

## Phylogenetic studies within Rubiaceae and relationships to other families based on molecular data

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**Abstract.** — Phylogenetic analyses of *rbcL* sequence data from 93 Rubiaceae genera and 62 genera from other Asteridae families were undertaken to answer the questions 1) Is the family Rubiaceae well supported as monophyletic? 2) What is its position within the angiosperm system, or which are its closest relatives? 3) Can or should one recognize subfamilies and what is the support for these? 4) Which tribes belong to the subfamily Rubioideae and how are these related? 5) Do changes in ovule number and fruit type represent unique events in the Rubioideae? From Rubiaceae 38 new species were sequenced, mainly from the subfamily Rubioideae, and also three species representing Gentianaceae, Loganiaceae, and Stilbaceae. The cladistic analyses show that the Rubiaceae are monophyletic, that the Rubiaceae are the sister group to the rest of the Gentianales, and that there are three groups of taxa more or less corresponding to the three subfamilies Rubioideae, Ixoroideae s.l., and Cinchonoideae s.str. There is no support for the subfamily Antirheoideae, the taxa of which are nested within Cinchonoideae s.str., Ixoroideae s.l., and Rubioideae. The analyses show that the tribes Ophiorrhizeae, Pauridiantheae, and Knoxieae are part of the Rubioideae as well as the genera *Danais*, *Manettia* and *Bouvardia*, which earlier have been proposed to form a link to the Cinchonoideae. As a result of the analyses the tribe Hedyotideae must be included in the Spermacoceae as the Spermacoceae s.str. are nested within Hedyotideae and the name Spermacoceae has priority. The evolution of ovule number and fruit types (fleshy or dry) was analysed within the Rubioideae. Ovule reduction has occurred at least seven times but in no case has a reduced ovule number reverted back to many ovules, and fleshy fruits have evolved at least five times in the subfamily. This latter character seems more plastic and in several lines there are reversals back to a dry fruit.

**Key words.** — *rbcL*; cladistic analysis; phylogeny; Gentianales; Rubiaceae; Rubioideae; Hedyotideae; Spermacoceae.

**Abbreviation.** — *rbcL*, ribulose-1,5-bisphosphate carboxylase.

### 1 Introduction

In his first attempt at a natural classification Linnaeus (1738) sorted the "Rubiaceae" into three different families ("ordo"), out of 65; *Rondeletia* into ordo XIX (with, e.g., *Viburnum*, *Sambucus*, *Evonymus*), *Catesbaea* into ordo LIV (with, e.g., *Rhamnus* and *Cestrum*), and *Rubia* and its six allied genera as well as *Spermacoce* into an ordo XLIV. Later in his sexual system, *Species Plantarum*, Linnaeus (1753) listed more than 20 genera, but due to the different arrangements of the stamens, they were placed in as many as five different classes (Tetrandria, Pentandria, Hexandria, Polygamia, Monoecia). However, several natural classification systems were soon to be presented and ever since Jussieu (1789) described the family, Rubiaceae have been accepted as a natural and monophyletic group. Few systematists (but see Baillon 1880 and Wernham 1912) have questioned the monophyly of the family. In many systematic works about Rubiaceae we find sentences like "the family Rubiaceae is a very well-defined group and its members are readily recognized" (Verdcourt 1958). The only more critical remark was delivered by Bremekamp (1966) "It is often assumed that the delimitation and the

subdivision of various families which have been distinguished in the angiosperms, do no longer offer serious difficulties ... These assumptions, however, are to be regarded as dangerous illusions". Regarding the Rubiaceae, however, it is clear that even Bremekamp accepted them as a monophyletic family with the exclusion of a few aberrant genera (e.g., *Henriquezia*, *Platycarpum*, *Carlemannia*, *Dialype-talanthus*). In many cases Bremekamp's general skepticism has been justified, not the least, after large cladistic analyses of molecular data sets when several plant families have proven to be paraphyletic/polyphyletic, e.g., Caprifoliaceae (Donoghue et al. 1992), Musaceae (Smith et al. 1993), Saxifragaceae (Morgan & Soltis 1993), Ericaceae (Kron & Chase 1993), and Scrophulariaceae (Olmstead & Reeves 1995). Despite the raised interest in the phylogeny of the angiosperms in general and also in the Rubiaceae, no one has, so far, made any thorough analyses showing that the family is monophyletic. It has been a matter of course or an axiom that the family Rubiaceae is natural and monophyletic.

The taxonomic position of Rubiaceae has not been very controversial. The family has been associated with the Dipsacales or with the Gentianales (Contortae). The reasons for these different positions are found among the characters of the Rubiaceae. A typical member of the family is characterized by opposite, entire leaves with stipules, often provided with colleters, an inferior ovary, absence of intraxylary phloem, presence of nuclear endosperm, and often of alkaloids. This combination of characters is unique among the angiosperms; no other family provides the same set of characters. When searching for its closest relationship different combinations of these characters will, of course, point to different relatives. Several of the characters that unite the family with the Gentianales were at the time of its establishment not known (alkaloids, colleters, nuclear endosperm) and this makes it easier to understand the former association with the Dipsacales. When Jussieu (1789) described the family it was placed close to Dipsacaceae and Caprifoliaceae. This placement was maintained into our century until Utzschneider (1947) and Wagenitz (1959) suggested a position close to or within Gentianales. However, Endlicher (1838) had already indicated affinities between the Rubiaceae and Loganiaceae of the Contortae (= Gentianales). After Wagenitz most systematists have accepted a position of the Rubiaceae within the Gentianales (Bremekamp 1966, Dahlgren 1980, Takhtajan 1987, Thorne 1983, 1992) except Cronquist (1981) who kept the family in the order Rubiales (together with Theligonaceae) putatively between the Dipsacales and the Gentianales and the Asterales.

The few Rubiaceae taxa that have been represented in larger phylogenetic analyses of angiosperms have all ended up together with the Gentianales (Downie & Palmer 1992, Chase et al. 1993, Olmstead et al. 1993), but their interrelationship to or within the Gentianales has been different in the different analyses and is so far not settled.

The subdivision of Rubiaceae into subfamilies has differed between different authors. Schumann (1891) divided the Rubiaceae into two subfamilies, Cinchonoideae and Coffeoidae, based on the number of ovules per locule. This character and Schumann's classification was almost totally rejected by later authors. Bremekamp (1954, 1966) instead emphasized testa structure, occurrence of albumen in the seeds, raphides, and secondary pollen presentation ("ixoroid pollen presentation mechanism"; Ixoroideae), and he recognized as many as eight subfamilies. Three of these, the Cinchonoideae, the Rubioideae, and the Guettardoideae (= Antirheoideae) were accepted by Verdcourt (1958). In the latest classification (Robbrecht 1988, 1993) the Rubiaceae are divided into four subfamilies: Cinchonoideae, Ixoroideae, Rubioideae, and Antirheoideae. Of these the last subfamily differs distinctly from the Guettardoideae/Antirheoideae of the earlier classifications, and includes more tribes than in Verdcourt's (1958) and Bremekamp's (1966) systems. Most characters for Antirheoideae (fide Robbrecht 1988, 1993) are variable but generally taxa have solitary pendulous seeds with large embryos. Several cladistic analyses covering the whole Rubiaceae based on molecular or/and morphological data (Bremer & Jansen 1991, Bremer & Struwe 1992, Bremer et al. 1995, Bremer 1996) have been published. These analyses have not included representatives of all described subfamilies. Representatives of Bremekamp's Gleasonioideae (1957) and Pomazotoideae (Darwin 1976) have not been analyzed cladistically, and a member of the subfamily Hillioideae of Bremekamp (1966) is included in one analysis only (Bremer et al. 1995). In that analysis it is placed within the Cinchonoideae as proposed by Robbrecht (1988). The various analyses differ in details but are in most parts congruent: all analyses support the subfamilies Rubioideae and Ixoroideae. The Cinchonoideae s.str. is also

supported (except in Bremer & Jansen 1991, due to different rooting); furthermore, all analyses reject a wide circumscription of a subfamily Antirheoideae (fide Robbrecht 1988, 1993).

**Table 1 – Tribes included in or associated with the Rubioideae**

Described tribes included in or associated with the subfamily by Verdcourt (1958, 1975), Bremekamp (1966), Robbrecht (1988) compared to the rbcL phylogeny. Tribal names in italics indicate that no taxa have been sequenced. Tribes are indicated with the first three letters of the tribal names; the subfamilies are indicated with the first four letters of the subfamilial names ANTIrheoideae, CINChonoideae, HILLioideae, POMAzotoideae, UROPhylloideae. "x": the tribe is accepted in the subfamily Rubioideae; "?": uncertain position according to the author; "in": the tribe is included in another tribe.

Tribes	Verdcourt	Bremekamp	Robbrecht	rbcL-support
<i>Anthospermeae</i>	x	x	x	x
<i>Argostemmatae</i>	x	x	x	new circumscription
<i>Coccocypseae</i>	x	x	x	x
<i>Coussareae</i>	x	x	x	new position
<i>Craterispermeae</i>	x	x	ANTI	
<i>Cruckshanksiae</i>	x	x	in HED	
<i>Gaertnereae</i>	x	x	in PSY	new position
<i>Hamelieae</i>	x	x	x	CINC
<i>Hedyotideae</i>	x	x	x	in SPE s. l.
<i>Hillieae</i>	?x	HILL	CINC	CINC
<i>Knoxieae</i>	x	x	ANTI	in SPE s. l.
<i>Lathraeocarpeae</i>		x	x	
<i>Maetticieae</i>	in HED	in HED	? in HED/CIN	in SPE s. l.
<i>Morindeae</i>	x	x	x	x
<i>Ophiorrhizae</i>	x	UROP	x	x
<i>Operculariaceae</i>		?	in ANT	in ANT
<i>Paederieae</i>	x	x	x	paraphyletic
<i>Pauridiantheae</i>	in URO	UROP	CINC	x
<i>Perameae</i>		x	?	
<i>Pomazoteae</i>	?	POMA	in HED	
<i>Psychotrieae</i>	x	x	x	x
<i>Rubieae</i>	x	x	x	x
<i>Schradereae</i>	x	x	x	
<i>Spermacoeae</i>	x	x	x	SPE s. l.
<i>Theligoneae</i>			x	x
<i>Triainolepideae</i>	in PSY	x	x	
<i>Urophyllaeae</i>	x	UROP	CINC	
<i>Virectarieae</i>	CINC (1975)	in OPH	in HED	

The Rubioideae are the most easily recognized and circumscribed subfamily within the Rubiaceae. As the name Rubioideae is an autonym the taxon is as old as the first published subfamilies (Rafinesque 1820), but Bremekamp (1952) was the first author who identified a core group of tribes which since then has been accepted as the natural subfamily Rubioideae. The subfamily is characterized by the presence of raphides, valvate aestivation, exotesta cells without perforated thickenings, and often articulate hairs. All modern classification schemes of Rubioideae include slightly less than 20 tribes; Verdcourt (1958) included 17, Bremekamp (1966) 19, and Robbrecht (1988, 1993) 16. Of these tribes, 12 have been accepted by all (*Anthospermeae*, *Argostemmatae*, *Coccocypseae*, *Coussareae*, *Hamelieae*, *Hedyotideae*, *Morindeae*, *Paederieae*, *Psychotrieae*, *Rubieae*, *Schradereae*, and *Spermacoeae*). Of the remaining tribes the opinion and circumscription differ between the authors, and if all tribes that have been associated with or included in the subfamily are counted (excluding nomenclatural synonyms), the total number of different tribes is 28 (table 1).

**Table 2 – New rbcL sequences reported in this paper**

Sources of material extracted for DNA. Rubiaceae species names are followed by a three (four) letter suffix indicating tribal (subtribal) position according to Robbrecht (1988, 1993). The source of the living material is first indicated followed by the collector and the herbarium where it is deposited.

Family	Species	Source/voucher information	Accession EMBL/GenBank
Gentianaceae	<i>Eustoma grandiflorum</i>	Cult. flowershop, Bremer 3307 (UPS)	Z68825
Loganiaceae	<i>Logania vaginalis</i>	Cult. K, Bremer 3013 (UPS)	Z68826
Stilbaceae	<i>Stilbe vestita</i>	South Africa, Goldblatt & Manning 10100 (MO, UPS)	Z68827
Rubiaceae	<i>Agathisanthemum bojeri</i> HED	Tanzania, Bremer 3060 (UPS)	Z68787
Rubiaceae	<i>Argostemma hookeri</i> ARG	Malaysia, Wanntorp s.n. (S)	Z68788
Rubiaceae	<i>Carphalea glaucescens</i> HED	Somalia, S. Med. Plant Project 215 (UPS)	Z68789
Rubiaceae	<i>Chasallia parviflora</i> PSY-P	Tanzania, Bremer 3067 (UPS)	Z68790
Rubiaceae	<i>Chazaliella abrupta</i> PSY-P	Tanzania, Bremer 3081 (UPS)	Z68791
Rubiaceae	<i>Conostomium quadrangulare</i> HED	Puff & Kelbessa 821222 (UPS)	Z68792
Rubiaceae	<i>Damnacanthus indicus</i> MIT	Cult. MO, Bremér 3107 (UPS)	Z68793
Rubiaceae	<i>Danals xanthorrhoea</i> HED	Tanzania, Bremer 3079 (UPS)	Z68794
Rubiaceae	<i>Didymaea alsinoides</i> RUB	Cult. CAS, Keller 1901 (CAS)	Z68795
Rubiaceae	<i>Faramea multiflora</i> COU	Ecuador, Bremer et al. 3331 (MO, QCA, QCNE, UPS)	Z68796
Rubiaceae	<i>Gaertnera</i> sp. PSY-G	Madagascar, Malcomber 2709 (MO)	Z68797
Rubiaceae	<i>Geophila repens</i> PSY-P	Cult. UPS, Bremér 3130 (UPS)	Z68798
Rubiaceae	<i>Hedyotis fruticosa</i> HED	Sri Lanka, Lundqvist 11106 (UPS)	Z68799
Rubiaceae	<i>Kohautia caespitosa</i> HED	Tanzania, Pettersson s.n. (UPS)	Z68800
Rubiaceae	<i>Ladenbergia pavonii</i> CIN	Ecuador, Delprete & Verduga 6404 (LL, TEX, UPS)	Z68801
Rubiaceae	<i>Lasianthus pedunculatus</i> PSY-P	Tanzania, Andreassen 71 (UPS)	Z68802
Rubiaceae	<i>Munellia bicolor</i> HED	Cult. SUNIV, Bremer 2716 (UPS)	Z68803
Rubiaceae	<i>Mitchella repens</i> MIT	Cult. MO, Bremer 2714 (UPS)	Z68805
Rubiaceae	<i>Mycetia malayana</i> ISE	Cult. AAU, Larsen et al. 42486 (AAU, UPS)	Z68806
Rubiaceae	<i>Neurocalyx zeylanicus</i> ARG	Sri Lanka, Bremer & Bremer 937 (PDA, S, US)	Z68807
Rubiaceae	<i>Oldenlandia goreensis</i> HED	Madagascar, Pettersson & Nilsson 728 (UPS)	Z68808
Rubiaceae	<i>Opercularia vaginata</i> ANT-O	Australia, Bremer & Gustafsson 25 (UPS)	Z68809
Rubiaceae	<i>Palicourea</i> sp. PSY-P	Ecuador, Bremer et al. 3332 (MO, QCA, QCNE, UPS)	Z68810
Rubiaceae	<i>Pauridiantha paucinervis</i> PAU	Tanzania, Bremer 3090 (UPS)	Z68811
Rubiaceae	<i>Pentanisia longituba</i> KNO	Ethiopia, Puff & Kelbessa 821220 (UPS)	Z68812
Rubiaceae	<i>Pentanopsis fragrans</i> HED	Ethiopia, Gilbert et al. 7458 (UPS)	Z68813
Rubiaceae	<i>Phyllis nobla</i> ANT-A	Cult. K, Bremer 3008 (UPS)	Z68814
Rubiaceae	<i>Placopoda virgata</i> HED	Yemen, Thulin & Gifri 8528 (UPS)	Z68815
Rubiaceae	<i>Plocama pendula</i> PAE	Canary Islands, Andreassen 1 (UPS)	Z68816
Rubiaceae	<i>Psychotria</i> cf. <i>borjensis</i> PSY-P	Cult. BR, Robbrecht s.n. (UPS)	Z68804
Rubiaceae	<i>Psychotria petersi</i> PSY-P	Tanzania, Bremer 3078 (UPS)	Z68817
Rubiaceae	<i>Psychotria poeppigiana</i> PSY-P	Ecuador, Bremer et al. 3330 (UPS, QCA, QCNE, UPS)	Z68818
Rubiaceae	<i>Psychotria</i> sp. PSY-P	Cult. Paris, Bremer 2722 (UPS)	Z68819
Rubiaceae	<i>Richardia pilosa</i> SPE	Cult. SUNIV, Bremer 2744 (UPS)	Z68820
Rubiaceae	<i>Rudgea</i> cf. <i>lorentensis</i> PSY-P	Ecuador, Bremer et al. 3346 (MO, QCA, QCNE, UPS)	Z68821
Rubiaceae	<i>Serissa foetida</i> PAE	Cult. CONN, Bremer 2717 (UPS)	Z68822
Rubiaceae	<i>Spermacoce laevis</i> SPE	Tanzania, Bremer 3062 (UPS)	Z68823
Rubiaceae	<i>Spermadictyon suaveolens</i> PAE	Cult. Paris, Bremer 3133 (UPS)	Z68824

Detailed relationships among the different tribes of the Rubioideae have never been proposed but it is clear from earlier discussions and from how tribes have been listed in the various classification schemes that the habit, the number of ovules, and type of fruit have played the most important roles in the "phylogenetic" considerations. Verdcourt (1958) grouped the woody tribes with erect solitary ovules together (*Psychotrieae*, *Morindeae*, *Coussareae*, and *Schradereae*) while he united the herbaceous or subshrubby tribes with dry usually dehiscent fruits into one group, including also the ones with solitary ovules (*Anthospermeae*, *Spermacoceae*, and *Hedyotideae*); the position of the remaining tribes was more vaguely indicated. Bremekamp (1966) did not discuss subfamily relationships in detail but he listed all tribes into two series, one with many and the other with solitary ovules. Robbrecht

Table 3 – Earlier published sequences extracted from DNA databases used in the analyses

Family	Species	Accession EMBL/ GenBank	Family	Species	Accession EMBL/ GenBank
Acanthaceae	<i>Aphelandra sinclairiana</i>	L01884	Rubiaceae	<i>Cephalanthus occidentalis</i>	X83629
Acanthaceae	<i>Thunbergia usambarica</i>	L12596	Rubiaceae	<i>Chiococca alba</i>	L14394
Adoxaceae	<i>Adoxa moschatellina</i>	L01884	Rubiaceae	<i>Cinchona pubescens (succirubra)</i>	X83630
Apiaceae	<i>Apium graveolens</i>	L01885	Rubiaceae	<i>Coffea arabica</i>	X83631
Apocynaceae	<i>Kopsia fruticosa</i>	L14402	Rubiaceae	<i>Crucianella angustifolia</i>	X81094
Aquifoliaceae	<i>Ilex crenata</i>	L01928	Rubiaceae	<i>Cubanola domingensis</i>	X83632
Araliaceae	<i>Aralia spinosa</i>	L11166	Rubiaceae	<i>Deppea grandiflora</i>	X83633
Argophyllaceae	<i>Corokia cotoneaster</i>	L11221	Rubiaceae	<i>Enterospermum coriaceum (Tarenno)</i>	X83634
Asteraceae	<i>Helianthus annuus</i>	L13929	Rubiaceae	<i>Erithalis fruticosa</i>	X83635
Asteraceae	<i>Dasyphyllum diacanthoides</i>	L13863	Rubiaceae	<i>Exostema caribaeum</i>	X83636
Aucubaceae	<i>Aucuba japonica</i>	L11210	Rubiaceae	<i>Galium album</i>	X81090
Boraginaceae	<i>Borago officinalis</i>	L11680	Rubiaceae	<i>Gardenia thunbergia</i>	X83637
Boraginaceae	<i>Heliotropium arborescens</i>	L14399	Rubiaceae	<i>Guettarda urugensis</i>	X83638
Brunoniaceae	<i>Berzelia lanuginosa</i>	L14391	Rubiaceae	<i>Haldina cordifolia</i>	X83639
Buddlejaceae	<i>Nicodemia diversifolia</i>	L14413	Rubiaceae	<i>Hallea (Mitragnya) rubrostipulata</i>	X83640
Buddlejaceae	<i>Buddleja davidii</i>	L14392	Rubiaceae	<i>Hamelia cuprea</i>	X83641
Caprifoliaceae	<i>Diervilla sessilifolia</i>	Z29672	Rubiaceae	<i>Hillia triflora</i>	X83642
Caprifoliaceae	<i>Lonicera orientalis</i>	X87389	Rubiaceae	<i>Hintonia latiflora</i>	X83643
Caprifoliaceae	<i>Sambucus racemosa</i>	L14066	Rubiaceae	<i>Hoffmannia refulgens x ghiesbreghtii</i>	X83644
Caprifoliaceae	<i>Viburnum acerifolia</i>	L01959	Rubiaceae	<i>Hydnophytum formicarum</i>	X83645
Convolvulaceae	<i>Convolvulus tricolor</i>	L11683	Rubiaceae	<i>Ixora coccinea</i>	X83646
Convolvulaceae	<i>Ipomea coccinea</i>	L14400	Rubiaceae	<i>Keetia zanzibarica</i>	X83647
Cornaceae	<i>Cornus canadensis</i>	L01898	Rubiaceae	<i>Luculia grandifolia</i>	X83648
Desfontainiaceae	<i>Desfontainia spinosa</i>	Z29670	Rubiaceae	<i>Meyna tetraphylla</i>	X83649
Dipsacaceae	<i>Dipsacus sativus</i>	L13864	Rubiaceae	<i>Mitrostigma axillare</i>	X83650
Ericaceae	<i>Rhododendron hippophaeoides</i>	L01949	Rubiaceae	<i>Morinda citrifolia</i>	X83651
Garryaceae	<i>Garrya elliptica</i>	L01919	Rubiaceae	<i>Mussaenda erythrophylla</i>	X83652
Gelsemiaceae	<i>Gelsemium sempervivens</i>	L14397	Rubiaceae	<i>Nauclea orientalis</i>	X83653
Gelsemiaceae	<i>Mostuea brunonis</i>	L14404	Rubiaceae	<i>Nertera granadensis</i>	X83654
Gentianaceae	<i>Anthocleista grandiflora</i>	L14389	Rubiaceae	<i>Oldenlandia cf. corymbosa</i>	X83655
Gentianaceae	<i>Exacum affine</i>	L11684	Rubiaceae	<i>Ophiorrhiza mungos</i>	X83656
Gentianaceae	<i>Fagraea sp.</i>	L14396	Rubiaceae	<i>Parapentas silvatica</i>	X83657
Gentianaceae	<i>Gentiana procera</i>	L14398	Rubiaceae	<i>Pentagonia macrophylla</i>	X83658
Griselinaceae	<i>Griselinia lucida</i>	L11225	Rubiaceae	<i>Pentas lanceolata</i>	X83659
Grossulariaceae	<i>Phyllonoma laticuspis</i>	L11201	Rubiaceae	<i>Pentodon pentandrus</i>	X83660
Helwingiaceae	<i>Helwingia japonica</i>	L11226	Rubiaceae	<i>Phuopsis stylosa</i>	X81103
Hydrophyllaceae	<i>Eriodictyon californicum</i>	L01916	Rubiaceae	<i>Pinckneya pubens</i>	X83661
Hydrophyllaceae	<i>Hydrophyllum virginianum</i>	L01927	Rubiaceae	<i>Pogonopus speciosus</i>	X83662
Lamiaceae	<i>Scutellaria bolanderi</i>	L01954	Rubiaceae	<i>Psychotria kirkii (bacteriophila)</i>	X83663
Lamiaceae	<i>Salvia divinorum</i>	L14407	Rubiaceae	<i>Rachicallis americana</i>	X83664
Lobeliaceae	<i>Lobelia erinus</i>	L13930	Rubiaceae	<i>Rogiera suffrutescens</i>	X83665
Loganiaceae	<i>Spigelia marilandica</i>	L14007	Rubiaceae	<i>Rubia tinctorum</i>	X83666
Loganiaceae	<i>Strychnos nux-vomica</i>	L14410	Rubiaceae	<i>Sarcocephalus latifolius</i>	X83667
Menyanthaceae	<i>Menyanthes trifoliata</i>	L14006	Rubiaceae	<i>Sherardia arvensis</i>	X81106
Montiniaceae	<i>Montinia caryophyllacea</i>	L11194	Rubiaceae	<i>Theligonum cynocrambe</i>	X83668
Nyssaceae	<i>Nyssa ogeche</i>	L11228	Rubiaceae	<i>Uncaria rynchophylla</i>	X83669
Oleaceae	<i>Ligustrum vulgare</i>	L11686	Rubiaceae	<i>Valantia muralis</i>	X81107
Pedaliaceae	<i>Proboscidea louisianica</i>	L01946	Rubiaceae	<i>Vangueria madagascariensis</i>	X83670
Pittosporaceae	<i>Pittosporum japonicum</i>	L11202	Sambucaceae	<i>Sambucus racemosa</i>	L14066
Primulaceae	<i>Anagallis arvensis</i>	M88343	Sapotaceae	<i>Manilkara zapota</i>	L01932
Rubiaceae	<i>Anthospermum herbaceum</i>	X83623	Scrophulariaceae	<i>Antirrhinum majus</i>	L11688
Rubiaceae	<i>Antirhea lucida</i>	X83624	Scrophulariaceae	<i>Digitalis purpurea</i>	L01902
Rubiaceae	<i>Asperula inervigata</i>	X81092	Solanaceae	<i>Petunia hybrida</i>	X04976
Rubiaceae	<i>Bertiera breviflora</i>	X83625	Solanaceae	<i>Nicotiana tabacum</i>	Z00044
Rubiaceae	<i>Bouvardia glaberrima</i>	X83626	Stilbaceae	<i>Euthystachys abbreviata</i>	Z29671
Rubiaceae	<i>Calycophyllum candidissimum</i>	X83627	Stilbaceae	<i>Retzia capensis</i>	Z29669
Rubiaceae	<i>Catesbaea spinosa</i>	X83628	Valerianaceae	<i>Valeriana officinalis</i>	L13934

(1988) further elaborated on Bremekamp's two series within Rubioideae, and discerned one group including tribes with numerous ovules on each placenta (Hedyotideae and the associated tribes Ophiorrhizeae, Coccosypseleae, Argostemmataeae, Hamelieae, and Schradereae) and another group with

solitary ovules (Psychotriaceae and associated tribes Triainolepideae, Lathraeocarpeae, Morindeae, Coussareeae, Paederieae, Anthospermeae, Theligoneae, Spermaceae, and Rubieae). In earlier cladistic analyses of sequence data of Rubiaceae in general (Bremer et al. 1995) or in Rubioideae (Natali et al. 1995), a minority of the described Rubioideae tribes have been investigated, but, in spite of the limited sampling, some congruent conclusions can be drawn from the two analyses, which contradict some earlier opinions. In particular, the tribes Hillieae and Hamelieae do not belong to the Rubioideae and the two groups of taxa based on ovule numbers (Bremekamp 1966, Robbrecht 1988) do not form two monophyletic groups.

Starting from the hitherto presented information and classifications and using molecular data as well as recent morphological analyses five hierarchically organized questions are asked about the Rubiaceae and their phylogeny. 1) Is the family Rubiaceae well supported as monophyletic? 2) What is its position within the angiosperm system, or which are its closest relatives? 3) Can monophyletic subfamilies be recognized and what is the support for these? 4) Which tribes belong to the subfamily Rubioideae and how are these related? 5) Do changes in ovule number and fruit type represent unique events in the Rubioideae?

## 2 Material and methods

In the first analysis testing the monophyly and position of the Rubiaceae 82 sequences representing all major clades of the "higher" Asteridae taxa were represented (asterid I-III fide Chase et al. 1993), including 21 Rubiaceae genera from 19 tribes. The taxa were sampled to represent from asterid I: Solanales, Boraginales, Gentianales, Lamiales s.l., *Garrya* clade; from asterid II: Asterales s.l., Apiales, Dipsacales, *Ilex* clade; from asterid III: Ericalean clade; and as outgroup the genera *Nyssa* and *Cornus* representing asterid IV were used.

In the second analysis, 59 taxa of Rubioideae were included, representing 19 of the described tribes (table 1). The intention was to include as many tribes as possible, as yet material of several tribes are lacking. *Ophiorrhiza* and *Neurocalyx* were used as a functional outgroup; the topology obtained with these genera as sister group to the rest of the tree is identical to the topology achieved if the rest of the Rubiaceae taxa are included. The other taxa were excluded from this analysis in order to minimize computing time.

The two analyses are based on sequence data from the *rbcl* gene including 443 and 317 phylogenetically informative characters, respectively. The *rbcl* gene has been sequenced from 41 species (table 2); sequences are accessioned in EMBL as Z68787, to Z68827. The remaining 114 sequences included in the analyses have been extracted from EMBL/GenBank databases (table 3).

DNA was extracted, amplified, and sequenced following the protocols in Bremer et al. (1995). The *rbcl* data matrices in the phylogenetic analyses comprise characters corresponding to each nucleotide position (27 to 1428, positions 1-26 are excluded as they are identical to one of the primers) of the *rbcl* sequence.

Parsimony analyses were conducted using PAUP version 3.1.1 (Swofford 1993) on a PowerMac 8100/80, with all character changes weighted equally. Only phylogenetically informative characters were included. The methods for the searches were heuristic, with random stepwise addition of sequences and 100 replications, and TBR branch swapping with MULPARS on and Steepest descent off. To estimate the support for each clade Bremer's branch support ( $b$  = the extra length needed to lose a branch in a consensus of near-most parsimonious trees; K. Bremer 1988) and bootstrap fractions (with 1000 replicates; Felsenstein 1985) were calculated.

## 3 Results

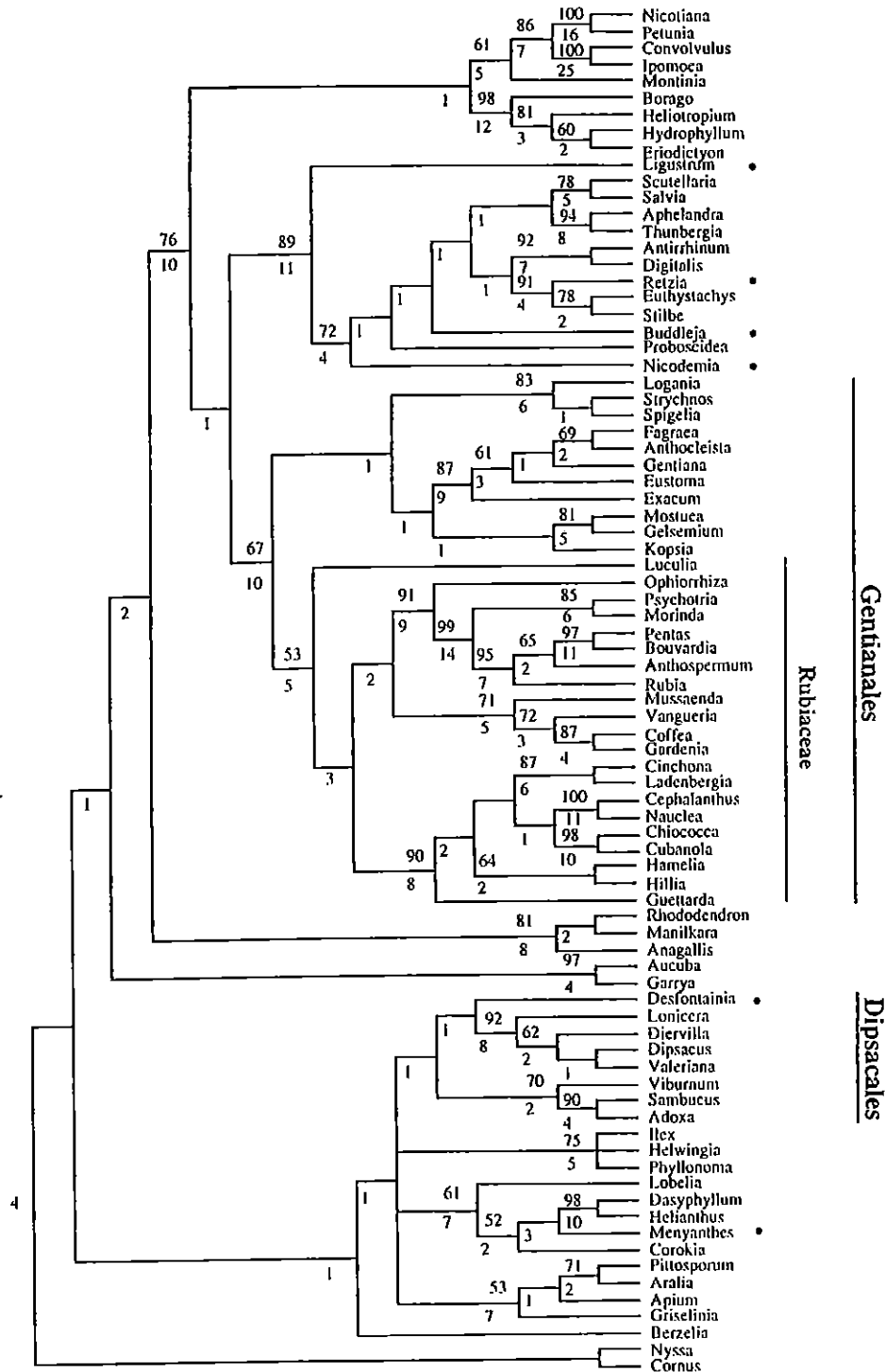
In the first analysis of 82 taxa, concerning the position and monophyly of the Rubiaceae (fig. 1), 676 nucleotide positions were variable and of these 443 were phylogenetically informative. The heuristic search with 100 random addition sequences including only the phylogenetically informative characters resulted in 2 equally parsimonious trees 2332 steps long (minimal possible steps 672) with a consistency index (ci) of 0.288 and a retention index (ri) of 0.578.

In the second analysis of 59 taxa, concerning the subfamily Rubioideae (figs. 2 & 3), 527 nucleotide positions were variable and of these 317 were phylogenetically informative. The heuristic search with 100 random addition sequences including only the phylogenetically informative characters resulted in 108 equally parsimonious trees 1108 steps long (minimal possible steps 429) with a consistency index (ci) of 0.387 and a retention index (ri) of 0.697.

Of all the nucleotide substitutions that have occurred only a few resulted in synapomorphic and non-homoplastic changes in amino acid composition. These unique changes in amino acids all occurred in subfamily Rubioideae (fig. 2). *Theligonum* and all genera of Rubieae share a change from 2Serine to

Glycine (nucleotides 28-30). All taxa of the *Pentas* group (fig. 2; *Carphalea*, *Parapentas*, *Pentanisia*, *Pentas*, and *Placopoda*; further discussed under 4.4.1) share a change from Valine to 2Serine (nucleotides 469-471). All taxa of Spermaceae s.l. share a unique amino acid change from Glutamine to Glutamic acid (nucleotides 445-447). All taxa of the Anthospermeae share a change from Methionine to Isoleucine (nucleotides 925-927) and have Threonine at the nucleotide positions 1445-1447.

Fig. 1.  
Strict consensus tree of two equally parsimonious trees illustrating the position and monophyly of Rubiaceae based on rbcL sequences. Black dots indicate taxa earlier included in the Gentianales. Numbers below branches indicate number of extra steps required to collapse the particular node. Numbers above nodes indicate bootstrap fractions above 50%.



## 4 Discussion

The discussion of the first two questions, concerning monophyly and the position of Rubiaceae is based on the first analysis (fig. 1), of the "higher" Asteridae. The resolution of the phylogenies in other parts of the Asteridae has been dealt with elsewhere, and will not be addressed here (cf. Olmstead et al. 1993, Gustafsson et al. 1996).

### 4.1 Rubiaceae – a monophyletic family

Although recent cladistic analyses of angiosperms and of the Rubiaceae in particular have included some phylogenetic conclusions concerning this family, the monophyly of Rubiaceae has not been demonstrated. In earlier analyses of the Rubiaceae (Bremer & Jansen 1991, Bremer & Struwe 1992, Bremer et al. 1995) the monophyly of the study group (in-group) has been taken for granted and with only a small outgroup monophyly can not be adequately tested; the outgroup is used only to root the resulting tree(s). To establish that a family is monophyletic a much larger sampling of taxa outside the study group must be included in the analysis. On the other hand, in analyses of large data sets representing many families of the angiosperms, the Rubiaceae have been represented by a single genus or too few genera (Downie & Palmer 1992, Chase et al. 1993, Olmstead et al. 1993, Bremer et al. 1994, Struwe et al. 1994).

In the present study 21 Rubiaceae genera representing various parts of the family were analyzed together with representatives of all major clades of the "higher" Asteridae taxa including, e.g., several Gentianales and Dipsacales. Although the analysis resulted in two equally parsimonious trees, with respect to the monophyly and the phylogeny of Rubiaceae these are identical in corroborating the phylogenetic hypothesis that the Rubiaceae are monophyletic.

### 4.2 The position of the Rubiaceae within the angiosperms – Gentianales

The present analysis corroborates the hypothesis advocated by Uetzschneider (1947), Wagenitz (1959), and most modern taxonomists, that the taxonomic position of the Rubiaceae is close to or within Gentianales and not close to Dipsacales. It also confirms, as shown by earlier analyses (Downie & Palmer 1992, Olmstead et al. 1993, Chase et al. 1993, Bremer et al. 1994), that Cronquist (1981) was wrong in his speculation that the Rubiaceae represent a link between Dipsacales, and Gentianales and Asterales. Another former putative "link" between Gentianales and Dipsacales was *Desfontainia*. The genus was placed in Loganiaceae (Gentianales) by, e.g., Lecuwenberg & Leenhouts (1980) but its position has long been questioned and a recent molecular investigation (Bremer et al. 1994) has shown that it is closer to Dipsacales and not included the Gentianales.

The hypothesized relationships within the Gentianales were rather diffusely expressed before phylogenetic analyses were performed, as illustrated by the sentence: "As with the tribes of Loganiaceae, the relationships between the families of the Gentianales are reticulate" (Leeuwenberg & Leenhouts 1980). Although the focus has been on issues other than relationships of the Gentianales, several recent phylogenetic analyses of molecular data of Asteridae have changed this view of the order. New relationships have been presented or earlier ideas have been confirmed, e.g., the Menyanthaceae are not close to the Gentianaceae (Downie & Palmer 1992, Olmstead et al. 1992) and the tribe Potalieae (e.g., *Anthoclesita*, *Fagraea*) of the Loganiaceae is a part of Gentianaceae (Olmstead et al. 1993). In several analyses with focus on the Loganiaceae, both morphological (Bremer & Struwe 1992, Struwe et al. 1994) and molecular (Bremer et al. 1994), new hypotheses about the relationships and circumscription of the Gentianales have been put forward: the Loganiaceae are paraphyletic (Olmstead et al. 1993, Bremer et al. 1994) and several genera earlier associated with the Loganiaceae are not parts of the Gentianales at all (Bremer et al. 1994, Struwe et al. 1994).

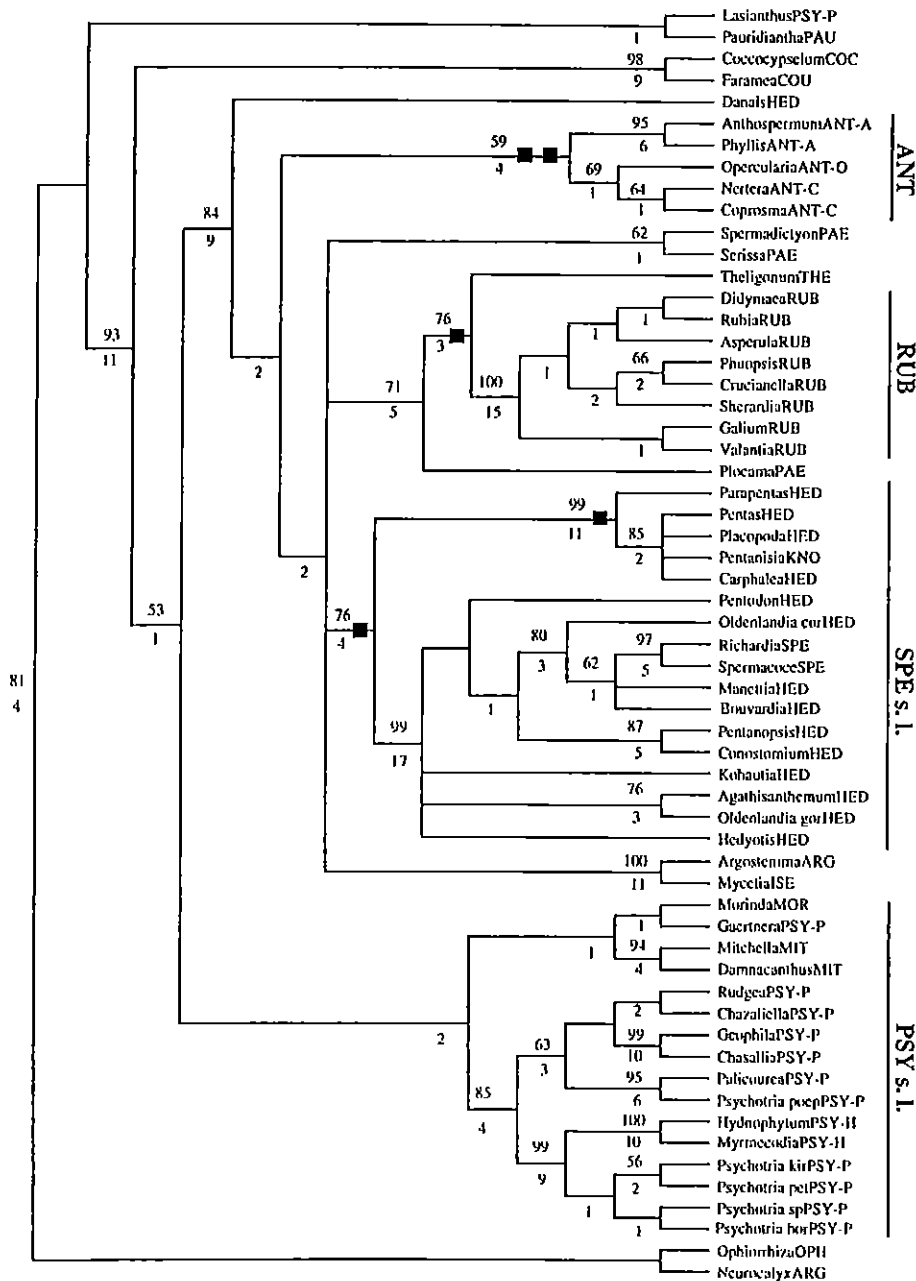
So far the most detailed morphological analyses (Struwe et al. 1994) of the Gentianales resulted in trees where the Rubiaceae are nested within Gentianales with *Gelsemium* + *Mostuea* as the sister group (also supported in an earlier morphological analysis by Bremer & Struwe 1992). However, the position of the Rubiaceae as nested within the Gentianales is contradicted by molecular investigations. In almost all investigations the Rubiaceae are the sister group to the rest of the Gentianales (Downie & Palmer 1992, Olmstead et al. 1993, Bremer et al. 1994). The present analysis also places the Rubiaceae



as the sister group to the rest of the Gentianales. The different result in the morphological analyses might be a result of high sensitivity to uneven sampling of taxa (Rubiaceae are represented by a single taxon or only three taxa, respectively) combined with the relatively small number of morphological characters as compared to the molecular data sets.

In Struwe et al. (1994) the former Loganiaceae are separated into four different families (Loganiaceae, Strychnaceae, Gelsemiaceae, and Geniostomaceae). The present study provides no support for the separation of Strychnaceae from the Loganiaceae as suggested there.

Fig. 2.  
 Strict consensus tree of  
 108 equally  
 parsimonious trees of  
 subfamily Rubioideae  
 based on rbcL  
 sequences.  
 Tribal (subtribal)  
 positions are indicated  
 by a three(four)-letter  
 suffix corresponding to  
 the tribes (subtribes) in  
 Robbrecht (1988, 1993).  
 Vertical bars and  
 corresponding letters  
 represent  
 ANT = Anthospermeae,  
 RUB = Rubioideae,  
 SPE s.l. =  
 Spermaceae s.l.,  
 PSY s.l. = Psychotriaceae  
 + Morindeae + the  
 Mitchella group.  
 Black squares indicate  
 synapomorphic and  
 non-homoplasic  
 changes in amino acid  
 composition (c.i. = 1.0).  
 Numbers below  
 branches indicate  
 number of extra steps  
 required to collapse the  
 particular node.  
 Numbers above nodes  
 indicate bootstrap  
 fractions above 50%.



4.3 Support for the three subfamilies: Rubioideae, Cinchonoideae s.str., and Ixoroideae. Classification is an ongoing process where new circumscriptions are made based upon the interpretation of newly available information. Today most systematists agree that new classifications should be based

on phylogenetic analyses and new groupings should be supported by relevant data. At present, the easiest way (perhaps not the cheapest) of obtaining data for a phylogenetic analysis is to sequence a piece of DNA. But should a new phylogenetic relationship shown by molecular data always result in a changed classification? I think not. If the discussion is limited to the division of subfamilies, it is important that the identified (circumscribed) and named subfamilies can be recognized by morphological diagnostic characters, for otherwise the division into subfamilies will be useless. So what is the situation within Rubiaceae? At this time relatively few (ca. 90) genera of Rubiaceae have been cladistically analyzed in a context comprising the whole family, where questions about subfamilial classification were or could be asked (Bremer & Jansen 1991, Bremer & Struwe 1992, Bremer et al. 1995, Bremer 1996, and in the present paper). It is definitely too early to present a revised subfamilial classification, but what is the support for the earlier presented subfamilies (Bremekamp 1952, 1954, 1966, Verdcourt 1958, Robbrecht 1988, 1993)?

The subfamily Rubioideae is strongly supported in the present study and in the various earlier published analyses (Bremer & Jansen 1991, Bremer & Struwe 1992, Bremer et al. 1995, Bremer 1996); the analyses are in most parts congruent. All support a monophyletic unit more or less identical to the Rubioideae (but excluding the tribe Hamelieae). The molecular data in the present analysis and in Bremer et al. (1995) also definitely show that the tribes Theligoneae (also proposed by Robbrecht 1988, Natali et al. 1995), Pauridiantheae (subfamily Cinchonoideae fide Robbrecht 1988), and *Ophiorrhiza* (excluded from the Rubioideae by Natali et al. 1995) are part of the subfamily Rubioideae. This group is highly supported by the molecular data (bootstrap fraction of 91% and a branch support value of 9; fig. 1), and is probably the most easily recognized subfamily based on diagnostic morphological characters. A typical Rubioideae species is characterized by a unique combination of characters, not found outside Rubioideae, e.g., herbaceous habit, raphides, valvate aestivation (probably the plesiomorphic state), and articulate hairs (however, many species are woody and not all have the articulate hairs). In a recent molecular study of the tribe Rubieae (Natali et al. 1995), a unique molecular marker (a 204 bp deletion in the *atpB* leader sequence) was found in the investigated taxa of the Rubioideae tribes Rubieae, Theligoneae, Anthospermeae, Hedyotideae, Spermacoceae, Psychotriaceae, Coccocypseleae, but not in the tribe Ophiorrhizeae. Natali et al. conclude that the tribe Ophiorrhizeae is not a part of the subfamily Rubioideae. I do not agree. In all analyses, including the one presented by Natali et al., Ophiorrhizeae are the sister group to the rest of the subfamily Rubioideae (*Ophiorrhiza* is the most basal taxon in the clade). This means that *Ophiorrhiza* cannot be included in any other of the existing subfamilies because it is more distantly related to those and if it is not included in the subfamily Rubioideae, it must be raised to a separate subfamily. Monotribal subfamilies should be avoided, if possible, as these will not simplify classification. Also, it would be very difficult to find any useful diagnostic characters for such a subfamily; the set of characters for Rubioideae excluding Ophiorrhizeae will not be unique as most characters typical for Rubioideae occur also in the Ophiorrhizeae, such as herbaceous habit, raphides, and valvate aestivation.

The subfamily Cinchonoideae s.str. (excluding *Mussaenda*, *Pogonopus*, *Pinckneya*, and *Calyco-phyllum*, and the tribes Pauridiantheae, but including the tribes Guettardeae, Chiococceae, and Hamelieae) is strongly supported by molecular data (90% bootstrap fraction and a branch support value of 8 in this study, 80% and 7, respectively, in Bremer 1996; 78% and 2, respectively, in Bremer et al. 1995). A typical member of the subfamily is woody with capsular fruits and entire stipules, but these characters are not unique nor do they occur in all taxa. The only unique character seems to be the imbricate aestivation (becomes unique to this subfamily when the following tribes are included: Hamelieae, Chiococceae, Cephalantheae, Guettardeae); however, not all taxa are imbricate, a few are valvate and in one of the clades, including Hamelieae and Hillieae, several species are right-contorted. Furthermore, the Cinchonoideae s.str. are supported by the occurrence of specific complex indole alkaloids (CIA; containing two structure elements, tryptamine/tryptophan with an indole nucleus and a C9- or C10-monoterpene moiety derived from secologanin); these CIAs are classified into different subgroups, three of which have been detected in Rubiaceae (cf. Kisakürek et al. 1983, Jensen 1992). The corynanthean and vallesiachotaman types have been found only in taxa belonging to Cinchonoideae s.str. and not in, e.g., *Pauridiantha*.

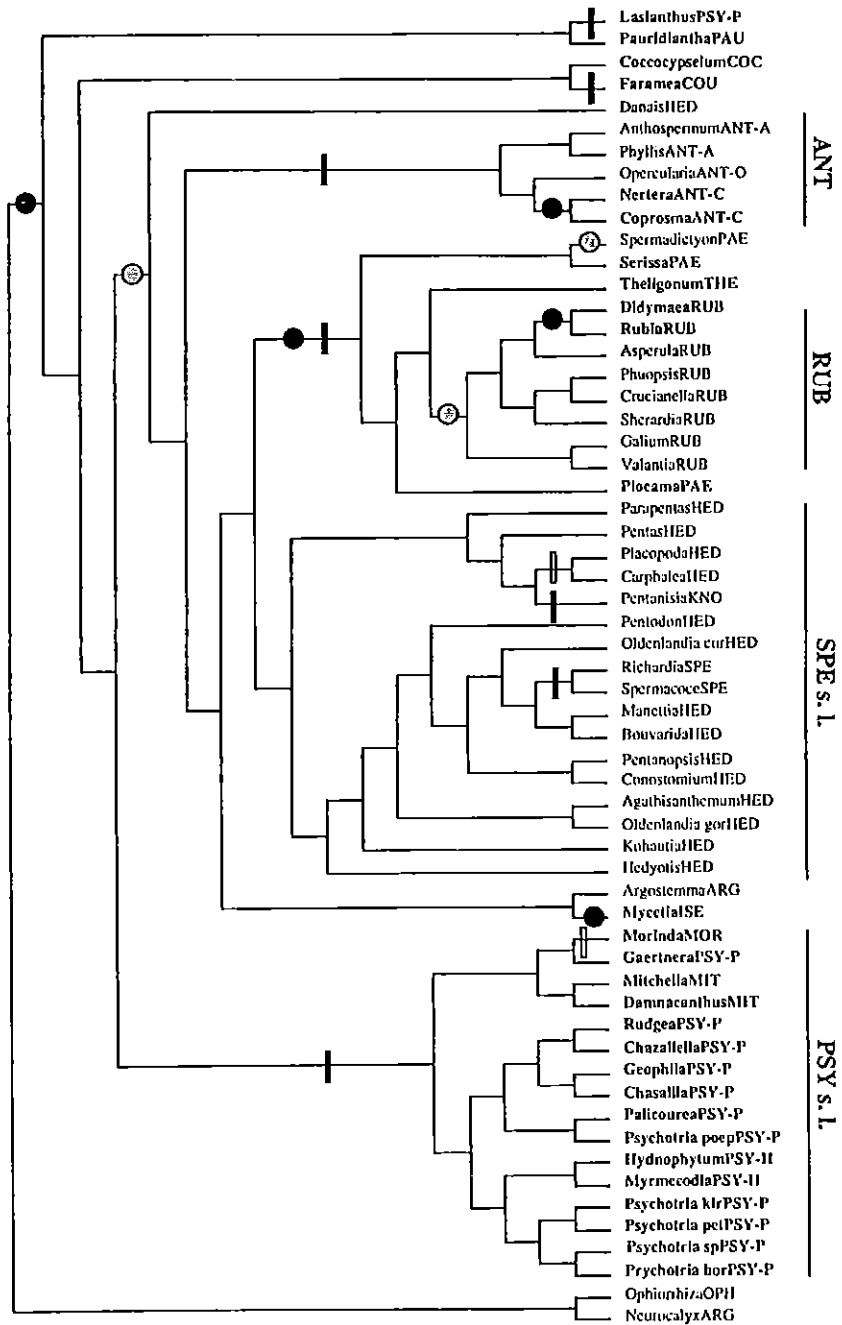
The Ixoroideae are supported as a monophyletic subfamily, but the delimitation is problematic. Bremekamp (1952, 1966) established the subfamily and included, as he said, all tribes with secondary pollen presentation, e.g., Gardenieae, Ixoreae, Cremasporae, and Vanguerieae, but he excluded the Naucleaeae. Later Robbrecht (1988) restricted the subfamily to include only the tribes with contorted (mainly left) aestivation, namely the relatives of the Gardenieae (Gardenieae, Pavetteae, Coffeae, Aulacocalyceae, and Hypobathreae), and, e.g., the Vanguerieae, with valvate aestivation, were moved to the Antirheoideae. All molecular analyses performed have shown that the Vanguerieae and the Ixoroideae s.str. are closest relatives (Bremer & Jansen 1991, Bremer et al. 1995, Bremer 1996, Andreasen & Bremer 1996). In the morphological analyses the position of Vanguerieae has differed; in the first study (Bremer & Struwe 1992) the Vanguerieae were placed close to Naucleaeae but in the later analysis (Bremer 1996) *Vangueria* was placed close to the Ixoroideae s.str. In both analyses the pollen presentation mechanism was coded as being the same in Ixoroideae s.str., Vanguerieae, and in the Naucleaeae although there is information showing that the secondary pollen presentation mechanism is much more complex and diverse (Skottsberg 1945, Nilsson et al. 1990, Imbert & Richards 1993, Puff et al. 1996). There seems to be a difference between species where pollen is deposited exclusively on the receptive surface of the stigma (Naucleaeae s.l.) and species where pollen is deposited on the outside of the stigma and/or style but not on the receptive surface (e.g. Vanguerieae and Ixoroideae s.str.; Igersheim 1993, Puff et al. 1996). This should further support the closeness of Vanguerieae to the Ixoroideae and not to the Naucleaeae. According to the molecular analyses there are other taxa from the tribes Isertieae, Condamineae, and Cinchoneae that seem to be closely related to the Ixoroideae, e.g., *Mussaenda*, *Pogonopus*, *Pinckneya*, and *Calycophyllum*, but so far no morphological data support inclusion of these taxa in the subfamily Ixoroideae.

The Antirheoideae were a monotribal subfamily (as Guettardoideae; Verdcourt 1958, Bremekamp 1966) before Robbrecht (1988) widened the circumscription to include not only the Guettardeae but also the tribes Retiniphyllae, Vanguerieae, Chiococceae, Alberteae, Cephalantheae, and Craterispermeae, and Knoxieae. The subfamily Antirheoideae as circumscribed by Verdcourt (1958) and Bremekamp (1966) is monophyletic but is nested within the subfamily Cinchonoideae. If it should be maintained as a separate subfamily, the Cinchonoideae become paraphyletic and must be split into several subfamilies without any morphological characters that could support these. Furthermore, the wide circumscription of Antirheoideae (Robbrecht 1988) is contradicted by all analyses (Bremer & Jansen 1991, Bremer & Struwe 1992, Bremer et al. 1995, Bremer 1996, and the present analysis) and according to these, Chiococceae and Cephalantheae are nested within the Cinchonoideae but not close to the Guettardeae or to each other. The Vanguerieae (discussed above and in Andreasen & Bremer 1996) and Alberteae (unpublished) are close to the Ixoroideae, the Knoxieae belong to subfamily Rubioideae (see below), and the remaining tribes, Retiniphyllae and Craterispermeae, have not been analyzed cladistically. The subfamily Antirheoideae can simply not be maintained.

#### 4.4 Phylogeny of the Rubioideae

**4.4.1 Rubioideae tribes and how they are related.** A core of Rubioideae tribes (table 1; Anthospermeae, Argostemmatae, Coccocypseae, Coussareae, Hamelieae, Hedyotideae, Morindeae, Paederieae, Psychotrieae, Rubieae, Schradereae, and Spermacoceae) has been accepted as a monophyletic group by most authors starting with Bremekamp (1952, 1954, 1966) and Verdcourt (1958). The present and earlier molecular studies (cf. Bremer et al. 1995, Natali et al. 1995) support this with the exception of the position of Hamelieae which has been shown to be part of the Cinchonoideae. The Schradereae have not been investigated. The molecular analyses also support the proposed inclusion of Theligoneae (Wunderlich 1971, Robbrecht 1988), and the exclusion of Hillieae (Robbrecht 1988). Of the tribes outside the core of described tribes only a few have been investigated molecularly, but the present analyses show that the Ophiorrhizeae, Pauridiantheae, and Knoxieae are part of Rubioideae, arrangements that have been questioned by some (Ophiorrhizeae by Bremekamp 1966, Natali et al. 1995; Pauridiantheae by Bremekamp 1966, Robbrecht 1988). The genera *Manettia* and *Bouvarida* (Manettieae) as well as *Danais* have been proposed to form links between Cinchoneae and Hedyotideae (Robbrecht 1988, but this is contradicted by the molecular data which show that these genera are nested within the Rubioideae).

Fig. 3.  
Sample cladogram, one of 108 equally parsimonious cladograms of subfamily Rubioideae based on rbcL sequences.  
Origins of fleshy fruits are indicated with black dots, origins of dry fruits are indicated with shaded dots, origins of ovule reduction to one ovule per carpel are indicated with black bars, and origin of few ovules per carpel is indicated with a white bar.  
All taxa with fleshy fruits are indicated with names in boldface.



In the following I will discuss the monophyletic groups identified in this study (figs. 2 & 3). Four larger clades can be identified, representing Anthospermeae, Rubieae, Spermaceae s.l. and Psychotriaceae s.l. Of these the Anthospermeae, Rubieae and Spermaceae s.l. are closely related. Some of the shown phylogenetic relationships are totally new and others support earlier proposed relationships.

Starting from the base of the tree (figs. 2 & 3), the two most basal clades are *Ophiorrhiza* + *Neurocalyx* and *Lasianthus* + *Pauridiantha*. None of these clades are supported by high bootstrap or branch support values and we should not put too much confidence in the relationships, but it is interesting to note that *Neurocalyx* (Argostemmaeae) is not close to *Argostemma* (see under *Argostem-*

*ma*). It is also interesting to note that *Lasianthus* (Psychotriaceae) does not come together with *Psychotria* or *Morinda* but with *Pauridiantha*. There are morphological similarities between *Lasianthus* and *Pauridiantha*, even if they differ in fruit type and color. The similarity in habit, flower shape, and inflorescences was also pointed out by Verdcourt (1958). A totally new, highly supported relationship is that between the drupaceous genus *Faramaea* (Coussareeae) and the baccate genus *Coccocypselum* (Coccocypseae). *Faramaea* is part of the Coussareeae and that tribe has always been connected to the Psychotriaceae.

The next split in the tree is between Psychotriaceae s.l. (Psychotriaceae + Morindeae + *Mitchella* group) and the rest of the subfamily. The relationship between Morindeae and Psychotriaceae is accepted by most systematists. In this analysis, *Morinda* together with *Gaertnera* and the *Mitchella* group is sister to the Psychotriaceae. Interestingly, *Gaertnera* is close to *Morinda* and not to *Psychotria* as usually suggested (e.g. Robbrecht 1988); note that it has recently been demonstrated that *Gaertnera* has wood anatomical similarities to *Morinda* (Jansen et al. 1996). This grouping needs more investigation as the support values in the DNA analysis are low; also, the delimitation of the Morindeae has been subject to much recent change (see Igersheim & Robbrecht 1993 for a survey of the segregation of the Prismatomerideae and the *Mitchella* group) and more taxa should be sequenced. A highly supported relationship is found between *Mitchella* and *Damnacanthus* and this relationship was first pointed out by Robbrecht et al. (1991). Furthermore, this analysis can also confirm that the relationship between the different species of *Psychotria* and other genera of the tribe Psychotriaceae is very complicated and needs much attention to be resolved. It is clear that the genus *Psychotria* is paraphyletic, e.g., *P. poeppigiana* is sister to a *Palicourea* species, and these are more closely related to *Chasallia*, *Geophila*, *Chazaliella*, and *Rudgea* than to the other investigated *Psychotria* species. Furthermore, the two genera *Hydnophytum* and *Myrmecodia*, both epiphytic, succulent myrmecophilous plants, are closely related and have also, together with three other genera, been described as a separate subtribe Hydnophytineae (Huxley & Jebb 1991). However, this group is closely related to only part of the Psychotriaceae (actually close to some *Psychotria* species) and the subtribe cannot be accepted unless the genus *Psychotria* is split into different subtribes as well.

The branch including the rest of the subfamily is a strongly supported clade including a majority of the dry-fruited taxa. At the most basal dichotomy in this clade we find the genus *Danais*. It is one of the genera with winged seeds that Bremekamp (1952) transferred from the Cinchoneae to the Hedyotideae, a position that was accepted by Verdcourt (1958). However, Robbrecht (1988) listed it under Cinchoneae and in the index with both tribes indicated; he is of the opinion that the Hedyotideae are a link between the Rubioideae and Cinchonoideae. In a detailed study of *Danais* and the closely related genera *Schismatoclada* and *Payera* (Buchner & Puff 1993) this group of taxa is proposed to occupy a "grey zone" in between the subfamilies. The present analysis clearly shows that *Danais* belongs to the Rubioideae, and is basal in the subfamily and not closest to the Hedyotideae.

The next clade in the tree (figs. 2 & 3) corresponds to the tribe Anthospermeae. So far only five genera of fourteen have been investigated but the branching of the tree is congruent with the division of the tribe into three subtribes (Anthosperminae, Coprosmineae and Operculariinae) by Puff (1982). The clade is supported by two unique amino acid changes; all taxa share a change from Methionine to Isoleucine (nucleotides 925-927) and all taxa have Threonine at the nucleotide positions 1445-1447.

One of the best supported branches, with a bootstrap fraction of 100%, indicates a totally new and unexpected relationship between *Argostemma* and *Mycetia*. *Argostemma* is the type genus of the tribe Argostemmatae, characterized by adnate anthers, a character that was supposed to be an almost unique apomorphy for the group. The proposed relationship of Argostemmatae to other tribes is interesting. "Argostemmatae" was identified by Bremekamp (1952, validly published by Verdcourt 1958). Bremekamp (1966) included both the here investigated genera *Argostemma* and *Neurocalyx* in the tribe. The closeness of *Argostemma* and *Neurocalyx* has never really been questioned. Bremekamp (1966) was doubtful about the section *Thyrsoideae* of *Neurocalyx*, but that part has later been described as a separate genus (*Steenisia*, Bakhuizen van den Brink 1952) and moved to the tribe Rondeletieae (Bremer 1984). In an early cladistic study of the Argostemmatae (Bremer 1987) all Rubioideae genera were "screened" for useful characters that could be used to find the sister group to Argostemmatae. Based on the false assumption (first showed by Bremer & Jansen 1991) that

Hamelieae are part of the Rubioideae, the conclusion in the analysis was that Argostemmatae and Hamelieae are closely related. However, the present analysis contradicts that *Neurocalyx* and *Argostemma* are closely related, although both belong to Rubioideae. *Argostemma*, a genus with capsular fruits, is here the sister group to *Mycetia*, a genus with baccate fruits; however, it should be noticed that the *Argostemma* capsules are operculate, often succulent and very different from Hedyotideae capsules. The genus *Mycetia* was included in Isertieae (Mussaendeae) by Schumann (1891) and Robbrecht (1988, 1993) but as *Mycetia* possesses raphides, it was transferred to "a somewhat isolated position in the Hedyotideae" by Bremekamp (1952), a position accepted by Verdcourt (1958).

The tribe Rubieae is the other clade supported by a 100% bootstrap fraction. The strong support for this group is congruent with the result of Natali et al. (1995) and the present study does not add much compared to their study. However, one interesting genus, associated with the Rubieae in this analysis is *Didymaea*. The genus comes close to *Rubia*, a position proposed before (cf. Verdcourt 1958), although questioned, as *Didymaea* differs from the Rubieae by having ordinary opposite leaves with well developed stipules and seeds which do not adhere to the pericarp (Bremekamp 1966). *Rubia* and *Didymaea* both have a fleshy pericarp. At the base of the Rubieae we find *Theligonum* (a position already indicated by Bremer et al. 1995 and Natali et al. 1995). The close relationship of the Rubieae and Theligoneae is supported by one unique change in amino acid composition; all genera share a change from 2Serine to Glycine (nucleotides 28-30). Also at the base of the Rubieae one finds at least parts of the Paederieae.

The tribe Paederieae was in Schumann's classification (1891) distinguished from the Psychotrieae by the long, enlarged stigma lobes. Bremekamp (1966) accepted the tribe but was critical to it: "In its present delimitation this tribe makes a rather unnatural impression". Later Puff (1982), who was of the opinion that this tribe is close to Theligoneae and Anthospermeae, transferred all insect-pollinated genera from Anthospermeae to the Paederieae. Today the tribe includes about 15 genera (Robbrecht 1988) but according to the tribal description it is difficult to identify characters that unite all taxa as most characters are variable. Four of the genera have been investigated molecularly, three in this study (*Plocama*, *Serissa*, and *Spermadictyon*) and one (*Putoria*) in Natali et al. (1995). Both analyses indicate that the taxa are closely related to the Rubieae and Theligoneae but not to Anthospermeae. In the present study there is no support for a natural group corresponding to the Paederieae; instead they constitute a so called grade (fig. 3). *Spermadictyon* is the sister group to *Serissa* and these together are the sister to *Plocama*, Theligoneae and Rubieae together. We have tried to sequence *Paederia* but not managed to get a complete sequence; however, the incomplete sequence (unpublished) attaches to the same unresolved node as *Serissa* and *Spermadictyon* in the consensus tree (fig. 2). This part of the tree has very little support and the *rbcL* gene is apparently not informative enough to clarify the status of the Paederieae.

The remaining taxa belong to a clade (figs. 2 & 3) supported by a bootstrap fraction of 76% and a branch support value of 4; there is a unique amino acid change for all taxa from Glutamine to Glutamic acid (nucleotides 445-447). This clade (here called SPE s.l.) includes all investigated taxa of the described tribes Hedyotideae (excluding *Danais* as noted above), Manettieae, Knoxieae, and Spermaceae. None of these tribes can be accepted as usually circumscribed. Within this group of taxa there is instead a deep basal split into two highly supported branches (99% bootstrap fractions and branch support values of 11 and 17, respectively), one representing the *Pentas* group and the other including the rest of the clade, here called the *Hedyotis/Spermaceae* group. These two groups were initially identified by morphological characters (Bremer 1987) and supported by molecular data (Bremer et al. 1995). The *Pentas* group is here further supported by a unique amino acid change from Valine to 2Serine (nucleotides 469-471). The *Pentas* group includes also *Parapentas*, *Placopoda*, *Carphalea*, and *Pentanisia*. The last genus is a part of the tribe Knoxieae which by Robbrecht (1988) has been transferred to the subfamily Antirrhoideae. If *Pentanisia* is a representative of the Knoxieae that placement was obviously wrong. One of the characters for Knoxieae is solitary pendulous ovules, but at least ovule reduction occurs several times in this part of the Rubioideae, also in the sister taxa *Placopoda* and *Carphalea*, both genera, however, with erect ovules. In the *Hedyotis/Spermaceae* group one finds many of the genera with many ovules and wing-less seeds, e.g., *Hedyotis*, *Oldenlandia*, *Kohautia*, and *Pentodon*, but also genera with many ovules and winged seeds earlier included in

Manettieae (*Bouvardia* and *Manettia*) as well as the genera of the tribe Spermaceae with solitary ovules. Interestingly but not unexpectedly the genus *Oldenlandia* is paraphyletic. The type species of the genus (*O. corymbosa*, subgenus *Oldenlandia*) is close to *Richardia*, *Spermaceae*, *Manettia*, and *Bouvardia*, while another species (*O. goreensis*, subgenus *Anotidopsis* sensu Bremekamp 1952) is closer to *Agathisanthemum*. The position of Spermaceae within Hedyotideae (pointed out by Bremer et al. 1995 and Natali et al. 1995) makes the Hedyotideae paraphyletic. To keep the tribe Spermaceae in its present restricted sense, one has to split Hedyotideae in many small tribes, but for such splitting there are no diagnostic characters, so the best approach is to unite all taxa into one tribe. One superficial problem with this solution is that the oldest name, which must be applied, is Spermaceae and the name refers to a seed with a sharp point, a character that is not relevant for most of the included taxa.

**Spermaceae** A. Richard ex Dumortier, *Analyse fam. plantes*: 33 (1829).

*Hedyotideae* Chamisso & Schlechtendal ex de Candolle, *Prodr. syst. nat. regni veg.* 4: 343, 401 (1830).

? *Knoxieae* Hooker f., in Bentham & Hooker, *Gen. plant.* 2: 9, 21 (1873).

*Manettieae* Bremekamp, *Rec. Trav. Bot. Neerl.* 31: 253 (1934).

Plants mostly herbaceous to subwoody, more rarely woody (a few species are small trees, cf. *Carphalea*; Puff 1988). Stipules interpetiolar, fimbriate. Raphides present. Corolla lobes valvate. Ovary usually 2-locular, with many to single ovules in each locule. Fruits dry, dehiscent or indehiscent, with many to solitary seeds. Flowers quite often heterostylous.

*Agathisanthemum*, *Bouvardia*, *Carphalea*, *Conostomium*, *Hedyotis*, *Kohautia*, *Manettia*, *Oldenlandia*, *Parapentas*, *Pentanisia*, *Pentanopsis*, *Pentas*, *Pentodon*, *Placopoda*, *Richardia*, *Spermaceae*.

A majority of the former Hedyotideae and Knoxieae genera should be included in this tribe but so far I have sequenced only the genera listed above. However, the morphology clearly supports the inclusion of at least the following genera: *Arcytophyllum*, *Diodia*, *Hemidiodia*, *Houstonia*, *Kadua*, *Knoxia*, *Lelya*, *Lucya*, *Manostachya*, *Neohymenopogon*, *Otomeria*, *Synaptantha*, *Thecorchus*.

**4.4.2 Changes in ovule number and fruit type do not represent unique events in the Rubioideae.**

Most systematists try to identify natural groups more or less in the spirit of one of Linnaeus' principles, "that a genus should furnish a character, not a character form a genus" (translation by Stearn 1960 in the preface to the facsimile edition of the *Genera Plantarum*). Nevertheless, many systematists still start to define markers or cardinal characters before the natural groups have been identified. Groups should be identified from phylogenies representing the most parsimonious solution considering all known characters, and the usefulness of a character as diagnostic can not be known a priori. Characters are useful when they are congruent with the phylogeny and when the specific hierarchic level is found where the character is informative.

In Rubiaceae ovule numbers and dry or fleshy fruits are well known cardinal characters and Schumann's classification (1891) was entirely based on these. However, later authors have strongly criticized the use of these characters, e.g., Bremekamp (1966) wrote: "the presence either of uni- or of pluriovular ovary cells, their kind of fruits, which to this end were divided in dry or fleshy ones ... even a superficial study of the groups that have these ... will show that most of them are entirely unnatural." For subfamilial classification Bremekamp totally rejected this kind of characters but within Rubioideae he, although not explicitly stated, grouped the genera after these characters. Later, Robbrecht (1988) in the evaluation of Bremekamp's Rubioideae identified two groups based on ovule numbers and he also stated "(ii) tribes with solitary ovules (numerous tribes, inter alia Psychotrieae and associated tribes)" ... "Group (ii) is surely a natural one"; this statement must be treated as a hypothesis of a single character evolution in an ancestor of a monophyletic group.

From this study of *rbcL* data compared to the distribution of solitary or many ovules (fig. 3), the hypothesis of a single evolutionary origin of the solitary ovule condition can be falsified. The most parsimonious optimization (same optimization with acctran or deltran, Swofford & Maddison 1987)

on the cladograms shows that the plesiomorphic or basal condition in the subfamily is a many-ovuled ovary and that solitary ovules have evolved at least seven times in the investigated taxa of the Rubioideae. Interestingly, within the Rubioideae there is not a single change from solitary ovules to many ovules. As the reduction of ovule number has happened several times, it is obvious that the ovule number is not a character that can be used to split the subfamily into two monophyletic halves. However, the character is informative at a lower level and certain groups can be characterized by this character together with other characters. There are single genera (*Lasianthus*, *Faramea*, and *Pentania*) or whole tribes or group of tribes with solitary ovules, e.g., Psychotrieae s.l. (with the exceptions of *Morinda* with two ovules), Rubieae + Theligoneae + the genera of the paraphyletic "Paederieae", Anthospermeae, and the Spermaceae s.str. In one case, in the *Pentas* group, we have ovule reduction both to few (*Placopoda* and *Carphalea*) and to solitary ovules (*Pentania*).

If the dry versus fleshy fruit character is optimized (fig. 3), the basal condition in the subfamily is a dry fruit, and fleshy fruits have evolved at least five times (cf. Eriksson & Bremer 1991, Bremer & Eriksson 1992). Compared to the ovule number this character is more plastic, and there are at least two reversal events, back to a dry fruit, and in one case also a subsequent change back to fleshy. If the co-occurrence of the two characters, number of ovules and dry versus fleshy fruits, is considered, we find that within evolutionary lines with solitary ovules in dry fruits, changes to fleshy fruits occur, and within lines with solitary ovules in fleshy fruits, changes to dry fruits occur, but in Rubioideae we have no case where many ovules in dry fruits have changed to fleshy fruits. As the dry versus fleshy fruit condition is more plastic than the ovule number, it is less useful for diagnosing larger groups. In this sample of taxa one large clade of taxa is characterized by fleshy fruits, namely the Psychotrieae s.l., but also the clades *Lasianthus* + *Pauridiantha*, *Coccocypselum* + *Faramea*, the genus *Mycetia*, and parts of the tribes Rubieae and Anthospermeae.

## 5 Conclusion

The cladistic analyses show that the Rubiaceae are monophyletic, and that the Rubiaceae are the sister group to the rest of the Gentianales. There are three major groups of taxa more or less corresponding to the subfamilies Rubioideae, Ixoroideae s.l., and Cinchonoideae s.str. There is no support for the subfamily Antirheoideae, the taxa of which are nested within Cinchonoideae s.str., Ixoroideae s.l., and Rubioideae. The analyses show that the tribes Ophiorrhizeae, Pauridiantheae, and Knoxieae are parts of the Rubioideae. The Hedyotideae must be sunk into the Spermaceae as the Spermaceae s.str. are nested within Hedyotideae and since the name Spermaceae has priority. The evolution of ovule number and fruit types may be analyzed within the Rubioideae, and ovule reduction has occurred at least seven times in the subfamily but in not a single evolutionary line has a reduced ovule number reverted back to many ovules. Fleshy fruits have evolved at least five times in the subfamily. This character seems to be more plastic and in several lines there are reversals back to a dry fruit.

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