

Deep divergences in the coffee family and the systematic position of *Acranthera*

Catarina Rydin · Kent Kainulainen ·
Sylvain G Razafimandimbison · Jenny E E Smedmark ·
Birgitta Bremer

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Abstract Despite extensive efforts, there are unresolved questions on evolutionary relationships in the angiosperm family Rubiaceae. Here, information from six loci and 149 Rubiaceae taxa provide new insights. *Acranthera* and *Coptosapelta* are strongly supported as sisters. Pollen grains of *Acranthera* possess several features common in Rubiaceae, but amongst potential similarities with the unusual grains of *Coptosapelta* are the nature of the apertures and the structure of the sexine. *Luculia*, *Acranthera* and *Coptosapelta* are excluded from the three subfamilies Ixoroideae, Cinchonoideae and Rubioideae. Sipaneeae and Condamineeae form a clade, sister to remaining Ixoroideae. Rondeletieae and Guettardeae are sisters to remaining Cinchonoideae. *Colletoeccema* is sister to remaining Rubioideae, followed by the Urophyllaeae–Ophiorrhizeae clade. Nuclear ITS provided structured information at all phylogenetic levels, but the main gain from adding nrITS was the increased resolution. Average support values also increased but were generally high also without nrITS and the increase was not statistically significant.

Keywords Anther-stigma complex · Cinchonoideae · *Coptosapelta* · Ixoroideae · *Luculia* · Rubioideae

Introduction

Rubiaceae is one of the largest families of flowering plants, comprising more than 13,000 species (Govaerts et al. 2006). Distribution is worldwide, with a particularly high diversity in the tropics and subtropics. The family is a well-defined monophyletic group that can be easily recognised by (generally) opposite branching and phyllotaxis, interpetiolar stipules and sympetalous and epigynous flowers (Schumann 1891; Hutchinson 1973).

Phylogenetic studies generally recognise three major lineages within Rubiaceae (Bremer et al. 1995; Bremer 1996b, 1999; Rova et al. 2002; Robbrecht and Manen 2006), often referred to as subfamilies Rubioideae, Ixoroideae and Cinchonoideae sensu Bremer et al. (1999). Subsequent studies have further investigated relationships within these subfamilies, for example Bremer and Manen (2000, Rubioideae), Andreasen and Bremer (2000, Ixoroideae) and Andersson and Antonelli (2005, Cinchonoideae).

However, despite these extensive efforts, several questions on evolutionary relationships within Rubiaceae, including deep divergences in the family, have remained unanswered. We introduce some of the unresolved questions here.

Acranthera

Acranthera (Arnott 1838) is distributed in India, South to Central China and Central Malesia and consists of about 40 species of sparsely branched shrubs (Bremekamp 1947; Govaerts et al. 2006). The flowers of *Acranthera* are unique within Rubiaceae and are characterised by the presence of united connective appendages, which in turn are united with the stigma by means of a columnar tissue (Puff et al. 1995). In the original description, Arnott (1838)

C. Rydin
Institute of Systematic Botany, University of Zürich,
Zollikerstrasse 107, 8008 Zürich, Switzerland

C. Rydin (✉) · K. Kainulainen · S. G. Razafimandimbison ·
J. E. E. Smedmark · B. Bremer
Department of Botany, Bergius Foundation, Royal Swedish
Academy of Sciences, Stockholm University,
106 91 Stockholm, Sweden
e-mail: Catarina.Rydin@botan.su.se

made a remark on a possible affinity to *Mussaenda* [now placed in the tribe Mussaendeae sensu Bremer and Thulin (1998) of Ixoroideae]. Bremekamp (1947) questioned this affinity in his monograph of the genus and considered the position of *Acranthera* unknown. He later classified *Acranthera* as a monogeneric tribe within Ixoroideae (Bremekamp 1966).

Since then, only a few studies have investigated this genus. Puff et al. (1995) described the pollination ecology, morphology and anatomy of the stamens in selected *Acranthera* species and Kiehn (1995) included *Acranthera* in a survey of chromosome data. Furthermore, *Acranthera* was assigned to the tribe Sabiceae (Cinchonoideae sensu Bremekamp 1966) based on the results of a morphological-based phylogenetic study by Andersson (1996). Bremer and Thulin (1998) did not include *Acranthera* in their molecular study but argued that its testa structure is different from that of *Sabicea*, being instead similar to that of *Amphidasya*. They postulated on a possible placement of *Acranthera* in Rubioideae.

One paper has addressed the phylogenetic position of *Acranthera* based on molecular data; Alejandro et al. (2005) analysed *trnT-L-F* chloroplast data and the genus was resolved as sister to the rest of subfamily Rubioideae, with a relatively high statistical support. This study focused, however, on *Mussaenda* and allied genera. *Luculia* (Rubiaceae) was used as outgroup, and the sampling within Rubioideae and Cinchonoideae was limited.

Coptosapelta and *Luculia*

Coptosapelta consists of 16 species from South East Asia (Valeton 1923; Govaerts et al. 2006). They are woody vines with axillary, pentamerous flowers (Chao 1978). The genus was originally described by Korthals (1851) and placed in the tribe Cinchoneae (subfamily Cinchonoideae), but the morphology and phylogeny of the genus were later re-investigated and debated by many authors (e.g. Verdcourt 1958; Bremekamp 1966; Robbrecht 1988; Andersson and Persson 1991). Bremekamp (1952, 1966) recognised the tribe Coptosapelteae in subfamily Ixoroideae.

Luculia comprises four species of trees or shrubs with showy flowers, distributed in Himalaya, northern Thailand and southern China (Polunin and Stainton 1984; Govaerts et al. 2006). *Luculia* was also placed in Cinchoneae by Schumann (1891), and subsequent authors (e.g. Verdcourt 1958; Bremekamp 1966; Robbrecht 1988) did not disagree. Based on the phylogenetic analysis of morphological data, Andersson and Persson (1991) included *Luculia* and several other genera in a much wider circumscription of Coptosapelteae, which was later shown to be highly polyphyletic (Razafimandimbison and Bremer 2001).

In phylogenetic studies based on molecular data, *Luculia* and *Coptosapelta* have typically had isolated, unresolved or poorly supported positions, often amongst the basal nodes within the family (Bremer and Jansen 1991; Bremer et al. 1995; Bremer 1996b; Bremer et al. 1999; Bremer and Manen 2000; Rova et al. 2002; Robbrecht and Manen 2006). The diversity of results that has been presented indicates that the position(s) of *Luculia* and *Coptosapelta* is not confidently resolved. The two genera form a clade in some studies (e.g. Andersson and Persson 1991; Robbrecht and Manen 2006), in others they appear more distantly related to each other (Bremer et al. 1999). In some studies, *Luculia* is sister to remaining Rubiaceae (Bremer and Jansen 1991, *Coptosapelta* not included), in others they are sister to Cinchonoideae–Ixoroideae (Robbrecht and Manen 2006).

Urophyllaeae and Ophiorrhizeae

The subfamily Rubioideae was proposed by Bremekamp (1952), based e.g. on the presence of raphide idioblasts, and was formally described by Verdcourt (1958). Andersson and Rova (1999) and Bremer and Manen (2000) addressed the phylogeny of the subfamily but some results were poorly supported and/or differed between the studies. For example, Andersson and Rova (1999) found a sister relationship between Urophyllaeae and *Ophiorrhiza*, this clade being the sister of remaining Rubioideae. Bremer and Manen (2000), who used a larger sample of species and more characters, found a basal grade within Rubioideae, with Ophiorrhizeae as the earliest diverging clade, followed by Urophyllaeae and Lasiantheae.

Aims of this study

After more than 60 phylogenetic studies during the last 18 years (adjusted from Bremer *in press*) many aspects of Rubiaceae evolution are now relatively well understood. There are, however, phylogenetic questions that remain unanswered, which hampers further studies addressing for example biogeography and geographical origin, molecular dating of divergences, ancestral state reconstruction and character evolution within the family. We address deep divergences in Rubiaceae with special emphasis on *Acranthera*, and we investigate the usefulness of nrITS for analysing deep divergences in Rubiaceae.

Materials and methods

Selection of species and laboratory procedures

We selected 149 taxa for the present study (Table 1), representing the major clades within Rubiaceae. We included

85 terminals (representing 16 tribes) from Rubioideae, 26 terminals (representing 13 tribes) of Ixoroideae, 11 terminals (representing 7 tribes) of Cinchonoideae, and in addition seven terminals of *Acranthera*, eight of *Coptosapelta* and four of *Luculia*. Eight outgroup taxa from the sister group of Rubiaceae (the other families within Gentianales, Backlund et al. 2000) were selected and sampled at the generic level. Ingroup sequences were sampled at the species level.

We utilised information from six loci: five chloroplast regions (*rbcL*, *rps16* intron, *ndhF*, *atpB-rbcL* spacer, *trnT-L-F* region) and the internal transcribed spacer of the nuclear ribosomal DNA (nrITS1, 5.8S, nrITS2). We used sequences from GenBank whenever available and we also produced 249 new sequences for this study. GenBank accession numbers are given in Table 1. DNA was extracted, amplified and sequenced using standard procedures outlined in Kårehed and Bremer (2007). References to primers are given in Table 2. Sequence fragments were assembled using the Staden package (Staden 1996).

Alignment

Alignments of *rbcL*, *rps16*, *ndhF*, *atpB-rbcL* spacer and *trnT-L-F* could easily be performed by eye using the software Se-Al v.2.0 (Rambaut 1996). Insertion/deletion events were visually inferred, following the alignment criteria outlined in Oxelman et al. (1997). Gaps were treated as missing data in the alignment and added as binominal characters (absent or present) at the end of the matrix.

In order to investigate if nrITS could be utilised for investigating deep divergences in Rubiaceae, we performed an initial alignment using Clustalx/Clustalw (Chenna et al. 2003). From the resulting alignment, it was obvious that most of the region could very easily be aligned over the entire family. Two short regions, one located in nrITS1, the other in nrITS2, were not properly aligned in Clustal and we edited the output from Clustal by eye. We made a simple parsimony analysis to evaluate the amount of information in nrITS. The resulting tree was partly collapsed in basal parts, but added valuable information on higher-level relationships. We continued by adding nrITS to the combined data set and compared results from bootstrap analyses including and excluding nrITS. We further conducted a bootstrap analysis on the combined six-gene data set where we removed the two regions (mentioned above), which were more difficult to align. Parts removed correspond to positions 173-236 and 537-541 in the nrITS sequence of *Luculia gratissima* (GenBank accession: EU145344).

Phylogenetic reconstruction

We analysed each gene separately, including and excluding information from indels. In order to evaluate the usefulness of nrITS, we performed combined analyses including and excluding nrITS (5-cp data set; six-gene data set). We further analysed the combined six-gene data set, including and excluding information from indels. All matrices were analysed with two approaches: Bayesian inference and parsimony.

Bayesian analyses were performed in MrBayes 3.1 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). For each single gene data set, the best performing evolutionary model was identified under three different model selection criteria: Akaike information criterion (AIC) (Akaike 1973), AICc (a second order AIC, necessary for small samples) and the Bayesian information criterion (BIC) (Schwartz 1978). We performed these calculations in software MrAIC ver. 1.4.3 (Nylander 2004). Indels were treated as a morphological partition.

For single gene analyses, one million generations were run, with a sample frequency of 1,000 and four parallel chains. Prior probabilities were specified as follows (according to output from MrAIC): a flat Dirichlet prior probability (all values set to 1.0) was selected for the substitution rates (revmatpr) and the nucleotide frequencies (statefreqpr). The prior probability for the shape parameter of the gamma distribution of rate variation (shapepr) was uniformly distributed in the interval (0.1, 50.0). For analyses using a gamma distribution with a proportion of invariable sites, we specified a prior probability for this proportion (pinvarpr), uniformly distributed on the interval (0.0, 1.0).

For combined analyses, five million generations were run. We partitioned the combined data set in several ways. First, we included all sequence data into a single partition and analysed it together with the morphological partition. Second, we included all chloroplast regions in one partition, and specified a separate partition for the nuclear ribosomal ITS. Indels constituted a separate morphological partition as before. We further excluded gap coding information and partitioned the molecular data into two partitions: chloroplast data and nrITS data. Finally, we specified seven partitions, one for each gene region, and one for indels. In all analyses, partitions were unlinked so that each partition was allowed to have its own set of parameters. Convergence of runs was assessed from the average standard deviation of split frequencies, chain swap information and potential scale reduction factors.

To investigate the usefulness of nrITS in the present study, we performed further analyses on the combined data set, (1) excluding nrITS, and (2) excluding potentially

Table 1 The data matrix

Taxon	Voucher (of previously unpublished sequences)	Classification	<i>rbcL</i>	<i>rps16</i>	<i>ndhF</i>	<i>atpB-rbcL spacer</i>	<i>trnT/F</i>	nrITS
<i>Acranthera</i> Arn. ex Meisn. (sp. 1)	Ridsdale 2470 (L)	- Coptosapelteae	AM117198 ³⁵	EU145477*	EU145400*	-	AJ847408 ⁴²	-
<i>Acranthera</i> Arn. ex Meisn. (sp. 2)	Bremer 1731 (UPS)	- Coptosapelteae	AM117199 ³⁵	EU145478*	-	EU145312*	EU145524*	-
<i>Acranthera atropella</i> Stapf	KH Kjeldsen 54 (AAU)	- Coptosapelteae	-	EU145480*	-	-	EU145525*	-
<i>Acranthera frutescens</i> Valetton	AD Poulsen 52 (AAU)	- Coptosapelteae	EU145449*	EU145475*	EU145398*	EU145310*	EU145522*	EU145345*
<i>Acranthera grandiflora</i> Bedd.	Klackenberg & Lundin 541 (S)	- Coptosapelteae	EU145448*	EU145474*	EU145397*	EU145309*	EU145521*	-
<i>Acranthera siamensis</i> (Kerr) Brenek.	Larsen 45665 (AAU)	- Coptosapelteae	EU145450*	EU145476*	EU145399*	EU145311*	EU145523*	EU145346*
<i>Acranthera siamensis</i> (?) (Kerr) Brenek.	Puff 990826-1/1 (WU)	- Coptosapelteae	AM117200 ³⁵	EU145479*	EU145401*	EU145313*	-	-
<i>Aidia micrantha</i> (K.Schum.) Bullock ex F. White		Ixo-Gardenieae	Z68844 ¹⁹	AF200974 ²⁹	-	-	AF201028 ²⁹	AJ224835 ¹⁷
<i>Alberta magna</i> E. Mey.	Tonkin 200 (UPS) ³⁶	Ixo-Albertae	Y18708 ¹⁷	EU145491*	AJ236282 ¹⁶	-	AJ620118 ⁴⁷	AJ224842 ¹⁷
<i>Alstonia scholaris</i> (L.) R.Br.		APOCYNACEAE	X91760 ⁷	AJ431032 ⁴	AJ011982 ⁵	DQ359161 ⁶	AJ430907 ⁴	DQ358880 ⁶
<i>Amphidasya ambigua</i> (Standl.) Standl.	Clark & Watt 736 (UPS)	RUB-Urophyllaeae	Y11844 ¹⁴	AF129271 ²⁴	-	EU145337*	EU145576*	EU145383*
<i>Anthocleista</i> Afzel. ex R.Br.		GENTIANACEAE	L14389 ⁸	-	AJ235829 ⁹	DQ131695 ⁶	AJ490190 ⁴⁴	AJ489864 ⁴⁴
<i>Anthospermum herbaceum</i> L.f.	Bremer, 3093 (UPS)	RUB-Anthospermeae	X83623 ¹	EU145496*	AJ236284 ¹⁶	AJ234028 ²	EU145544*	EU145355*
<i>Arcytophyllum aristatum</i> Standl.		RUB-Spermacoceae	AJ288595 ²	AF333348 ²⁰	-	-	AF333349 ²⁰	AM182061 ⁵⁷
<i>Argostemma hookeri</i> King	Malaysia, Wanmtorp s.n. (S)	RUB-Argostemmatae	Z68788 ²¹	EU145497*	EU145419*	AJ234032 ²	EU145545*	EU145356*
<i>Batopedina pulvinellata</i> Robbr.		RUB-Knoxiae	AJ288596 ²	AM266813 ³⁷	-	-	AM266902 ³⁷	AM266989 ³⁷
<i>Berteria guianensis</i> Aubl.		Ixo-Bertiariae	AJ224845 ¹⁷	AF200983 ²⁹	-	-	AF152670 ¹²	AJ224841 ¹⁷
<i>Bouvardia ternifolia</i> (Cav.) Schldl.) (syn. <i>Bouvardia glaberrima</i>)		RUB-Spermacoceae	X83626 ¹	AF002758 ¹¹	-	X76478 ⁴¹	DQ359165 ⁶	DQ358884 ⁶
<i>Calycophyllum candidissimum</i> (Vahl) DC.		Ixo-Condamineae	X83627 ¹	AF004030 ¹¹	AJ236285 ¹⁶	DQ131708 ⁶	AF152646 ¹²	DQ358886 ⁶
<i>Dirichletia glaucescens</i> Hiern (syn. <i>Carphalea glaucescens</i>)		RUB-Knoxiae	Z68789 ²¹	AM266817 ³⁷	AJ236287 ¹⁶	-	AM266906 ³⁷	AM266993 ³⁷
<i>Catesbaea spinosa</i> L.		CINCH-Chiococceae	X83628 ¹	AF004032 ¹¹	AM117343 ³⁵	-	AF152706 ¹²	AY763880 ¹³
<i>Cephalanthus occidentalis</i> L.		CINCH-Naucleaeae	X83629 ¹	AF004033 ¹¹	AJ236288 ¹⁶	DQ131710 ⁶	AF152692 ¹²	AJ346883 ³
<i>Chiococca alba</i> (L.) Hitchc.		CINCH-Chiococceae	L14394 ⁸	AF004034 ¹¹	AJ130835 ¹⁶	DQ131711 ⁶	AY763813 ¹³	DQ358887 ⁶
<i>Cinchona pubescens</i> Vahl		CINCH-Chiococceae	X83630 ¹	AF004035 ¹¹	AJ235843 ⁹	AJ233990 ²	AJ346963 ³	AY538356 ¹⁵
<i>Coccocypselum condalia</i> Pers.	Pirani & Bremer 4891 (SPF)	RUB-Coussareae	AM117217 ³⁵	EU145499*	EU145420*	EU145324*	EU145547*	EU145358*
<i>Coccocypselum hirsutum</i> Bartl. ex DC.	CT 908, Bremer 2700 (S)	RUB-Coussareae	X87145 ²²	EU145500*	EU145421*	EU145325*	EU145548*	EU145359*
<i>Coffea arabica</i> L.		Ixo-Coffeae	X83631 ¹	AF004038 ¹¹	AJ236290 ¹⁶	X70364 ⁴⁰	DQ153845 ⁴⁶	DQ153609 ⁴⁶

Table 1 continued

Taxon	Voucher (of previously unpublished sequences)	Classification	<i>rbcL</i>	<i>rps16</i>	<i>ndhF</i>	<i>atpB-rbcL spacer</i>	<i>trnT/F</i>	<i>mtTS</i>
<i>Colletocena devevrei</i> (De Wild.) E.M.A. Petit	S Lisowski 47195 (K)	Rub-Colletocemateae	EU145457 ⁶¹	AF129272 ²⁴	EU145409 ⁶¹	DQ131713 ⁶	EU145532 ⁶¹	EU145353*
<i>Condaminea corymbosa</i> (Ruiz & Pav.) DC.		Ixo-Condamineae	Y18713 ¹⁶	AF004039 ¹¹	AJ236291 ¹⁶	–	AF102406 ⁴³	–
<i>Coptosapelta diffusa</i> (Champ.) Steenis (specimen 1)	Bartholomew et al. 847 (AAU)	–Coptosapelteae	EU145452*	EU145482*	EU145403*	EU145315*	EU145527*	EU145347*
<i>Coptosapelta diffusa</i> (Champ.) Steenis (specimen 2)	Steward et al. 594 (S)	–Coptosapelteae	EU145453*	EU145483*	EU145404*	AJ233987 ²	DQ359166 ⁶	DQ358882 ⁶
<i>Coptosapelta flavescens</i> Korth. (specimen 1)	Puff 950720-1/2 (WU)	–Coptosapelteae	Y18714 ¹⁶	EU145484*	AJ236292 ¹⁶	EU145316*	AM117354 ³⁵	EU145348*
<i>Coptosapelta flavescens</i> Korth. (specimen 2)	Gardette et al. EG1716 (K)	–Coptosapelteae	EU145454*	EU145485*	EU145405*	EU145317*	EU145528*	EU145349*
<i>Coptosapelta flavescens</i> Korth. (specimen 3)	Larsen et al. 31147 (AAU)	–Coptosapelteae	–	EU145488*	EU145408*	–	EU145531*	EU145352*
<i>Coptosapelta montana</i> Korth. ex Valeton	Clemens & Clemens 40864 (K)	–Coptosapelteae	EU145451*	EU145481*	EU145402*	EU145314*	EU145526*	–
<i>Coptosapelta tomentosa</i> Valeton ex K. Heyne (specimen 1)	Beusekom & Charoenpol 1741 (AAU)	–Coptosapelteae	EU145455*	EU145486*	EU145406*	EU145318*	EU145529*	EU145350*
<i>Coptosapelta tomentosa</i> Valeton ex K. Heyne (specimen 2)	Beusekom & Charoenpol 1933 (AAU)	–Coptosapelteae	EU145456*	EU145487*	EU145407*	EU145319*	EU145530*	EU145351*
<i>Coussarea hydrangeifolia</i> (Benth.) Benth. & Hook.f. ex Müll. Arg.	Fuentes 5504 (GB)	Rub-Coussareae	EU145460*	EU145501*	EU145422*	EU145326*	EU145549*	EU145360*
<i>Coussarea macrophylla</i> (Mart.) Müll. Arg.		Rub-Coussareae	Y11847 ¹⁴	AF004040 ¹¹	–	–	AF152612 ¹² (C. sp)	–
<i>Cremaspora triflora</i> (Thonn.) K.Schum.		Ixo-Cremasporaeae	Z68856 ¹⁹	AF200990 ²⁹	–	DQ131718 ⁶	AF201040 ²⁹	AJ224824 ¹⁷
<i>Cremocarpon lantzii</i> Bremek.	Razafimandimbison 517 (UPS)	Rub-Psychotrieae	AM117222 ³⁵	AM117296 ³⁵	–	–	AM117356 ³⁵	–
<i>Cruckshanksia hymenodon</i> Hook. & Arn.	Rodriguez 10 (K)	Rub-Coussareae	AJ288599 ²	EU145502*	–	AJ234004 ²	EU145550*	–
<i>Cubanela domingensis</i> (Britton) Aiello		CINGH-Chiococceae	X83632 ¹	AF004044 ¹¹	AM117345 ³⁵	DQ131720 ⁶	AF152701 ¹²	AY763891 ¹³
<i>Dannacanthus indicus</i> C.F. Gaertn.		Rub-Morindeae	Z68793 ²¹	AF331647 ²⁰	–	AJ234015 ²	–	AY514061 ⁵³
<i>Danais xanthorrhoea</i> (K. Schum.) Bremek.	Bremer 3079 (UPS)	Rub-Danaideae	Z68794 ²¹	AM117297 ³⁵	AJ236293 ¹⁶	AJ234019 ²	DQ662138 ³²	EU145364*
<i>Declieuxia cordigera</i> Mart. & Zucc. ex Schult. & Schult.f.	Pirani & Bremer 4893 (SPF)	Rub-Coussareae	AM117224 ³⁵	AM117298 ³⁵	EU145423*	EU145327*	EU145551*	EU145361*
<i>Declieuxia fruticosa</i> (Willd. ex Roem. & Schult.) Kuntze	B. Hammel 20875 (MO, CR)	Rub-Coussareae	AJ002177 ²³	EU145503*	–	DQ131721 ⁶	EU145552*	EU145362*

Table 1 continued

Taxon	Voucher (of previously unpublished sequences)	Classification	<i>rbcL</i>	<i>rps16</i>	<i>ndhF</i>	<i>atpB-rbcL</i> spacer	<i>trnT/F</i>	nrITS
<i>Dentella repens</i> (L.) J.R.Forst. & G.Forst.		RUB-Spermacoceae	–	AF333370 ²⁰	–	–	AF381540 ⁴⁹	–
<i>Dibrachionostylus kaessneri</i> (S.Moore) Bremek.	Strid 2564 (UPS)	RUB-Spermacoceae	AJ616211 ²⁸	AF002761 ¹¹	–	–	EUI145574*	–
<i>Didymaea alstinoides</i> (Cham. & Schldl.) Standl.	Keller 1901 (CAS)	RUB-Rubieae	Z68795 ²¹	–	–	AJ234036 ²	EUI145570*	–
<i>Diplospora polysperma</i> Valetton	Ridsdale IV.E.130 (L)	Ixo-Coffeaeae	AJ286703 ¹⁸	AM117301 ³⁵	–	–	EUI145538*	–
<i>Dunnia sinensis</i> Tutcher (Specimen 1)	Yangchun 10, Ge et al. 2002	RUB-Dunnieae	EUI145467 ⁶¹	EUI145515 ⁶¹	EUI145442 ⁶¹	EUI145339 ⁶¹	EUI145583 ⁶¹	EUI145390*
<i>Dunnia sinensis</i> Tutcher (Specimen 2)	Taishan 10, Ge et al. 2002	RUB-Dunnieae	EUI145468 ⁶¹	EUI145516 ⁶¹	EUI145443 ⁶¹	EUI145340 ⁶¹	EUI145584 ⁶¹	EUI145391*
<i>Dunnia sinensis</i> Tutcher (Specimen 3)	Zhuhai 12, Ge et al. 2002	RUB-Dunnieae	EUI145469 ⁶¹	EUI145517 ⁶¹	EUI145444 ⁶¹	EUI145341 ⁶¹	EUI145585 ⁶¹	EUI145392*
<i>Dunnia sinensis</i> Tutcher (Specimen 4)	Longmen 12, Ge et al. 2002	RUB-Dunnieae	EUI145470 ⁶¹	EUI145518 ⁶¹	EUI145445 ⁶¹	EUI145342 ⁶¹	EUI145586 ⁶¹	EUI145393*
<i>Dunnia sinensis</i> Tutcher (Specimen 5)	Xinhui 16, Ge et al. 2002	RUB-Dunnieae	EUI145471 ⁶¹	EUI145519 ⁶¹	EUI145446 ⁶¹	EUI145343 ⁶¹	EUI145587 ⁶¹	EUI145394*
<i>Emmenopteryx henryi</i> Oliv.		Ixo-Condamineae	Y18715 ¹⁶	AM117302 ³⁵	AJ236294 ¹⁶	DQ131728 ⁶	AF152637 ¹²	–
Unknown Rubiaceae (GenBank name: <i>Ernodea littoralis</i> Sw.)		RUB-Spermacoceae	AJ288601 ²	AF002763 ¹¹	–	AJ234025 ²	–	–
<i>Faramaea multiflora</i> A Rich.	Bremer et al. 3331 (UPS)	RUB-Coussareae	Z68796 ²¹	AF004048 ¹¹	EUI145424*	EUI145328*	AF102422 ⁴³	EUI145363*
<i>Ferdinandusa speciosa</i> Pohl	Malme 2442 (UPS)	Ixo-Condamineae	AM117226 ³⁵	AM117304 ³⁵	EUI145412*	DQ131735 ⁶	EUI145534*	–
<i>Feretia aeruginescens</i> Stapf	Bremer 3137 (UPS)	Ixo-Octotropideae	Z68857 ¹⁹	AM117305 ³⁵	–	–	EUI145539*	–
<i>Fernelia buxifolia</i> Lam.	de Block s.n. (BR)	Ixo-Octotropideae	AJ286704 ¹⁸	AM117306 ³⁵	–	DQ131736 ⁶	EUI145540*	–
<i>Galium album</i> Mill.		RUB-Rubieae	X81090 ²⁷	AF004050 ¹¹	–	X76459 ⁴¹	–	–
<i>Gelsemium</i> Juss.		GELSEMIACEAE	L14397 ⁸	AJ431033 ⁴	AJ011984 ⁵	AJ233985 ²	AF102428 ⁴³	DQ358881 ⁶
<i>Gentiana</i> L.		GENTIANACEAE	L14398 ⁸	AJ431034 ⁴	L36400 ³⁸	DQ398604 ³⁹	X77893 ⁴⁵	DQ398639 ³⁹
<i>Geophila obvallata</i> Didr.	Q Luke 9037 (FR)	RUB-Psychotrieae	AM117228 ³⁵	AF369845 ²⁶	–	–	EUI145569*	–
<i>Guettarda uruguensis</i> Cham. & Schldl.	X5-127, Gillis 9575 (FTG)	CINGH-Guettardeae	X83638 ¹	EUI145489*	AJ236297 ¹⁶	DQ131739 ⁶	EUI145533*	AY730294 ³⁰
<i>Gynochthodes coriacea</i> Blume		RUB-Morindeae?	AJ288603 ²	AM117311 ³⁵	–	–	AJ847407 ⁴²	–
<i>Hedyotis fruticososa</i> L.		RUB-Spermacoceae	Z68799 ²¹	–	–	AJ234026 ²	AF381539 ⁴⁹	–
<i>Hillia triflora</i> (Oerst.) C.M. Taylor		CINGH-Hillieae	X83642 ¹	AM117315 ³⁵	AJ236298 ¹⁶	AJ233993 ²	AM117362 ³⁵	–
<i>Houstonia caerulea</i> L.		RUB-Spermacoceae	AJ288604 ²	AF333379 ²⁰	–	–	AF381524 ⁴⁹	DQ012706 ⁵⁸
<i>Hydnophytum formicarum</i> Jack		RUB-Psychotrieae	X83645 ¹	AF001339 ¹¹	–	X76480 ⁴¹	–	DQ012774 ⁵⁸
								AF034912 ³³

Table 1 continued

Taxon	Voucher (of previously unpublished sequences)	Classification	<i>rbcL</i>	<i>rps16</i>	<i>ndhF</i>	<i>atpB-rbcL</i> spacer	<i>trnT/F</i>	nrITS
<i>Hymenodictyon floribundum</i> (Hochst. & Steud.) Rob.	Puff 861109-3/1 (WU)	CINCH-Hymenodictyoneae	AJ347015 ³	AF004058 ¹¹	EU145411*	DQ131742 ⁶	AY538454 ¹⁵	AJ346905 ³
<i>Ixora coccinea</i> L.		Ixo-Ixoreae	X83646 ¹	AM117321 ³⁵	AJ236299 ¹⁶	–	AJ620117 ⁴⁷	AJ224826 ¹⁷
<i>Kohautia caespitosa</i> Schnizl.	Bremer et al. 42566B (UPS)	RUB-Spermacoceae	Z68800 ²¹	AM117324 ³⁵	–	–	EU145573*	–
<i>Kopsia fruticosa</i> (Roxb.) A.DC.		APOCYNACEAE	X91763 ⁸	–	AJ235824 ⁹	–	AM295091 ¹⁰	–
<i>Kraussia floribunda</i> Harv.		Ixo-Octotropeae	Z68858 ¹⁹	AM117325 ³⁵	–	DQ131746 ⁶	AM117368 ³⁵	–
<i>Lasianthus kilimandscharicus</i> K.Schum.	H. Lantz 119 (UPS)	RUB-Lasiantheae	AM117237 ³⁵	AM117327 ³⁵	EU145426*	EU145330*	DQ662147 ³²	EU145366*
<i>Lasianthus lanceolatus</i> (Griseb.) Urb.	Taylor 11719 (MO)	RUB-Lasiantheae	AM117238 ³⁵	AF004062 ¹¹	–	EU145331*	EU145554*	EU145367*
<i>Lasianthus pedunculatus</i> E.A. Bruce	Andreasen 71 (UPS)	RUB-Lasiantheae	Z68802 ²¹	EU145504*	EU145427*	AJ234003 ²	EU145555*	EU145368*
<i>Lasianthus strigosus</i> Wight	Bremer & Bremer 3902 (UPS)	RUB-Lasiantheae	AM117239 ³⁵	EU145505*	EU145428*	–	EU145556*	EU145369*
<i>Lerchea bracteata</i> Valetton	Axelius 343 (S)	RUB-Ophiorrizeae	AJ288610 ²	EU145508*	EU145433*	AJ233997 ²	EU145561*	EU145374*
<i>Luculia grandifolia</i> Ghose	Bremer 2713 (S)	-Luculieae	X83648 ¹	AM900593 ⁶⁰	AM117346 ³⁵	AJ233986 ²	AJ346929 ³	AJ346896 ³
<i>Luculia gratissima</i> (Wall.) Sweet	Cult in Univ. Conn. Storres 870064 (no voucher)	-Luculieae	AM117243 ³⁵	AJ431036 ⁴	AJ011987 ⁵	EU145308*	AJ430911 ⁴	EU145344*
<i>Luculia intermedia</i> Hutch.	Howick et al. HOMC1524 (K)	-Luculieae	–	EU145473*	EU145396*	–	EU145520*	–
<i>Luculia pinceana</i> Hook.	NN Thin et al. 3061 (AAU)	-Luculieae	EU145447*	EU145472*	EU145395*	DQ131749 ⁶	AM117371 ³	–
<i>Manostachya ternifolia</i> E.S. Martins	Bamps & Martins 4410 (UPS)	RUB-Spermacoceae	AJ616213 ²⁸	AM117328 ³⁵	–	–	EU145572*	–
<i>Margaritopsis nudiflora</i> (Griseb.) K. Schum. (Syn. <i>Margaritopsis acufolia</i>)	Ekman 10248 (UPS)	RUB-Psychotrieae	AM117247 ³⁵	AF001340 ¹¹	–	–	EU145568*	–
<i>Maschalocorymbus corymbosus</i> (Blume) Brenek.	Ridsdale 2471 (L)	RUB-Urophyllaeae	AJ288611 ²	AM900611 ⁶⁰	–	–	EU145577*	EU145384*
<i>Mitchella repens</i> L.		RUB-Morindeae	Z68805 ²¹	AF001441 ¹¹	–	–	–	AB103535 ⁵⁴ AB103536
<i>Mitrasacmopsis quadrivalvis</i> Jovet	Kayombo et al. (UPS)	RUB-Spermacoceae	AJ616214 ²⁸	AM117329 ³⁵	EU145439*	EU145336*	EU145575*	EU145382*
<i>Morinda citrifolia</i> L.		RUB-Morindeae	AJ318448 ²⁵	AJ320078 ²⁵	AJ236300 ¹⁶	AJ234013 ²	AFI52616 ¹²	AY762843 ⁵⁵
<i>Mostuea brunonis</i> Didr.		GELSEMIACEAE	L14404 ⁸	–	AJ235828 ⁹	DQ131697 ⁶	–	–
<i>Mussaenda erythrophylla</i> Schumacher & Thonn.	Gillis 10838 (FTG)	Ixo-Mussaendeae	X83652 ¹	EU145493*	AJ130836 ¹⁶	DQ131754 ⁶	EU145535*	AJ846858 ⁴²
<i>Mycetia malayana</i> (G. Don) Craib		RUB-Argostemmateae	Z68806 ²¹	AF002771 ¹¹	–	AJ234033 ²	AFI52622 ¹²	–
<i>Naucllea orientalis</i> (L.) L.	Novotny et al. (2002)	CINCH-Nauclleae	X83653 ¹	AJ320080 ²⁵	EU145410*	EU145320*	AJ346958 ³	AJ346897 ³
<i>Coprosma granadensis</i> Mutis ex L.f. (syn. <i>Nertera granadensis</i>)		RUB-Anthospermeae	X83654 ¹	AF002741 ¹¹	–	–	AFI52623 ¹²	AF257927 ³¹

Table 1 continued

Taxon	Voucher (of previously unpublished sequences)	Classification	<i>rbcL</i>	<i>rps16</i>	<i>ndhF</i>	<i>atpB-rbcL</i> spacer	<i>trnT/F</i>	nrITS
<i>Neurocalyx championii</i> Benth. ex Thwaites	Thor 601 (S)	RUB-Ophiorrhizeae	EU145463*	EU145509*	EU145435*	–	EU145563*	EU145376*
<i>Neurocalyx zeylanicus</i> Hook.	B & K Bremer 937 (S)	RUB-Ophiorrhizeae	Z68807 ²¹	AM900594 ⁶⁰	EU145434*	AJ233995 ²	EU145562*	EU145375*
<i>Normandia neocaledonica</i> Hook.f.	Munzinger 532 (MO)	RUB-Anthospermeae	AM117250 ³⁵	AF257931 ³¹	–	–	EU145543*	AF257930 ³¹
<i>Oldenlandia corymbosa</i> L.		RUB-Spermatocoeae	X83655 ¹	AF333381 ²⁰	AJ130837 ¹⁶	–	AF381537 ⁴⁹	AY854053 ⁵⁹
<i>Ophiorrhiza elmeri</i> Merr.	Kjeldsen & Poulsen 233 (AAU)	RUB-Ophiorrhizeae	EU145464*	EU145510*	EU145436*	–	EU145564*	EU145378*
<i>Ophiorrhiza mungos</i> L.	Bremer 3301 (UPS)	RUB-Ophiorrhizeae	X83656 ¹	AF004064 ¹¹	AJ130838 ¹⁶	–	DQ662151 ⁶	EU145377*
<i>Oreopolus glacialis</i> (Poepp.) Ricardi		RUB-Coussareae	AJ288612 ²	AF004042 ¹¹	–	–	–	–
<i>Paederia foetida</i> L.		RUB-Paederiaceae	AF332373 ²⁰	AF004065 ¹¹	–	AJ234006 ²	AF152619 ¹²	–
<i>Palicourea crocea</i> (Sw.) Schult		RUB-Psychotriaceae	AM117253 ³⁵	AF147510 ³³	–	–	–	AF149322 ³³
<i>Palicourea guianensis</i> Aubl.		RUB-Psychotriaceae	–	AF001345 ¹¹	–	–	AF152615 ¹²	AY635554 ⁵⁶
<i>Parapentias sylvatica</i> (K. Schum.) Bremek.		RUB-Knoxieae	X83657 ¹	AM266849 ³⁷	–	AJ234021 ²	AM266937 ³⁷	AM267023 ³⁷
<i>Pauridiantha symplocoides</i> (S. Moore) Bremek.	Lantz 123 (UPS)	RUB-Urophyllaeae	AY538502 ¹⁵	AF004068 ¹¹	EU145440*	EU145338*	AF102467 ⁴³	EU145386*
<i>Pauridiantha paucinervis</i> (Hiem) Bremek.	Bremer 3090 (UPS)	RUB-Urophyllaeae	Z68811 ²¹	AM900600 ⁶⁰	AJ236302 ¹⁶	AJ233998 ²	EU145578*	EU145385*
<i>Pentas lanccolata</i> (Forssk.) Defters		RUB-Knoxieae	X83659 ¹	AM266875 ³⁷	AJ236304 ¹⁶	X76479 ⁴¹	AM266963 ³⁷	AB247275 ⁵²
<i>Pentodon pentandrus</i> (Schumach. & Thonn.) Vatke, Oesterr.		RUB-Spermatocoeae	X83660 ¹	AF003612 ¹¹	–	AJ234024 ²	–	–
<i>Pouchetia baumanniana</i> Büttner (syn. <i>Pouchetia gillettii</i>) Praravina suberosa (Merr.) Bremek.	Kiehn HBV sub RR-81-31 (WU) Sabah: Ridsdale no voucher	Ixo-Octotropideae	Z68859 ¹⁹	AM117336 ³⁵	–	–	EU145541*	–
<i>Pravinaria leucocarpa</i> Bremek.	Beaman 7950 (S)	RUB-Urophyllaeae	AJ288616 ²	EU145514*	–	–	EU145579*	EU145387*
<i>Psychotria kirkii</i> Hiern		RUB-Urophyllaeae	AJ288617 ²	AM900613 ⁶⁰	EU145441*	AJ234001 ²	EU145580*	EU145388*
<i>Psychotria pittieri</i> Standl.		RUB-Psychotriaceae	X83663 ¹	AF410728 ³⁴	AJ236307 ¹⁶	X76481 ⁴¹	AY338469 ¹⁵	AF072038 ²³
<i>Psychotria poeppigiana</i> Müll. Arg.		RUB-Psychotriaceae	–	AF002746 ¹¹	–	–	AF152614 ¹²	AF071998 ²³
<i>Pyrostria hystrix</i> (Bremek.) Britson	Bremer 3791 (UPS)	Ixo-Vanguerieae	Z68818 ²¹	AF002748 ¹¹	–	AJ234018 ²	–	AF149400 ³³
<i>Retinophyllum pilosum</i> (Spruce ex Benth.) Müll.Arg.	Wurdack & Adderley 43270 (S)	Ixo-Retiniophylleae	AF331654 ²⁰	AF004076 ¹¹	–	–	AJ620168 ⁴⁷	AJ315114 ⁵⁰
<i>Rhachicallis americana</i> (Jacq.) Hitchc.		CINCH-Rondeletieae	X83664 ¹	AF004073 ¹¹	–	–	AF152747 ¹²	AY730301 ³⁰

Table 1 continued

Taxon	Voucher (of previously unpublished sequences)	Classification	<i>rbcL</i>	<i>rps16</i>	<i>ndhF</i>	<i>atpB-rbcL spacer</i>	<i>trnT/F</i>	nrITS
<i>Rondeletia odorata</i> Jacq.	Bremer & Andreasen 3504 (UPS)	CINCH-Rondeletieae	Y11857 ¹⁴	EU145490*	AJ235845 ⁹	EU145321*	AF152741 ¹²	AY730307 ³⁰
<i>Rubia tinctorum</i> L.		RUB-Rubieae	X83666 ¹	–	DQ359167 ⁶	X76465 ⁴¹	–	DQ358885 ⁶
<i>Sabicea aspera</i> Aubl.	Andersson et al. 1941 (NY)	Ixo-Sabiceae	AY538508 ¹⁵	AF004079 ¹¹	EU145416*	–	AY538475 ¹⁵	AM409008
<i>Sabicea diversifolia</i> Pers.	Bremer et al. 4018-B18 (UPS)	Ixo-Sabiceae	EU145459*	EU145494*	EU145415*	DQ131781 ⁶	AJ847396 ⁴²	AJ846883 ⁴²
<i>Saldinia</i> A. Rich. ex DC. (specimen 1)	Bremer & al 4038-BB38 (UPS)	RUB-Lasiantheae	AM117269 ³⁵	AF129275 ²⁴	EU145429*	EU145332*	EU145557*	EU145370*
<i>Saldinia</i> A. Rich. ex DC. (specimen 2)	Kårehed et al. 257 (UPS)	RUB-Lasiantheae	EU145461*	EU145506*	EU145430*	EU145333*	EU145558*	EU145371*
<i>Schismatoclada</i> sp. Baker	Razafimandimbison & Ravelonarivo 373 (MO)	RUB-Danaideae	AM117271 ³⁵	AM117341 ³⁵	EU145425*	EU145329*	EU145553*	EU145365*
<i>Schizocolea linderi</i> (Hutch. & Dalziel) Bremek.	Adam 20116 (UPS)	RUB-Schizocoleae	AM117272 ³⁵	EU145498 ⁶¹	–	EU145323 ⁶¹	EU145546 ⁶¹	EU145357*
<i>Schradera</i> sp K. Krause		RUB-Schradereae	Y11859 ¹⁴	AF003617 ¹¹	–	AJ234014 ²	AF152613 ¹²	–
<i>Stherardia arvensis</i> L.	K. Andreasen 345 (SBT)	RUB-Rubieae	X81106 ²⁷	AF004082 ¹¹	–	X76458 ⁴¹	EU145571*	–
<i>Sipanea biflora</i> (L.f.) Cham. & Schldt.	Rova et al. 2005 (S)	Ixo-Sipaneae	AY538509 ¹⁵	AF004085 ¹¹	EU145413*	DQ131788 ⁶	AF152675 ¹²	AY555116 ⁴⁸
<i>Sipanea hispida</i> Benth. ex Wernham	Irwin et al. 34756 (UPS)	Ixo-Sipaneae	EU145458*	EU145492*	EU145414*	EU145322*	AY555107 ⁴⁸	AY555122 ⁴⁸
<i>Sipanea pratensis</i> Aubl.		Ixo-Sipaneae	–	AF243022 ³⁰	–	–	AF152677 ¹²	AY555115 ⁴⁸
<i>Spermacoce remota</i> Lam.		RUB-Spermacoceae	Z68823 ²¹	–	AJ236309 ¹⁶	–	–	–
<i>Spigelia</i> L.		LOGANIACEAE	Y11863 ¹⁴	AF004093 ¹¹	AJ235840 ⁹	–	–	AF178004 ⁵¹
<i>Spiradiclis bifida</i> Kurz	J. B. H. 55 (S)	RUB-Ophiorrhizeae	EU145465*	EU145511*	EU145437*	–	EU145565*	EU145379*
<i>Strychnos</i> L.		LOGANIACEAE	L14410 ⁸	AF004094 ¹¹	AJ235841 ⁹	DQ131691 ⁶	AF102484 ⁴³	–
<i>Thecorchus wauensis</i> (Schweinf. ex Hiem) Bremek.		RUB-Spermacoceae	AM117282 ³⁵	AM266901 ³⁷	–	–	AM266987 ³⁷	AM267070 ³⁷
<i>Theligonum cynocrambe</i> L.		RUB-Theligoneae	X83668 ¹	AF004087 ¹¹	–	X81680 ⁴⁰	AF152621 ¹²	–
<i>Tricalysia cryptocalyx</i> Baker		Ixo-Coffeae	Z68854 ¹⁹	AF004088 ¹¹	–	–	AF152669 ¹²	AJ224827 ¹⁷
<i>Trichostachys aurea</i> Hiem	Andersson & Nilsson 2304 (GB)	RUB-Lasiantheae	EU145462*	EU145507*	EU145431*	EU145334*	EU145559*	EU145372*
<i>Trichostachys</i> sp. Hook.f.	B. Sonké 1725 (UPS)	RUB-Lasiantheae	AJ288626 ²	AM900595 ⁶⁰	EU145432*	DQ131792 ⁶	EU145560*	EU145373*
<i>Urophyllum arboreum</i> (Reinw. ex Blume) Korth.	Boeea 7887 (S)	RUB-Urophyllaeae	–	AM900617 ⁶⁰	–	DQ131793 ⁶	EU145582*	–
<i>Urophyllum ellipticum</i> (Wight) Thwaites	Lundqvist 11085 (UPS)	RUB-Urophyllaeae	AJ288627 ²	AM900619 ⁶⁰	–	AJ234002 ²	EU145581*	EU145389*
<i>Vangueria madagascariensis</i> J.F. Gmel.	Bremer 3077 (UPS)	Ixo-Vanguerieae	X83670 ¹	–	AJ130840 ¹⁶	–	EU145542*	AJ224839 ¹⁷

Table 1 continued

Taxon	Voucher (of previously unpublished sequences)	Classification	<i>rbcL</i>	<i>rps16</i>	<i>ndhF</i>	<i>atpB-rbcL</i> spacer	<i>trnT/F</i>	nrITS
<i>Virectaria major</i> (K. Schum.) Verdc.	Reekmans 10916 (UPS)	Ixo-Sabiaceae	Y11861 ^{1,4}	EU145495*	EU145417*	AJ233989 ²	EU145537*	EU145354*
<i>Xanthophyllum borneense</i> (Valeton) Axelius	Axelius 316 (S)	RUB-Ophiorrhizaceae	EU145466*	EU145513*	EU145438*	EU145335*	EU145567*	EU145381*
<i>Xanthophyllum capitellatum</i> Ridl.	Ridsdale 2473 (L)	RUB-Ophiorrhizaceae	AJ288628 ²	EU145512*	–	AJ233996 ²	EU145566*	EU145380*
Total number of taxa in single gene data sets			141	141	91	97	135	105
Total number of characters in single gene data sets			1402	1602 + 23	2243 + 7	1098 + 18	3219 + 18	925 (677)**
Number of variable characters			527	1029	1172	605	1837	608 (386)**
Number of phylogenetically informative characters			404	648	856	395	1145	504 (309)**
Evolutionary model employed (AICc weights)			GTRIG	GTRG	GTRG	GTRG	GTRIG	GTRIG
Conflicts between Bayesian and parsimony analyses			No	No	No	No	No	No
Conflicts between results including/excluding indels			–	No	No	No	No	–

Notes. Classification: SUBFAMILY ABBREVIATION-Tribe. For outgroup taxa, only the FAMILY name is given. New classification in bold. Detailed information on methods and results is presented in the text

* Previously unpublished sequence. ** Numbers within brackets represent values when parts of the nrITS alignment were removed. Published sequences: 1: Bremer et al. (1995). 2: Bremer and Manen (2000). 3: Razafimandimbison and Bremer (2002). 4: Bremer et al. (2002). 5: Oxelman et al. (1999). 6: J-F Manen (GenBank unpublished). 7: Sennblad and Bremer (1996). 8: Olmstead et al. (1993). 9: Backlund et al. (2000). 10: ME Endress et al. (GenBank unpublished). 11: Andersson and Rova (1999). 12: Rova et al. (2002). 13: Motley et al. (2005). 14: Bremer et al. (1998). 15: Andersson and Antonelli (2005). 16: Bremer et al. (1999). 17: Andreassen et al. (1999). 18: Andreassen and Bremer (2000). 19: Andreassen and Bremer (1996). 20: L Andersson (GenBank unpublished). 21: Bremer (1996b). 22: Bremer (1996a). 23: Nepokroeff et al. (1999). 24: Piesschaert et al. (2000a). 25: Novotny et al. (2002). 26: Andersson (2001). 27: Manen and Natali (1995). 28: Thulin and Bremer (2004). 29: Persson (2000). 30: JHE Rova (GenBank unpublished). 31: CL Anderson et al. (GenBank unpublished). 32: M Backlund (GenBank unpublished). 33: L Andersson and C Taylor (GenBank unpublished). 34: Andersson (2002). 35: B Bremer (in prep.). 36: A Mouly (unpublished). 37: Kårehed and Bremer (2007). 38: Olmstead and Reeves (1995). 39: XL Zhang et al. (GenBank unpublished). 40: Natali et al. (1994). 41: Manen et al. (1995). 42: Alejandro et al. (2005). 43: Struwe et al. (1998). 44: Yuan et al. (2003). 45: Gielly and Taberlet (1996). 46: O Maurin et al. (GenBank unpublished). 47: Lantz and Bremer (2004). 48: Delprete and Cortes-B (2004). 49: Church (2003). 50: Lantz et al. (2002). 51: Gould and Jansen (1999). 52: Nakamura et al. (2006). 53: P Ding et al. (GenBank unpublished). 54: J Yokoyama et al. (GenBank unpublished). 55: AD Projansky and DL Stern (GenBank unpublished). 56: CW Dick and E Bermingham (GenBank unpublished). 57: D Wolff and S Liede-Schumann (GenBank unpublished). 58: Church and Taylor (2005). 59: CI Yuan (GenBank unpublished). 60: Smedmark et al. (2008). 61: Rydin et al. (2008)

Table 2 Primers

DNA region	Primer names	Sequence 5'–3'/Reference
<i>rbcL</i>	5'F, 3'R and 427F	Bremer et al. (2002)
<i>rbcL</i>	Z895R	Zurawski, DNAX Research institute
<i>rps16</i>	F and 2R	Oxelman et al. (1997)
nrITS	ITSForwRub	CCTTATCATTTAGAGGAAGGAG
nrITS	ITSRevRub	CCTCCGCTTATTGATATGC
nrITS	P17 and 26S-82R	Popp and Oxelman (2001)
nrITS	P25	Oxelman (1996)
<i>ndhF</i>	2F	Rydin et al. (2008)
<i>ndhF</i>	1000R	Rydin et al. (2008)
<i>ndhF</i>	720F	Rydin et al. (2008)
<i>ndhF</i>	1700R	Rydin et al. (2008)
<i>ndhF</i>	1320F	Rydin et al. (2008)
<i>ndhF</i>	2280R	Rydin et al. (2008)
<i>atpB-rbcL</i> spacer	<i>rbcL5'R</i>	Rydin et al. (2008)
<i>atpB-rbcL</i> spacer	<i>atpB5'R</i>	Rydin et al. (2008)
<i>trnT–L–F</i>	A1	Bremer et al. (2002)
<i>trnT–L–F</i>	940R	Rydin et al. (2008)
<i>trnT–L–F</i>	820F	Rydin et al. (2008)
<i>trnT–L–F</i>	IR	Bremer et al. (2002)
<i>trnT–L–F</i>	1250F	Rydin et al. (2008)
<i>trnT–L–F</i>	D	Taberlet et al. (1991)
<i>trnT–L–F</i>	1880F	Rydin et al. (2008)
<i>trnT–L–F</i>	2670R	Rydin et al. (2008)

ambiguous parts of nrITS (specified above). We used Wilcoxon-signed rank tests implemented in VassarStats (Lowry 2008) to test for significant changes in posterior probabilities and bootstrap estimates between analysis including or excluding nrITS.

Parsimony analyses were performed in Paup* version 4.0b10 for Unix (Swofford 1998), for single gene data sets, as well as for combined data sets including and excluding

nrITS. Most parsimonious trees were calculated using the heuristic search option, 500 random sequence additions, tree bisection reconnection branch swapping. Support values were obtained by using bootstrap in Paup*, performing 1,000 bootstrap replicates, each with 10 random sequence additions with settings as before. A majority rule consensus tree was produced from the resulting trees, in which nodes with a bootstrap support <50% were collapsed.

Pollen morphology

Anthers with in situ pollen of *Acranthera tomentosa* R.Br. ex Hook.f., voucher: Vidal 5001 (P), were mounted on cleaned aluminium stubs and initially investigated under a stereomicroscope. For scanning electron microscopy (SEM), the material was coated with gold for 90 s in a sputter coater, and examined with a Hitachi Field Emission scanning electron microscope at 5 kV.

Results

Data

The aligned six-gene data set included 149 terminals and 10,555 characters, from which 1,402 derived from *rbcL*, 1,602 from *rps16*, 2,243 from *ndhF*, 1,098 from *atpB-rbcL* spacer, 3,219 from *trnT–L–F*: 3,219, 925 from nrITS and 66 from indels (see also Table 1). The nrITS alignment with potentially ambiguous parts removed contained 677 characters. The number of variable and informative characters, number of supported nodes and average support values are given for single gene analyses in Table 1 and for combined analyses in Table 3.

Model choice

For each single gene analysis, the best performing model according to the corrected Akaike information criterion

Table 3 Results of selected combined analyses

	5 regions + indels	6 regions + indels	6 regions + indels (parts of nrITS removed)	6 regions (no indels)
Number of characters in matrix	9,630	10,555	10,307	10,489
Number of variable characters	5,228	5,778	5,614	5,712
Number of informative characters	3,449	3,952	3,757	3,886
Number of supported nodes (bootstrap)	120	129	128	128
Number of supported nodes (Bayesian)	133	136	–	133
Average support (bootstrap)	90.84	92.15	89.56	90.60
Average support (Bayesian)	96.60	97.15	–	96.66

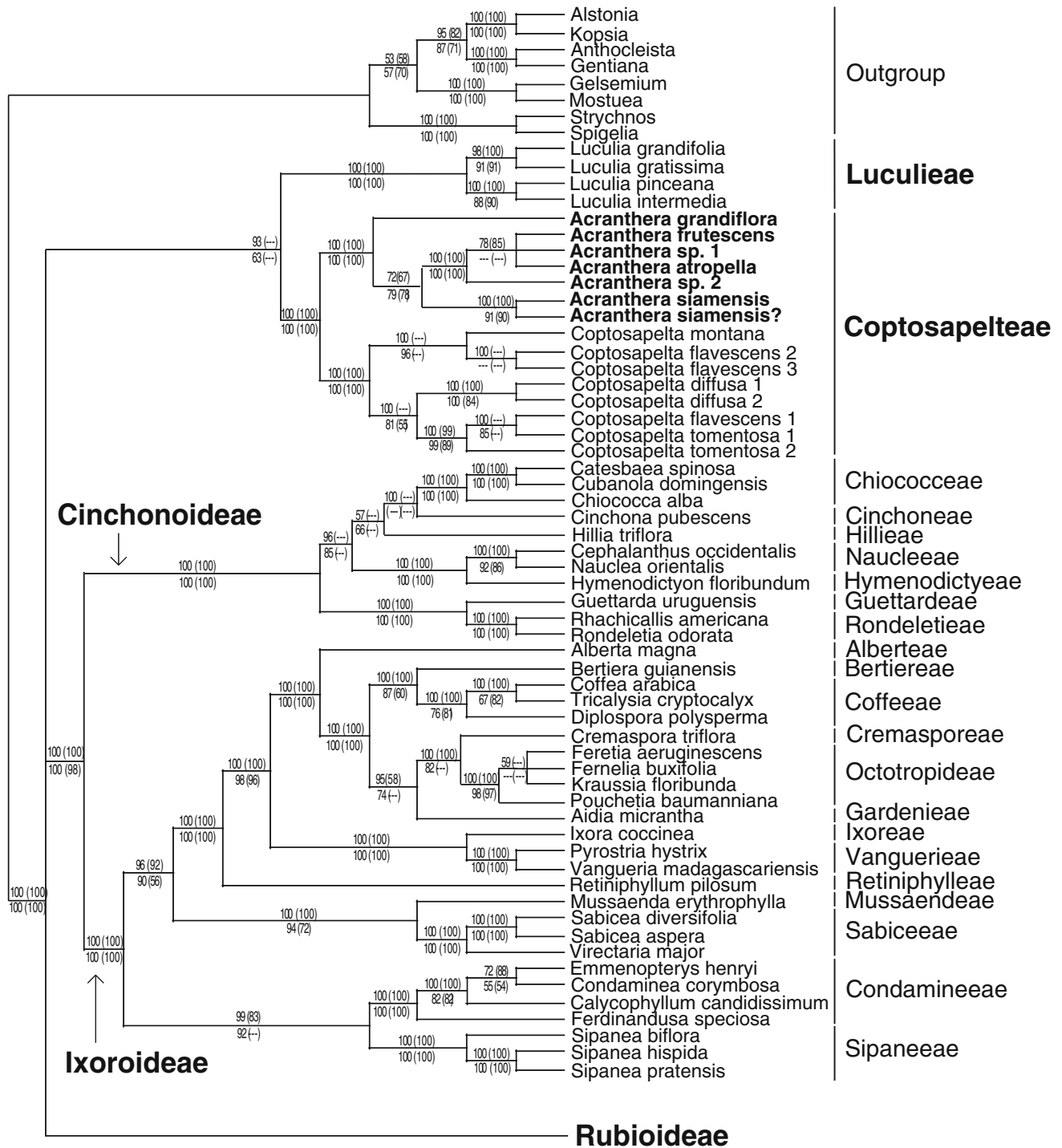


Fig. 1 Relationships within the tribes Luculieae and Coptosapelteae; and the subfamilies Cinchonoideae and Ixoroideae, estimated using Bayesian inference of phylogeny based on molecular data from chloroplast regions *rbcL*, *rps16* intron, *ndhF*, *atpB-rbcL* spacer,

trnT-L-F, the nuclear ribosomal ITS and indels. Posterior probabilities are given *above branches*, bootstrap values (under parsimony) *below*. Support values from the analyses of chloroplast data (excluding nrITS) are given (*in brackets*)

(AICc, Akaike 1973) was selected. AICc is appropriate when the ratio between sample size and number of parameters is small ($n/K < 40$, Burnham and Anderson 2003, p. 66), but also for higher ratios because AICc will

then converge to AIC (Posada and Buckley 2004). Empirically, the three criteria indicated the same best performing model for all matrices. For the *rbcL*, *trnT-L-F* and nrITS data, the general time reversible model (Tavare

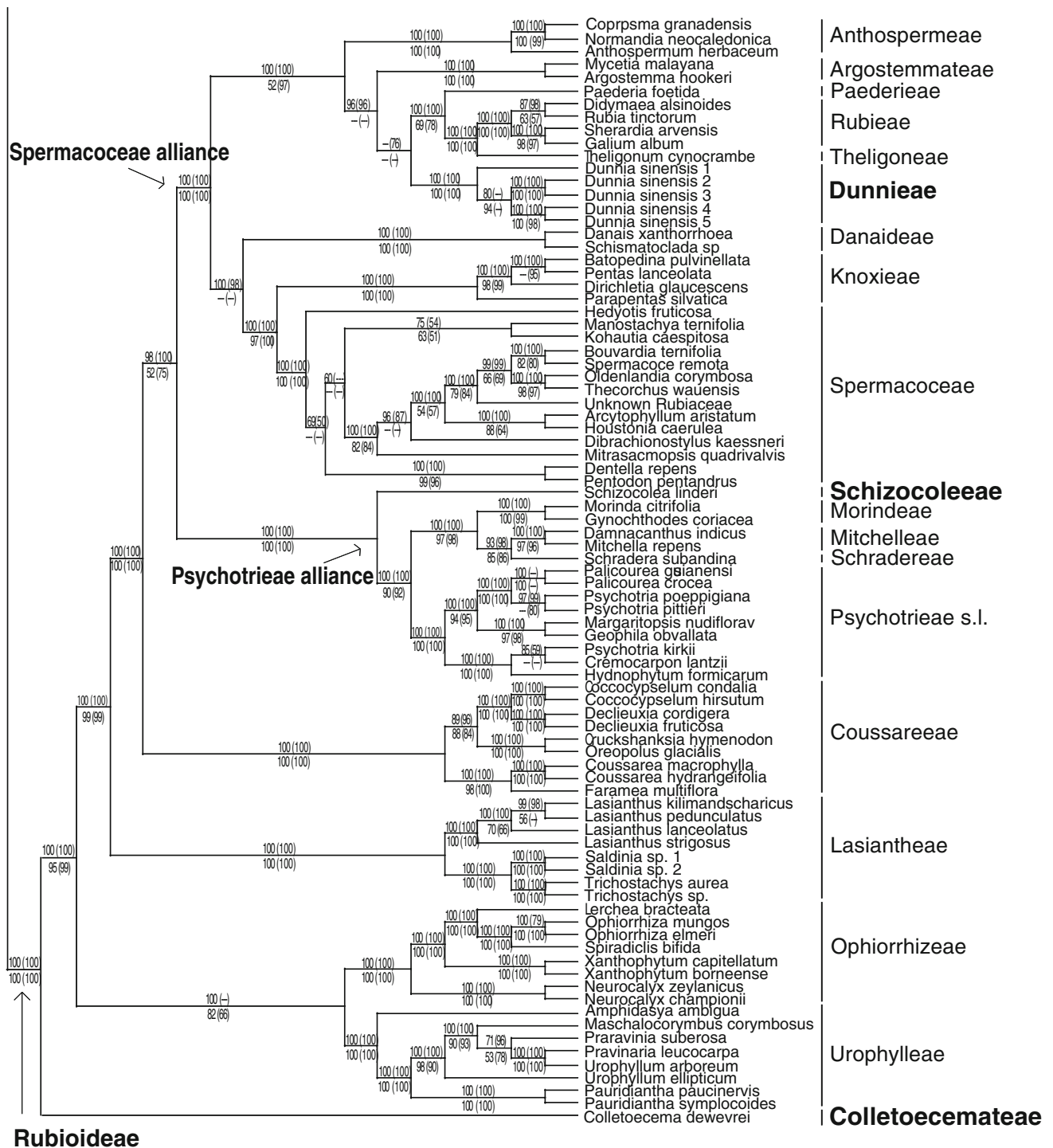


Fig. 2 Relationships within subfamily Rubioideae, estimated using Bayesian inference of phylogeny based on molecular data from chloroplast regions *rbcl*, *rps16* intron, *ndhF*, *atpB-rbcL* spacer, *trnT-L-F*, the nuclear ribosomal ITS and indels. Posterior

probabilities are given *above branches*, bootstrap values (under parsimony) *below*. Support values from the analyses of chloroplast data (excluding nrITS) are given (*in brackets*)

1986) with gamma distributed rates (Yang 1993) and a proportion of invariable sites was selected (GTR + I + Γ). For the *rps16*, *ndhF* and the *atpB-rbcL* spacer, GTR + Γ

was selected (Table 1). For combined analyses with less than seven partitions, GTR + Γ was selected for the chloroplast partition.

Analyses

Combined data set

As described in “Materials and methods”, the combined data set was analysed several times, partitioning the data set in different ways. These analyses resulted in nearly identical topologies, but with slight differences in resolution and support values. We observed no supported (i.e. $\geq 50\%$ posterior probability and/or bootstrap support) conflicts between results obtained from the different combined analyses. Figures 1, 2 show the results from the Bayesian analysis including information from indels (two data partitions: nucleotide data and indels). Bootstrap values of 50% or more are plotted on the Bayesian tree. We have further indicated (within brackets) support values from the 5-cp gene analysis (excluding nrITS).

Usefulness of nrITS for addressing deep divergences in Rubiaceae

Including nrITS generally increased resolution and support values over the entire phylogeny (Figs. 1, 2; Table 3). Some nodes received a lower support when nrITS was added, but overall resolution and average support (arithmetic mean) increased. The phylogeny based on *rbcl*, *rps16*, *ndhF*, the *atpB-rbcL* spacer and *trnT-L-F* (excluding nrITS) had 120 supported nodes with an average bootstrap value of 90.84%. The tree also based on nrITS data had 129 supported nodes with an average bootstrap support of 92.15%. For Bayesian analyses, the analysis excluding nrITS had 133 supported nodes with an average posterior probability of 96.60%. Including nrITS yielded 136 supported nodes with an average posterior probability of 97.15%. However, the increase in mean support values was not statistically significant (Table 4), neither in Bayesian analyses ($z = 0.98$, $P = 0.327$), nor in bootstrap analyses ($z = 0.92$, $P = 0.358$). For subfamily Rubioideae, mean bootstrap support was slightly lowered when including nrITS, but the difference was not significant ($z = -0.46$, $P = 0.6455$). In Bayesian analyses, support values increased also in Rubioideae when including nrITS, but again not significantly ($z = 0.16$, $P = 0.8729$).

The topology from the analysis of six genes, excluding potentially ambiguous sites in nrITS, was basically the same as for the complete six-gene topology but support values generally decreased and some resolution was lost (Table 3). The sister relationship between *Luculia* and the *Acranthera-Coptosapelta* clade was for example collapsed in this tree, as was the case in the 5-cp analysis, excluding nrITS altogether (Fig. 1).

Table 4 Test for significance of differences in support values, when including/excluding nrITS

Including/excluding nrITS	z	P (two-tailed)
Bayesian posterior probabilities (Rubiaceae)	$z = 0.98$	$P = 0.3271$
Bayesian posterior probabilities (clade A ^b)	– ^a	– ^a
Bayesian posterior probabilities (clade B ^b)	– ^a	– ^a
Bayesian posterior probabilities (clade C ^b)	$z = 0.16$	$P = 0.8729$
Bootstrap values (Rubiaceae)	$z = 0.92$	$P = 0.3576$
Bootstrap values (clade A)	– ^a	– ^a
Bootstrap values (clade B)	$z = 1.55$	$P = 0.1211$
Bootstrap values (clade C)	$z = -0.46$	$P = 0.6455$

Wilcoxon signed-rank test

^a $n_{s/r}$ too small

^b Clade A: *Luculia-Coptosapelta-Acranthera*; clade B: Cinchonoideae-Ixoroideae; clade C: Rubioideae

Single gene analyses

We found no major conflicts between single gene data sets and no conflicts within each region (between parsimony and Bayesian analyses, or when including or excluding gap information, see also Table 1). The position of a few taxa varied between single gene data sets and supported deviations are presented below.

Phylogeny—the combined data set

Deep divergences and the *Luculia-Acranthera-Coptosapelta* clade

All ingroup taxa were resolved in three (or four) major clades (Figs. 1, 2). 1: The *Luculia-Acranthera-Coptosapelta* clade (which collapsed in the 5-cp analysis into one *Luculia* clade and one *Acranthera-Coptosapelta* clade); 2: the Cinchonoideae-Ixoroideae clade; 3: the Rubioideae clade. Support was very high for the latter two groups (support values are presented as follows [Bayesian posterior probability including nrITS (posterior probability excluding nrITS)/bootstrap support including nrITS (bootstrap support excluding nrITS)]: Cinchonoideae-Ixoroideae [100 (100)/100 (98)] and Rubioideae [100 (100)/100 (100)]. *Luculia*, *Acranthera* and *Coptosapelta* fell outside these groups. *Acranthera* and *Coptosapelta* were sister groups in all analyses [100 (100)/100 (100)], a result which to our knowledge has not been presented before. *Luculia* was sister to the *Acranthera-Coptosapelta* clade with relatively high Bayesian posterior probability, but low bootstrap support and only recovered when information from the entire nrITS was included [93 (–)/63 (–)].

All currently recognised species of *Luculia* were included in this study and we show that the genus is monophyletic [100 (100)/100 (100)]. Our results also support the monophyly of *Acranthera* [100 (100)/100 (100)] and *Coptosapelta* [100 (100)/100 (100)].

Results within the Cinchonoideae–Ixoroideae clade

Support values for Cinchonoideae and Ixoroideae (Fig. 1) were high [100 (100)/100 (100)]. Within Cinchonoideae, Rondeletiae–Guettardeae [100 (100)/100 (100)] was sister to a large clade comprising Hymenodictyeae, Naucleaeae, Hilliaeae, Cinchoneae and Chiococceae [96 (–)/85 (–)]. Hymenodictyeae and Naucleaeae formed a clade [100 (100)/100 (100)]. Hilliaeae was sister to Cinchoneae and Chiococceae [100 (–)/– (–)].

Within Ixoroideae, Sipaneeae and Condamineeae were sister groups [99 (83)/92 (–)], and this clade was sister to remaining Ixoroideae [96 (92)/90 (56)]. Sabiceae and Mussaendeae [100 (100)/94 (72)] comprise the next diverging clade, followed by *Retiniphyllum*. The position of *Retiniphyllum* was strongly supported. Within remaining Ixoroideae, Vanguerieae and Ixoreae [100 (100)/100 (100)] were sister to a clade comprising *Alberta*, Coffeaeae, Bertiereae, Cremasporeae, Octotropideae and *Aidia* [100 (100)/100 (100)], within which *Alberta* was sister to the two sister clades [100 (100)/100 (100)]: Coffeaeae–Bertiereae [100 (100)/87 (60)] and Cremasporeae–Octotropideae–*Aidia* [95 (58)/74 (–)]. Within the latter, *Aidia* was sister to a Cremasporeae–Octotropideae clade [100 (100)/82 (–)].

Results within Rubioideae

Subfamily Rubioideae (Fig. 2) was well supported [100 (100)/100 (100)]. *Colletocema dewevrei* was sister to remaining Rubioideae with high support [100 (100)/95 (99)]. The next diverging clade consisted of Urophyllaeae [100 (100)/100 (100)] and Ophiorrhizeae [100 (100)/100 (100)], which grouped together with relatively high support [100 (–)/82 (66)]. Lasiantheae [100 (100)/100 (100)] was the next diverging group, followed by Coussareae [100 (100)/100 (100)], which was sister group to the Psychotrieae and Spermaceae alliances [98 (100)/52 (75)].

The Psychotrieae alliance [100 (100)/100 (100)] was here represented by 15 species. *Schizocolea linderi* was highly supported as sister to a clade comprising the remaining sampled taxa [100 (100)/90 (92)]. The remaining species comprised two sister groups: 1) Mitchellaeae–Schradereae [93 (98)/85 (86)], sister to Morindeae [100 (100)/100 (99)], and 2) Psychotrieae s.l. [100 (100)/100 (100)].

The Spermaceae alliance [100 (100)/100 (100)] comprised two major clades: The first was here represented

by Anthospermeae, Argostemmatae, Paederieae, Rubieae, Theligoneae and Dunnieae [100 (100)/52 (97)]. Amongst these, Anthospermeae [100 (100)/100 (100)] was the earliest diverging group, followed by Argostemmatae [100 (100)/100 (100)]. The next diverging clade [– (76)/ –(–)] comprised Dunnieae [100 (100)/100 (100)] and its sister clade [100 (100)/69 (78)], which consisted of Paederieae and Rubieae–Theligoneae [100 (100)/100 (100)]. Within the second major clade of the Spermaceae alliance [100 (98)/ –(–)], Danaideae [100 (100)/100 (100)] was sister to Knoxiaceae–Spermaceae [100 (100)/97(100)].

Phylogeny—single gene data sets

Generally, single gene analyses produced the same topologies as those obtained from the combined data set. There are some minor deviations and we arbitrarily decided that differences with a Bayesian posterior probability higher than 85%, and/or a bootstrap support higher than 50% can be considered “supported”. Such differences are presented here (posterior probability/bootstrap index).

rbcL

The results from the Bayesian analysis of the *rbcL* data resolved Ophiorrhizeae and Urophyllaeae as a basal grade (instead of a clade) within Rubioideae (95/– for Rubioideae except Ophiorrhizeae). The result was not supported in the bootstrap analysis of the *rbcL* data.

rps16

The analyses of the *rps16* data resolved *Luculia*, *Acranthera* and *Coptosapelta* as sister to the Cinchonoideae–Ixoroideae clade (93/76).

ndhF

In the *ndhF* tree, the *Acranthera*–*Coptosapelta* clade was sister to remaining Rubiaceae including *Luculia* (98/85). *Colletocema* was sister to Lasiantheae (90/–). This relationship was not supported in bootstrap analyses. Sipaneeae and Condamineeae formed a basal grade (not a clade) within Ixoroideae. Support for Condamineeae and remaining Ixoroideae was low (55/80).

trnT–L–F

In analyses based on the *trnT–L–F* data, the *Acranthera*–*Coptosapelta* clade was sister to Rubioideae (88/80). There were some differences amongst major clades in the Spermaceae alliance, regarding the positions of Danaideae, Anthospermeae and Argostemmatae. The differences had

a Bayesian posterior probability of 80–90% but were not present in the bootstrap tree. These results are further investigated elsewhere.

nrITS

The nrITS data resolved *Colletocema* as sister to the Urophyllaeae–Ophiorrhizeae clade (82/76). Coussareeae grouped together with a collapsed Anthospermeae (95/–). This relationship was not supported in the bootstrap analysis.

Pollen morphology

Because our results strongly support *Acranthera* as sister to *Coptosapelta*, which has unique pollen morphology (Verellen et al. 2004), we made a preliminary SEM study of *Acranthera* pollen. *Acranthera* pollen (Fig. 3) is triangular (rarely quadrangular) in shape and spheroidal to subspheroidal, with a polar axis of about 17 μm and equatorial diameter of 18–22 μm . They have three (rarely four) apertures positioned at the angles. The apertures are of a compound, colpate type. The ectoaperture is a short colpus (6–8 μm long), with acute to obtuse endings. The mesoaperture is a pore with a diameter of about 3–4 μm . Each mesoaperture is covered by an apertural

protrusion. The sexine is (micro)reticulate-perforate but differs probably between mesocolpial and apocolpial areas. Structures tentatively interpreted as aborted grains (ovoid, about 3 μm long, roughly undulating-palliate surface and apertures, not shown), were numerous present amongst the grains. Note: this SEM study represents a preliminary overview of characters found in grains (not acetolysed) from one specimen. Further studies are needed to provide more details and detect potential inter and intraspecific variation in *Acranthera* pollen.

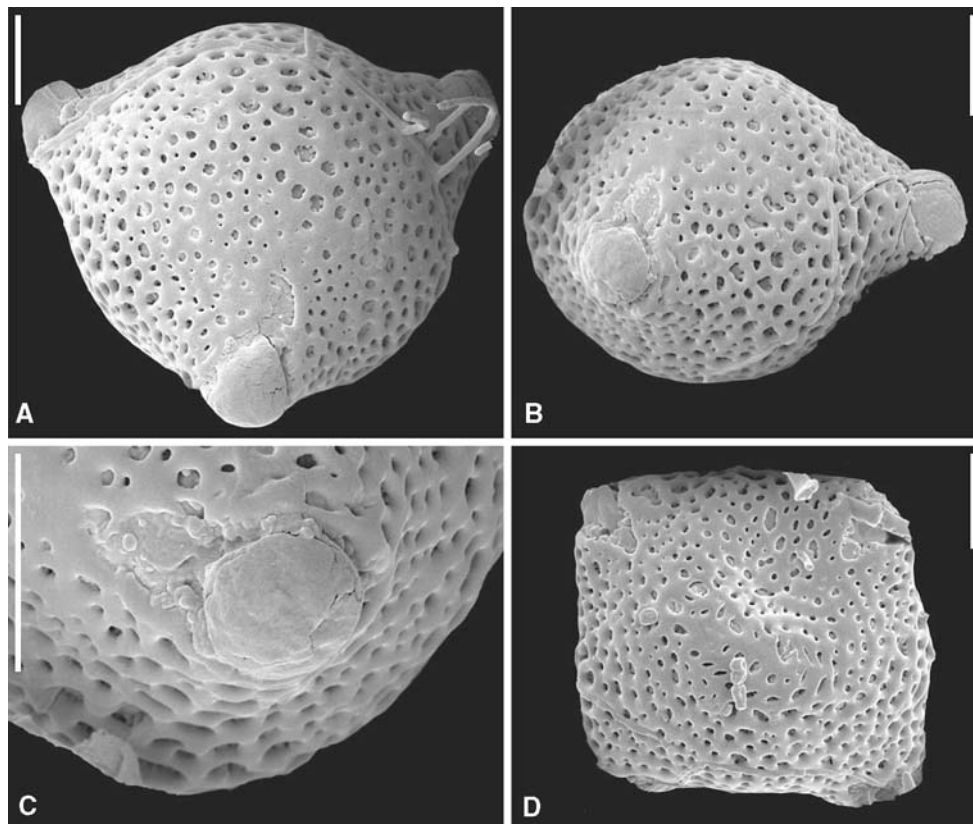
Taxonomic implications

Based on the results, we describe four new tribes and one new tribal circumscription. Our decisions are based on the principles of classification outlined in Backlund and Bremer (1998).

Acranthera is strongly supported as sister to *Coptosapelta* and we have included *Acranthera* in the tribe Coptosapelteae.

Considering the persisting difficulties to find support for a close relationship between *Luculia* and other species of Rubiaceae, we have chosen to describe the new monogeneric tribe Luculieae. Luculieae and Coptosapelteae are clearly excluded from the three subfamilies Ixoroideae,

Fig. 3 Pollen grains of *Acranthera tomentosa* (SEM): **a** Polar view. *Acranthera* pollen is generally triangular in shape, spheroidal to subspheroidal and about 18–22 μm in equatorial diameter. The sexine is (micro)reticulate-perforate. The grains have three apertures positioned at the angles. **b** Equatorial view. **c** The apertures are of a compound, colpate type; the ectoaperture is a short colpus and the mesoaperture is a pore. Each mesoaperture is covered by an apertural protrusion. **d** Polar view. *Acranthera* grains are rarely quadrangular with four apertures positioned at the angles. Scale bars 5 μm



Cinchonoideae and Rubioideae, but we do not propose a new subfamily for the *Luculia–Coptosapelta–Acranthera* clade at this point. The clade is relatively well supported (93%) in the Bayesian analysis of the six-gene data set, but poorly supported in bootstrap analysis (63%), and collapsed in five-gene data sets. Further studies are needed to confirm the monophyly of the *Luculia–Acranthera–Coptosapelta* clade.

Three genera, *Colletocema*, *Schizocolea* and *Dunnia*, are lone sister lineages of large clades comprising several well-defined tribes. They cannot be implemented in any of the existing tribes and we have therefore described the new monogeneric tribes Colletocemateae, Schizocoleae and Dunnieae (see below).

Discussion

In order to address deep divergences in Rubiaceae, we sampled a large data set comprising 149 terminals and nearly 11,000 characters. The project has thus had potential to address a number of previously unresolved relationships and conflicting results throughout the family. Morphology and character evolution are discussed but obvious morphological support for major groups defined by molecular data may be difficult to find.

The usefulness of nrITS

Nuclear ribosomal ITS has previously been used for resolving higher-level relationships within Rubiaceae (e.g. Andreasen et al. 1999) but not for addressing the phylogeny of the entire family. A comparison of the topologies from analyses including and excluding nrITS shows that when nrITS is included, resolution and/or support increase for relationships within several groups of interest here, for example, the sister relationships between Urophyllae and Ophiorrhizeae, between Sipaneeae and Condamineeae and between *Luculia* and the *Coptosapelta–Acranthera* clade (Figs. 1, 2).

There are also nodes (for example in the Spermaceae alliance), for which support values decrease when nrITS is included and we conducted a bootstrap analysis on the combined six-gene data set, excluding two short regions of nrITS where homology assessments were difficult and potentially ambiguous. The resulting topology was nearly identical to that obtained from the complete six-gene data set, but slightly less well resolved and with a distinctly lower average support value (Table 3). In the present study, nrITS thus provided structured information, which resulted in increased resolution. Nuclear ITS also contributed to an increase in average support, however, many nodes were well-supported also without information from

nrITS and the increase in support values was not statistically significant.

New insights into evolutionary relationships—*Acranthera*

The sister relationship between *Acranthera* and *Coptosapelta* is very well supported in all combined and single gene analyses except in the analysis of nrITS, where the node is present but less well supported (94/–). Our results further support the monophyly of the two genera. To our knowledge, these results have not been presented before.

Although the *Acranthera–Coptosapelta* clade is well supported by molecular data, we find no unambiguous morphological support for the relationship. Bremekamp (1947, p. 273) discussed a potential synapomorphy for *Acranthera* and *Coptosapelta*: the style functioning as a temporary depository for pollen, a “receptaculum pollinis”. However, Puff et al. (1995) considered such a structure in *Acranthera* a misconception and they consequently refuted this synapomorphy for *Coptosapelta* and *Acranthera*. Further, even though Bremekamp (1947) suggested secondary pollen presentation as a potential synapomorphy for *Acranthera* and *Coptosapelta*, he argued that the united apical connective appendage in *Acranthera* is a feature unique within Rubiaceae and similar to the morphology of stamens in Apocynaceae. Puff et al. (1995) also considered the “anther–style and stigma complex” of *Acranthera* unique within Rubiaceae, in structure as well as function.

Pollen grains of *Coptosapelta* possess several features unique within Rubiaceae (Verellen et al. 2004). They are pororate and may have up to 10 apertures (even if 3–4 apertures are most common), they lack columellae and they have “droplets” on the inner nexine (Verellen et al. 2004). *Acranthera* pollen has so far not been thoroughly documented (but see Mathew and Philip 1983) and in order to get an indication on whether *Acranthera* pollen shares some of the features of *Coptosapelta* grains, we performed a preliminary SEM study of the outer surface of the grains and the nature of the apertures (Fig. 3).

Several characters of *Acranthera* pollen are common in Rubiaceae and probably plesiomorphic. *Acranthera* grains are not pororate (like *Coptosapelta* grains) but colporate, which is considered the plesiomorphic condition in the family (Dessein et al. 2005). The size of *Acranthera* grains (18–22 µm in equatorial diameter) fits within the 20–40 µm, which is most common in Rubiaceae (Dessein et al. 2005). The triangular (rarely quadrangular) shape is more unusual but occurs according to Dessein et al. (2005) for example in *Tapiphyllum* Robyns (i.e. *Vangueria* Juss.) and *Psydrax* Gaertn. (Vanguerieae, Ixoroideae). Apertural protrusions (papillae-forming onci), pollen buds and

structures that cover the aperture (opercula) have been reported for several genera of Rubiaceae, but to our knowledge, not for *Coptosapelta*.

There are some potential similarities between *Acranthera* and *Coptosapelta* pollen. The short ectocolpi of *Acranthera* could perhaps be compared with the ectopores of *Coptosapelta* and the microreticulate to perforate sexine in *Acranthera* is similar to that described for some species of *Coptosapelta* (Verellen 2002). However, pollen characters in *Acranthera* need to be further studied (e.g. the presence or absence of columellae, “droplets” on the inner nexine, the nature of the apertural protrusions) before any hypotheses on synapomorphies can be put forward.

The enigmatic *Luculia*

Our study included all four currently recognised species of *Luculia* (Govaerts et al. 2006) and we show that the genus is monophyletic, but its relationship to other species of Rubiaceae remains uncertain. The clade comprising *Luculia*, *Acranthera* and *Coptosapelta* is here only supported in some of the single gene analyses (*atpB-rbcL* spacer and nrITS) and in combined analyses including nrITS. However, no analysis resulted in a well-supported alternative position for *Luculia*. Further, there is biogeographical support for a relationship between these three South East Asian genera and a relationship between *Luculia* and *Coptosapelta* has been indicated in other recent studies (Robbrecht and Manen 2006).

The *Luculia*–*Acranthera*–*Coptosapelta* clade is equally puzzling from a morphological perspective as is the *Acranthera*–*Coptosapelta* clade. Korthals (1851) very briefly mentioned some similarities between *Luculia* and *Coptosapelta* regarding the form of the seed, but he did not specify this further. Bremekamp (1947, p. 261) considered corolla aestivation, insertion of the stamens in the corolla tube and many-seeded fruits important regarding the systematic position of *Acranthera*, but these characters provide no support for the *Luculia*–*Acranthera*–*Coptosapelta* clade. Corolla aestivation is imbricate in *Luculia* (Bremer and Struwe 1992), valvate in *Acranthera* (Bremekamp 1947) and contorted in *Coptosapelta* (Andersson and Persson 1991). Filaments are inserted at the base of the corolla tube in *Acranthera* (Bremekamp 1947), but at about one-third from the mouth of the corolla tube in *Coptosapelta* and *Luculia* (Andersson and Persson 1991). All three genera have many-seeded fruits (Sweet 1826; Korthals 1851; Bremekamp 1947), but this character is common in Rubiaceae and probably plesiomorphic.

Pollen characters also show little resemblance between the three genera. *Luculia* grains are small to medium-sized, 22–24 µm in polar axis (Murray 1990), spheroidal, 3(–4)-colporate and with a reticulate tectum (Dessein et al. 2005).

These character states probably represent primitive states within the family (Dessein et al. 2005) so even though grains of *Coptosapelta* are (oblate)spheroidal (Verellen et al. 2004), and *Acranthera* grains are (tri)colporate (the present study), these respective similarities with *Luculia* grains are likely plesiomorphic. The more specialised respective features of *Coptosapelta* and *Acranthera* pollen, e.g. the pororate pollen of *Coptosapelta* and the triangular shape of *Acranthera* grains, are not present in *Luculia*.

Early divergences within the family

Despite that we have used a relatively extensive sampling of taxa and characters in this study, the major clades of the family form a basal trichotomy: (1) the *Luculia*–*Acranthera*–*Coptosapelta* clade, (2) a clade consisting of the subfamilies Cinchonoideae and Ixoroideae, (3) subfamily Rubioideae (Figs. 1, 2).

Robbrecht and Manen (2006) argued, based on parsimony analyses of 15 selected species and eight gene regions, that *Luculia* and *Coptosapelta* (*Acranthera* was not investigated) are “basal to the rest of Cinchonoideae” (i.e. sister to the Cinchonoideae–Ixoroideae clade). However, this conclusion is not supported by their results. Their combined analysis had no support for the position of these genera (Robbrecht and Manen 2006, Fig. 2) and the super tree analysis placed *Luculia* and *Coptosapelta* as sister to the rest of the family, not sister to the Cinchonoideae–Ixoroideae clade (Robbrecht and Manen 2006, Fig. 4a). Results from super tree analyses are difficult to evaluate; trees from the literature often contain some poorly supported nodes, which consequently may decrease accuracy of the super tree. Further, some information in the original data sets is lost, because the character information is simplified into a phylogeny (de Queiroz and Gatesy 2007). When analysing a combined data set, it is possible to get increased support for relationships that are not supported, perhaps not even present, in the single gene analyses (see e.g. Kluge 1989; Olmstead and Sweere 1994). This has, however, not been the case regarding basal relationships in Rubiaceae. Different gene regions produce contradicting (poorly supported) results and the combined analyses are unresolved (the present study and Robbrecht and Manen 2006).

Ixoroideae

Sipaneeae and Condamineeae form a strongly supported clade, which is sister to the remaining Ixoroideae. Sabiceae and Mussaendeae are sisters (see also Alejandro et al. 2005) and comprise the next diverging clade, followed by Retiniphyllae. Two additional well-supported relationships within Ixoroideae have not been presented

before: Retiniphyllae sister to the (Vanguerieae–Ixoreae) + (Alberteae–remaining Ixoroideae) clade (Fig. 1).

It should be noted, however, that no representatives of Posoqueriidae and Henriqueziidae are included in the present study. Further, Sipaneeae and Condamineeae are not sisters but form a grade to remaining Ixoroideae in our *ndhF* analyses and this is consistent with results found in Kainulainen et al. (in press).

Cinchonoideae

In our study, Rondeletiae and Guettardeae form a clade, sister to the remaining Cinchonoideae. The result is well supported but differs from that reported in Andersson and Antonelli (2005), where Naucleaeae and Hymenodictyeae constituted the sister clade to the remaining Cinchonoideae. The sister-group relationship between Naucleaeae and Hymenodictyeae, previously shown by Razafimandimbison and Bremer (2001) and later endorsed by Andersson and Antonelli (2005), is further supported by our analyses, as well as by pollen morphology (Verellen et al. 2007). However, an extended sampling in Cinchonoideae is needed to further address the relationships and evolution of the group (see Manns and Bremer 2008).

Rubioideae

The sister relationship between *Colletocema dewevrei* and remaining Rubioideae is here confirmed with high support (see also Robbrecht and Manen 2006; Rydin et al. 2008). The next diverging clade comprises the East Asian Ophiorrhizeae and the pantropical Urophyllaeae. This is consistent with Andersson and Rova (1999), but the tribes have otherwise often had an unresolved position at the base of Rubioideae or they have formed a basal grade, being subsequent sister groups to the rest of the subfamily (Bremer and Manen 2000; Robbrecht and Manen 2006; Razafimandimbison et al. 2008). The sister-group relationship between Ophiorrhizeae and Urophyllaeae is well supported, but as often is the case for major groups in Rubiaceae, obvious morphological support is difficult to find.

Spiradiclis bifida, is here sister to *Ophiorrhiza* (Fig. 2), but a *rps16* sequence (Rydin et al. 2006) nested *Spiradiclis caespitosa* Blume within *Ophiorrhiza*. The monophyly of the two genera needs to be investigated further.

Coussareae is a morphologically variable group, restricted to the New World. Most species occur in lowland rainforests, but the monotypic genus *Oreopolus* inhabits the Andean regions. Several studies have contributed to our understanding of relationships between the genera in Coussareae (Andersson and Rova 1999; Bremer and Manen 2000), but they were based on the less amounts of

data and did not include representatives from all genera. We show that *Coussareae–Faramaea* constitutes the sister clade to remaining genera. *Oreopolus* and *Cruckshanksia* have long been considered related based on morphology (Taylor 1996), but few phylogenetic studies have included *Cruckshanksia*. We confirm, with high support, the close relationship between *Oreopolus* and *Cruckshanksia*. *Heterophyllaea* Hook.f. also belongs to this group (Andersson and Rova, 1999), sister to the *Oreopolus–Cruckshanksia* clade (Rydin et al. 2006). These three genera are all restricted to the western parts of South America. The Neotropical genera *Coccocypselum* and *Declieuxia* are sisters and results from Rydin et al. (2006) highly support the inclusion of *Hindsia* Benth. ex Lindl. in this clade, as sister to *Declieuxia*. Piesschaert et al. (2000b) discussed morphological as well as biogeographical support for the *Coccocypselum + Declieuxia–Hindsia* clade.

The tribe Danaideae is here sister to the Knoxiidae–Spermacoceae clade. The posterior probability for this relationship is high, but the clade is collapsed in bootstrap consensus trees. In Bremer and Manen (2000) Danaideae was sister to the remaining Spermacoceae alliance (with very low bootstrap support). More research is needed to further assess the position of Danaideae.

Conclusions

The systematic position of *Acranthera*, a long-debated question, is resolved; *Acranthera* and *Coptosapelta* are sisters. *Acranthera* is considered unique within Rubiaceae in reproductive characters and obvious morphological synapomorphies for the *Acranthera–Coptosapelta* clade are currently not known, but the well-supported result in all our analyses leaves little doubt about their close relationship. We performed a preliminary study of the pollen grains of *Acranthera* in an attempt to find synapomorphies with the unique pollen of *Coptosapelta*, but most characters of *Acranthera* grains (for example size, the colpate grains with three apertures positioned at angles and the reticulate sexine) are common in Rubiaceae and probably plesiomorphic. There are some potential (derived) similarities though; future studies may reveal new insights on morphological features of the clade.

Luculia is sister to *Acranthera–Coptosapelta* but the clade is only well-supported in Bayesian analyses including nrITS. Nuclear ITS has traditionally been utilised mainly for studying higher-level relationships, e.g. within a genus, but it cannot be a priori assumed that homology assessments are impossible for certain loci at certain taxonomic levels. Here, nrITS provided structured information on deep divergences, as well as on higher-level relationships in Rubiaceae, and appear particularly useful in Cinchonoideae and Ixoroideae.

Basal relationships within the three subfamilies Rubioideae, Cinchonoideae and Ixoroideae are indicated in the present study, but deep divergences in the family were not resolved. Single gene regions produced contradicting (poorly supported) results and combined analyses resulted in a basal polytomy consisting of (1) *Luculia*–*Acranthera*–*Coptosapelta*, (2) an Ixoroideae–Cinchonoideae clade, (3) Rubioideae. Like for example major relationships amongst seed plants (Burleigh and Mathews 2007a, 2007b); mosses and worts (Qiu et al. 2006); the position of *Equisetum* (Schuettpelz et al. 2006) and relationships within the angiosperm clades Ericales (Schoenenberger et al. 2005), Lamiales (Wortley et al. 2005) and Malpighiales (APGII 2003), early radiation patterns within Rubiaceae have not been unambiguously resolved despite that large amounts of data have been analysed. In cases when molecular markers produce conflicting results, other kinds of data, for example structural rearrangements in the genomes, developmental biology and comparative morphology, may be useful when discriminating between alternative hypotheses.

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Appendix: FAMILY—RUBIACEAE JUSS.

Tribe *Luculieae* Rydin and B. Bremer, tribus nov.

Type: *Luculia* Sweet

Diagnosis: Arbuscula. Calyx 5-merous, corolla 5-mera, tubo longo vix supra dilatato. Flores heterostyli. Antherae intra tubum subsessiles semiexsertae. Stigmata 2, ovarium 2-loculare, loculis polyspermis. Fructus baccatus, semina minuta.

Description: Small trees or shrubs, opposite phyllotaxis. Stipules deciduous, lanceolate to linear. Flowers large, showy, pentamerous, heterostylous. Stamens inserted in narrow corolla tube, filaments short. Ovary bilocular, fruit baccate, seeds small, numerous.

Genus included: *Luculia* Sweet

Useful publications: Murray (1990); Bremer et al. (1999).

Tribe *Coptosapelteae* Bremek. ex S. Darwin, Taxon 25: p. 600, (Darwin 1976), emend. Rydin and B. Bremer

Type: *Coptosapelta* Kort.

Description: Sparsely branched subshrubs or vines. Flowers usually pentamerous (rarely 4 or 6 parted). Ovary bilocular, fruit a capsule, seeds numerous. Chromosome basic number 10–11, *Acranthera* x10 (Kiehn 1995), *Coptosapelta* x11 (Verdcourt 1958; Puangsomlee and Puff 2001).

Note: The new circumscription is based on molecular evidence presented in this paper. Morphological synapomorphies are not known at this point.

Genera included: *Coptosapelta* Kort., *Acranthera* Arn. ex Meisn.

Useful publications: Alejandro et al. (2005); Verellen et al. (2004); Puangsomlee and Puff (2001); Bremer et al. (1999); Kiehn (1995); Puff et al. (1995); Bremekamp (1947); Valetton (1923); Rydin et al. (this study).

SUBFAMILY—RUBIOIDEAE VERDC.

Bull. Jard. Bot. État Brux. 28: 280 (1958)

Tribe *Colletocemateae* Rydin and B. Bremer, tribus nov.

Type: *Colletocema* E.M.A. Petit

Diagnosis: Arbores vel fructices, stipulis integris. Inflorescentiae axillares floribus multis conglomeratis. Flores heterostyli, 5-meri. Calyx cupuliformis, corolla tubiformis, stamina filamentis longis sub sinibus corollae adfixis. Ovarium 2-loculare, ovulo 1. Fructus drupaceus, pyrena 2-loculare, semina albumine satis molli et oleoso, embryo teres.

Description: Small trees or shrubs. Inflorescences axillary, flowers pentamerous, heterostylous. Stamens inserted in corolla tube. Ovary bilocular, one ovule per locule. Embryo long and narrow. Fruit a drupe, pyrenes bilocular.

Genus included: *Colletocema* E.M.A. Petit

Useful publications: Petit (1963); Piesschaert et al. (2000a); Robbrecht and Manen (2006); Rydin et al. (2008).

***Schizocoleae* Rydin and B. Bremer, tribus nov.**

Type: *Schizocolea* Bremek.

Diagnosis: Arbuscula. Stipulae in vaginam longam et angustam in fimbriis plerumque 8 fissam connatae. Flores in axillis foliorum dispositi. Calyx 5-merous, lobis e basi triangulari-setiformibus, hirsutis. Corolla hypocrateriformis, tubo calycem longitudine multo excedente. Stamina parte dilatata tubi inserta. Ovarium biloculare, loculis septo tenui separatis. Fructus baccatus, monospermus.

Description: Small trees, stipules bordered with fine hairs. Flowers pentamerous, calyx triangular at base. Corolla extends beyond calyx, stamens inserted in corolla tube. Ovary bilocular with thin dissepiments separating the locules. Fruit a berry, one-seeded, surmounted by persistent calyx.

Genus included: *Schizocolea* Bremek.

Useful publications: Bremekamp (1950); Razafiman-dimbison et al. (2008); Rydin et al. (2008).

Dunnieae Rydin and B. Bremer, tribus nov.

Type: *Dunnia* Tutcher

Diagnosis: Frutex. Inflorescentiae terminales, cymosae, floribus multis conglomeratis, bracteis magnis albis circumdatae. Flores 5-meri, calycis lobi minuti, persistentes. Corolla tubiformis, tubo calycem longitudine multo excedente. Fructus capsularis, 2-valvis, valvis 2-partitis. Semina numerosa.

Description: Woody shrubs, stipules pubescent. Inflorescences terminal cymes, surrounded by enlarged, petaloid bracts. Flowers pentamerous, corolla tube extends out of calyx. Stamens inserted in corolla lobe. Pistil distylous. Fruit a capsule, seeds numerous. Diagnosis and description are based on the original publication of *Dunnia* (Tutcher 1905) and on observations made by C. Taylor (Missouri Botanical Garden, pers. com.).

Genus included: *Dunnia* Tutcher

Useful publications: Tutcher (1905); Ge et al. (2002); Chiang et al. (2002); Rydin et al. (2008).

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