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Phylogenetic relationships in Apocynaceae based on nuclear PHYA and plastid *trnL-F* sequences, with a focus on tribal relationships

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Abstract. To date, most molecular phylogenetic studies of Apocynaceae have been based on plastid DNA regions or nuclear ribosomal DNA. In this study, we used part of the PHYA (phytochrome A) exon, a low-copy nuclear gene, and combined it with the *trnL-F* region (intron and spacer) to investigate placement of Periplocoideae, intergeneric relationships of Asclepiadoideae and relationships within Rauvolfioideae. We included 112 taxa representing most major clades of Apocynaceae. The study confirms that both subfamilies Apocynoideae and Rauvolfioideae are paraphyletic and that Periplocoideae are nested within Apocynoideae. The APSA clade (Apocynoideae, Periplocoideae, Secamonoideae and Asclepiadoideae) is strongly supported here, but the crown clade of Apocynaceae (comprised of subfamilies Asclepiadoideae, Secamonoideae, Periplocoideae and Echiteae, Mesechiteae, Odontadenieae and Apocyneae of Apocynoideae) has only moderate support. The present study places Periplocoideae as part of the sister group to the rest of the crown clade. This contrasts with results from the previous only PHYA and plastid marker–based studies in which periplocoids appeared as sister to a clade comprising Baisseeae (Apocynoideae) plus Secamonoideae and Asclepiadoideae. Old World Cynanchinae form a well-supported clade with the New World MOG (Metastelmatinae, Oxypetalinae and Gonolobinae) tribes rather than with the largely Old World. Asclepiadinae and Tylophorinae, as suggested by earlier studies. In our combined analyses, resolution among most groups is improved as compared to previous plastid-only analyses.

Keywords. Apocynaceae, Asclepiadeae, Baisseeae, Periplocoideae, Phylogeny, Phytochrome A.

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1. INTRODUCTION

Since Endress and Bruyns (2000), Apocynaceae sensu lato have been investigated with molecular data, mostly plastid, to evaluate relationships among various groups proposed in their classification (Potgieter and Albert 2001; Rapini et al. 2003 and 2006; Livshultz et al. 2007; Simões et al. 2007; Livshultz 2010). Subfamilies Rauvolfioideae and Apocynoideae have been recovered as non-monophyletic (e.g., Sennblad et al. 1998; Potgieter and Albert 2001; Livshultz et al. 2007; Simões et al. 2004). In more recent classifications (Endress et al. 2007a; Simões et al. 2007; Endress et al., 2014)), ten tribes have been proposed in Rauvolfioideae: Tabernaemontaneae, Alstonieae, Alyxieae, Carisseae, Hunterieae, Melodineae, Plumerieae, Vinceae, Willughbeieae, Aspidospermeae and Amsonieae. Monophyly of most tribes in the subfamily has always remained suspect (Potgieter and Albert 2001; Sennblad and Bremer 2002); however, in the recent phylogenetic analysis by Simões et al. (2007), six clades (out of nine tribes sensu Endress and Bruyns 2000) were identified in Rauvolfioideae, which could be referred to Willughbeieae, Tabernaemontaneae, Hunterieae, Alyxieae, Plumerieae, and Carisseae, while Melodineae, Alstonieae and Vinceae were polyphyletic

Similarly, in Apocynoideae, five tribes were recognized by Endress and Bruyns (2000): Wrightieae, Malouetieae, Apocyneae, Echiteae and Mesechiteae. Since this publication, five more tribes, Nerieae (Sennblad and Bremer 2002), Odontadenieae (Endress et al. 2007a), Baisseeae (Endress et al. 2007a) and Rhabdadenieae (Endress et al., 2014) have been recognized in this subfamily. Baisseeae are considered a sister group of the milkweeds (Asclepiadoideae-Secamonoideae) rather than subfamily Periplocoideae on the basis of various phylogenetic studies (Sennblad and Bremer 1996, 2000 and 2002; Potgieter and Albert 2001; Lahaye et al. 2005; Livshultz et al. 2007). Also phylogenetic analyses firmly support placement of Periplocoideae in the APSA (Apocynoideae, Periplocoideae, Secamonoideae, Asclepiadoideae) clade (Judd et al. 1994; Sennblad and Bremer 1996, 2002; Civeyrel et al. 1998; Potgieter and Albert 2001). Periplocoideae were recognized until the last decades of the 20th century as members of Asclepiadaceae (Kunze 1990 and 1993; Venter et al. 1990; Dave and Kuriachen 1991; Liede and Kunze 1993; Nilsson et al. 1993; Swarupanandan et al. 1996). In subfamily Asclepiadoideae five tribes have been recognized: Asclepiadeae, Ceropegieae, Marsdenieae, Fockeeae and Eustegieae (Endress et al. 2007a; Endress et al., 2014)). Endress and Bruyns (2000) delimited the tribes of Asclepiadoideae on the basis of the orientation of pollinia in pollen sacs: upwardly directed in Ceropegieae-Marsdenieae and pendulous in Asclepiadeae. Erect pollinia are considered a primitive character and also found in Secamonoideae and Fockeeae (Kunze 1993). Recognition of Fockeeae as a tribe separate from Marsdenieae in Asclepiadoideae by Kunze et al. (1994) is disputed by Endress and Bruyns (2000) due to insufficient taxon sampling in Marsdenieae. The isolated basal position of Fockeeae has been confirmed by subsequent phylogenetic analyses (Potgieter and Albert, 2001; Rapini et al., 2003; Verhoeven et al., 2003).

Rapini et al. (2003) identified three main clades in Asclepiadeae that could be referred to as subtribes: Astephaninae and two multiple subtribe clades, ACTG (Asclepiadinae, Cynanchinae, Tylophorinae and Glossonematinae) and MOG. Subtribe Glossonematinae was later dissolved by Liede et al. (2002), *Glossonema* and *Odontanthera* were included in Cynanchineae and a third genus of the tribe *Solenostemma* belongs to none of the subtribes presently recognized in the Asclepiadeae (Endress et al. 2007a).

Cynanchinae within the ACT clade are divided into a monophyletic Old World succulent group (containing Malagasy *Cynanchum* species), but New World sections of the subtribe are polyphyletic (Liede and Taüber 2002; Rapini et al. 2006). Furthermore, cladistic analyses of Goyder et al. (2007) and Fishbein et al. (2011) have emphasized that generic delimitation of subtribe Asclepiadineae is problematic. These studies concluded that Asclepiadoideae still needs further investigation to identify monophyletic groups and find morphological characters by which to recognize them. To date, almost all broader molecular phylogenetic studies of Apocynaceae have been based on plastid DNA, either alone or in combination with morphological datasets.

Livshultz (2010) presented a study using the low-copy nuclear gene, PHYA (phytochrome A, exon 1) for a number of Apocynaceae groupings. Her approach proved useful in describing the status of tribe Baisseeae as the sister group of the milkweeds (i.e. Asclepiadoideae and Secamonoideae) rather than Periplocoideae. However, there are still many other areas within the family where resolution/support is low. For this study, we sequenced the same region of PHYA 1 (first exon) as in Livshultz (2010) for a broader dataset sampled across the family and combined these data with the widely sampled plastid *trnL-F* (intron/spacer) region. Our main goals are to: 1) further improve resolution in the primary clades of Asclepiadoideae (one of the groups from the crown clade defined by Livshultz 2010), 2) examine the position of Periplocoideae in Apocynaceae and 3) improve resolution within the subfamily Rauvolfioideae.

2. MATERIALS AND METHODS

2.1. DNA extraction and amplification

Taxa of Apocynaceae used here were either collected from the field in Pakistan or sampled from the DNA Bank at The Royal Botanic Gardens, Kew (https://dnabank.science.kew.org/). A complete list of taxa including voucher details, taxonomic treatment and provenance are provided in Table 1.

Total genomic DNA was extracted from silica-dried field collections following the 2 ×CTAB protocol of Richard (1997) with modifications described by Nazar and Mahmood (2010). DNA from herbarium specimens was isolated by pulverising dry material in tubes containing plastic beads (using a Genogrinder 2010, SPEX CertiPrep Ltd, Harrow, Middlesex, UK) and then following a modified Doyle and Doyle (1987) 2 ×CTAB method. To isolate DNA from these samples, we used precipitation in chilled ethanol (-20 °C) for at least 24 hr and then resuspended in 1.55 g/ml caesium chloride/ethidium bromide. Samples were then purified using a density gradient, followed by removal of the ethidium and caesium chloride with butanol/dialysis and storage in Tris EDTA.

Primers (PHYA 2059F, 2745F, 2971R, 3560R) used to amplify the first exon of PHYA are those of Livshultz (2010). The region was amplified using ReddyMix PCR Mastermix (Thermo Scientific, Epsom, Surrey, UK) in a 25 µl reaction volume. Degraded DNA (and/or possibly impure DNA), in some samples caused problems for amplification using the ReddyMix PCR Mastermix. To amplify the target regions from degraded DNA, especially from herbarium samples, Platinum® taq DNA polymerase (Invitrogen) was used. The reaction mix (25 µl total volume) consisted of 2.5 µl 10 ×PCR buffer, 2 µl MgCl2 (50 mM/ml), 1 µl of BSA (50 mg/ml), 0.6 µl of each primer (0.1 ng/µl), 0.2 µl of 5 U/µl of Platinum taq DNA polymerase, made up to volume with nuclease free water. The following PCR program was used for amplification: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 20 sec, annealing at 50 °C for 30 sec and extension at 72 °C for 2 min. A final extension was carried out at 72 °C for 7 min. Higher annealing temperatures reduced yields, and we found that using 50 °C did not cause amplification of more than one region (i.e. the sequencing reactions were free from obvious polymorphisms.

PCR products were cleaned using NucleoSpin® Extract II mini-columns (Macherey-Nagel, Duren, Germany) following the manufacturer's protocols. For cleaning of cycle sequencing products, we used precipitation in ethanol (using EDTA). Samples were sequenced on an ABI 3730 automated sequencer according to the manufacturer's protocols (Applied Biosystems, Inc.). Electropherograms were edited and assembled using Sequencher version 4.5 (Gene Codes, Ann Arbor, Michigan, USA); these sequences were easily aligned by eye in PAUP following the suggestions of Kelchner (2000).

2.2. Data analysis

Incongruence between *trnL-F* and PHYA results was assessed by looking for contradictory clades in both PHYA and *trnL-F* Bayesian and parsimony trees by following the same criteria regarding bootstrap support used by Livshultz (2010). Several studies have shown that the incongruence length test (ILD) proposed by Ferris et al. (1994) is too sensitive and unreliable for detection of incongruence (Darlu and Lecointre, 2002), so we did not use it here and have instead relied on inspection for well supported but different tree topologies as the basis for assessing incongruence (which we did not observe here). For the Bayesian results, we considered posterior probabilities (PP) > 0.95 as well-supported; < PP 0.95 is considered weakly supported and not indicative of incongruence. For the parsimony results, we considered bootstrap percentages (BP) of 80 as the cut-off for assessing incongruence. The separate analyses did not produce any clear evidence for incongruent clades, so we produced combined analyses of *trnL-F* and PHYA.

The combined dataset (*trnL-F* and PHYA) comprises of 112 sequences — 47 sequences from study of Livshultz (2010) are included (Table 2). Phylogenetic analyses were performed using maximum parsimony (MP; PAUP version 4.0b10, Swofford 2002) and Bayesian methods (Mr. Bayes ver.3.1, Huelsenbeck and Ronquist, 2001). Gaps were treated as missing data. For the parsimony analyses, the combined data matrix was analysed using tree bisection-reconnection (TBR) swapping and 1000 replicates of random taxon-addition, holding 10 trees at each step to reduced time searching islands of equally parsimonious trees. DELTRAN character optimization was used to illustrate branch lengths (due to reported errors with ACCTRAN optimization in PAUP version 4.0b).

For Bayesian analysis, a HKY85 model was specified in which all transitions and transversions have potentially different rates. More complex models were also tested, but these yielded the same tree with similar PP. The analysis was performed with 500,000 generations of Markov chain Monte Carlo with equal rates and a sampling frequency of 10. Microsoft excel was used to plot

Table 1. A list of the samples from Pakistan and The Royal Botanic Gardens Kew, London with vouchers information and place of collection are given.

Taxa	Voucher detail	Country	Regions sequenced
Asclepiadoideae – Asclepiadeae: Metastelmatinae			
Blepharodon lineare (Decne.) Decne.	Forzza *et al.* 2027	Argentina	*trnL-F* and *PHYA*
Asclepiadoideae – Asclepiadeae: Oxypetalinae			
Araujia sericifera Brot.	Forster 7656	Australia	*PHYA*
Funastrum clausum (Jacq.) Schltr.	Mello- Silva *et al.* 1919	Argentina	*PHYA*
Oxypetalum capitatum Mart.	Mello- Silva *et al.* 1924	Argentina	*PHYA*
Philibertia discolor (Schltr.) Goyder	Mello- Silva *et al.* 1887	Argentina	*PHYA*
Philibertia lysimachioides (Wedd.) T. Mey.	Mello- Silva *et al.* 1886	Argentina	*PHYA*
Asclepiadoideae – Asclepiadeae: Gonolobinae			
Matelea pseudobarbata (Pitter) Woodson	M. Endress 97-08	Costa Rica	*PHYA*
Asclepiadoideae – Asclepiadeae: Asclepiadinae			
Calotropis procera (Aiton) W. T. Aiton	Naz001*	Pakistan	*trnL-F* and *PHYA*
Kanahia laniflora (Forssk.) R. Br.	Goyder *et al.* 3931	Tanzania	*PHYA*
Pergularia daemia (Forssk.) Chiov.	Naz024*	Pakistan	*PHYA*
Pergularia tomentosa L.	Naz012*	Pakistan	*trnL-F* and *PHYA*
Stenostelma corniculatum (E. Mey.) Bullock	Balkwill 10908	South Africa	*PHYA*
Xysmalobium parviflorum Harv. ex Scott-Elliot	Killick & Vahrmeijer 3658	South Africa	*PHYA*
Asclepiadoideae – Asclepiadeae: Cynanchinae			
Cynanchum viminale (L.) Bassi	Chase 731	**	*PHYA*
Cynanchum jacquemontianum Decne.	Naz010*	Pakistan	*trnL-F* and *PHYA*
Cynanchum obtusifolium L.f.	P. Bruyns Vch	South Africa	*PHYA*
Asclepiadoideae – Asclepiadeae: Tylophorinae			
Tylophora hirsuta (Wall.) Wight	Naz014*	Pakistan	*trnL-F* and *PHYA*
Unplace genus			
Oxystelma esculentum (L. f.) Sm.	Naz020*	Pakistan	*trnL-F* and *PHYA*
Asclepiadoideae – Asclepiadeae: Astephaninae			
Eustegia minuta (L. F.) N. E. Br.	P. Bruyns 4357	South Africa	*PHYA*
Oncinema lineare (L. F.) Bullock	P. Bruyns Vch?	South Africa	*PHYA*
Schubertia grandiflora Mart.	Irwin *et al.* 31285	Brazil	*PHYA*
Asclepiadoideae – Marsdenieae			
Dischidia lanceolata Decne.	Chase 734	Indonesia	*PHYA*
Dregea abyssinica K.Schum.	Goyder *et al.* 3918	Tanzania	*PHYA*
Gymnema sylvestre (Retz.) Schultz.	Chase 3902	India	*trnL-F* and *PHYA*
Hoya finalasonii Wight	Chase 17138	India	*PHYA*
Hoya manipurensis Deb.	Chase 733	Thailand	*PHYA*
Marsdenia carvalhoi Morillo & Carnevali	Chase 3904	Brazil	*trnL-F* and *PHYA*
Rhyssolobium dumosum E. Mey.	P. V. Bruyns 3948	South Africa	*PHYA*
Staphanotis floribunda Brongn.	Chase 732	Senegal	*trnL-F* and *PHYA*
Wattakaka volubilis (Linn.f.) Stapf.	Naz006*	Pakistan	*trnL-F* and *PHYA*
Asclepiadoideae – Ceropegieae			
Caralluma tuberculata N.E. Br.	Naz019*	Pakistan	*trnL-F* and *PHYA*
Ceropegia sandersonii Decne.ex Hook.	Chase 17507	**	*PHYA*
Duvalia polita N. E. Br.	Kew	**	*PHYA*
Boucerosia indica Dalzell	Chase 2861	India	*PHYA*
Leptadenia pyrotechnica	Naz018*	Pakistan	*trnL-F* and *PHYA*
Neoschumannia kamerunensis	Chase 3903	Cameroon	*PHYA*
Quaqua incarnata (L. f.) Bruyns	Chase 9818	South Africa	*PHYA*
Heterostemma acuminatum Decne.	Forster 5090	**	*PHYA*

Taxa	Voucher detail	Country	Regions sequenced
Secamonoideae			
Secamone alpini Schult.	P. Bruyns Vch	South Africa *trnL-F* and *PHYA*	
Periplocoideae			
Cryptolepis buchananii Roemer & Schult.	Naz002*	Pakistan	*trnL-F* and *PHYA*
Cryptolepis decidua (Planch. ex Benth.) N. E. Br.	P. V. Bruyns s.n. (east of Fish R.)	Namibia	*trnL-F* and *PHYA*
Hemidesmus indicus (L.) R.Br. ex Schult.	Chase 725	Tamil Nadu	*PHYA*
Periploca aphylla Decne.	Naz004*	Pakistan	*trnL-F* and *PHYA*
Raphionacme hirsuta (E.Mey.sec.N.E.Brown) R.A.Dyer	CFR 15	South Africa	*PHYA*
Schlechterella abyssinicum (Chiov.) Venter & R. L. Verh.	Chase 720	Ethopia	*trnL-F* and *PHYA*
Apocynoideae - Malouetieae			
Kibatalia gitingensis (Elmer) Woodson	Liede 3268	**	*trnL-F* and *PHYA*
Pachypodium leallii Welw.	Chase 735	South Africa *trnL-F* and *PHYA*	
Apocynoideae - Nerieae			
Adenium obesum (Forssk.) Roem. & Schult.	Chase 727	Somalia	*trnL-F* and *PHYA*
Nerium oleander L.	Naz015*	Pakistan	*trnL-F* and *PHYA*
Apocynoideae - Apocyneae			
Beaumontia grandiflora (Roxb.) Wall.	Naz008*	Pakistan	*trnL-F* and *PHYA*
Trachelospermum jasminoides (Lindl.) Lem.	Naz022*	Pakistan	*trnL-F* and *PHYA*
Apocynoideae - Echiteae			
Fernaldia pandurata (A.DC.) Woodson	M Endress, Zurich	**	*PHYA*
Rauvolfioideae - Wrightieae			
Pleioceras barteri Baill.	Endress, P. 99-10	Ivory Coast	*PHYA*
Rauvolfioideae - Carisseae			
Carissa spinarum L.	Naz017*	Pakistan	*trnL-F* and *PHYA*
Rauvolfioideae - Plumerieae			
Anechites nerium Urb.	Bremer *et al.* 3386 UPS	**	*PHYA*
Skytanthus acutus Meyen	M. Endress, Zurich	**	*PHYA*
Thevetia peruviana (Pers.) K. Schum.	Naz013*	Pakistan	*trnL-F* and *PHYA*
Rauvolfioideae - Vinceae			
Amsonia hurbritchii Woodson	Chase 19252	USA	*trnL-F* and *PHYA*
Petchia ceylanica (Wight) Livera	R. Olmor s. n	Germany	*trnL-F* and *PHYA*
Rauvolfia serpentina (L.) Benth.	Naz003*	Pakistan	*trnL-F* and *PHYA*
Rhazya orientalis A.DC.	M. Endress s.n.	Zurich	*trnL-F* and *PHYA*
Vinca major L.	Naz025*	Pakistan	*trnL-F*
Rauvolfioideae - Tabernaemontaneae			
Tabernaemonta divericata (L.) R. Br. exRoem.Schult	Chase 5571	Bangladesh	*PHYA*
Rauvolfioideae - Hunterieae			
Gonioma kamassi E.Mey.	Chase 5806	South Africa *trnL-F* and *PHYA*	
Rauvolfioideae - Alyxieae			
Alyxia buxifolia R. Br.	Smith, R.J. (RJS202)	Australia	*PHYA*
Rauvolfioideae - Alstonieae			
Alstonia scholaris (L.) R. Br.	Naz007*	Pakistan	*trnL-F* and *PHYA*

*Vouchers specimens are preserved in the Plant Biochemistry and Molecular Biology Laboratory of Quaid-i-sAzam University, Islamabad, Pakistan.
** Information not present in Kew's databases.

generation number against InL to find the 'burn in'. Trees of low PP were deleted, and all remaining trees were imported into PAUP 4.0b10. A Bayesian tree (i.e., a majority-rule consensus tree) was produced showing frequencies of all observed bi-partitions (i.e. the posterior probabilities for each node).

Table 2. A list of taxa with GenBank accession numbers used in *trnL-F* and *PHYA* analyses, sequenced in present study, previously published in Rapini et al. (2003), Sennblad and Bremer (1998) and Livshultz (2010) with updated nomenclature (Endress et al., 2014).

Species Name	*PHYA*	*trnL-F*	Subtribe	Tribe	Subfamily
Adenium obesum (Forssk.) Roem. & Schult.	LT972249	HE805526		Nerieae	Apocynoideae
Aganosma wallichii G. Don.	GU901319	EF456127	Ichnocarpinae	Apocyneae	Apocynoideae
Alstonia scholaris (L.) R. Br.	LR027092	HE805532		Alstonieae	Rauvolfioideae
Alyxia buxifolia R. Br.	LT972244	AF214152		Alyxieae	Rauvolfioideae
Amsonia hurbritchii Woodson	LR027376			Amsonieae	Rauvolfioideae
Anechites nerium Urb.	LT972245	AM295087	Thevetiinae	Plumerieae	Rauvolfioideae
Angadenia berteroi (A.DC.) Miers	GU901358	EF456246		Echiteae	Apocynoideae
Anodendron paniculatum A. DC.	GU901327	EF456194	Papuechitinae	Apocyneae	Apocynoideae
Apocynum androsaemifolium L.	GU901328	AF214308	Apocyinae	Apocyneae	Apocynoideae
Araujia sericifera Brot.	LT972246	AJ704332	Oxypetalinae	Asclepiadeae	Asclepiadoideae
Artia balansae (Baillon) Pichon ex Guillaumin	GU901372	EF456142		Echiteae	Apocynoideae
Baissea multiflora A. DC.	GU901330	EF456199		Baisseeae	Apocynoideae
Beaumontia grandiflora (Roxb.) Wall.	LR027094	HE805527	Beaumontiinae	Apocyneae	Apocynoideae
Blepharodon linere (Decne.) Decne.	LR026999	AY163668	Metastelmatinae	Asclepiadeae	Asclepiadoideae
Boucerosia indica Dalzell	HF969013	AF214202		Ceropegieae	Asclepiadoideae
Calotropis procera (Aiton) W. T. Aiton	LT972247	HE805509	Asclepiadinae	Asclepiadeae	Asclepiadoideae
Caralluma tuberculata N.E. Br.	LT972248	HE805510		Ceropegieae	Asclepiadoideae
Carissa spinarum L.	LR027375	HE805533		Carisseae	Rauvolfioideae
Ceropegia sandersonii Decne.ex Hook.	HF969012	AF214179		Ceropegieae	Asclepiadoideae
Chonemorpha fragrans (Moon) Alston	GU901332	EF456132	Chonemorphinae	Apocyneae	Apocynoideae
Cleghornia malaccensis (Hook. f.) King & Gamble	GU901333	EF456241	Apocyinae	Apocyneae	Apocynoideae
Cryptolepis buchananii Roemer & Schult.	HG004619	HE805522			Periplocoideae
Cryptolepis decidua (Planch. ex Benth.) N. E. Br.	HG004618	HE805523			Periplocoideae
Cycladenia humilis Bentham	GU901367	EF456140		Odontadenieae	Apocynoideae
Cynanchum jacquemontianum Decne.	LR027368	HE805511	Cynanchinae	Asclepiadeae	Asclepiadoideae
Cynanchum obtusifolium L.f.	HF969010	AJ428692	Cynanchinae	Asclepiadeae	Asclepiadoideae
Cynanchum viminale (L.) Bassi	HG004632	AJ290912	Cynanchinae	Asclepiadeae	Asclepiadoideae
Dischidia lanceolata Decne.	LR028004			Marsdenieae	Asclepiadoideae
Dregea abyssinica K.Schum.	HG004620			Marsdenieae	Asclepiadoideae
Duvalia polita N. E. Br.	HF969009	AJ488374		Ceropegieae	Asclepiadoideae
Echites umbellatus Jacq.	GU901387	EF456186		Echiteae	Apocynoideae
Elytropus chilensis Müll. Arg.	GU901398	EF456171		Odontadenieae	Apocynoideae
Epigynum cochinchinense (Pierre) D.J. Middleton	GU901340	EF456147	Ichnocarpinae	Apocyneae	Apocynoideae
Eustegia minuta (L.f.) N.E.Br.	LR027089	AJ410207	Eustegieae	Asclepiadeae	Asclepiadoideae
Fernaldia pandurata (A.DC.) Woodson	GU901329	EF456209		Echiteae	Apocynoideae
Finlaysonia insularum (King & Gamble) Venter	GU901341	EF456105			Periplocoideae
Fockea edulis K. Schum.	LR027374	AF214199		Fockeeae	Asclepiadoideae
Forsteronia guyanensis Müll.Arg.	GU901359	EF456153		Mesechiteae	Apocynoideae
Funastrum clausum (Jacq.) Schltr.	HG004645	AJ428794	Oxypetalinae	Asclepiadeae	Asclepiadoideae
Gonioma kamassi E.Mey.	HG004623	HE805535		Hunterieae	Rauvolfioideae
Gymnanthera oblonga (Burm. f.) P.S. Green	GU901348	EF456106			Periplocoideae
Gymnema sylvestre (Retz.) Schultz.	HG004637	HE805512		Marsdenieae	Asclepiadoideae
Hemidesmus indicus (L.) R.Br. ex Schult.	HG004617	DQ916877			Periplocoideae
Heterostemma acuminatum Decne.		AJ574827		Ceropegieae	Asclepiadoideae
Hoya finalasonii Wight	HG004636			Marsdenieae	Asclepiadoideae
Hoya manipurensis Deb.	LR027373	AF214227		Marsdenieae	Asclepiadoideae
Ichnocarpus frutescens R. Br.	GU901356	EF456136	Ichnocarpinae	Apocyneae	Apocynoideae
Kanahia laniflora (Forssk.) R. Br.	HG004642	AY163695	Asclepiadinae	Asclepiadeae	Asclepiadoideae
Kibatalia gitingensis (Elmer) Woodson	HG004629	HE805528		Malouetieae	Apocynoideae

Species Name	*PHYA*	*trnL-F*	Subtribe	Tribe	Subfamily
Laubertia contorta (Mart.& Galeotti) Woodson	GU901375	EF456180		Echiteae	Apocynoideae
Leptadenia pyrotechnica	HG004614	HE805513		Ceropegieae	Asclepiadoideae
Mandevilla boliviensis Decne.	GU901343	EF456134		Mesechiteae	Apocynoideae
Marsdenia carvalhoi Morillo & Carnevali	LR027091	DQ334521		Marsdenieae	Asclepiadoideae
Marsdenia glabra Costantin	LR027370	EF456114		Marsdenieae	Asclepiadoideae
Matelea pseudobarbata (Pitter) Woodson	HG004621		Gonolobinae	Asclepiadeae	Asclepiadoideae
Microloma tenuifolium (L.) Kuntze	LR027371	AJ410230	Astephaninae	Asclepiadeae	Asclepiadoideae
Motandra guineensis A. DC.	GU901361	EF456210		Baisseeae	Apocynoideae
Neoschumannia kamerunensis Schltr.	HG004613	AJ410054		Ceropegieae	Asclepiadoideae
Nerium oleander L	LR027093	HE805529		Nerieae	Apocynoideae
Odontadenia perrotteti (A. DC.) Woodson	GU901335	EF456211		Odontadenieae	Apocynoideae
Oncinema lineare (L. F.) Bullock	LR027090	AJ428827	Astephaninae	Asclepiadeae	Asclepiadoideae
Oncinotis tenuiloba Stapf	GU901368	EF456141		Baisseeae	Apocynoideae
Orthanthera jasminiflora Schinz		AJ574827		Ceropegieae	
Oxypetalum capitatum Mart.	HG004644	AY163710	Oxypetalinae	Asclepiadeae	Asclepiadoideae
Oxystelma esculentum (L. f.) Sm.	HF969014	AJ290887		Asclepiadeae	Asclepiadoideae
Pachypodium leallii Welw.	HG004628	HE805530		Malouetieae	Apocynoideae
Papuechites aambe Markgr.	GU901370	EF456189	Papuechitinae	Apocyneae	Apocynoideae
Parameria laevigata (Juss.) Mold.	GU901371	EF456197	Urceolinae	Apocyneae	Apocynoideae
Parsonsia eucalyptophylla F. Muell.	GU901380	EF456215		Echiteae	Apocynoideae
Peltastes isthmicus Woodson	GU901324	EF456129		Echiteae	Apocynoideae
Pentalinon luteum (L.) B.F. Hansen & R.P. Wunderlin	HG004631	EF456191		Echiteae	Apocynoideae
Pergularia daemia (Forssk.) Chiov.	HG004641	JN205300	Asclepiadinae	Asclepiadeae	Asclepiadoideae
Pergularia tomentosa L.	HG004640	HE805514	Asclepiadinae	Asclepiadeae	Asclepiadoideae
Periploca aphylla Decne.	HG004616	HE805524			Periplocoideae
Petchia ceylanica (Wight) Livera	HG004624	AM295093	Catharanthinae	Vinceae	Rauvolfioideae
Petopentia natalensis (Schltr.) Bullock	GU901376	EF456107			Periplocoideae
Philibertia discolor (Schltr.) Goyder	LR027369	AY163700	Oxypetalinae	Asclepiadeae	Asclepiadoideae
Philibertia lysimachioides (Wedd.) T. Mey.	HG004643	AJ290900	Oxypetalinae	Asclepiadeae	Asclepiadoideae
Phyllanthera grayi (P.I. Forst.) Venter	GU901377	EF456103			Periplocoideae
Pinochia corymbosa (Jacq.) M.E. Endress & B.F. Hansen	GU901378	EF456167		Odontadenieae	Apocynoideae
Pleioceras barteri Baill.	LR027096	EF456251		Wrightieae	Apocynoideae
Prestonia lagoensis (Müll. Arg.) Woodson	GU901337	EF456237		Echiteae	Apocynoideae
Quaqua incarnata (L. f.) Bruyns	HG004612	AJ488455		Ceropegieae	Asclepiadoideae
Raphionacme hirsuta (E.Mey.sec.N.E.Brown) R.A.Dyer	HG004615	AJ581825			Periplocoideae
Rauvolfia serpentina (L.) Benth.	HG004625	HE805539	Rauvolfiinae	Vinceae	Rauvolfioideae
Rhabdadenia biflora Müll.Arg.	LR028003			Rhabdadenieae	Apocynoideae
Rhazya orientalis A.DC.		AM295095		Vinceae	Rauvolfioideae
Rhodocalyx rotundifolius Müll. Arg.	GU901396	EF456238		Echiteae	Apocynoideae
Rhyssolobium dumosum E. Mey.	HG004635	AM233378		Marsdenieae	Asclepiadoideae
Schlechterella abyssinicum (Chiov.) Venter & R. L. Verh.	HG004611	HE805525			Periplocoideae
Schubertia grandiflora Mart.	HG004622	AJ428827	Astephaninae	Asclepiadeae	Asclepiadoideae
Secamone alpini Schult.	LR027095	HE805519			Secamonoideae
Secamone elliptica R. Br.	GU901389	EF456116			Secamonoideae
Secondatia densiflora A. DC.	GU901339	EF456228		Odontadenieae	Apocynoideae
Sindechites chinensis Oliv. & Tsiang:	GU901393	EF456244	Amphineuriinae	Apocyneae	Apocynoideae
Skytanthus acutus Meyen	HG004627	AF214269	Thevetiinae	Plumerieae	Rauvolfioideae
Staphanotis floribunda Brongn.	HG004634	HE805517		Marsdenieae	Asclepiadoideae
Stenostelma corniculatum (E. Mey.) Bullock	HG004639	AY163722	Asclepiadinae	Asclepiadeae	Asclepiadoideae
Stipecoma peltigera Müll. Arg.	GU901394	EF456193		Echiteae	Apocynoideae
Tabernaemonta divericata (L.) R. Br. exRoem.Schult		AF214399		Tabernaemontaneae	Rauvolfioideae

Species Name	*PHYA*	*trnL-F*	Subtribe	Tribe	Subfamily
Temnadenia odorifera (Vell.) J.F. Morales:	GU901373	EF456179		Echiteae	Apocynoideae
Thevetia peruviana (Pers.) K. Schum.	LR027097		Thevetiinae	Plumerieae	Rauvolfioideae
Thyrsanthella difformis (Walter) Pichon	GU901391	EF456177		Odontadenieae	Apocynoideae
Toxocarpus villosus(Blume) Decne	GU901399	EF456117			Secamonoideae
Trachelospermum jasminoides (Lindl.) Lem.	HG004630	HE805531	Chonemorphinae	Apocyneae	Apocynoideae
Tylophora hirsuta (Wall.) Wight		HE805515	Tylophorinae	Asclepiadeae	Asclepiadoideae
Urceola lucida Benth. & Hook. f.	GU901400	EF456226	Urceolinae	Apocyneae	Apocynoideae
Vallaris solanacea (Roth) *O. Kuntze*	GU901401	EF456162	Beaumontiinae	Apocyneae	Apocynoideae
Vinca major L.	LR028005	HE805541	Vincinae	Vinceae	Rauvolfioideae
Wattakaka volubilis (Linn.f.) Stapf.	HF969011	HE805516		Marsdenieae	Asclepiadoideae
Xysmalobium parviflorum Harv. ex Scott-Elliot	HG004638	AM295674	Asclepiadinae	Asclepiadeae	Asclepiadoideae
Zygostelma benthamii Baill	GU901404	EF456109			Periplocoideae

3. RESULTS

3.1. Incongruence

As mentioned above, well-supported clades incongruent between the Bayesian and parsimony results were not observed. In some cases, *trnL-F* provided higher support for certain clades than did PHYA, but in other cases the reverse was true. Overall resolution produced by *trnL-F* for both Bayesian and parsimony analyses was lower than for PHYA. We will not describe the results of the separate analyses (they are highly similar), but we do include figures here for comparison (Supplementary data (Figures 1a, 1b, 2a, 2b)); we confine our discussion to only the combined results because the individual gene trees are congruent and the combined results are better resolved and have higher support.

3.2. Combined trnL-F *and PHYA analyses*

The dataset comprises 112 taxa and 2325 characters, of which 1400 are contributed by PHYA and 975 from *trnL-F*. In the parsimony analysis, 701 characters (479 from PHYA and 222 from *trnL-F*) proved to be parsimony informative. Analysis produced 13960 equally most-parsimonious trees with 3284 steps and a consistency index of 0.49 and retention index of 0.71. Mr Modeltest indicated that the best fit model was a general time reversible model with an alpha parameter for the shape of the gamma distribution to account for rate heterogeneity among sites (GTR+G+I). A burn in period of 2 10^6 generations per run was removed. The Bayesian tree (Figure 2) generally depicts more resolved groups as compared to the parsimony tree (Figure 1). Rauvolfioideae and Apocynoideae are non-monophyletic, but the subfamilies of the traditional Asclepiadaceae are strongly supported. The APSA clade receives high support (BP 99; PP 1.0), and Wrightieae emerge as sister to the rest of the clade.

In Rauvolfioideae, resolution of groups is low in both analyses. Monophyly of Plumerieae receives low support in both analyses (BP 59; PP 0.93), whereas Vinceae are paraphyletic in the MP analysis and poorly supported in the Bayesian tree (PP 0.83). In both analyses *Tabernaemontana* falls with Vinceae, whereas Hunterieae cluster with the *Amsonia-Rhazya* clade (BP 100; PP 1.0), but this relationship is not well supported. The position of Carisseae is found here as sister to the APSA clade with weak support (BP 59; PP 0.91).

APSA is well supported (BP 100; PP 1.0). *Rhabdadenia*, the only genus of Rhabdadenieae, forms a weakly supported clade with members of Malouetieae in the MP analysis (Figure 1), whereas in the Bayesian tree *Rhabdadenia* appears elsewhere (Figure 2). Periplocoideae receive good support in both analyses (BP 100; PP 1.0), but they are embedded in Apocynoideae. The clade comprising Odontadenieae, Mesechiteae, Echiteae and Apocyneae (all Apocynoideae) is well supported (BP 99; PP 1.0).

Baisseeae (sensu Endress et al. 2007a) are supported (BP 99; PP1.0) with *Dewevrella* as their sister. This clade forms a strongly supported sister to the milkweeds in the Bayesian tree (PP 1.0) and is relatively less well supported in the parsimony tree (BP 78).

The Secamonoideae-Asclepiadoideae clade receives strong support in both analyses (BP 100; PP 1.0), and the position of Fockeeae as sister to the rest is confirmed (BP 100; PP1.0). Members of Ceropegieae form a well-supported clade (BP 99; PP 1.0) in Asclepiadoideae. The monophyly of Marsdenieae receives strong support only in the Bayesian analysis (PP 0.99; BP 78). The close relationship between these tribes receives strong support also only in the Bayesian tree (PP 1.0; BP 53).

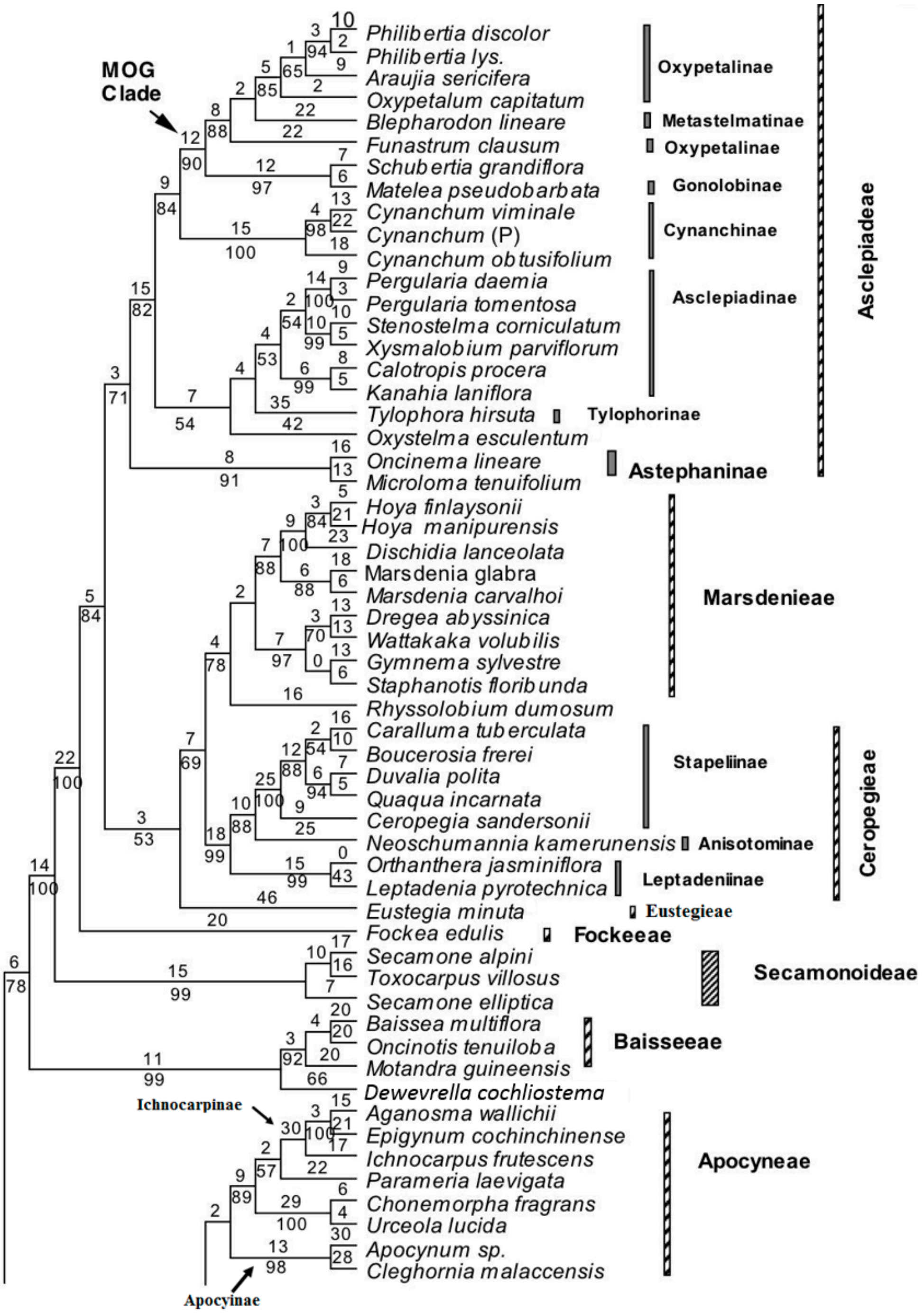

Fig. 1. One of the most parsimonious trees for Apocynaceae based on sequences of the combined dataset (PHYA and *trnL-F*). Bootstrap percentages > 50 and consistent with the strict consensus tree are indicated below branches. *Cynanchum* (P) = *Cynanchum jacquemontianum*.

Eustegia of the new tribe Eustegieae is recovered as sister to the combined Marsdenieae-Ceropegieae clade (BP 53; PP 1.0). The major clades in Asclepiadeae receive strong support in the Bayesian analysis, whereas resolution is relatively poor in the parsimony analysis. Subtribe Astephaninae (PP 1.0) is sister to rest of Asclepiadeae. The informally named ACT clade is not recovered here due to the position of Cynanchineae (Figures 1, 2).

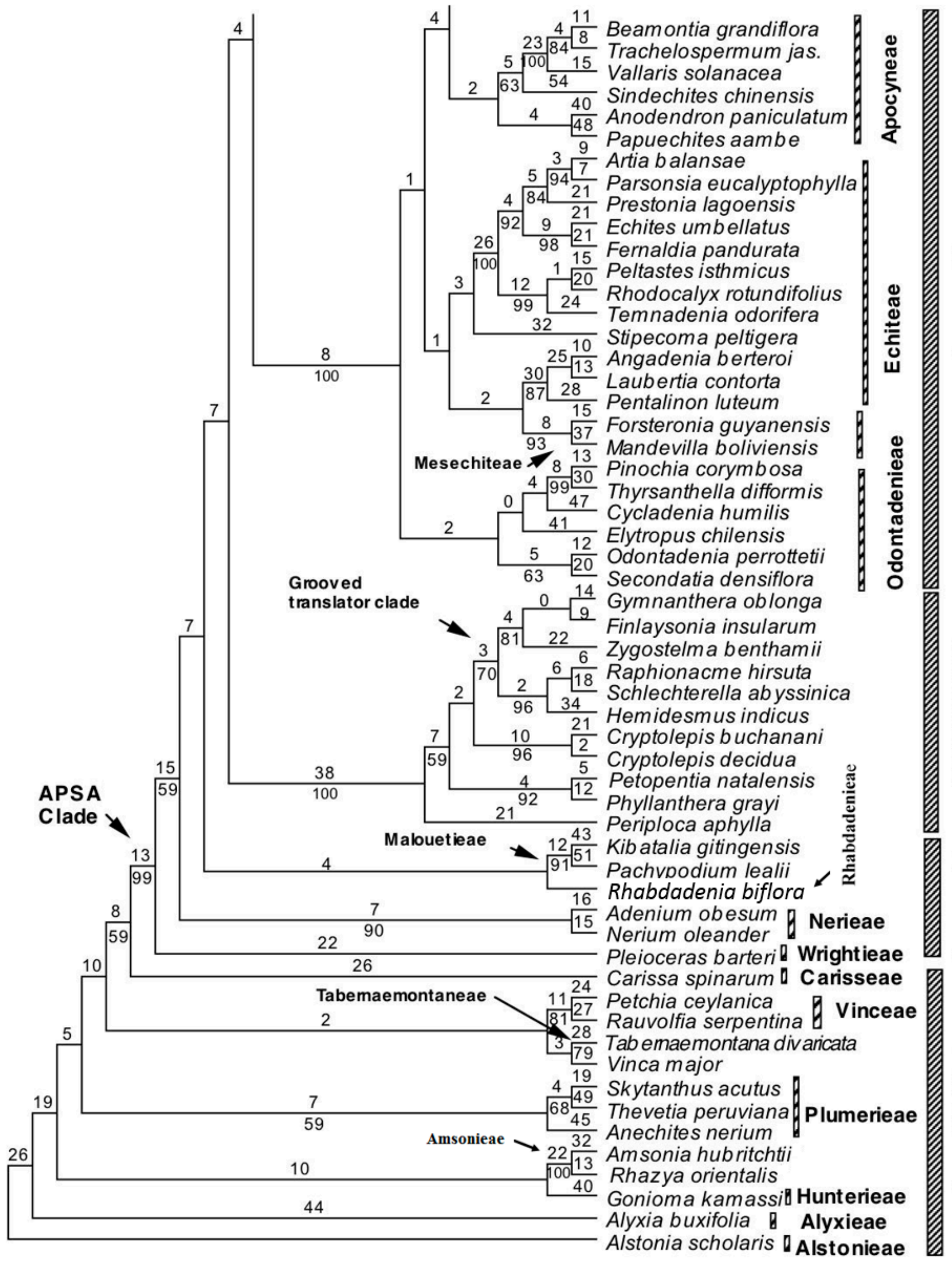

Fig. 1. (Continued).

Asclepiadineae and Tylophorinae form a well-supported clade (the AT clade) with *Oxystelma* as their sister (PP 1.0). Cynanchineae here comprise just the Old World genus *Cynanchum*, which forms a strongly supported clade sister to the MOG clade (BP 84; PP 1.0).

The MOG clade (all New World) receives strong support only in the Bayesian tree (PP 1.0). Within MOG, Gonolobineae are monophyletic (PP 1.0). *Blepharodon* (Metastelmatinae) is weakly supported (PP 0.61) as sister to *Funastrum* (Oxypetalinae), resulting in Oxypetalinae not being monophyletic. Within Oxypetalinae, *Araujia*, *Philibertia* and *Oxypetalum* form a well-supported clade (PP 1.0).

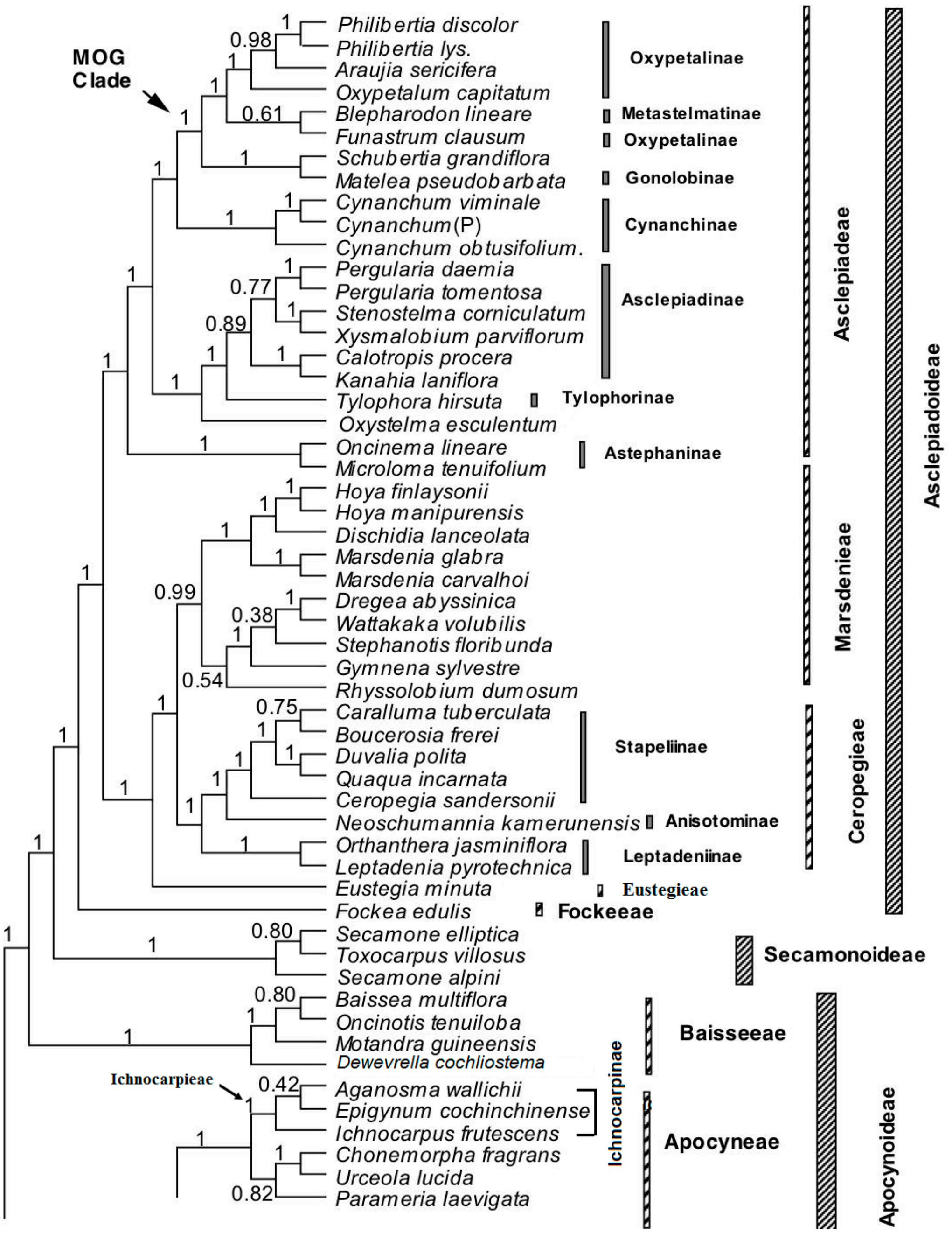

Fig 2. Bayesian analysis of Apocynaceae using combined datasets (PHYA and *trnL-F*). Posterior probabilities are shown along branches. *Cynanchum* (P) = *Cynanchum jacquemontianum*.

4. DISCUSSION

In the separate Bayesian and MP analyses, well-supported incongruent nodes are not observed, which was also reported by Livshultz (2010). Relationships are better supported in the combined results (Figures 1, 2) compared to the separate *trnL-F* and PHYA trees. Here, we confine our discussion of results to the combined analyses.

These results are broadly congruent with previously published phylogenetic studies of Apocynaceae (Livshultz et al. 2007; Simões et al. 2007; Endress et al. 2007a), and like these both subfamilies of Apocynaceae sensu stricto are not resolved as monophyletic. On the

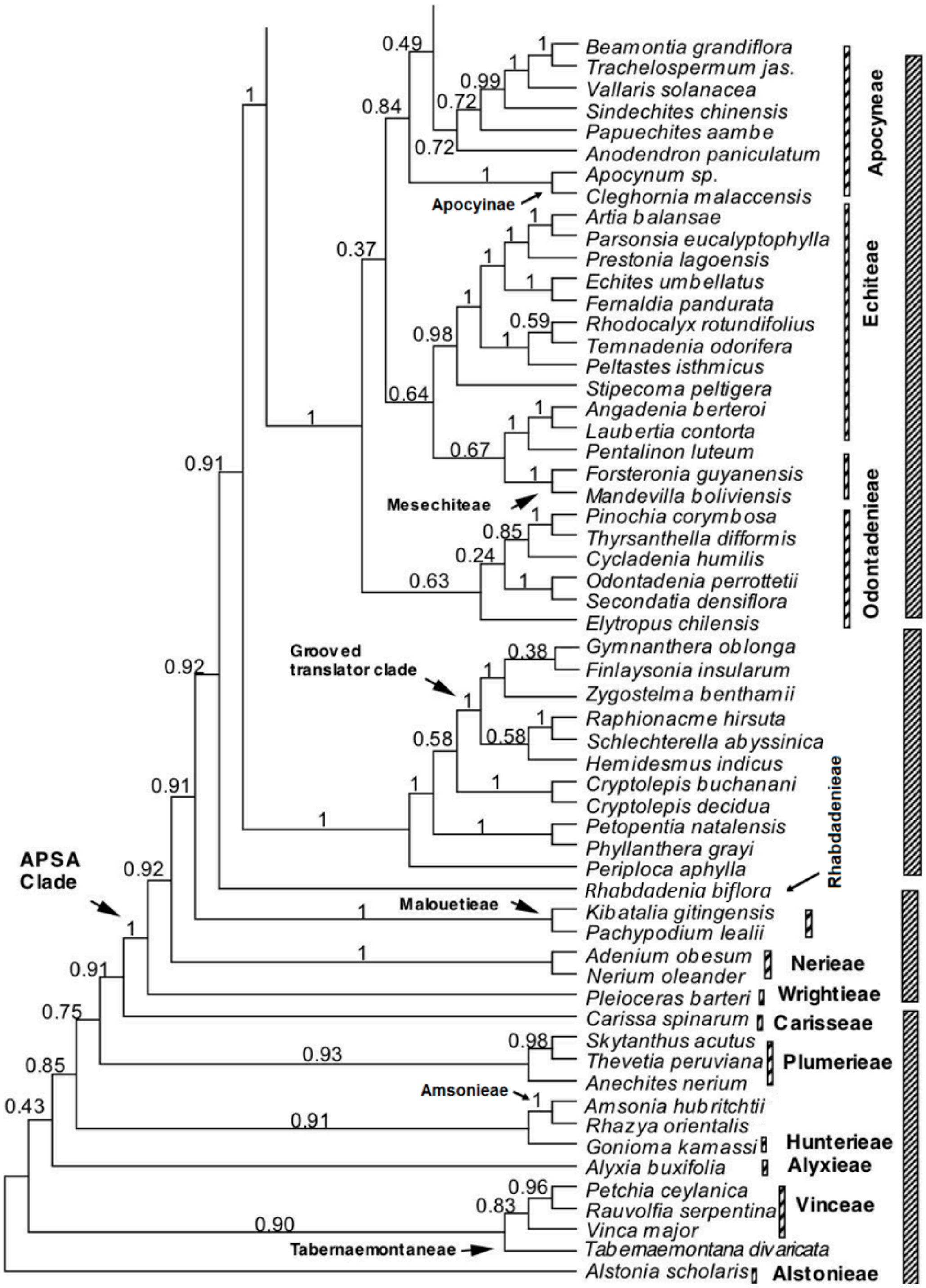

Fig. 2. (Continued).

basis of evidence from previous studies (Sennblad and Bremer 2002; Simões et al. 2004 and 2007), members of Alstonieae are sister to the rest of Apocynaceae. In our study, *Alstonia* was designated as the outgroup (Figures 1, 2). The separate position of *Amsonia* and *Rhazya* from the rest of Vinceae is in agreement with earlier DNA studies (Potgieter and Albert 2001; Endress et al. 2007b; Simões et al. 2007). However, a floral study conducted by Endress et al. (2007b) on *Amsonia* and *Rhazya* suggested that these two genera are more similar to *Catharanthus* and *Vinca*, but our results place the former pair with Hunterieae. Endress and Bruyns (2000) treated *Rhazya* as a synonym of *Amsonia* on the basis of similar fruits, seeds and floral morphology (Pichon 1949; Nilsson 1986), and this relationship is also strongly supported in our study (BP 100; PP 1.0). As in Simões et al. (2007), monophyly of Plumerieae did not receive strong support in our analyses. Carisseae emerge as a sister group to the APSA clade, corresponding with the results of Civeyrel et al. (1998), Potgieter and Albert (2001), Simões et al. (2007) and Livshultz et al. (2007).

4.1 APSA clade

Wrightieae of subfamily Apocynoideae is sister to the rest of the APSA clade as was the case in other phylogenetic analyses of Apocynaceae (Sennblad and Bremer 1996 and 2002; Sennblad et al. 1998; Potgieter and Albert 2001; Livshultz et al. 2007; Livshultz 2010).

A strongly supported clade termed as the 'crown clade' by Livshultz et al. (2007) received less support in our Bayesian tree (PP 0.92) and is weakly supported in our MP analysis as compared to Livshultz et al. (2007) and Livshultz (2010). However the moderately supported (PP 0.91) sister-group relationship of Malouetieae with the crown clade, as illustrated in recent studies (Livshultz et al. 2007; Livshultz 2010), is also confirmed in our analyses. *Pachypodium* has traditionally been included in Echiteae (Pichon 1950), but Endress and Bruyns (2000) transferred this genus into Malouetieae, and this change was supported by Livshultz et al. (2007) and our study.

Old World Apocyneae form a well-supported clade with the New World tribes (Odontadenieae, Echiteae and Mesechiteae) of Apocynoideae in both analyses. In a recent phylogenetic analysis (Livshultz 2010), this clade received less support: BP 68 compared to BP 100/ PP 1.0 here. Monophyly of Apocyneae is not supported by the MP analysis as compared to 100 BP in Livshultz (2010), but in the Bayesian tree they receive low support (PP 0.84). Our sampling of more taxa may be responsible for the shift in support observed in our results relative to those of Livshultz (2010). The topology in Apocyneae is somewhat inconsistent with that in Livshultz (2010), only by adding *Trachelospermum*, a basal clade (PP 0.72) emerges comprising of *Beaumontia*, *Trachelospermum*, *Vallaris*, *Sindechites*, *Papuechites* and *Anodendron*. In previous phylogenetic studies (Potgieter and Albert 2001; Sennblad and Bremer 2002; Simões et al. 2004 and 2007) *Beaumontia* and *Trachelospermum* form a clade with *Chonemorpha*, but here *Chonemorpha* from subtribe Chonemorphinae is sister to *Urceola* from subtribe Urceolinae (BP 100; PP 1.0; Figures 1, 2). In the present study, only two subtribes Apocyinae and Ichnocarpinae of the tribe Apocyneae described in updated classification of Apocynaceae (Endress et al., 2014) appeared monophyletic.

New World Apocynoideae (Echiteae, Mesechiteae and Odontadenieae) do not form a well-supported clade in our analyses as observed by Livshultz et al. (2007) and Livshultz (2010). In our analyses we did not add more taxa to the New World Apocynoideae group. Therefore intergeneric relationships of New World Apocynoideae are similar to those observed in the studies of Livshultz (2010) (Figures 1, 2). Endress et al. (2014) recently described subtribe Rhabdadenieae, which is sister to the crown clade (as a separate clade in the Bayesian analysis and with members of Malouetieae in MP) as observed by Livshultz (2010).

4.2 Baisseeae (African clade)

Endress et al. (2007a) defined a new tribe Baisseeae comprising three African genera – *Baissea*, *Oncinotis* and *Motandra* – and Livshultz et al. (2007) stated that Baisseeae are sister to the milkweeds rather than subfamily Periplocoideae. This relationship was originally suggested by Macfarlane (1933) on the basis of their geography (Livshultz et al. 2007). In previous phylogenetic analyses, this relationship has frequently been noted, but with weak support (Sennblad et al. 1998; Potgieter and Albert 2001; Sennblad and Bremer 2002) and more recently with stronger support (Lahaye et al. 2007; Livshultz et al. 2007; Simões et al. 2007). In our analysis, this sister relationship of Baisseeae receives strong support in the Bayesian analysis (PP 1.0) and comparatively weak bootstrap support (BP 78). In contrast, tetrad bearing Periplocoideae are most closely related to pollinium-bearing milkweeds, and Baisseae in the present study and previous molecular studies (Sennblad and Bremer, 2000; Livshultz et al. 2007) received strong support as sister to the pollinium-bearing milkweeds. In the classification of Endress and Bruyns (2000), *Baissea* and *Motandra* are grouped with *Prestonia* and *Cycladenia* on the basis of corona characters (particularly finger-like projections above the stamens).

In molecular phylogenetic analyses *Prestonia* forms a group with the 'core Echiteae', and *Baissea* and *Motandra* form a separate clade (Baisseeae; Livshultz 2010). Recently, Livshultz et al. (2007) identified these genera as having colleters on the adaxial surface of their petiole (rarely extending onto the base). However, this character is shared by *Farquharia* (Malouetieae), *Isonema* and *Nerium* (Nerieae). Therefore, morphologically, the African clade still needs additional characters to justify its separate tribal identity as the sister group of the milkweeds.

4.3 Periplocoideae

In both these analyses (Bayesian and MP), the position of Periplocoideae in Apocynoideae differs from the analyses of Livshultz (2010). However, this result has been observed in other previous studies (Sennblad and Bremer 2000; Potgieter and Albert 2001; Livshultz et al. 2007; Livshultz 2010). On the basis of floral morphology (Table 3), the subfamily is regarded as an intermediate stage in a transition series between characters typical of Apocynoideae and those of milkweeds (Demeter 1922; Safwat 1962; Cronquist 1981; Rosatti 1989; Endress 1994, 2001 and 2004; Endress and Bruyns 2000; Wyatt et al. 2000). *Apocynum* has pollen in tetrads with simple translators, which is frequently considered to be the first stage in this series (Demeter 1922; Safwat 1962; Nilsson et al. 1993 and also cited by Livshultz et al. 2007). This is followed by pollen in tetrads with spoon-shaped translators in some Periplocoideae and then further aggregation leading to a pollinia in some Periplocoideae (Nilsson et al. 1993; Verhoeven and Venter 1998; Livshultz et al. 2007). Therefore, Periplocoideae as sister to the milkweeds is a common concept in the literature, but results of phylogenetic analyses have shown that Periplocoideae are more closely related to Apocynaceae sensu stricto; instead, Baisseeae are the sister of the milkweeds (Kunze 1996; Judd et al. 1994; Struwe et al. 1994; Sennblad and Bremer 1996; Endress 1997; Sennblad 1997; Potgieter and Albert 2001; Sennblad and Bremer 2002; Livshultz et al. 2007). Pollen in tetrads and pollinia have evolved in parallel in the APSA clade (Livshultz et al. 2007).

In this analysis, Periplocoideae are well supported (BP 100; PP 1.0) as observed in Livshultz et al. (2007) and Livshultz (2010). The grooved translator clade described by Ionta and Judd (2007) is also well supported in the Bayesian tree (PP 1.0) and receives relatively less support in the MP analysis (BP 70). These results show *Periploca* (the type genus of subfamily Periplocoideae) is sister to the rest of the subfamily, which can be contrasted with the findings of Ionta and Judd (2007), in which *Phyllanthera* is sister to the rest of Periplocoideae. Note that *Phyllanthera* is sister to *Petopentia* (BP 92; PP 1.0) with these data.

4.4 Asclepiadoideae-Secamonoideae (milkweed clade)

Secamonoideae have commonly been observed as sister of Asclepiadoideae (Sennblad and Bremer 1996, 2000 and 2002; Civeyrel et al. 1998; Civeyrel and Rowe

Table 3. Key morphological characters in subfamilies of family Apocynaceae.

Subfamily	Key Characters	Reference
Rauvolfioideae	Corolla with sinistrorse aestivation in bud; anthers free from style head; staminal filaments free; sclerified anther wings absent; pollen granular; stylar head secretions not differentiated; fruit a berry drupe or follicle; seeds lacking a coma	Sennblad (1997); Endress and Bruyns (2000)
Apocynoideae	Corolla with dextrorse aestivation in bud; anthers adnate to style head; staminal filaments free; sclerified anther wings absent; pollen granular; stylar head secretions not differentiated; fruit a follicle; seeds comose	Endress et al. (1996); Endress and Bruyns (2000)
Periplocoideae	Corolla with dextrorse to valvate aestivation in bud; anthers adnate to style head; staminal filaments free; sclerified anther wings absent; pollen in tetrads, sometimes clumped into pollinia lacking waxy coating; stylar head secretions forming spoonlike translators with sticky basal viscidium; pollinia if present 4 per translator; fruit a follicle; seeds comose	Verhoeven and Venter (1998); Endress and Bruyns (2000); Goyder et al. (2012)
Secamonoideae	Corolla with dextrorse or sinistrorse to valvate aestivation in bud; anthers and style head fused to form gynostegium; staminal filaments fused into a tube; sclerified anther wings present; pollen in tetrads clumped into pollinia lacking waxy coating; stylar head secretions differentiated into pale soft translator lacking clearly structured translator arms (pollinia fused directly to corpusculum or on short stalks); pollinarium with 4(-5) pollinia; fruit a follicle; seeds comose	Civeyral (1996); Verhoeven and Venter (1998); Endress and Bruyns (2000); Goyder et al. (2012)
Asclepiadoideae	Corolla with dextrorse to valvate aestivation in bud; anthers and style head fused to form gynostegium; staminal filaments fused into a tube; sclerified anther wings present; pollen in tetrads clumped into pollinia encased in waxy coating; stylar head secretions differentiated into dark hard translator with translator arms (pollinia (mostly) linked to corpusculum via variously structured translator arms); pollinarium with 2 pollinia; fruit a follicle; seeds comose	Klackenberg (1995b); Civeyral (1996); Endress and Bruyns (2000); Goyder et al. (2012)

2001; Fishbein 2001; Potgieter and Albert 2001; Lahaye et al. 2005 and 2007; Livshultz et al. 2007). In our study, this clade receives strong support (BP 100; PP 1.0). Although not broadly sampled here, the included taxa confirm monophyly of Secamonoideae with high support (BP 99; PP 1.0) *Secamone* is not recovered here as monophyletic, which is congruent with the results of Lahaye et al. (2007).

Asclepiadoideae, the largest subfamily of Apocynaceae, comprises ~3000 species distributed worldwide (Goyder, 2006). Currently, five tribes are recognized in the subfamily: Fockeeae, Ceropegieae, Marsdenieae,Asclepiadeae (Endress et al., 2007a) and Eustegieae (Endress et al., 2014). The position here for Fockeeae is consistent with previous analyses (Civeyrel et al. 1998; Fishbein 2001; Potgieter and Albert 2001; Rapini et al. 2003; Livshultz et al. 2007; Livshultz 2010). *Eustegia* is a monotypic genus with pendent pollinia, placed in to separate tribe of Asclepiadoideae (Goyder 2006), but phylogenetic studies based on plastid markers (Liede 2001; Rapini et al. 2003; Goyder et al. 2007) have placed *Eustegia* sister to the Marsdenieae-Ceropegieae clade, a result confirmed by our results

4.5 Ceropegieae-Marsdenieae clade

Meve and Liede (2004) recognized four subtribes in Ceropegieae based on anatomical characters: Anisotominae, Heterostemminae, Leptadeniinae and Stapeliinae. In our study Leptadeniinae are sister to the rest of Ceropegieae. Stapeliinae receive strong support (BP 100; PP 1.0), and Anisotominae are sister to Stapeliinae with strong support in the Bayesian analysis (PP 1.0) and moderate support in the parsimony analysis (BP 88).

Both subtribes have overlapping morphological features (Meve 1995; Meve and Liede 2001a, 2001b and 2004). The *Hoya*/*Dischidia* group forms a well-supported subclade in both analyses (BP 100; PP 1.0) and along with members of the genus *Marsdenia* they receive strong support in the Bayesian analysis (PP 1.0) and moderate MP support (BP 88). The association of *Hoya* and *Dischidia* has previously been supported by Potgieter and Albert (2001), Livshultz (2002 and 2003), Rapini et al. (2003), Meve and Liede (2004) and Wanntorp et al. (2006a and 2006b). There is little molecular phylogenetic data available for Marsdenieae; however, recently a few studies have focused on *Hoya* (Wanntorp and Forster 2007; Wanntorp and Kunz 2009; Wanntrop et al. 2011).

Another well-supported subclade (BP 97; PP 1.0) in Marsdenieae is comprised of *Dregea*, *Gymnema*, *Stephanotis* and *Wattakaka*. However, the position of *Rhyssolobium* seems unclear in both analyses. In the Bayesian analysis this genus is sister to the subclade that is sister to the rest, whereas with MP it is sister to other members of Marsdenieae; in both analyses, the position of this genus is poorly supported. This result is congruent with Meve and Liede (2004) and Wanntorp et al. (2006a).

Monophyly of Ceropegieae-Marsdenieae (which possess erect pollinia, regarded as a primitive condition in Asclepiadoideae; Kunz, 1993) is well supported in Bayesian analysis (PP 1.0). In earlier studies (Orbigny, 1843; Decaisne, 1844) Ceropegieae and Marsdenieae sensu Endress and Bruyns (2000) were considered a single entity.

However Endress and Bruyns (2000) treated Marsdenieae and Ceropegieae as two tribes, due to the lack of hyaline insertion crest on outer surface of pollinium and absence of an outer corona and milky latex in former (Bruyns and Forster 1991; Omlor 1998; Meve and Liede 2004). However, Swarupanandan et al. (1996) again united these two tribes, and this idea was later supported by molecular phylogenetic analyses (Potgieter and Albert 2001; Rapini et al. 2003; Meve and Liede 2004). Both tribes have also been observed to have the lowest level of polyploidy compared to other member of Asclepiadoideae (Albers and Meve, 2001).

4.6 Asclepiadeae

Asclepiadeae, the largest tribe of Asclepiadoideae having pendent pollinia (Table 3) and reduced chromosome number ($x=10$, $x=9$) from basic number ($x=11$) (Albers and Meve, 2001), are recovered here as monophyletic. The African genus *Eustegia* appearing as sister to the Ceropegieae-Marsdenieae clade is now recognized as separate tribe Eustegieae in Asclepiadoideae (Endress et al., 2014). Higher levels of intergeneric resolution in Asclepiadeae are recovered in the Bayesian analysis as compared to parsimony. In a broad overview of Apocynaceae conducted by Rapini et al. (2003), three main clades were defined — Astephaninae comprising of only three genera *Astephanus*, *Microloma* and *Oncinema* sensu Liede (2001), ACTG (Asclepiadinae, Cynanchinae, Tylophorinae and Glossonematinae) and MOG (Metastelmatinae, Oxypetalinae and Gonolobinae). In the present study, *Oncinema* and *Microloma* of Astephaninae are well supported as sister to the rest of Asclepiadeae, a result similar to previous molecular studies (Liede 2001; Rapini et al. 2003; Figures 1, 2). Of the other two clades recovered by Rapini et al. (2003), the MOG clade is resolved as monophyletic, whereas the ACT clade remains non-monophyletic with these data. *Oxystelma* is recovered here as sister to the Asclepiadineae-Tylophorinae clade (AT clade) with strong support in the Bayesian analysis (PP 1.0). *Oxystelma* was among the *incertae sedis* of Asclepiadoideae (Liede and Taüber 2000; Endress et al. 2007a), and previously Liede (1997) included it in Metastelmatinae. In subsequent molecular phylogenetic analyses (e.g., Potgieter and Albert 2001; Liede and Taüber 2002; Liede et al. 2002; Rapini et al. 2003) the genus failed to a form a clade with members of Metastelmatinae. Instead, this genus occupied a position sister to the rest of the AT clade, as also observed here; however, in previous molecular phylogenetic analyses using plastid loci this close relationship was not well-supported. In the updated classification of Apocynaceae by Endress et al. (2014), *Oxystelma* was placed in subtribe Asclepiadineae. Cynanchineae here comprised of only Old World taxa (*Cynanchum viminale*, C. *jacquemontianum* and *C. obtusifolium*) appear as sister of the MOG clade (PP 1.0), which is comprised of members from the New World. However, these results can be contrasted with Rapini et al. (2003) where Cynanchinae are embedded in the ACT clade (but without support).

The MOG clade (New World) is recovered here with high support (PP 1.0) as observed in previous studies (Liede and Taüber 2000, 2002; Rapini et al. 2003; Liede-Schumann et al. 2005; Rapini et al. 2006). *Blepharodon lineare* and *Funastrum clausum* were resolved taxa in the study of Rapini et al. (2006) and appeared as sister to Metastelmatinae and Oxypetalinae, respectively. According to Liede (1997) *Funastrum clausum* was previously included in Metastelmatinae on the basis of morphological characters, but in the most recent classification (Endress et al. 2007a; Endress et al., 2014) and also various molecular studies (Rapini et al. 2006) it is placed in Oxypetalinae. However here in the Bayesian analysis the relationship between *Blepharodon lineare* and *Funastrum clausum* is unclear, but their sister-group position to the rest of Oxypetalinae is well supported (PP 1.0; Figure 2). The MP analysis fails to produce good resolution in the MOG clade. In the present study, *Oxypetalum* is sister to *Araujia-Philbertia*, similar to the result of Rapini et al. (2006). However, in earlier studies (with fewer data) a close relationship between *Philbertia* and *Blepharodon* (Liede and Taüber 2000) or *Philbertia* and *Funastrum* (Rapini et al. 2003) was observed. Gonolobineae receive strong support with these data (BP 97; PP 1.0).

Our study included a low-copy nuclear region and shows better resolution within some key clades in Apocynaceae when compared to previous studies, but the relationships recovered are not in particular markedly divergent from those obtained previously with just plastid data. The present analyses concluded that Rauvolfioideae, Apocynoideae and the traditional Asclepiadaceae are all non-monophyletic groups and that, in contrast, the APSA clade is well supported. The crown clade of Livshultz et al. (2007) and Livshultz (2010) received only moderate support here. Our studies confirm that Periplocoideae are nested within Apocynoideae, in a position comparable to that in Livshultz et al. (2007). Periplocoideae should be placed in Apocynoideae rather than thought of as the sister group of the milkweeds. The sister group relationship between Baisseeae and the milkweeds is also confirmed by our analyses. The ACT clade was not monophyletic, whereas the MOG clade was. Old World Cynanchineae forms a well-supported group within the New World MOG clade.

In the present study support for clades are comparatively better than in studies where plastid regions alone were sequenced. In the future, there is a need to sequence greater numbers of taxa of Apocynaceae to further refine the relationships in the family. There is also a need to be increased field collection of material so that high-quality DNA can be recovered from a wider range of Apocynaceae taxa.

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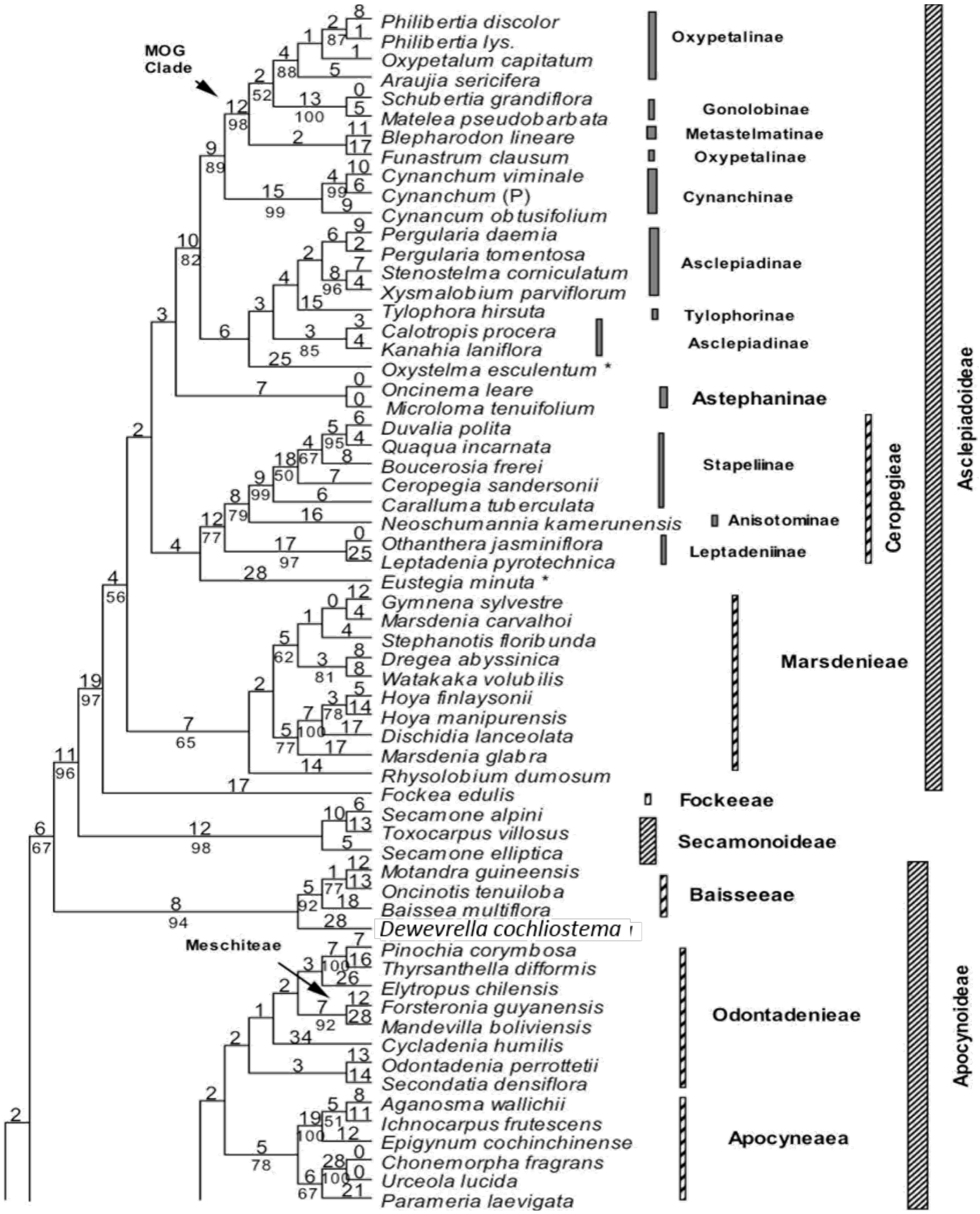
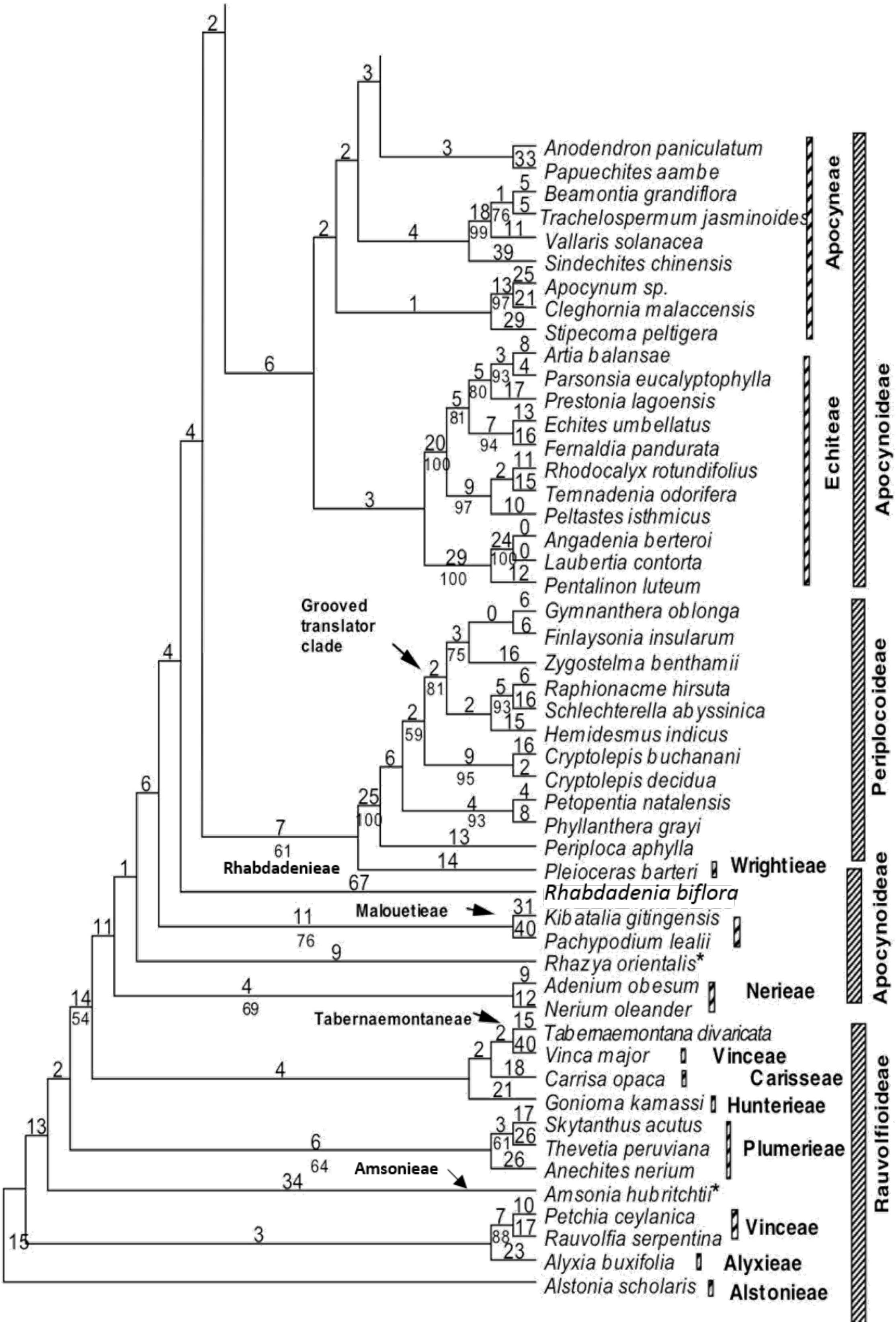


Fig. 1a. Parsimony analysis of only PHYA sequences from Apocynaceae. Bootstrap percentages > 50 and consistent with the strict consensus tree are indicated below branches. *Cynanchum* (P) = *Cynanchum jacquemontianum*.



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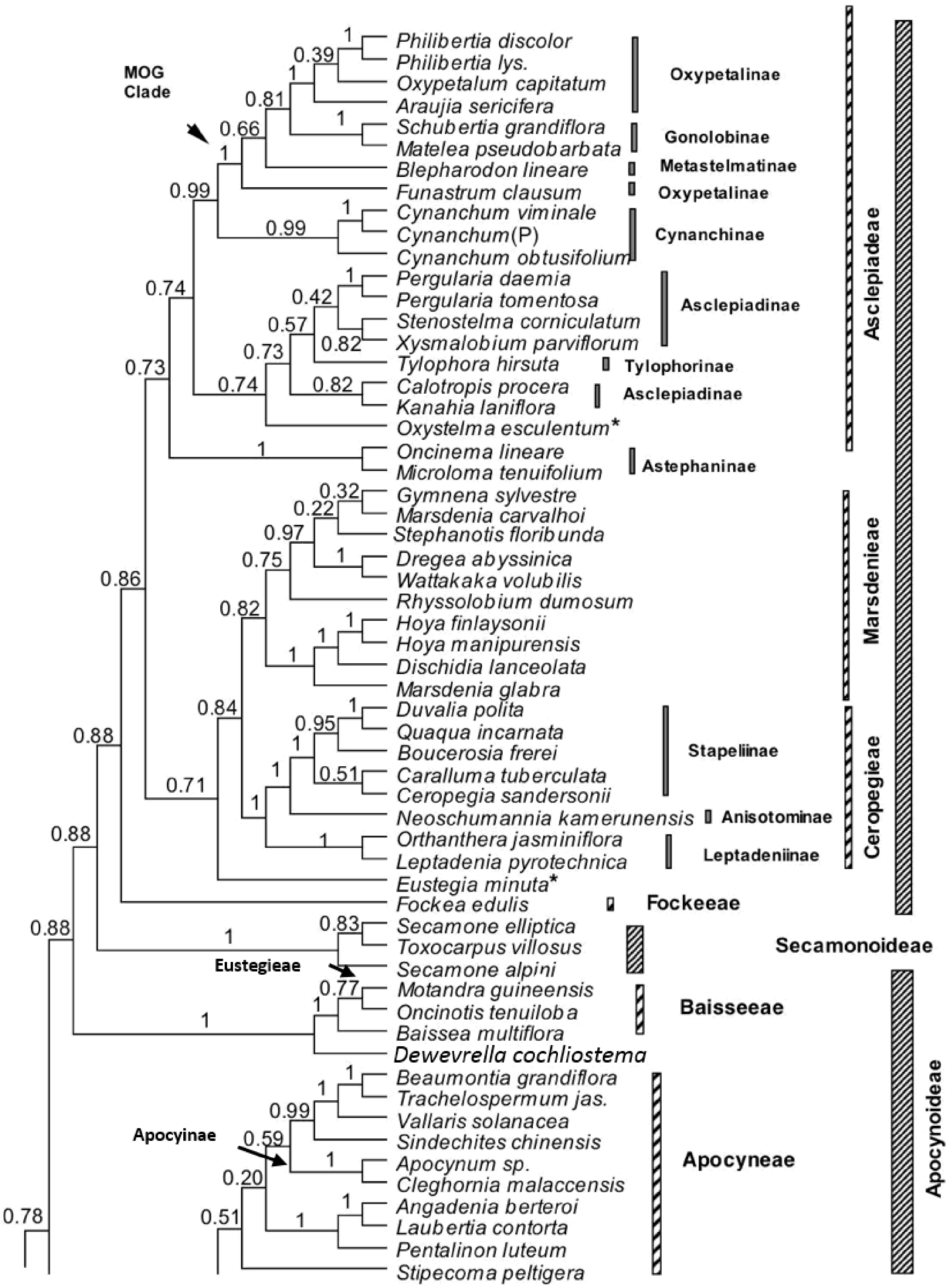

Fig. 1b. Bayesian tree based on only PHYA sequences. PP values are indicated above the branches. Values > 0.95 are considered as strong support. *Cynanchum* (P) = *Cynanchum jacquemontianum*.

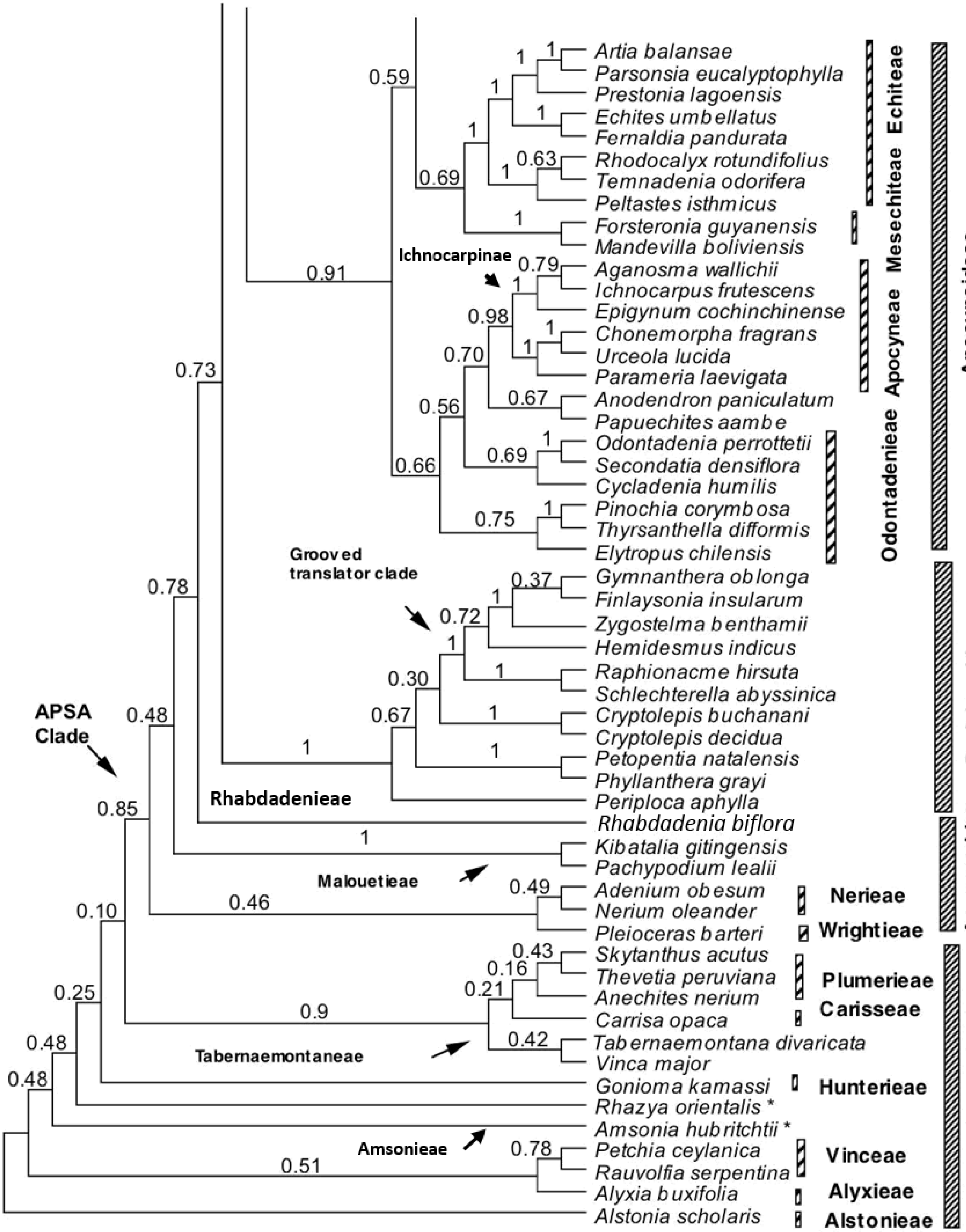

Fig. 1b. (Continued).

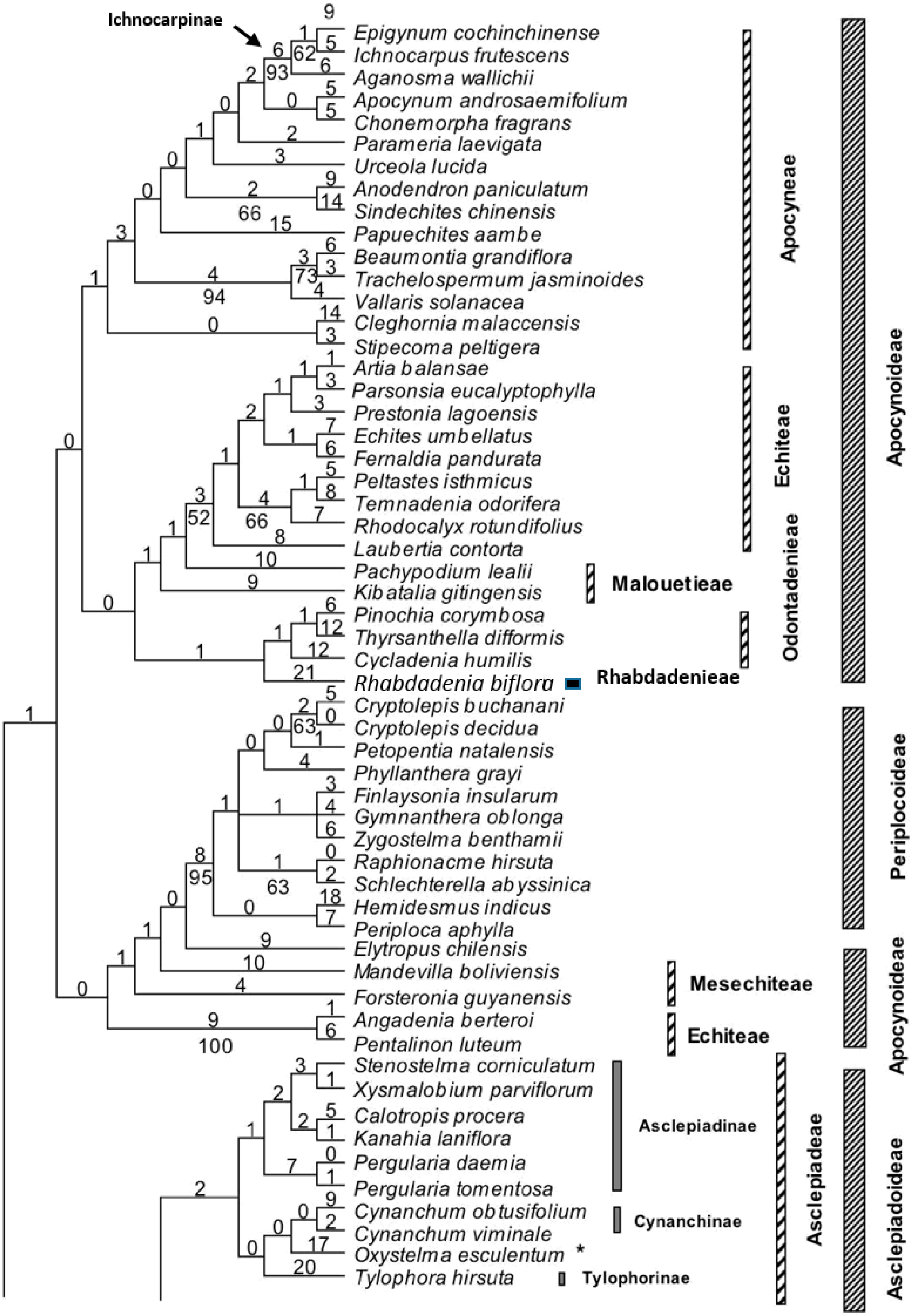

Fig. 2a. Parsimony analysis of *trnL-F* sequences of taxa present in combined analyses. Bootstrap percentages > 50 are indicated below branches. *Cynanchum* (P) = *Cynanchum jacquemontianum*.

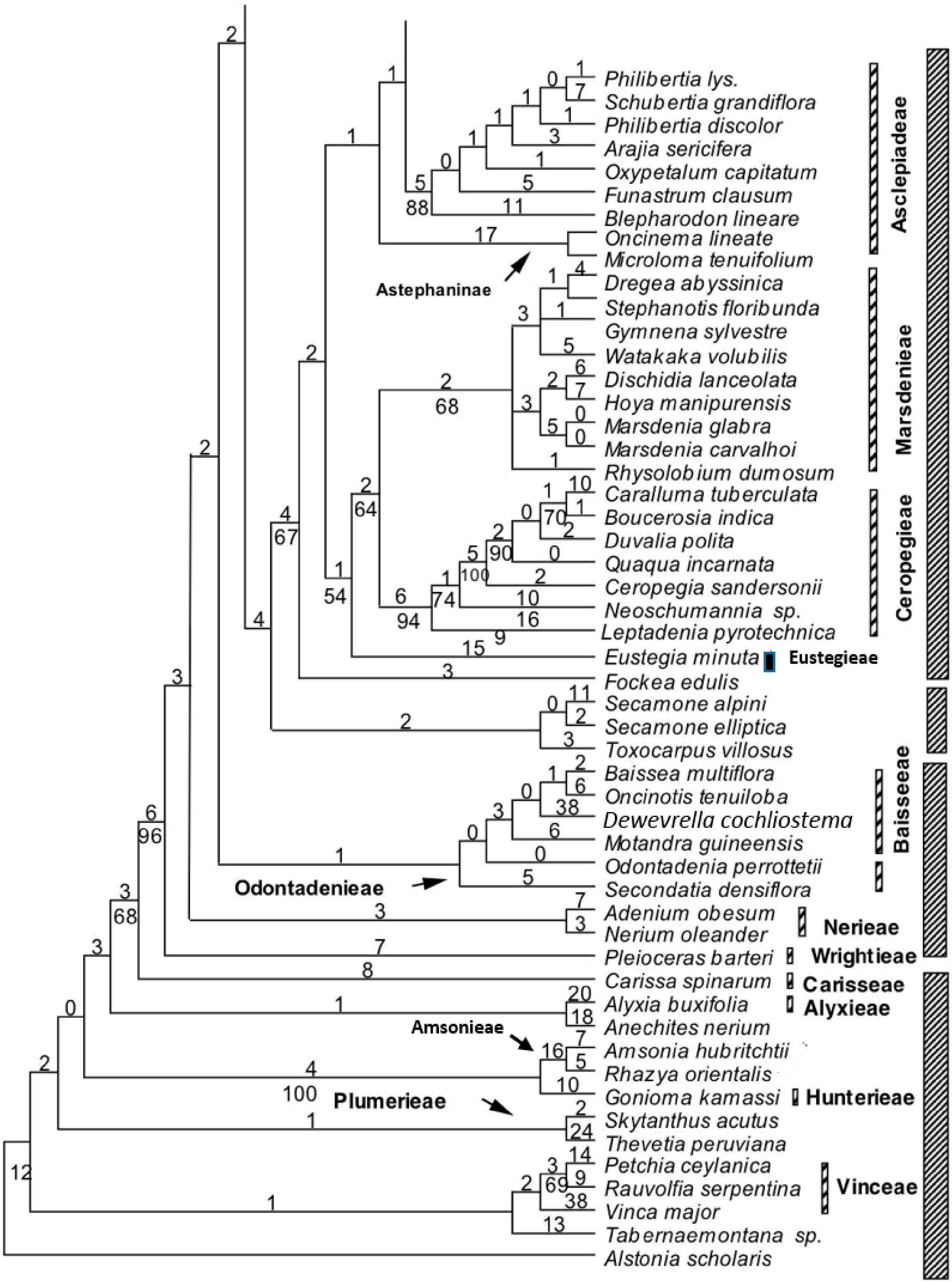

Fig. 2a. (Continued).

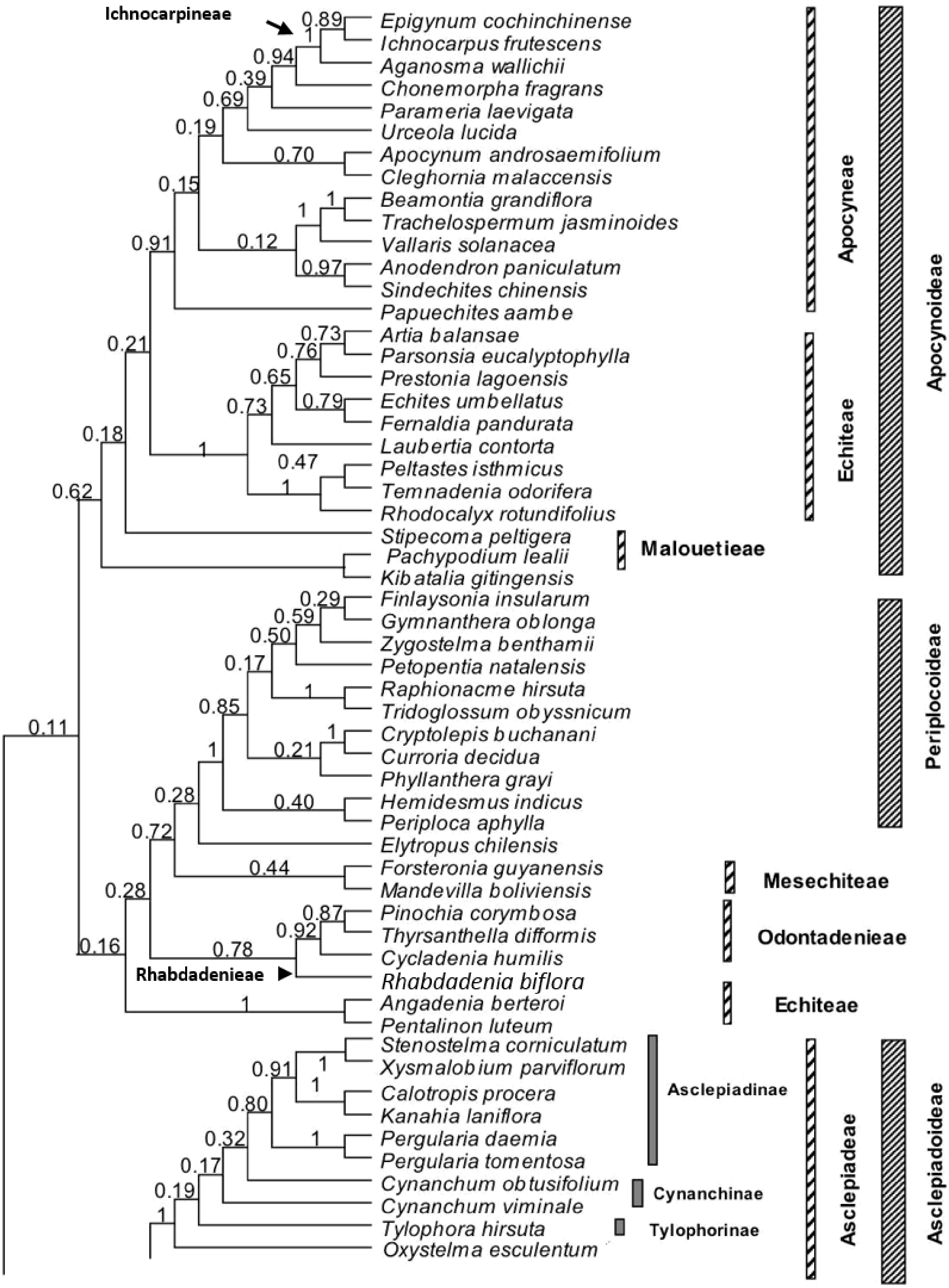

Fig. 2b. Bayesian analysis of *trnL-F* sequences of taxa included in combined analyses. Values > 0.95 are considered as strong support. *Cynanchum* (P) = *Cynanchum jacquemontianum*.

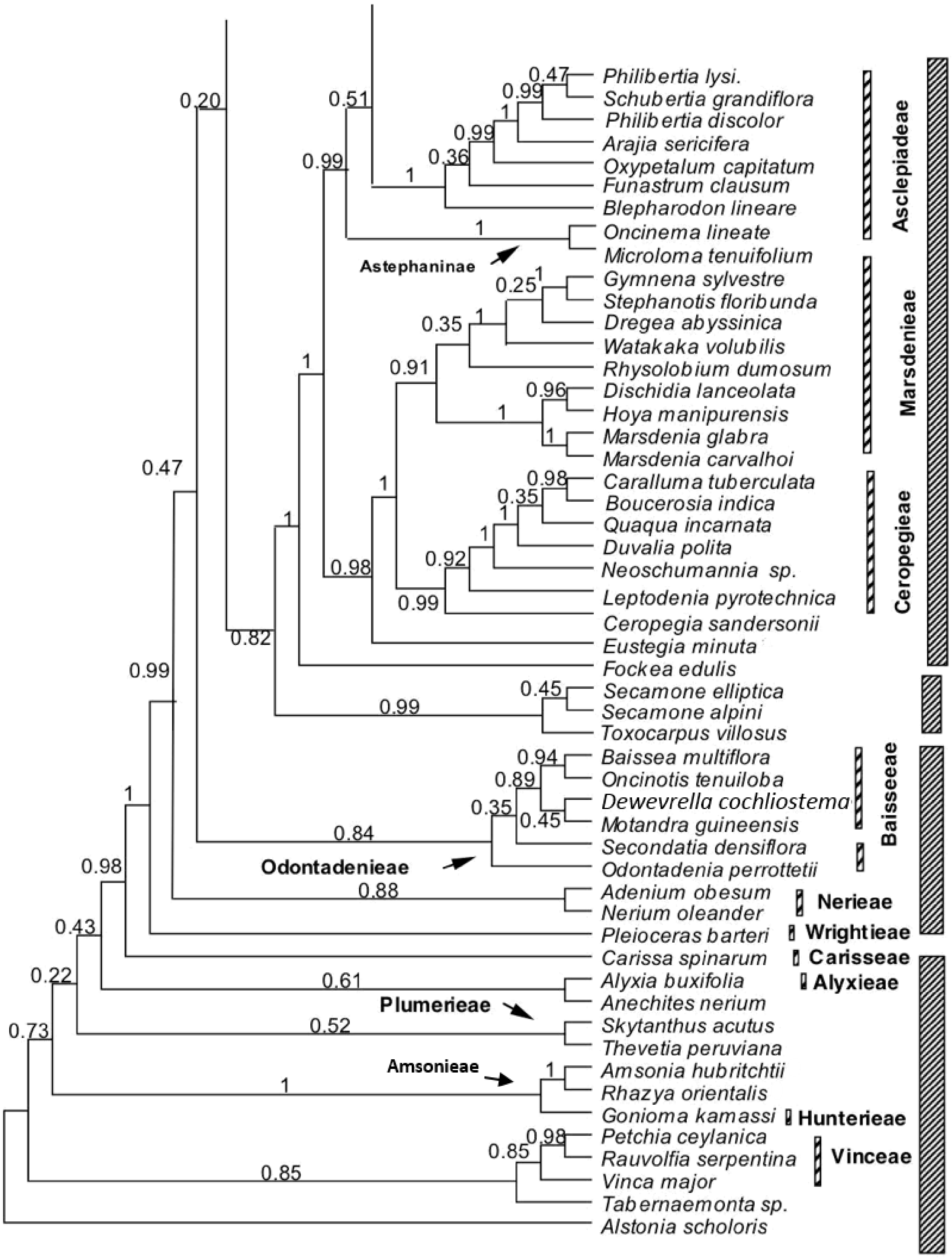

Fig. 2b. (Continued).