A phylogeny of Urophylleae (Rubiaceae) based on *rps16* intron data

Jenny E.E. Smedmark[1], Catarina Rydin[1], Sylvain G. Razafimandimbison[1], Saleh A. Khan[2], Sigrid Liede-Schumann[2] & Birgitta Bremer[1]

[1] *The Bergius Foundation at the Royal Swedish Academy of Sciences and Department of Botany, Stockholm University, 106 91 Stockholm, Sweden. jenny@bergianska.se (author for correspondence)*
[2] *Department of Plant Systematics, University of Bayreuth, Universitätsstr. 30, 95440 Bayreuth, Germany*

This is the first study of phylogenetic relationships within the pantropical group Urophylleae. Previous studies have included few representatives from this group and little is known about its phylogeny. Here we use sequence data from the *rps16* intron to address the question where the four genera *Temnopteryx*, *Pentaloncha*, *Pleiocarpidia*, and *Poecilocalyx*, which have sometimes been classified in this group, belong. By using different outgroups we show that there is conflict regarding the resolution among lineages in Rubioideae, which partly affects the support for relationships within Urophylleae. Urophylleae is shown to consist of two sister groups, one consisting of Old World taxa and one smaller including the New World genera *Amphidasya* and *Raritebe*, and as sister of these two groups the African monotypic genus *Temnopteryx*. *Pentaloncha*, *Pleiocarpidia*, and *Poecilocalyx* all belong in the large Old World clade, which only comprises taxa included in the original circumscription of Urophylleae. Relationships within this group are not completely resolved, but *Poecilocalyx* is found to be the sister of *Stelechantha*, and *Pleiocarpidia* to be the sister of *Urophyllum leucophleum*. *Urophyllum* is paraphyletic as it seems to include *Maschalocorymbus*, *Pleiocarpidia*, *Pravinaria*, and *Pravinaria*. It is not clear from the present analysis whether *Pauridiantha* is monophyletic or not.

KEYWORDS: molecular phylogenetics, *Pentaloncha*, *Pleiocarpidia*, *Poecilocalyx*, *Temnopteryx*, Urophylleae

INTRODUCTION

Urophylleae Bremek. ex Verdc. is a group in the coffee family (Rubiaceae) including about 200 species (Govaerts, 2006), which are usually woody and have indehiscent fruits with multiovulate locules. The name Urophylleae was first introduced by Bremekamp (1952) who suggested to include 22 genera (Table 1), characterized by exotestal cells with thick walls traversed by large numbers of narrow pit-canals. He refuted the usefulness of characters previously used to circumscribe tribes in Rubiaceae and suggested to segregate Urophylleae from Mussaendeae, where previous workers had placed these genera based on the number of ovules in each locule (Hooker, 1873; Schumann, 1891). Bremekamp (1952) could, however, not decide in what subfamily to place Urophylleae. For example, he did not consider the species in Urophylleae to have raphides and the group could therefore not be a part of Rubioideae. The name Urophylleae was not validly published until the treatment by Verdcourt (1958), who did not agree that they lack raphides and placed the group with other raphidiate taxa in Rubioideae. Verdcourt did not explicitly state what taxa he included in Urophylleae, but judging from the description and his expressed intention to validate taxon names of Bremekamp, he accepted Bremekamp's (1952) circumscription. Breme-

kamp (1966) segregated Pauridiantheae from Urophylleae based on, among others, their usually bilocular instead of plurilocular ovaries, bisexual heterostylous rather than dioecious flowers, and the shape and position of the placenta. These two groups were classified in a separate subfamily, Urophylloideae (Bremekamp, 1966). In addition, he also transferred *Acranthera*, originally included in Urophylleae (Bremekamp, 1952), to the monotypic tribe Acranthereae. The segregation of Pauridiantheae was accepted by Robbrecht (1988), who placed the two tribes in Cinchonoideae. Later studies (Bremer & Manen, 2000; Robbrecht & Manen, 2006) have returned to the original, wide circumscription of Urophylleae (Bremekamp, 1952; Verdcourt, 1958) and placed it in Rubioideae.

The first molecular phylogenetic study to indicate that Urophylleae belongs in Rubioideae (Bremer & Thulin, 1998) showed that *Pauridiantha* and *Amphidasya* are sister groups. In contrast to taxa traditionally placed in Urophylleae (Bremekamp, 1952), which all occur in the Old World, *Amphidasya* is restricted to central and southern tropical America. The latter genus had previously been classified in Isertieae (Kirkbride, 1979; Robbrecht, 1988) or Sabiceeae (Andersson, 1996). Morphological characters supporting its position in Urophylleae are the presence of raphides, the absence of ornamentation on the inner surface of the exotesta, and the presence of fringed stipules

(Bremer & Thulin, 1998). The first molecular phylogenetic study to include a representative of Urophylleae (Bremer, 1996) showed that *Pauridiantha* belongs in Rubioideae. This genus was later shown to form a group with *Urophyllum* and *Raritebe* (Andersson & Rova, 1999), referred to as Urophylleae. Like *Amphidasya*, *Raritebe* is neotropical and has been classified in Isertieae (Kirkbride, 1979; Robbrecht, 1988). Together with *Ophiorrhiza*, Urophylleae was indicated to be the sister lineage of the rest of Rubioideae (Andersson & Rova, 1999). In addition to *Pauridiantha* and *Urophyllum*, Urophylleae was subsequently shown to include *Maschalocorymbus*, *Commitheca*, *Pravinaria* and *Praravinia* (Bremer & Manen, 2000). Piesschaert & al. (2000) confirmed that *Amphidasya* and *Raritebe* belong in Urophylleae and showed that these two genera are sisters. Based on a supertree analysis of the entire Rubiaceae (Robbrecht & Manen, 2006), Urophylleae comprises the genera *Pauridiantha*, *Pravinaria*, *Praravinia*, *Urophyllum*, *Stelechantha* Bremek., *Maschalocorymbus*,

Commitheca Bremek., *Amphidasya*, and *Raritebe*. Khan & al. (2008) show that *Pentaloncha* and *Temnopteryx*, both included in the original circumscription of Urophylleae (Bremekamp, 1952), belong in Rubioideae but do not resolve their position within the group.

The present study includes representatives of twelve genera that have been classified in Urophylleae at some point. Four of these, *Temnopteryx*, *Pentaloncha*, *Pleiocarpidia*, and *Poecilocalyx*, have never been shown by a phylogenetic study to belong to Urophylleae. The first two are small genera with petaloid sepals that both occur in western central tropical Africa. *Temnopteryx* is a shrub with relatively large flowers having purple sepals and a pink or red cylindrical corolla tube, while *Pentaloncha* is a trailing suffrutescent with anisophyllous leaves. Both of them were originally classified in Mussaendeae (Hooker, 1873; Schumann, 1891) and transferred to the new tribe Urophylleae by Bremekamp (1952). He expressed uncertainty, however, about their affinities and later included the two

Table 1. Tribal classifications of genera that have at some point been included in Urophylleae.

Genus	Bremekamp (1952)	Verdcourt (1958)	Hallé (1961)	Hallé (1966)	Bremekamp (1966)	Robbrecht (1988)	Andersson (1996)	Andersson & Rova (1999)	Bremer & Manen (2000)	Robbrecht & Manen (2006)
Acranthera Arn. ex Meisn.	Uro	–	–	–	Acr	Ise	Sab	–	–	–
Amphidasya Standl.	?	–	–	–	–	Ise	Sab	–	Uro	Uro
Antherostele Bremek.	Uro	Uro	–	–	Uro	Uro	–	–	–	–
Commitheca Bremek.	Uro	Uro	–	Mus	Pau	Pau	–	–	Uro	Uro
Crobylanthe Bremek.	Uro	Uro	–	–	Uro	Uro	–	–	–	–
Didymopogon Bremek.	Uro	Uro	–	–	Uro	Uro	–	–	–	–
Lepidostoma Bremek.	Uro	Uro	–	–	Uro	Uro	–	–	–	–
Leucolophus Bremek.	Uro	Uro	–	–	Uro	Uro	–	–	–	–
Maschalocorymbus Bremek.	Uro	Uro	–	–	Uro	Uro	–	–	Uro	Uro
Pauridiantha Hook. f.	Uro	Uro	Mus	Mus	Pau	Pau	?	Uro	Uro	Uro
Pentaloncha Hook. f.	Uro?	Uro?	Mus	Mus	Pau?	Inc. sed.	Sab	–	–	–
Pleiocarpidia K. Schum.	Uro	Uro	–	–	Uro	Uro	–	–	–	–
Poecilocalyx Bremek.	Uro	Uro	–	Mus	Pau	Pau	–	–	–	–
Praravinia Korth.	Uro	Uro	–	–	Uro	Uro	–	–	Uro	Uro
Pravinaria Bremek.	Uro	Uro	–	–	Uro	Uro	–	–	Uro	Uro
Raritebe Wernham	?	Uro	–	–	–	Ise	?	Uro	–	Uro
Rhaphidura Bremek.	Uro	Uro	–	–	Uro	Uro	–	–	–	–
Rhipidantha Bremek.	Uro	Uro	–	–	Pau	Pau	–	–	–	–
Stelechantha Bremek.	Uro	Uro	–	Mus	Pau	Pau	–	–	–	Uro
Stichianthus Valeton	Uro	Uro	–	–	Uro	Uro	–	–	–	–
Temnopteryx Hook. f.	Uro?	Uro?	Mus	Mus	Pau?	Ise	Sab	–	–	–
Urophyllum Jack ex Wall.	Uro	Uro	–	–	Uro	Uro	?	Uro	Uro	Uro

Note: Genus names in bold are represented in the present study.

Abbreviations: Acr, Acranthereae; Ise, Isertieae; Mus, Mussaendeae; Pau, Pauridiantheae; Sab, Sabiceae; Uro, Urophylleae; –: not included in the study; ?, no clear conclusion regarding relationships.

tentatively in the segregate Pauridiantheae (Bremekamp, 1966). No later workers have followed Bremekamp's treatment of *Pentaloncha* and *Temnopteryx*. For example, Hallé (1961) considered *Pentaloncha* and *Temnopteryx* to be typical Mussaendeae because of their large, petaloid, often unequally sized calyx lobes, while Robbrecht (1988) placed *Temnopteryx* in Isertieae, but was unsure about the placement of *Pentaloncha*. Based on a morphological phylogenetic study, Andersson (1996) placed *Pentaloncha* and *Temnopteryx* in Sabiceeae. Characters that supported this were the sparsely branched shrubby habit, large corollas, and the presence of a sterile process on the anther tip. Dessein & al. (2001) agreed that *Pentaloncha* seems to be closely related to *Sabicea* based on morphological data.

Neither *Pleiocarpidia* nor *Poecilocalyx* have been included in a molecular phylogenetic study before. *Pleiocarpidia* is a monotypic genus occurring in western Malesia. It is a small tree with characteristic thick and disc-like stigmas. It was originally described as *Aulacodiscus* by Hooker (1873), a name which is unvalid because it had already been used for a genus of diatoms, and was therefore renamed *Pleiocarpidia* (Schumann, 1891). Both Hooker (1873) and Schumann (1891) placed it in Mussaendeae. Bremekamp (1952) included *Pleiocarpidia* in Urophylleae, which has been followed in later treatments (Bremekamp, 1966; Robbrecht, 1988). The other genus, *Poecilocalyx*, includes four species that occur in western and central tropical Africa. They are shrubs with horizontal branches and have few flowered inflorescences subtended by involucral bracts. Like *Pleiocarpidia*, *Poecilocalyx* was also included in Urophylleae under the original circumscription (Bremekamp, 1952), but was later transferred to the segregate Pauridiantheae (Bremekamp, 1966; Robbrecht, 1988). Hallé (1966), on the other hand, placed it in Mussaendeae. None of these four genera were included in the most recent taxonomic treatments (e.g., Bremer & Manen, 2000; Robbrecht & Manen, 2006), since these were based on molecular phylogenetic data, which has not been available for these taxa until now.

The main aim of this study, therefore, is to determine the phylogenetic position of *Pentaloncha*, *Temnopteryx*, *Pleiocarpidia*, and *Poecilocalyx*. Do they belong in Urophylleae, or are their closest relatives to be found elsewhere in Rubioideae? To address this question we use DNA sequence data from the *rps16* intron of the chloroplast for a taxon sample including representatives of all major lineages in Rubiaceae. In order to explore the effect of rooting and outgroup size on levels of support in Urophylleae, five additional datasets, where the more distantly related outgroup taxa had been removed, were also analyzed. We also want to get a first estimate of phylogenetic relationships within the group. Are, for example, the two large genera *Urophyllum* and *Pauridiantha* monophyletic, and does molecular data support clades corresponding to Uro-

phylleae s.str. and Pauridiantheae (Bremekamp, 1966)? Previous phylogenetic studies (Bremer, 1996; Andersson & Rova, 1999; Bremer & Manen, 2000; Piesschaert & al., 2000; Robbrecht & Manen, 2006) have all relied exclusively on parsimony analyses. In some cases when evolutionary rates are not uniform parsimony analyses have been shown to fail in finding the correct topology (Felsenstein, 1985). In this study, we use model based Bayesian inference, in addition to parsimony, in order to evaluate whether the result is influenced by the method of analysis.

■ MATERIALS AND METHODS

Plant material and selection of taxa. — Species were selected to represent as many as possible of the genera included in Urophylleae; type species were included when available. The sample was limited by access to herbarium specimens and our success in amplifying *rps16*. A total of 53 terminals, representing 50 putatively different species, were included in the study. Of these, 36 are classified in genera that have been shown in previous phylogenetic studies to belong to Urophylleae (Andersson & Rova, 1999; Bremer & Manen, 2000; Piesschaert & al. 2000), and 4 in genera that have been associated with this group but not based on phylogeny. Since *Luculia* has been indicated to belong to the sister lineage of all other Rubiaceae (Bremer, 1996), a species from this genus was used to root the tree (dataset *a*). Ten other species in genera outside Urophylleae were also included in these analyses, two from each of the other three basal Rubioideae tribes (Bremer & Manen, 2000) Coussareeae, Ophiorrhizeae, and Lasiantheae, one from each of the *Psychotria* and Spermacoceae alliances (Bremer & Manen, 2000), as well as one from each of Cinchonoideae and Ixoroideae (Bremer & al., 1995). This sample of outgroups was based on a phylogenetic analysis of 260 *rps16* sequences, mainly from Rubioideae (not shown).

Five smaller datasets were also constructed. In one of these matrices (*b*), *Luculia*, *Mussaenda*, *Spermacoce*, and *Psychotria* had been removed, and *Cinchona* was used to root the resulting trees. Placing the root inside Rubioideae and still keeping a good sample of outgroup species is not possible based on our current knowledge of phylogenetic relationships within the group, unless the tree is rooted using a taxon that is in a more derived position than Urophylleae. Ophiorrhizeae and Urophylleae have been shown to have been the first lineages to diverge within Rubioideae, although their exact interrelationships have not yet been clarified. They have either been found to be sister groups with moderate support (Andersson & Rova, 1999) or unresolved (Bremer & Manen, 2000; Piesschaert & al., 2000) in phylogenetic analyses. There-

fore, there is no known lineage within Rubioideae that is basal to Urophylleae that can be used for rooting. In order to evaluate whether other branches within Rubioideae affect the support for relationships within Urophylleae, a dataset including only the Rubioideae taxa in the original dataset was analyzed, placing the root on the branch to the derived *Spermacoce* (dataset *c*). The other three matrices included only two outgroup species representing one of the other basal lineages in Rubiodeae; Coussareeae (*d*), Ophiorrhizeae (*e*), and Lasiantheae (*f*), respectively. Leaf material was sampled from herbarium specimens in most cases, but for a few species silica gel dried material was used. Voucher specimens are listed in the Appendix.

Molecular methods and dataset construction. — DNA extractions were carried out using a slightly modified version of the CTAB extraction method (Doyle & Doyle, 1990). The *rps16* intron was amplified using the rpsF and rpsR2 primers (Oxelman & al., 1997), following standard PCR procedures, and sequenced using the same two primers. Sequences were assembled and edited using the phred (Green & Ewing, 2002) and phrap (Green, 1999) modules in Pregap4 and Gap4 (Staden & al., 1998). All new sequences have been submitted to EMBL. Accession numbers are presented in the Appendix. Sequence alignment was performed by eye, in the sequence alignment editor Se-Al (Rambaut, 1996).

Model selection and phylogenetic analysis. — An evolutionary model was selected based on the Akaike information criterion (AIC; Akaike, 1973) and the Bayesian information criterion (BIC; Schwartz, 1978), which were calculated with MrAIC (ver. 1.4; Nylander, 2004). Both criteria favoured the General time reversible (GTR; Tavaré, 1986) substitution model with gamma distributed rate variation among sites (+Γ) for all datasets. In MrBayes (ver. 3.1.1; Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003), the Markov chain was run for 3 million generations, sampling phylogenetic hypotheses every 1,000 generations. Because mixing among heated chains was poor, the temperature was decreased to 0.12 (0.15 for the pruned datasets) and the number of chains in each analysis increased to eight. Convergence of the Markov chain was assumed to be reached when plots of the overall likelihood, as well as individual parameters of the model, were fluctuating around stable values. At this point the average standard deviation of split frequencies for two analyses run in parallel was less than 0.01 and the potential scale reduction factor was 1.00 for all parameters. The first 1,000,000 generations were discarded as "burn-in" and the last 2,000 trees from both of the parallel analyses were used to construct a majority rule consensus tree and calculate posterior probabilities of clades (PPs). To make sure that the Markov chain really had been sampling from the posterior distribution, three independent analyses, each starting from a random tree, were performed. Since

the topologies of the resulting majority rule consensus trees contained the same nodes with posterior probabilities above 0.95 and were free from supported incongruence, this was considered to be the case.

A bootstrap analysis (Felsenstein, 1985) with 10,000 pseudoreplicates was performed with PAUP* (ver. 4.0b10, Swofford, 2002). Each pseudoreplicate was analyzed with heuristic search, creating ten start trees by random addition and improving these by TBR branch swapping. One tree was saved each time and used to construct a majority rule consensus tree and calculate bootstrap proportions (BPs). The analysis was repeated three times to make sure that very similar BPs were obtained in independent runs.

RESULTS

Data. — Forty-four new *rps16* sequences were produced in this study (Appendix). Sequences ranged in length from 704 to 972 basepairs. EMBL accession numbers are shown in the Appendix. The large matrix (*a*) consisted of 53 terminals and 1,412 aligned DNA characters and included 1.5% missing data. Of the 358 variable characters, 174 were parsimony informative. The one reduced dataset from which the resulting tree is presented, that with only Ophiorrhizeae as outgroup (*e*), consisted of 44 terminals and 969 aligned DNA characters and included 2.1% missing data. Of the 177 variable characters, 91 were parsimony informative.

Phylogenetic relationships. — The majority rule consensus tree from the Bayesian phylogenetic analysis of the dataset with a larger sample of outgroup taxa (*a*) is shown in Fig. 1. In this tree, there is low support (Fig. 1, clade M, PP = 0.78, BP < 50) for Urophylleae including *Temnopteryx*. The support for this node is likewise low when *Luculia*, *Mussaenda*, *Spermacoce*, and *Psychotria* are removed from the analysis, and the tree rooted using *Cinchona* (dataset *b*: PP = 0.69, BP < 50, not shown), as well as when all non-Rubioideae taxa are left out and the tree rooted using *Spermacoce* (*c*: PP = 0.71, BP < 50, not shown). This relationship is, however, strongly supported in all three analyses with an outgroup restricted to a single other lineage within Rubioideae. When the tree is rooted on *Neurocalyx* in Ophiorrhizeae (dataset *e*: Fig. 2) instead of the more distantly related *Luculia* (*a*: Fig. 1), the Bayesian PP increases from 0.78 to 0.99. Placing the root in Lasiantheae (dataset *f*) or Coussareeae (*d*) also yielded high support for *Temnopteryx* being the sister of the remainder of Urophylleae, with BPs of 1.00 in both cases (not shown). We choose to present the tree rooted on Ophiorrhizeae (dataset *e*), rather than those rooted on Coussareeae (*d*) or Lasiantheae (*f*) because several analyses have indicated that Ophiorrhizeae is most closely
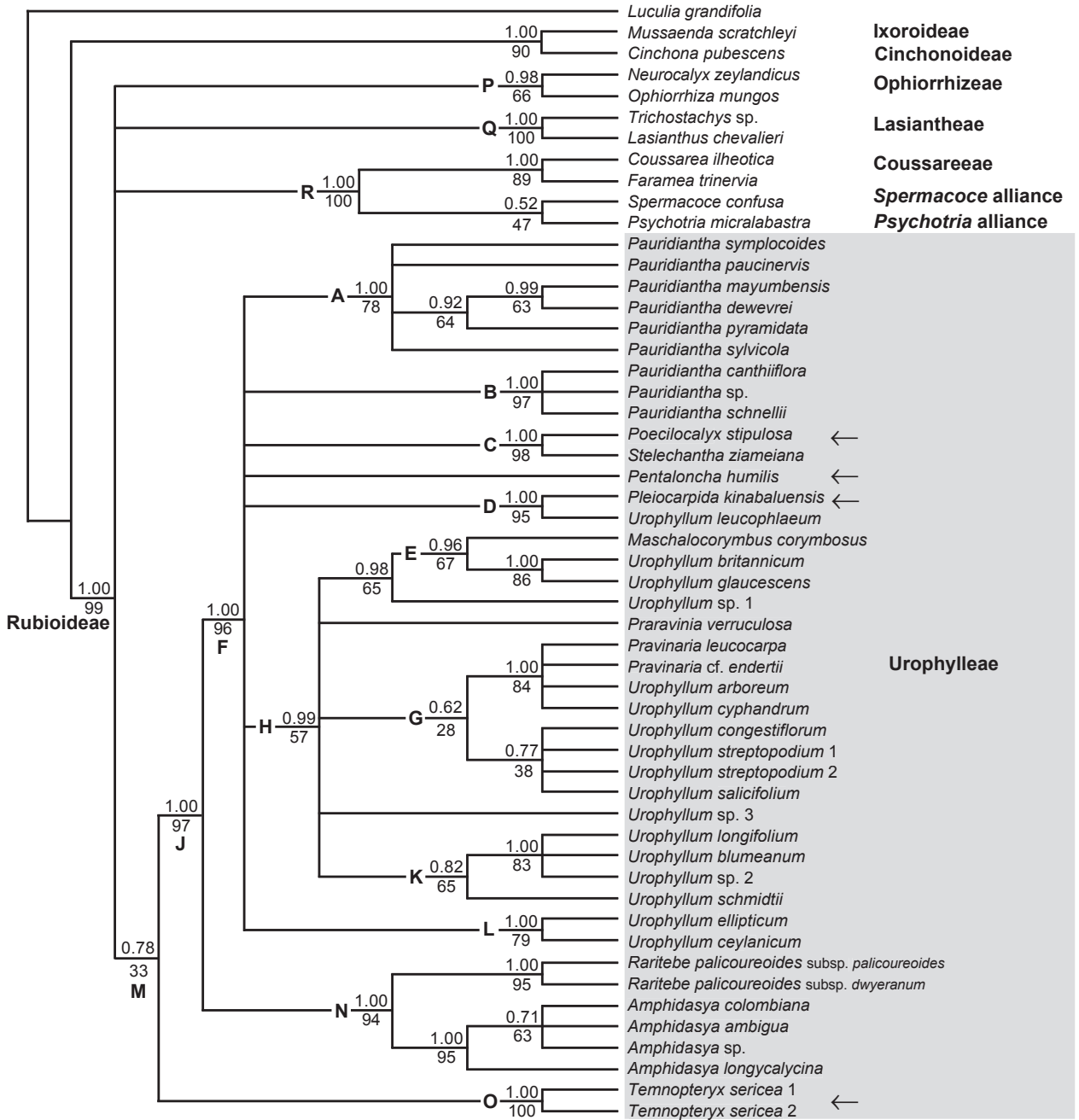
Fig. 1. Majority rule consensus tree from Bayesian analysis under the GTR+Γ model. Posterior probabilities of clades are given above branches and parsimony bootstrap proportions below. Clades mentioned in the text are labeled with capital letters and arrows indicate the position of the four previously unplaced genera *Temnopteryx*, *Pentaloncha*, *Pleiocarpidia*, and *Poecilocalyx*.

related to Urophylleae (Andersson & Rova, 1999; Bremer & Manen, 2000; Piesschaert & al., 2000). In both trees (Figs. 1–2), Urophylleae includes two strongly supported clades; one consisting entirely of Old World taxa, all of which were originally placed in Urophylleae (Bremekamp, 1952; Figs. 1–2, clade F), and the other of the Neotropical *Amphidasya* and *Raritebe* (Figs. 1–2, clade N). These

two lineages form a clade with strong support (Figs. 1–2, clade J). The other three genera, besides *Temnopteryx*, whose phylogenetic affinities were to be examined in this study, *Pentaloncha*, *Poecilocalyx*, and *Pleiocarpidia*, all belong in clade F (Figs. 1–2). *Poecilocalyx* is strongly supported to be the sister of *Stelechantha* (Figs. 1–2, clade C), the position of *Pentaloncha* is unresolved, and *Pleio-*
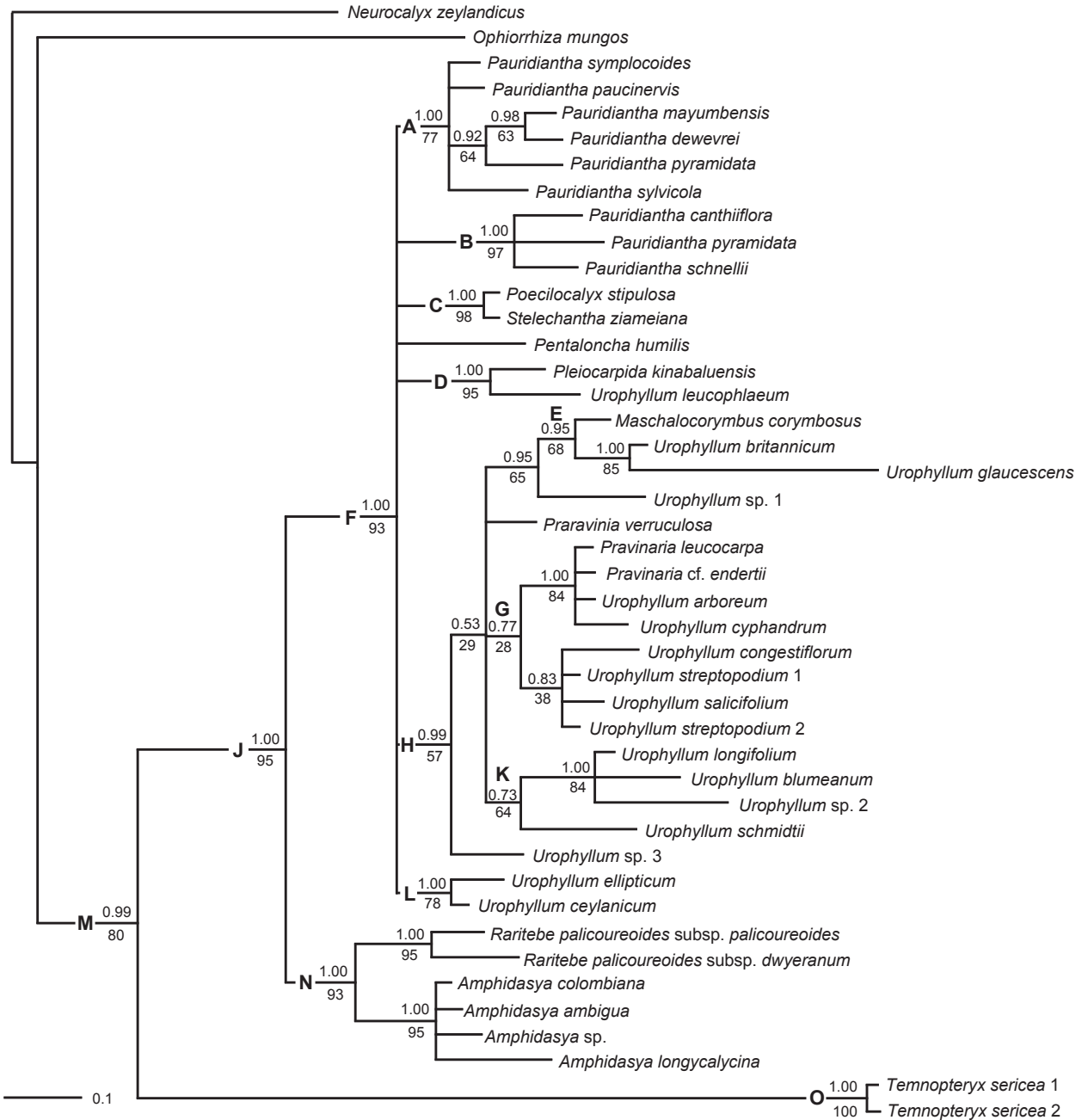
Fig. 2. Phylogram from Bayesian analysis under the GTR+Γ model of a dataset with a reduced number of outgroups. Posterior probabilities of clades are given above branches and parsimony bootstrap proportions below. Clades mentioned in the text are labeled with capital letters.

carpidia is strongly supported to be the sister of *Urophyllum leucophleum* (Figs. 1–2, clade D). *Urophyllum* species are found in three unresolved lineages (Figs. 1–2, clades D, H, and L) and the genus is paraphyletic, since *Maschalocorymbus*, *Praravinia*, *Pravinaria*, and maybe also *Pleiocarpidia*, are all nested inside it. The analysis does not answer the question of whether *Pauridiantha* is monophyletic. Species in this genus belong to two lineages

whose relationships are not resolved (Figs. 1–2, clades A and B). Pauridiantheae (fide Bremekamp, 1966), here represented by *Pauridiantha*, *Poecilocalyx*, *Stelechantha*, *Pentaloncha*, and *Temnopteryx*, is shown not to be monophyletic, since *Temnopteryx* (Figs. 1–2, node O) belongs to a separate lineage that is indicated to be the sister of the rest of Urophylleae (Fig. 2, node M). It is not possible to conclude from the present analysis whether Urophylleae

s.str. (fide Bremekamp, 1966), represented by *Urophyllum*, *Pleiocarpidia*, *Maschalocorymbus*, *Praravinia*, and *Pravinaria*, is monophyletic or not, since the three lineages where these genera are found (Figs. 1–2, clades D, H, and L) are unresolved.

DISCUSSION

Phylogenetic relationships. — There are no contradictions between clades supported by Bayesian phylogenetic inference and parsimony bootstrapping (Figs. 1–2). Both types of analyses support the same topology, despite the fact that a couple of branches are distinctly longer than the rest (Fig. 2, *Urophyllum glaucescens* and *Temnopteryx*). This agreement makes it less likely that there are errors in the phylogenetic reconstruction and increases the confidence in the topologies presented.

This study corroborates Verdcourt's opinion that *Pentaloncha*, *Temnopteryx*, *Pleiocarpidia*, and *Poecilocalyx* belong in Rubioideae (1958). All other workers have placed them in either Cinchonoideae (Hooker, 1873; Schumann, 1891; Hallé, 1961; Hallé 1966; Robbrecht, 1988) or in a separate subfamily, Urophylloideae (Bremekamp, 1966). The analysis of the large *rps16* dataset (*a*), including representatives of all major lineages in Rubiaceae (Fig. 1), showed that *Temnopteryx* belongs in Rubioideae, but did not provide convincing support for its position within this group. Both analyses rooted on a taxon outside Rubioideae (datasets *a* and *b*), as well as that including multiple outgroup taxa from within Rubioideae (*c*), gave weak support for *Temnopteryx* being the sister of the remainder of Urophylleae (e.g., Fig. 1). It is possible that this modest support is caused by conflict regarding the resolution among lineages within Rubioideae rather than conflict regarding the actual placement of *Temnopteryx*. To test this, we performed analyses where all outgroup taxa except representatives of a single other lineage within Rubioideae had been removed. The fact that rooting on Ophiorrhizeae (dataset *e*; Fig. 2), Coussareeae or Lasiantheae (*d* and *f*, not shown) gave high support (PPs of 0.99 or 1.00) for *Temnopteryx* being the sister of the remainder of Urophylleae indicates that conflicting signals regarding the relationships among the four major lineages in Rubioideae (Fig. 1, clades M, P–R) affect the support for the position of *Temnopteryx*. The branch leading to *Temnopteryx* is quite long (Fig. 2), which could contribute to the low support, given that there is conflict among the other branches, in the same way that homoplasy can contribute to long branch attraction (Felsenstein, 1978). We conclude from these results that *Temnopteryx* does indeed belong in Urophylleae. The other three genera, *Pentaloncha*, *Poecilocalyx*, and *Pleiocarpidia*, were found to be nested within a strongly supported group (Figs. 1–2,

clade F) that only comprises taxa included in the original circumscription of Urophylleae (Bremekamp, 1952, see Table 1). Bremekamp (1952, 1966) characterized this group by an exotesta with large, thick-walled cells and an inner surface covered with minute pits. *Amphidasya* and *Raritebe*, that make up the sister group of clade F (Figs. 1–2), also have this type of exotesta while that of *Tempnopteryx* has a different structure (Andersson, 1996). This combination of testal characters could therefore be a synapomorhy for clade J. These characters do, however, need to be studied more closely in order to evaluate whether they have any diagnostic value. The basic chromosome number of *Pauridiantha* and *Urophyllum* is $x = 9$ (Kiehn, 1995), which differs from those of other groups in Rubioideae (Coussareeae $x = 10$ or 11, Lasiantheae $x = 11$, and Ophiorrhizeae $x = 11$ or 12, *Psychotria* alliance $x = 10$ or 11, *Spermacoce* alliance $x = 8$, 10, 11, 14, 15 or 17). They also have a heteromorphic karyotype with both long and short chromosomes, a feature that is unique in Rubiaceae (Kiehn, 1995). These two karyological characteristics are probably synapomorphies for clade F, or perhaps for Urophylleae as a whole (Figs. 1–2, clade M), although the chromosomes of *Amphidasya*, *Raritebe*, and *Temnopteryx* have not yet been studied. *Poecilocalyx* is found to be the sister of *Stelechantha* (Figs. 1–2, clade C), which like *Poecilocalyx* is a small genus occurring in western tropical Africa. Both genera lack domatia, a character that is common in closely related taxa but otherwise differ considerably morphologically. They do, however, share several characters that are also found in for example *Pauridiantha*, for example heterostylous flowers, upright stigmata, ovaries with a false septum dividing the upper part of each locule, and obcordate placentas that are attached to the middle of the septum. *Pleiocarpidia*, which is distributed in western Malesia, is found to be the sister of *Urophyllum leucophleum* (Figs. 1–2, clade D) from peninsular Malaysia. Morphological characters supporting the affinity of *Pleiocarpidia* to *Urophyllum* are the dioecious flowers, flattened hairs in the corolla tube, paired axile placentas, and spreading stigmata. *Urophyllum* species are also found in two other unresolved clades H, and L (Figs. 1–2). Since the type species of *Urophyllum*, *U. villosum*, was not included in the analysis, it is not possible to determine whether *Pleiocarpidia*, *Pravinaria*, *Praravinia*, and *Maschalocorymbus*, which are all nested inside *Urophyllum*, should be included in this genus or whether *Urophyllum* should perhaps be divided into smaller genera. The other large genus in Urophylleae, *Pauridiantha*, is not supported to be monophyletic either and the type species, *P. canthiiflora*, is found in clade B. Like in the case of *Urophyllum*, the clades of *Pauridiantha* species are unresolved (Figs. 1–2, clades A and B) but there is no indication that either of these two genera are polyphyletic. Future research in our laboratory will

strive to add additional molecular markers and widen the taxon sample in order to produce a more robust phylogeny that may resolve these issues. Such a study will hopefully also answer the question whether Pauridiantheae (excluding *Temnopteryx*) and Urophylleae s.str. (Bremekamp, 1966) constitute evolutionary groups within clade F. Biogeographical and morphological evidence (Bremekamp, 1966; Robbrecht, 1988; Ntore & al. 2003) suggests that at least one of them may be monophyletic, but neither one is supported in the present analyses.

◼ ACKNOWLEDGEMENTS

◼ LITERATURE CITED

Akaike, H. 1973. Information theory as an extension of the maximum likelihood principle. Pp. 267–281 in: Petrov, B.N. & Csaki, F. (eds.), *Second International Symposium on Information Theory.* Akademiai Kiado, Budapest.

Andersson, L. 1996. Circumscription of the tribe Isertieae (Rubiaceae). *Opera Bot. Belg.* 7: 139–164.

Andersson, L. & Rova, J.H.E. 1999. The *rps16* intron and the phylogeny of the Rubioideae (Rubiaceae). *Pl. Syst. Evol.* 214: 161–186.

Bremekamp, C.E.B. 1952. The African species of *Oldenlandia* L. sensu Hiern et K. Schumann. *Verh. Kon. Ned. Akad. Wetensch., Afd. Natuurk., Sect. 2* 18: 1–297.

Bremekamp, C.E.B. 1966. Remarks on the position, the delimitation and the subdivision of the Rubiaceae. *Acta Bot. Neerl.* 15: 1–33.

Bremer, B. 1996. Phylogenetic studies within Rubiaceae and relationships with other families based on molecular data. *Opera Bot. Belg.* 7: 33–50.

Bremer, B., Andreasen, K. & Olsson, D. 1995. Subfamilial and tribal relationships in the Rubiaceae based on *rbcL* sequence data. *Ann. Missouri Bot. Gard.* 82: 383–397.

Bremer, B. & Manen, J.-F. 2000. Phylogeny and classification of the subfamily Rubioideae (Rubiaceae). *Pl. Syst. Evol.* 225: 43–72.

Bremer, B. & Thulin, M. 1998. Collapse of Isertieae, re-establishment of Mussaendeae, and a new genus of Sabiceeae (Rubiaceae); phylogenetic relationships based on *rbcL* data. *Pl. Syst. Evol.* 211: 71–92.

Dessein, S., Andersson, L., Robbrecht, E. & Smets, E. 2001. *Hekistocarpa* (Rubiaceae): a member of an emended tribe Virectarieae. *Pl. Syst. Evol.* 229: 59–78.

Doyle, J.J. & Doyle, J.L. 1990. Isolation of plant DNA from fresh tissue. *Focus* 12: 13–15.

Felsenstein, J. 1978. Cases in which parsimony or compatibility methods will be positively misleading. *Syst. Zool.* 27: 401–410.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.

Govaerts, R. 2006. World Checklist of Rubiaceae. The Board of Trustees of the Royal Botanic Gardens, Kew. Published on the Internet: http://www.kew.org/wcsp/

Green, P. 1999. *Phrap*, vers. 0.990329. Available at: www.phrap.org

Green, P. & Ewing, B. 2002. *Phred*, vers. 0.020425c. Available at: www.phrap.org.

Hallé, F. 1961. Contribution a l'étude biologique et taxonomique des Mussaendeae (Rubiaceae) d'Áfrique tropicale. *Adansonia* 1: 266–298.

Hallé, N. 1966. Famille des Rubiacées (1re partie). Vol. 12 of: Aubréville, A. (ed.), *Flore du Gabon*. Muséum National d'Histoire Naturelle, Laboratoire de Phanérogamie, Buffon, Paris.

Hooker, J.D. 1873. Rubiaceae. Pp. 7–151 in: Bentham, G. & Hooker, J.D. (eds.), *Genera Plantarum*. Reeve & Co., London.

Huelsenbeck, J.P. & Ronquist, F. 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754–755.

Khan, S.A., Razafimandimison, S.G., Bremer, B. & Liede-Schumann, S. 2008. Sabiceeae and Virectarieae (Rubiaceae, Ixoroideae): one or two tribes? New tribal and generic circumscriptions of Sabiceeae and biogeography of *Sabicea* s.l. *Taxon* 57: 7–23.

Kiehn, M. 1995. Chromosome survey of the Rubiaceae. *Ann. Missouri Bot. Gard.* 82: 398–408.

Kirkbride, M.C.G. 1979. Review of the neotropical Isertieae (Rubiaceae). *Brittonia* 31: 313–332.

Novotny, V., Basset, Y., Miller, S.E., Weiblen, G.D., Bremer, B., Cizek, L. & Drozd, P. 2002. Low host specificity of herbivorous insects in a tropical forest. *Nature* 416: 841–844.

Ntore, S., Robbrecht, E., Smets, E. & Dessein, S. 2003. Révision de *Pauridiantha paucinervis* (Rubiaceae-Pauridiantheae) et des espèces voisines. *Belg. J. Bot.* 136: 73–90.

Nylander, J.A.A. 2004. *MrAIC.pl*, version 1.4. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Oxelman, B., Lidén, M. & Berglund, D. 1997. Chloroplast *rps16* intron phylogeny of the tribe Sileneae (Caryophyllaceae). *Pl. Syst. Evol.* 184: 393–410.

Piesschaert, F., Andersson, L., Jansen, S., Dessein, S., Robbrecht, E. & Smets, E. 2000. Searching for the taxonomic position of the African genus *Colletoecema* (Rubiaceae): morphology and anatomy compared to an *rps16*-intron analysis of the Rubioideae. *Canad. J. Bot.* 78: 288–304.

Rambaut, A. 1996. *Se-Al: Sequence Alignment Editor*, version 2.0a11. Available at: http://evolve.zoo.ox.ac.uk/.

Robbrecht, E. 1988. Tropical woody Rubiaceae. *Opera Bot. Belg.* 1: 1–271.

Robbrecht, E. & Manen J.-F. 2006. The major evolutionary lineages of the coffee family (Rubiaceae, angiosperms). Combined analysis (nDNA and cpDNA) to infer the position of *Coptosapelta* and *Luculia*, and supertree construction based on *rbcL*, *rps16*, *trnL-trnF* and *atpB-rbcL* data. A new classification in two subfamilies, Cinchonoideae and Rubioideae. *Syst. Geogr. Pl.* 76: 85–146.

Ronquist, F. & Huelsenbeck, J.P. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574.

Schumann, K. 1891. Rubiaceae. Pp 1–156 in: Engler, A. & Prantl, K. (eds.), *Die natürlichen Pflanzenfamilien*, part 4. W. Engelmann, Leipzig.

Schwartz, G. 1978. Estimating the dimensions of a model. *Ann. Stat.* 6: 461–464.

Staden, R., Beal, K.F. & Bonfield, J.K. 1998. The Staden Package. Pp. 115–130 in: Misener, S. & Krawetz, S.A. (eds.), *Computer Methods in Molecular Biology*, vol. 132, *Bioinformatics Methods and Protocols*. The Humana Press Inc., Totowa.

Swofford, D.L. 2002. *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods)*, ver. 4.0b10. Sinauer, Sunderland, Massachusetts.

Tavaré, S. 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. Pp. 57–86 in: Miura, R.M. (ed.), *Some Mathematical Questions in Biology— DNA Sequence Analysis*. American Mathematical Society, Providence.

Verdcourt, B. 1958. Remarks on the classification of the Rubiaceae. *Bull. Jard. Bot. État. Bruxelles* 28: 209–290.

Appendix. Material used in the study.

Species; origin; collector, collection number and herbarium; EMBL accession.

Amphidasya ambigua (Standl.) Standl.; Ecuador; *Ståhl & al. 3542* (GB); AF129271[1]. *Amphidasya colombiana* (Standl.) Steyerm.; Ecuador; *Ståhl & al. 3542* (GB); AF242906[2]. *Amphidasya longicalycina* (Dwyer) C.M. Taylor; Costa Rica; *Huber 2963* (CR); AM900637. *Amphidasya* sp.; Peru; *Morawetz & Wallnöfer 114-11888* (GB); AM900636. *Cinchona pubescens* Vahl; Cult. Göteborg Botanical Garden; *Andersson 2214* (GB); AF004035[3]. *Coussarea ilheotica* Müll.Arg.; Brazil; *De Carvalho & al. 4081* (K); AM900597. *Faramea trinervia* K. Schum. & Donn.Sm.; Costa Rica; *Gomez-Laurito 8374* (CR); AM900598. *Lasianthus chevalieri* Pit.; Vietnam; *Averyanov & al. VH2673* (AAU); AM900596. *Luculia grandifolia* Ghose; Cult. Stockholm Univ.; *Bremer 2713* (S); AM900593. *Maschalocorymbus corymbosus* (Blume) Bremek.; Sabah; *Ridsdale 2471* (L); AM900611. *Mussaenda scratchleyi* Wernham; New Guinea; *Drozd & Molem 118-11-13*; AJ320079[4]. *Neurocalyx zeylanicus* Hook.; Sri Lanka; *B. & K. Bremer 937* (S); AM900594. *Ophiorrhiza mungos* L.; Cult. Uppsala University; *Bremer 3301* (UPS); AF004064[3]. *Pauridiantha canthiiflora* Hook. f.; Gabon; *Breteler 6746* (AAU); AM900604. *Pauridiantha dewevrei* (De Wild. & T. Durand) Bremek.; Congo; *Champluvier 5083* (K); AM900603. *Pauridiantha mayumbensis* (R.D. Good) Bremek.; Gabon; *Diabata 1158* (UPS); AM900601. *Pauridiantha paucinervis* (Hiern) Bremek.; Tanzania; *Bremer 3090* (UPS); AM900600. *Pauridiantha pyramidata* (K. Krause) Bremek.; Central African Republic; *Harris & Fay 644* (K); AM900607. *Pauridiantha schnellii* N. Hallé; Liberia; *Adanes 829* (UPS); AM900606. *Pauridiantha* sp. (K. Krause) Bremek.; Congo; *Devred 2297* (UPS); AM900605. *Pauridiantha sylvicola* (Hutch. & Dalziel) Bremek.; Cameroon; *Leeuwenberg 9711* (UPS); AM900602. *Pauridiantha symplocoides* (S. Moore) Bremek.; Malawi; *Lantz 123* (UPS); AM900599. *Pentaloncha humilis* Hook. f.; Gabon; *Breteler & al. 10985* (WAG); AM900632. *Pleiocarpidia kinabaluensis* Bremek.; Borneo; *Beaman 8841* (S); AM900610. *Poecilocalyx stipulosa* (Hutch. & Dalziel) N. Hallé; Ivory coast; *Wilde & Leeuwenberg 3515* (UPS); AM900608. *Praravinia verruculosa* Bremek.; Borneo; *Beaman 9670* (S); AM900612. *Pravinaria* cf. *endertii* Bremek.; Brunei; *Sands 5262* (K); AM900614. *Pravinaria leucocarpa* Bremek.; Borneo; *Beaman 7950* (S); AM900613. *Psychotria micralabastra* (Lauterb. & K. Schum.) Valeton; New Guinea; *Drozd & Molem 13 Nov. 1998*; AJ320084[4]. *Raritebe palicoureoides* Wernham subsp. *palicoureoides*; Ecuador; *Jaramillo & Rivea 195* (NY); AF004075[3]. *Raritebe palicoureoides* subsp. *dwyeranum* J.H. Kirkbr.; Panama; *Antonio 1697* (AAU); AM900635. *Spermacoce confusa* Rendle ex Gillis; Colombia; *Andersson & al. 2136* (GB); AF003619[3]. *Stelechantha ziamaeana* (Jacq.-Fél.) N. Hallé; Liberia; *Adam 20999* (UPS); AM900609. *Temnopteryx sericea* 1 Hook. f.; Gabon; *Tabak 99* (WAG); AM900633. *Temnopteryx sericea* 2 Hook. f.; Equatorial Guinea; *Wieringa & Haegens 2266* (WAG); AM900634. *Trichostachys* sp; Cameroon; *Sonké 1725* (UPS); AM900595. *Urophyllum arboreum* (Reinw. ex Blume) Korth.; Sumatra; *Boeea 7887* (S); AM900617. *Urophyllum blumeanum* (Wight) Hook. f.; Thailand; *Puff & Sridith 930724* (AAU); AM900629. *Urophyllum britannicum* Wernham; Papua New Guinea; *Gideon & al. 76915* (K); AM900623. *Urophyllum ceylanicum* (Wight) Thwaites; Sri Lanka (S); *Klackenberg 214* (S); AM900620. *Urophyllum congestiflorum* Ridl.; Brunei; *Wong 1057* (K); AM900621. *Urophyllum cyphandrum* Stapf; Borneo; *Beaman & al. 9549* (K); AM900618. *Urophyllum ellipticum* (Wight) Thwaites; Sri Lanka; *Lundqvist 11085* (UPS); AM900619. *Urophyllum glaucescens* Valeton; Papua New Guinea; *Damas 58925* (K); AM900625. *Urophyllum leucophlaeum* Ridl.; Malaysia; *Stone 12658* (AAU); AM900626. *Urophyllum longifolium* (Wight) Hook. f; Thailand; *Larsen & Larsen 33340* (AAU); AM900616. *Urophyllum salicifolium* Stapf; Borneo; *Beaman 11523* (K); AM900624. *Urophyllum schmidtii* C.B. Clarke; Thailand; *Geesink & al. 6592* (AAU); AM900627. *Urophyllum* sp. 1; Borneo; *Beaman 7501* (S); AM900630. *Urophyllum* sp. 2; Singapore; *Axelius 177* (S); AM900631. *Urophyllum* sp. 3; Philippines; *Ingle 725* (AAU); AM900615. *Urophyllum streptopodium* 1 Wall. ex Hook. f.; Sumatra; *Boeea 7766* (S); AM900622. *Urophyllum streptopodium* 2 Wall. ex Hook. f.; Malaysia; *Maxwell 81-141* (AAU); AM900628.

1(Piesschaert & al., 2000) 2(Rova, 1999) 3(Andersson & Rova, 1999) 4(Novotny & al., 2002)