu* APG, 1998) that has been identified in most recent phylogenetic analyses of relationships among angiosperm families (see A below, from Magallón *et al.*, 1999 and refs. therein).



A. General eudicot phylogenetic pattern (from Magallón *et al.*, 1999).

Within this clade, three subclades have been identified: cornalean, ericalean and core asterid, the latter again divisible into smaller clades, termed Asteridae *s.l.* and Lamiidae *s.l.* by Magallón *et al.* Bruniaceae has been placed in Asteridae *s.l.* (=euasterids II *sensu* APG, 1998) by these authors, together with Dipsacales, Apiales and Asterales, which lineages comprise families of Cronquist's Asteridae and Rosidae. Ericaceae and Epacridaceae have on the other hand been included in the ericalean clade (=asterid III *sensu* Chase *et al.*, 1993; Ericales *sensu* APG, 1998) according to Magallón *et al.* (1999), along with Diapensiaceae, Theaceae, Grubbiaceae and Clethraceae.



B. Relationships within the Asterid clade: core asterids (from Magallón *et al.*, 1999)

The different sister group relationships indicated for Bruniaceae by the results of present study and of those discussed above may be related to several factors:

a) Current lack of agreement as to relationships within asterid clade

The phylogenetic pattern within the asterid clade (see B above) is not universally supported, although apparent in many analyses, mostly based on rbcL sequence data (Magallón *et al.*, 1999). In the analyses of Olmstead *et al.* (1993), Soltis and Soltis (1997) and Soltis *et al.* (1997) for instance, the ericalean and cornalean clades and Asteridae *s.l.* appeared to be non-monophyletic. In some studies these clades appear in unexpected positions e.g the cornalean clade as sister to Lamiidae *s.l.* + *Garrya* (Downie and Palmer, 1992) or Lamiidae *s.l.* at the base of the asterid clade (Olmstead *et al.*, 1993).

The ericalean clade, though retrieved in analyses based on both *rbc*L and 18S sequences (Olmstead *et al.*, 1993; Chase *et al.*, 1993; Soltis and Soltis, 1997), has also appeared as a grade closely linked with the caryophyllid clade within an asterid assemblage (Soltis *et al.* 1997). The placement of the ericalean clade as sister to the core asterids (Hufford, 1992; Chase *et al.*, 1993) is not universally supported. Soltis and Soltis (1997) found this clade to be sister to Hydrangeaceae, together sister taxon to the core asterids. Olmstead *et al.* (1993) found the ericalean clade to be sister to asterids excluding Lamiidae *s.l.*, while Downie and Palmer (1992) found it to be the most basal clade within the asterids. Other studies, discussed in Magallón *et al.* (1999), have discovered yet other placements of the ericalean clade.

b) Scale of the analyses

The six studies discussed above were all, with the exception of that of Olmstead *et al.* (1993), aimed at exploring relationships at ordinal level and above. The present study focused on relationships within the Euasterid II clade (APG, 1998). Different sampling densities were used, which is likely to have influenced the topologies of the trees discovered. It has been noted (Soltis *et al.*, 1993; Olmstead *et al.*, 1993; Hillis, 1998; Poe, 1998) that uneven and/or insufficient taxon sampling may result in incorrect topologies or anomalous placements in molecular (and presumably also other) phylogenies. Bruniaceae was represented in all six studies discussed above by a single species: *Berzelia lanuginosa*. As a sample of a palaeoendemic and morphologically, anatomically and palynologically extemely diverse family, this was probably inadequate. If, as has been suggested by Hall (1987), the diversity exhibited by the family is the product of great age, giving Bruniaceae "time to accumulate the results of numerous evolutionary events", then it may be that more extensive sampling of the lineages identified in the phylogenetic analysis of within-family relationships is necessary, in order to accurately reflect molecular diversity.

The fact that none of the recent phylogenetic studies have associated Bruniaceae with Ericaceae or Epacridaceae may also have to do with uneven sampling of taxa in these lineages. The representation of these families was sparse and/or uneven in all the analyses except for that of Chase *et al.* and was probably inadequate (see discussion above) for the purposes of reflecting within-family diversity. This could be seen particularly in the non-molecular analyses, where characters polymorphic for the family were coded as having a single state.

c) Sampling strategy

Some of the studies discussed above e.g. Hufford (1992) and Soltis *et al.* (1997) selected representative taxa according to Cronquist's classification (Cronquist, 1981), while Hoot *et al.* (1999) followed Tahktajan's system (Tahktajan, 1997). Nandi *et al.* (1998) sampled according to existing macrotaxonomic studies as well as lineages discovered in previous cladistic studies. It seems possible that a

biased approach may result from too-rigid adherence to any one taxonomic system or topology. A good example is provided by the present study; for purposes of flavonoid analysis, taxa were selected according to the classifications of Cronquist (1988), Dahlgren and van Wyk (1988) and G. Dahlgren (1989) because molecular studies were not at the time available as a guide to possible sister taxa. These systems suggested families now included in an ericalean clade (APG, 1998). On the other hand, taxa used in the cladistic study to explore sister relationships of Bruniaceae were selected from the Asterid II (= euasterid II; APG, 1998) and III lineages discovered in the analysis of Chase *et al.* (1993). Had Tahktajan's system been followed throughout, selection of taxa would have been different again, as Bruniaceae is placed by him in Superorder Ericanae of Subclass Dilleniidae. Very different relationships may have been suggested, depending on the sampling strategy followed.

d) Sources of evidence

Molecular data (DNA restriction sites and sequences) have been extensively used in recent phylogenetic studies on account of the large number of characters offered for analysis and the availability of comparable data across all major seed-plant lineages. This represents an advantage over non-molecular data which may not be comparable e.g. absence of floral characters in gymnosperms, or may give rise to empty cells in a data matrix e.g. secondary compounds present in only some taxa. Molecular data also offer the advantage that, depending on the rate of evolution of the chosen gene sequence, it is theoretically possible to examine relationships at any taxonomic level (Hillis, 1987). Sequence data from the slowly-evolving plastid gene *rbc*L, which codes for the large subunit of ribulose-1, 5-biphosphate carboxylase/oxygenase (RuBisCO), have been most often applied to exploration of relationships at ordinal level (e.g. Chase *et al.*, 1993, for the seed plants) but have also proved useful at a lower level (e.g. Kron

and Chase, 1993, for the systematics of Ericaceae, Epacridaceae, Empetraceae + related taxa). Sequences of the nuclear gene 18S ribosomal DNA have likewise been used both at family level and above (e.g. Soltis and Soltis, 1995, for relationships within Saxifragaceae; Soltis *et al.*, 1997 for the angiosperms), as have those of *atp*B (Hoot *et al.*, 1995 for relationships in Lardizabalaceae; Hoot *et al.*, 1999, for relationships among lower eudicots).

Ideally the gene sequence chosen should be appropriate to the taxonomic level of the problem addressed. Concern was expressed initially that 18S rDNA might be too evolutionarily conservative for elucidating phylogenetic relationships at family level and above (see Soltis *et al.*, 1997). Nickrent and Soltis (1995), however, compared the rate of evolution and phylogenetic resolution of 18S rDNA sequences with those of *rbc*L and concluded that seqences of the former gene were sufficiently variable for studies at higher levels among the angiosperms.

However, the fact that there exist areas of conflict between between the results of various phylogenetic studies based on different gene sequences suggests that no single gene will provide the perfect phylogeny and may not be appropriate to all levels of the taxonomic hierarchy. The large-scale analyses of Chase *et al.* (1993) and Soltis *et al.* (1997) have provided a valuable framework for more focused studies. In dealing with problem areas, the approach has often been to combine data sets from different gene sequences (Hoot *et al.*, 1999) or from molecular and non-molecular evidence (Nandi *et al.*, 1998). The current view is that the best phylogenetic information will be obtained by cladistic analysis of all available data, molecular and non-molecular i.e. using total evidence (Hillis, 1987; Kluge, 1989; Vane-Wright *et al.*, 1992; de Queiroz, 1993; Olmstead and Sweere, 1994; Nandi *et al.*, 1998).

No such analysis of the sister relationships of Bruniaceae has been possible. The conflict between the results presently available may well be resolved by an analysis using *rbc*L, 18S rDNA and *atp*B sequence data in combination with non-molecular evidence and based on adequate sampling of possible sister taxa.

Agreement between results of the present study and others

Despite the conflicting views as to sister relationships of Bruniaceae discussed above, there are areas of agreement between the results of the various studies. The topology of the single tree obtained in the present analysis (Figure 14) of non-molecular data is in agreement with topologies (see Figures 15-17) obtained in the molecular phylogenetic analyses of Olmstead *et al.* (1993), Chase *et al.* (1993), and Soltis *et al.* (1997) in their common retrieval of:

1. An Asteraceae/Goodeniaceae/Campanulaceae clade
2. A Dipsacaceae/Valerianaceae clade
3. A Pittosporaceae/Apiaceae clade
4. A Cornaceae/Aquifoliaceae clade

A further study of interest is Anderberg's analysis of the circumscription of Ericales (1992), based, as is the present analysis, on non-molecular data. Although Anderberg did not include Bruniaceae in his study, it focused on relationships that are relevant to the results of the present one. As can be seen from Figure 18, the topology of the strict consensus tree obtained by Anderberg closely resembles that of the tree shown in Figure 14. Broad support for the results of the present analysis is therefore forthcoming from recent phylogenetic studies based on both molecular and non-molecular data.

1.2 Comparison of the results of the present analysis with existing classifications

When viewed against the concept of an Asteridae *s.l.*, the different views regarding affinities of Bruniaceae (see Table 8, reproduced from Chapter 1) are reconciled. Brongniart (1826) allied the family with Araliaceae and Cornaceae, as did van Tieghem (1897). Lindley (1853), Niedenzu (1891), Thorne (1976), Dahlgren (1980), Dahlgren and van Wyk (1988), G. Dahlgren (1989) and Carlquist (1991) all proposed an affinity with Grubbiaceae, but in different orders. Takhtajan (1969) and Cronquist (1981) allied Bruniaceae with Pittosporaceae, again in different orders. Placement of Bruniaceae within an ericalean framework was suggested by Dahlgren and van Wyk (1988), Cronquist (1988) and G. Dahlgren (1989).

The inclusion within a newly-defined Asteridae *s.l.* of families that were formerly part of Cronquist's Dilleniidae (Ericaceae, Epacridaceae, Grubbiaceae, Diapensiaceae) or Rosidae (Pittosporaceae, Cornaceae, Aquifoliaceae, Araliaceae, Apiaceae), is consistent with the opinions of all the above authors regarding the placement of Bruniaceae within an asterid lineage. This is a tribute to the utility of the framework provided by molecular phylogenies, within which existing classifications can be re-examined.

1.3 The position of Grubbiaceae

This small Cape Floristic Region endemic family has been allied with Bruniaceae in some traditional classifications (see Table 8), but not included in any of the large-scale molecular phylogenetic analyses. In phylogenetic studies of Ericales by Anderberg (1992, 1993), Grubbiaceae was placed basal to an Ericaceae/Epacridaceae clade i.e. a similar relationship to that found in the present study. Anderberg hypothesised that Grubbiaceae belonged among the lower representatives of the Ericales. The association of Grubbiaceae with Aquifoliaceae in the present analysis may provide a basis for future investigations of the precise affinities of this family. The Grubbiaceae + Aquifoliaceae + Cornaceae clade identified was supported by two parallellisms: perianth tetramery and tri/multilacunar nodal anatomy. Bootstrap support for this

clade was below 50% and estimates of Bremer support showed the clade to collapse in trees one step longer than the single most parsimonious tree obtained. The inclusion of more characters in the analysis may increase confidence in the robustness of the clade, but Grubbiaceae, like Bruniaceae, suffers from a lack of detailed information regarding secondary chemistry, embryology and floral anatomy. Molecular studies of Grubbiaceae and other endemic southern African taxa are urgently needed.

1.4. Homoplasy in the data

There is significant homoplasy in the data, as evidenced by the consistency index and levels of bootstrap support. The fact that all branches collapsed in cladograms only 1 or 2 steps longer than the most parsimonious cladogram indicated limited support for the tree topology discovered in this analysis.

The consistency index associated with the single cladogram obtained in analysis 1 (0.34) is not dissimilar from indices obtained in other non-molecular phylogenetic analyses such as those of Hufford, 1992 (0.18); Anderberg, 1992 (0.30); Anderberg, 1993 (0.40) and Judd and Kron, 1993 (0.46). According to Quicke (1993), a consistency index in the range 0.25-0.35 is quite common for analyses based on non-molecular data. Sanderson and Donoghue (1989) found consistency index (ci) to be directly correlated with the number of taxa included in a cladistic analysis, ci decreasing and homoplasy increasing as more taxa were added. This could be related to an increase in the average number of character state changes per character with the addition of more taxa. Their conclusion was that the ci was not a measure of quality but merely of the overall level of homoplasy. These authors did not find a significant difference between levels of homoplasy in molecular and non-molecular data, a finding supported by the results of recent molecular phylogenetic studies e.g. Olmstead *et al.*, 1992 (ci=0.29).

Bremer (1988) noted the common occurrence of homoplasy in morphological data at higher taxonomic levels and Soltis and Soltis (1995) made the

TABLE 8 AFFINITIES OF BRUNIACEAE ACCORDING TO VARIOUS AUTHORS

Date	Author	Allied taxa	Order or clade
1818	Brown	Corneae, Hamamelideae	
1825	De Candolle	Rhamnaceae	
1826	Brongniart	Cornaceae (Myrtes), Hamamelidaceae	
		Haloragaceae, Araliaceae	
1836-40	Endlicher		Hamamelideae, Corneae
1853	Lindley	Hamamelideae , Grubbia	
1861-2	Sonder	Saxifragaceae Hamamelidaceae	
1865	Hooker	Saxifragaceae	
1868	Harvey		Hamamelideae
1872	Baillon	Saxifragaceae	Hamamelideae
1891	Niedenzu	Grubbiaceae	
1897	Van Tieghem	Cornaceae	"Umbelliferae"(Rosalean)
1912	Haller		Rosales
1915	Bessey		Rosales
1930	Niedenzu & Harms		Rosales (isolated position)
1935	Wettstein		Rosales
1953	Soó		Hamamelidales
1964	Schulze-Menz		Rosales
1969	Hutchinson		Hamamelidales
1969	Takhtajan	Pittosporaceae, Escalloniaceae, Roridulaceae	Saxifragales
1987		Geissolomaceae	
1997		Grubbiaceae	Bruniales, superorder Ericanae
1976	Thorne	Grubbiaceae, Roridulaceae, Geissolomaceae, Myrothamnaceae, Hydrostachyaceae	Pittosporales, suborder Brunineae
1992			Bruniales, superorder Rosanae
1981	Cronquist	Cunoniaceae, Pittosporaceae, Roridulaceae	Rosales
1988			Ericales
1980	Dahlgren	Grubbiaceae	Cunoniales
1983		Cunoniaceae, some non-South African families	
1988	Dahlgren & van Wyk	Grubbiaceae	Bruniales, near Ericales
1989	Dahlgren, G.		Bruniales, part of Ericales
1991	Carlquist	Grubbiaceae	Rosales Hamamelidales
1992	Hufford, L.		Corniflorae-asterid group of Rosidae
1993	Chase *et al.*	Apiaceae, Pittosporaceae, Valerianaceae, Dipsacaceae	Asteridae *s.l.*
1993	Olmstead *et al.*	Escalloniaceae, Cornaceae	Asteridae *s.l.*
1997	Soltis *et al.*	Lamiaceae, Solanaceae, Boraginaceae, Apocynaceae	Asteridae *s.l.*
1998	Nandi *et al.*	Eucommiales, Icacinaceae	Asteridae *s.l.*
1998	APG	Apiales, Asterales, Aquifoliales	Euasterids 2
1999	Hoot *et al.*	Hydrangeaceae, Araliaceae	Asterids

observation that "perhaps one of the most significant discoveries of molecular phylogenetics is the high frequency of homoplasy suggested for some morphological and chemical features" (of plants).

Inspection of the cladogram obtained in this analysis reveals that most of the chemical, morphological and embryological characters used are homoplastic. As regards secondary chemistry, the capacity to synthesise foliar ellagitannins for example appears to be the plesiomorphous condition for the ingroup, but is lost in Aquifoliaceae and the majority of other taxa examined. A reversal to the plesiomorphous state occurs in Diapensiaceae and Vaccinioideae. Presence of foliar proanthocyanidins is a plesiomorphy for the entire group, with losses of the biosynthetic step leading to these compounds occurring in several lineages: Aquifoliaceae/Cornaceae, Clade F and the grouping comprising Campanulaceae, Calyceraceae and Goodeniaceae. The loss of capacity to synthesise myricetin in leaves is a synapomorphy for Clade A, with a reversal to the plesiomorphous state in Clade D. The biosynthetic pathway leading to synthesis of polyacetylenes is a synapomorphy for Clade E, but is lost in Dipsacaceae and Calyceraceae. Iridoids arise in several lineages: Cornaceae, Actinidiaceae/Escalloniaceae, Clade F and Calyceraceae/Goodeniaceae.

Of the morphological characters, perianth pentamery appears to be the ancestral state for the ingroup, with reduction to tetramery in the Grubbiaceae + Aquifoliaceae + Cornaceae lineage and in Ericoideae. Free stamens represent the plesiomorphic condition, with stamen adnation having multiple origins in Theaceae, Lonchostomoideae, Diapensiaceae, two out of three epacrid lineages and the taxa above Apiaceae (node 28). A reversal to the ancestral state occurs in Campanulaceae. Hypogyny has multiple origins, in Brunioideae, Grubbiaceae, Vaccinioideae and Clade E above node 29. Haplostemony defines Aquifoliaceae/Cornaceae as well as Clade C, with reversals to the diplostemonous condition in the lineages of Ericaceae. Anther dehiscence by apical pores has two origins: in Actindiaceae and Ericaceae.

Anatomical features likewise appear to demonstrate multiple origins and reversals. The presence of endosperm haustoria appears to be plesiomorphous

for the group, with loss of this character in Theaceae as well as in Clade E. A reversal to the plesiomorphous state occurs in Clade G. Nodal anatomy is plesiomorphically unilacunar with tri- to multi-lacunar nodal anatomy occurring in the Grubbiaceae + Aquifoliaceae + Cornaceae lineage as well as in Clade E above Diapensiaceae. A reversal to unilacunar nodal anatomy occurs in Clade F above Asteraceae.

D. RESULTS OF ANALYSIS 2

INFRAFAMILIAL RELATIONSHIPS IN BRUNIACEAE

1. Analysis using Hennig 86

The Hennig command h*; bb* generated 1442 equally parsimonious trees, each 148 steps in length, with a consistency index (ci) of 0.28 and a retention index (ri) of 0.76. The strict consensus tree of these (Figure 19) was 189 steps long, with ci=23 and ri=68. The topology of the tree suggests that the species of Bruniaceae constitute a monophyletic lineage (Clade A), with *Dracophyllum* (Epacridaceae) as sister to the group. Within Bruniaceae, *Lonchostoma* is sister to the remaining taxa, which form a clade (B) in which *Thamnea hirtella* and *Audouinia capitata* are basally placed. *Thamnea* species, other than *T.diosmoides/T.massoniana*, do not form a distinct lineage; *T. thesioides* is shown as sister to *Tittmannia* and *Audouinia* is nested within the remaining currently recognised species. *Tittmannia* is sister to a large clade (C) comprising the species of *Linconia, Brunia, Nebelia, Berzelia, Staavia, Pseudobaeckia, Mniothamnea* and *Raspalia.* Within this lineage, *Linconia* is sister to the remaining taxa (Clade D). Within Clade D, the species of *Nebelia*, together with *Brunia nodiflora*, constitute a sister lineage to *Berzelia, Staavia, Mniothamnea, Pseudobackia, Raspalia*, with the remaining *Brunia* species at the base of the clade (E). Within Clade E, *Berzelia* species constitute a sister group to the other

taxa, while *Pseudobaeckia* and *Mniothamnea* are basal to a lineage comprising *Raspalia* species, which are placed at the apex of the tree. The results suggest that *Lonchostoma* is closest to the ancestors of the family and *Raspalia* the most evolutionarily advanced.

The monophyly of 7 out of the 12 currently recognised genera is supported; species of *Lonchostoma, Tittmannia, Linconia, Nebelia, Berzelia, Staavia* and *Raspalia* constitute monophyletic lineages, but there is little within-clade resolution, except for *Raspalia* in which two sub-clades are distinguishable (*R. trigyna/R.dregeana* and *R. barnardii/R. variabilis)* and *Staavia*, amongst which *S.capitella/S.radiata* and *S. zeyheri/S. dregeana* form subgroups. *Brunia* species do not appear to constitute a monophyletic group; *B. nodiflora* occupies a sister position with respect to *Nebelia*, while *B. stokoei* is sister to a *Berzelia + Staavia + Raspalia + Pseudobaeckia + Mniothamnea* clade, which includes also *Brunia albiflora* and *B. alopecuroides.* The remaining *Brunia* species are basally placed in Clade E. The two *Pseudobaeckia* species included in the analysis do not form a clade but are placed, together with *Mniothamnea*, as sister to *Raspalia*. Paraphyly is suggested for *Thamnea*, unless merged with *Audouinia* and for *Brunia*, if not merged with *Nebelia*.

2. Analysis using PAUP

PAUP retained 4774 trees from analysis of the unweighted data set, each 150 steps in length, with a ci of 0.29 and a ri of 0.76. The strict consensus tree of these (Figure 20) was similar to that generated by Hennig in suggesting the monophyly of the family and of 7 generic groupings, as well as a sister relationship of *Dracophyllum* with Bruniaceae, a basal placement of *Lonchostoma* within Bruniaceae and a position at the apex of *Raspalia*. The topology of the PAUP strict consensus tree differed from that of Hennig in the following respects:

1. The *Nebelia* clade was placed higher on the tree as sister (together with *Brunia nodiflora*) to a clade comprising *Raspalia, Staavia, Pseudobaeckia* and *Mniothamnea* species. *Berzelia*, together with *Brunia albiflora*, was sister to a clade comprising all of these taxa. *Brunia alopecuroides* was sister to this grouping, instead of part of it, as suggested by the Hennig analysis.
2. Resolution was changed in the *Raspalia, Staavia* and *Nebelia* clades: *Raspalia staavioides, Staavia zeyheri* and *Nebelia paleacea* were placed as sister taxa to the remaining species in their respective clades.
3. *Pseudobaeckia africana* and *Mniothamnea callunoides* formed a sub-clade with *P. cordata* as sister. This lineage was sister to a clade comprising the species in *Raspalia.*

The different topologies of the strict consensus trees obtained using Hennig and PAUP may be related to the fact that exact solutions were not sought in either analysis on account of the large size of the data set. Both results are reported here as a basis for future investigation. One of the Hennig most parsimonious trees was used for a preliminary exploration of character evolution in the family on the grounds that the trees generated were two steps shorter than those obtained in the PAUP analysis.

3. Weighted data

The topology of the strict consensus (Figure 21) of all the most parsimonious trees generated by PAUP following successive weighting of the data was in agreement with that of that obtained from unweighted data in the following respects: the basal placement of *Lonchostoma* within Bruniaceae, the sister relationship of *Dracophyllum* and Bruniaceae and the monophyly of 7 generic groupings i.e. *Lonchostoma, Tittmannia, Linconia, Nebelia, Berzelia, Staavia* and *Raspalia.* With respect to the ingroup taxa, the topology of the consensus tree based on weighted data was almost identical to that generated from the unweighted data set, except in the case of the *Lonchostoma* clade which was

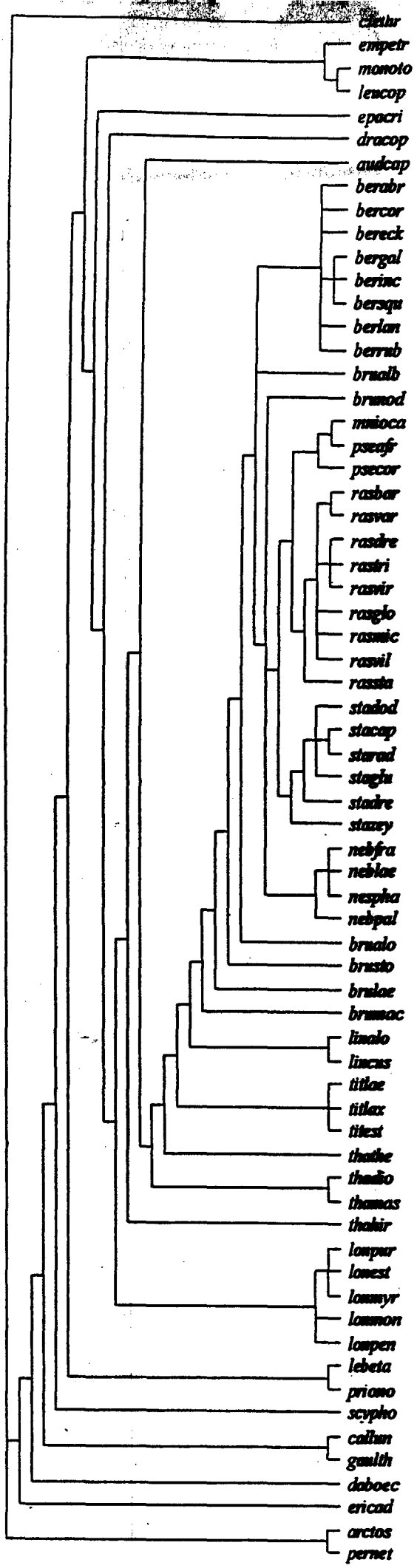


Figure 21. Strict consensus of set of most parsimonious trees obtained in PAUP analysis following successive weighting of the data.

further resolved in the weighted tree to include a sub-clade comprising *L. purpureum, L. myrtoides* and *L. esterhuyseniae.*

The weighted data consensus tree differed from that obtained from unweighted data mainly in respect of the outgroup taxa; in the weighted tree *Arctostaphylos/Pernettya* formed a grouping and *Empetrum* was sister to the *Monotoca/Leucopogon* lineage, with *Lebetanthus/Prionotes* as sister to this clade. The taxa representing Ericaceae were all basally placed on the cladogram, while those representing Epacridaceae and Empetraceae were more closely allied with Bruniaceae.

4. Analysis with Character 30 (pollen morphology) coded as polymorphic

Analysis of unweighted data using the PAUP option for coding polymorphic characters in respect of character 30 generated 32 700 trees, each 150 steps long, with a ci of 0.293 and a ri of 0.776. The topology of the strict consensus tree of these (Figure 22) differed from that obtained from analysis of the weighted data set in resolution of the *Berzelia, Raspalia* and *Lonchostoma* clades. Two additional subclades were detected in the *Berzelia* lineage: *B. lanuginosa* + *B.rubra* and *B. ecklonii* + *B.abrotanoides* + *B. cordifolia.* In the *Raspalia* lineage *R. staavioides and R. villosa* were basally placed with a *R. barnardii* + *R. variabilis* + *R. virgata* clade sister to a grouping comprising *R. dregeana* + *R. trigyna* +*R. globosa* + *R. microphylla* at the apex of the lineage. The latter clade represents the most recently evolved species in the family.

In the *Lonchostoma* grouping at the base of the tree, *L. pentandrum* was sister to the remaining species, in which *L. myrtoides* was basal to *L. purpureum* +*L. esterhuyseniae* + *L. monogynum.*

The position of the *Nebelia* lineage in this analysis was different from that in the other two PAUP analyses in its more basal placement with respect to *Brunia nodiflora.*

5. Bootstrap analysis: unweighted data

Bootstrap analysis of the unweighted data (Figure 23) indicated moderate to good support for 6 out of 7 generic groupings i.e. those comprising *Berzelia* (50%), *Tittmannia* (51%), *Staavia* (66%), *Raspalia* (66%), *Linconia* (75%) and *Nebelia* (84%) species. A *Lonchostoma* clade was not supported. The clade comprising all the taxa in Bruniaceae was supported at the 78% level, while a Bruniaceae + Epacridaceae + Empetraceae lineage received 67% support. The confidence level associated with this clade together with *Scyphogyne* was 60%. Within *Berzelia*, a sub-group comprising *B. squarrosa*, *B. galpinii* and *B. incurva* received 58% support.

6. Bootstrap analysis: weighted data

Bootstrap analysis of the weigted data set (Figure 24) indicated support for the *Nebelia*, *Staavia* and *Tittmannia* clades at the 75%, 54% and 52% levels respectively. Amongst the outgroups, 80% and 77% support was shown for the *Monotoca/Leucopogon* and *Lebetanthus/Prionotes* lineages of Epacridaceae. Amongst the ingroup taxa two clades comprising species pairs were supported: *Staavia capitella/S.radiata* (52%) and *Raspalia barnardii/R.variabilis* (51%).

7. Constraint analyses

7.1. Constraining *Audouinia* to a basal position within Bruniaceae

Constraint analysis of the unweighted data generated a single tree 151 steps long i.e. 3 steps longer than the set of most parsimonious trees obtained in the Hennig analysis and 1 step longer than those obtained in the PAUP analysis.

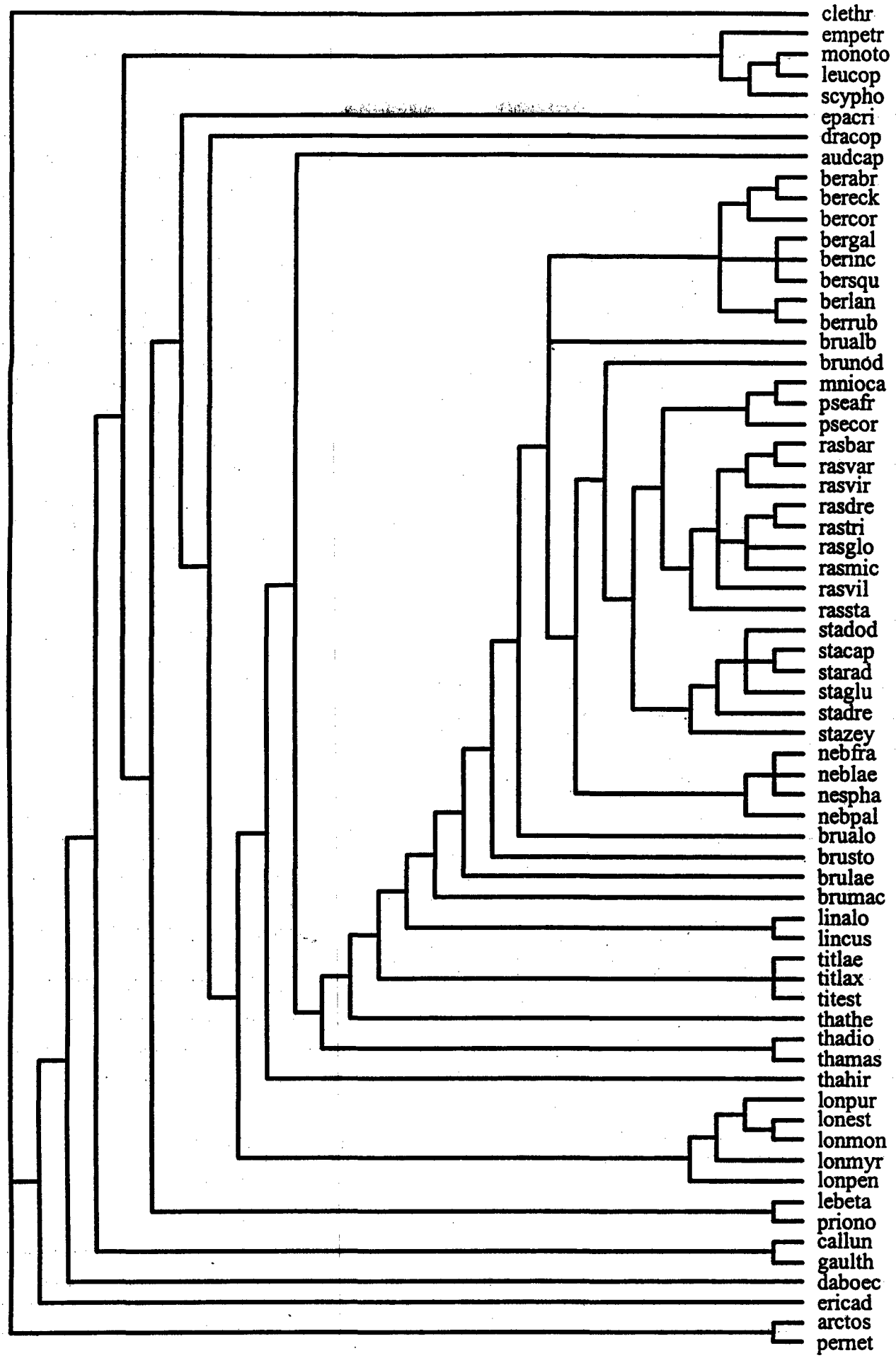


Figure 22. Strict consensus of set of most parsimonious trees obtained in PAUP analysis of unweighted data set, with Character 30 (pollen colpus number) coded as polymorphic.

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available in the published literature. Four of these characters were generated by the phytochemical analyses carried out in the present study. The results suggested that Ericaceae and Epacridaceae are most likely the sister groups of Bruniaceae and therefore the most appropriate out-groups for inclusion in the major analysis, aimed at exploring within-family relationships. Grubbiaceae was shown to be closer to Aquifoliaceae than to Bruniaceae and was not included in the second analysis.

In the second cladistic analysis, a total of 52 species of Bruniaceae, 6 genera in Epacridaceae and 7 in Ericaceae were scored for 32 characters; 5 of these were chemical characters generated by the present study and the remainder were obtained from published work on the morphology, anatomy, cytology and palynology of Bruniaceae and its sister-groups. The results showed that 7 out of 12 currently recognised genera comprise monophyletic lineages. There was no support either for the recognition of subfamilial lineages or for the suggested merging of *Nebelia/ Brunia*, *Tittmannia/Thamnea*, *Raspalia/Pseudobaeckia* and *Berzelia/Mniothamnea*. *Lonchostoma* was shown to be basal within the family, a finding that is consistent with the flavonoid chemistry of the genus. The most significant result was the identification of *Dracophyllum* (Epacridaceae) as sister to Bruniaceae. The latter finding is supported by the microfossil record, which indicates the presence of Epacridaceae at three sites in southern Africa at the Cretaceous/Tertiary boundary. Bruniaceae may be regarded as an ancient Gondwanan relict family with possible Cretaceous origins and closer links with Australian rather than African lineages.

members of Grubbiaceae, Diapensiaceae and Geissolomaceae surveyed in this study. Bruniaceae is thus distinct from the last three families as regards tannin synthesis. The methylated flavonol isorhamnetin was found in 70% of species in all genera of Bruniaceae except *Lonchostoma* and *Audouinia*, but not in any other species analysed except *Galax urceolata* (trace amounts). The flavonols myricetin and kaempferol were found in 57% and 54% respectively of species surveyed and quercetin in 100%. Flavonoids having tri-hydroxylated B-rings were not found in *Audouinia*, *Tittmannia*, *Thamnea*, *Linconia* or *Nebelia*. The rarer flavonol gossypetin, was detected in *Erica arborea*, *Galax aphylla* and *Shortia soldanelloides*, but not in Bruniaceae, Grubbiaceae, Retziaceae or Geissolomaceae. Quercetin 3-O-methyl ether was detected in some *Nebelia*, *Staavia*, *Brunia* and *Berzelia* species, but not in the remainder of species surveyed. The flavone luteolin was found in some *Nebelia*, *Raspalia* and *Grubbia* species and chrysoeriol in *Nebelia* and *Raspalia*. The presence of glycosides lacking a 5-hydroxy function was recorded in *Raspalia* and may be based on fisetin (5-deoxy quercetin), which characterised the aglycone profile of most species in this genus. Other genera in which 5-hydroxy function was lacking included *Pseudobaeckia*, *Staavia*, *Brunia and Berzelia*. The presence of C-glycosides was suggested in a few species, while flavonoid sulphates and glucuronides were not detected in the 4 species analysed. Biflavonoids were not identified in any of the species investigated.

On the basis of the aglycone patterns discovered, a phylogeny for Bruniaceae could be proposed, in which *Lonchostoma* was basal in the family and two subfamilial lineages could be recognised, based on loss of B-ring trihydroxylation, replacement of flavonols by flavones and gain of B-ring methylation. *Lonchostoma* species were noteworthy for the simplicity of their flavonoid profiles in comparison with those of other species in Bruniaceae. The flavonoid and tannin profile of Bruniaceae did not suggest a close relationship with Grubbiaceae, Diapensiaceae or Geissolomaceae but rather with Ericaceae.

Critical to cladistic analysis of within-family relationships is the identification of sister group(s) to the taxon under examination. Bruniaceae is regarded as taxonomically isolated and traditional phenetic classifications have allied the family with representatives of several Cronquistian subclasses. Recent opinion has favoured Dilleniidae, in particular Ericales, while the southern African endemic family Grubbiaceae is thought to be the closest relative of Bruniaceae.

Molecular phylogenetic studies carried out during the past few years have on the other hand suggested a position for Bruniaceae within Asteridae *s.l.*, a monophyletic lineage identified by several independent research groups. The latter studies were not in agreement as to the identity of sister taxa to Bruniaceae, but provided a guide to the selection of families for inclusion in a cladistic analysis of its extra-familial relationships. A preliminary analysis, aimed at identifying sister group(s) to Bruniaceae, utilised 24 taxa and 22 characters obtained from morphology, chemistry, anatomy and palynology and mostly

SUMMARY

A CHEMOSYSTEMATIC AND CLADISTIC STUDY OF THE SOUTHERN AFRICAN ENDEMIC FAMILY BRUNIACEAE DC

Bruniaceae is one of the southern African endemic plant families. The last revision of the family was completed in 1947 by Pillans, and 76 species in 12 genera are currently recognised. Since 1947, proposals have been made by various systematists for the recognition of subfamilial groupings as well as for the merging of several genera, but the relationships among genera are still poorly understood. Cladistic method is ideally suited to exploring such questions but calls for a broad base of molecular and/or non-molecular data. Very few molecular data are currently available for Bruniaceae, but recent studies of the palynology and chromosome cytology as well as the wood and leaf anatomy of the family have provided several non-molecular characters for phylogenetic analysis. Many phylogenetic studies today include information from secondary chemistry, on the grounds that these data are independent from morphology or anatomy but also that assessment of relationships should take into account all available data. Phytochemical information for Bruniaceae is scanty, but suggested that flavonoid distribution patterns in the family were likely to provide useful systematic data. This was confirmed by the results of a pilot study. A central goal of the present study was an examination of foliar flavonoid profiles of 58 species representing all genera in Bruniaceae. Eight species representing Grubbiaceae, Diapensiaceae, Ericaceae, Retziaceae and Geissolomaceae, families with which Bruniaceae has been allied in recent classifications, were included in the phytochemical survey. To ensure adequate sampling, an initial investigation of infraspecific variation in flavonoid profiles of three representative species was carried out. The results suggested that sampling from one or two populations of each species would be sufficiently representative of its flavonoid chemistry.

The phytochemical survey involved methanol extraction of fresh leaf material, yielding a mixture of glycosides which were separated by means of paper chromatography and analysed using mainly UV spectroscopy. The identity of one glycoside was confirmed by proton NMR spectroscopy. Leaf extract hydrolysates were separated by means of paper and thin layer chromatography, and the aglycones identified by co-chromatography. The occurrence of flavonoid sulphates and/or glucuronides was investigated using paper electrophoresis.

The results of the survey showed Bruniaceae to be characterised by the presence of proanthocyanidins; in *Audouinia*, *Tittmannia*, *Thamnea*, *Linconia* and *Nebelia* only procyanidin was detected, while in the remaining genera of Bruniaceae and in *Erica arborea* prodelphinidin was also present. The other species tested either had no proanthocyanidins or only procyanidin in trace amounts. The hydrolysable tannin aglycones gallic and ellagic acids were not recorded from Bruniaceae, Retziaceae or *Erica arborea*, but characterised all

species. Thirdly, the placement of Bruniaceae within a general ericalean context, as proposed in several recent classifications, is supported and paves the way for future studies of relationships between Ericaceae (now including Epacridaceae) and Bruniaceae.

The results of phylogenetic analysis of the family, although based on a low character to taxon ratio in the data matrix, were cladograms with fairly well-resolved topologies which suggested the monophyly of 7 of the 12 currently recognised genera in Bruniaceae. The positions of *Nebelia* and *Brunia* require clarification however. The sister group of Bruniaceae having been identified as Epacridaceae, specifically *Dracophyllum,* a more closely-focused analysis of Ericaceae is now possible. Additional characters are needed from phytochemistry (Bruniaceae, Epacridaceae and the southern African Ericaceae) and leaf anatomy, chromosome cytology and palynology (Epacridaceae). This highlights a third difficulty that applies to all characters used in phylogenetic analysis but particularly to phytochemical data at generic or specific level (Richardson, 1982) i.e. that for many taxa the sister group is unknown and can only be identified by cladistic study. Once this is accomplished, phytochemical studies can be undertaken at the required taxonomic level and relationships re-examined.

In a recent study of angiosperm phylogeny, based on combined *rbc*L and non-molecular data, Nandi *et al.* (1998) emphasised the need for biochemical (specifically secondary metabolite) studies in 23 plant families, Bruniaceae among them, in order to increase the data base available for future cladistic analyses. The present survey of foliar flavonoid patterns has taken a step towards meeting this need and contributed to elucidation of relationships at both generic and family level. It is hoped that the results of the present study, by providing a framework for more focused analysis, will lead to a better understanding of the evolution and relationships of this interesting Cape endemic family.

orcinol, gentisic acid and salicylic acid have a variable distribution in Ericaceae but tend to characterise particular subfamilies. Ericaceae is represented in southern Africa by only two genera: *Erica* (800+ species, which now include those of the former "minor genera" of the Ericoideae) and *Vaccinium* (1 species). The secondary chemistry of these endemic species is poorly understood (Oliver, pers. comm.). Future studies of the relationships between Bruniaceae and the African Ericaceae would benefit from investigation of flavonoids in the latter and of phenols in both.

The flavonoid chemistry of Epacridaceae has not been much studied (Hegnauer, 1966-1992). Flavonoid and phenol patterns in leaves of 27 species were investigated by Harborne and Williams (1973), who found a simple syndrome based on quercetin and kaempferol. Other phytochemical studies of the family have focused on fruit anthocyanin pigments (Jarman and Crowden, 1973) and flavonol arabinosides of leaf, flower and fruit of 30 species (Jarman and Crowden, 1977; Menadue and Crowden, 1983). Epacridaceae appears to be characterised by myricetin, quercetin and kaempferol arabinosides, particularly foeniculin (quercetin-3-arabinoside). A survey of Bruniaceae and the southern African Ericaceae for the occurrence of these glycosides would provide additional phytochemical data for cladistic analysis of the relationships between the three families.

The present study contributed to establishing the identity of sister groups to Bruniaceae, up to now a matter of controversy. The association of Bruniaceae with the largely Australian family Epacridaceae is of interest for several reasons. Firstly, it redresses to some extent the situation referred to by Linder *et al.* (1992) as "a curious lack of Gondwanan and particularly Australian sister relationships for Cape endemic families". Secondly, a Bruniaceae-Epacridaceae alliance provides support for the view that Bruniaceae is an ancient family, with possible Gondwanan origins. This is consistent with its lack of anatomical specialisation, palaeoendemic status and Red Data categorisation of approximately 25% of its

The fact that more of the data generated by the present flavonoid survey were not used in the cladistic analysis of Bruniaceae phylogeny highlights two difficulties associated with the use of flavonoid data in systematics and their use in cladistic studies at or below family level in particular. A practical difficulty is the time needed to collect adequate material, extract and separate the glycosides present and identify them with a reasonable degree of certainty. Paper chromatography is cheap but laborious. Several runs in different solvents are necessary in order to purify individual glycosides to the stage when identification can be made. Many of the glycosides isolated in the course of the present study could be only tentatively identified and potentially useful data were under-utilised. The use of high performance liquid chromatography (HPLC), which was not widely-available at the time the flavonoid analyses were done in this study (1991-2), may solve this problem in the future. The discovery in Bruniaceae of glycosides lacking a 5-hydroxy function is of particular interest in relation to the known occurrence in Ericaceae of the flavonol 5- and 3,5-methyl ethers azaleatin and caryatin and their glycosides.

Aglycone patterns were more easily established, yielding four characters for incorporation into the cladistic analysis of extra-familial relationships of Bruniaceae and five for that of within-family relationships. Other potential characters based on agycone patterns e.g. the presence of flavones and fisetin in some genera, could not be incorporated into the present analyses either because the identity of some of the aglycones required confirmation or because corresponding data were not available for many of the outgroup taxa. The latter problem might be partly solved by a careful scouring of the literature but for many of the taxa concerned the data do not exist. The leaf flavonoid and phenol chemistry of many genera in Ericaceae is well known (Harborne, 1969; Harborne and Williams, 1969, 1971 and 1973). Both the 5-methyl ethers and dihydroflavonols of quercetin, myricetin and kaempferol occur in the family, particularly in Rhododendroideae, and the yellow flavonol gossypetin in both Rhododendroideae and Vaccinioideae. The simple phenols hydroquinone,

CONCLUSION

The central goal of the present study was to undertake a phylogenetic study of the southern African endemic family Bruniaceae as a contribution to the revision of the family, currently in progress. This approach, i.e. the uncoupling of revisionary from evolutionary studies, was suggested by Raven (1974) and Goldblatt (1978), who did not imply by this that these were separate topics but rather that evolutionary insight could be gained (into a particular taxon) even if alpha-taxonomic work was incomplete.

The first objective of the study - an investigation of foliar flavonoid patterns in Bruniaceae - provided information concerning an aspect that has not until now received much attention. The results obtained did not suggest a close relationship between Bruniaceae and any of the other families included in the survey except Ericaceae (represented by *Erica arborea*). It was possible, using the chemical data generated, to propose a phylogeny for Bruniaceae, based on current theories regarding flavonoid evolution. This suggested a basal position in the family for *Lonchostoma* and support for the proposed merging of *Tittmannia* and *Thamnea* but not of *Berzelia* and *Mniothamnea*, *Brunia* and *Nebelia*, or *Raspalia* and *Pseudobaeckia*. Uniformity in chemical profile suggested the monophyly of 7 out of 12 genera in Bruniaceae. Humphries and Richardson (1980) have argued however that correct evolutionary interpretation of phytochemical data can only be achieved by cladistic analysis. The phylogeny proposed on the basis of the chemical data gathered in the course of the present study could thus be treated as an hypothesis to be objectively tested by combining these data with all other available evidence. Despite the fact that only 5 of several potential characters generated by the survey of foliar flavonoids were used in the cladistic analysis, the resultant phylogeny agreed with that obtained from chemical data in a basal placement within the family of *Lonchostoma*, as well as in the monophyly of some of the genera.

major higher dicot lineages, during a short period of time in the Late Cretaceous (80-95 my).

Not all of the fossil pollen records unequivocally confirm the presence of Epacridaceae however. The microfossils from southern Africa have affinities with *Dicrotetradites clavatus* Couper or *Paripollis ochesis* Partridge, both of which are considered to indicate Epacridaceae (Jordan and Hill, 1996). The occurrence of Epacridaceae in southern Africa at the Cretaceous/Tertiary boundary has been accepted without question (Coetzee *et al.*, 1983; Scholtz, 1985). The two relevant records are from Namaqualand and Botswana, regions which today are arid to semi-arid but appear from the Palaeogene microfossil record to have supported a dry subtropical forest, of which Epacridaceae is thought to have been an understory component. A third record, also from Namaqualand (de Villiers, 1997), is based on *Ericipites longisulcatus* Wodehouse and *Dicotetradites clavatus* Couper. This collection, perhaps less reliable owing to the very low numbers of grains identified to Epacridaceae, suggested that this family formed part of the understory in humid-temperate forest vegetation.

Some of the Australian records however are of *Ericipites scabratus*, which is referable to Ericaceae, Epacridaceae or Empetraceae. When recorded from Australia, *Ericipites* is usually taken to represent Epacridaceae on grounds of present day distribution, whereas in southern Africa (Coetzee *et al.*, 1983; Scholtz, 1985) this form is attributed rather to Ericaceae, again on grounds of present-day distribution.

A critical analysis of the variation in pollen morphology of the Bruniaceae-Ericales lineage, associated with an evaluation of the Tertiary fossil grains, in the context of the phylogeny of the lineage, may cast further light on the geographical history of Bruniaceae.

The apparent similarity in floral and leaf morphology between modern *Lonchostoma* and some *Epacris* and *Dracophyllum* species is interesting. *Epacris crassifolia*, herbarium specimens of which were inspected at Kew, is the subject of a recent paper by Crowden and Menadue (1996). Its resemblance to *Lonchostoma esterhuyseniae* seems remarkable. In addition, the two species occupy similar rock crevice habitats on moist sandstone cliffs, *E. crassifolia* in the mountains of New South Wales and *L. esterhuyseniae* in the Riviersonderend Mountains of the southern Western Cape Province. It is possible that the vegetative and floral features of these species have persisted unchanged in protected habitats, supporting the hypothesis of a moist-temperate origin for ancestral forms. Conversely it is possible that similarity in vegetative features is the result of convergent evolution, since the two species occupy similar habitats.

7.2 The fossil record

The fossil record for Bruniaceae is limited to a single Pleistocene pollen collection from the Cedarberg, south Western Cape Province (Meadows and Sugden, 1991). The microfossil record for Epacridaceae appears to be better and pollen grains attributed to this family have been recorded from the Late Cretaceous (71-64-mya) and early Tertiary of southern Africa (Scholtz and Deacon, 1982; Scholtz, 1985); the Palaeocene-Eocene (65-40 mya) of southern Africa (de Villiers, 1997), the Campanian-Maastrichtian (76-72 mya) of Antarctica (Dettman and Jarzen, 1990; Crame, 1992) and of Australia (Dettmann, 1994), the Eocene (55-38 mya) of New Zealand (Crosbie and Clowes, 1980) and the Oligocene (38-25 mya) of south-east Australia (Stover and Partridge, 1973).
If these attributions are correct, the almost contemporaneous occurrence of Epacridaceae in southern Africa and Australia/Antarctica at the Late Cretaceous/Tertiary boundary suggests that this lineage may have arisen early in the history of Asteridae *s.l.* Olmstead *et al.* (1992) concluded, on the basis of evidence from the fossil record and the results of their phylogenetic study of Asteridae *s.l.*, that the latter group had originated and diversified, along with other

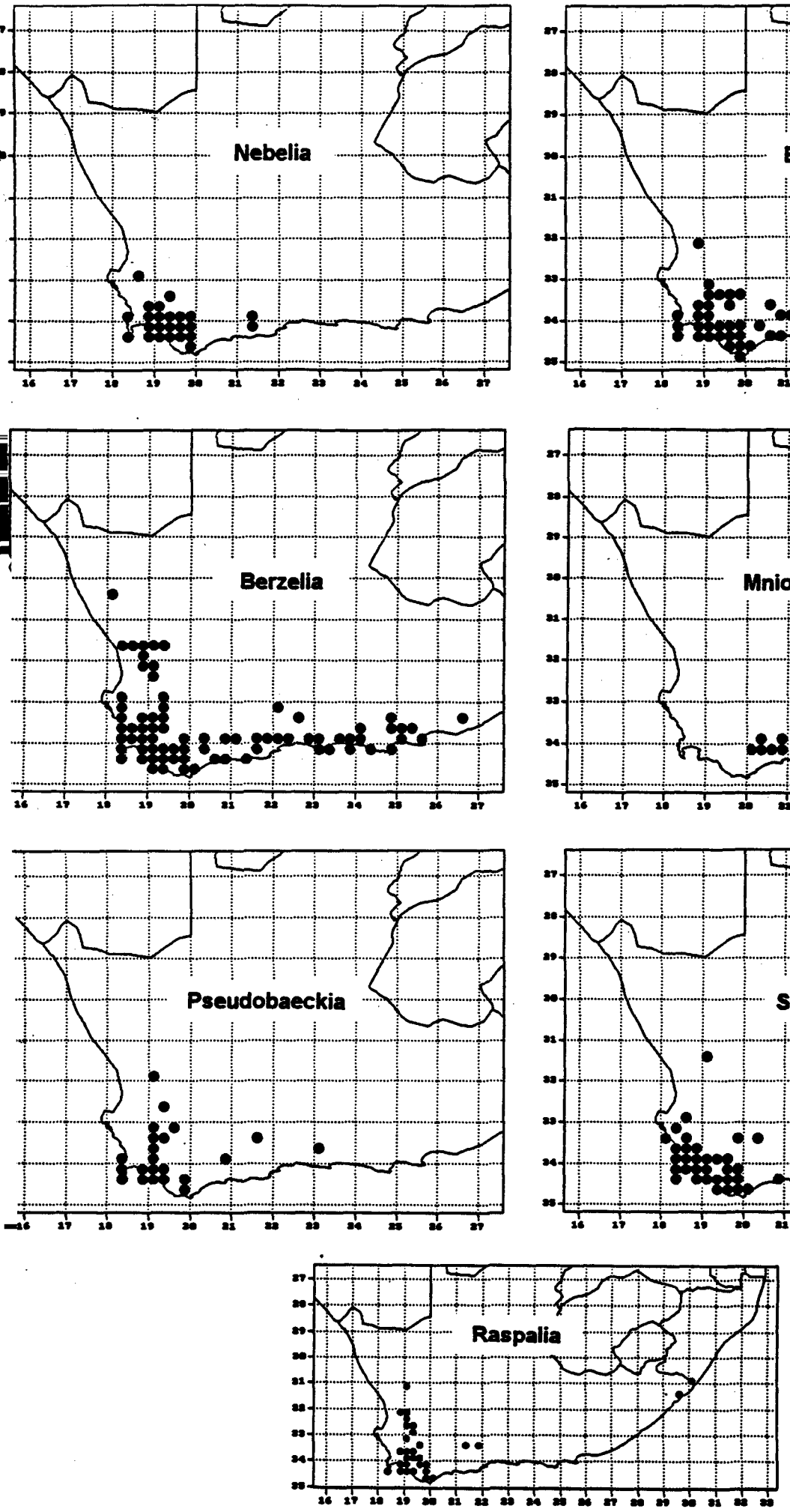
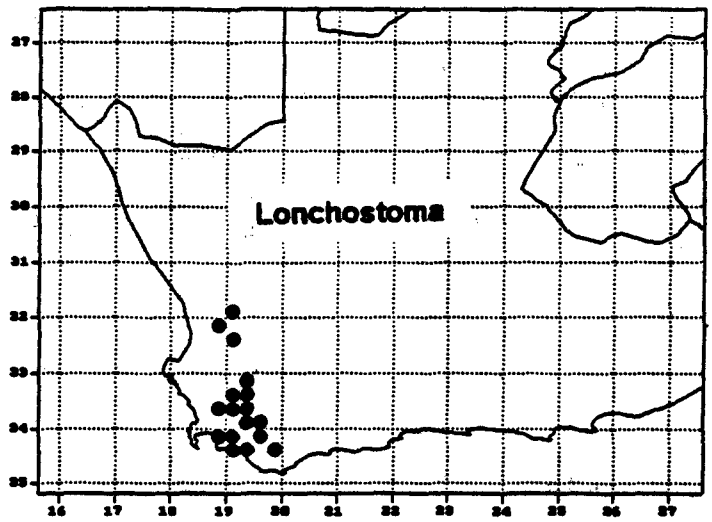
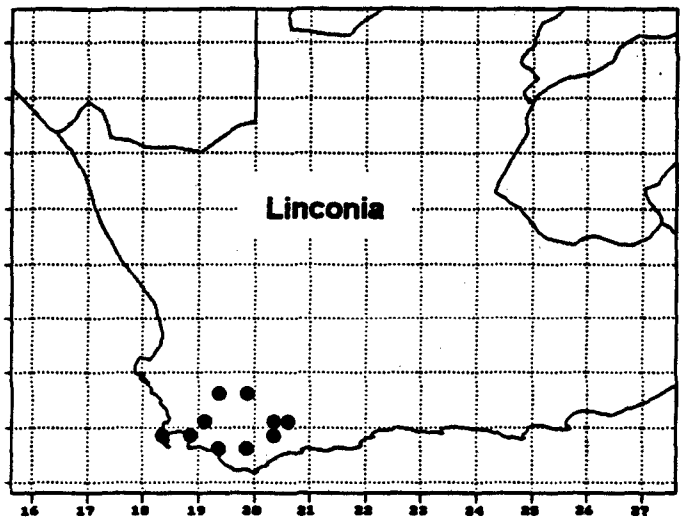
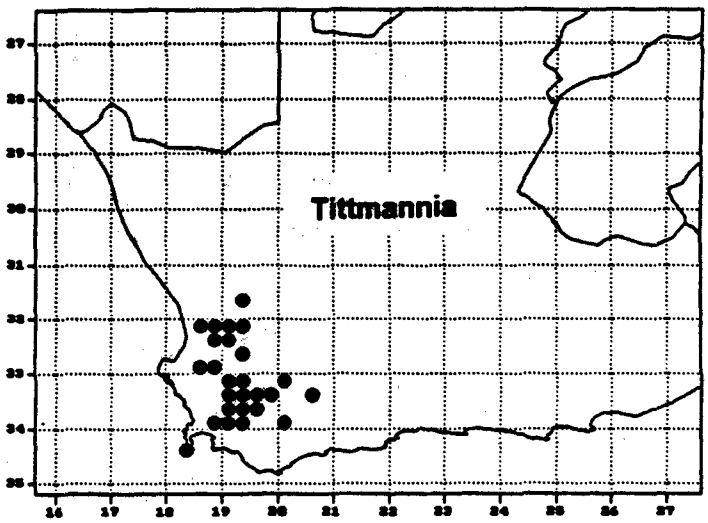
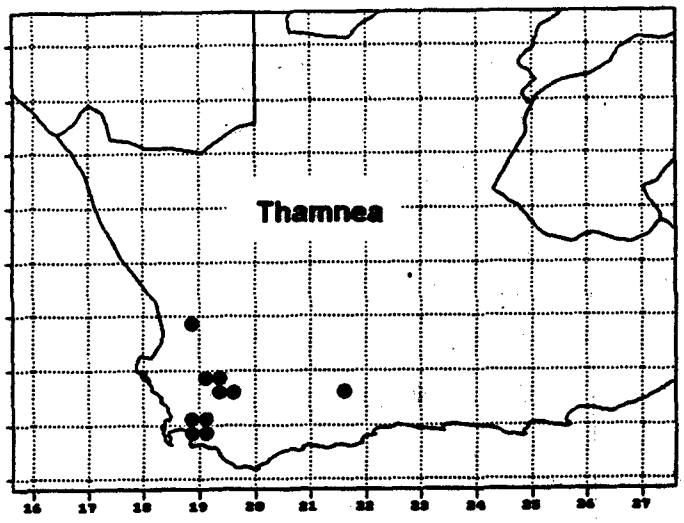


Figure 28. Geographical distribution of *Nebelia*, *Brunia*, *Staavia*, *Berzelia*, *Mniothamnea*, *Pseudobaeckia* and *Raspalia*.









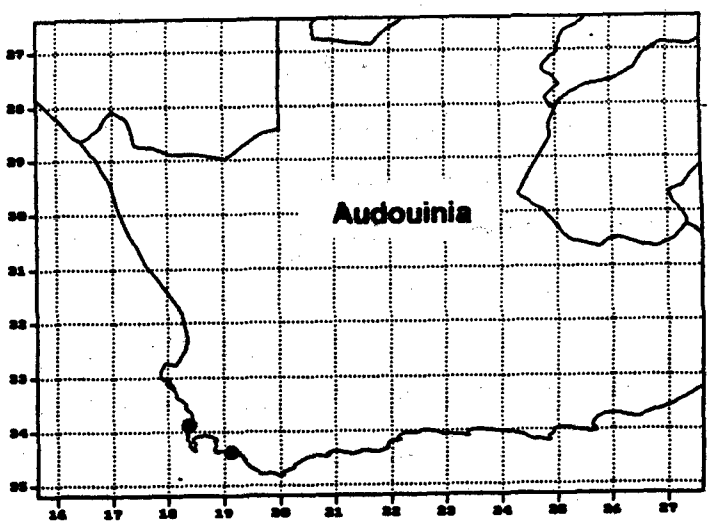


Figure 27. Geographical distribution of *Lonchostoma*, *Linconia*, *Tittmannia*, *Thamnea* and *Audouinia*.

7. A scenario for the origin and evolution of Bruniaceae

An hypothesis regarding the origin and evolution of Bruniaceae can be advanced, based on the results of the present study, together with evidence from the fossil record and modern distribution of Bruniaceae (Figures 27 and 28) and its sister group.

7.1 Distribution

The combined distribution of Bruniaceae and Epacridaceae today encompasses southern South America, South Africa, Australia, New Zealand, Tasmania and Malesia i.e. a Gondwanan "track" (Linder *et al.*, 1992). Bruniaceae (± 76 species) is endemic to southern Africa while Epacridaceae (± 400 species) is largely Australian but occurs on all Gondwanan fragments except Africa, Madagascar and India. Genera considered basal in each family, according to the results of phylogenetic analysis, are *Lonchostoma* in Bruniaceae (this study) and *Prionotes* or *Lebetanthus* in Epacridaceae (Powell *et al.;* Crayn *et al.*, 1996). All three genera are endemic to their respective areas of distribution and two are monotypic (*Lebetanthus* and *Prionotes).* All occupy moist-temperate habitats, in the south Western Cape Province of South Africa, Tasmania and southern Patagonia respectively. This suggests that the ancestors of both lineages may have evolved under mild and mesic conditions, while the modern distribution of these taxa suggests an ancient Gondwanan origin.

The role of mesic sites as refugia for southern African and Australian palaeoendemic or relict taxa has been suggested by Adamson (1958), Levyns (1962), Stebbins and Major (1965), Melville (1975), Goldblatt (1978) and Specht and Dettmann (1995). The modern occurrence of most Bruniaceae (palaeoendemic) as well as many endemic or relict Epacridaceae (Hill and Read, 1987; Dettmann, pers. comm.) in moist temperate habitats supports the hypothesis that the ancestors of a Bruniaceae-Epacridaceae lineage originated under such a climatic regime.

Bruniaceae by reference to seasonal growth unit rather than according to the typological concept of a synflorescence (Troll, 1964, 1969).
There is nonethless some agreement between Pillans' hypothetical tree and those discovered in the present analysis in that *Lonchostoma*, *Linconia*, *Audouinia* and *Tittmannia* are basally placed in both.

6. Sister relationship of Bruniaceae and Epacridaceae

The identification of *Dracophyllum* as sister to Bruniaceae is of particular interest and recalls Hooker's comment (1865) that "*Audouinia* is an epacridaceous subshrub". Assuming that Epacridaceae *sensu* Watson (1967) is a monophyletic lineage, as suggested by Anderberg, Judd and Kron, Kron and Chase (all 1993) as well as by Powell *et al.* and Kron (both 1996), then Bruniaceae and Epacridaceae can be regarded as sister families. The results of a phylogenetic analysis by Crayn *et al.* (1996) suggested however that *Lebetanthus* was closer to taxa of Stevens' Vaccinioideae (Ericaceae) than to other lineages in Epacridaceae, and that the retention of *Lebetanthus* rendered the latter family paraphyletic.
Assuming that Bruniaceae and Epacridaceae are sister families, the question arises as to whether this lineage is derived from within Ericaceae. The current view suggests that "the evolutionary origin of the Epacridaceae lies among Ericaceae of the tribes Andromedeae and Gaultheriae" (Kron and Chase, 1993) and that Epacridaceae "form a robust clade that occupies a derived position among Ericaceae as sister to an Andromedeae/Daboecieae clade" (Kron, 1996). The sinking of Epacridaceae in Ericaceae is currently being formalised, although Allaway (1996) and Kron (1996) both noted that paraphyly could be avoided by splitting Ericaceae into smaller families.
The need for investigation of the relationships between Epacridaceae, Bruniaceae and Ericaceae (particularly Ericoideae and Vaccinioideae) is suggested by the results of the present analysis.

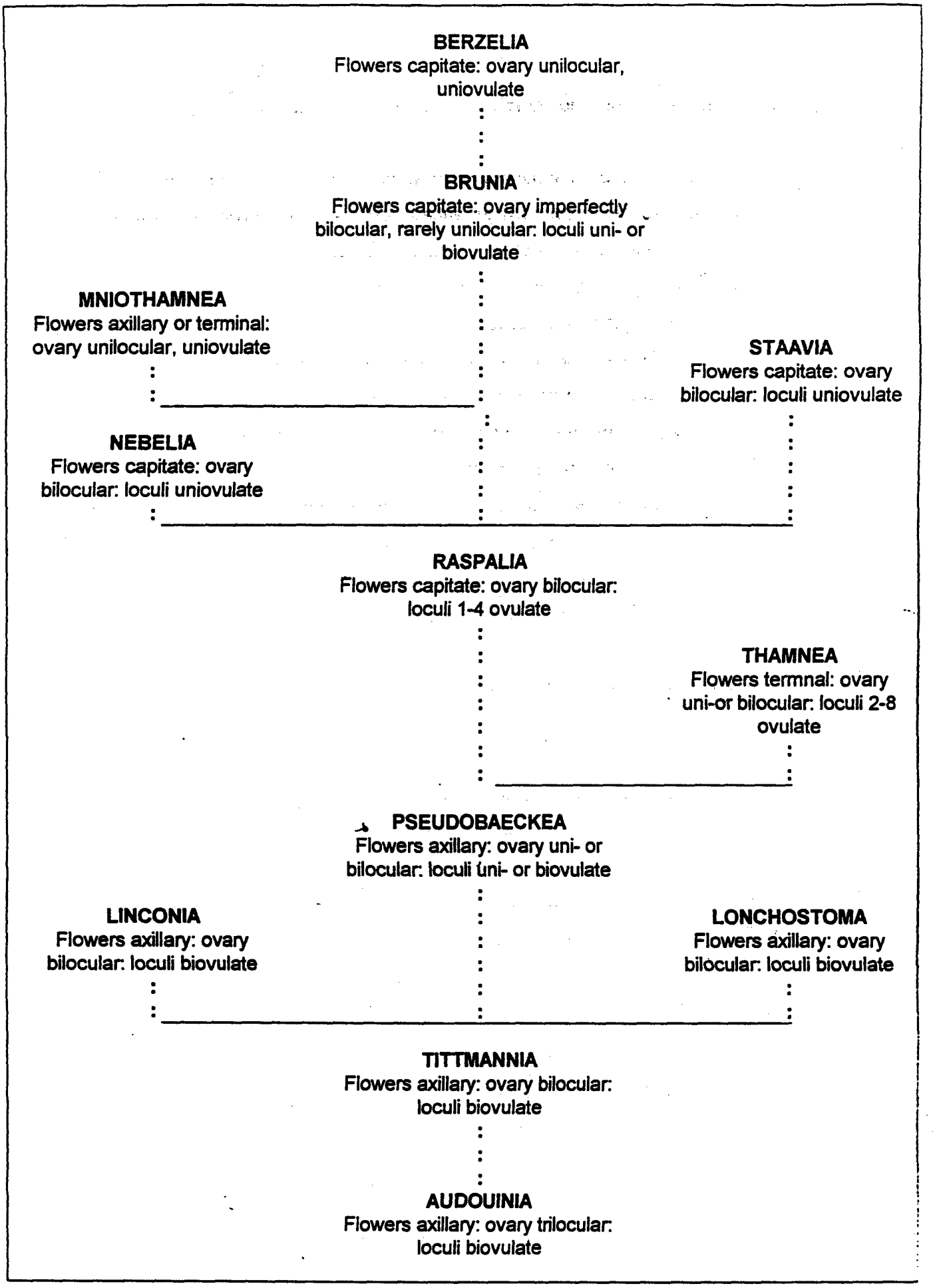


Figure 26. Pillans' proposed phylogeny of Bruniaceae (1947).

Comparison of this tree with the strict consensus trees obtained in cladistic analyses of Bruniaceae reveals agreement in the following features:

1. Basal placement of *Lonchostoma.*
2. Absence of support for the proposed merging (Powrie, unpublished MS) of various genera, namely *Raspalia* + *Pseudobaeckia*, *Mniothamnea* + *Brunia*, *Tittmannia* + *Thamnea* and *Nebelia* + *Brunia.*

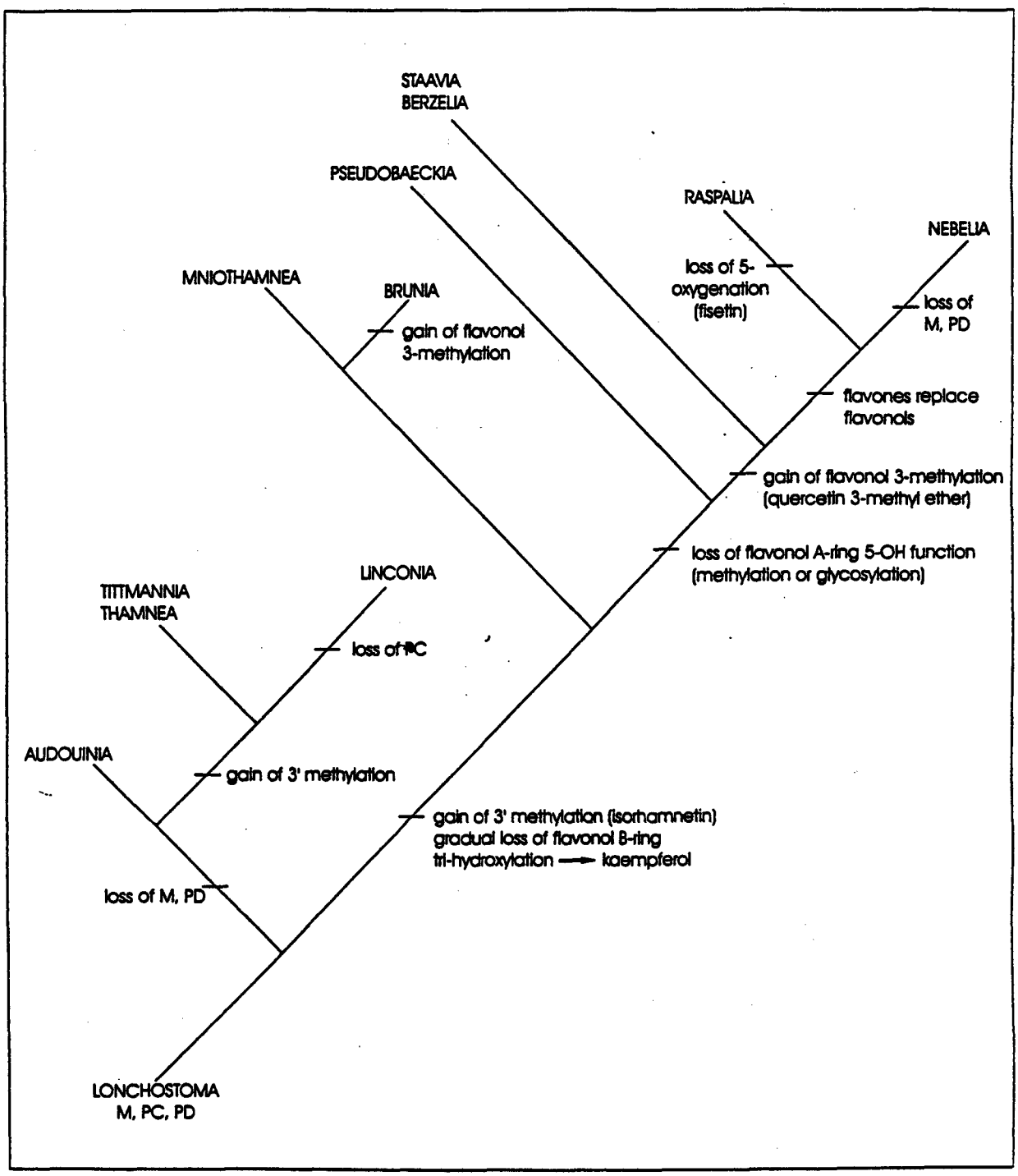
5. Comparison of results of cladistic analysis with Pillans' (1947) phylogeny of Bruniaceae, based on morphological criteria

Current theories regarding family phylogeny are not based on cladistic methods but on observations regarding the distribution of characters generally regarded as primitive or advanced. Pillans (1947) was able, using morphological criteria, to suggest a probable direction in evolutionary development in Bruniaceae, from a trilocular to unilocular ovary and toward a reduction in the number of ovules per ovary chamber. These modifications were usually accompanied by changes in the inflorescence, whereby axillary flowers became terminal and finally crowded into heads. Progress in development appeared to Pillans to have been uneven in that the inflorescence of several genera exhibited a greater degree of advancement than did the ovary (Figure 26). In Pillans' view (supported by Dahlgren and van Wyk, 1988) the monotypic *Audouinia* was the least advanced member of the family, by virtue of its trilocular ovary and axillary flowers borne on short shoots. *Berzelia*, in which the ovary is unilocular and the flowers crowded into terminal heads, was considered most advanced.

The characters used by Pillans in his proposed phylogeny of Bruniaceae were both limited in number and open to alternative interpretation. Unilocular ovaries are for instance present in some *Thamnea* species and may have a different origin to those of *Berzelia* and *Mniothamnea* (Brongniart, 1826). Classen-Bockhoff (in prep.) has called into question many currently-accepted concepts of inflorescence morphology in woody plants and has re-interpreted that of

4. Comparison of the results of cladistic analysis with proposed phylogeny based on foliar flavonoid patterns

The results of the chemical investigations carried out in the present study were used to construct a hypothetical "tree" (reproduced here from Chapter 4) depicting the possible evolution of flavonoid characters in Bruniaceae.



Hypothesis for the evolution of Bruniaceae, based on foliar flavonoid patterns.

M = myricetin, PC = procyanidin, PD = prodelphinidin

while the merging of *Mniothamnea* with *Pseudobaeckia* was suggested by the PAUP analyses. Despite a low character to taxon ratio, good cladogram resolution was achieved, particularly in the PAUP analysis with Character 30 coded as polymorphic. Further phylogenetic studies, using additional characters, may resolve the differences between the results obtained in Hennig and PAUP analyses and further improve resolution of individual clades. Inflorescence morphology and the occurrence of fungal symbionts are two potentially informative characters that could not be used in the present analysis because of uncertain or inadequate information. Pollination biology and seed dispersal mechanisms of species in Bruniaceae have been little studied and would provide additional characters for phylogenetic analysis. Better information is required as to cytology, resprouting vs. reseeding capacity, presence/absence of stipules and identity of some of the flavonoids isolated in this study. The results obtained in the present study are not regarded as sufficiently conclusive to suggest formal taxonomic changes, but as a framework for future more focused investigation.

• a decrease in the number of ovules/ovary loculus, from up to 10 in *Thamnea* and *Lonchostoma* to one in *Berzelia, Nebelia* and *Staavia.*

Brongniart (1826) considered the unilocular ovary in *Thamnea*, with 10 pendulous ovules attached to a central column, to be equivalent to the five biovulate locules occasionally seen in *Audouinia.* This is the state for the majority of Epacridaceae and further supports a basal position in Bruniaceae for these two genera. In Brongniart's opinion the bilocular ovary of *Tittmannia*, which he regarded as very close in floral structure to *Thamnea*, had arisen via a flattening of the central column in *Thamnea* to give a partition, followed by a reduction in the number of ovules/ovary chamber to two (the state for the majority of Bruniaceae). Baillon (1874) expressed a similar view of *Tittmannia*, which he referred to as "a somewhat unusual *Thamnea*" in which the septum between the two biovulate cells of the ovary was less rapidly and completely destroyed. A comparable although less clear-cut development could be proposed for *Lonchostoma,* the only other genus in the family with multiovulate ovary chambers. In *L. esterhuyseniae* there are 2(1) ovary chambers, with 5-8 ovules /chamber; in *L. purpureum* there are also 2(1) chambers, but only 1-2 ovules/chamber (Rebelo, 1980). A detailed investigation of ovary structure in this genus, as has been done for *Audouinia* (de Lange, 1992), may reveal the persistence in some populations of the plesiomorphic 5-locular ovary.

3. Relevance of results to revision of Bruniaceae

A revision of Bruniaceae by Professor A. V. Hall (formerly of the Department of Botany, University of Cape Town) is currently in progress and the results of the present phylogenetic analysis may assist in establishing a framework for formal classification of the family.

The topologies of the four strict consensus trees supported the monophyly of 7 out of the 12 currently recognised genera in Bruniaceae. *Brunia* as recognised by Pillans (1947) was not found to be a monophyletic group in any of the analyses

the PAUP analysis using Character 30 coded as polymorphic, *Brunia nodiflora* retained this sister relationship, but *Nebelia* was placed basal to the entire clade. The placement of *Brunia macrocephala, B. laevis* and *B. stokoei* was similar in all four analyses, while that of *B. albiflora* and *B. alopecuroides* differed in the Hennig and PAUP analyses, with *B. albiflora* placed higher on the PAUP trees than *B. alopecuroides*. The proposal of Baillon (1874) and Powrie (in Dyer, 1975) that *Brunia* and *Nebelia* be merged was not supported by the results of the present study.

2. Character evolution

It is difficult, given the multiple origins and reversals of many of the characters used in the analysis, to interpret their evolution in the family. The plesiomorphous condition for the family appears from the present phylogenetic analysis to include hypogyny, sympetaly, epipetalous stamens, loculicidal capsular fruits, capacity to regenerate from a subterranean rootstock, synthesis of foliar flavonoids having tri-hydroxylated B-rings, absence of biochemical pathways leading to B-ring methylation and a conspicuous corolla (> 5mm long and brightly coloured). Pillans' proposed phylogeny of Bruniaceae relied heavily on inflorescence morphology, which was not used as a character in the present analysis because of the doubtful homology of capitate (*Staavia*), globose *(Berzelia, Brunia, Nebelia)* and spicate (*Pseudobaeckia*) inflorescences as opposed to flowers borne singly on short shoots (*Audouinia, Tittmannia, Thamnea*). Classen-Bockhoff (in prep.) concluded for instance that each flower in *Audouinia, Thamnea* and *Linconia* was comparable to an entire lateral inflorescence.

Other key characters in Pillans' phylogeny were:

- a decrease in the number of ovary chambers from 3 in *Audouinia* to 1 in *Berzelia, Mniothamnea and Thamnea*

ovary (occasionally 4-5 locular), haploid chromosome number and single flowers borne on short shoots, was basally placed on all four strict consensus trees among the 4 *Thamnea* species included in the present analysis, above *Lonchostoma*. A basal position in the family was supported by the results of constraint analysis. The merging of *Audouinia* and *Thamnea* has not been proposed by any author, although many regard these two taxa, together with *Tittmannia*, as a natural grouping. The concept of a *Tittmannia* + *Audouinia* + *Thamnea* alliance was formalised by Niedenzu (1891) as subfamily Audouineae, a classification followed by Thonner (1915) and Niedenzu and Harms (1930). Tahktajan (1987) also recognised this grouping, as subfamily Audouinioideae, in his classification of Bruniaceae. Such a distinct lineage was not detected in any of the tree topologies obtained in the present analysis.

Mniothamnea, regarded as close to *Berzelia* on grounds of common possession of a unilocular ovary (Powrie, unpublished MS), was shown in the Hennig analysis to be sister (together with the two *Pseudobaeckia* species) to a *Raspalia* clade. In the PAUP analyses, *Pseudobaeckia cordata* was sister to a *P. africana* + *Mniothamnea callunoides* subclade. All tree topologies support Niedenzu's (1891) proposed close relationship between *Pseudobaeckia and Raspalia* but not Powrie's proposals that *Pseudobaeckia* be merged with *Raspalia* and *Mniothamnea* with *Berzelia*.

Berzelia species, identified as a monophyletic lineage in both analyses, differed as regards placement on cladograms generated by Hennig and PAUP. In the Hennig analysis this grouping was sister to a *Staavia* + *Mniothamnea* + *Pseudobaeckia* + *Raspalia* clade at the apex of the cladogram, while in the PAUP analyses it was located further down the trees, as sister to a lineage comprising *Nebelia* + *Staavia* + *Raspalia* + *Mniothamnea* + *Pseudobaeckia* + *Brunia nodiflora*.

Brunia species did not constitute a monophyletic lineage in any of the analyses. In the Hennig analysis *Brunia nodiflora* was sister to a *Nebelia* lineage, while in two of the PAUP analyses this species and the *Nebelia* lineage were sisters to a large clade comprising *Mniothamnea* + *Pseudobaeckia* + *Staavia* + *Raspalia*. In

ovule/ovary loculus (character 20, state 2) and pollen shed in pseudomonads (character 23, state 1).

1.2.8. Homoplasy in the data

As in the case of the preceding analysis there was evidence of considerable homoplasy in the data, particularly in respect of flavonoid characters which showed multiple origins and reversals in both ingroup and outgroup taxa. Morphological features such as corolla colour, presence or absence of lignotubers, number of ovary chambers, fruit dehiscence and stamen inclusion/exsertion also appeared to be homoplastic, as did some anatomical characters e.g. stomatal distribution and leaf crystal type. This was reflected in a low consistency index (0.28) and moderate bootstrap support. As discussed by Sanderson and Donoghue (1989) consistency index is correlated with number of taxa included in a cladistic analysis and it was to be expected that the value obtained in analysis 2 would be lower than that in analysis1.

E. DISCUSSION OF RESULTS OF ANALYSIS 2

INFRAFAMILIAL RELATIONSHIPS IN BRUNIACEAE

1. Comparison with existing classifications

The topologies of the strict consensus trees obtained in all four analyses suggest that *Lonchostoma* is sister to the rest of the family. Pillans (1947), although he was uncertain as to evolutionary directions within Bruniaceae, placed *Lonchostoma* near the base of his hypothetical tree (Figure 26) together with *Linconia*, *Tittmannia* and *Audouinia*.

The monotypic *Audouinia*, generally regarded as the least advanced taxon in the family (Pillans, 1947; Dahlgren and van Wyk, 1988) on account of its trilocular

C. CLADES REPRESENTING SUB-GENERIC LINEAGES IN BRUNIACEAE

1. *Lonchostoma purpureum* + *L. myrtoides* (node 97), supported by a single parallelism: character 19 (stamens not epipetalous).

2. *Berzelia abrotanoides* + *B. cordata* + *B.ecklonii* (node 74), diagnosed by one reversal: capacity to regenerate from a subterranean stem (character 5).

3. *Berzelia galpinii* + *B. squarrosa* + *B. incurva* (node 75), diagnosed by one reversal: character 1 (lack of capacity to synthesise foliar isorhamnetin).

4. *Staavia capitella* + *S. radiata* (node 79), diagnosed by one reversal: character 5 (capacity to regenerate from a subterranean stem).

5. *Staavia dregeana* + *S. zeyheri* (node 70), diagnosed by one parallelism: synthesis of foliar kaempferol (character 4).

6. *Raspalia barnardii* + *R. variabilis* (node 69), diagnosed by one parallelism: capacity for synthesis of foliar isorhamnetin (character 1).

7. *Raspalia dregeana* + *R. trigyna* (node 66), diagnosed by one parallelism: absence of foliar calcium oxalate (character 24,state 3).

D. CLADES OCCURRING AMONG OUTGROUP TAXA

1. A *Scyphogyne* + *Empetrum* clade (node 114), supported by three parallelisms: corolla length of <5mm, stamen number < / = the number of perianth segments and one ovule/ovary loculus (characters 11, 16 and 20, state 2).

2. *Erica* + *Daboecia* (node 116), diagnosed by one reversal; synthesis of foliar myricetin (character 3).

3. *Calluna* + *Gaultheria* (node 113), diagnosed by a single reversal; presence of stomata on both leaf surfaces (character 25).

4. *Lebetanthus* + *Prionotes* (node 111), diagnosed by two reversals: synthesis of foliar myricetin (character 3) and leaves having serrate margins (character 6).

5. *Monotoca* + *Leucopogon* (node 106), diagnosed by four parallelisms: a corolla of < 5mm in length (character 11), indehiscent fruits (character 15, state 2), one

B. CLADES CORRESPONDING TO CURRENTLY RECOGNISED GENERA IN BRUNIACEAE

1. *Lonchostoma* species (node 104), supported by 2 reversals: synthesis of leaf myricetin (character 3) and stamen epipetaly (character 19).
2. *Tittmannia* species (node 95), supported by one parallelism [apical placentation (character 22, state 1)] and one reversal [presence of stomata on both leaf surfaces (character 25)].
3. *Linconia* species (node 93), supported by one synapomorphy [divergent anther thecae (character 28)] and one parallelism [leaf calcium oxalate druses (character 24, state 1)]
4. *Nebelia* species (node 89), supported by one parallelism [lack of capacity ot regenerate from subterranean stem (character 5)], one reversal [lack of capacity for synthesis of leaf kaempferol (character 4)] and one synapomorphy [scarious extended bracts subtending the flower (character 31)].
5. *Berzelia* species (node 80), supported by two parallelisms: indehiscent fruits (character 15, state 2) and a single ovary chamber (character 21, state 3).
6. *Staavia* species (node 82), supported by one synapomorphy [inflorescence subtended by an involucre of white conspicuous bracts (character 13)] and one reversal [brightly coloured perianth (character 12)].
7. *Raspalia* species (node 77), supported by a single synapomorphy: calyx lobes adjacent at the base (character 14).
8. *Pseudobaeckia* species (node 71), supported by one parallelism [absence of foliar myricetin (character 3)] and one reversal: stomata present on both leaf surfaces (character 25).
9. *Mniothamnea* + *Pseudobaeckia* species (node 76), supported by a single parallelism: indehiscent fruits (character 15, state 2).

7.2. Constraining monophyly of *Brunia*

Analysis of the unweighted data generated 100 trees, the shortest of which was 160 steps long i.e. 12 steps longer than the unconstrained trees.

8. Character optimisation

Characters were plotted on to one of the most parsimonious trees generated in the Hennig analysis (Figure 25), to be used as a basis for exploring character evolution in Bruniaceae. The following clade categories were distinguished:

A. MAJOR CLADES

1. Epacridaceae + Bruniaceae (node 112), supported by one synapomorphy and two parallelisms: anther dehiscence by longitudinal slits (character 18), stamens = or < number of perianth segments (character 16) and lack of anther ornamentation (character 27).
2. Bruniaceae (node 107), supported by one synapomorphy (leaf apex modified to a black apiculus-character 7), one parallelism (two ovary chambers-character 21, state 2) and one reversal (pollen release in monads-character 23, state 0).
3. Bruniaceae minus *Lonchostoma* (node 105), supported by four parallelisms [presence of leaf procyanidins only (character 2), epigyny (character 9), indehiscent fruits (character 15, state 2), calcium oxalate rhomboids in the leaves (24, state 2)] and one reversal [choripetaly(character 10)].
4. 12. A *Staavia* + *Raspalia* + *Pseudobaeckia* + *Mniothamnea* clade (node 84), supported by two parallelisms: leaf calcium oxalate druses (character 24, state 1) and 4-5-colporate pollen grains (character 30, state 1) plus two reversals: lack of capacity to synthesise foliar kaempferol (character 4) and included stamens (character 17).

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APPENDIX 1 VOUCHER SPECIMENS

SPECIES	DATE	LOCALITY	COLLECTION NUMBER
BRUNIACEAE			
Audouinia capitata (L.) Brong.	26/11/90	Karbonkelberg	324
	1/3/91	Hemel-en-Aarde	336
	18/2/89	Cape Point	292
Berzelia abrotanoides (L.) Brongn.	24/3/92	Pella Mission station	359
	16/7/89	Cape Point	95
Berzelia cordifolia Schldl.	15/2/89	near Cape Infanta	280
	19/8/91	De Hoop	346
Berzelia ecklonii Pill.	23/9/88	Kogelberg	250
Berzelia galpinii Pill.	4/8/87	Lemoenshoek	J.P.R. SN
	20/12/90	Fouriesberg	F.J.P. 377
Berzelia incurva Pill.	18/7/87	Vogelgat	102
	22/2/89	Vogelgat	296
Berzelia lanuginosa (L.) Brongn.	18/6/87	Kirstenbosch.L 14	108
	16/7/87	Cape Point	93
	5/7/87	Cedarberg Sneeuberg	112
	19/8/87	Storms River	125
	7/2/89	Zuurvlakte	267
	28/1/91	Du Toit's Kloof	329
	18/7/87	Vogelgat	97
	6/7/87	Cedarberg,Uitkyk	113
	3/10/87	Cedarberg,Eikeboom	137
	15/2/89	Grootvadersbos	276
	3/8/87	Hottentots Holland	124
	11/7/88	Cedarberg,Pakhuis	207
	16/12/90	Du Toit's Kloof	325
Berzelia rubra (Willd.) Schldl.	18/7/87	Vogelgat	101
Berzelia squarrosa (Thunb.) Sond.	31/1/90	Cedarberg,Donkerkloof	AH 361
	3/8/87	Landdroskop	126
	28/3/88	Somerset Sneeukop	191
Berzelia intermedia Schldl.	16/4/91	Gysmanshoek Pass	FJP 391
Brunia albiflora E Phillips	30/7/88	Kogelberg	213
	20/3/91	Betty's Bay	340
Brunia alopecuroides Thunb.	3/8387	Landdroskop	119
	26/10/87	Betty's Bay	152
Brunia laevis Thunb.	14/10/87	Kirstenbosch,L4	141
	26/10/87	Betty's Bay	148
Brunia nodiflora L.	5/3/91	Kalk Bay Mts	338
	16/4/91	Gysmanshoek Pass	FJP 395
	18/6/87	Kirstenbosch estate	107
	26/10/87	Hemel-en-Aarde	154
	29/8/89	Blaauwkrantz	FJP 164
	23/9/88	Steenbras Dam,	248
	26/3/89	Bain's Kloof	302
	20/4/89	Villiersdorp	304
	26/11/90	Karbonkelberg	323
Brunia stokoei E. Phillips	20/3/91	Betty's Bay	341
Brunia stokoei E. Phillips	30/3/90	Betty's Bay	317
Brunia macrocephala Willd.	20/4/88	Keeromsberg	193
Lonchostoma purpureum Pill.	4/8/88	Somerset Sneeukop	219
Lonchostoma esterhuyseniae Strid	1/12/88	Pilaankop	255
Lonchostoma monogynum (Vahl) Pill.	20/4/88	Keeromsberg	198
	25/3/89	Limietberg	297

SPECIES	DATE	LOCALITY	COLLECTION NUMBER
Lonchostoma myrtoides (Vahl) Pill.	11/10/89	Ceres Flower Show	311
Lonchostoma pentandrum (Thunb.) Swartz	7/2/89	Zuurvlakte	262
Linconia alopecuroides L.	15/12/87	Grootvadersbos	166
Linconia cuspidata (Thunb.) Swartz	14/11/89	Landdroskop	310
Mniothamnea callunoides	14/12/87	Grootvadersbos	155
(Oliver) Niedenzu	15/2/89	Grootvadersbos	288
Nebelia fragarioides (Willd.)	28/3/88	Landdroskop	192
Kuntze	3/8/87	Landdroskop	123
Nebelia laevis (E. Meyer) Kuntze	30/7/87	Jonaskop	JPR SN
	27/11/90	Jonaskop	H. Crous 113
Nebelia paleacea (Bergius) Sweet	26/10/87	Hemel-en-Aarde	149
	7/2/88	Perdeberg, Kleinmond	172
Nebelia sphaerocephala (Sonder)	11/1/88	Keeromsberg	178
Kuntze	28/3/88	Somerset Sneeukop	190
Pseudobaeckia africana	24/10/87	Groot Winterhoek	142
(Burm.f.) Pill.	28/1/91	Du Toit's Kloof	330
Pseudobaeckia cordata (Burm.f)	25/3/89	Limietberg	298
Niedenzu	5/1/91	Outeniqua Mts	326
Pseudobaeckia cordata var. monostyla Pill.	14/12/88	Witteberg	258
	31/12/89	Bain's Kloof	314
Raspalia barnardii Pill.	18/3/90	Misty Point, Swellendam	316
Raspalia dregeana (Sond.) Niedenzu	15/1/90	Matroosberg	315
Raspalia globosa (Lam.) Pill.	4/8/88	Landdroskop	217
Raspalia microphylla (Thunb.) Brongn.	23/9/88	Steenbras Dam	244
	1/12/88	Pilaarkop	253
Raspalia staavioides (Sonder) Pill.	30/10/89	Krakadouwpoort	FJP 189
Raspalia trigyna (Schltr.) Dümmer	20/11/89	Southern Natal, Egossa	313
Raspalia villosa Presl.	23/10/90	Turret Peak, Koue Bokkeveld	EEE 37070
	20/12/90	Watervalberg	FJP 354
Raspalia variabilis Pil.l	1/12/88	Pilaarkop	254
Staavia dodii Bolus	16/7/87	Cape Point, Okifantskop	90
	22/8/90	Cape Point, Sirkelsvlei	FJP 235
Staavia capitella (Thunb.) Sonder	2/12/91	Wolfieskop	FJP 425
Staavia dregeana Presl.	30/1/91	Table Mt, Nursery Buttress	332
Staavia glutinosa (L.) Dahl	8/9/89	Table Mt, Grootkop	RS SN
	17/3/88	Table Mt, Nursery Ravine	NBG 091/88
Staavia radiata (L.) Dahl	16/7/87	Cape Point, Olifantskop	91
	5/5/92	Rondeberg	363
Thamnea diosmoides Oliver	21/9/88	Gydo Pass, Ceres	233
Thamnea hirtella Oliver	2/9/88	Witzenberg, Inkruip	234
Thamnea massoniana Dümmer	29/7/88	Victoria Peak	211
Thamnea thesioides Dümmer	7/10/89	Ceres Nature Reserve	309
Tittmannia esterhuyseniae E. Powrie	16/5/88	Stettynskloof	201
Tittmannia laxa (Thunb.) Presl.	5/7/87	Cedarberg Sneeuberg	114
	3/10/87	Cedarberg Sneeuberg	135
Tittmannia laevis Pill.	23/10/90	Turret Peak, Koue Bokkeveld	EEE 37069
GRUBBIACEAE			
Grubbia rosmarinifolia Berg.	15/12/87	Grootvadersbosch	165
Grubbia rourkei Carlq.	23/9/88	Steenbras Dam	246
Grubbia tomentosa (Thunb.) Harms	15/12/87	Grootvadersbosch	167
	23/9/88	Steenbras Dam	243

SPECIES	DATE	LOCALITY	COLLECTION NUMBER
DIAPENSIACEAE			
Galax urceolata	18/7/91	Edinburgh Botanic Garden	694463
Shortia soldanelloides	18/7/91	Edinburgh Botanic Garden	700731
ERICACEAE			
Erica arborea ssp. alpina	13/9/91	Chelsea Physic Garden	348
RETZIACEAE			
Retzia capensis Thunb.	23/9/88	Steenbras Dam	249
	7/12/88	Perdeberg, Kleinmond	171
GEISSOLOMACEAE			
Geissoloma marginata (L.) A.Juss.	14/2/89	Swellendam Mts, 12 O'Clock Peak	271
PENAEACEAE			
Endonema retzioides Sond.	18/6/92	Riviersonderend	FJP 505
Penaea mucronata L.	15/2/89	nr Cape Infanta	284
Penaea dahlgrenii Rourke	23/10/89	Boosmansbos, Heidelberg	308

All collectors numbers are the author's. Voucher specimens are held at NBI except for E.E.E. collections (BOL) and the Diapensiaceae collections (Edinburgh Botanic Garden)

F.J.P. = F.J. Powrie
A.H. = A. Hitchcock
R.S. = R. Saunders
J.P.R = J.P.Rourke
E.E.E = E.E. Esterhuysen

APPENDIX 2

COMPOSITION OF SOLVENT SYSTEMS (PAPER CHROMATOGRAPHY), BUFFER SOLUTIONS (ELECTROPHORESIS) AND SPRAY REAGENTS (IDENTIFICATION).

A: SOLVENT SYSTEMS

1) BAW (BUTANOL -ACETIC ACID-WATER)

n-Butyl alcohol	4	Shake and discard lower layer
Acetic acid (glacial)	1	
Water	5	

2) CAW (CHLOROFORM-ACETIC ACID-WATER)

Chloroform	30	
Acetic acid (glacial)	15	Shake and discard surplus water
Water	2	

3) FORESTAL

Glacial acetic acid	30
Hydrochloric acid conc.	3
Water	10

4) ROUX SOLVENT (FORMIC)

Hydrochloric acid conc.	2
Formic acid	5
Water	3

5) ACH 50% (15%)

Acetic acid (glacial)	1 (15)
Water	1 (85)

6) PhOH (Phenol saturated with water)

Phenol	3	500g	Shake and discard
Water	1	125ml	aqueous layer

B: BUFFER SOLUTIONS

1) ACETATE BUFFER pH 4,4 (PAPER ELECTROPHORESIS: FLAVONOID GLUCURONIDES)

0,2M	acetic acid	305 ml
0,2M	sodium acetate	195ml
Water		500ml

2) FORMATE-ACETATE BUFFER pH 2,2 (PAPER ELECTROPHORESIS: FLAVONOID SULPHATES)

Formic acid	2,5%	250ml
Acetic acid	8,0%	250ml

C: SPRAY REAGENTS (CHROMATOGRAPHY)

1) NATURSTOFFREAGENZ A (NA)

1% solution in methanol of 2-amino-ethyl diphenylborinate (diphenyl-boric acid-ethanolamine complex; diphenylboric acid,β-aminoethyl ester)

Flavones and flavonols having 3',4'-dihydroxy pattern appear as orange spots in both visible and ultra-violet light; their 4'-hydroxy equivalents appear yellow-green.

2) ALUMINIUM CHLORIDE SOLUTION

5% solution of $AlCl_3$ in ethanol

All 5-hydroxy flavonoids appear as fluorescent yellow spots on dried chromatograms viewed in UV light (365nm)

3) PAULY'S REAGENT (DIAZOTISED SULPHANILIC ACID)

Solution A: 0,3% sulphanilic acid in 8% HCl 25ml

Solution B: 5% aqueous sodium nitrite 1,5ml

The two solutions are mixed just before use and applied as a spray to the dried chromatogram. A 20% aqueous sodium carbonate solution is sprayed onto the PC before drying. Compounds having free phenolic hydroxyl groups appear as yellow, orange or red spots.

4) VANILLIN-HCl

Vanillin 5% in ethanol 4

Hydrochloric acid conc. 1

The two reagents are mixed prior to use and sprayed onto the PC, which is then warmed with a hair dryer. Catechins and proanthocyanidins appear as red to purple-red spots immediately following the application of heat, while flavonones and dihydroflavonols react similarly but more slowly. The regent detects flavonoids having an A-ring oxidation pattern as in phloroglucinol, combined with a saturated C-ring.

APPENDIX 3 Rf VALUES IN VARIOUS SOLVENTS, APPEARANCE IN ULTRA-VIOLET LIGHT (365nm) AND COLOUR REACTIONS OF COMMON FLAVONOIDS/PHENOLIC ACIDS

	Rf VALUES								COLOUR REACTIONS IN UV/VIS/NA			
	CHEMICAL CLASS	BAW	CAW	PhOH	FOR.	ACH 50%	H_2O	ROUX	U.V.	UV/NH_3	VIS	NA/UV
Cyanidin	A	0,68	0,03	-	0,49	0,4	0,0	0,22	mag	blue	mag	-
Delphinidin	A	0,42	0,01	0,08	0,32	0,3	0,0	0,13	purp	blue	purp	-
Pelargonidin	A	0,8	-	-	0,68	-	0,0	0,33	red	blue	red	-
Myricetin	Fl	0,43	0,06	0,13	0,28	0,13	0,0		Y	Br Y	P	O/O
Quercetin	Fl	0,64	0,19	0,29	0,41	0,15	0,0		Y	Br Y	inv.	P/O
Kaempferol	Fl	0,83	0,58	0,58	0,55	0,18	0,0		Y	Br Y-G	inv.	Y/G
Isorhamnetin	Fl	0,74	0,71	0,66	0,52	0,15	0,0		pale Y	Br Y-G	inv.	Y/G
Gossypetin	Fl	0,31	-	0,12	0,26	0,18			dull	dull	Y	P/O-
Fisetin	Fl	0,76	0,26	0,5	0,45	0,2	0,0				inv.	P/O
Apigeni	F	0,89	0,78	0,88	0,83	0,27	0,0				inv.	Y/G
Luteolin	F	0,78	0,39	0,66	0,66	0,23	0,0		dull	T/Oc	inv.	P/O
Diosmetin	F	0,85	0,85	0,89	0,63	0,26	0,0			bright	inv.	Y/G
Chrysoeriol	F	0,82	0,84	0,9	0,77	0,26	0,0		Y/	Y-G	inv.	Y/G
Orientin	C	0,31	0,07	0,43	0,65	0,38	0,02				inv.	P/O
Vitexin	C	0,41	0,18	0,63	0,78	0,49	0,06				inv.	Y/G
Ellagic acid	P	0,34	0,04	0,17	0,26	0,1	0,0		pale lilac	pale yellow	inv.	-
Gallic acid	P	0,68	0,16	0,12	0,64	0,6	0,0		bright blue	brilliant blue	inv.	-
Daidzein	I	0,92					0,04		light blue	B-G	inv.	-
Rutin	G	0,45		0,46			0,23		deep mauve	Y-G	inv.	Y/G

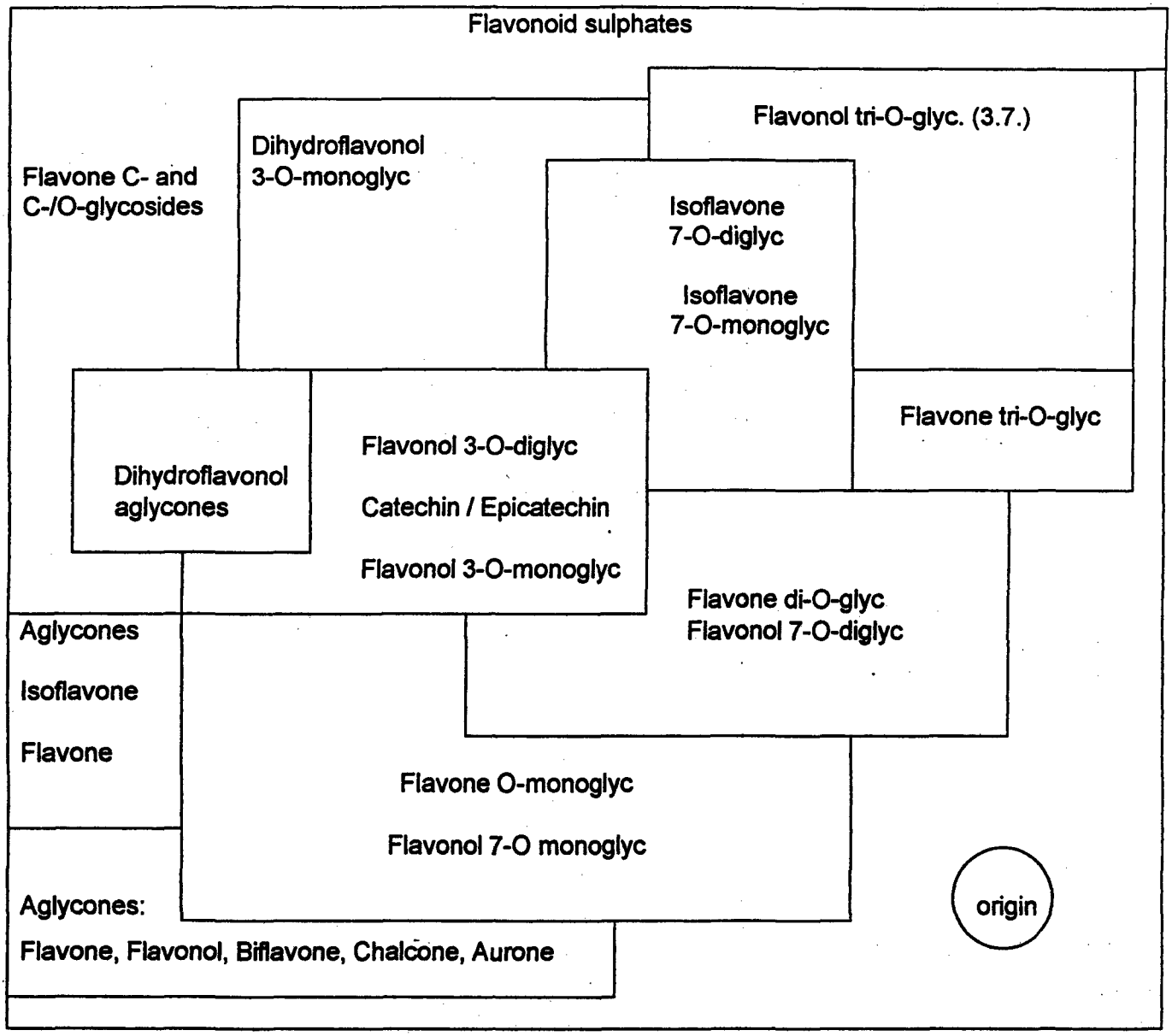
CHEMICAL CLASS
Fl = flavonol
F = flavone
A = anthocyanidin
C = flavone C-glycoside
P = phenolic acid
I = isoflavone
G = flavonol glycoside

Br = bright

COLOUR REACTION
mag = magenta
purp = purple
Y = fluorescent yellow
Y-G = fluorescent yellow-green
B-G = fluorescent blue-green
P = peach
O = orange
T = taupe
Oc = ochre
inv. = invisible
vis. = colour in daylight

NA: Naturstoffreagenz A

APPENDIX 4A



A guide to the distribution of flavonoid types on a chromatogram developed in TBA/15% HOAc.

APPENDIX 4B

Interpretation of spot colour in terms of flavonoid structure

Spot colour in ultraviolet light		Possible flavonoid type
UV light without NH_3	UV light with NH_3	
Dark purple	Yellow, yellow-green or green	a. Commonly 5-OH flavones or flavonols (3-O-substituted with 4'-OH) b. Occasionally 5-OH flavanones and 4'-OH chalcones with no B-ring-OHs
	Little or no colour change	c. Commonly flavones or 3-O- substituted flavonols with 5-OH but lacking a d. *free* 4'-OH e. Some 6- or 8-OH flavones and 3-O-substituted flavonols with 5-OH f. Isoflavones, dihydroflavonols, biflavonyls and some flavanones with 5-OH g. Chalcones with 2'- or 6'-OH but without a free 2- or 4-OH
	Light blue	Some 5-OH flavones
	Red or orange	Chalcones with a free 2- and/or 4-OH
Fluorescent	Fluorescent yellow-green or fluorescent blue-green	Flavones and flavanones lacking a free 5-OH, e.g. 5-O-glycosides Flavonols lacking a free 5-OH but with the 3-OH substituted
	Little or no colour change	Isoflavones lacking a free 5-OH
	Bright fluorescent light blue	Isoflavones lacking a free 5-OH

Spot colour in ultraviolet light		Possible flavonoid type
UV light without NH_3	UV light with NH_3	
Invisible	Fluorescent light-blue	Isoflavones lacking a free 5-OH
Dull yellow and yellow, or orange fluorescence	Little of no colour change	Flavonols with a free 3-OH and with or without a free 5-OH (sometimes originating from the dihydroflavonol)
Fluorescent yellow	Orange or red	Aurones with a free 4'-OH and flavanones lacking a free 5-OH
Yellow-green blue-green or green	Little or no colour change	a. Aurones lacking a free 4'-OH and flavanones lacking a free 5-OH b. Flavononls with a free 3-OH and with or without a free 5-OH
Dull orange red or mauve	Blue	Anthocyanidin 3-glycosides
Cerise pink or fluorescent yellow	Blue	Most Anthocyanidin 3,5 diglycosides

```
B ink
320 mesh
OBSERVE H1
 Frequency 399.958 MHz
 Spectral width 4752.6 Hz
 Acquisition time 5.759 sec
 Relaxation delay 0.000 sec
 Pulse width 28.1 degrees
 Temperature 25.0 deg. C / 298.1 K
 No. repetitions 256
DECOUPLE H1
 High power 26
  Decoupler gated off during acquisition
 Decoupler gated on during delay
 Single frequency
Double precision acquisition
DATA PROCESSING
 Line broadening 0.3 Hz
 FT size 65536
Total acquisition time 15 minutes
```

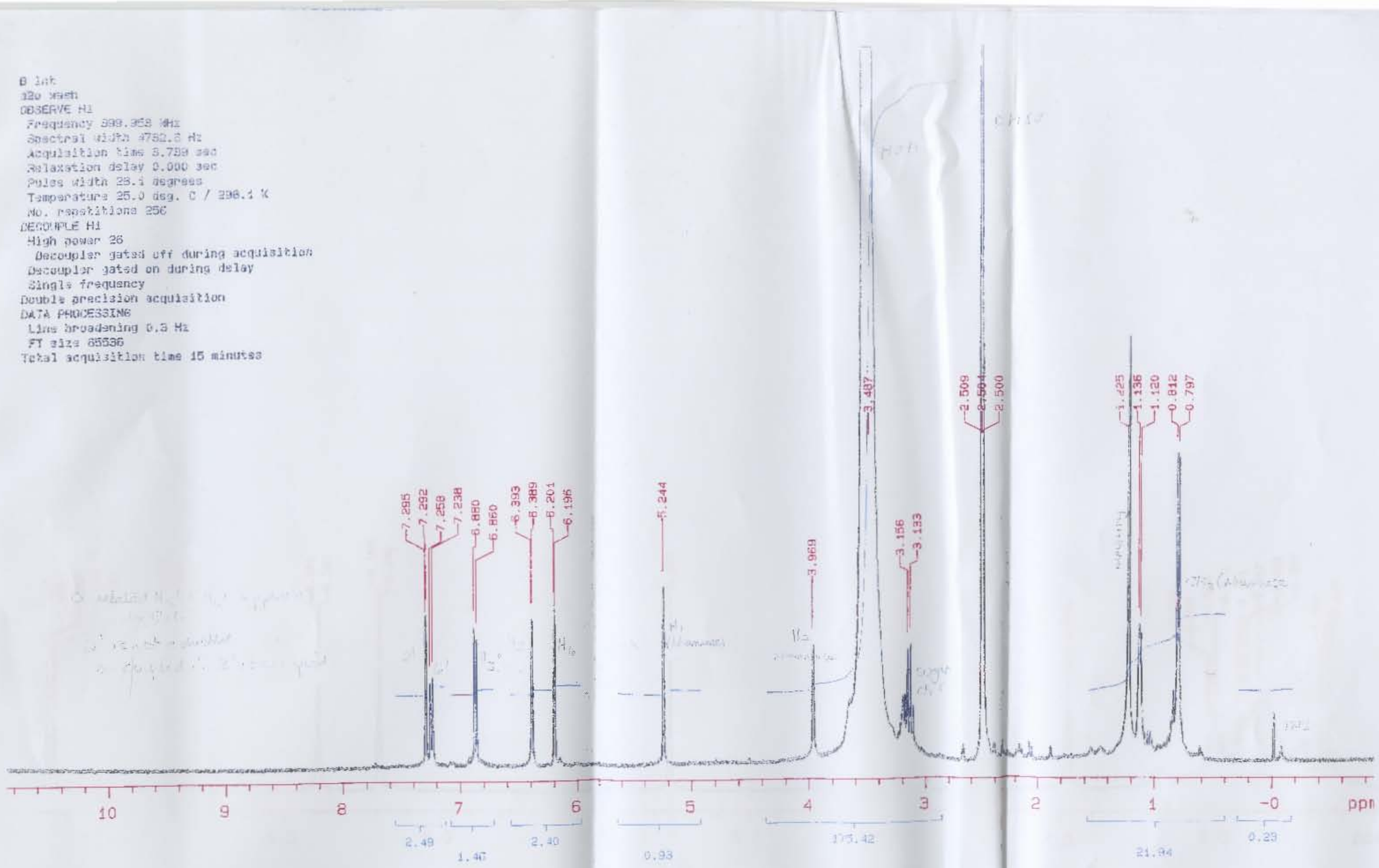
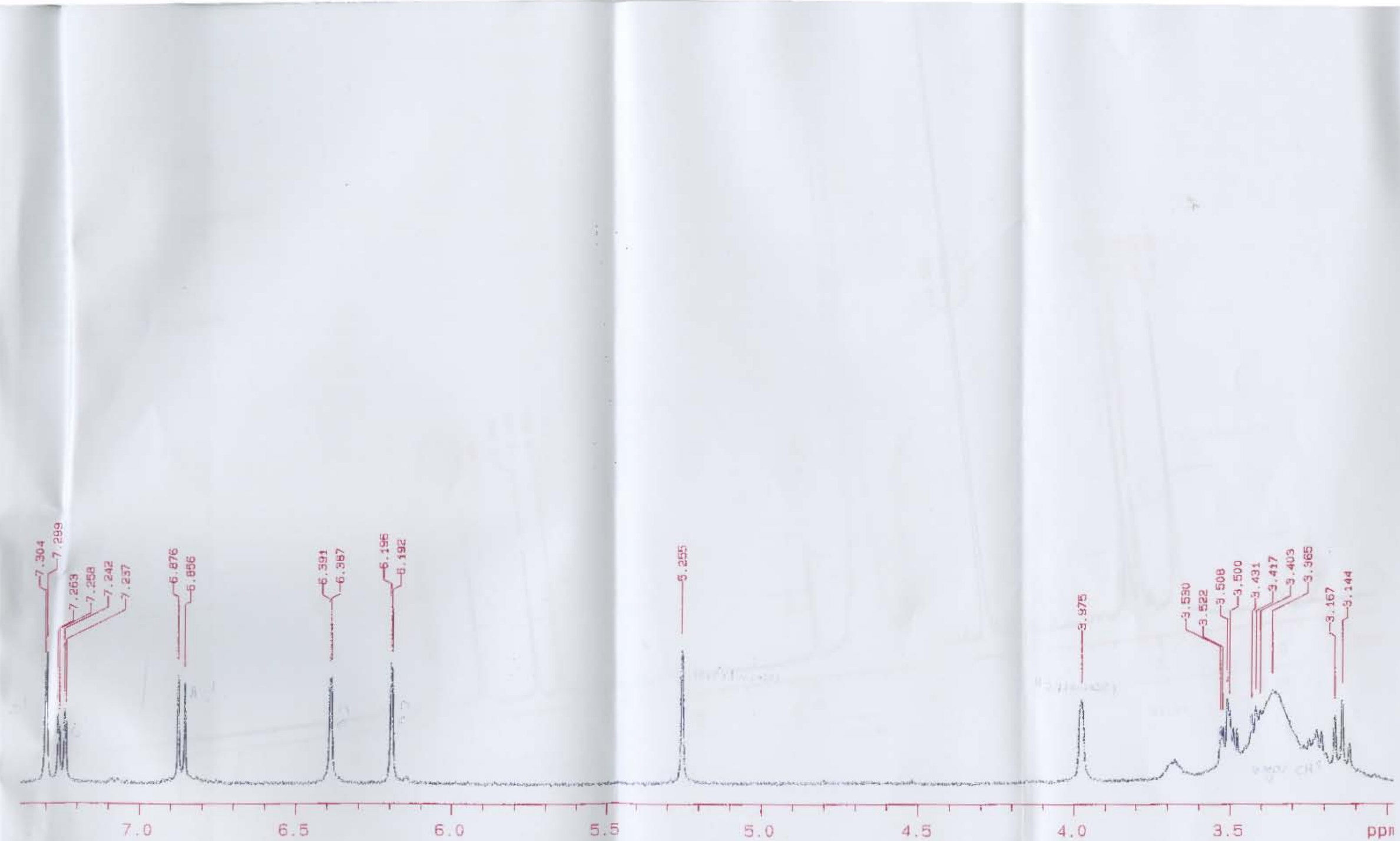


Figure 12. Proton nuclear magnetic resonance spectrum for *Berzelia cordifolia* fraction 6 (quercitrin).


7.304
7.29
7.263
7.258
7.242
7.237
6.876
6.866
6.391
6.387
6.196
6.192
5.255
3.975
3.530
3.522
3.508
3.500
3.431
3.417
3.403
3.365
3.167
3.144
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
ppm

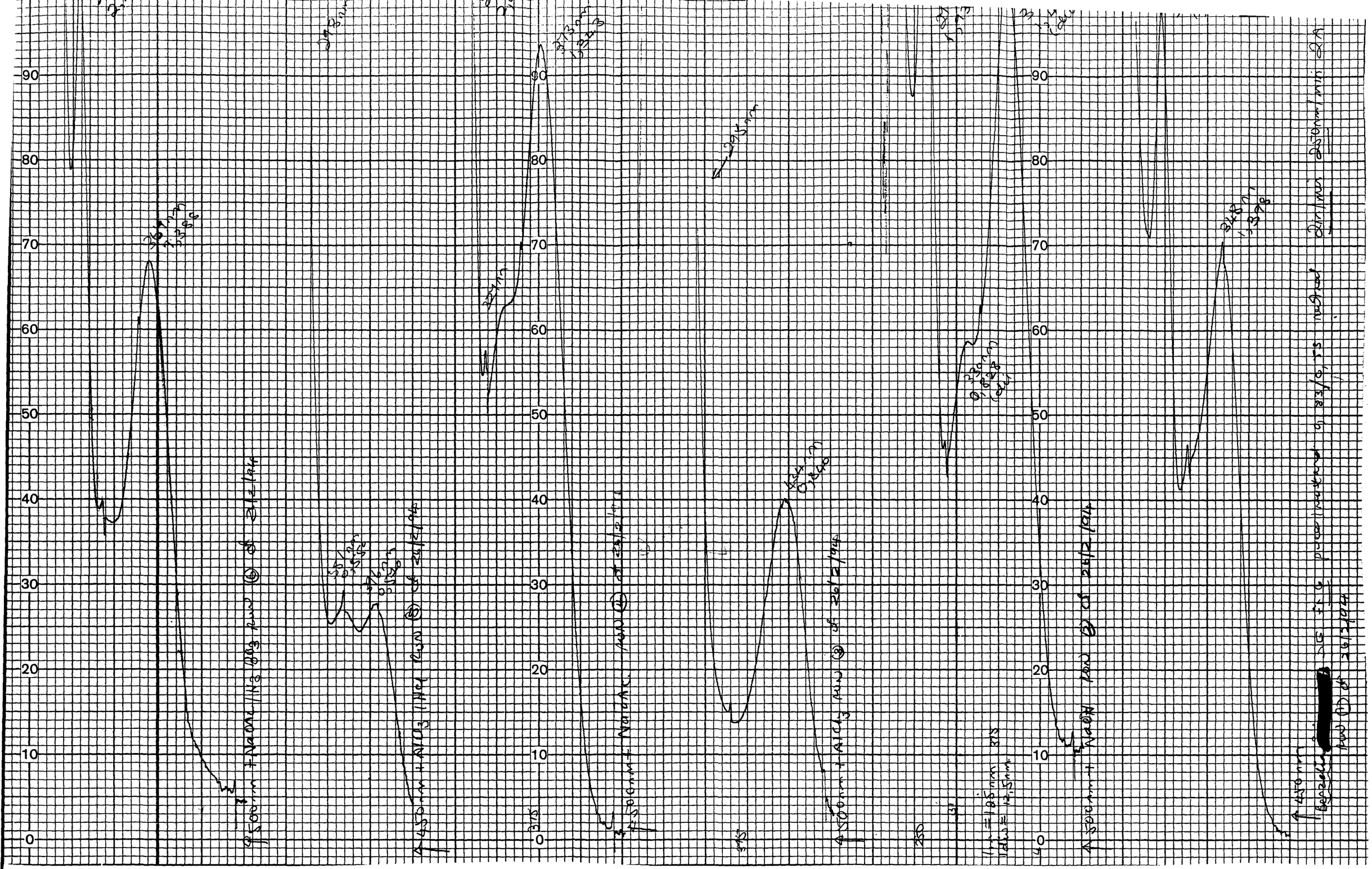


Figure 11. UV spectra for *Berzelia cordifolia* fraction 6 (quercitrin).

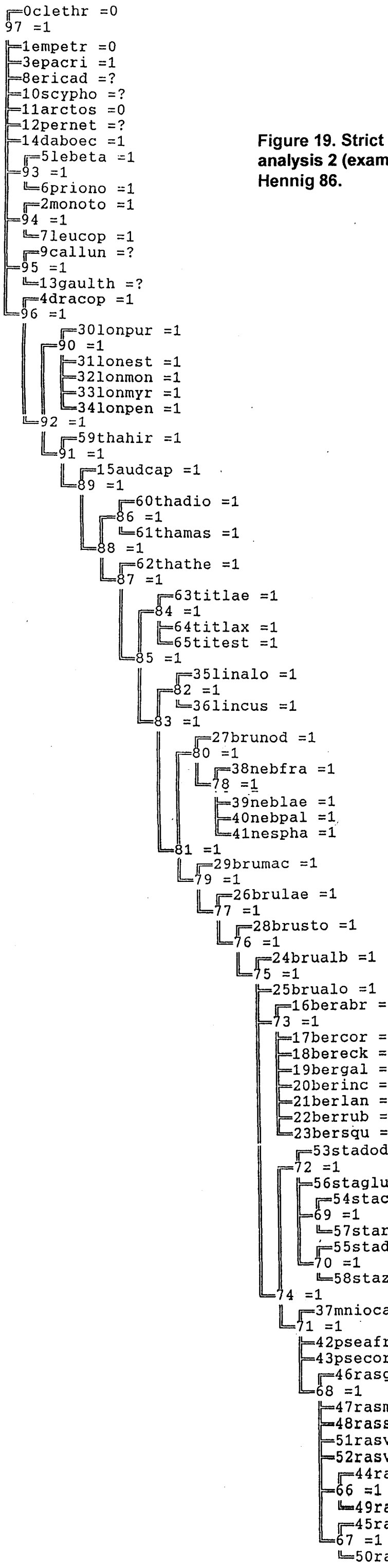


Figure 19. Strict consensus of 1442 most parsimonious trees, obtained in analysis 2 (examination of within family relationships in Bruniaceae), using Hennig 86.

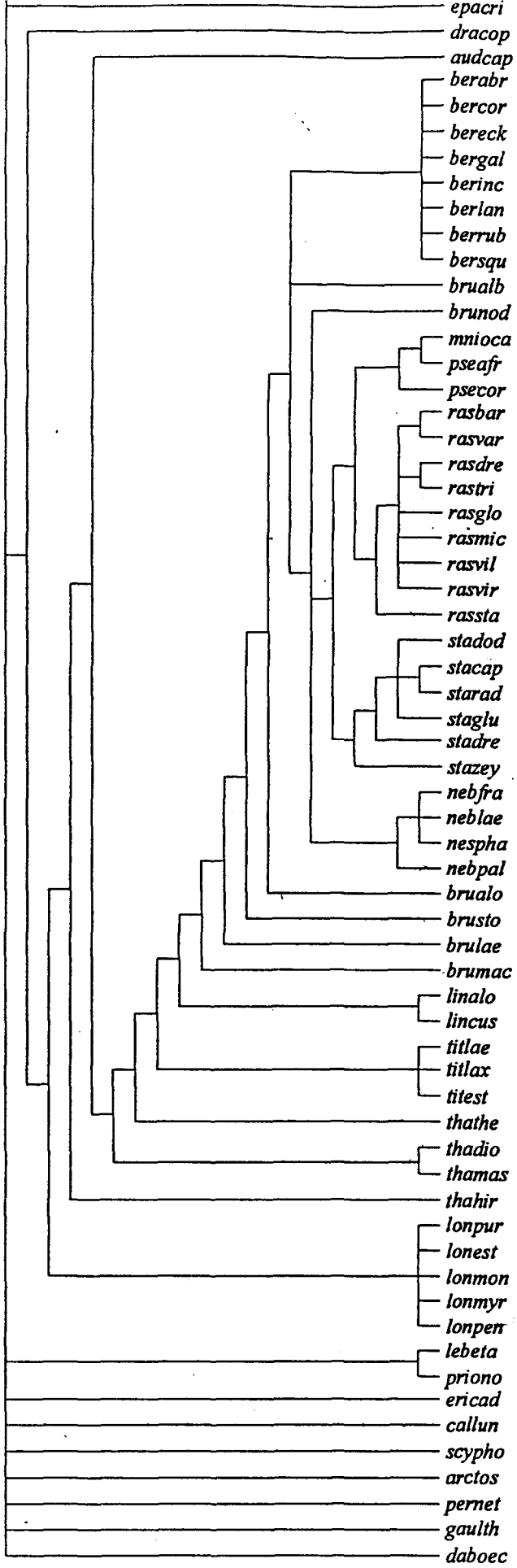


Figure 20. Strict consensus of 4774 most parsimonious trees obtained in PAUP analysis of unweighted data set.

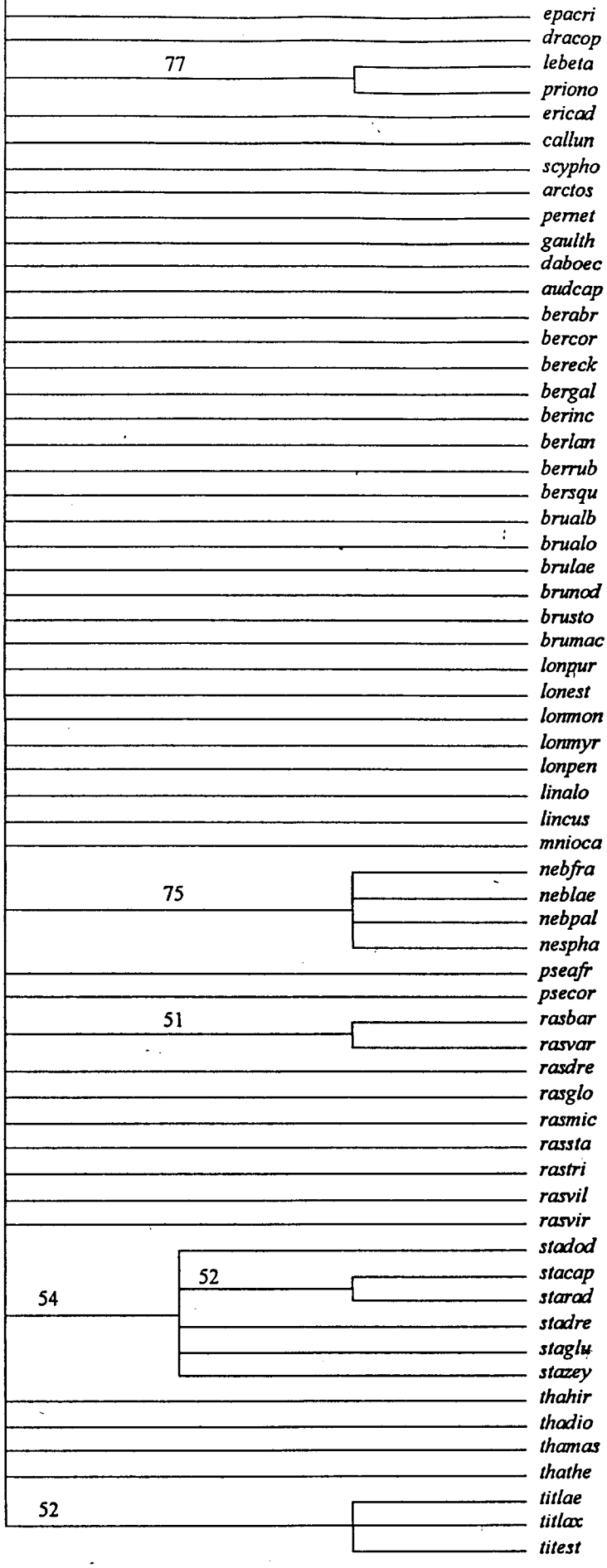


Figure 24. Bootstrap analysis (weighted data set).

br y	259	304	360	268	decomp		268	365	401	258		365	Iso-7-glycoside
br y	258		358	272	330	408	269	360	400	262	294	376	Q-3-glycoside
br green	267		352	275	328	408	276	351	399	267	300	357	Iso/K3-glycoside
y	260		361	270	331	408	269	360	401	262	299	378	Q-3-glycoside
y-gr	268		352	276	327	403	276	346	395	268	299	354	Iso/K3-glycoside
y-gr	253sh		356	271		411	267	364	400sh	257sh	315	365	Iso/K3-glycoside
y	269		353	decomp.		361	269	406	357	258		360	Q-3-diglycoside
y-gr	263		361	271		408	269	358	397	-		367	K-3'-glycoside
gr	258		357	272		407	269	358	401	262		370	Q-3-glycoside
y-gr	259		359	272	331	408	270	364	402	263		374	Q-3-glucuronido
y-gr	257		357	273		411	269	362	401	260		368	Iso-3-glycoside
y/p/y-gr	268		357	273	328	415	270	363	403	257		360	Iso-3,7-diglycoside
y/p/y-gr	255		356	271	328	413	267	359	398	258		362	Iso-3-galactoside
y-gr	262		359	271		409	271	363	398	264		378	Q-3-glycoside
y/p/y-gr	266		363	272	328	408	271	367	399	264		381	Q-3-glycoside
y/p/y-br	266		372	270		409	270	365	399	264		381	Q-3-rutinoside
y/p/y-gr	265		360	272		408	271	363	399	264		378	Q-3-glycoside
y-gr	256		357	273		407	269	362	402	257		362	Iso-3-rutinoside
y/dk/y-gr	257		358	272	330	414	270	362	402	263		364	Iso-3-galactoside
y/dk/y-gr	257		356	273		417	269	362	398	263		358	Iso-3- glycoside
e visy/p/br y	257		357	272	329	415	269	358	396	265		366	Iso-3- glycoside
e visy/p/br y	257		357	271	325	416	268	359	398	264		361	Iso-3- glycoside
-gr	270		356	270	325	411	270	408sh	362	260		359	kaempferol 5-0-Me ether 3,7-diglycoside
-gr	257		356	271	325	414	270	362	400	258		361	Iso-3- 0-glycoside
-gr	256		354	268		417	270	356	393	268		359	Iso-3- glycoside
apricot/p/y-gr	258		358	269		415	268	400sh	361	269		415	Fisetin 3-glycoside Azaleatin-3-glycoside
	257		347	270		391	264		344	261		353	
-gr	255		353	267		411	269		402	258sh		362	Iso/K3- glycoside
y/p/y-gr	258		359	271		413	272	362	400	259		367	Iso/K3- glycoside
-gr	256		346	268		398	267		355	257		361	Luteolin 7-glycoside
y/y/br y	259		356	271		415	275	303	403	288	259	368	Iso-3- glycoside
r y	255		357	272		411	269	360	400sh	261		359	
r y	259		357	272	325	410	269	362	394	262		370	Q-3- glycoside
y/p/y-gr	257		359	272		408	268		361			363	
y/p/y-gr	257		357	289		413	272	400sh	359	260		362	K 5 - Me ether 3 glycoside
e-p/y-gr	257	272	357	274		415	268	395	359			358	Iso-3,7 diglycoside
/p/y-gr	271		357	273		413	271	359	400	271		362	Iso-7,3 diglycoside
/y/br y	267		358	271		416	270	362	400	261		368	Iso-3,7 diglycoside
gr	258		356	270		406	270	358	395	263		371	Q-3-galactoside
/p/y-gr	268		356	272	315	415	270	359	399	259		361	Iso-3,7 diglycoside
/y/br y	270		353	273		403	272	400sh	358	275		375	Aza -3,7 diglycoside
gr	268		354	270		408	267		355	267		373	Aza/Fis 3 glycoside
/p/br.y	266		354	271		404	268		353	267		370	Aza/Fis 3,7 diglycoside
y	264		355	272		405	268		356	265		378	Aza/Fis 3,7 diglycoside
gr	263		366	271		410	268		363	265		382	Aza/Fis 3 glycoside
y	258		344	269		393	265		363sh	266		363	Luteolin 5 glycoside
gr	257		356	272	328	410	269	355	398	261		368	Q-3- glycoside
/p/y-gr	256		358	272	330	412	268	359	397	261		369	Q-3-rutinoside
gr	258		358	269		411	270	356	398	262		373	Q-3- glycoside
y	261		359	272		409	271	398sh	356	262		369	Q-5-Me
gr	259		358	272		417	269		363	263		370	Q-5-Me 3 glycoside Fisetin
p/br y	266		353	-		398	274	352	398	263		369	Q+ Q-3-Me
gr	265		357	273		399	270		356	266		373	Q-5-Me/Fis
p/y-gr	276		353	274		391	276		350	273		350	K-5-Me
-gr	263		352	269		404	267		348	262		365	Aza/Fis 3 glycoside

y-gr	259		359	269		401	266	354	395	260sh	291	372	Q-3-glycoside
k/br y	259	304	360	268	decomp		268	365	401	258		365	Iso-7-glycoside
k/br y	258		358	272	330	408	269	360	400	262	294	376	Q-3-glycoside
k/br green	267		352	275	328	408	276	351	399	267	300	357	Iso/K3-glycoside
k/y	260		361	270	331	408	269	360	401	262	299	378	Q-3-glycoside
k/y-gr	268		352	276	327	403	276	346	395	268	299	354	Iso/K3-glycoside
k/y-gr	253sh		356	271		411	267	364	400sh	257sh	315	365	Iso/K3-glycoside
k/y	269		353	decomp.		361	269	406	357	258		360	Q-3-diglycoside
k/y-gr	263		361	271		408	269	358	397	-		367	K-3'-glycoside
y gr	258		357	272		407	269	358	401	262		370	Q-3-glycoside
k/y-gr	259		359	272	331	408	270	364	402	263		374	Q-3-glucuronido
k/y-gr	257		357	273		411	269	362	401	260		368	Iso-3-glycoside
sy/p/y-gr	268		357	273	328	415	270	363	403	257		360	Iso-3,7-diglycoside
sy/p/y-gr	255		356	271	328	413	267	359	398	258		362	Iso-3-galactoside
k/y-gr	262		359	271		409	271	363	398	264		378	Q-3-glycoside
sy/p/y-gr	266		363	272	328	408	271	367	399	264		381	Q-3-glycoside
sy/p/y-br	266		372	270		409	270	365	399	264		381	Q-3-rutinoside
sy/p/y-gr	265		360	272		408	271	363	399	264		378	Q-3-glycoside
k/y-gr	256		357	273		407	269	362	402	257		362	Iso-3-rutinoside
sy/dk/y-gr	257		358	272	330	414	270	362	402	263		364	Iso-3-galactoside
sy/dk/y-gr	257		356	273		417	269	362	398	263		358	Iso-3- glycoside
ale visy/p/br y	257		357	272	329	415	269	358	396	265		366	Iso-3- glycoside
ale visy/p/br y	257		357	271	325	416	268	359	398	264		361	Iso-3- glycoside
y-gr	270		356	270	325	411	270	408sh	362	260		359	kaempferol 5-0-Me ether 3,7-diglycoside
y-gr	257		356	271	325	414	270	362	400	258		361	Iso-3- 0-glycoside
y-gr	256		354	268		417	270	356	393	268		359	Iso-3- glycoside
s apricot/p/y-gr	258		358	269		415	268	400sh	361	269		415	Fisetin 3-glycoside Azaleatin-3-glycoside
y	257		347	270		391	264		344	261		353	
y-gr	255		353	267		411	269		402	258sh		362	Iso/K3- glycoside
s y/p/y-gr	258		359	271		413	272	362	400	259		367	Iso/K3- glycoside
y-gr	256		346	268		398	267		355	257		361	Luteolin 7-glycoside
sy/y/br y	259		356	271		415	275	303	403	288	259	368	Iso-3- glycoside
br y	255		357	272		411	269	360	400sh	261		359	
br y	259		357	272	325	410	269	362	394	262		370	Q-3- glycoside
sy/p/y-gr	257		359	272		408	268		361			363	
sy/p/y-gr	257		357	289		413	272	400sh	359	260		362	K 5 - Me ether 3 glycoside
ue-p/y-gr	257	272	357	274		415	268	395	359			358	Iso-3,7 diglycoside
sy/p/y-gr	271		357	273		413	271	359	400	271		362	Iso-7,3 diglycoside
sy/y/br y	267		358	271		416	270	362	400	261		368	Iso-3,7 diglycoside
/-gr	258		356	270		406	270	358	395	263		371	Q-3-galactoside
sy/p/y-gr	268		356	272	315	415	270	359	399	259		361	Iso-3,7 diglycoside
sy/y/br y	270		353	273		403	272	400sh	358	275		375	Aza -3,7 diglycoside
-gr	268		354	270		408	267		355	267		373	Aza/Fis 3 glycoside
y/p/br.y	266		354	271		404	268		353	267		370	Aza/Fis 3,7 diglycoside
ır y	264		355	272		405	268		356	265		378	Aza/Fis 3,7 diglycoside
-gr	263		366	271		410	268		363	265		382	Aza/Fis 3 glycoside
r y	258		344	269		393	265		363sh	266		363	Luteolin 5 glycoside
-gr	257		356	272	328	410	269	355	398	261		368	Q-3- glycoside
y/p/y-gr	256		358	272	330	412	268	359	397	261		369	Q-3-rutinoside
-gr	258		358	269		411	270	356	398	262		373	Q-3- glycoside
r y	261		359	272		409	271	398sh	356	262		369	Q-5-Me
-gr	259		358	272		417	269		363	263		370	Q-5-Me 3 glycoside Fisetin
/p/br y	266		353	-		398	274	352	398	263		369	Q+ Q-3-Me
gr	265		357	273		399	270		356	266		373	Q-5-Me/Fis
/p/y-gr	276		353	274		391	276		350	273		350	K-5-Me
/-gr	263		352	269		404	267		348				

													rhamnoside
-gr	268		354	269		398	275	390sh	359	265		376	Fisetin/azaleatin 3 glycoside
nustard	266		351	273		395	275	355 decomp	402	264		369	
y/dk/ stard y	269		356	272		403	272	400sh	363	266		372	Fisetin/azaleatin 3 glycoside
rill.y	259		362	270		425	270	373	407	260	373	369	Q -3-7 diglycoside
right y	255sh		362	270		427	270	369	406	267sh		370	
r y	256		357	270		414	270	364	404	253		364	Iso/K3- glycoside
y/p/y-gr	261		378	268		421	263	364	412	264		396	Q -7- glycoside
-gr	255	270sh	347	268		403	273	355	385	270		368	Lu.-7- glycoside
-gr	270		346	270		397	276	353	389	decomp			
r y	276		348	269		410	275	355	390sh	270		350	
-gr	256		357	272		410	270	361	401	259		365	Iso-3- diglycoside
y/p/ mustard y	286		343	278		391	282	352	403sh	286		345	
o/y-gr	275	260sh	380	265		419	277	362	420	275		382	
-gr	262		343	270		393	261		343	261		345	
-gr	267		351	271		399	271	340	398sh	269		352	K-5-Me ether 3,7 diglycoside
dky/p/ stard y	269	287	351	276	325	395	277	353	399	266	340	367	Q -7- glycoside
dky/p/brill y	270		384	274		433	274	420sh	362	266		392	
-gr	258		356	273	320	403	270	355	400	262		368	Q -3- glycoside
y/p/y-gr	268		357	274	325	404	273	392sh	359	267		365	No 3,5OH function No O-di OH
y/p/y-gr	258	300sh	357	272	330	410	273	360sh	401	261		371	Q-3-rutinoside
-gr	267	330	348	274		396	275	346	395	268		354	Iso-3- glycoside
r y	259		355	268		405	268	356	397	262		367	Q -3- glycoside
rill y	256		359	- decomp		418	269	361	404	-		375	Q-3- glycoside
rill y	268		355	267	300	411	275	356	400	260		375	Q-3-Me7 glycoside
-gr	269		363	270		410	266	390sh	359	263		375	
r y	257		355	271		403	267	357	397	262		375	Q3 Me ether
/bl-gr	254		350	268	323	399	266		353	257		368	Lu-5- glycoside
y	269		354	272		399	272	354	398	263		366	Lu-7- glycoside
/lt bl/y-gr	255		357	268		391	283sh		349	-		365	carytin 3 glycoside caryatin -3'-
/y	258		359	272	324	404	270	362	401	262	300 sh	378	Q derivative
	257		360	273	278	404	269	363	398	276	324	389	Q-3- glycoside
-y/p/y-gr	259		358	274	300	408	268	400sh	364	264	323	370	
ry/p/y-gr	258		360	272	312sh	401	270	363sh	400	261		376	Q-3- glycoside
gr	268		354	272	324	409	272	359	395	265		374	Q-3- glycoside
p/mustard y	258		348	272		386	270	351	395	262		365	Q-3 rhamnoside
p/y	248		353	270		402	249		367	decomp		359	5 substitution or 5 -deoxy

fis = fisetin
Lu = luteolin