

Paraphyly and phylogenetic relationships in *Lasianthus* (Rubiaceae) inferred from chloroplast *rps16* data

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ABSTRACT. A phylogenetic analysis of *Lasianthus* and some representatives of tribes in subfamily Rubioideae based on the chloroplast *rps16* data indicates that *Lasianthus* as currently circumscribed is paraphyletic, because *Saprosma crassipes*, representative of the species with two locules per ovary developing into two pyrenes per drupe with a thin wall, and *Litosanthes biflora*, the species in the monotypic genus *Litosanthes*, are nested within the highly supported *Lasianthus* clade. The present delimitation of the tribe Lasiantheae, which includes *Saldinia* and *Trichostachys*, is supported by our results. Finally, our results are inconclusive for evaluating the monophyly of the infrageneric classification of *Lasianthus*.

Keyword: *Lasianthus*; Lasiantheae; *Litosanthes*; Paraphyly; *rps16* intron.

INTRODUCTION

Lasianthus Jack is a large pantropical genus in Rubiaceae comprising more than 180 species. Of these, c. 160 species occur in tropical Asia, with one extending to Australia, c. 20 in tropical Africa and three in tropical America. The members of *Lasianthus* are exclusively confined to primary rainforests throughout their geographic ranges. The distribution pattern of *Lasianthus* appears to be important for understanding biogeography and speciation in tropical rainforests (Zhu, 2002).

Some regional taxonomic revisions have been made for *Lasianthus* [e.g., Verdcourt (1976); Denys (1981) for Africa, Wong (1989) for the Malay Peninsula; Deb and Gangopadhyay (1991) for India, Zhu (2001) for Thailand, and Zhu (1994, 1998, 2002) for Eastern Asia]. However, the delimitation of *Lasianthus* has always been controversial and remains unsettled. Jack (1823) originally described *Lasianthus* as a 4-locular ovary bearing a single basally erected ovule per locule, and a drupe with four pyrenes. Blume (1826) enlarged Jack's original circumscription to include species with 4-9 locular ovaries and drupes with 4-9 pyrenes. Wight (1846) and Korthals (1851) added some species with 2-locular ovaries, developing into 2-pyrene drupes. Later these species were transferred to *Saprosma* (Schumann, 1891; Boerlage, 1899). In addition, the Madagascar genus *Saldinia*, with 2-locular ovaries and drupes with 1-pyrene, was once placed under *Lasianthus* as a subgenus, (Baillon, 1880). Furthermore, Bremekamp (1957) proposed a new classification of *Lasianthus* as species with two or more locules per ovary and two or more pyrenes per drupe with

a thick wall. He also restored *Saldinia* as a separate genus, and merged part of the species with 2-locular ovaries developing into 2-pyrene drupes in *Lasianthus*.

The tribal position of *Lasianthus* has also been controversial. Traditionally, *Lasianthus* was placed in the tribe Psychotrieae based on aestivation of the corolla lobes and the position, attachment, and types of its ovules (Hooker, 1880; Schumann, 1891). Petit (1964) proposed new circumscriptions for Psychotrieae and Morindeae and transferred *Lasianthus* to Morindeae based on its seeds, which have soft oily endosperm and large embryos. However, molecular data based on a few samples (Bremer, 1996; Andersson and Rova, 1999; Piesschaert et al., 1999; Bremer and Manen, 2000) indicated that *Lasianthus* appeared to be related to *Pauridiantha*, *Perama*, *Trichostachys*, and *Saldinia*. Bremer and Manen (2000) placed *Lasianthus*, along with *Saldinia* and *Trichostachys*, in the tribe Lasiantheae. In addition, the only available comprehensive infrageneric classification of *Lasianthus* was Hooker's classification (1880) based mainly on quantitative characters, such as the size of stipules, the occurrence of bracts, and peduncles. Hooker divided *Lasianthus* into four sections: *Bracteatae*, *Nudiflorae*, *Stipulares*, and *Pedunculatae*.

The identity of the Asian monotypic genus *Litosanthes*, *L. biflorus*, has also been controversial. *Litosanthes* is characterised by its imbricate corolla, forked stipules, and pedunculate inflorescences. Some Asian *Lasianthus* with pedunculate inflorescences were transferred to *Litosanthes* (Deb and Ganopadhyay, 1989; 1991) and recently returned to a section of *Lasianthus* (Gangopadhyay and Chakrabarty, 1992). In the Flora of China, however, *Litosanthes biflorus* is treated as a monotypic genus (Lo, 1999).

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Here we present a ribosomal protein S16 (*rps16*) intron phylogenetic analysis with 11 *Lasianthus* from the tropical Africa, America, and Asia, and 28 representatives from the recognized tribes in the subfamily Rubioideae based on the classification proposed by Bremer and Manen (2000). The *rps16* intron was chosen because the marker has proven useful for inferring phylogenetic relationships at generic or higher levels (e.g. Andersson and Rova, 1999; Bremer and Manen, 2000; Nie et al., 2005). Additionally, many published Rubioideae *rps16* sequences are available for our studies. Pairwise comparisons of the 17 chloroplast introns shared between tobacco (*Nicotiana tabacum* L.) and rice (*Oryza sativa* L.) indicate that the *rps16* intron is one of the most divergent, with 67% sequence similarity (Downie et al., 1996). The following questions are to be addressed in particular: (1) Is the current circumscription of the genus *Lasianthus* monophyletic? (2) What are the relationships of *Lasianthus* with other Lasiantheae genera? (3) What are the infrageneric relationships of *Lasianthus* from tropical America, Africa and Asia? (4) And, finally, is this phylogeny consistent with Hooker's classification?

MATERIALS AND METHODS

Eleven species representing *Lasianthus* from tropical Africa, tropical America, and tropical Asia, and 28 species representing all recognized tribes in the subfamily Rubioideae (except Spermacoceae and Theligoneae) (Bremer and Manen, 2000) were sampled as ingroups. The outgroups were designated as *Ixora amplexicaulis* (Ixoroideae) and *Cinchona pubescens* (Cinchonoideae) based on the identification of the monophyly of subfamily Rubioideae (Bremer et al., 1995). The materials collected in this study were identified by the second author, Dr Zhu, H., a specialist on *Lasianthus*. All sequences have been deposited in GenBank. (For accession numbers for the *rps16* intron sequences and vouchers/references information see Table 1.)

Total genomic DNA was extracted from silica-dried or fresh leaves using a modified CTAB procedure (Doyle and Doyle, 1987). The primers *rpsF* and *rpsR*₂ described by Oxelman et al. (1997) were used for amplifying the *rps16* intron from the genomic DNA. PCR reaction volumes (30 μ l) contained 1.5 U of Ampli *Taq* DNA polymerase (Perkin-Elmer 9600). Reactions were incubated at 95°C for 3 min, then cycled 35 times (95°C for 1 min, 55°C for 1 min, 72°C for 1.5 min), followed by a final extension for 10 min at 72°C. Double-stranded products were purified using the E.Z.N.A. Cycle-Pure Kit (OmegaBio-tek, USA). Sequencing reactions were performed using PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.). The products of the sequencing reaction were electrophoresed on an ABI 3700 automated sequencer.

Contiguous DNA sequences were edited using SeqMan (DNASTAR package) and subsequently adjusted manually. All sequences were aligned using MEGALIGN

(DNASTAR package) and then adjusted manually. Deletions were coded as missing data.

Maximum parsimony (MP) analysis was performed using PAUP 4.0b10 (Swofford, 2001) treating gaps as missing data using heuristic search options with 1,000 random replications of stepwise data addition and TBR swapping and Multrees on no tree limit with all characters weighted equally and unordered. Bootstrap analysis (Felsenstein, 1985) was performed with 1,000 replicates to evaluate internal support.

RESULTS

All the newly acquired sequences were submitted to GenBank (Table 1). The total length of 1,191 nucleotides of the *rps16* intron sequences in the data matrix, including 41 species, was determined, and 327 were parsimony-informative (27.5%). A parsimony analysis of the *rps16* intron data matrix resulted in 970 equally most parsimonious trees, each with 819 steps, CI = 0.6119, and RI = 0.807. The strict consensus tree is shown in Figure 1. *Saprosma crassipes*, *Litosanthes biflorus*, and all sequenced *Lasianthus* species formed a strongly supported (BP = 92) monophyletic group. This mostly *Lasianthus* clade was resolved as sister to a highly supported (BP = 98) clade containing *Saldinia* and *Trichostachys*. However, the support for this sister-group relationships was weak (BP = 58). The Lasiantheae clade was in turn resolved as sister to the tribe Perameae, represented by *Perama hirsuta*. The highly supported (BP = 100) Lasiantheae-Perameae clade was resolved with strong support (BP = 100) as sister to a highly supported (BP = 98) large clade containing *Saprosma ternatum* and the remaining sequenced Rubioideae taxa, formally classified into eleven tribes (Morindeae, Gaertnereae, Schradereae, Psychotrieae, Craterispermeae, Anthospermeae, Paederieae, Argostemmeae, Danaideae, Coussarieae, Urophyllaeae, and Ophiorhizeae). The two studied species of the genus *Saprosma*, *S. crassipes* and *S. ternatum*, did not form a clade. Finally, the African *Lasianthus batangensis* and the Neotropical *L. lanceolatus* formed a monophyletic group while the sequenced Asian *Lasianthus* did not group together as a clade.

DISCUSSION

Delimitation of *Lasianthus*

Lasianthus as presently delimited is not monophyletic, unless *Litosanthes* and *Saprosma crassipes* are included. In other words, the species with two locules per ovary developing into two pyrenes per drupe with a thin wall should be transferred to *Lasianthus*, and *Litosanthes biflorus* should not be separated from *Lasianthus*.

Our results further support the placement of *Lasianthus* in Lasiantheae as proposed by Bremer and Manen (2000). However, we find no support for the position of the genus in Psychotrieae (e.g., Schumann, 1891) or Morindeae

Table 1. GenBank accession, Vouchers or references information and the species sampled in this study.

Species	Tribe	Vouchers /References	Origin	GenBank accession number
<i>Lasianthus hirsutus</i> (Roxb.) Merr.	Lasiantheae	Gong, 04298	Vietnam	*DQ282637
<i>Lasianthus attenuatus</i> Jack	Lasiantheae	Zhu, 03122	Malaysia	*DQ282638
<i>Lasianthus sikkimensis</i> Hook. f.	Lasiantheae	Zhu, 03155	China	*DQ282644
<i>Lasianthus rhinocerotis</i> Bl.	Lasiantheae	Zhu,03123	Malaysia	*DQ282639
<i>Lasianthus chinensis</i> (Champ.) Benth.	Lasiantheae	Xiao, 04010	China	*DQ282641
<i>Lasianthus verticillatus</i> (Lour.) Merr.	Lasiantheae	Zhu, 03156	China	*DQ282640
<i>Lasianthus hookeri</i> Clarke ex Hook. f.	Lasiantheae	Zhu, 03157	China	*DQ282643
<i>Lasianthus chrysoneurus</i> (Korth) Miq.	Lasiantheae	Zhu, 03159	China	*DQ282642
<i>Lasianthus batangensis</i> Schum.	Lasiantheae	Andersson & Antonelli, 2005	Congo	AY538439
<i>Lasianthus lanceolatus</i> (Griseb.) Urb.	Lasiantheae	Andersson & Rova, 1999	Puerto Rico	AF004062
<i>Lasianthus coffeoides</i> Fyson	Lasiantheae	Andersson & Rova, 1999	India	AF004061
<i>Litosanthes biflorus</i> Bl.	Lasiantheae	Zhou, 2655	China	*DQ282649
<i>Saldinia</i> sp.	Lasiantheae	Piesschaert et al., 1999	Madagascar	AF129275
<i>Trichostachys microcarpa</i> Schum.	Lasiantheae	Piesschaert et al., 1999	Congo	AF191491
<i>Perama hirsuta</i> Aubl.	Lasiantheae	Andersson & Rova, 1999	Guiana	AF004070
<i>Coussarea</i> sp.	Coussareeae	Andersson & Rova, 1999	Guiana	AF004041
<i>Declieuxia dusenii</i> Standl.	Coussareeae	Andersson & Rova, 1999	Brazil	AF004045
<i>Craterispermum laurinum</i> (Poirot) Benth.	Craterispermeae	—	?	AF331645
<i>Gaertnera paniculata</i> Benth.	Gaertnereae	Andersson & Rova, 1999	Congo	AF002736
<i>Morinda angustifolia</i> Roxb.	Morindeae	Zhu, 03160	China	*DQ282648
<i>Gynochthodes epiphytica</i> AC Sm. & S.Darwin	Morindeae	Andersson & Rova, 1999	Fiji	AF001440
<i>Psychotria peduncularis</i> (Salisb.) Steyerl.	Psychotrieae	Andersson, 2002	?	AF410742
<i>Amaracarpus kochii</i> Valetton	Psychotrieae	Andersson, 2002	?	AF410679
<i>Saprosma crassipes</i> Lo.	?	Xiao, 04009	China	DQ282645
<i>Saprosma ternatum</i> Hook. f.	?	Zhu, 03161	China	DQ282646
<i>Schradera</i> sp.	Schradereae	Andersson & Rova, 1999	Colombia	AF003617
<i>Danais</i> sp.	Danaideae	Andersson, 2000	?	AF331648
<i>Anthospermum tricostatum</i> Sond	Anthospermeae	—	?	AF257898
<i>Galopina crocylloides</i> Schinz	Anthospermeae	Andersson & Rova, 1999	South Africa	AF002764
<i>Argostemma rupestre</i> Ridl	Argostemmataeae	Andersson & Rova, 1999	Malaysia	AF002756
<i>Mycetia malayana</i> Craib	Argostemmataeae	Andersson & Rova, 1999	?	AF002771
<i>Paederia scandens</i> (Lour.) Merr.	Paederieae	Zhu, 03162	China	*DQ282647
<i>Plocama pendula</i> Aiton	Paederieae	Andersson & Rova, 1999	?	AF004071
<i>Rubia fruticosa</i> Aiton	Rubieae	Andersson & Rova, 1999	?	AF004078
<i>Didymaea mexicana</i> Hook. f.	Rubieae	Andersson & Rova, 1999	Mexico	AF004047
<i>Pauridiantha lyallii</i> (Baker) Bremek.	Urophyllaeae	Andersson & Rova, 1999	Madagascar	AF004067
<i>Urophyllum glabrum</i> Jack	Urophyllaeae	Andersson & Rova, 1999	Singapore	AF004089
<i>Amphidasya colombiana</i> (Standl.) Steyerl.	Urophyllaeae	Andersson & Rova, 1999	Angola	AF242906
<i>Ophiorrhiza mungos</i> L.	Ophiorrhizeae	Andersson & Rova, 1999	?	AF004064
<i>Cinchona pubescens</i> Vahl.		Andersson & Rova, 1999	?	AF004035
<i>Ixora amplexicaulis</i> Gillespie		—	?	AF242969

Accession numbers marked with* represent the samples which were sequenced in this study.

(Petit, 1964). Our analysis also supports the monophyly of Lasiantheae sensu Bremer and Manen (2000) and the exclusion of *Saldinia* from *Lasianthus* (Figure 1).

The relationships of *Lasianthus*

Lasianthus has been postulated to be closely related to the genera *Psychotria*, *Morinda*, *Saprosma*, *Pauridiantha*, *Perama*, *Saldinia* and *Trichostachys* (Schumann, 1891; Petit, 1964; Verdcourt, 1976; Robbrecht, 1988; Bremer, 1996; Andersson and Rova, 1999; Bremer and Manen, 2000). Our present data confirm that *Saldinia* and

Trichostachys are the closest relatives of *Lasianthus*, and they constitute the tribe Lasiantheae together, as sister to Perameae. This is largely congruent with most previous molecular phylogenetic analyses with few and different samples (Andersson and Rova, 1999; Piesschaert et al., 1999; Bremer and Manen, 2000). However, a previous *rbcL* phylogenetic analysis suggested that *Lasianthus* and *Pauridiantha*, possessing completely different fruit types respectively, come into a single clade (Bremer, 1996). This may result from long branch attraction for the too sparse samples in that analysis. Morphologically,

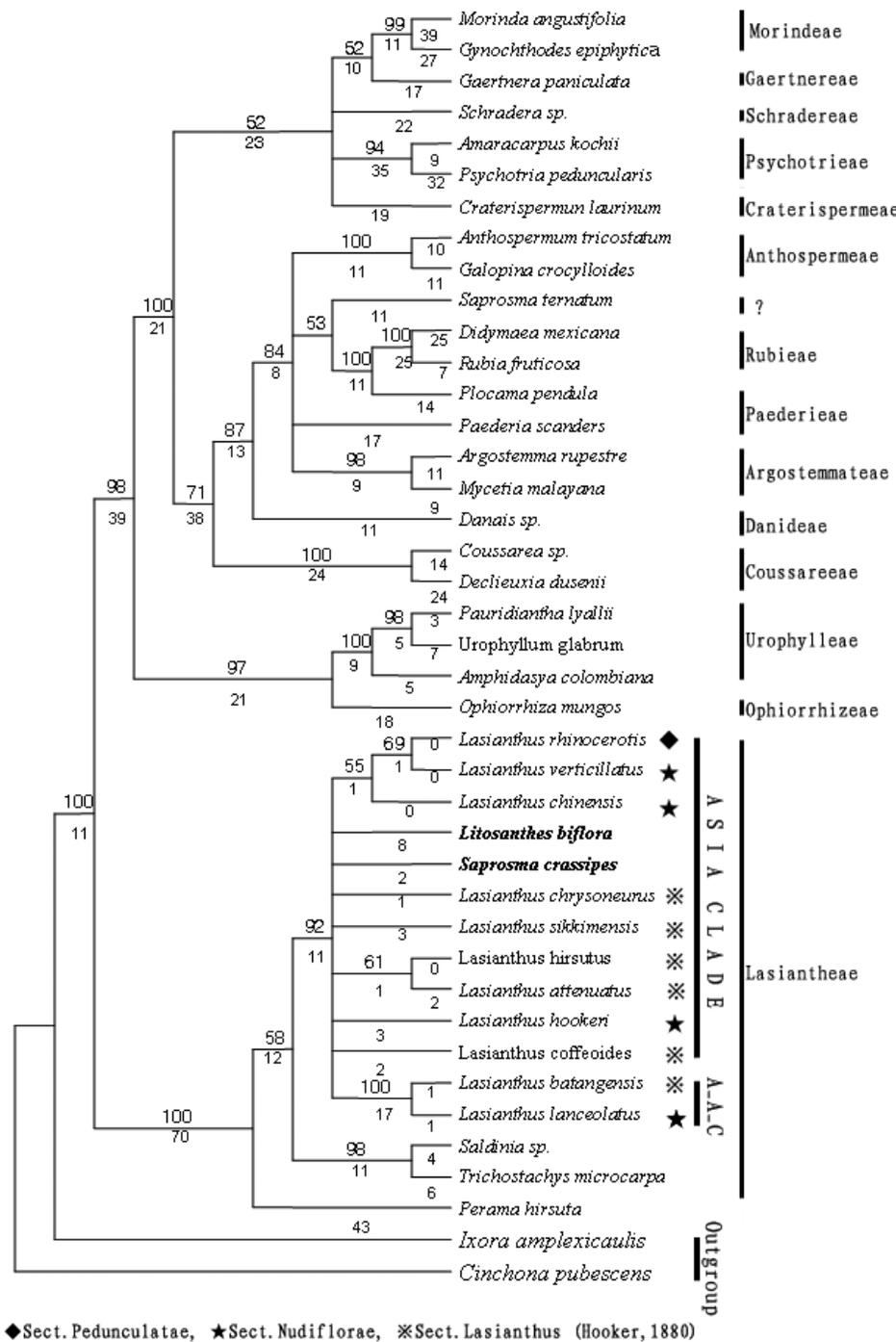


Figure 1. The strict consensus tree of 970 equally parsimonious trees based on *rps16* intron sequences. Length = 819, CI=0.6119, RI=0.8070. Numbers above braches indicate bootstrap percentage (BP), and branch lengths are below branches. Names of the major clades are shown on the right. A-A-C = tropical America-Africa Clade.

Lasianthus, *Saldinia*, and *Trichostachys* share the same seed morphology and wood structure, with features such as fibre tracheids and solitary vessels (Piesschaert et al., 1999).

The infrageneric relationships of *Lasianthus*

In traditional taxonomic treatment (Hooker, 1880), *Lasianthus* was divided into four sections, i.e., *Bracteatae*, *Nudiflorae*, *Stipulares* and *Pedunculatae*, determined by the size of stipules and by the occurrence of bracts and peduncles. Our chloroplast DNA phylogenetic tree does not resolve the infrageneric classification well, but the species from tropical America and tropical Africa form a clade with a full bootstrap percentage. They were, however, placed into Section *Nudiflorae* and Section *Lasianthus*, respectively (Hooker, 1880). Our results are thus inconclusive for testing the monophyly of Hooker's section of *Lasianthus*. Morphologically, the species from tropical America and tropical Africa share common characters, having eight or more locules per ovary and pyrenes per drupe while the others possess fewer than eight locules per ovary and pyrenes per drupe (with only one exception not sampled in this analysis). Further insight into the infrageneric classification of *Lasianthus* will require more extensive taxa sampling for comprehensive analyses through molecular data combined with morphological characters.

The *rps16* sequences have shown much higher divergence (1.722-1.825%) than some other chloroplast markers (*atpB-rbcL*: 0.551-0.735%; *rbcL*: 0.376-0.601%) between the species in *Kelloggia*, which is a rather small genus in Rubiaceae with only two species (Nei et al., 2005). It is thus interesting to mention that all members of the *Lasianthus* clade have short branch lengths (Figure 1). This indicates that their pan-tropical distribution may result from a relatively recent inter-continent dispersal and that these species may have undergone a recent rapid radiation in tropical Asia, perhaps related to the tropical rain forest fragmentation and secondary sympatry. *Lasianthus* has limited potential for developmental and physiological acclimation to intense light. Consequently, the individuals of *Lasianthus* are absent in forest gaps and exclusive found in the understory of primary forests (Cai, 2005). Therefore, the lack of fierce species competition for lots of vacant ecological niches in the understory, coupled with the infrequent migration between isolative forest patches, has contributed to the rapid speciation. This is also implied by the sympatric occurrence of some tropical Asian *Lasianthus* species in relative narrow habitats (Personal observation by Zhu), and by the very asymmetric species richness between continents (twenty species in tropical Africa, three in tropical America, and 160 in tropical Asia, respectively), which shows the marked differences in their species diversification rates between continents. However, a decrease of nucleotide substitution in the *rps16* intron sequence, remains difficult to exclude as an alternative explanation for the observed diversification pattern in *Lasianthus*.

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粗葉木屬 (*Lasianthus*) 的界定及其系統關係的分析： 來自葉綠體 *rps16* 的證據

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基於葉綠體 *rps16* 內含子序列對粗葉木屬 (*Lasianthus*) 和茜草亞科各個族的代表進行系統發育分析，結果表明：1) 粗葉木屬是一個並系類群，因為厚梗染木 (*Saprosma crassipes*) 作為子房有二室、核果含薄壁間開的兩個小堅果而歸屬上有爭議的一些種類的代表，以及單種屬的石核木 (*Litosanthes biflora*) 嵌入其中形成一個強烈支援的分支；2) 粗葉木族由粗葉木屬 *Lasianthus*、*Saldinia* 和 *Trichostachys* 組成；3) 粗葉木屬的分組關係沒有得到支持。

關鍵詞：粗葉木屬；粗葉木族；石核木屬；並系；*rps16* 內含子。