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

Pruesapan, K.

Chapter 3

Phylogenetic reconstruction in *Breynia*, *Sauropus* and related genera (Phyllanthaceae) based on noncoding chloroplast and nuclear DNA sequences [*]

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Abstract

The preliminary molecular phylogeny of *Sauropus* sensu lato (Phyllanthaceae) does not corroborate earlier morphological, intuitive inter- or infra-generic classifications. To increase and optimize the phylogenetic signal, four nuclear and non-coding chloroplast DNA markers and sequences were analysed under maximum parsimony and Bayesian inference. More highly resolved trees were obtained from nuclear data than from chloroplast data. The results confirm the position of monophyletic *Breynia* nested within *Sauropus* sensu stricto (s.s.) and should be named as *Breynia* sensu lato (s.l.). Two subclades clearly shown within *Breynia* s.l.: i) *Breynia* forming a distinct group together with the former *Sauropus* section *Hemisauropus* and *S.* sect. *Cryptogynium* and ii) sister to the former group is a clade consisting of all other sampled species of *Sauropus* s.s., the former *S.* sect. *Glochidioidei*, *S.* sect. *Sauropus* and *S.* sect. *Schizanthi*. The genus *Synostemon*, formerly included in *Sauropus*, is sister to *Breynia/Sauropus* and should be reinstated to generic rank.

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Introduction

Kathriarachchi et al. (2006) produced a skeletal phylogeny of *Phyllanthus* L. and related genera, from which it is apparent that *Phyllanthus* is only monophyletic when the embedded genera (*Breynia* J.R.Forst. & G.Forst., *Glochidion* J.R.Forst. & G.Forst., *Sauropus* Blume and *Reverchonia* A.Gray) are synonymised with it. Hoffmann et al. (2006) more or less formalized a new classification, treating these genera under *Phyllanthus* within tribe *Phyllantheae*, subfamily Phyllanthoideae of family Phyllanthaceae. However, we consider that the establishment of an unwieldy, large *Phyllanthus* (s.l.) would be uninformative. Therefore, more detailed phylogenetic studies must show which parts of *Phyllanthus* s.l. are clades and readily morphologically diagnosable.

Sauropus, if treated in a broad sense, is a large genus distributed widely from tropical Southeast Asia to Australia and Indian Ocean islands (Webster, 1994; Govaerts et al., 2000; Radcliffe-Smith, 2001). However, recent studies have demonstrated that *Sauropus* sensu lato (s.l.) is not monophyletic (Hoffmann et al., 2006; Kathriarachchi et al., 2006; Pruesapan et al., 2008) and should be segregated into at least two taxa (Pruesapan et al., 2008). One of these two taxa is *Synostemon* F.Muell., predominantly Australian, first described at the generic level (Mueller, 1858) and later transferred to *Sauropus* (Airy Shaw 1980a, b). The other is *Breynia* J.R.Forst. & G.Forst. s.l., which includes the mainly Asian species of *Sauropus*, referred to as *Sauropus* sensu stricto (s.s.), and *Breynia* (Pruesapan et al., 2008). The name *Breynia* (Forster & Forster, 1775) has priority over *Sauropus* (Blume, 1825).

Pruesapan et al. (2008) looked into the delimitation of *Sauropus*, *Breynia* and related taxa. The present study continues to pursue this topic, and investigates infrageneric groupings with sufficient taxa added. The phylogeny study by Pruesapan et al. (2008) found the DNA sequences of Internal Transcribed Spacer (ITS) of the nuclear ribosomal showed weakly support for the possible subgroups and recovered less resolved using DNA sequences of chloroplast *matK* within the *Sauropus* s.s. and *Breynia* clade and *Synostemon* clade.

To confirm and achieve better phylogenetic resolution both across and within clades of the study group, a mix of rather conservative markers (to provide basal resolution in the cladogram) together with more fast-evolving regions (for resolution in the upper parts of branches) is needed. Therefore, a combination of markers was selected, which comprises two

noncoding chloroplast DNA markers, *trnS-trnG* and *accD-psaI* intergenic spacers (IGS) and two nuclear DNA markers, *Phytochrome C* (*PHYC*) and ITS. The noncoding chloroplast markers *trnS-trnG* and *accD-psaI* IGS have been used to resolve the relationships within the Angiosperms, just like the low-copy nuclear gene *PHYC*. The *trnS-trnG* has also been used in a phylogeographic approach to deal with intraspecific genetic variation in Angiosperm plant populations (Hamilton, 1999). The *accD-psaI* IGS has been successfully used to distinguish closely related species in Orchidaceae (Barkman & Simpson, 2002) and was more variable than *atpB-rbcL* and *trnL-trnF* (Small et al., 1998; Kimura et al., 2003). The sequence data of *PHYC* not only provided a high degree of resolution within the higher order Angiosperm phylogeny (Mathews et al., 1995; Davis & Chase, 2004), but it was also used to evaluate tribal and generic delimitation within the Phyllanthaceae (Samuel et al., 2005). Nuclear ribosomal ITS based phylogenies have been constructed for many organismal groups, including angiosperms (Baldwin, 1992). Pruesapan et al. (2008) also obtained good results with ITS and, therefore, this DNA marker will again be used to unravel the evolution of nuclear and noncoding chloroplast markers in *Breynia*, *Sauropus*, *Synostemon* and related genera in the Phyllanthaceae.

The purposes of this paper are (i) to more soundly reconstruct the phylogeny of *Breynia*, *Sauropus* and *Synostemon* and related genera by assessing the molecular evolution of nuclear and noncoding chloroplast DNA; (ii) and to explore the generic boundaries of *Breynia–Sauropus* s.l., *Glochidion* and *Phyllanthus*; (iii) to look for possible infrageneric taxa in the groups under study.

Materials and methods

Taxon sampling

A total of 303 accessions (Appendix 3.1) representing 11 species (16 taxa) of *Breynia*, 58 species (69 taxa) of *Sauropus* s.l. (Pax & Hoffmann, 1922; Airy Shaw, 1969, 1974, 1980a, b; Hunter & Bruhl, 1997a, b, c; Van Welzen, 2003) with among them 15 species representing *Synostemon* (Mueller, 1858; Webster, 1960; Airy Shaw, 1978, 1981; Airy Shaw & Kalotas, 1981; Telford et al., in prep.), together with of the related genera 13 species (16 taxa) of *Glochidion* and 7 species of *Phyllanthus*. *Flueggea virosa* (Roxb. ex Willd.) Royle and *Notoleptopus decaisnei* (Benth.) Voronts. & Petra Hoffm. were used as outgroups. Due to

difficulties with amplification, *Flueggea virosa* could not be used as outgroup for *trnS-trnG* and, instead, *Notoleptopus decaisnei*, obtained from GenBank (Vorontsova et al., 2007; Vorontsova & Hoffmann, 2008), was used as outgroup for ITS.

DNA extraction, amplification and sequencing

In addition to the DNA samples used in previous studies (Kathriarachchi et al., 2006; Vorontsova et al., 2007; Pruesapan et al., 2008; Vorontsova & Hoffmann, 2008; Appendix 3.1), genomic DNA was extracted from silica-dried samples and from herbarium specimens using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) following manufacturer instructions. For most herbarium specimens a modified protocol was used (a prolonged lysis step with proteinase K and ß-mercaptoethanol added; Wurdack et al., 2004). Collection and voucher data are presented in Appendix 3.1.

The conditions for Polymerase chain reaction (PCR) were performed with 10--100 ng of genomic DNA, 1X PCR buffer (Qiagen, Hilden, Germany), 0.2 mM dNTPs, 0.2 µM of each primers, 3 µM $MgCl_2$, 0.4% of BSA (Promega, Madison, Wisconsin, USA) and 0.5 U of Taq DNA Polymerase (Qiagen, Hilden, Germany) in a total volume of 50 µl. The following PCR program 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 at the temperature for each primer see Table 3.1 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.

PCR and sequencing amplification of the *accD-psaI* were performed with primers *accD* and *psaI*-75R. The primers *trnS*F and *trnG*R were used to amplify and sequenced the *trnS-trnG* IGS. Internal transcribed spacer (ITS) region 1 and 2 and the 5.8S gene were amplified with primers ITS4 and ITS5. The amplification and sequencing for the *PHYC* gene using primers *PHYC*-F and *PHYC*-R. The primer sequences for all markers are shown in Table 3.1.

Sequences were initially edited and sequence contigs assembled, using Sequencher 4.7 (Gene Codes Corp., Ann Arbor, Michigan, USA). All sequences were submitted to GenBank (see Appendix 3.1 for accession numbers).

DNA sequence alignment and gap coding

Sequence alignments were initially viewed in MacClade v4.08 (Maddison & Maddison, 2001) using pairwise alignment option and manual adjustment where necessary. Two different ways of treating gap characters were employed: (i) gaps were treated as missing data and (ii) gaps were manually added as additional binary characters in accordance with the principles specified by Anderson & Chase (2001).

Phylogenetic analyses

Optimal topologies were sought while using Maximum parsimony (MP) and Bayesian Inference (BI). Datasets were analyzed separately and in combination. All characters were unordered, equally weighted, and gaps treated as missing data.

Parsimony analyses were conducted with PAUP version 4.0b10 (Swofford, 2003) using Fitch parsimony (Fitch, 1971), heuristic search with a 1,000 replicates with random taxon addition, in combination with tree-bisection reconnection (TBR) branch swapping and the MulTrees option active, with no more than ten trees saved per replicate. All trees obtained were used as starting trees for another round of swapping with a tree limit of 10,000. The strict consensus was computed on the remaining trees. Support for each node was assessed by performing 1,000 bootstrap replicates (Felsenstein, 1985) and 10 random taxon addition using TBR branch-swapping and no more than ten trees saved per replicate. Bootstrap percentages (BP) are described as high (85--100%), moderate (75--84%), or low (50--74%).

The nucleotide substitution model was determined with the AIC and hLRT as implemented in Modeltest v.2.2 (Nylander, 2004) and always selected the same evolutionary models for each partition or marker. The chosen models were used for individual data and combined dataset as shown in Table 3.2. The best-fitting models were used in Bayesian analyses. Bayesian Inference was conducted with MrBayes v.3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). BI was performed with four Marcov chains, each initiated with a random tree. Each run was composed of one cold and tree heated chains with the temperature parameter T set to 0.05 to ensure good mixing. An analysis was run for 24

million generations, sampling every 100 generations. Likelihood values were checked for stationarity and to determine for burn-in using Tracer v. 1.3 (Rambaut & Drummond, 2004). Generally, ten percent of the trees was discarded as burn-in. Posterior probability values (PP; Ronquist & Huelsenbeck, 2003)≥ 0.95 were used to determine the confidence support in Bayesian trees.

Testing incongruence between datasets

The congruence between the individual results of the nuclear and chloroplast DNA analyses and the combined datasets was determined in two ways. The incongruence length difference tests (ILD, implemented in PAUP* as the partition homogeneity test as both implemented in PAUP*, Farris et al., 1994, 1995) were used to test the incongruence in the phylogenetic signal of the datasets. The ILD test was conducted with 1000 replicates, saving 10 trees per replicates, TBR branch swapping and MulTrees off.

In addition, we studied the level of incongruence between the nuclear and chloroplast datasets using a conditional combination approach as outlined by Kellogg et al. (1996), Mason-Gamer & Kellogg (1996) and Johnson & Soltis (1998). We used a posterior probability of 0.95 and a bootstrap value of 70% as cut-off level for assessing hard incongruences between the total noncoding chloroplast and nuclear datasets.

Results

Sequence variation

The aligned sequence variation is shown in Table 3.2. The amplified ITS regions are between 637 base pairs (bp) (*Phyllanthus sikkimensis* Müll.Arg.) and 683 bp (*Notoleptopus decaisnei*) in length. The *PHYC* has a constant length of 607 bp for most species except *Flueggea virosa* that has 610 bp. The length of *accD-psaI* IGS varies from 445 bp (*Notoleptopus decaisnei*) to 813 bp (*Flueggea virosa*). The *trnS-trnG* has a lenght of 675 (*Notoleptopus decaisnei*) to 896 bp (*Sauropus* "*lithophila*" sp. nov.) for the species sequenced in this study. Some species could not be sequenced completely due to amplification problems.

The results with all data and sequence characters/gap characters dataset returned the same topologies of the trees for the main clades. The dataset with all data combined is used for the discussion. Information on trees and there statistics for individual and combined datasets are

given in Table 3.2. *Phyllanthus* species are present at the base of the tree in all analyses (Figs. 3.1—2). As we have limited sampling of *Phyllanthus* species in our analyses and as the results largely agree with those of a previous study focusing on *Phyllanthus* (Kathriarachchi et al., 2006), we will focus the results only on the relationships among *Breynia*, *Glochidion*, *P. mirabilis* Müll.Arg., *Sauropus* s.s. and *Synostemon*.

Combined analyses of nuclear dataset

The MP strict consensus tree of the nuclear analysis (Fig. 3.1a) shows the support for the clades (Table 3.2), which varies between weak to high support. Only 14 clades are supported with bootstrap values of less than 70%, whereas the nodes with higher bootstrap values are up to 39 nodes and 20 of which have bootstrap values of 95% or more (Table 3.2).

The MP strict consensus tree of 1320 trees (Fig. 3.1a) is largely congruent with the topology of the BI, but the supported values are lower than in the BI tree. *Glochidion* and *Phyllanthus mirabilis* form a sister clade (A) with high support (PP 1.0, BP 100), high support is as well present for the *Glochidion* clade alone. *Synostemon* forms a strongly supported clade (B, PP 1.0, BP 99). The MP and BI analyses agree with the separation of *Sauropus* s.s. into two groups, *S.* sect. *Glochidioidei* Airy Shaw, *S.* sect. *Sauropus* and *S.* sect. *Schizanthi* Pax & K.Hoffm. form a Clade C1 (largely unresolved, PP 0.98, BP 76) and *S.* sect. *Cryptogynium* Müll.Arg. and *S.* sect. *Hemisauropus* Müll.Arg. form a clade (PP 0.95, BP <50) plus the *Breynia* (PP 1.0, BP 94) in Clade C2 form another clade with high support (PP 1.0, BP 94).

Combined analyses of chloroplast dataset

The MP strict consensus tree (Fig. 3.1b) of the chloroplast analysis shows mainly clades that are weakly to moderately supported, only seven clades have a support of BP ≥ 95.

The MP strict consensus tree of 6800 trees (Fig. 3.1b) shows the same topology for the main clades as in the BI tree (not shown) with fewer supported branches. The results of MP and BI analyses show high support for *Glochidion* and *Phyllanthus mirabilis* as sister groups (Clade A, PP 1.0, BP 100), as well as for *Glochidion* (PP 1.0, BP 99). *Synostemon* (Clade B, PP 1.0, BP 98) is largely unresolved, just like *Sauropus* s.s. with *Breynia* embedded in it (Clade C, PP 0.86, BP 76), whereby *Breynia* always forms a monophyletic group (PP 1.0, BP 59)

Table 3.1. Information of amplification primers used in this study.

Locus	Primer	Primer sequence (5'→ 3')	Tm (° C)	References
Nuclear regions				
ITS-5.8S-ITS2	ITS5	GGAAGTAAAAGTCGTAACAAGG	52.5	White et al. (1990
	ITS4	TCCTCCGCTTATTGATATGC		
PHYC	*PHYC*-F	CCAGCTACTGATATACCTCAAGCTTC	48	Samuel et al. (2005)
	PHYC-R	CCAGCTTCCATAAGGCTATCAGTACT		
Chloroplast regions				
psaI-accD IGS	*accD*	AATYGTACCACGTAATCYTTTAAA	49	Shaw et al. (2007)
	psaI-75R	AGAAGCCATTGCAATTGCCGGAAA		Small et al. (1998)
*trn*S$^{(GCU)}$-*trn*G$^{(UCC)}$ IGS	*trn*SF	GCCGCTTTAGTCCACTCAGC	49-52	Hamilton (1999)
	*trn*GR	GAACGAATCACACTTTTACCAC		

Table 3.2. Values and statistics from phylogenetic analyses of the individual data and combined datasets. Consistency index values excluded uninformative characters.

Regions	ITS	*PHYC*	*accD-psaI*	*trnS-trnG*	Combined nuclear	Combined chloroplast	Combined dataset
Number sequenced ingroup species	102	62	57	75	107	80	108
Aligned length	710	610	1057	1312	1320	2370	3690
Number of variable site (%)	108 (15.2)	73 (12)	71 (6.7)	171 (13)	181 (13.7)	233 (9.8)	706 (19.1)
Informative characters (%)	246 (34.6)	102 (16.7)	58 (5.5)	90 (6.8)	348 (26.4)	143 (6.0)	547 (14.8)
Number of trees	4410	9920	8850	7310	1320	6800	2460
Number of steps	1206	405	309	525	1621	824	2482
Consistency index	0.53 (0.46)	0.68 (0.54)	0.88 (0.71)	0.89 (0.71)	0.57 (0.48)	0.89 (0.71)	0.66 (0.51)
Retention index	0.82	0.85	0.91	0.90	0.83	0.91	0.83
Model selected	GTR+I+G	GTR+G	GTR+G	GTR+G	GTR+I+G	GTR+G	GTR+I+G
Number of nodes with BP 50-69%	18	7	13	10	14	16	18
Number of nodes with BP ≥ 70%	42	18	9	7	39	16	46
Number of nodes with BP ≥ 95%	25	8	5	3	20	7	27

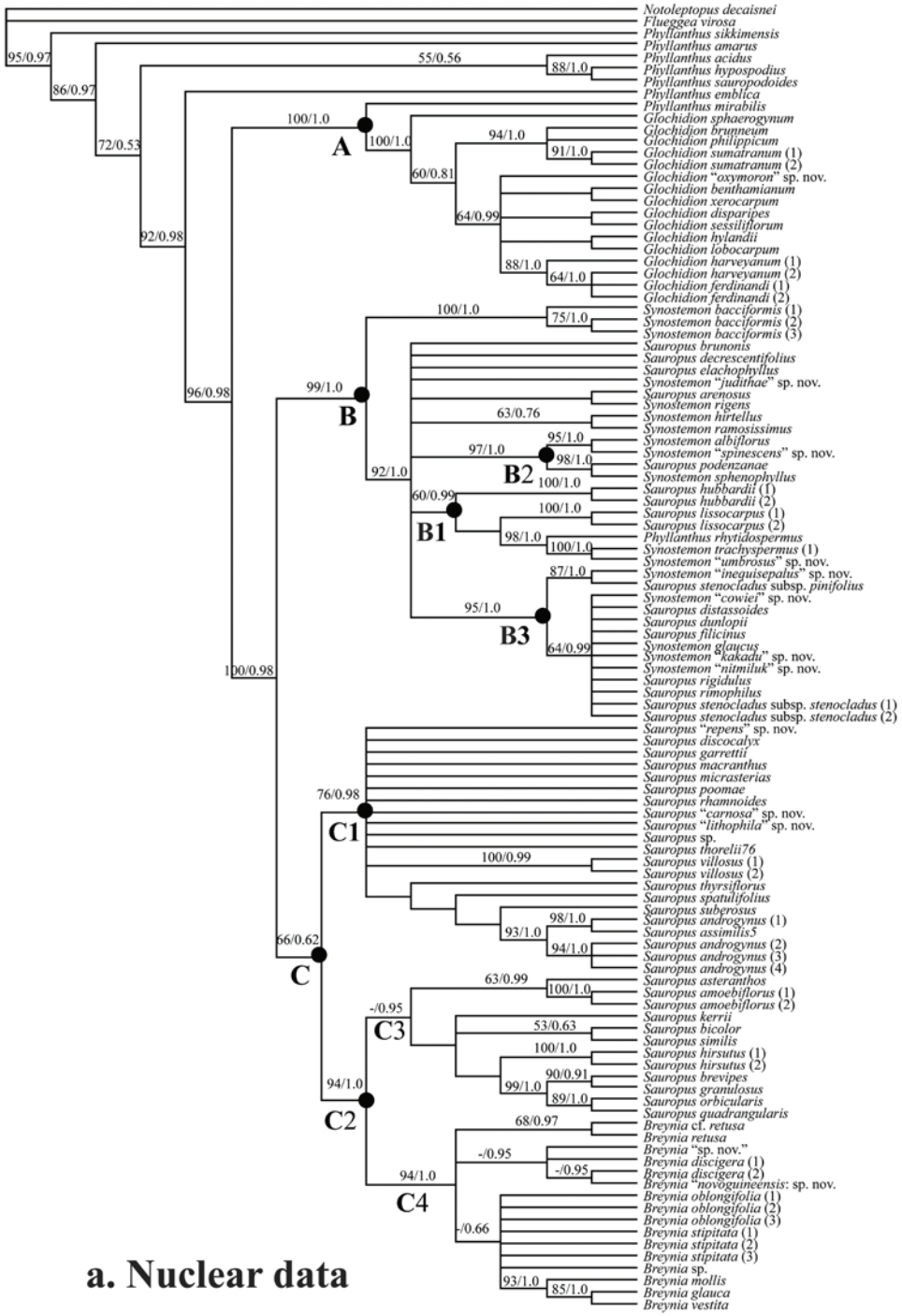
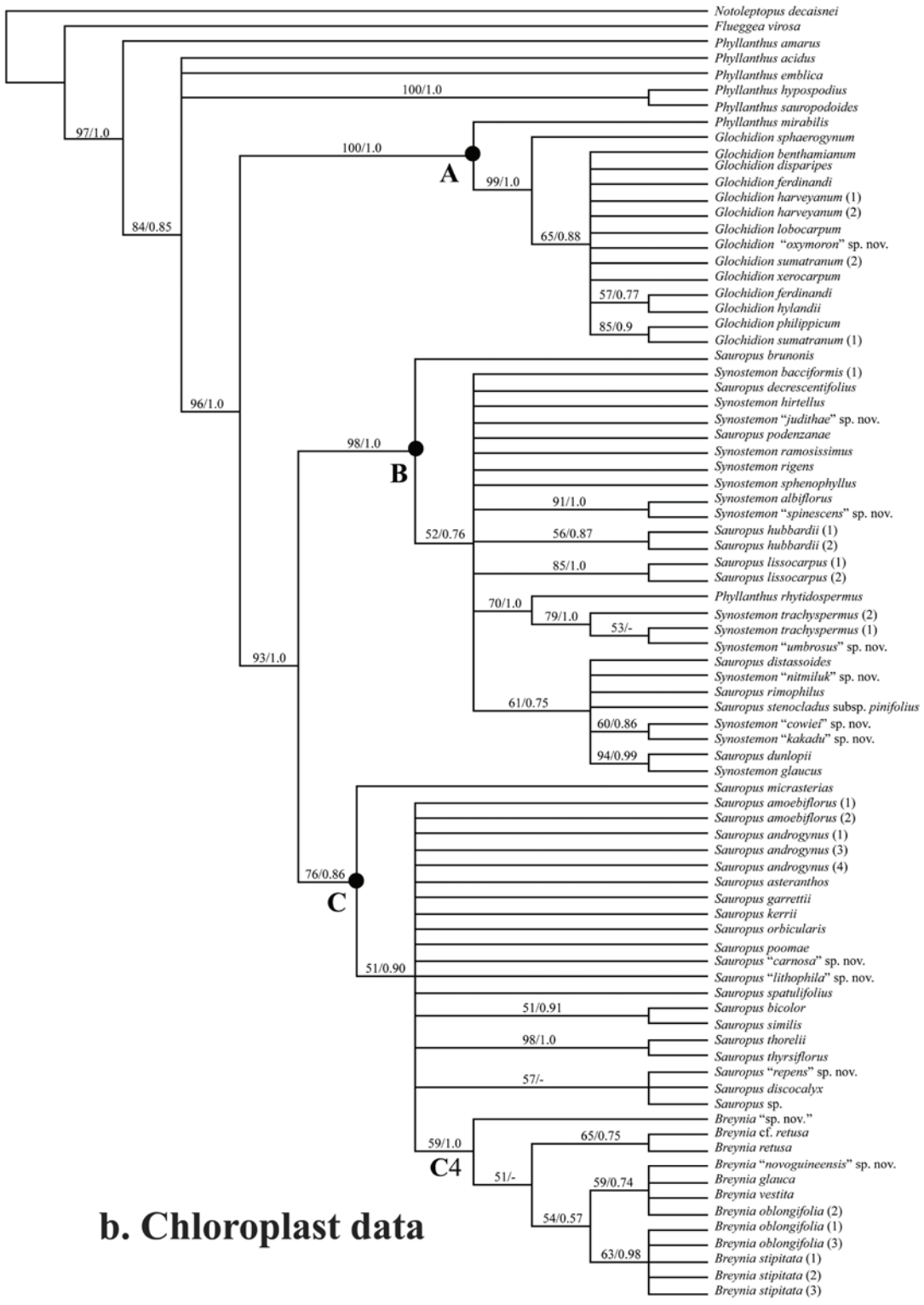


Fig. 3.1. Strict consensus cladograms under maximum parsimony of the nuclear (ITS and *PHYC*) dataset (a) and chloroplast (*accD-psaI* and *trnS-trnG*) dataset; (b) Posterior probabilities and bootstrap percentage values are indicated. Black circles and letters indicated the nodes of the major clades. A: *Phyllanthus mirabilis-Glochidion* clade; B, B1--3: *Synostemon* clade; C, C1--2: *Breynia* sensu lato clade.


Notoleptopus decaisnei
Flueggea virosa
Phyllanthus amarus
Phyllanthus acidus
Phyllanthus emblica
Phyllanthus hypospodius
Phyllanthus sauropodoides
Phyllanthus mirabilis
Glochidion sphaerogynum
Glochidion benthamianum
Glochidion disparipes
Glochidion ferdinandi
Glochidion harveyanum (1)
Glochidion harveyanum (2)
Glochidion lobocarpum
Glochidion "oxymoron" sp. nov.
Glochidion sumatranum (2)
Glochidion xerocarpum
Glochidion ferdinandi
Glochidion hylandii
Glochidion philippicum
Glochidion sumatranum (1)
Sauropus brunonis
Synostemon bacciformis (1)
Sauropus decrescentifolius
Synostemon hirtellus
Synostemon "judithae" sp. nov.
Sauropus podenzanae
Synostemon ramosissimus
Synostemon rigens
Synostemon sphenophyllus
Synostemon albiflorus
Synostemon "spinescens" sp. nov.
Sauropus hubbardii (1)
Sauropus hubbardii (2)
Sauropus lissocarpus (1)
Sauropus lissocarpus (2)
Phyllanthus rhytidospermus
Synostemon trachyspermus (2)
Synostemon trachyspermus (1)
Synostemon "umbrosus" sp. nov.
Sauropus distassoides
Synostemon "nitmiluk" sp. nov.
Sauropus rimophilus
Sauropus stenocladus subsp. pinifolius
Synostemon "cowiei" sp. nov.
Synostemon "kakadu" sp. nov.
Sauropus dunlopii
Synostemon glaucus
Sauropus micrasterias
Sauropus amoebiflorus (1)
Sauropus amoebiflorus (2)
Sauropus androgynus (1)
Sauropus androgynus (3)
Sauropus androgynus (4)
Sauropus asteranthos
Sauropus garrettii
Sauropus kerrii
Sauropus orbicularis
Sauropus poomae
Sauropus "carnosa" sp. nov.
Sauropus "lithophila" sp. nov.
Sauropus spatulifolius
Sauropus bicolor
Sauropus similis
Sauropus thorelii
Sauropus thyrsiflorus
Sauropus "repens" sp. nov.
Sauropus discocalyx
Sauropus sp.
Breynia "sp. nov."
Breynia cf. retusa
Breynia retusa
Breynia "novoguineensis" sp. nov.
Breynia glauca
Breynia vestita
Breynia oblongifolia (2)
Breynia oblongifolia (1)
Breynia oblongifolia (3)
Breynia stipitata (1)
Breynia stipitata (2)
Breynia stipitata (3)
100/1.0
97/1.0
100/1.0
A
99/1.0
84/0.85
65/0.88
57/0.77
85/0.9
96/1.0
98/1.0
B
91/1.0
52/0.76
56/0.87
85/1.0
70/1.0
79/1.0
53/-
93/1.0
61/0.75
60/0.86
94/0.99
76/0.86
C
51/0.90
51/0.91
98/1.0
57/-
59/1.0
C4
65/0.75
51/-
59/0.74
54/0.57
63/0.98
b. Chloroplast data

← Fig. 3.1.

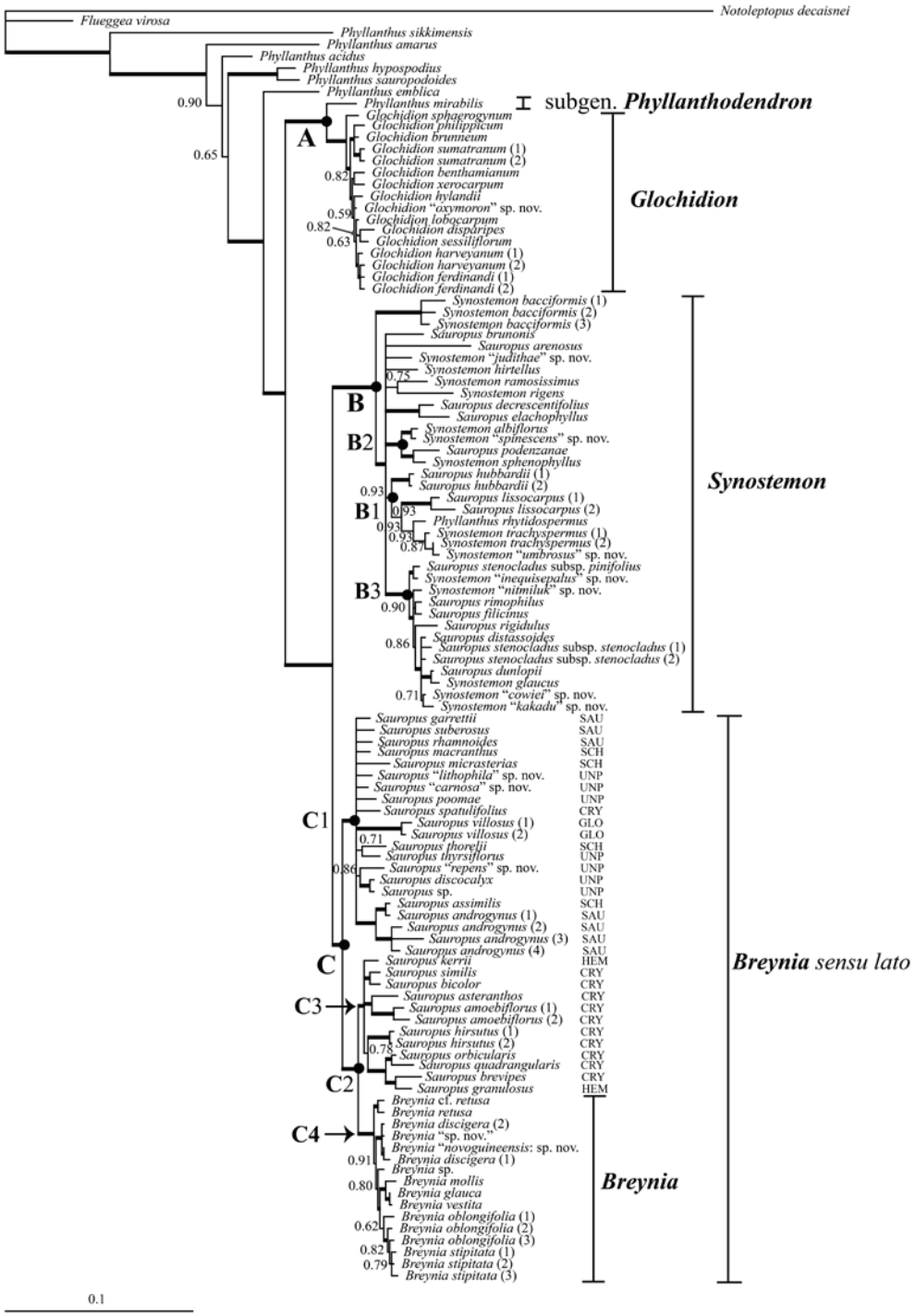


Fig. 3.2. Bayesian majority rule consensus tree of the combined nuclear and chloroplast datasets. Posterior probabilities (PP) are displayed at the nodes. Thick branches indicate PP = 1.0. Black circles and letters indicate the nodes of the major clades. A: *Phyllanthodendron-Glochidion* clade; B: *Synostemon* clade; C, C1--2: *Breynia* sensu lato clade. The abbreviations show the previously recognized sections of *Sauropus* sensu stricto: CRY = *Cryptogynium*, GLO = *Glochidioidei*, HEM = *Hemisauropus*, SAU = *Sauropus*, SCH = *Schizanthi*, and UNP = not placed.

Incongruence between datasets

The combined nuclear and chloroplast datasets were checked with the ILD test and showed significant incongruence among the partitions with $P = 0.01$.

Visual observation of our separate analyses of the nuclear and chloroplast datasets mainly shows areas of incongruence in the *Synostemon* clade (B, Fig. 3.1a--b). The basal species present in the *Synostemon* clade of the nuclear analyses is *Synostemon bacciformis* (L.) G.L.Webster with high support in the BI and MP analyses (PP 1.0, BP 92; Fig. 3.1a), whereas *Sauropus brunonis* (S.Moore) Airy Shaw is basal in the chloroplast analyses with only weak support in the BI analysis (PP 0.76, BP 52, Fig. 3.1b).

In fact, the incongruent areas are weakly supported with BP < 70 and therefore considered to be insignificant (Hillis & Bull, 1993). The nuclear and chloroplast datasets then were combined.

Combined analyses of nuclear and chloroplast datasets

The MP and Bayesian analyses returned the same tree topology, but the Bayesian one provided higher overall branch support. Higher posterior probability values when compared with bootstrap values is normal in this type of analysis (Suzuki et al., 2002). The Bayesian majority rule consensus tree was used for the interpretation of the results in Fig. 3. 2.

The MP strict consensus tree of 2460 cladograms (not shown) has mostly moderate to high support for the clades. The 46 nodes with BP ≥70 and 27of which have BP ≥ 95, whereas 18 nodes have BP 50-69 (Table 3.2, tree not shown). The MP (not shown) and BI phylogenetic analyses of the combined dataset (Fig. 3.2) give better resolved cladograms with higher support than the cladograms resulting from the separate analyses of the nuclear and chloroplast datasets. Therefore, we use the combined tree (Fig. 3.2) in our discussion of the major clades.

The results of the MP (not shown) and BI analyses of the combined dataset (Fig. 3.2) shows several strongly resolved major clades (A--C). Clade A combines *Phyllanthus mirabilis* with *Glochidion* (PP 1.0). Clade B comprises *Synostemon*, including *Synostemon bacciformis* (PP 1.0). Clade C contains *Sauropus* s.s. and *Breynia* (PP 1.0) and splits into two subclades, Subclade C1 (PP 1.0), largely unresolved, including *S.* sect. *Glochidioidei*, *S.* sect.

Sauropus, *S.* sect. *Schizanthi*, and Clade C2 (PP 1.0) of *S.* sect. *Cryptogynium*, *S.* sect. *Hemisauropus* (PP 1.0) and *Breynia* (PP 1.0).

Discussion

Phylogenetic utility of the DNA sequences

The four sequenced DNA markers showed significant differences in the sequence variation between the species and in the number of potentially phylogenetic informative positions (Table 3.2). The *accD-psaI* has many more conservative positions (only 6.7% variable positions, VPs) than *PHYC*, *trnS-trnG* and ITS (12%, 13% and 15.2% VP, respectively). These findings are uncorrelated with the differences in the number of potentially phylogenetic informative positions, as the chloroplast has less positions (between 5.5% in *accD-psaI* and 6.8% in *trnS-trnG*) than the nuclear DNA (16.7% and 34.6% for *PHYC* and ITS, respectively). On average, the chloroplast dataset contains 6% potentially phylogenetic informative positions, whereas the nuclear dataset contains 26.4% of potentially phylogenetic informative positions. These differences are also reflected in the results of the MP (Fig. 3.1a, b) and BI (not shown) analyses of the chloroplast and nuclear datasets as the nuclear dataset yields more resolved cladograms than the chloroplast dataset. However, the characters of the chloroplast dataset show less homoplasy (CI of 0.89 and RI of 0.91) than the nuclear dataset (CI of 0.57 and RI of 0.83).

The incongruence between the nuclear DNA and chloroplast DNA might be caused by the different biological sources and molecular evolution (Wendel & Doyle, 1998). As far as our results are concerned, the chloroplast DNA evolved slower than the nuclear DNA, which is especially shown in the chloroplast data that yielded only 143 (6%) potential phylogenetic informative characters out of an aligned length of 2370 base pairs, whereas the nuclear data yielded 384 (26.4%) potential phylogenetic informative characters out of an aligned length of 1320 base pairs only.

Clades and their synapomorphies

Most early divergent lineages of *Phyllanthus* (Kathriarachchi et al., 2006) are still grossly undersampled and will form the basis of further studies of study group: e.g. *P.* subgen. *Gomphidium* (2 of c. 100).

Our present study clarifies more details for the embedded genera *Glochidion*, *Synostemon*, *Sauropus* s.s., and *Breynia* (Figs. 3.1--2) of Clade M in the phylogenetic study of *Phyllanthus* by Kathriarachchi et al. (2006). In this study, we confirm the close relationship between *P. mirabilis* of subgen. *Phyllanthodendron* and *Glochidion* (Clade A, Figs. 3.1--2) as shown by Kathriarachchi et al. (2006) based on *matK* only and the *Sauropus* s.l. (*Sauropus* s.s. and *Synostemon*) and *Breynia* clade (B plus C in Figs. 3.1--2) as shown by Pruesapan et al. (2008) based on *matK* and ITS. The cladograms clearly prove that *Sauropus* s.l. has to be split again in *Synostemon* (Clade B) and *Sauropus* s.s. (Clade C minus *Breynia*, Fig. 3.2) and that the latter should be united with *Breynia*. The distribution areas with the highest numbers of species are Australia for *Synostemon* and Southeast Asia for *Sauropus*; these foci are more or less separate, only two species show overlap (*Synostemon bacciformis* and *Sauropus macranthus* Hassk. both range from Southeast Asia up to Australia). *Breynia* shows radiation in tropical eastern Asia and Southeast Asia, and in New Guinea and Australia (Govaerts et al., 2000). Most Australian species are limited to East Australia. Morphologically, these genera are not easily recognizable. In fact, *Breynia* and *Sauropus* s.s. have very different types of androecium, but both types are present in *Synostemon*. Styles are often used to distinguish the genera:

Recent pollination studies by Kawakita & Kato (2009), building on their previous studies (Kato et al., 2003; Kawakita & Kato, 2004a, b) show a coevolved obligate pollination mutualism between several large groups of Phyllantheae (Phyllanthaceae) and *Epicephala* moths (Gracillariidae). The species of Phyllantheae that are pollinated by moths have a small degree of stigma spreading (apical/basal stigma width < 1.87; styles are reduced and fused to form a narrow apical cavity into which moths actively deposit pollen), whereas the species pollinated by the nectar-seeking insects have larger stigmas that split and spread (apical/basal stigma width ≥ 1.87; bifid styles spreading horizontal, which assists passive pollen receipt from insect bodies). The studies showed that about half of the species of *Phyllanthus*, and almost all species of *Glochidion* and *Breynia* are actively pollinated by the moths, whereas the other half of the species of *Phyllanthus*, *Sauropus* s.s. and *B. retusa* (Dennst.) Alston are not visited at all by these moths, just as in *Flueggea* and *Margaritaria*. The pollination mutualism arose several times in *Phyllanthus*, once in *Glochidion* and once in *Breynia* (Kawakita & Kato, 2009). This is confirmed by the morphological differences in the style reductions.

Species of *Glochidion* have the stigmas united into a pyramidal cone (except *G. sericeum* (Blume) Zoll. & Moritzi with well-developed spreading stigmas, which may be pollinated by different insects). In *Breynia* the stigmas are generally very short, well separated from each other, and they lack stigmatic papillae.

Cytological studies (Punt, 1962; Thongpuban, 2002) have shown *Breynia*, *Sauropus* s.s., *Synostemon* and *Glochidion* to be the diploid with 2n = 48—52, whereas *Phyllanthus* is more variable with diploid and polyploid numbers between 2n = 26 to 8n = 104. Pollen morphology indicates *P. mirabilis* of subgen. *Phyllanthodendron* and *Glochidion* (Clade A, Figs. 3.1--2) to have distinctive monoporate pollen, whereas *Synostemon* (Clade B, Figs. 3.1--2), *Sauropus* s.s. and *Breynia* (Clade C, Figs. 3.1--2) share diploporate pollen. However, both pollen characters are present in *Phyllanthus* (Webster & Carpenter, 2002; Sagun & Van der Ham, 2003; Webster & Carpenter, 2008). Palynology of the ingroup is clearly worth further study.

The discussion below will focus on the relationships of *Phyllanthus mirabilis*, *Glochidion*, *Synostemon*, *Breynia* (including *Sauropus* s.s.) and their synapomorphies are shown in Table 3.3.

-The relationship of Phyllanthus mirabilis and Glochidion

Clade A (Fig. 3.2) combines *Phyllanthus mirabilis* (*P.* subgen. *Phyllanthodendron*) and *Glochidion* with strong support. With about 300 species (Radcliffe-Smith, 2001) *Glochidion* is the largest genus embedded within *Phyllanthus* based on molecular phylogenetic studies (Hoffmann et al., 2006; Kathriarachchi et al., 2006). An earlier study (Kathriarachchi et al., 2006) already showed the strong relationship between *Glochidion* and *P. mirabilis*, but this was only based on a single gene, the coding chloroplast *matK*. Our present study uses four DNA markers, *accD-pasI*, ITS, *phyC*, and *trnS-G*, and confirms the relationship between *P. mirabilis* and *Glochidion.*.

Phyllanthodendron Hemsl. has been accepted as a distinct genus by various authors (Hemsley, 1898; Croizat, 1942; Li, 1994). Croizat (1942) and Webster (1967) suggested that (*P.* subgen.) *Phyllanthodendron*'s characters resemble those of *Glochidion*, like the absence of a floral disc (seemingly overlooking the linear disc glands), the thick and undivided style grooves, an androecium of three connate stamens with long apiculate anthers, and a ventral excavation of the seeds. Webster & Carpenter (2008) reported similarities between the pollen

of *P.* subgen. *Phyllanthodendron* and *P.* subgenus *Emblica*; both have pollen with a subprolate shape, short narrow colpi, and a brochate exine reticulum, but *P.* subgen. *Phyllanthodendron* has lalongate rather than circular pores as in *P.* subgen. *Emblica*. Webster and Carpenter discussed the possibilities to treat *P.* subgen. *Phyllanthodendron* as a subgenus, genus, or as part of *P.* subgen. *Emblica*. *Glochidion* also shares character states with *P.* subgen. *Phyllanthodendron* and *P.* subgen. *Emblica* like 3-6-colporate pollen with monoporate colpi, but *P.* subgen. *Emblica* also has up to 10-colporate pollen with diploporate colpi. According to our molecular phylogenies and those by Kathriarachchi et al. (2006) *P.* subgen. *Phyllanthodendron* is more closely related to *Glochidion* than to *P.* subgen. *Emblica*. Hence, subsuming *P.* subgen. *Phyllanthodendron* into *P.* subgen. *Emblica* is out of the question. It is more likely that *P.* subgen. *Phyllanthodendron* deserves generic status next to *Glochidion*. Both groups have distinct characters. However, this is not the place to decide for a new generic circumscription, because only 1 of 12 species of *P.* subgen. *Phyllanthodendron* was present in our study and, just like 13 species of c. 300 of *Glochidion* and 6 spp. of c. 833 spp. of *Phyllanthus*. Thus, future research is much needed in this difficult group.

-Species relationship within Synostemon

A total of 30 species (36 specimens) included in our study again prove the generic status of *Synostemon*. This reinstatement has to wait till the revision of *Synostemon* is finished, this revision is still on going by Ian Telford and co-authors. They will make all new combinations necessary, we will only use *Synostemon* names when combinations exist, where lacking we use the names under *Sauropus* (Appendix 3.1, Figs 3.1--2). Forthcoming descriptions of new species are already indicated under their future name, nomenclatorally they are not introduced here.

Clade B represents all species of *Synostemon* (Fig. 3.2). The molecular phylogeny shows some distinct groups in *Synostemon*. We found three further monophyletic groups in *Synostemon* (Fig. 3.2 Clades B1, B2, and B3). Clade B1 contains *Sauropus hubbardii*, *S. lissocarpus*, *S. rhytidospermus*, *Synostemon trachyspermus*, and *S.* "*umbrosus*" (sp. nov. 7). Clade B2 (Fig. 3.2) contains *Sauropus podenzanae*, *Synostemon albiflorus*, *S. sphenophyllus*, and *S.* "*spinescens*" (sp. nov. 6). Clade B3 (Fig. 3.2) is a large, resolved group comprising *Sauropus distassoides*, *S. filicinus*, *S. dunlopii*, *S. stenocladus* ssp. *pinifolius*, *S. rigidulus*, *S.*

rimophilus, *S. stenocladus* ssp. *stenocladus*, *Synostemon* "*cowiei*" (sp. nov. 1), *S. glaucus*, *S.* "*inaequisepalus*" (sp. nov.2), *S.* "*kakadu*" (sp. nov.4), *S.* "*nitmiluk*" (sp. nov. 5). However, morphological characters are not clear-cut to distinguish these three clades. The rest of *Synostemon* species are polytomies with *Sauropus elachophyllus* and *S. decrescentifolius* a sister clade with strong support by sharing anther connectives partly joined on the androphore, leaving the anther apices free and slightly divergent. *Synostemon stenocladus* ssp. *stenocladus* and *S. stenocladus* ssp. *pinifolius* are not recovered as sister taxa; the subspecies should be raised to the rank of species. The wide spread *Synostemon bacciformis* splits off basally in *Synostemon* with strong support. The morphological phylogeny misplaced this species within Asian *Sauropus* s.s. (Van Welzen, 2003) and this has been solved by our previous study (Pruesapan et al., 2008) and is confirmed again in this present study with more DNA markers used (Fig. 3.2).

Our previous study (Pruesapan et al., 2008) did not clarify the morphological differences between *Synostemon* and *Breynia* (including *Sauropus*). Here we indicate clearly the synapomorphies of the groups (Fig. 3.3, Table 3.3). All species of *Synostemon* can be distinguished from *Breynia* (including *Sauropus*) by the ovate ovary with the obtuse or lobed apex; the lobes surround a depressed area where the stigmas are inserted; the stigmas are generally erect, not split or slightly bifid to mostly split less than halfway, the stigma branches are not coiled (Fig. 3.3d). The fruits of *Synostemon* (Fig. 3.3e) are more or less ovoid, and higher than wide (generally, especially in *Sauropus* s.s., wider than high), the apex is usually obtuse, but in some species lobed [flattened in *Breynia* (including *Sauropus*), Fig. 3.3b] and the seeds (Fig. 3.3f) are more or less crescentiform and three to four times as long as wide and usually strongly ornamented, the hilum is hollow for about half the length of the seed (the seeds are more or less smooth and about twice as long as wide, with the adaxial hollow part much larger in *Breynia* (including *Sauropus* s.s.) (Fig. 3.3c).

-Species relationship within the Breynia sensu lato clade

Breynia and *Sauropus* s.s. form a single clade (C), which can be recognized as the monophyletic genus *Breynia* s.l. in our previous study (Pruesapan et al., 2008; see introduction). Our previous study showed that the resolution within *Sauropus* s.s. was poor, but did not support the classifications of Pax & Hoffmann (1922), Beille (1927) and Airy Shaw (1969). We used four additional DNA markers to increase the resolution in the

phylogeny. Unfortunately, the results obtained were highly similar to our previous study (Pruesapan et al., 2008; Chapter 2). The two obtained Subclades C1 and C2 of *Breynia* s.l. (Clade C, Fig. 3.2) are strongly supported. Subclade C1 comprises most species of *Sauropus* sect. *Glochidioidei*, *S.* sect. *Sauropus* and *S.* sect. *Schizanthi* and other unplaced species. Subclade C2 comprises of *S.* sect. *Cryptogynium* and *S.* sect. *Hemisauropus* and the genus *Breynia*.

Table 3. 3. Typical characters of the main clades present in this study.

Clade	**Taxa**	Typical characters
A	*Glochidion* plus *Phyllanthus mirabilis*	Stamens with (long) apiculate anthers. Pollen monoporate.
B + C	*Synostemon* plus *Breynia* sensu lato	Stamens without apiculate anthers. Pollen diploporate.
B	*Synostemon*	Ovary apex obtuse or lobed; stigmas not split or split less than halfway, branches not coiled. Fruit ovoid, longer than wide. Seed crescentiform, strongly ornamented, hilum cavity half of seed length. Male sepal scales usually absent.
C	*Breynia* sensu lato (*Sauropus* sensu stricto plus *Breynia*)	Ovary apex flattened; stigmas deeply split or completely split, branches coiled. Fruit subglobose or depressed globose, wider than long. Seed smooth; hilum with larger adaxial cavity. Male sepal scales usually present.

Sauropus spatulifolius Beille was generally considered to be a member of section *Cryptogynium* (Beille, 1927) placed here in Subclade C1 (Fig. 3.2), whereas other member of this section placed in Subclade C2 (Fig. 3.2). Leaving this species in section *Cryptogynium* (major part in Subclade C2, Fig. 3.2) will render Subclade C1 paraphyletic, thus *S. spatulifolius* needs to be reassigned. All species in Clade C (Fig. 3.2) of *Breynia* s.l. show some distinct characters from *Synostemon* species in Clade B (see Table 3.3). *Breynia* (including *Sauropus*) species share a subglobose ovary, often flattened apically, and the stigmas are split from halfway to completely (Fig. 3.3a). In *Breynia*, *Sauropus kerrii*, and *S. quadrangularis* (Willd.) Müll.Arg. the stigmas are vertical (like in *Synostemon*) and not or somewhat coiled; in the remaining *Sauropus* s.s. species they are horizontal and coiled (Fig. 3.3a). The fruit character for the species in Clade C of *Breynia* (including *Sauropus*) (Fig. 3.3b) is subglobose or depressed globose, wider than long and the seeds (Fig. 3.3c) are more or less smooth and about twice as long as wide, with the adaxial cavity of the hilum much larger than that of *Synostemon* (Fig. 3.3f).

The results from this study agree with Croizat's suggestion (1940) that *Sauropus* and *Breynia* are closely related, but they are (natural) groups that are difficult to circumscribe. Subdivision of *Breynia* s.l. is still problematic based on molecular data and requires further study.

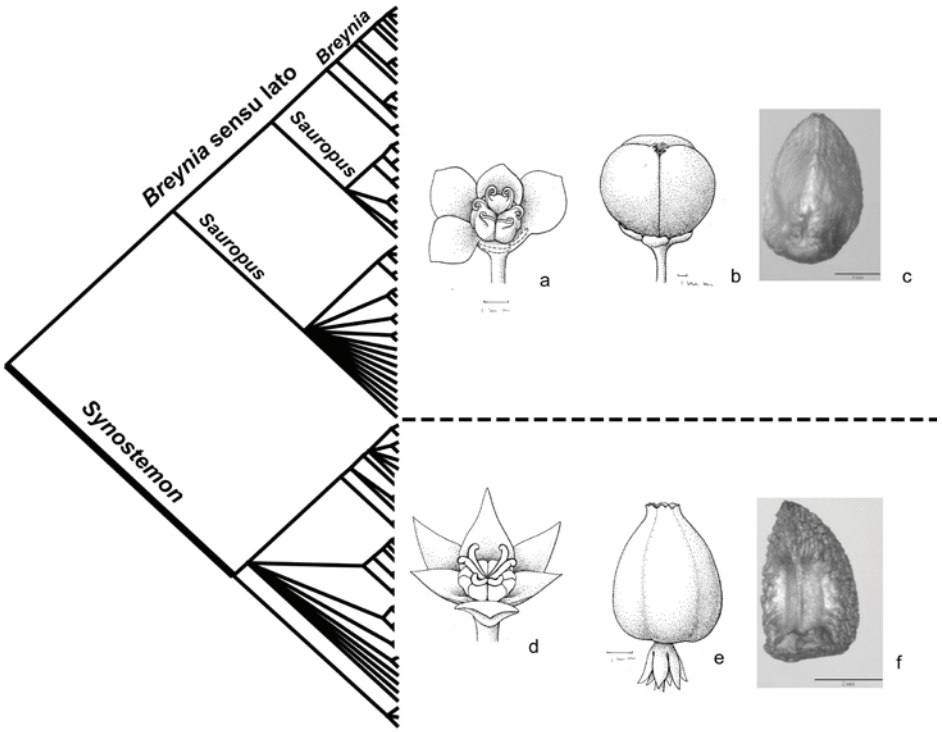


Fig. 3.3. Characters used to distinguish *Synostemon* and *Breynia* sensu lato. a: pistillate flower and b: fruit of *Sauropus androgynus* (L.) Merr. (Pruesapan 2009-9, L); c: seed of *Sauropus kerrii* Airy Shaw (Pooma et al. 2209, L); d: seed and e: fruit of *Synostemon bacciformis* (L.) G.L.Webster (Pruesapan 2009-9, L); f: seed of *Synostemon albiflorus* (F. Muell. ex Müll.Arg.) Airy Shaw (Foster 21362, L).

Conclusions

The results of this study show that the nuclear DNA evolved faster than the non-coding chloroplast DNA in the Phyllanthaceae and provides a higher resolution in the cladograms. The DNA markers are suitable to assess the species composition of *Synostemon* and *Breynia* s.l. and also confirm the position of *Breynia* and suggest a preliminary picture for *Glochidion*. The relationship between all closely related species could not be satisfactorily resolved due to

the low level of sequence variation. There is a close relationship between *Glochidion* and *Phyllanthus mirabilis* of subgen. *Phyllanthodendron* and it seems like that the latter should be retained at generic rank. *Glochidion* needs more analysis to resolve the infrageneric relationships and to test the sections proposed by Airy Shaw (unpubl.). The molecular phylogeny shows that the boundaries between *Glochidion*, *Breynia* (including *Sauropus*), and *Synostemon* are clearly resolved and differ from the assemblage of *Phyllanthus* included here.

The present study reinforces the conclusions from our previous study (Pruesapan et al., 2008) that *Synostemon* should be recognized at generic rank, Further morphological study is needed to make the groups identifiable. Suggestions for infrageneric groups in *Synostemon* are possible, coinciding with their distribution in Australia, but morphological characters still overlap for the groups. *Sauropus* s.s. should be subsumed under *Breynia*. Infrageneric subdivision of *Breynia* s.l. is still problematic based on molecular data and requires further study, which we are undertaking.

Therefore, we suggest maintaining *Glochidion*, *Breynia* s.l., and *Synostemon* at generic rank and to continue working on the *Phyllanthus* assemblage till this large genus can be classified on a sound phylogenetic basis.

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Appendix 3.1. List of sequence samples, data of origin and GenBank accession number used in the phylogenetic analyses. $ Species that are part of *Synostemon*, but the new combination within *Synostemon* does not exist yet, therefore, still treated under *Sauropus*. * Published by Pruesapan et al. in 2008 as *Sauropus*; ** Published by Vorontsova et al. in 2007 under *Leptopus*; *** Published by Kathriarachchi et al. in 2006 under *S. retroversus*; GenBank accession number in bold was published by Pruesapan et al. in 2008; [1]Misdentification as *Breynia* cf. *cernua* (Poir.) Müll.Arg. in Pruesapan et al. (2008); [2]Esser & Stuppy (in prep.); [3] Van Welzen and Pruesapan (in press), names will be published under *Breynia*; [4]Telford et al. (in prep.).

Taxon	Voucher	Origin	ITS	*PHYC*	*accD-psaI*	*trnS-trnG*
Breynia cf. *retusa* (Dennst.) Alston	Van Welzen 2006-3 (L)	Chiang Rai, Thailand	**EU623554**	---	GQ503473	GQ503531
Breynia discigera Müll.Arg. (1)	Takeuchi et al. 18786 (L)	N. Sumatra, Indonesia	GQ503354	---	---	---
Breynia discigera Müll.Arg. (2)	Takeuchi et al. 18873 (L)	N. Sumatra, Indonesia	**EU623550**	GQ503410	---	---
Breynia glauca Craib	Pooma et al. 2702 (L)	Nong Khai, Thailand	**EU623551**	GQ503411	---	GQ503532
Breynia mollis J.J.Sm.	Sands 1076 (L)	Papua New Guinea	**EU623552**	GQ503412	---	---
Breynia "*novoguineensis*" sp. nov. 1[1,2]	Baker et al. 37 (L)	Papua, Indonesia	**EU623549**	GQ503409	GQ503472	GQ503530
Breynia oblongifolia (Müll.Arg.) Müll.Arg. (1)	Forster 31931 (NE)	Australia	---	GQ503413	GQ503474	GQ503533
Breynia oblongifolia (Müll.Arg.) Müll.Arg. (2)	Forster 32745 (NE)	Australia	GQ503355	GQ503414	GQ503475	GQ503534
Breynia oblongifolia (Müll.Arg.) Müll.Arg. (3)	Hunter 1973 (BRI)	Queensland, Australia	**EU623577**	---	GQ503479	GQ503539
Breynia retusa (Dennst.) Alston	Soejarto & Southavong 10783 (L)	Vientiane, Laos	GQ503358	GQ503417	GQ503477	GQ503536
Breynia sp.	Hoogland & Pullen 5327 (P)	Papua New Guinea	GQ503361	---	---	---
Breynia sp. nov. 2[2]	Ambri et al. AA1468 (L)	East Kalimantan, Indonesia	GQ503357	GQ503416	GQ503476	---
Breynia stipitata Müll.Arg. (1)	Bruhl 2478 (NE)	Australia	GQ503359	GQ503418	GQ503478	GQ503537
Breynia stipitata Müll.Arg. (2)	Bruhl 2541 (NE)	Australia	GQ503360	---	---	GQ503538
Breynia stipitata Müll.Arg. (3)	Telford 12998 (NE)	Australia	GQ503356	GQ503415	---	GQ503535
Breynia vestita Warb.	Barker & Beaman 70 (L)	Papua, Indonesia	**EU623553**	GQ503419	GQ503480	GQ503540
Flueggea virosa (Roxb. ex Willd.) Voigt	Larsen et al. 45328 (L)	Thailand	GQ503362	GQ503420	GQ503481	---
Glochidion benthamianum Domin.	Bruhl 1026 (NE)	Australia	GQ503363	---	GQ503482	GQ503541

Appendix 3.1. Continued.

Taxon	Voucher	Origin	ITS	*PHYC*	*accD-psaI*	*trnS-trnG*
Glochidion brunneum Hook.f.	Lestari HL 26 (L)	East Kalimantan, Indonesia	GQ503364	---	---	---
Glochidion disparipes Airy Shaw	Hunter 1547 (NE)	Australia	GQ503365	---	GQ503483	GQ503542
Glochidion ferdinandi (Müll.Arg.) Pax & Hoffm. (1)	Bruhl 2457 (NE)	Australia	GQ503366	GQ503421	GQ503484	GQ503543
Glochidion ferdinandi (Müll.Arg.) Pax & Hoffm. (2)	Bruhl 2556 (L)	New South Wales, Australia	GQ503367	GQ503422	GQ503485	GQ503544
Glochidion harveyanum Domin (1)	Bruhl 2527 (NE)	Australia	GQ503368	GQ503423	GQ503486	GQ503545
Glochidion harveyanum Domin (2)	Hyland 9155 (L)	Queensland, Australia	GQ503369	---	---	GQ503546
Glochidion hylandii Airy Shaw	Bruhl 837 (NE)	Australia	GQ503370	---	GQ503487	GQ503547
Glochidion lobocarpum (Benth.) F.M.Bailey	Bruhl 1146 (NE)	Australia	GQ503371	GQ503424	GQ503488	GQ503548
Glochidion "oxymoron" sp. nov.	Bruhl 1112 (NE)	Australia	GQ503372	GQ503425	GQ503489	GQ503549
Glochidion philippicum (Cav.) C.B.Rob.	Forster 29379 (NE)	Australia	GQ503373	GQ503426	GQ503490	GQ503550
Glochidion sessiliflorum Airy Shaw	Bruhl 1109 (NE)	Australia	GQ503374	---	---	---
Glochidion sphaerogynum (Müll.Arg.) Kurz	Van Welzen 2003-21 (L)	Nakhon Ratchasima, Thailand	**EU623555**	GQ503427	---	GQ503551
Glochidion sumatranum Miq. (1)	Bruhl 863 (NE)	Australia	GQ503375	GQ503428	---	GQ503552
Glochidion sumatranum Miq. (2)	Bruhl 13058 (NE)	Australia	GQ503376	GQ503429	---	GQ503553
Glochidion xerocarpum (O.Schwarz) Airy Shaw	Bruhl 1271 (NE)	Australia	GQ503377	GQ503430	---	GQ503554
Notoleptopus decaisnei (Benth.) Voronts. & Petra Hoffm.	Fraser 267 (L)	Queensland, Australia	---	GQ503431	GQ503491	GQ503555
Notoleptopus decaisnei (Benth.) Voronts. & Petra Hoffm.	Evans 3222 (K)	Australia	AM745832**	---	---	---
Phyllanthus acidus (L.) Skeels	Van Welzen 2003-14 (L)	Saraburi, Thailand	**EU623556**	GQ503432	GQ503492	GQ503556
Phyllanthus amarus Schumach. & Thonn.	Van Welzen 2006-5 (L)	Chachoengsao, Thailand	**EU623557**	GQ503433	GQ503493	GQ503557
Phyllanthus emblica L.	Van Welzen 2003-11 (L)	Saraburi, Thailand	GQ503378	GQ503434	GQ503494	GQ503558
Phyllanthus hypospodius F.Muell.	Bruhl et al. 1123 (L)	Queensland, Australia	---	GQ503435	GQ503495	GQ503559

Appendix 3.1. Continued.

Taxon	Voucher	Origin	ITS	*PHYC*	*accD-psaI*	*trnS-trnG*
Phyllanthus mirabilis Müll.Arg.	Sirichamorn YSM 2009-05 (L)	Phrae, Thailand	HM132100	HM132101	HM132099	HM132102
Phyllanthus rhytidospermus F.Muell. ex Müll.Arg. $	Cumming 14160 (NE)	Australia	GQ503398	GQ503460	GQ503518	GQ503589
Phyllanthus sauropodoides Airy Shaw	Forster 29857 (L)	Queensland, Australia	**EU623558**	GQ503436	GQ503496	GQ503560
Phyllanthus sikkimensis Müll.Arg.	Pooma et al. 5233 (L)	Phetchaburi, Thailand	**EU623559**	---	---	---
Sauropus amoebiflorus Airy Shaw (2)	Maxwell 90-721 (L)	Chiang Mai, Thailand	**EU623561**	---	GQ503499	---
Sauropus androgynus (L.) Merr. (1)	Kathriarachchi et al. 40 (K)	Sri Lanka	AY936747***	GQ503459	GQ503517	GQ503588
Sauropus androgynus (L.) Merr. (2)	Middleton et al. 1496 (L)	Surat Thani, Thailand	**EU623562**	---	---	---
Sauropus androgynus (L.) Merr. (3)	Telford & Bruhl 13056 (L)	Queensland, Australia	GQ503380	GQ503438	---	GQ503563
Sauropus androgynus (L.) Merr. (4)	Van Welzen 2006-4 (L)	Chachoengsao, Thailand	**EU623563**	GQ503439	GQ503500	GQ503564
Sauropus arenosus J.T.Hunter & J.J.Bruhl $	George 15563 (NSW)	Western Australia, Australia	**EU623564**	---	---	---
Sauropus assimilis Thwaites	Kostermans 27871 (L)	Pelawatte, Sri Lanka	GQ503381	---	---	---
Sauropus asteranthos Airy Shaw	Esser 99-13 (L)	Nakhon Sawan, Thailand	**EU623565**	---	GQ503501	---
Sauropus bicolor Craib	Esser 99-21 (L)	Chiang Mai, Thailand	**EU623567**	---	GQ503503	**---**
Sauropus brevipes Müll.Arg.	Middleton et al. 974 (L)	Phetchaburi, Thailand	**EU623568**	---	---	---
Sauropus brunonis (S.Moore) Airy Shaw $	Forster 6105 (L)	Northern Territory, Australia	GQ503384	GQ503441	GQ503504	GQ503565
Sauropus "*carnosa*" sp. nov.[3]	Middleton et al. 4070 (L)	Surat Thani, Thailand	GQ503401	---	---	GQ503594
Sauropus decrescentifolius J.T.Hunter & J.J.Bruhl $	Telford 13094 (NE)	Australia	GQ503386	GQ503443	GQ503505	GQ503568
Sauropus discocalyx Welzen	Beusekom & Phengklai 566 (L)	Ranong, Thailand	GQ503387	---	---	GQ503569
Sauropus distassoides (Müll.Arg.) Airy Shaw $	Byrnes 1308 (L)	Northern Territory, Australia	GQ503388	---	---	GQ503570
Sauropus dunlopii J.T.Hunter & J.J.Bruhl $	Hunter et al. 1570 (L)	Northern Territory, Australia	**EU623569**	---	GQ503506	GQ503571

Appendix 3.1. Continued.

Taxon	Voucher	Origin	ITS	*PHYC*	*accD-psaI*	*trnS-trnG*
Sauropus elachophyllus (F.Muell. ex Benth.) Airy Shaw $	Clarkson & Neldner 9204 (L)	Queensland, Australia	AY936745***			
Sauropus filicinus J.T.Hunter & J.J.Bruhl $	Johnson 4673 (BRI)	Northern Territory, Australia	GQ503389	---	---	---
Sauropus garrettii Craib	Sino-American Guizhou Botanical Expedition 1872 (L)	Guinzhou, China	**EU623570**	GQ503444	GQ503507	GQ503572
Sauropus granulosus Airy Shaw	Pooma *et al*. 4257 (L)	Sakon Nakhon, Thailand	GQ503390	---	---	---
Sauropus hirsutus Beille (1)	Larsen *et al*. 33993 (P)	Thailand	GQ503391	GQ503445	---	---
Sauropus hirsutus Beille (2)	Van Beusekom & Phengklai 1241 (L)	Chiang Mai, Thailand	**EU623572**	GQ503446	---	---
Sauropus hubbardii Airy Shaw (1) $	BT 3340 (NE)	Australia	GQ503392	GQ503448	---	GQ503575
Sauropus hubbardii Airy Shaw (2) $	Mitchell 3226 (NE)	Australia	GQ503393	GQ503449	---	GQ503576
Sauropus kerrii Airy Shaw	Van Beusekom & Phengklai 1065 (P)	Tak, Thailand	**EU623574**	GQ503452	---	GQ503579
Sauropus lissocarpus (S.Moore) Airy Shaw (1) $	Hunter et al. 1561 (L)	Northern Territory, Australia	**EU623575**	GQ503453	GQ503511	GQ503580
Sauropus lissocarpus (S.Moore) Airy Shaw (2) $	Johnson 5103 (NSW)	Queensland, Australia	**EU623576**	GQ503454	GQ503512	GQ503581
Sauropus "*lithophila*" sp. nov.[3]	Phonsena et al. 5594 (L)	Chon Buri, Thailand	---	GQ503464	GQ503522	GQ503595
Sauropus macranthus Hassk.	Telford & Bruhl 13107 (L)	Queensland, Australia	GQ503396	---	---	---
Sauropus micrasterias Airy Shaw	Erwin & Chai S27479 (L)	Sarawak, Malaysia	**EU623578**	GQ503455	---	GQ503582
Sauropus orbicularis Craib	Soejarto & Southavong 10792 (L)	Vientiane, Laos	**EU623580**	GQ503456	GQ503513	GQ503584
Sauropus podenzanae (S.Moore) Airy Shaw $	Blake 23210 (L)	Queensland, Australia	**EU623581**	---	GQ503514	GQ503585
Sauropus poomae Welzen & Chayam.	Phonsena et al. 5245 (L)	Chiang Rai, Thailand	**EU623582**	GQ503457	GQ503515	GQ503586
Sauropus quadrangularis (Willd.) Müll.Arg.	Maxwell 99-116 (L)	Chiang Mai, Thailand	**EU623583**	**---**	**---**	**---**

Appendix 3.1. Continued.

Taxon	Voucher	Origin	ITS	*PHYC*	*accD-psaI*	*trnS-trnG*
Sauropus "*repens*" sp. nov.[3]	Middleton et al. 2287 (L)	Thailand	GQ503385	---	---	GQ503566
Sauropus rhamnoides Blume	Esser 2001-4 (L)	Chanthaburi, Thailand	**EU623584**	---	---	---
Sauropus rigidulus (F.Muell. ex Müll.Arg.) Airy Shaw $	Johnson MRS787 (BRI)	Queensland, Australia	**EU623586**	---	---	---
Sauropus rimophilus J.T.Hunter & J.J.Bruhl $	Bruhl et al. 1246 (BRI)	Northern Territory, Australia	**EU623587**	GQ503461	---	GQ503591
Sauropus similis Craib	Larsen et al. 46639 (L)	Chiang Mai, Thailand	GQ503399	GQ503462	GQ503520	GQ503592
Sauropus sp.1	Phonsena et al. s.n.	Kaeng Krachan NP, Thailand	GQ503400	GQ503463	GQ503521	GQ503593
Sauropus spatulifolius Beille	Wong s.n. (L)	Honolulu, U.S.A.	**EU623588**	---	GQ503523	GQ503596
Sauropus stenocladus (Müll.Arg.) J.T.Hunter & J.J.Bruhl subsp. *pinifolius* J.T.Hunter & J.J.Bruhl $	Bruhl et al. 1278A (L)	Northern Territory, Australia	GQ503405	GQ503467	GQ503525	GQ503599
Sauropus stenocladus (Müll.Arg.) J.T.Hunter & J.J.Bruhl subsp. *stenocladus* $	Hunter et al. 1579 (L)	Northern Territory, Australia	GQ503406	---	---	---
Sauropus stenocladus (Müll.Arg.) J.T.Hunter & J.J.Bruhl subsp. *stenocladus* $	Latz 6132 (L)	Northern Territory, Australia	GQ503404	---	---	---
Sauropus suberosus Airy Shaw	Chin 827 (L)	Perak, Malaysia	**EU623589**	---	---	---
Sauropus thorelii Beille	Van Welzen 2006-1 (L)	Chiang Mai, Thailand	**EU623590**	GQ503468	GQ503526	GQ503600
Sauropus thyrsiflorus Welzen	Kostermans 765 (L)	Kanchanaburi, Thailand	**EU623591**	GQ503469	GQ503527	GQ503601
Sauropus villosus (Blanco) Merr. (1)	Mcgregor 32398 (L)	Panay, Philippines	**EU623593**	---	---	---
Sauropus villosus (Blanco) Merr. (2)	Phengklai et al. 12122 (BKF)	Thailand	**EU623592**	---	---	---
Synostemon albiflorus (Müll.Arg.) Airy Shaw*	Forster 21362 (L)	Queensland, Australia	**EU623560**	---	GQ503497	GQ503561
Synostemon bacciformis (L.) G.L.Webster (1)	Cowie I 3418 (L)	Northern Territory, Australia	GQ503382	---	GQ503502	---
Synostemon bacciformis (L.) G.L.Webster (2)*	Kerr 8350 (L)	Ubon Ratchatani, Thailand	**EU623566**	---	---	---

Appendix 3.1. Continued.

Taxon	Voucher	Origin	ITS	*PHYC*	*accD-psaI*	*trnS-trnG*
Synostemon bacciformis (L.) G.L.Webster (3)	Pruesapan 2009-4 (L)	Bangkok, Thailand	GQ503383	GQ503440	---	---
Synostemon "*cowiei*" sp.nov.1[4]	Cowie 11606 (NE)	Australia	---	GQ503442	---	GQ503567
Synostemon glaucus F.Muell.*	Hunter et al. 1565 (L)	Northern Territory, Australia	**EU623571**	---	---	GQ503573
Synostemon hirtellus F.Muell.*	Bean 15558 (BRI)	Queensland, Australia	**EU623573**	GQ503447	GQ503508	GQ503574
Synostemon "*inequisepalus*" sp.nov.2[4]	Cowie 8679 (BRI)	Northern Territory, Australia	GQ503394	---	---	---
Synostemon "*judithae*" sp.nov.3[4]	Barrett 3905 (NE)	Australia	---	GQ503450	GQ503509	GQ503577
Synostemon "*kakadu*" sp.nov.4[4]	Bruhl 1270 (NE)	Australia	GQ503395	GQ503451	GQ503510	GQ503578
Synostemon "*nitmiluk*" sp. nov.5*[,4]	Bruhl & Hunter 1238 (L)	Northern Territory, Australia	**EU623579**	---	---	GQ503583
Synostemon ramosissimus F.Muell	Latz & Albrecht_20135 (BRI)	Northern Territory, Australia	GQ503397	GQ503458	GQ503516	GQ503587
Synostemon rigens F.Muell.*	Kraehenbuehl 6007 (L)	South Australia, Australia	**EU623585**	---	GQ503519	GQ503590
Synostemon sphenophyllus Airy Shaw	Gray 08597 (BRI)	Queensland, Australia	GQ503402	GQ503465	---	GQ503597
Synostemon "*spinescens*" sp.nov.6[4]	Bean 20738 (NE)	Australia	GQ503403	GQ503466	GQ503524	GQ503598
Synostemon trachyspermus (F.Muell.) Airy Shaw	Bell 547 (NE)	Australia	GQ503407	GQ503470	GQ503528	GQ503602
Synostemon trachyspermus (F.Muell.) Airy Shaw	Chippendale & ConsAppendix 19076 (L)	New South Wales, Australia	---	---	---	GQ503603
Synostemon "*umbrosus*" sp.nov.7[4]	Barrett 3262 (NE)	Australia	GQ503408	GQ503471	GQ503529	GQ503604