The rise and fall of Sauropus (Phyllanthaceae) : a molecular phylogenetic analysis of Sauropus and allies

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Chapter 2

Delimitation of *Sauropus* (Phyllanthaceae) based on plastid *matK* and nuclear ribosomal ITS DNA sequence data*

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Abstract

A recent molecular phylogenetic study showed that *Sauropus* is deeply embedded within *Phyllanthus* together with its allies, *Breynia* and *Glochidion*. As relationships within *Sauropus* are still problematic and the relationship with *Breynia* has long been doubted, more molecular data are needed to test/corroborate such a broad definition of *Phyllanthus*. This study aims to clarify the status and delimitation of *Sauropus* and establish its position within Phyllanthaceae. Plastid *matK* and nuclear ribosomal ITS DNA sequence data for *Sauropus* and its allies were used to construct phylogenetic trees using maximum parsimony and Bayesian methods. Within *Phyllanthus*, *Sauropus* can be split into the mainly Southeast Asian *Sauropus* sensu stricto (s.s.) plus *Breynia* and the mainly Australian *Sauropus* (formerly *Synostemon*). *Sauropus* s.s. plus *Breynia* comprise two distinct clades; one is the combination of *Sauropus* sections *Glochidioidei*, *Sauropus* and *Schizanthi* and the other is the combination of *S.* sect. *Cryptogynium* and *Hemisauropus* and the monophyletic genus *Breynia*. Molecular data indicate that *Synostemon* should be reinstated at the same level as *Sauropus* s.s. and that *Sauropus* s.s. should be united with *Breynia* under the latter, older name. The molecular data corroborate only two of the five infrageneric groups of *Sauropus* recognized on the basis of morphological data.

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Introduction

The genus *Sauropus* Blume (Blume, 1825) contains monoecious and dioecious woody herbs to small shrubs. Most of the species commonly occur in monsoonal tropical woodlands and rain forests (Van Welzen, 2003; Hunter, 2005). *Sauropus* is closely related to *Breynia*, *Glochidion* and *Phyllanthus*. Distinguishing morphological characters are not always clear-cut for these genera.

Molecular phylogenetic studies of *Phyllanthus*, the largest genus in Phyllanthaceae, found three out of its eight subgenera to be polyphyletic and the genus in its traditional circumscription to be paraphyletic (Kathriarachchi et al., 2005, 2006). *Breynia*, *Glochidion*, *Reverchonia* and *Sauropus* are embedded in *Phyllanthus*. If all these genera are united with *Phyllanthus*, then the number of *Phyllanthus* species increases from 833 to 1269 (Govaerts et al., 2000) and a giant and morphologically heterogeneous genus is created. Many nomenclatural changes would be necessary to obtain a classification that conforms to the molecular results. Kathriarachchi et al. (2005, 2006) suggested the possibility of maintaining a paraphyletic *Phyllanthus* or recognizing more than 20 clades in *Phyllanthus* at generic rank. However, Hoffmann et al. (2006) argued for uniting *Phyllanthus* sensu lato (s.l.) and avoiding a paraphyletic construct. The non-monophyletic subgenera and problem genera deeply embedded within *Phyllanthus* are in need of analysis to resolve the issues of the *Phyllanthus* classification.

Sauropus is one of these problem genera (morphologically difficult to recognize; e.g. Van Welzen, 2000) apparently deeply embedded within *Phyllanthus* (Kathriarachchi et al., 2006). Traditionally the genus was classified in Euphorbiaceae subfamily Phyllanthoideae (Webster, 1994; Radcliffe-Smith, 2001). Later, Euphorbiaceae was segregated into five families based on molecular phylogenetic studies (APG II, 2003); *Sauropus* is now placed in Phyllanthaceae (Wurdack et al., 2004; Kathriarachchi et al., 2005; Samuel et al., 2005; Hoffmann et al., 2006). The genus comprises 83 species found in the Mascarenes, India, Southeast Asia, Malesia and Australia (Govaerts et al., 2000; Van Welzen, 2003). There are two centres of diversity, one in Thailand-Indochina, *Sauropus* sensu stricto (s.s.), and one in Australia, where most species formerly placed in *Synostemon* (Airy Shaw, 1980a; Radcliffe-Smith, 2001; Van Welzen, 2003) are found. We use *Sauropus* s.l. for the combination of Southeast

Asian *Sauropus* and *Synostemon*, *Sauropus* s.s. for the mainly Southeast Asian part of *Sauropus* and *Synostemon* for the mostly Australian species.

The placement of *Synostemon* within *Sauropus* has long been under doubt. Airy Shaw (1980a) considered these genera to resemble each other closely in habit, with the differences between them supposedly too small to recognize both groups at the generic rank (Airy Shaw, 1971, 1975, 1980a). He stated (1980a): "Their bifocal development in Southeast Asia and Australia is curious and without an obvious parallel. It does not seem possible to utilize the subgenera and sections proposed by Müller Arg. … (1866) and by Pax & Hoffmann… (1922), in order to systematize the genus as a whole, including the Australian species. The so-called section (or subgenus) *Hemisauropus* Müll.Arg. (cf. Kew Bull. 23:55 (1969)) appears to be unrepresented in Australia, and is in any case doubtfully tenable as a natural group, since the distinctive floral character seems to be uncorrelated with vegetative or other features." Airy Shaw suggested placing the Australian species into section *Schizanthi*, but at the same time he noted the increased morphological problems within this section. Radcliffe-Smith (2001) stated that Airy Shaw might have a good reason for transferring the Australian species of *Synostemon* to *Sauropus*. However, he also indicated the problematic demarcation of *Sauropus* from *Breynia*, because the latter resembles *Synostemon* in floral characters.

The presence of diploporate pollen suggests a close relationship between *Sauropus* s.l. and *Breynia* (Sagun & Van der Ham, 2003), and there is also a great resemblance in seed morphology (Stuppy, 1996; Tokuoka & Tobe, 2001). A phylogenetic study based on morphological and palynological data showed *Sauropus* to be paraphyletic with diploporate *Phyllanthus* species embedded within the genus, and *Sauropus* s.s. distinct from *Synostemon* (Van Welzen, 2003). Only one species formerly included in *Synostemon*, *Sauropus bacciformis* (L.) Airy Shaw, was found to be better placed within *Sauropus* s.s. of Southeast Asia. *Breynia* formed a polytomy with two groups of *Sauropus*. However, Van Welzen (2003) found no bootstrap support for these results. More recently molecular phylogenetic studies by Kathriarachchi et al. (2006) confirmed the paraphyletic nature of *Sauropus*, with *Breynia* embedded in the largely unresolved *Sauropus*. The sample of *Sauropus* species used by Kathriarachchi et al. was insufficient to confirm the separation of the Southeast Asian *Sauropus* and *Breynia* from *Synostemon*. Further molecular work is needed to clarify relationships in and around *Sauropus*. Here we carry out molecular phylogenetic analyses

using nuclear and plastid DNA markers to elucidate the limits of *Sauropus*, and to confirm its position within Phyllanthaceae.

Materials and methods

Taxon sampling

Data for 125 accessions, including 97 accessions from this study and 28 accessions already in GenBank (http://www.ncbi.nlm.nih.gov/Genbank), were used in this study (Appendix 2.1). Ingroup sampling focused on the representatives of all sections of *Sauropus* recognized by Pax & Hoffmann (1922) and Airy Shaw (1969) with 47 specimens (42 species) presented here. Other ingroups included representatives of the related genera *Breynia* (12 species), *Glochidion* (four species), and *Phyllanthus* (seven species) inferred from the studies of Hoffmann et al. (2006), Kathriarachchi et al. (2006), and Webster (1994). *Margaritaria rhomboidalis* was used as the outgroup (see Kathriarachchi et al., 2006).

The analyses used plastid *matK* sequences from 66 ingroup accessions (61 species), 52 of which were newly generated for this study. The internal transcribed spacer (ITS) data set contained 57 ingroup accessions (52 species), 45 of which were generated for this study.

DNA extraction, amplification, and sequencing

Herbarium specimens were available for most taxa, and these were supplemented with a few silica-dried samples. DNA was isolated using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany). For silica-dried material the manufacturer's instructions were followed. For most herbarium specimens a modified protocol was used with a prolonged lysis step with proteinase K and ß-mercaptoethanol (Wurdack et al., 2004).

The plastid *matK* and the flanking *trnK* intron were amplified using all primers described by Samuel et al. (2005). Most degraded DNA from herbarium specimens was amplified in four or five fragments that were sequenced separately and then combined into a single contig. Amplification of the nuclear ribosomal ITS region was carried out using the primer pairs ITS5 and ITS4 (White et al., 1990).

Amplifications were performed in a volume of 50 µl containing 10--100 ng genomic DNA, 50× PCR Buffer (Qiagen, Hilden, Germany), 20 pmol of each primer, 5 mM dNTPs, 25 mM $MgCl_2$, 0.5 µg bovine serum albumin (BSA; Promega, Madison, Wisconsin, USA),

and 2 units Taq DNA polymerase (Qiagen, Hilden, Germany). The following temperature profile was used: an initial denaturation for 2 min at 94°C followed by 35--40 cycles of: denaturation for 1 min at 94°C, annealing for 30 s at 48°C for *matK* and 52.5°C for ITS and elongation for 1 min at 72°C. There was a final elongation step of 10 min at 72°C.

PCR fragments were checked for length and yield by gel electrophoresis on 1% agarose gels and cleaned with either the Promega PCR cleaning kit (Promega, Madison, Wisconsin, USA) or Nucleospin Extract II (Macherey-Nagel, Düren, Germany) columns. The cleaned PCR products were analyzed on either an ABI 3730xl automated sequencer (Applied Biosystems, Forster City, California, USA) using ABI BigDye terminator chemistry or a MegaBACE 1000 automated sequencer (Amersham Bioscience) using DYEnamic™ ET Dye Terminators chemistry following the manufacturers' protocols. Each PCR template was sequenced in both directions using the respective amplification primers. Sequence contigs were assembled and edited using Sequencher v4.1.4 or v4.7 (Gene Codes Corp., Ann Arbor, Michigan, USA). These sequences have been deposited in GenBank under accession numbers EU623549--EU623593 and EU643735--EU643786.

Sequence alignment and phylogenetic analyses

Sequence alignments were initially made using pairwise alignment in MacClade v4.08 (Maddison & Maddison, 2001) and improved by eye. If obviously overlapping nucleotide peaks were detected in both forward and reverse chromatograms, then the site was coded with IUPAC ambiguity codes. Gaps in *matK-trnK* (1--19 bp in length) occurred mostly in the intron of the *trnK* intron, but a few in multiples of three (6--15 bp in length) were found in the coding region. In the ITS alignment, gaps occurred in the non-coding regions only. Gaps were treated as missing data in our analyses and indels with uncertain homologies were excluded from the alignment.

Parsimony (MP) analyses were performed in PAUP* v.4.0b1 (Swofford, 2003). All characters were treated as unordered (Fitch parsimony; Fitch, 1971), equally weighted, and gaps were treated as missing data. Parsimony analyses were conducted using heuristic search methods with 1000 replicates of random taxon addition combined with tree-bisection-reconnection branch swapping (TBR) and the MulTrees option active, with no more than 10 trees saved per replicate to save time instead of swapping on large numbers of potentially

suboptimal trees. To assess support for each clade, bootstrap analyses (Felsenstein, 1985) were performed with 1000 bootstrap replicates, TBR swapping of all replicates consisting each of 10 random taxon additions, and no more than 10 trees saved per replicate. Bootstrap percentages (BP) are described as high (85--100%), moderate (75--84%), low (50--74%) or no (<50%) support. The consistency index (CI) including uninformative characters is used to discuss the results.

Bayesian inference was conducted with MrBayes v.3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003) to determine the simplest model of sequence evolution that best fits the data for the combined *matK* and ITS matrix. MrModeltest v.2.2 (Nylander, 2004) was used to find the best-fitting substitution model. The models of molecular evolution were selected using the Akaike Information Criterion (AIC). The chosen models were GTR+G (nst=6, rate=gamma) for *matK* and SYM+I+G (nst=6, rate=invgamma) for ITS. For each analysis two simultaneous runs were done starting from random trees for 10,000,000 generations, having three heated and one cold chain. Markov chains were sampled every 100 generations. Analyses were run until the average standard deviation of split frequencies approached 0.01, indicating the convergence of two runs. The plot of generation vs. log probability was inspected after the run to ensure that stationarity was reached and to determine the burn-in. Typically, about 10% of trees were discarded as burn-in. The majority-rule consensus tree (not shown) containing posterior probabilities (PP) was built from the remaining sampled trees.

Results

Due to difficulties in amplifying and sequencing *matK* and ITS from degraded herbarium specimens, only partial sequences could be obtained for several taxa. Five taxa present for *matK* were completely missing for ITS and 13 taxa present for ITS were completely missing for *matK*.

Information on the analyses of individual and combined datasets is given in Table 2.1. Here we report only the cladograms based on the analyses including indels because the inclusion or exclusion of indels in the analyses had no or little effect on the phylogenetic results. The trees produced by both parsimony (Figs. 2.1—2.3) and Bayesian inferences (BA; not shown) were largely congruent with respect to the groups recovered. The results of the

combined analysis (Fig. 2.3) are used to discuss phylogenetic relationships within *Sauropus* and the bootstrap values are used to discuss support.

Table 2.1. Summary of data properties and parsimony analyses for the three alignments.

Sequence characteristic	*matK*+*trnK*	ITS	Combined-reduced taxon sampling
Taxon sampling			
No. of accessions (ingroups)	67 (66)	58 (57)	53 (52)
No. of species (ingroups)	62 (61)	53 (52)	50 (49)
Length of sequences (bp)	479-1888	636-678	not determined
Length of alignment (bp)	1959	708	2661
No. of variable characters	217	121	325
No. of potentially informative sites (%)	135 (6.9)	225 (31.8)	316 (11.9)
No. of gap positions (%)	101 (5.1)	100 (14.1)	167 (6.3)
No. of missing data (%)	398-1409 (21-75)	N/A	not determined
No. of MPTs	9860	4834	7270
Length of MPTs	450	971	1297
Consistency index (CI), excluding uninformative characters	0.71	0.50	0.54
Consistency index (CI), all characters	0.85	0.57	0.67
Retention index (RI)	0.90	0.73	0.76
Tree topology	Fig. 2.1	Fig. 2.2	Fig. 2.3

Analysis of matK

In the *matK* dataset, complete sequences were obtained for 31%. For the remaining taxa 25--79% of the sequence was obtained. The *matK* data included the *matK* gene with 1512--1542 base pairs (bp) and the flanking *trnK* intron at 5′ and 3′ ends with 317--346 bp from completed sequences. The incomplete sequences varied from 479--1490 bp. The *matK* alignment was 1959 bp long. Maximum parsimony analysis of the plastid *matK* produced 9860 most-parsimonious trees (MPTs) of 450 steps with 135 potentially parsimony-informative characters, CI = 0.85, RI = 0.90. The strict consensus with bootstrap percentages and Bayesian posterior probabilities are shown in Fig. 2.1. *Sauropus* s.l. and *Breynia* form a clade (clade A) with strong support (BP 93; Fig. 2.1). Within this clade, there are two subclades *Synostemon* (B) and *Sauropus* s.s. plus *Breynia* (C). Clade B is strongly supported (BP 97), whereas Clade C has low support (BP 67). Most species within Clades B and C form polytomies, but *Breynia* (Clade D) forms a strongly supported monophyletic group (BP 91). Clade A is sister to *Glochidion* with strong support (BP 91). Clade A and *Glochidion* are

embedded within *Phyllanthus* with moderate support (BP 81). Most of the above mentioned BP-supported relationships have PP values 1.0.

Analysis of ITS

The ITS region (ITS1 + 5.8S + ITS2) varied from 557 to 599 bp in length, including 187--217 bp for ITS1 and 206--218 bp for ITS2. The ITS alignment was 708 bp long. The ITS analysis recovered 4834 MPTs of 971 steps (CI = 0.57, RI = 0.73) with 225 potentially parsimony-informative characters.

There is high support (BP 100) for the *Sauropus* s.l. plus *Breynia* clade (A; Fig. 2.2). Within this clade, there are three subclades (B, C and D). Clade B includes all *Synostemon* spp. (BP 99). Clade C includes *Sauropus* s.s. sect. *Glochidioidei*, *Sauropus* and *Schizanthi* and unplaced species (BP 55). Clade D (BP 87) comprises *Sauropus* s.s. sect. *Cryptogynium* and *Hemisauropus* (forming a polytomy) and *Breynia* (Clade E, strong support, BP 93). *Sauropus* s.l. plus *Breynia* (Clade A) is sister to *Glochidion* (strong support, BP 89) and both are embedded within *Phyllanthus* (strong support, BP 92). The results of BA are largely congruent with MP, although in BA Clade A has two subclades (not shown), one of *Synostemon* (Clade B) with high support (PP 1.0), and the other of *Sauropus* s.s. plus *Breynia* (Clades C+D) with support less than 0.95 PP. In the BA the *Sauropus* s.s. plus *Breynia* clade is made up of two subclades with high support (PP 0.99), i.e. the same main clades in MP.

Combined analysis

Seventy two taxa (65 species) were included in the combined dataset. The MP and BA (not shown) resulted in a tree topology largely congruent with the *matK* tree (Fig. 2.1), but BA showed an uncertain placement of the taxa completely missing for *matK* or ITS, causing reduced resolution and/or support values. The taxa completely missing for *matK* or ITS were removed from the final analyses with the combined dataset (Fig. 2.3), which resulted in increased resolution and support.

The combined analysis with a reduced taxon sampling of 53 specimens (50 species) resulted in 7270 shortest trees with 1297 steps (CI = 0.67, RI = 0.76). The aligned data consisted of 2661 bp with 316 potentially parsimony-informative characters. The percentage of potentially informative characters was higher for ITS (31.8%) than *matK* (6.9%). The CI

and RI were much higher for *matK* (CI = 0.85, RI = 0.90) than for ITS (CI = 0.57, RI = 0.73) or the combined data (CI = 0.67, RI = 0.76).

The strict consensus tree of the combined dataset showed many polytomies (the resolved branches are indicated as thick line in Fig. 2.3). It corroborates the results from the individual analyses. *Glochidion*, *Sauropus* s.l. and *Breynia* are embedded within *Phyllanthus* (moderate support, BP 82), and *Glochidion* is sister to *Sauropus* s.l. plus *Breynia* (strong bootstrap support, BP 99). The *Sauropus* s.l. plus *Breynia* clade (A, high support, BP 100) contains two clades (B and C) as in the *matK* analysis (Fig. 2.1): Clade B consisting of *Synostemon* (high support, BP 100) and Clade C consisting of *Sauropus* s.s. plus *Breynia* (strong support, BP 89). Clade C contains two subclades: Clade D comprising *Sauropus* s.s. sect. *Cryptogynium* and *Hemisauropus* and *Breynia* (strong support, BP 96) and Clade E comprising *Sauropus* s.s. sect. *Glochidioidei*, *Sauropus* and *Schizanthi* and some unplaced species (weak bootstrap support, BP 62, but high Bayesian support, PP 1.0 (not shown)). The *Breynia* clade (F) with high support (BP 100) forms a polytomy with *Sauropus* sect. *Cryptogynium* and *Hemisauropus* in Clade D. The BA (not shown) has the same topology as the MP with posterior probabilities (PP 0.99 and 1.0) for the main clades in the MP.

Discussion

The previous study by Hoffmann et al. (2006) showed cladograms with a largely unresolved *Sauropus*. Here we report more resolution within *Sauropus* with representatives of all sections recognized by Pax & Hoffman (1922) and Airy Shaw (1969). Moreover, our results solved the problem of unclear placement of former *Synostemon*. *Sauropus bacciformis* is part of *Synostemon*, although its morphology in a previous phylogenetic study pointed at inclusion in *Sauropus* s.s. (Van Welzen, 2003). The main groups identified in our study support recognition of monophyletic subgroups within *Phyllanthus* in future classifications as suggested by Hoffmann et al. (2006).

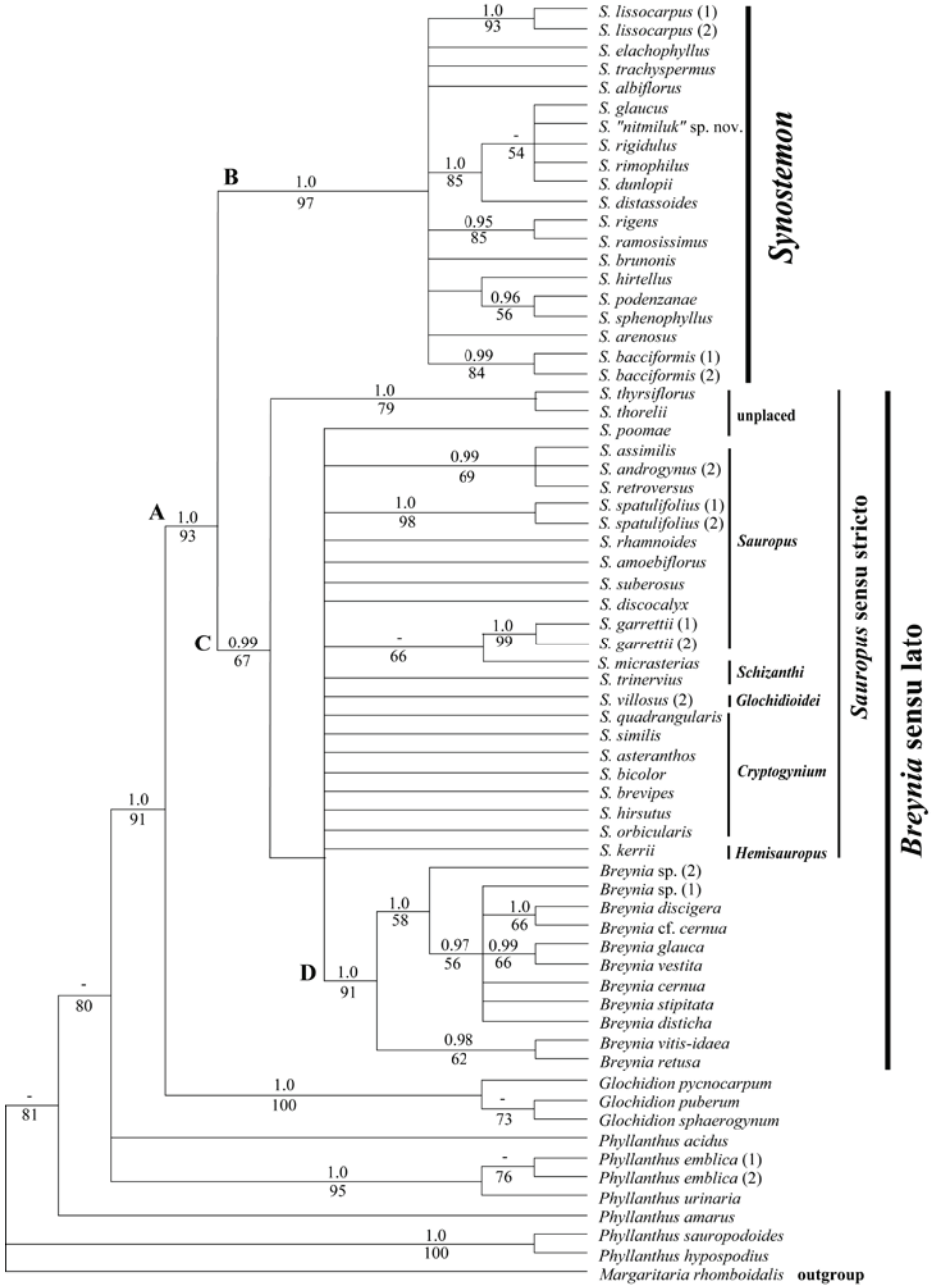

Fig. 2.1. Strict consensus of 860 most-parsimonious trees (450 steps, CI = 0.85, RI = 0.90) of *Sauropus* and allies based on plastid *matK* gene and partial *trnK* intron data. Bayesian posterior probabilities ≥0.95 and bootstrap.

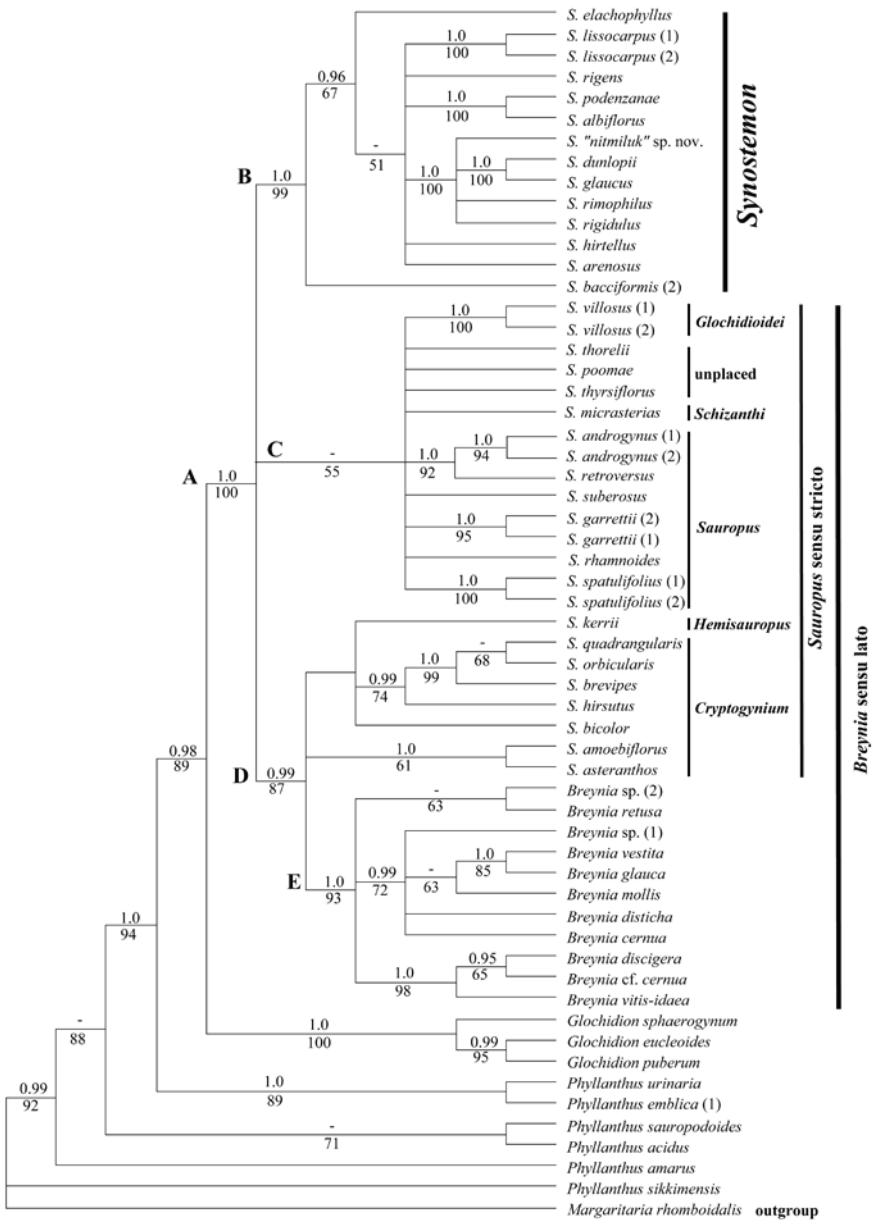

Fig. 2.2. Strict consensus of 8581 most-parsimonious trees (971 steps, CI = 0.57, RI = 0.73) of *Sauropus* and allies based on nuclear ribosomal ITS data. Bayesian posterior probabilities ≥0.95 and bootstrap percentage ≥50 are shown above and below branches, respectively. '-' indicates Bayesian posterior probabilities <0.95.

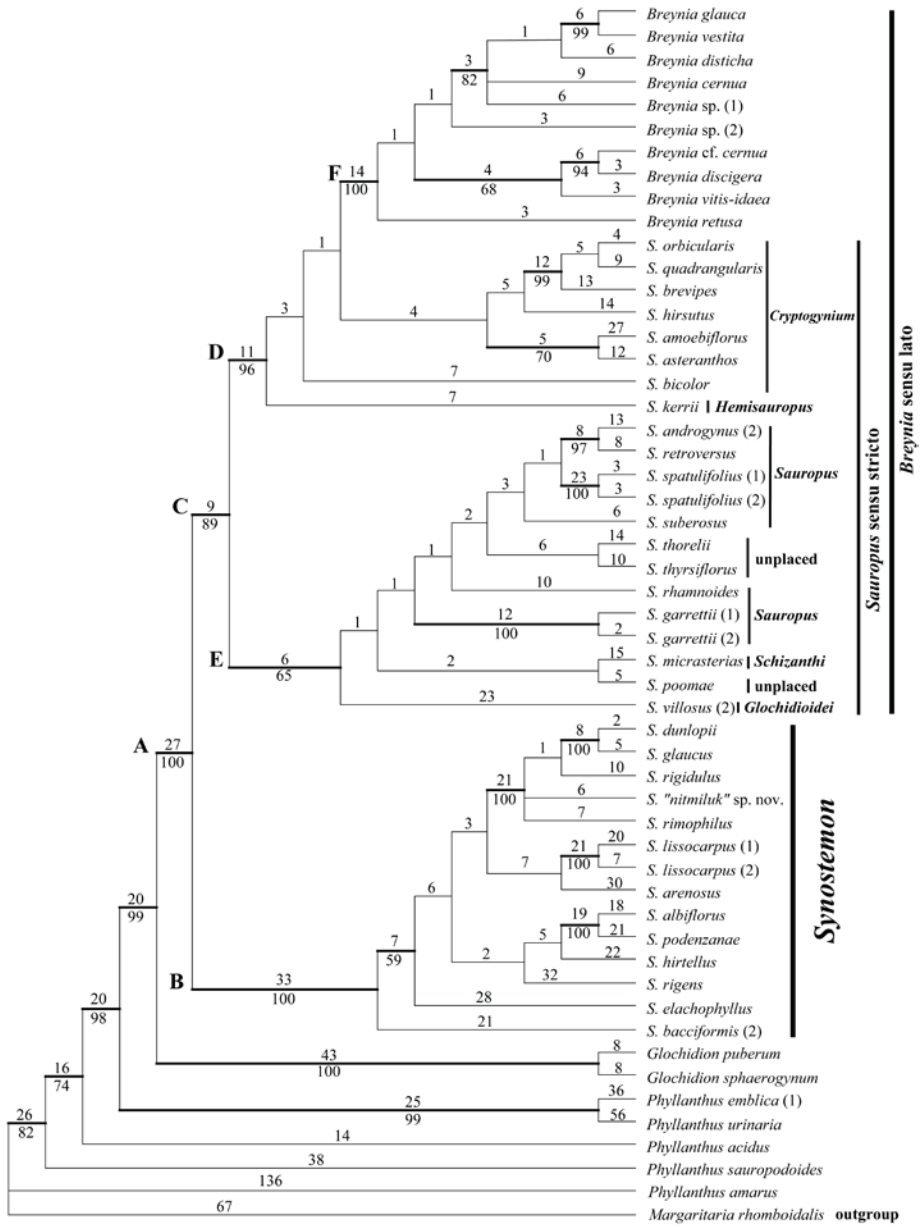

Fig. 2.3. One of 7270 most-parsimonious trees (1297 steps, CI = 0.67, RI = 0.76) of *Sauropus* and its allies based on combined plastid *matK* gene data and nuclear ribosomal ITS. Branch lengths and bootstrap percentage ≥50 are shown above and below branches, respectively. The strict consensus of the 7270 MPTs is indicated by the bold branches. Branches that collapse in the strict consensus tree are indicated by the thinner lines.

Paraphyly of Sauropus sensu lato

Our results from the combined analysis of *matK* and ITS sequences confirm the paraphyly of *Sauropus* s.l. reported in molecular phylogenetic analyses focusing on *Phyllanthus* (Kathriarachchi et al., 2006). *Breynia* is shown to be deeply embedded in *Sauropus* s.s. This paraphyly in the molecular analyses contradicts the results of phylogenetic analyses based on morphological and palynological data, that recover a monophyletic *Sauropus* s.s. embedded within diploporate *Phyllanthus* species, both within *Sauropus* s.l. (Van Welzen, 2003). Airy Shaw (1980b) and Radcliffe-Smith (2001) noted that *Breynia* is scarcely distinct from *Sauropus.* Our results support their view. Mennega (1987) showed that the wood anatomy of *Phyllanthus* and related genera (subtribe Fluggeinae) is quite similar. She too stressed the similarity between *Breynia* and *Sauropus*, which both deviate from the other genera in having small intervascular and vessel-ray pits. Levin (1986) suggested a grouping of *Breynia* with *Sauropus*, *Synostemon*, *Glochidion* and *Phyllanthus* because of similarities in leaf anatomy, including a shared stomatal development pattern. Morphologically *Breynia* is more similar to *Sauropus* s.s. in its microphyllous leaves, whereas *Synostemon* has nanophyllous leaves. Airy Shaw (1980b) reported that the leaves of *Breynia* blacken on drying, but this is not true for all species. Tokuoka & Tobe (2001) reported similarity in the inner integument thickness and oblong, multi-cell-layered exotegmen of the ovules of both genera. The palynological study of Sagun & Van der Ham (2003) also supported the merging of *Sauropus* and *Breynia* based on similar pollen ornamentation, completely endexinous exine and diploporate colpi.

According to Radcliffe-Smith (2001), *Breynia* and *Sauropus* share a bifid or emarginated style (but see also below), non-apiculate anthers and three locular ovaries, although the fruit is more drupaceous in *Breynia* (not or only tardily dehiscent) and generally capsular in *Sauropus*. *Breynia* forms a distinct group within *Sauropus* s.s. (see *Paraphyly of Southeast Asian Sauropus* below). The differences between the two genera are mainly in the staminate flowers. The staminate calyx is usually discoid in *Sauropus* and turbinate in *Breynia*. The morphology of the androecium is usually also different (see below). There are also some differences in the stigmas. Those of *Breynia* are generally short and indistinct, whereas in *Sauropus* s.s. the stigmas divide distally and form crescent-shaped branches which are held either erect or horizontal. Japanese researchers (Kato et al., 2003; Kawakita & Kato, 2004b) observed a close, probably co-evolutionary, relationship between *Epicephala* moths and

several species in *Glochidion*, *Breynia* and *Phyllanthus*. The relationship is comparable to that between *Yucca* and the yucca moths, in which the female moths actively seek pollen and pollinate the pistillate flowers while depositing eggs. Species of Phyllanthaceae species involved in the Japanese studies mainly showed stigmas to which pollen does not attach, although in various ways (the stigmas of *Glochidion* and *Breynia* are different, stigmatic tissue in *Glochidion* being hidden by the development of a cone-like structure by the stigmas, whereas in *Breynia* the stigmas are often extremely short and devoid of papillae). *Sauropus* s.l. species were not included in these studies. In fact, no information about pollination of *Sauropus* s.l. flowers is available; the flowers may be pollinated by various pollinators or they may also be part of the *Epicephala*–Phyllanthaceae pollination complex.

Monophyly of Australian Sauropus (former Synostemon)

Our results show that the Australian *Synostemon* is monophyletic (Figs. 2.1--3). The results agree with the morphological and palynological phylogenetic analyses (Van Welzen, 2003) except for *Sauropus bacciformis*, which Van Welzen placed in *Sauropus* s.s. In our analyses *S. bacciformis* is sister to the rest of *Synostemon* (Fig. 2.3). Its morphological based placement with *Sauropus* s.s. might be due to plesiomorphic character states. The results also indicate that the placement of *Synostemon* in section *Schizanthi* as suggested by Airy Shaw (1980a) is incorrect. The species of *Sauropus* section *Schizanthi* group with species of other sections in *Sauropus* s.s. and *Breynia* (see *Paraphyly of Southeast Asian Sauropus*). The genus *Synostemon* was described by Mueller (1858) based on *Synostemon ramosissimus* F.Muell. (type) and *S. glaucus* F.Muell. Several species of *Synostemon* were incorrectly placed in *Glochidion* and *Phyllanthus* (Hunter & Bruhl, 1997a). Airy Shaw's (1980a, b) reason for transferring *Synostemon* to *Sauropus* remains unclear to us. Our analyses (Figs. 2.1–3) show *Synostemon* to be a well supported clade, distinct from *Sauropus* s.s. and *Breynia* (Figs. 2.1–3). *Sauropus bacciformis*, however, blurs the morphological distinction between *Sauropus* s.s. and *Synostemon*, because it has the same type of androecium as *Sauropus* s.s. Airy Shaw (1975) stated that specimens of *S. bacciformis* from Borneo are scarcely distinct from *Sauropus* s.s. It had seemed curious that this widespread species is absent from Australia (Airy Shaw, 1980a), but we are now able to report its presence in Australia from at least five specimens from coastal tropical Australia hitherto identified as '*Sauropus* sp.'. *Sauropus*

bacciformis is similar to *Sauropus* s.s. in its connate sepals with scales, whereas most other *Synostemon* have free sepals and no scales. However, study of seed coats showed a closer resemblance between *S. bacciformis* and Australian *S. huntii* than between *S. bacciformis* and most species of *Sauropus* s.s. (Stuppy, 1996).

Apart from the staminate calyx similar to that of *Sauropus* s.s., *S. bacciformis* has an androphore typical of *Sauropus* s.s.; this branched androphore is also present in *Synostemon* species, *S. lissocarpus* (S.Moore) Airy Shaw and *S. salignus* J.T.Hunter & J.J.Bruhl (not represented in our analysis). *Sauropus anemoniflorus* J.T.Hunter & J.J.Bruhl (not represented in our analysis) from north-eastern Queensland has sepals that are fused, forming a lobed cup with a scale-like swelling at the base of each lobe, but otherwise it has an androphore typical of *Synostemon*. Other species of *Synostemon* with staminate flowers with fused sepals include *S. huntii* Airy Shaw*, S. rigens* (F.Muell.) Airy Shaw, *S. ramosissimus*, *S. sphenophyllus* (Airy Shaw) Airy Shaw and *S. hirtellus* (F.Muell.) Airy Shaw*,* but these lack basal scales, which may indicate secondary fusion of the sepals.

Telford and Bruhl (in prep.) are redefining the limits of many species of *Synostemon*. Their study should provide a framework for a detailed molecular analysis of the genus and aid further assessment of morphological homology/homoplasy across *Synostemon* and *Sauropus* s.s.

Paraphyly of Southeast Asian Sauropus

The cladogram from the resulting combined analyses (Fig. 2.3) shows paraphyly of *Sauropus* s.s. due to the inclusion of *Breynia*. Trees from the combined *matK* and ITS sequence data show that only two groups can be recognized with *Sauropus* s.s., in contrast to the sections proposed by Pax & Hoffmann (1922) and Airy Shaw (1969). A distinct and strongly supported group is the combination of *S.* sect. *Cryptogynium* and *Hemisauropus* and *Breynia*. Although *Breynia* is always monophyletic, its recognition renders the rest of the clade paraphyletic. Our results indicate the need to unite *Breynia* and *Sauropus* under *Breynia*, as the name *Breynia* J.R.Forst. & G.Forst. (Forster & Forster, 1775) predates *Sauropus* Blume (Blume, 1825).

Most species of *Sauropus* sect. *Glochidioidei*, *Sauropus* and *Schizanthi* form a polytomy with some unplaced taxa. Apart from the difference in staminate calyx shape, the androecium

in *Breynia* is also different. *Breynia* has a robust androphore with anthers arranged along it, whereas the androphore in (most) species of *Sauropus* s.s. is slender and splits into three horizontal rays with the anthers hanging underneath. The only exception to the latter type is shown by the species in section *Hemisauropus*. This section has more robust stamens pointing diagonally upwards. The staminate calyx of section *Hemisauropus* is also different: it lacks scales and half of the lobes are folded inwards and grown together with the rest of the sepal; moreover, all species except *S. granulosus* have the same type of pollen. The morphological and palynological phylogenetic analyses (Van Welzen, 2003) demonstrated that section *Hemisauropus* may need special status. The present analysis cannot address this issue, as we were only able to sample one species of this section.

Conclusions

Morphological characters traditionally used to distinguish species in *Sauropus* and *Breynia* have focused on leaf, staminate and pistillate characters (Pax & Hoffmann, 1922; Airy Shaw, 1969; Van Welzen, 2003). Our molecular analyses show that these characters do not support a division into monophyletic genera. Our data suggest that *Synostemon* should be reinstated at the generic level and *Sauropus* s.s. must be united with *Breynia* under *Breynia*. As *Breynia* s.s. appears to be monophyletic and morphologically recognizable, it merits infrageneric recognition within the proposed *Breynia* s.l. These taxonomic changes should be postponed until a larger sample of *Sauropus* s.s. has been analysed and robust estimations of phylogeny have been obtained.

In our opinion, the placement of *Glochidion*, *Breynia* (including *Sauropus* s.s.) and *Synostemon* within *Phyllanthus* remains tentative, because the unification does not resolve the relationships between the different recognizable groups. Unification only displaces the problem to infrageneric levels. With the present state of knowledge, maintaining the different genera is practical; it prevents numerous name changes and provides nomenclatural stability. More variable DNA markers are needed to resolve the species relationships and prior to formal revision of the generic and infrageneric classification of *Phyllanthus*. Also, further detailed micromorphological studies across the group are needed to better assess the morphological homology and covariation/corroboration of molecular and morphological data to elucidate practical, morphological diagnostic features of the genera.

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Appendix 2.1. Specimens used in the present study. GenBank accession numbers of new sequences are shown in bold.

Taxa	Voucher/Herbaria	Source	GenBank accession number	
			matK	ITS
Ingroups				
Breynia cernua (Poir.) Müll.Arg.	Wightman 1810 (K)	Australia	AY552423	AY936650
B. cf. *cernua* (Poir.) Müll.Arg.	Baker et al. 37 (L)	Papua, Indonesia	**EU643735**	**EU623549**
B. discigera Müll.Arg.	Takeuchi et al. 18873 (L)	N. Sumatra, Indonesia	**EU643736**	**EU623550**
B. disticha J.R.Forst. & G.Forst.	Chase 14458 (K)	RBG Kew, Living collection (1973-12222)	AY936564	AY936651
B. glauca Craib	Pooma et al. 2702 (L)	Nong Khai, Thailand	**EU643737**	**EU623551**
B. mollis J.J.Sm.	Sands 1076 (L)	Papua & New Guinea, Indonesia	N/A	**EU623552**
B. retusa (Dennst.) Alston	Kathriarachchi et al. 43 (K)	Sri Lanka	AY936565	AY936652
B. stipitata Müll.Arg.	Chase 14461 (K)	RBG Kew, Living collection from Queensland, Australia	AY552422	N/A
B. vestita Warb.	Barker & Beaman 70 (L)	Papua, Indonesia	**EU643738**	**EU623553**
B. vitis-idaea (Burm.f.) C.E.C.Fisch.	Kathriarachchi et al. 7 (K)	Sri Lanka	AY936566	AY936653
Breynia sp. (1)	Hunter 1973 (BRI)	Queensland, Australia	**EU643767**	**EU623577**
Breynia sp. (2) [*]	Van Welzen 2006-3 (L)	Chiang Rai, Thailand	**EU643739**	**EU623554**
Glochidion eucleoides S.Moore	Utteridge 249 (K)	New Guinea, Indonesia	N/A	AY936657
G. puberum (L.) Hutch.	Chase 14460 (K)	RBG Kew, Living collection from Guizhou, China	AY552428	AY936659
G. pycnocarpum (Müll.Arg.) Bedd.	Kathriarachchi et al. 44 (K)	Sri Lanka	AY936570	N/A
G. sphaerogynum (Müll.Arg.) Kurz	Van Welzen 2003-21 (L)	Nakhon Ratchasima, Thailand	**EU643740**	**EU623555**
Phyllanthus acidus (L.) Skeels	Van Welzen 2003-14 (L)	Saraburi, Thailand	**EU643741**	**EU623556**
P. amarus Schumach. & Thonn.	Van Welzen 2006-5 (L)	Chachoengsao, Thailand	**EU643742**	**EU623557**
P. emblica L. (1)	Chase 14459 (K)	RBG Kew, Living collection (1984-4527) from India	AY936594	AY936689
P. emblica L. (2)	Van Welzen 2003-11 (L)	Saraburi, Thailand	**EU643743**	N/A
P. hypospodius F.Muell.	Bruhl et al. 1123 (L)	Queensland, Australia	**EU643744**	N/A
P. sauropodoides Airy Shaw	Forster 29857 (L)	Queensland, Australia	**EU643745**	**EU623558**
P. sikkimensis Müll.Arg.	Pooma et al. 5233 (L)	Phetchaburi, Thailand	N/A	**EU623559**
P. urinaria L.	Ralimanana et al. 271 (K)	Mayotte, Comoro Islands	AY936637	AY936736
Sauropus albiflorus (F.Muell. ex Müll.Arg.) Airy Shaw	Forster 21362 (L)	Queensland, Australia	**EU643746**	**EU623560**

[*] This specimen was identified as *Breynia* cf. *retusa* (Dennst.) Alston in Chapter 3, Appendix 3.1

Appendix 2.1. Continued.

Taxa	Voucher/Herbaria	Source	GenBank accession number	
			matK	ITS
S. amoebiflorus Airy Shaw	Maxwell 90-721 (L)	Chiang Mai, Thailand	**EU643747**	**EU623561**
S. androgynus (L.) Merr. (1)	Middleton et al. 1496 (L)	Surat Thani, Thailand	N/A	**EU623562**
S. androgynus (L.) Merr. (2)	Van Welzen 2006-4 (L)	Chachoengsao, Thailand	**EU643748**	**EU623563**
S. arenosus J.T.Hunter & J.J.Bruhl	George 15563 (NSW)	Western Australia	**EU643749**	**EU623564**
S. assimilis Thwaites	Kostermans 27871 (L)	Pelawatte, Sri Lanka	**EU643750**	N/A
S. asteranthos Airy Shaw	Esser 99-13 (L)	Nakhon Sawan, Thailand	**EU643751**	**EU623565**
S. bacciformis (L.) Airy Shaw (1)	Cowie I 3418 (L)	Northern Territory,Australia	**EU643752**	N/A
S. bacciformis (L.) Airy Shaw (2)	Kerr 8350 (L)	Ubon Ratchatani, Thailand	**EU643753**	**EU623566**
S. bicolor Craib	Esser 99-21 (L)	Chiang Mai, Thailand	**EU643754**	**EU623567**
S. brevipes Müll.Arg.	Middleton et al. 974 (L)	Phetchaburi, Thailand	**EU643755**	**EU623568**
S. brunonis (S.Moore) Airy Shaw	Forster 6105 (L)	Northern Territory,Australia	**EU643756**	N/A
S. discocalyx Welzen	Beusekom & Phengklai 566 (L)	Ranong, Thailand	**EU643757**	N/A
S. distassoides (Müll.Arg.) Airy Shaw	Byrnes 1308 (L)	Northern Territory, Australia	**EU643758**	N/A
S. dunlopii J.T.Hunter & J.J.Bruhl	Hunter et al. 1570 (L)	Northern Territory, Australia	**EU643759**	**EU623569**
S. elachophyllus (F.Muell. ex Benth.) Airy Shaw	Clarkson & Neldner 9204 (L)	Queensland, Australia	AY936644	AY936745
S. garrettii Craib* (1)	Chase 14464 (K)	RBG Kew, Living collection from China	AY552450	AY936744
S. garrettii Craib (2)	Sino-American Guizhou Botanical Expedition 1872 (L)	Guinzhou, China	**EU643760**	**EU623570**
S. glaucus (F.Muell.) Airy Shaw	Hunter et al. 1565 (L)	Northern Territory, Australia	**EU643761**	**EU623571**
S. hirsutus Beille	Van Beusekom & Phengklai 1241 (L)	Chiang Mai, Thailand	**EU643762**	**EU623572**
S. hirtellus (F.Muell.) Airy Shaw	Bean 15558 (BRI)	Queensland, Australia	**EU643763**	**EU623573**
S. kerrii Airy Shaw	Van Beusekom & Phengklai 1065 (L)	Tak, Thailand	**EU643764**	**EU623574**
S. lissocarpus (S.Moore) Airy Shaw (1)	Hunter et al. 1561 (L)	Northern Territory,Australia	**EU643765**	**EU623575**
S. lissocarpus (S.Moore) Airy Shaw (2)	Johnson 5103 (NSW)	Queensland, Australia	**EU643766**	**EU623576**
S. micrasterias Airy Shaw	Erwin & Chai S 27479 (L)	Sarawak, Malaysia	**EU643768**	**EU623578**
S. "*nitmiluk*" sp. nov.	Bruhl & Hunter 1238 (L)	Northern Territory, Australia	**EU643769**	**EU623579**
S. orbicularis Craib	Soejarto & Southavong 10792 (L)	Vientiane, Laos	AY936645	**EU623580**
S. podenzanae (S.Moore) Airy Shaw	Blake 23210 (L)	Queensland, Australia	**EU643770**	**EU623581**

Appendix 2.1. Continued.

Taxa	Voucher/Herbaria	Source	GenBank accession number	
			matK	ITS
S. poomae Welzen & Chayam.	Phonsena et al. 5245 (L)	Chiang Rai, Thailand	**EU643771**	**EU623582**
S. quadrangularis (Willd.) Müll.Arg.	Maxwell 99-116 (L)	Chiang Mai, Thailand	**EU643772**	**EU623583**
S. ramosissimus (F.Muell) Airy Shaw	Latz & Albrecht 20135 (BRI)	Northern Territory, Australia	**EU643773**	N/A
S. retroversus Wight	Kathriarachchi et al. 40 (K)	Sri Lanka	AY936646	AY936747
S. rhamnoides Blume	Esser 2001-4 (L)	Chanthaburi, Thailand	**EU643774**	**EU623584**
S. rigens (F.Muell.) Airy Shaw	Kraehenbuehl 6007 (L)	South Australia, Australia	**EU643775**	**EU623585**
S. rigidulus (F.Muell. ex Müll.Arg.) Airy Shaw	Johnson MRS787 (BRI)	Queensland, Australia	**EU643776**	**EU623586**
S. rimophilus J.T.Hunter & J.J.Bruhl	Bruhl et al. 1246 (BRI)	Northern Territory, Australia	**EU643777**	**EU623587**
S. similis Craib	Larsen et al. 46639 (L)	Chiang Mai, Thailand	**EU643778**	N/A
S. spatulifolius Beille (1)	Wong s.n. (L)	Honolulu, U.S.A.	**EU643779**	**EU623588**
S. spatulifolius Beille (2)	Xia et al. s.n. (K)	China	AY936647	AY936748
S. sphenophyllus (Airy Shaw) Airy Shaw	Gray 08597 (BRI)	Queensland, Australia	**EU643780**	N/A
S. suberosus Airy Shaw	Chin 827 (L)	Perak, Malaysia	**EU643781**	**EU623589**
S. thorelii Beille	Van Welzen 2006-1 (L)	Chiang Mai, Thailand	**EU643782**	**EU623590**
S. thyrsiflorus Welzen	Kostermans 765 (L)	Kanchanaburi, Thailand	**EU643783**	**EU623591**
S. trachyspermus (F.Muell.) Airy Shaw	Chippendale & Constable 19076 (L)	New South Wales, Australia	**EU643784**	N/A
S. trinervius Hook.f. & Thomson ex Müll.Arg.	Koelz 30060 (L)	Assam, India	**EU643785**	N/A
S. villosus (Blanco) Merr. (1)	Phengklai et al. 12122 (BKF)	Thailand	N/A	**EU623592**
S. villosus (Blanco) Merr. (2)	Mcgregor 32398 (L)	Panay, Philippines	**EU643786**	**EU623593**
Outgroup				
Margaritaria rhomboidalis (Baill.) G.L.Webster	Rabenantoandro et al. 656 (MO)	Madagascar	AY936571	AY936665

* Listed in GenBank under *Sauropus androgynus* but redetermined by Bruhl and van Welzen 22 Mar 2008 based on the original living and herbarium material at K.