CHAPTER III
PHENETIC AND PHYLOGENETIC STUDIES

1. Introduction

The large and widespread tribe Vernonieae (Compositae) are well represented in Thailand. Sixteen genera and 48 species, including many endemic species in *Camchaya* and *Acilepis,* are currently recognized. However, the relationships among these taxa are poorly understood, as are their relationships with Vernonieae outside of Thailand (Robinson, 2007; Keeley & Robinson, 2009). Difficulty in delimiting genera and species and placing them within subtribes is due in large part to the highly variable and overlapping nature of many of the morphological characters on which taxonomic treatments have been based (Koyama, 1984, 1993, 1997, 1998, 2003, 2004, 2005; Robinson, 1999a, 1999b; Keeley *et al.*, 2007; Robinson & Skvarla, 2006, 2007, 2009). Molecular studies using chloroplast and nuclear DNA (cpDNA and nDNA) have overcome many of these limitations, clarifying subtribal relationships, and when coupled with morphology provide clear characters to define genera and species in monophyletic groups (Keeley & collaborators, 1994, 2007, 2009; Robinson 1999a, 1999b; Robinson *et al.*, 2008). In 2007, Keeley *et al.* provided the first phylogeny for the tribe worldwide and clearly demonstrated the monophyly of the Vernonieae. The tribal phylogeny also showed that the Old and New World subtribal lineages were almost entirely distinct from each other with the Old World taxa basal to the New World taxa. The tribe originated in Madagascar/east Africa (Keeley *et al.*, 1994, 2007) as well. Two closely related southeast Asian taxa (*Strobocalyx arborea*, *Tarlmounia elliptica*) did show a cross-hemispheric relationship, however. They were most closely related to a small American group of taxa in subtribes Leiboldiinae and Stokesiinae. For the most part, however, the two hemispheres have distinct and separate evolutionary lines.

Robinson (1999a, 1999b) revised the subtribes and genera of the Old and New World Vernonieae in separate treatments. This work was based on studies of pollen, morphological characters and combined with existing data on secondary chemistry

and chromosome numbers (Keeley & Robinson, 2009). Of greatest significance in Robinson's 1999 treatment was the new concept of the core genus, *Vernonia.* Of the >1000 species originally placed in *Vernonia* ("the *Vernonia* problem" of Bremer, 1994) fewer than 30 species remain. The genus now includes only the New World species, primarily from eastern North America where the type species, *V. noveboracensis*, is found (Robinson, 1999b), plus a small number of species in Mexico and South America (Keeley & Robinson, 2009). Since the New World species were much better studied and detailed taxonomic treatments available for most areas it was possible to erect robust new subtribes and genera in this hemisphere. The same is not true for the Old World, however, and many treatments are considered preliminary and subject to change (Robinson, 1999b; 2007, Keeley & Robinson, 2009). Thus even though the name *Vernonia* is still applied to species in Africa, India, Asia and Malaysia they must eventually be better studied and then placed in other genera (Keeley & Robinson, 2009). Since taxonomic treatments of the Old World species are lacking for many areas, however, it may be some time before new generic reassignments can be made with confidence.

It was the purpose of this study to establish a phylogeny for the Vernonieae of Thailand using information from cpDNA and nDNA sequences, morphology and palynology. Morphology and palynology used alone, and sequence data used alone failed to resolve generic and species relationships.

2. Materials and methods

2.1 Phenetic analysis

Twenty-six binary and 20 multistate characters for 42 species were obtained from studies of morphology, micro-hairs and pollen (Table 3.1). At least 15 specimens per taxon were examined. A number of these characters had previously been shown to be phylogenetically informative by Bunwong & Chantaranothai (2008) and thus their results were included in the final data matrix. The data were analyzed using MacClade 4.03 (Maddison & Maddison, 2001) and imported into PAUP* 4.0b10 (Swofford, 2002) for UPGMA cluster analysis.

Table 3.1 Morphological characters and character states included in the study.

No.	Characters and character states
1	Habit; erect herb (0), scandent (1), shrub (2), tree (3)
2	Stem; acauline (0), cauline (1)
3	Stem rib; absent (0), present (1)
4	Stem vestiture; puberulous (0), tomentose (1), villose-pilose (2), sericeous (3)
5	Petiole length; 0-1 mm (0), > 1 mm (1)
6	Leaf shape; ovate or lanceolate (0), obovate or oblanceolate (1), elliptic or oblong (2)
7	Leaf margin; crenate (0), serrate (1), entire or undulate (2)
8	Leaf apex; obtuse or rounded (0), acute or acuminate (1), apiculate or cuspidate (2), caudate or aristate (3)
9	Leaf base; attenuate (0), cuneate or obtuse (1)
10	Leaf texture; coriaceous (0), chartaceous (1)
11	Leaf hair: whip-shaped with long terminal cell; absent (0), present (1)
12	Leaf hair: whip-shaped with short terminal cell; absent (0), present (1)
13	Leaf hair: filiform; absent (0), present (1)
14	Leaf hair: flagellate; absent (0), present (1)
15	Leaf hair: cylindrical; absent (0), present (1)
16	Leaf hair: T-shaped; absent (0), present (1)
17	Vestiture on upper leaf surface; puberulous (0), tomentose (1), villose-pilose (2), sericeous (3), scabrous (4)
18	Vestiture on lower leaf surface; puberulous (0), tomentose (1), villose-pilose (2), sericeous (3), scabrous (4)
19	Gland on upper leaf surface; absent (0), present (1)
20	Gland on lower leaf surface; absent (0), present (1)
21	Capitulescence type; spicate (0), paniculate (1), solitary (2), scapose (3), corymbose (4)
22	Phyllary rows; 1-2 (0), 3-5 (1), > 5 (2)
23	Phyllary arrangement; decussate (0), imbricate (1)
24	The middle phyllary shape; ovate (0), lanceolate (1), oblong (2)
25	The outer and the middle phyllary apex; obtuse (0), acute or acuminate (1), apiculate or aristate (2), spinose (3)
26	The outer and the middle phyllary with reflexed apex; absent (0), present (1)
27	Phyllary margin; ciliate (0), filiferous (1), spinulose (2), entire (3)
28	Phyllary vestiture; puberulous (0), tomentose (1), villose-pilose (2), arachnoid (3), sericeous (4)
29	Capitate gland on phyllaries; absent (0), present (1)
30	Number of floret per a capitulescence; 1-4 (0), 5-30 (1), >30 (2)
31	Corolla symmetry; actinomorphic (0), zygomorphic (1)
33	Corolla hairs; absent (0), present (1)
34	Pollen type; echinate (0), sublophate (1), lophate (2)
35	Pollen furrow; absent (0), present (1)
36	Pollen pore shape; circular (0), semicircular (1)
37	Number of pollen pore; 3 pores (0), 6 pores (1)

Table 3.1 Morphological characters and character states included in the study. (Cont.)

38	Pollen spine length; 0 μm (0), >0-5 μm (1), >5 μm (2)
39	Pollen columella; prominent (0), inconspicuous (1)
40	Pollen micropuncta; absent (0), present (1)
41	Achene shape; turbinate (0), clavate (1), terete (2)
42	Achene hairs; absent (0), present (1)
43	Achene glands; absent (0), present (1)
44	Number of achene rib; 1-9 (0), ≥10 (1)
45	Carpopodium; absent (0), present (1)
46	Pappus; absent (0), present in every floret (1), present in some florets (2)

Table 3.2 Thai Vernonieae sequenced in this study. Collection localities, voucher information and GenBank accession numbers follow each taxon. S. Bunwong's collections are deposited in KKU, QBG and US, those of other collectors in QBG.

Species	Locality (Province)	Voucher information	GenBank Accession Number		
			ITS	ndhF	trnL
Acilepis attenuata (I)	Udon Thani	S. Bunwong 347	HQ158368	HQ158470	HQ158418
A. attenuata (II)	Khon Kaen	S. Bunwong 351	HQ158369	HQ158471	HQ158419
A. attenuata (III)	Loei	S. Bunwong 373	HQ158370	HQ158472	HQ158420
A. attenuata (IV)	Sakon Nakon	S. Bunwong 354	HQ158371	HQ158473	HQ158421
A. chiangdaoensis	Chiang Mai	S. Bunwong 78	-	HQ158474	HQ158422
A. divergens (I)	Chiang Mai	S. Bunwong 366	HQ158372	HQ158475	HQ158423
A. divergens (II)	Ciang Mai	S. Bunwong 377	HQ158373	HQ158476	HQ158424
A. kingii	Chiang Mai	S. Bunwong 77	HQ158374	HQ158477	HQ158425
A. namnaoensis	Chaiyaphum	S. Bunwong 385	HQ158375	HQ158478	HQ158426
A. ngaoensis	Ranong	S. Bunwong 386	HQ158376	HQ158479	HQ158427
A. peguensis	Loei	S. Bunwong 372	HQ158377	HQ158480	HQ158428
A. pseudosutepensis	Tak	S. Bunwong 388	HQ158378	HQ158481	HQ158429
A. saligna	Mae Hong Son	S. Bunwong 357	HQ158379	HQ158482	HQ158430
A. silhetensis	Chiang Mai	S. Bunwong 364	HQ158380	HQ158483	HQ158431
A. sutepensis (I)	Chiang Mai	S. Bunwong 361	HQ158381	HQ158484	HQ158432
A. sutepensis (II)	Chiang Mai	S. Bunwong 367	HQ158382	HQ158485	HQ158433
Camchaya gracilis	Ubon Ratchathani	S. Bunwong 346	HQ158383	HQ158486	HQ158434
C. loloana (I)	Khon Kaen	S. Bunwong 330	HQ158384	HQ158487	HQ158435
C. loloana (II)	Ubon Ratchathani	S. Bunwong 339	HQ158385	HQ158488	HQ158436
C. loloana var. *mukdahanensis* (I)	Mukdahan	S. Bunwong 338	HQ158386	HQ158489	HQ158437
C. loloana var. *mukdahanensis* (II)	Ubon Ratchathani	S. Bunwong 343	HQ158387	HQ158490	HQ158438
C. pentagona	Ubon Ratchathani	S. Bunwong 344	HQ158388	HQ158491	HQ158439
C. spinulifera (I)	Udon Thani	S. Bunwong 327	HQ158389	HQ158492	HQ158440
C. spinulifera (II)	Sakon Nakon	S. Bunwong 332	HQ158390	HQ158493	HQ158441
C. spinulifera (III)	Nong Khai	S. Bunwong 336	HQ158391	HQ158494	HQ158442
C. tenuiflora	Loei	S. Bunwong 348	HQ158392	HQ158495	HQ158443
Camchaya sp.1	Udon Thani	S. Bunwong 328	HQ158393	HQ158496	HQ158444

Table 3.2 Thai Vernonieae sequenced in this study. Collection localities, voucher information and GenBank accession numbers follow each taxon. S. Bunwong's collections are deposited in KKU, QBG and US, those of other collectors in QBG. (Cont.)

Species	Locality (Province)	Voucher information	GenBank Accession Number		
			ITS	ndhF	trnL
Cyanthillium cinereum	Loei	S. Bunwong 350	HQ158470	HQ158497	HQ158445
Cyanthillium hookerianum	Unon Ratchathani	S. Bunwong 341	HQ158471	HQ158498	HQ158446
Decaneuropsis cumingiana	Petchaboon	S. Bunwong 74	HQ158472	HQ158499	HQ158447
D. eberhardtii	Chaiyaphum	S. Bunwong 384	HQ158473	HQ158500	HQ158448
D. garrettiana	Chiang Mai	S. Bunwong 75	HQ158474	HQ158501	HQ158449
Elephantopus mollis	Ubon Ratchathani	S. Bunwong 340	HQ158475	HQ158504	HQ158452
E. scaber (I)	Udon Thani	S. Bunwong 325	HQ158476	HQ158505	HQ158453
E. scaber (II)	Sakon Nakon	S. Bunwong 334	HQ158477	HQ158506	HQ158454
E. scaber var. *penicillatus*	Ubon Ratchathani	S. Bunwong 345	HQ158478	HQ158507	HQ158455
Gymnanthemum cylindriceps	Chiang Mai	S. Bunwong 378	HQ158479	HQ158508	HQ158456
Iodocephalopsis eberhardtii	Chiang Mai	S. Bunwong 335	HQ158480	HQ158509	HQ158457
Koyamasia calcarea	Chiang Mai	P. Suksathan 2847	HQ158481	HQ158510	HQ158458
Kurziella gymnoclada	Khon Kaen	S. Bunwong 391	HQ158407	HQ158511	HQ158459
Monosis volkameriifolia	Chiang Mai	S. Bunwong 362	HQ158408	HQ158512	HQ158460
Pseudelephantopus spicatus (I)	Ubon Ratchathani	S. Bunwong 342	HQ158409	HQ158513	HQ158461
P. spicatus (II)	Chiang Rai	S. Bunwong 352	HQ158410	HQ158514	HQ158462
Strobocalyx arborea	Loei	M. Norsangsri 1052	HQ158411	HQ158515	HQ158463
S. solanifolia	Loei	S. Bunwong 395	HQ158412	HQ158516	HQ158464
Tarlmounia elliptica (I)	Khon Kaen	S. Bunwong 390	HQ158413	HQ158517	HQ158465
T. elliptica (II)	Khon Kaen	S. Bunwong 392	HQ158414	HQ158518	HQ158466
Vernonia cinerea var. *montana* (I)	Mae Hong Son	S. Bunwong 356	HQ158415	HQ158519	HQ158467
V. cinerea var. *montana* (II)	Chiang Mai	S. Bunwong 371	HQ158416	HQ158520	HQ158468
V. curtisii	Chiang Rai	S. Watthana 875	HQ158417	HQ158521	HQ158469

Table 3.3 Morphological data matrix of 44 species of Thai Vernonieae. The Asterisk (*) indicates species not included in the molecular study.

Taxon/Node	1111111111222222222233333333334444444 12345678901234567890123456789012345678901234567890123456
Acilepis attenuata	00101111101000004401321031130201120001110111111
A. chiagdaoensis	01121011100100104401111120131101020001111111111
A. divergens	01121011100100004411411000131101020001101011111
A. kingii	01101011100100004411111000130101120001100111111
A. namnaoensis	01121111100100004411111030131101020001000111111
A. ngaoensis	01101111100100004411221030130101020001002111111
A. peguensis	01131111000100004411111010131101020001000011111
*A. principis**	01101011100100004401111020130101020001000101111
A. pseudosutepensis	01131111101000004401111010131101020001000111111
A. saligna	01130011100100004411111020130101120001100111111
A. silhetensis	01120111100100004411421011130201020001100010111
*A. squarrosa**	01120110100100004411221010130201120001100101111
A. sutepensis	01120010100100004??1110101311010200011101111
Camchaya gracilis	01020220000100004401411010331101120111101110000
C. loloana	01020010010100104411111131231201120111103001102
C. loloana var. *mukdahanensis*	01020010010100104411111130231201120111103001102
C. pentagona	01021010010100104411121130231201120111103000002
C. sp.	01001210010000110011411010301201120111103001102
C. spinulifera	01020010010100114411221130230201120111103001102
C. tenuiflora	01021010010100104411111130231201120111103011102
Cyanthillium cinereum	01131011010000113301111110131101120001011111111
Cy. hookerianum	01131210010000010311111130131201120001010010111
Decaneuropsis cumingiana	11011221100100100311111010110101121002011101111
D. eberhardtii	11001221100000100000111010100100021002010101111
D. garrettiana	11001221110100100001121100100101011002103101111
Elephantopus mollis	01021201010010102211100210120010021001101101111
El. scaber var. *penicillatus*	00021100000010102201300210121010020001103101111
El. scaber var. *scaber*	00031100010010102201300210120010020001101101111
Gymnanthemum cylindriceps	31001111010100100001421000100101011002010111111
Iodocephalopsis eberhardtii	01001211010100100011411010131101121002110011100
Koyamasia calcarea	01000011010100004401211010301201020111101001102
Kurziella gymnoclada	01100110110100004411011100100100001002010100111
Monosis parishii	21011211000001000101111010130101021000100111111
M. volkameriifolia	31021111000001000001111010130101021000100111111
Pseudelephantopus spicatus	00001110000010000001000210300000020001101101111
Sparganophoros sparganophora	01001211010000100011211010100201020001010010011
Strobocalyx arborea	31001223100010000011111000100101001002010110111
St. solanifolia	11011011110011000101111000110101101002010111111
Tarlmounia elliptica	11031211100000010300111000131101001002010010111
Vernonia curtisii	01001011010100000011221111300201120001101011111
V. cinerea var. *montana*	01121011010100010211111111012010002000101011111
*V. pulicarioides**	01120211100100004401221110140201120031000100111

2.2 Molecular Data

2.2.1 Taxon sampling and nomenclature

Thirty-five species in 16 genera of Thai Vernonieae were sampled using herbarium and silica gel preserved specimens (Table 3.2). Nomenclature is based on that of Robinson (1990, 1999b), Robinson & Skvarla (2006, 2007, 2009) and Robinson *et al.* (2008). Two species of *Distephanus* Cass. were chosen as the outgroup following the work of Keeley & Jansen (1994) and Keeley *et al.* (2007).

2.2.2 DNA extraction, amplification and sequencing

Total genomic DNA was extracted using Qiagen DNeasy Plant Mini Kits following the instructions supplied. Primer ITS5A (Downie & Katz-Downie, 1996). Primers used to amplify and sequence the trnL-F region of cpDNA were designed by Taberlet *et al.* (1991) and those used for the 3′ end of the ndhF region were designed by Jansen (1992). All primer sequences are given in Table 3.3. For the PCR amplification reactions, each 25µl PCR reaction cocktail contained 14 µl of sterile water, 2.5 µl of 10x NH_4 PCR reaction buffer (Bioline), 2 µl of 20 mM dNTPs (Promega) in an equimolar ratio, 1.25 µl of 50 mM magnesium chloride, 0.5 µl of 10 mg/ml Bovine Serum Albumin (Sigma), 1 µl of a 10 mM concentration of the forward primer, 1 µl of a 10 mM concentration of the reverse primer, 0.25 µl of Biolase Red DNA polymerase enzyme (1 unit/µl from Bioline), and 2.5 µl of template DNA. The amount of template DNA was adjusted when necessary to generate sufficient PCR products for DNA sequencing.

The amplification reactions were conducted in a PTC-100 thermal cycler (MJ Research, USA). The PCR profile consisted of an initial preheating at 94 °C for 2 minutes followed by 35 cycles of 1 min at 94 °C, followed by 1 minute at 48 °C (54 °C for cpDNA) and 2 minutes at 72 °C. Primer extension time was increased by 4 second (7 second for cpDNA) for each subsequent reaction cycle. An additional seven minutes extension at 72 °C was added for completion of unfinished DNA strands. All PCR products were quantified by agarose gel electrophoresis in comparison to an aliquot of product with a known quantity of a HyperladderII (Bioline; Ohio, USA). The remainder was stored at 4 °C until utilized. PCR products were purified for sequencing using a mixture of Shrimp alkaline phosphatase and exonuclease (ExoSap) following recommendations from the manufacturer. The cycle

presequencing reactions were done using PTC-100 thermal cycler (MJ Research, USA), incubation at 37 °C for 15 minutes and enzymatic inactivation at 80 °C for 15 minutes. The purified products were sequenced on ABI 3730XL capillary-based DNA sequencers at the center of Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB), University of Hawaii at Manoa.

Table 3.4 Primer sequences used for PCR and cycle sequencing

Name	Sequence (5′-3′)
ITS5A	GGA AGG AGA AGT CGT AAC AAG G
ITS4	TCC TCC GCT TAT TGA TAT GC
trnL C	CGA AAT CGG TAG ACG CTA CG
trnL F	ATT TGA ACT GGT GAC ACG AG
ndhF 1603	CCT YAT GAA TCG GAC AAT ACT ATG C
ndhF +607	CCT YAT GAA TCG GAC AAT ACT ATG C

2.2.3 Sequence alignment

For each accession, contiguous sequences were compiled with Sequencer 4.8 (Gene Codes, Ann Arbor, Michigan, USA) and edited manually. All sequences were deposited in GenBank (Table 3.2). Sequence alignments were generated by the program MUSCLE, Multiple Sequence Comparison by Log-Expectation, version 3.7 (Edgar, 2004) and adjusted manually in Se-Al sequence alignment editor (Rambaut, 1996).

2.2.4 Phylogenetic analyses

Data sets were analyzed individually and in combination. Similarly, the morphological data were analyzed separately and then in combination with the combined (concatenated) sequences. Analyses were conducted using Maximum Parsimony (MP) and Bayesian Analysis (BA) criteria as employed in PAUP* 4.0b10 (Swofford, 2002) and MrBayes 3.0b4 (Ronquist & Huelsenbeck, 2003), respectively. MP trees were generated with the following conditions; gaps treated as missing data, full heuristic search, tree-bisection-and-reconnection (TBR), 1000 random additions, one tree held at each step, and 1000 bootstrap replicates (Felsenstein, 1985). Bayesian

analysis was performed using the General Time Reversible model (GTR) using Markov Chain Monte Carlo (MCMC) simulations (Geyer, 1991) with four heated chains for one million generations. Four runs were conducted and each run was sampled every 100th generation. The first 1250 trees were discarded as the burn-in period, the number determined by generations to convergence. All trees saved from the independent runs (excluding burn-in) were used to construct 50% majority-rule consensus trees. Trees were drawn in PAUP*4.01b.10 (Swofford, 2002) or with the program MEGA 3.0 (Molecular Evolutionary Genetics Analysis) (Kumar *et al.*, 2004).

3. Results

3.1 Phenetic analysis

UPGMA analysis of the 46 characters scored for external morphological characters and for pollen revealed five clusters (Figure 3.1):

Group I. Subtribe Elephantopinae; *Elephantopus scaber*, *E. mollis* and *Pseudelephantopus spicatus*.

Group II. Subtribe Centrapalinae; *Camchaya gracilis, C. loloana, C. pentagona, C. spinulifera, C. tenuiflora* , *C.* sp., and *Koyamasia calcarea.*

Group III. Subtribe Gymnantheminae; *Decaneuropsis cumingiana, D. eberhardtii, D. garrettiana, Gymnanthemum cylindriceps, Monosis parishii, M. volkameriifolia, Strobocalyx arborea, S. solanifolia* and *Tarlmounia elliptica.*

Group IV. Uncertain placement; *Iodocephalopsis eberhardtii, Sparganophoros sparganophora* and *Vernonia curtisii.*

Group V. Subtribe Erlangeinae; *Acilepis attenuata, A. chiangdaoensis, A. divergens, A. kingii, A. namnaoensis, A. ngaoensis, A. peguensis, A. principis, A. pseudosutepensis, A. saligna, A. silhetensis, A. squarrosa*, *A. sutepensis*, *Cyanthillium cinereum, C. hookerianum*, *Vernonia cinerea* var. *montana*, *Kurziella gymnoclada* and *Vernonia pulicarioides.*

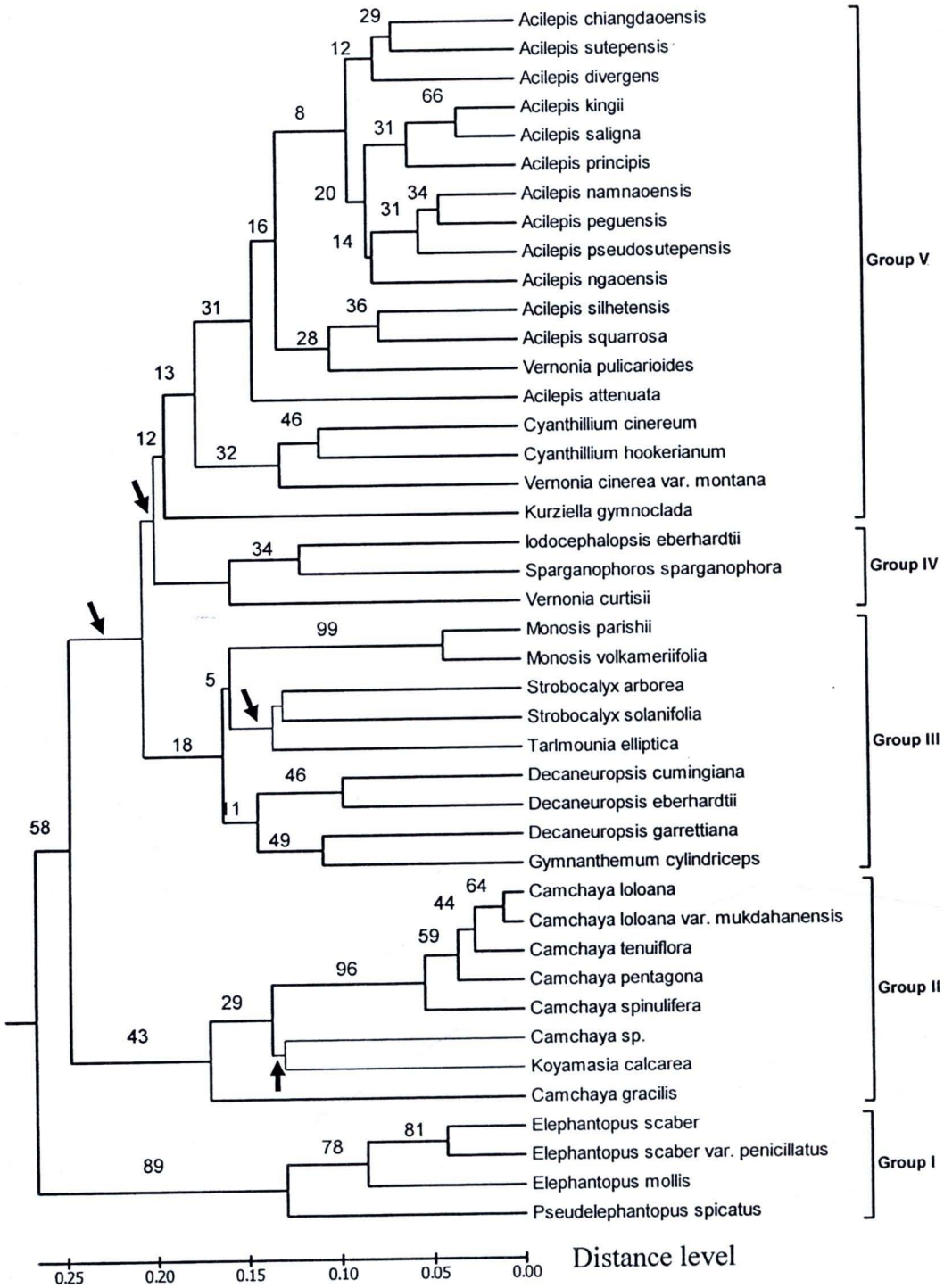

Figure 3.1 UPGMA tree of 42 Thai Vernonieae based on morphological characters Numbers above the lines indicate bootstrap support (1000 replications). Arrows indicate that line lack of support in the bootstrap consensus tree.

3.2 Phylogeny

3.2.1 Maximum Parsimony analyses

The results of the MP analyses of the individual and the combined data sets (ITS, ndhF, trnL, morphological characters) show only slight differences between taxa. Multiple polytomies also prevent understanding of the larger scale subtribal relationships. This is especially true for the tree generated from trnL-F sequences alone and the tree resulting from morphological characters alone (Table 3.5, Figure 3.2). Even with the better resolved clades of the individual ITS and ndhF trees (Figures 3.2; A, B) and those produced by analyzing all the data sets in combination (Figure 3.3) relationships among the cladesshown by MP remain unclear. Moderately- to well-supported monophyletic groups recognized in the latter combined analysis included *Acilepis/Koyamasia/Vernonia curtisii, Camchaya, Decaneuropsis, Elephantopus, Tarlmounia/Strobocalyx* and *Cyanthillium/Vernonia*, respectively. These generic groupings are consistent with those recognized in recent revisionary treatments by Robinson & Skvarla, 2006, 2007, 2009; Robinson *et al.* 2008)

Table 3.5 Summary of statistics for each and combined data sets resulting from MP analyses.

Partition	Aligned length	Informative character	% of informative character	Tree length	CI	RI
ITS	769	408	53.06	1461	0.548	0.845
ndhF	900	98	10.89	317	0.868	0.903
trnL-F	997	82	8.22	335	0.558	0.668
Morphology	46	46	100	261	0.287	0.728
ITS + ndhF + trnL-F	2666	588	22.05	2254	0.506	0.808
Molecular + Morphology	2712	634	23.38	2562	0.522	0.791

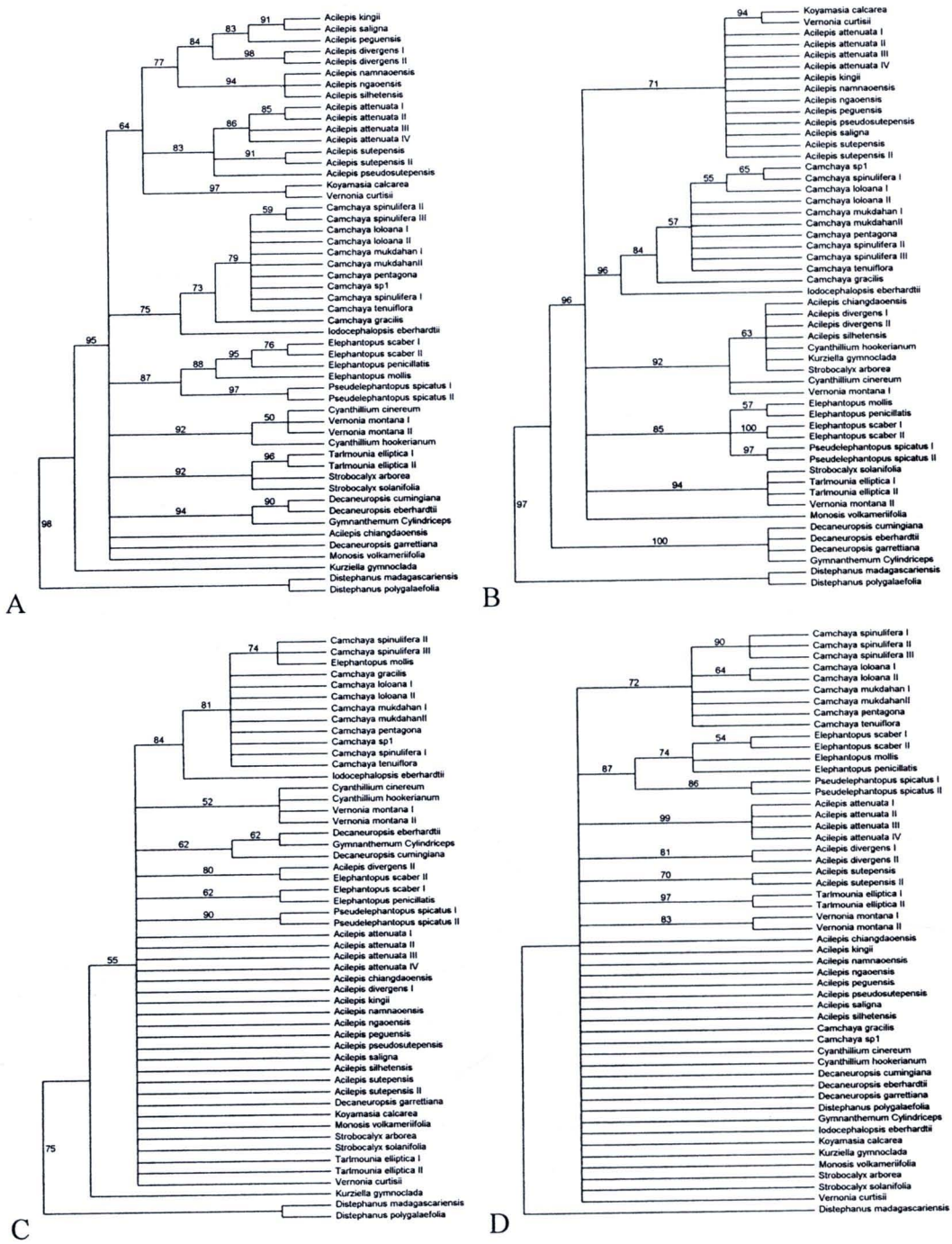

Figure 3.2 Consensus trees of MP analyses from single data sets: A. ITS, tree length 1461, CI 0.548, RI 0.845; B. ndhF, tree length 317, CI 0.868, RI 0.903; C. trnL-F, tree length 335, CI 0.558, RI 0.668; D. Morphology tree length 261, CI 0.287, RI 0.728. Numbers above the branches are bootstrap support > 50% (1000 replicates).

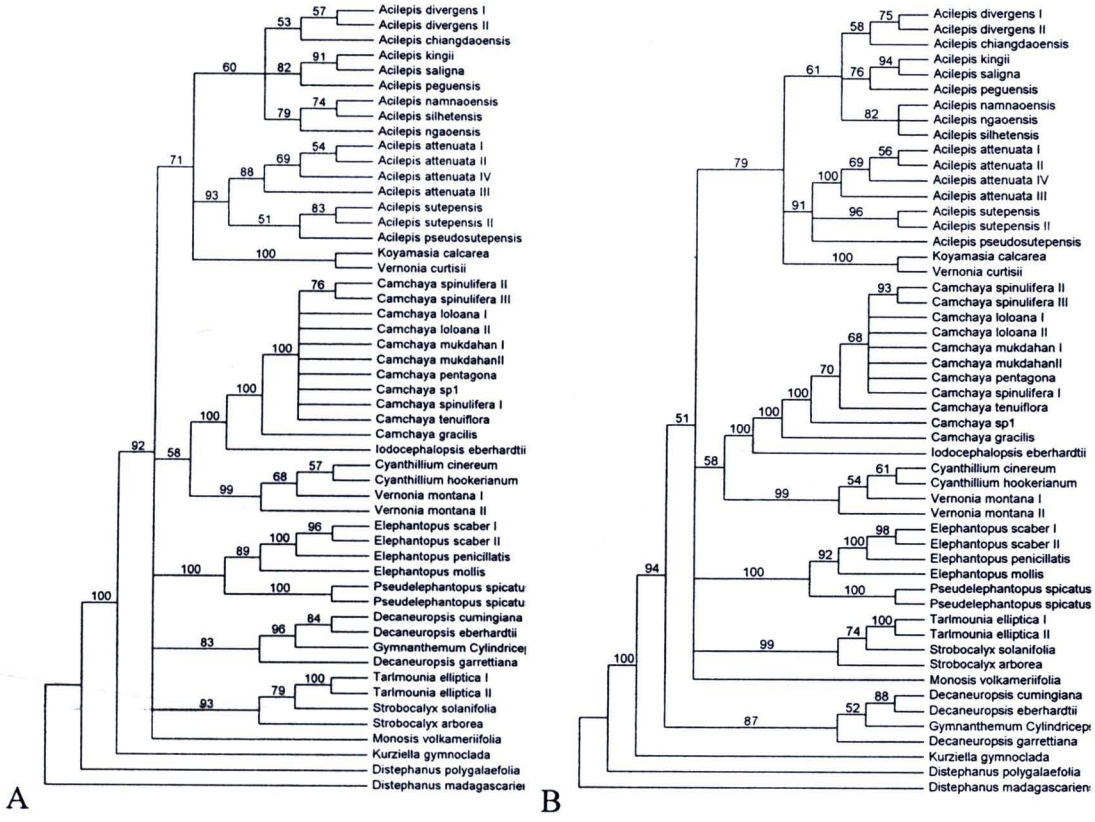

Figure 3.3 Consensus trees of the MP analyses: A. Concatenated molecular sequences (ITS + ndhF + trnL), tree length 2254, CI 0.506, RI 0.808; B. Combined molecular and morphological data sets, tree length 2562, CI 0.522, RI 0.791. The numbers above the branches are >50% bootstrap support (1000 replicates).

3.2.2 Bayesian Analysis

Fully resolved trees were obtained using BA with the combined data sets (Figure 3.4); the same monophyletic groups and genera (above) were identified as were shown in the MP analyses (Figure 3.3). *Kurziella gymnoclada*, a taxon not yet assigned to tribe, was basal to all other Thai Vernonieae, as was the case with the combined-data-sets MP trees. The most significant results of the BA analyses were the paraphyly of both the Gymnantheminae and the Erlangeinae. *Decaneuropsis* and *Tarlmounia/Strobocalyx*, subtribe Gymnantheminae (Robinson & Skvarla, 2007; Robinson *et al.*, 2008), are not closely related to each other as would be expected if they were, in fact, members of the same subtribe (Figure 3.4). Similarly, the

Erlangeinae is also paraphyletic with most members in the *Acilepis* group (Erlangeinae I) while *Cyanthillium* spp. and *Vernonia cinerea* var. *montana,* also thought to belong to the Erlangeinae, are more closely related to taxa in the subtribe Centrapalinae (Figure 3.4). *Monosis volkameriifolia* is sister to both the Erlangeinae (I & II) and the Centrapalinae. *Decaneuropsis*, on the other hand, holds a basal position similar to that of the African Gymnantheminae in Keeley *et al.*, 2007. The taxonomic implications of these relationships are discussed below.

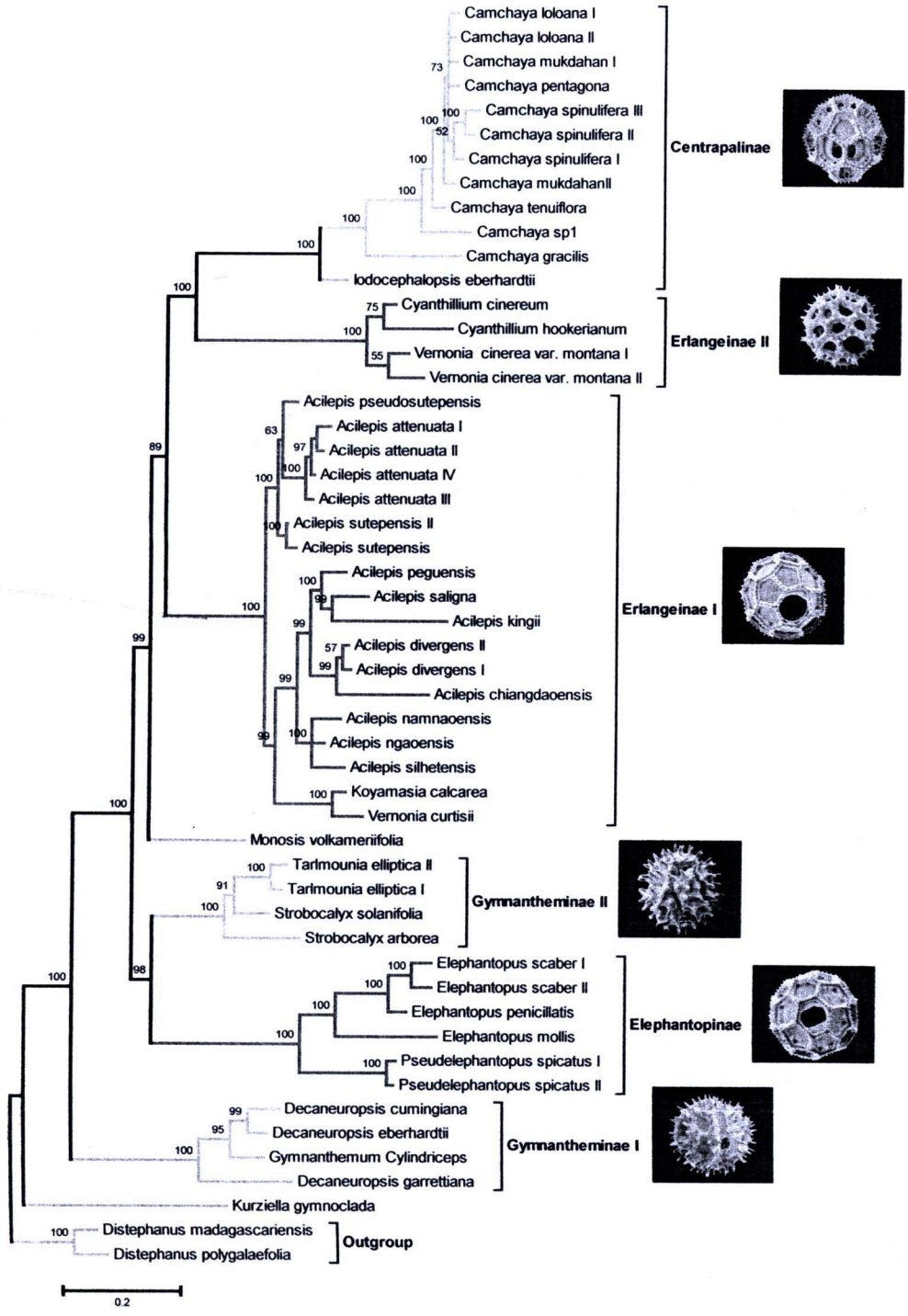

Figure 3.4 Consensus tree of BA analysis from a combined molecular and morphological data sets; run for $1x10^6$ generations, model GTR, log-likelihood score -17253. Posterior probability values are given above lines. Color of lines indicate type of pollen; Blue = 3-colporate, Green = 3-porate, Purple = 6-porate.

4. Discussion

4.1 Taxonomic implications

The six clades identified in the combined data MP and BA analyses (Figures 3.3, 3.4) and the five groups revealed by UPGMA (Figure 3.1) are highly congruent. However, the paraphyly of both the Gymnantheminae and the Erlangeinae make new subtribal assignments likely, especially if relationships seen here are confirmed within the framework of a global tribal analysis such as that of Keeley *et al.* (2007). The lack of modern taxonomic treatments for the majority of the Old World Vernonieae is the principal reason for tribal misassignments as data on which to place taxa are sparse. As a result, it is inevitable that there will be (major) changes in subtribal boundaries among Old World taxa, and in the circumscription of genera and species within them. A tantalizing aspect to the relationships shown in this study, however, is the likelihood that there are separate (south/east) Asia-centered and Africa-centered Vernonieae lineages. These would derive from different sources/areas and likely evolved in isolation from each other. Several different lineages have also been identified within the New World species (Keeley *et al.*, 2007), but such smaller monophyletic divisions were not apparent among Old World species until now.

4.1.1 Subtribe Centrapalinae

On the basis of both morphology and DNA sequence data the genus *Camchaya* (Centrapalinae) is well supported. The genus is characterized by an annual habit, erect stems with cauline leaves, achenes without a carpopodium, a deciduous pappus, and echinolophate six-porate pollen. Bunwong *et al.* (2009) distinguished the genus *Iodocephalopsis* from *Camchaya* based on the absence of a spine at the tip of the involucral bracts, differences in bract shape, and on the tricolporate pollen of *Iodocephalopsis* rather than the six-porate grains of *Camchaya*. Such a distinction is strongly supported (p.p. 100%) by the phylogenetic relationships revealed in the BA tree. Although both genera are clearly within a monophyletic Thai Centrapalinae Robinson (pers. comm.) expressed doubt about the existence of true Centrapalinae in Thailand because the taxa are morphologically different from those in Africa. As a result, Thai genera will need to be put into an analysis in combination with African Centrapalinae taxa from before a final decision can be made as to whether *Camchaya* and *Iodocephalopsis* belong in this subtribe or another possibly a new one.

4.1.2 Subtribe Elephantopinae

The subtribe Elephantopinae is clearly monophyletic like the Centrapalinae. *Elephantopus* and *Pseudelephantopus* are also strongly supported as distinct from each other (Figures 3.1, 3.3, 3.4). The taxa in the Elephantopinae are characterized by an annual to biennial herbaceous/subshrubby habit, liguliform zygomorphic corollas, capitula clustered within foliose bracts, filiform hairs on the leaf surfaces and echinolophate triporate pollen. Keeley *et al.* (2007) found that *Elephantopus* is New World in origin. There are multiple species of *Elephantopus* in eastern North America and Brazil, but little is known of Old World taxa. Additionally, *Elephantopus mollis* and *E. scaber*, as well as *Pseudelephantopus spicatus,* are found widely across tropical regions worldwide as weeds of disturbed areas. In the analyses conducted in this study the Elephantopinae are sister to two southeast Asian/Malayasian Gymnantheminae, *Tarlmounia elliptica*, a monotypic genus with a shrubby, scandent habit, and *Strobocalyx arborea*, a timber tree up to 30 m tall (Keeley *et al.*, 2007, Robinson *et al.*, 2008). These findings differ substantially from those of Keeley *et al.* (2007), however. In the latter study, *Tarlmounia* and *Strobocalyx* together were sister to the relict American subtribes Stokesiinae and Leiboldiinae. The Elephantopinae, on the other hand, were in a separate distantly related clade with a sister group whose numerous taxa are much more widespread throughout the Americas (the subtribe Lepidaploinae). The close relationship of *Tarlmounia/Strobocalyx* to taxa in the Leiboldiinae and Stokesiinae supports a connection between Old and New World Vernonieae as proposed by Keeley *et al.* (2007) to represent a transatlantic dispersal from the Old to the New World. However, the ultimate phylogenetic position of the Elephantopinae remains to be confirmed as there are putatively six other species in east Asia and Malaysia that have not yet been sequenced. Adding these to geographically inclusive analyses may alter currently perceived relationships within and between hemispheres.

4.1.3 Subtribe Gymnantheminae

The constitution of the Gymnantheminae has been in flux since the initial establishment of the subtribe by Robinson in 1999. In a number of cases new taxa such as *Decaneuropsis* and *Tarlmounia* have been added, (*i.e.*, Robinson, 2007; Robinson *et al.*, 2007, 2008) and equally, some have been removed and placed in their

own subtribes *i.e.*, *Distephanus*, subtribe Distephaneae (Keeley & Robinson, 2009). The Gymnantheminae is well represented in Africa and are among the basal members of the tribe (Keeley *et al.*, 2007). In addition to the large tree species of the Old World, *i.e.*, *Strobocalyx arborea,* there are also shrubs/treelets, and less commonly, herbs in this subtribe. Taxa frequently have deciduous involucral bracts and are characterized by blunt sweeping hairs on the style, an indurate appendage on the anthers, reflexed and deeply divided corolla lobes and tricolporate, non-lophate pollen (Robinson, 2007; Robinson *et al.*, 2008; Keeley & Robinson, 2009).

There are two distinct clades of Thai Gymnantheminae, making the subtribe as now constituted paraphyletic (Figure 3.4). The Gymnantheminae I is composed of species of the recently erected genus, *Decaneuropsis,* (Robinson & Skvarla, 2007) plus one species of *Vernonia* that has yet to be transferred into it. *Decaneuropsis* also holds a basal position within the Thai Vernonieae, similar to the position of the African Gymnantheminae species in the tribal phylogeny (Keeley *et al.*, 2007). *Decaneuropsis* differs from African Gymnantheminae, however, in that it is characterized by a low-growing scandent habit, differences in corolla morphology and has thrysiform rather than corymbiform inflorescences (Robinson *et al.*, 2007). Whether this genus should be placed in a separate tribe from the other Gymnantheminae will depend on the results of analyses that include putative members of both this subtribes in Thailand and Africa.

The Gymnantheminae II taxa (Figure 3.4) may also need to be recognized as a separate subtribe. In the study by Keeley *et al.* (2007) *Strobocalyx arborea* and *Tarlmounia elliptica* plus the New World subtribes Stokesiinae and Leiboldiinae formed a clade sister to all other New World Vernonieae (Keeley *et al.*, 2007, Figure 2) or, alternatively, were included within the New World clades (their Figure 3). In both cases *Strobocalyx/Tarlmounia* plus Stokesiinae/Leiboldiinae were distant from the (basal) African Gymnanthemiae. More analyses with New and Old World Vernonieae will be needed in order to establish the correct position of these taxa, however.

4.1.4 Subtribe Erlangeinae

The Erlangeinae, like the Gymnantheminae, are paraphyletic (Figure 3.4). Also like the Gymnantheminae, no African taxa were included in analyses

performed during this study and hence the relationships to other members of the subtribe, including the African type genus *Erlangea*, are unknown. There are 21 genera in the Erlangeinae, all of which occur in Africa; only two (those included in this study) have ranges that extend beyond Africa (Keeley *et al.*, 2007). It seems highly likely that Thai taxa may not belong to the Erlangeinae, but rather to a tribe of their own.

Within the Thai Erlangeinae, as presently classified, (Figure 3.4) are two paraphyletic subclades. One includes the genus *Acilepis* + (*Koyamasia calcarea* and *Vernonia curtisii* (Erlangeinae I)) and the other *Cyanthillium* + (*Vernonia cinerea* var. *montana* (Erlangeinae II)). The Erlangeinae I is strongly monophyletic and because of this does not support maintaining *Koyamasia* as a separate genus, but rather transferring it to *Acilepis*. Supporting this conclusion are low rates of sequence divergence (Table 3.5) that are well within the range found for other Compositae genera. Additionally, if *Koyamasia* is retained as a separate genus it will force *Acilepis* to be paraphyletic, an untenable solution (Keeley *et al.*, 2007; Funk *et al.*, 2009). *Koyamasia calcarea* and *V. curtisii* are morphologically distinct from most other *Acilepis,* the likely result of their limestone soil-restricted distribution in the mountains of Thailand. It may be that a lineage of limestone-adapted taxa evolved within *Acilepis* in specific areas where this soil type predominates and where soil types more common in the rest of Thailand are not found.

The subtribal position of *Cyanthillium* is also unclear, especially as it relates to the otherwise African Erlangeinae (II) (Figure 3.4). If the sister group relationship of *Cyanthillium* and *Camchaya/Iodocephalopsis* holds up when other New and Old World taxa are added to the analysis then it would be more logical to either a) create a new subtribe for *Cyanthillium* or b) to include it in the Centrapalinae. Which of these approaches is best overall will depend on any further evidence of paraphyly or its lack among *Cyanthillium* species in the New World, as well as refining subtribal concepts for Thai Vernonieae overall. At this point final decisions must, as in many of the other cases, wait on information yet to be obtained.

5. Conclusions

Neither molecular nor morphological data alone can provide a resolved phylogeny for the Thai Vernonieae. Instead all the data must be used together. Additionally, it is only BA that provides a resolved phylogeny (*i.e.*, Figures 3.2, 3.3 versus Figure 3.4), similar to the results of Keeley *et al.* (2007). It is also clear that for Thai Vernonieae some subtribal assignments are in need of clarification, especially for the Erlangeinae and the Gymnantheminae. *Cyanthillium* is not closely related to *Acilepis*, both of which are currently assigned to the Erlangeinae (Robinson, 2007), and in the Gymnantheminae, *Decaneuropsis* is only distantly related to *Tarlmounia* and *Strobocalyx*. Additionally, the latter two taxa were found to be most closely related to the New World not the Old World species by Keeley *et al.* (2007). Given that the Erlangeinae, the Gymnantheminae, and the Centrapalinae are found predominantly in Africa, but are also distributed elsewhere in the Old World, the location of the closest relatives of Thai taxa is as yet unknown. It is equally unclear if there are any true Gymnantheminae in Thailand (Robinson, pers. comm.). This makes it likely that new subtribes will need to be created to reflect the monophyly of the Thai species groups and their differences from African taxa, for example. *Kurziella gymnoclada*, currently unplaced, will also need to be included in a broader analysis of Vernonieae within the Old World in order to ascertain its affinities.

Despite current gaps in our knowledge of relationships within the tribe it is an exciting time for research in the Vernonieae of southeast Asia. There is now a regional phylogeny where none existed before that will allow for testing of hypotheses of relationships over a wider geographic area (the Old World). Of particular interest are the relationships of taxa now putatively in the same subtribe, but found in different areas of Asia and Africa. Thailand is important in tracing these relationships because it is located at the crossroads of biotic migrations westward from Malaysia and eastward from India and Africa. Understanding these historical pathways of dispersal will tell us much about the evolution and radiation of the Vernonieae in the Old World.