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REGULAR PAPER

Non-monophyly of the woody bamboos (Bambuseae; Poaceae): a multi-gene region phylogenetic analysis of Bambusoideae s.s.

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Abstract The taxonomy of Bambusoideae is in a state of flux and phylogenetic studies are required to help resolve systematic issues. Over 60 taxa, representing all subtribes of Bambuseae and related non-bambusoid grasses were sampled. A combined analysis of five plastid DNA regions, *trnL* intron, *trnL-F* intergenic spacer, *atpB-rbcL* intergenic spacer, *rps16* intron, and *matK*, was used to study the phylogenetic relationships among the bamboos in general and the woody bamboos in particular. Within the BEP clade (Bambusoideae s.s., Ehrhartoideae, Pooideae), Pooideae were resolved as sister to Bambusoideae s.s. Tribe Bambuseae, the woody bamboos, as currently recognized were not monophyletic because Olyreae, the herbaceous bamboos, were sister to tropical Bambuseae. Temperate Bambuseae were sister to the group consisting of tropical Bambuseae and Olyreae. Thus, the temperate Bambuseae would be better treated as their own tribe Arundinarieae than as a subgroup of Bambuseae. Within the tropical Bambuseae, neotropical Bambuseae were sister to the palaeotropical and Austral Bambuseae. In addition, Melocanninae were found to be sister to the remaining palaeotropical and Austral Bambuseae. We discuss phylogenetic and morphological patterns of diversification and interpret them in a biogeographic context.

Keywords *trnL-F* · *atpB-rbcL* · *rps16* · *matK* · Bambusoideae · Woody bamboos

Introduction

There are approximately 80–90 genera and about 1,000–1,500 bamboo species in the world (Clark 1995; Dransfield and Widjaja 1995; Judziewicz et al. 1999; Ohrnberger 1999; McClure 1966; Soderstrom and Ellis 1987). Their natural range includes all continents except Antarctica and Europe, from lowlands up to about 4,000 m in altitude. Biogeographically, bamboos can be categorized into two groups, tropical or temperate (Ohrnberger 1999). In woody bamboos, however, molecular evidence (Clark et al. 1995; Ní Chonghaile 2002) has suggested that they could be divided into at least three major lineages. These can be defined as temperate, palaeotropical, and neotropical.

Bamboo classification has been revolutionized by DNA sequence evidence. The first molecular DNA phylogenetic study to include a broad sample of bamboo diversity (Clark et al. 1995) clearly demonstrated the non-monophyly of Bambusoideae s.l. sensu Clayton and Renvoize (1986). Since this landmark paper, several other studies have supported these results including GPWG (2001) and Bouchenak-Khelladi et al. (2008). In these studies several taxa previously classified in Bambusoideae s.l. were

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resolved as the most outlying lineages within the grass family (these taxa are now recognized at subfamily rank and include Anomochlooideae, Pharoideae, and Puelioideae). Other taxa included in Bambusoideae s.l. sensu Clayton and Renvoize (1986) have also been shown to be more closely related to Pooideae including the tribes Brachyelytreae, Diarrheneae, and Phaenospermatideae (Clark et al. 1995; GPWG 2001; Bouchenak-Khelladi et al. 2008).

In contrast, the Bambusoideae s.s. sensu Clark et al. (1995) and the GPWG (2001) are well supported as monophyletic (Bouchenak-Khelladi et al. 2008). About 90% of Bambusoideae s.s. are woody bamboos (Ohrnberger 1999). Traditional methods have established classifications within the bamboos and allowed detailed floristic work, but these studies are sometimes limited, because of the lack of morphological characteristics (Stapleton 1997). Phylogenetic relationships at tribal and subtribal levels within Bambusoideae are still unclear and several classifications of woody bamboos are incongruent (Clayton and Renvoize 1986; Soderstrom and Ellis 1987; Dransfield and Widjaja 1995; Li 1998; Ohrnberger 1999; Table 1). There is, therefore, a need for taxonomic stability and it is hoped that molecular DNA data may help identify major groupings. Some studies on taxa of woody bamboos based on molecular phylogenetic analyses have been undertaken such as for the one-flowered, determinate, genera of Bambuseae using *rpl16* intron sequence data and morphological characteristics (Clark et al. 2007), 16 Asian genera using restriction site mutations of cpDNA (Watanabe et al. 1994), *Bambusa* using ITS nuclear rDNA sequences (Sun et al. 2005), *Chusquea* using chloroplast *rpl16* sequence data (Kelchner and Clark 1997), *Phyllostachys* using different techniques such as RFLP markers (Friar and Kochert 1994), RAPD markers (Gielis et al. 1997), ITS nuclear rDNA sequence data and AFLP markers (Hodkinson et al. 2000), and *Schizostachyum* using *GBSSI* and *trnL-F* sequences (Yang et al. 2007). Combined data-set analyses to study phylogenetic relationships among woody bamboos are rare. There is particularly a need for large multi-gene/sequence phylogenetic trees of Bambusoideae. Plastid DNA variation within the subfamily is low in comparison with several of the other grass subfamilies (Ní Chonghaile 2002) and single-gene analyses of plastid DNA have been found to be insufficient to adequately resolve phylogenetic pattern needed for detailed classification. Few phylogenetic studies have attempted to combine sequences. Ní Chonghaile (2002) applied *trnL* intron, *trnL-trnF* intergenic spacer, *rpl16* intron sequence data, and ITS nuclear rDNA sequences to study relationships among woody bamboos. However her study focussed mainly on temperate woody bamboos.

In this study, representatives from all subtribes of Bambuseae according to Clayton and Renvoize (1986) and Ohrnberger (1999) were sequenced for five plastid DNA regions (*trnL* intron, *trnL-F* intergenic spacer (the names of these two regions are hereafter combined and called *trnL-F* as they are continuous tandemly arranged sections of DNA sequence), *atpB-rbcL* intergenic spacer, *rps16* intron, and *matK* gene region) for combined analysis (Table 2). The *trnL-F* and *atpB-rbcL* regions are commonly used for phylogenetic study of plants (from species to family levels; Soltis and Soltis 1998), while *matK* is commonly used for species to order levels. Several studies have shown these genes to be useful for phylogenetic study of grasses and bamboos: *trnL-F* (Hodkinson et al. 2002; Ní Chonghaile 2002; Yang et al. 2007); *matK* (e.g. Liang and Hilu 1996; Hilu et al. 1999). The *atpB-rbcL* and *rps16* regions have not previously been used to study bamboo phylogenetics. However, *rps16* has proven useful for plant molecular systematics both for dicots, for example Caryophyllaceae (Oxelmann et al. 1997), and for monocots, for example Palmae (Asmussen et al. 2000) and Marantaceae (Andersson and Chase 2001). Combined analysis of plastid DNA regions are often useful for improving phylogenetic resolution and support (Reeves et al. 2001; Hodkinson et al. 2007a). Plastid DNA is generally non-recombining and maternally inherited in most angiosperms. Different sequences found on the plastid genome should therefore share the same evolutionary history and provide congruent phylogenetic trees. The justification to combine datasets in the analyses in this study was based on an examination of groupings (and support for these) found in the single-gene analyses (data not shown). No major and well supported incongruences were found between the results from single gene region analyses and it was deemed appropriate to combine datasets (a total evidence approach).

The objective of this study was to resolve major phylogenetic groupings within Bambusoideae s.s. and evaluate the currently used classifications. More specifically, the objective was:

1 to define major bamboo groups and assess the monophyly of existing taxa;
2 to study molecular variation in different plastid gene sequence regions to assess their usefulness in bamboo phylogenetics;
3 to study the relationships of woody bamboos in comparison with other closely related bamboos and grasses and, especially, to examine the relationship between Bambuseae and Olyreae; and
4 to assess the monophyly, inter-relationships, and biogeography of taxa within Bambuseae.

Table 1 Subtribes of woody bamboos (Bambuseae) according to five classifications[a]

Subtribe	Clayton and Renvoize (1986)	Soderstrom and Ellis (1987)	Dransfield and Widjaja (1995)[b] (=Clark 1995)	Li (1998)	Ohrnberger (1999)
1. Arthrostylidiinae (established 1929)	Not recognized, genera are either under Arundinariinae or Bambusinae	11/Confined to the New World	13/Confined to the New World	13/New World	13/New World
2. Arundinariinae (established 1834)	20/Pantropic	12/Essentially Old World (one extending to N. America)	14/Old World (one extending to N. America)	14/Essentially Old World	16/S. and SE. Asia, E. Asia (one confined to south-eastern N. America)
3. Bambusinae (established 1830)	25/Pantropic	6/Old World tropics	13/Mostly Tropical Asia	10/Old World tropics	17/S. and SE. Asia, E. Asia (*Bambusa* also found in Madagascar, Pacific, and Australia; *Dendrocalamus* also found in Pacific; *Oreobambos* and *Oxytenanthera* only found in Tropical Africa)
4. Chusqueinae (established 1929)	Not recognized, genera are under Arundinariinae	1/New World tropics	2/New World tropics	2/New World tropics	2/New World tropics
5. Guaduinae (established 1987)	Not recognized, genera are either under Arundinariinae or Bambusinae	5/New World tropics	5/New World tropics	5/New World tropics	5/New World tropics
6. Hickeliinae (established 1924 = Nastinae, established 1987)	Not recognized, genera are either under Arundinariinae or Bambusinae	4/E. Africa (Tanzania) and Madagascar to Indonesia and New Guinea	6/Mostly in southern hemisphere of the Old World tropics	6/Tropical Africa and Madagascar to Tropical Asia	7/Tropical Africa to Madagascar and Pacific to S. Asia
7. Melocanninae (established 1881 = Schizostachydinae, established 1987)	4/Old World tropics	7/Old World tropics	8/Mostly tropical Asia	9/Old World tropics	9/S. and SE. Asia, E. Asia (*Cephalostachyum* and *Ochlandra* also found in Madagascar; *Schizostachyum* also found in Madagascar and Pacific)
8. Neurolepidinae (established 1987)	Under Arundinariinae	New World at high elevation from Costa Rica to Peru	Under Chusqueinae	Under Chusqueinae	Under Chusqueinae
9. Racemobambosinae (established 1994)	Not recognized, genera are under Bambusinae	Uncertain placement	3/Himalayas and Tropical Asia	1/Tropical Asia	3/S. and SE. Asia, E. Asia (one also found in Pacific)
10. Shibataeinae (established 1987)	Not recognized, genera are either under Arundinariinae or Bambusinae	7/Western Himalayas to Japan	5/Temperate Asia	7/E. Asia forests	8/S. and SE. Asia, E. Asia
11. Thamnocalaminae (established 1992)	Not recognized, genera are under Arundinariinae	Not recognized, genera are under Arundinariinae	Not recognized, genera are under Arundinariinae	Not recognized, genera are under Arundinariinae	8/S. and SE. Asia, E. Asia (two also found in Africa and Madagascar)

[a] Below each classification is the number of genera and their distribution

[b] Classification system based on Soderstrom and Ellis (1987), with some modifications developed as a collaborative effort by C.M.A. Stapleton, S. Dransfield, L.G. Clark, and K.M. Wong

Table 2 Taxa, vouchers, and GenBank no. of all sequences

Taxon*	Genus size	Voucher/herbarium	GenBank no.			
			trnL-F	*atpB-rbcL*	*rps16*	*matK*
Bambusoideae						
Arundinarieae						
Borinda sp.[a]	23	Stapleton 1347/K	EU434043	EU434107	EU434171	EU434235
Chimonobambusa quadrangularis (Fenzi) Makino	36	SS and AT 105/THNHM** and KUFF**	EU434046	EU434110	EU434174	EU434238
Chimonocalamus pallens Hsueh and Yi[b]	13	Stapleton 1340/K	EU434044	EU434108	EU434172	EU434236
Chimonocalamus sp.[b]		Piya s.n./KUFF	EU434045	EU434109	EU434173	EU434237
Menstruocalamus sichuanensis (Yi) Yi[c,T]	1	Stapleton 1319/K	EU434048	EU434112	EU434176	EU434240
Oligostachyum glabrescens (Wen) P.C. Keng and Z.P. Wang[d]	16	Stapleton 1302/K	EU434042	EU434106	EU434170	EU434234
Phyllostachys edulis (Carriere) Houzeau De Lehaie	51	SS and AT 107/THNHM and KUFF	EU434050	EU434114	EU434178	EU434242
Phyllostachys nigra (Loddiges ex Lindley) Munro		SS and AT 106/THNHM and KUFF	EU434049	EU434113	EU434177	EU434241
Pseudosasa cantorii (Munro) P.C. Ken	18	Stapleton 1/K	EU434041	EU434105	EU434169	EU434233
Bambuseae						
Neotropical Bambuseae						
Arthrostylidium glabrum R.W. Pohl (=A. *merostachyoides* R.W. Pohl)	31	Hodkinson 572/TCD	EU434072	EU434136	EU434200	EU434264
Chusquea patens L.G. Clark	129	Hodkinson 571/TCD	EU434047	EU434111	EU434175	EU434239
Guadua angustifolia Kunth subsp. *chacoensis* (N. Rojas Acosta) S.M. Young and W. Judd	24	Stapleton1308/K	EU434071	EU434135	EU434199	EU434263
Rhipidocladum racemiflorum (Steudel) McClure	17	Hodkinson 576/TCD	EU434073	EU434137	EU434201	EU434265
Palaeotropical and Austral Bambuseae						
Melocanninae						
Cephalostachyum pergracile Munro[e]	15	SD 1435/K	EU434082	EU434146	EU434210	EU434274
Neohouzeaua fimbriata S. Dransf., Pattan. and Sungkaew[e]	8	SS and RP 12/K and BKF	EU434083	EU434147	EU434211	EU434275
Neohouzeaua kerriana S.Dransf., Pattan. and Sungkaew[e]		SS and RP 13/K and BKF	EU434084	EU434148	EU434212	EU434276
Pseudostachyum polymorphum Munro[e,T]	1	SS and AT 176/THNHM and KUFF	EU434085	EU434149	EU434213	EU434277
Schizostachyum grande Ridley	57	SS and AT 100704-6/THNHM and KUFF	EU434086	EU434150	EU434214	EU434278
Schizostachyum jaculans Holttum		SS and AT 307/THNHM and KUFF	EU434087	EU434151	EU434215	EU434279
Schizostachyum zollingeri Steudel		SS and AT 090704-1/THNHM and KUFF	EU434088	EU434152	EU434216	EU434280
The rest of palaeotropical and Austral Bambuseae						
Bambusa bambos (L.) Voss[T]	129	SS and AT 030704-16/THNHM and KUFF	EU434051	EU434115	EU434179	EU434243
Bambusa beecheyana Munro		Stapleton 1313/K	EU434052	EU434116	EU434180	EU434244
Bambusa malingensis McClure		Stapleton 1332/K	EU434053	EU434117	EU434181	EU434245
Bambusa oldhamii Munro [*Sinocalamus oldhamii* (Munro) McClure[T]]		SS and AT 111/THNHM and KUFF	EU434054	EU434118	EU434182	EU434246
Bambusa oliveriana Gamble		Stapleton 1321/K	EU434055	EU434119	EU434183	EU434247
Bambusa tulda Roxburgh		Stapleton 1328/K	EU434056	EU434120	EU434184	EU434248
Dendrocalamus asper (J.H. Schultes) Backer ex K. Heyne	54	BAM[n] 1	EU434057	EU434121	EU434185	EU434249
Dendrocalamus giganteus Munro		BAM[n] 45	EU434058	EU434122	EU434186	EU434250
Dendrocalamus latiflorus Munro [*Sinocalamus latiflorus* (Munro) McClure]		SS and AT 113/THNHM and KUFF	EU434059	EU434123	EU434187	EU434251

Table 2 continued

Taxon*	Genus size	Voucher/herbarium	GenBank no.			
			trnL-F	*atpB-rbcL*	*rps16*	*matK*
Dendrocalamus membranaceus Munro		SS and AT 020704-4/THNHM and KUFF	EU434060	EU434124	EU434188	EU434252
Dendrocalamus minor (McClure) Chia and H.L. Fung		Stapleton 1317/K	EU434061	EU434125	EU434189	EU434253
Dendrocalamus strictus (Roxburgh) Nees^{T}		SS and AT 718/THNHM and KUFF	EU434062	EU434126	EU434190	EU434254
Dendrocalamopsis valida Q.H. Dai^{f}	–	SS and AT 625/THNHM and KUFF and TCD	EU434063	EU434127	EU434191	EU434255
Dinochloa malayana S. Dransfield	33	SD 1412/K	EU434067	EU434131	EU434195	EU434259
Gigantochloa ligulata Gamble	54	SS and AT 090704-4/THNHM and KUFF	EU434065	EU434129	EU434193	EU434257
Gigantochloa scortechinii Gamble		SS and AT 309/THNHM and KUFF	EU434066	EU434130	EU434194	EU434258
Melocalamus compactiflorus (Kurz) Bentham^{T}	6	SS and AT 175/THNHM and KUFF	EU434068	EU434132	EU434196	EU434260
Mullerochloa moreheadiana (F.M. Bailey) K.M. Wong^{g,T}	1	Hodkinson NSW33/TCD	EU434079	EU434143	EU434207	EU434271
Neololeba atra (Lindley) Widjaja^{h,T}	3	Hodkinson NSW32/TCD	EU434078	EU434142	EU434206	EU434270
Neosinocalamus affinis (Rendle) P.C. Keng^{i,T} [*Bambusa affinis* Rendle]	–	SS and AT 624/THNHM and KUFF and TCD	EU434064	EU434128	EU434192	EU434256
Oreobambos buchwaldii K. Schumann^{T}	1	Kare s.n./Uganda, TCD	EU434080	EU434144	EU434208	EU434272
Oxytenanthera abyssinica (A. Richard) Munro^{T}	1	Stapleton 1307/K	EU434081	EU434145	EU434209	EU434273
Phuphanochloa speciosa Sungkaew and A. Teerawa.^{j,T}	1	SS and AT 191/THNHM and KUFF and TCD	EU434070	EU434134	EU434198	EU434262
Temburongia simplex S. Dransfield and K.M. Wong^{k,T}	1	Ahmed et al. 20038 (dnaBank no. 21774)	EU434077	EU434141	EU434205	EU434269
Temochloa liliana S. Dransfield^{l,T}	1	SS and AT 100704-15/THNHM and KUFF	EU434076	EU434140	EU434204	EU434268
Thyrsostachys siamensis Gamble	2	SS and AT 020704-3/THNHM and KUFF	EU434069	EU434133	EU434197	EU434261
Vietnamosasa ciliata (A. Camus) Nguyen^{m}	3	SS and AT 208/THNHM and KUFF	EU434074	EU434138	EU434202	EU434266
Vietnamosasa pusilla (A. Chevalier and A. Camus) Nguyen^{m}		SD 1466/K	EU434075	EU434139	EU434203	EU434267
Olyreae						
Cryptochloa granulifera Swallen	8	Hodkinson 554/TCD	EU434089	EU434153	EU434217	EU434281
Lithachne pauciflora (Swartz) Palisot De Beauvois ex Poiret	4	Mejia s.n./TCD	EU434090	EU434154	EU434218	EU434282
Olyra latifolia L.^{T}	25	Hodkinson 614/TCD	EU434091	EU434155	EU434219	EU434283
Piresia sp.	4	Hodkinson 601/TCD	EU434092	EU434156	EU434220	EU434284
Ehrhartoideae						
Ehrharteae						
Ehrharta calycina Sm.	36	Hodkinson G25/TCD	EU434096	EU434160	EU434224	EU434288
Oryzeae						
Leersia hexandra Sw.	18	Hodkinson 636/TCD	EU434093	EU434157	EU434221	EU434285
Oryza rufipogon Griff.	18	AT and SS 164/THNHM	EU434094	EU434158	EU434222	EU434286
Oryza sativa L.^{T}		Hodkinson 46/TCD	EU434095	EU434159	EU434223	EU434287
Pooideae						
Lygeeae						
Lygeum spartum Loefl. ex L.^{T}	1	Hodkinson 18/TCD	EU434098	EU434162	EU434226	EU434290
Nardeae						
Nardus stricta L.^{T}	1	Hodkinson and Curran 5/TCD	EU434097	EU434161	EU434225	EU434289
Poeae						

Table 2 continued

Taxon*	Genus size	Voucher/herbarium	GenBank no.			
			trnL-F	*atpB-rbcL*	*rps16*	*matK*
Lolium perenne L.^{T}	7	Hodkinson 29/TCD	EU434099	EU434163	EU434227	EU434291
Aveneae						
Alopecurinae						
Alopecurus pratensis L.^{T}	40	Hodkinson 30/TCD	EU434101	EU434165	EU434229	EU434293
Aveninae						
Arrhenatherum elatius (L.) P. Beauv. ex J. Presl and C. Presl^{T}	7	Hodkinson 27/TCD	EU434100	EU434164	EU434228	EU434292
Panicoideae						
Paniceae						
Setariinae						
Panicum virgatum L.	468	Hodkinson 120/TCD	EU434102	EU434166	EU434230	EU434294
Andropogoneae						
Saccharinae						
Saccharum officinarum L.^{T}	37	Hodkinson and Renvoize 104/K	EU434103	EU434167	EU434231	EU434295
Miscanthus sinensis Anderss	15	Hodkinson and Renvoize 5/K	EU434104	EU434168	EU434232	EU434296

Abbreviations are as follows; *K* Kew herbarium, UK; *KUFF*** Herbarium of Faculty of Forestry, Kasetsart University, Bangkok, Thailand; *TCD* Herbarium, School of Botany, Trinity College, Dublin, Eire; *THNHM*** Thailand Natural History Museum, National Science Museum, Techno Polis, Pathum Thani, Thailand; *AT* A. Teerawatananon, *RP* R. Pattanavibool, *SD* S. Dransfield, *SS* S. Sungkaew

*Classification of bamboos based on this study, those of other grasses based on Clayton and Renvoize (1986)

**Abbreviations used in this study as there are no international herbarium acronyms for these herbaria

[a] Genus established in 1994, in Thamnocalaminae in Ohrnberger (1999), but *Thamnocalamus* in Arundinariinae in Clayton and Renvoize (1986)

[b] Genus under *Sinarundinaria*, Arundinariinae in Clayton and Renvoize (1986)

[c] Genus established in 1992, in Arundinariinae in Ohrnberger (1999)

[d] Genus under *Arundinaria*, Arundinariinae in Clayton and Renvoize (1986)

[e] Genus under *Schizostachyum* in Clayton and Renvoize (1986)

[f] Genus under *Bambusa* (Clayton and Renvoize 1986)

[g] Genus established in 2005, no subtribe applied

[h] Genus established in 1997, no subtribe applied

[i] Genus under *Dendrocalamus* in Clayton and Renvoize (1986), but under *Bambusa* in Ohrnberger (1999)

[j] New genus and a new species (Sungkaew et al., in press)

[k] Genus established in 1996, in Hikeliinae in Ohrnberger (1999), but *Hickelia* in Bambusinae in Clayton and Renvoize (1986)

[l] Genus established in 2000, no subtribe applied

[m] Genus established in 1990, not present in Clayton and Renvoize (1986), but in Racemobambosinae in Ohrnberger (1999), but *Racemobambos* placed in Bambusinae by Clayton and Renvoize (1986)

[n] Bambusetum, Rimba Ilmu Botanic Garden, University of Malaya, Kuala Lumpur, Malaysia; specimen collected by K.M. Wong

[T] Nomenclatural type

Materials and methods

Plant materials

Sixty-four species from three subfamilies sensu Clayton and Renvoize (1986), Bambusoideae, Pooideae, and Panicoideae, were sampled (Table 2). Panicoid grasses were selected as a suitable outgroup because they lie outside the BEP clade (GPWG 2001) and because we were primarily concerned with establishing relationships of taxa within Bambusoideae s.s.. Bambusoideae s.s. are a robust clade with high levels of support in most recent analyses (Hilu et al. 1999; Zhang 2000; GPWG 2001; Salamin et al. 2002; Bouchenak-Khelladi et al. 2008). We also wanted to determine the closest relatives to Bambusoideae s.s. from within the BEP clade. For this reason an outgroup that outlies the BEP clade was required. Because of the incongruence between infra-subfamilial classifications of Bambusoideae s.s., representatives from all bamboo subtribes according to Clayton and Renvoize (1986) and Ohrnberger (1999) were included. The number of accepted species included per genus (Clayton et al. 2008) is shown in Table 2. For genera not recognized in the website, species number follows Ohrnberger (1999). *Phuphanochloa* is a bamboo genus new to science (S. Sungkaew et al., in press). Plant material was collected in silica gel to rapidly desiccate the material and minimize DNA degradation (following Chase and Hills 1991). Some samples, however, were obtained from herbarium specimens.

Isolation of total genomic DNA

Total genomic DNA (tDNA) was extracted from ca 0.2 g of leaf using the modified 2XCTAB method of Doyle and Doyle (1987) as outlined in Hodkinson et al. (2007b) and precipitated in isopropanol for at least 1 week or longer at −20°C (Hodkinson et al. 2007b). The tDNA was then pelleted, washed with 70% ethanol, and purified using a JetQuick PCR product-purification kit (Genomed). All DNA samples were processed in this study, except for that from *Oreobambos buchwaldii*, which was processed by Ní Chonghaile (2002). DNA was then stored in TE buffer (10 mM *Tris*-HCl; 1 mM EDTA; pH 8.0) at −20°C until use.

DNA amplification and sequencing

The polymerase chain reaction (PCR) was used to amplify each of the five gene regions. Using the primers “c” and “f” designed by Taberlet et al. (1991), the PCR amplification protocol of *trnL-F* consisted of a pre-heat of 95°C for 1 min, and 30 cycles of the following: 95°C for 45 s of denaturation, 50°C for 45 s of annealing, 72°C for 2 min of extension. A final extension of 72°C for 7 min was also included. The primers used to amplify the *atpB-rbcL* were from Samuel et al. (1997) while those for the *rps16* were from Oxelmann et al. (1997). The protocols for *atpB-rbcL* and *rps16* were similar to *trnL-F* but used a higher annealing temperature (52°C). Four primers were used to amplify the *matK*, they were: “19F” (Molvray et al. 2000), “9R” (Hilu et al. 1999), “390F” (Cuènoud et al. 2002), and “*trnK*2R” (Johnson and Soltis 1994). The PCR amplification protocol of *matK* consisted of a pre-heat of 94°C for 3 min, and 30 cycles of the following: 94°C for 1 min of denaturation, 52°C for 1 min of annealing, 72°C for 2.5 min of extension. A final extension of 72°C for 7 min was also employed. All successful PCR products were purified using the same procedure to tDNA purification but used ultra-pure sterile water instead of TE buffer as the elution buffer. DNA was sequenced using Applied Biosystems BigDye terminator kits, v.1.1, on an Applied Biosystems 310 automated DNA sequencer. The full sequences of all taxa listed in Table 2 were obtained. Only partial sequence of one taxon, *Olyra latifolia*, was used, because of difficulties with the *matK* 19F primer during sequencing.

DNA sequence editing, assembly, and phylogenetic analysis

DNA sequences were edited and assembled using AutoAssembler Software, version 2.1 (Applied Biosystems). The sequences were then imported to PAUP 4.0* Beta 2 (Swofford 1998) for alignment. Sequences were aligned by eye. Gaps were scored as additional binary characters (scoring gaps of identical size and position only). The resulting sequences were subjected to maximum-parsimony analysis using the heuristic search options in PAUP 4.0* Beta 2 (Swofford 1998). Searches included 1,000 replicates of random stepwise addition saving no more than 100 trees for tree bisection reconstruction branch swapping per replicate. Bootstrapping included 1,000 replicates and the same heuristic search settings as the individual searches, except that simple addition sequence was used instead of random stepwise addition.

Bayesian analyses were performed using MrBayes version 3.2 (Huelsenbeck and Ronquist 2001). The GTR + G model of substitutions was selected for each gene partition following hierarchical likelihood ratio tests (Huelsenbeck et al. 1996). Substitution rate matrix parameters and the shape of the Gamma distribution were estimated independently for each gene partition. The Markov chain Monte-Carlo (MCMC) algorithm was run for ten million generations over eight parallel chains, sampling every 1,000 generations on the Vital-it cluster of the Swiss Institute of

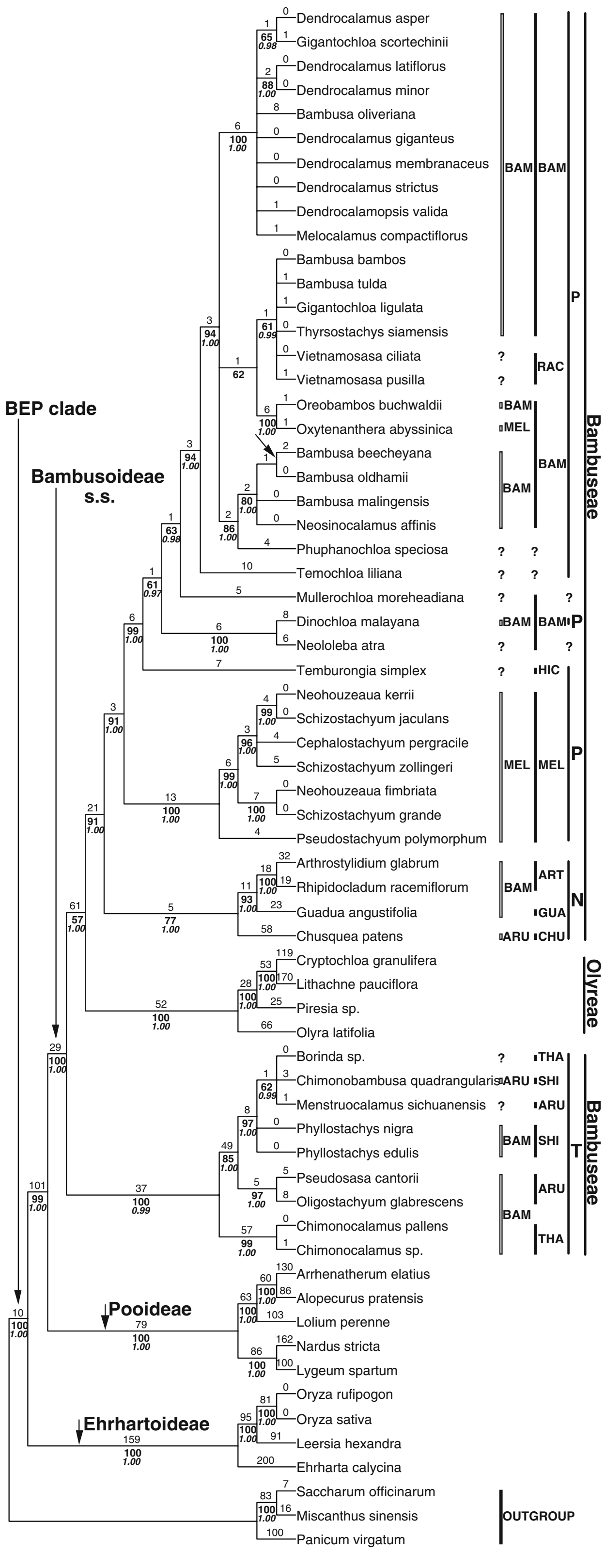

Fig. 1 One of three equally most parsimonious trees obtained from ▶ comparative sequence analysis of combined *trnL-F, atpB-rbcL*, *rps16*, and *matK* sequence data. *Values above branches* represent the number of steps supporting each branch. *Values below branches* represent the percentages of bootstrap supporting each branch. *Numbers in italics* represent the posterior probability value (PP). The BEP clade, the subfamial classification, and the tribal classification (first column on far *right*) are according to GPWG (2001). The geographical classification of woody bamboos (second column from far *right*) is shown according to Clark et al. (1995) and Ní Chonghaile (2002). The two subtribal classifications shown (the first and the second from far *left*) are according to Clayton and Renvoize (1986) and Ohrnberger (1999), respectively. *N*, neotropical woody bamboos; *P*, palaeotropical woody bamboos; *T*, temperate woody bamboos; *ART*, Arthrostylidiinae; *ARU*, Arundinariinae; *BAM*, Bambusinae; *CHU*, Chusqueinae; *GUA*, Guaduinae; *HIC*, Hickeliinae; *MEL*, Melocanninae; *RAC*, Racemobambosinae; *SHI*, Shibataeinae; *THA*, Thamnocalaminae

Bioinformatics. Convergence of the MCMC was assessed using the Gelman and Rubin (1992) test as implemented in the R package Coda (Plummer et al. 2006) and the generations before convergence were discarded as burnin.

Results

Multi-gene region phylogenetic analysis

The matrix used for the multi-gene analysis was obtained from *trnL-F, atpB-rbcL*, *rps16*, and *matK* sequences. The sequences have been deposited in GenBank, EMBL, GDBJ under the accession numbers (EU434041–EU434104). The aligned matrix was 4,983 characters long, 3,326 were constant, 738 were variable but parsimony-uninformative, and 919 were parsimony informative. The tree search using maximum parsimony found three equally most parsimonious trees, of 2,688 steps (Fig. 1). CI and RI were 0.72 and 0.79, respectively. Bootstrap (BS) percentages (≥50%BS) are described as low (50–74%), moderate (75–84%), and high (85–100%). The Bayesian analysis produced the same topology as the parsimony analysis. Posterior probability (PP) values from the Bayesian analyses are provided in italics below the bootstrap value in Fig. 1.

The BEP clade was highly supported (100%BS, 1.00PP) as monophyletic. The monophyly of each subfamily was also highly supported (100%BS, 1.00PP). Pooideae were sister to Bambusoideae s.s. with 99%BS (1.00PP) and Ehrhartoideae were sister to the lineage consisting of Pooideae and Bambusoideae s.s. (100%BS, 1.00PP).

The analysis showed that Olyreae (a monophyletic group with 100%BS, 1.00PP) were sister only to the tropical Bambuseae and not to the whole of Bambuseae. This suggests that Bambuseae are not monophyletic. There is 57%BS and 1.00PP for the sister-group status of Olyreae to the tropical Bambuseae. Division of the Bambuseae into temperate, neotropical, and palaeotropical groups was also evident. The temperate Bambuseae were highly supported (100%BS, 0.99PP) as monophyletic and were sister to the group consisting of Olyreae and the tropical woody bamboos. The tropical woody bamboos were also highly supported (91%BS, 1.00PP) as monophyletic, consisting of neotropical and the palaeotropical Bambuseae plus *Mullerochloa moreheadiana* from Australia and another species *Neololeba atra* from South Mindanao in the Philippines extending to Australia. The neotropical woody bamboos were moderately supported (77%BS, but had a PP value of 1.00) and the palaeotropical Bambuseae together with *Mullerochloa moreheadiana* and *Neololeba atra* were monophyletic with high support (91%BS, 1.00PP).

None of the subtribes of temperate woody bamboos, whether according to Clayton and Renvoize (1986) or Ohrnberger (1999), were monophyletic. However, several tropical subtribes according to Ohrnberger (1999) were monophyletic. Arthrostylidiinae, represented by *Arthrostylidium* and *Rhipidocladum* were monophyletic with high support (100%BS, 1.00PP). *Guadua* (Guaduinae) was sister to Arthrostylidiinae with high support (93%BS, 1.00PP). *Chusquea* (Chusqueinae) was sister to the group consisting of Arthrostylidiinae and Guaduinae (77%BS, 1.00PP). The analysis has also shown that subtribe Melocanninae (Schizostachydinae) was highly supported (100%BS, 1.00PP). Furthermore, Melocanninae were sister to the rest of the palaeotropical Bambuseae together with *Mullerochloa moreheadiana* and *Neololeba atra*, with high support (91%BS, 1.00PP). *Temburongia* represents subtribe Hickeliinae according to Ohrnberger (1999) and it was sister to the remaining palaeotropical woody bamboos plus *Mullerochloa moreheadiana* and *Neololeba atra* (99% BS, 1.00PP). The subtribe Bambusinae according to Ohrnberger (1999) was not monophyletic because the genus *Vietnamosasa* (representing Racemobambosinae of Ohrnberger 1999) was embedded within Bambusinae. Subtribe Bambusinae according to Clayton and Renvoize (1986) was also not monophyletic (Fig. 1).

Discussion

Phylogenetics of Bambusoideae s.s.

The subfamily Bambusoideae s.s., as defined by the GPWG (2001), was resolved as monophyletic, consisting of members from the herbaceous bamboos (tribe Olyreae) and a non-monophyletic assemblage of woody bamboos (tribe Bambuseae). The sister group to the Bambusoideae s.s. was Pooideae. This confirms the monophyly of Bambusoideae s.s. as found in previous analyses (Clark et al. 1995; GPWG 2001; Bouchenak-Khelladi et al. 2008). The secondary gain of the pseudopetiole and the secondary loss of the lamina of the first seedling leaf were recognized by the GPWG (2001) as synapomorphies for Bambusoideae s.s. The presence of strongly asymmetrically invaginated arm cells, as suggested by Zhang and Clark (2000), may also be a potential synapomorphy. The natural distribution of Bambusoideae s.s. is wide, ranging approximately from 46°N to 47°S latitude and from sea level to as much as 4,300 m in equatorial highlands (Judziewicz et al. 1999).

One tribe of Bambusoideae s.s., Olyreae, was supported as monophyletic with high bootstrap support. However, the combined analysis failed to support the monophyly of the other bambusoid tribe, Bambuseae. Bambuseae were paraphyletic and can be divided into two clades based on their molecular variation and geographical distribution. The tropical woody bamboos were sister to Olyreae (57%BS, 1.00PP, Fig. 1) while the temperate woody bamboos were sister to the group consisting of Olyreae and tropical woody bamboos (100%BS, 1.00PP). The non-monophyly of Bambuseae was also found in a combined analysis of taxa spanning the whole of the grass family (Bouchenak-Khelladi et al. 2008). This combined study, those of Bouchenak-Khelladi et al. (2008), and the Bamboo Phylogeny Group (BPG; recent personal communication) showed that Olyreae are sister to the tropical Bambuseae. There is, therefore, strong evidence that Bambuseae (sensu Clayton and Renvoize 1986; Ohrnberger 1999) are not monophyletic and that Olyreae are the sister group of the tropical Bambuseae. Thus, the temperate woody bamboos are best accommodated at tribal level as Arundinarieae Nees ex Ascherson and Graebner (name validated in 1902). Under this new scenario the Bambusoideae s.s. should include three tribes: Olyreae, Bambuseae s.s., and Arundinarieae (Fig. 2). In terms of character evolution, there are two equally parsimonious scenarios for the evolution of woodiness, one involving parallel evolution and the other reversal. Either the Bambuseae s.s. and Arundinarieae independently evolved woodiness from a herbaceous ancestral state or, alternatively, the ancestor of the Bambuseae s.s., Arundinarieae, and Olyreae group was woody and Olyreae subsequently reversed to a herbaceous state of their non-bambusoid sister group. We can only speculate which of these two scenarios is the most likely. Woodiness has evolved a number of times independently in the grasses, for example in some Panicoideae and Arundinoideae. It is therefore not unlikely that there has been parallel gain of woodiness in Bambusoideae s.s.

The geographical division of woody bamboos into temperate, palaeotropical, and neotropical groups (Clark et al. 1995) could be generally applied to our results. The temperate woody bamboos were highly supported. Palaeotropical woody bamboos plus *Mullerochloa moreheadiana* and *Neololeba atra* (palaeotropical and Austral woody bamboos), were supported (91%BS, 1.00PP, Fig. 1). This is the first time an Australian Bambuseae genus, *Mullerochloa,* and a genus extending to Australia, *Nelololeba,* have been included in phylogenetic analyses. The topology of having

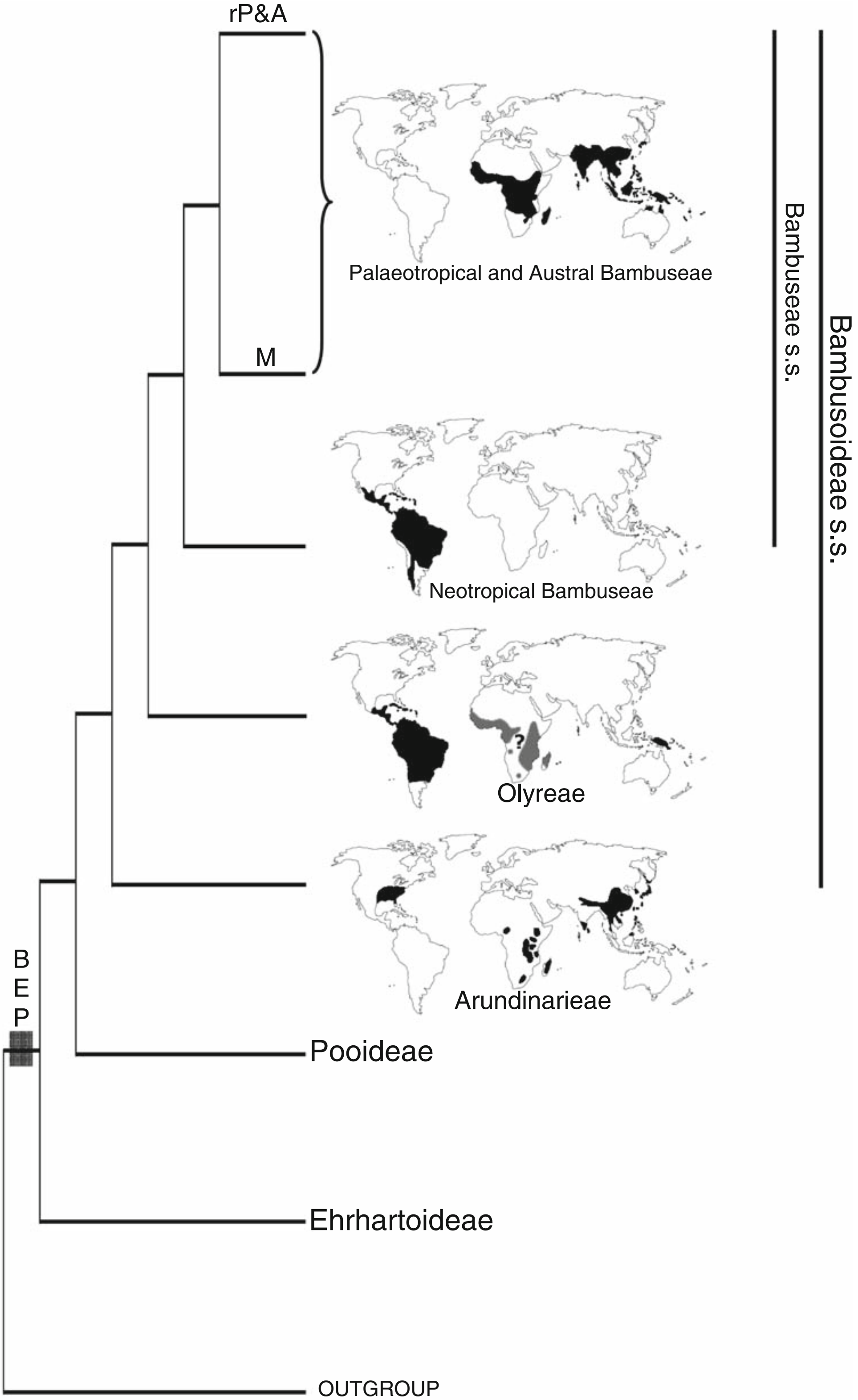

Fig. 2 Cladogram showing relationships within Bambusoideae s.s. and other grasses. The *grey box* indicates the BEP clade (Bambusoideae, Ehrhartoideae, Pooideae) according to GPWG (2001). The tribal classification (Arundinarieae, Bambuseae s.s., and Olyreae) is according to this study where Arundinarieae and Bambuseae s.s. are redefined whereas Olyeae (herbaceous bamboos) is as defined by GPWG (2001). The geographical classification of woody bamboos is shown according to Clark et al. (1995) and Ní Chonghaile (2002). *M*, Melocanninae according to Ohrnberger (1999) is sister to rP&A (= the rest of palaeotropical plus Austral woody bamboo subtribes). For the distribution of Olyreae, the *lighter shade of black colour* indicates uncertainty about whether it is truly native to these areas. The distribution of Arundinarieae found in the tropical zone is from the high elevation, usually from 1,000 m to as high as 3,630 m (adapted from Ohrnberger 1999). All the distribution maps were adapted from http://www.eeob.iastate.edu/research/bamboo/maps.html, with permission from Dr Lynn Clark

Neololeba sister to *Dinochloa* and *Mullerochloa* is a novel result. *Temburongia* is a monotypic genus (*T. simplex*) from Brunei (Dransfield and Wong 1996), representing subtribe Hickeliinae according to Ohrnberger (1999). It was the most outlying group within the palaeotropical and Austral woody bamboos. Thus a lineage represented by *Temburongia*, a lineage represented by a group consisting of *Neololeba* and *Dinochloa*, and another lineage represented by *Mullerochloa* are successively sister to the rest of the palaeotropical and Austral Bambuseae s.s. The neotropical woody bamboos were also resolved as monophyletic (77%BS, 1.00PP). The previous phylogenetic analyses of this group (e.g. Clark et al. 1995; Kelchner and Clark 1997; Zhang 2000) are congruent with our study. The temperate woody bamboos (here recognized as Arundinarieae) were supported as monophyletic, but none of their subtribes according to Clayton and Renvoize (1986) or Ohrnberger (1999) were supported. A limited amount of supported resolution was found within Arundinarieae in our analyses. For example, *Borinda, Chimonobambusa*, and *Menstruocalamus* group together (62%BS, 0.99PP).

Neotropical woody bamboos

A group containing Arthrostylidiinae, Chusqueinae, and Guaduinae sensu Ohrnberger (1999) were resolved. Arthrostylidiinae, represented by *Arthrostylidium* and *Rhipidocladum,* were positioned in Bambusinae by Clayton and Renvoize (1986), and were highly supported in our analysis (100%BS, 1.00PP). *Guadua,* representing Guaduinae, was sister to Arthrostylidiinae (93%BS, 1.00PP). *Guadua* was treated under *Bambusa* in Bambusinae by Clayton and Renvoize (1986). There was no evidence from the results of single or combined analyses to support this placement. Other molecular studies, that sampled Guaduinae according to Ohrnberger (1999), have shown that they were also sister to Arthrostylidiinae (Kelchner and Clark 1997; Zhang 2000; Bouchenak-Khelladi et al. 2008). Chusqueinae, represented by *Chusquea,* were sister to the group consisting of Arthrostylidiinae and Guaduinae (77%BS, 1.00PP). This relationship is generally congruent with previous studies (Kelchner and Clark 1997; Zhang 2000; Bouchenak-Khelladi et al. 2008). All three of these subtribes are from Central and South America (Ohrnberger 1999).

Palaeotropical and Austral woody bamboos

Subtribe Melocanninae (Schizostachydinae) according to Ohrnberger (1999) was well supported and Melocanninae were sister to the rest of the palaeotropical and Austral woody bamboos (91%, 1.00PP). This relationship is a novel result. Melocanninae are generally found at lower elevations in South, South-East, and East Asia (Table 1). Morphologically, this subtribe has pseudo-spikelets with a distinctive glabrous ovary that bears an elongated and persistent style usually divided into three short stigmas (Soderstrom and Ellis 1987). Anatomically, they differ from other subtribes in having larger microhairs, in the presence of refractive papillae, and a pronounced S-shaped keel with complex vasculature in its leaf-blade (Soderstrom and Ellis 1987). These are synapomorphic characters that set Melocanninae apart from the rest of the palaeotropical and Austral woody bamboos. Genus *Pseudostachyum* was placed under *Schizostachyum* by Clayton and Renvoize (1986). However, our analyses showed that *Pseudostachyum* was sister to the remaining Melocanninae (100%BS, 1.00PP) and support the generic status of *Pseudostachyum*. This finding is congruent with the classifications adopted by Soderstrom and Ellis (1987), Dransfield and Widjaja (1995), Clark (1995), Li (1998) and Ohrnberger (1999). *Cephalostachyum, Neohouzeaua* and *Schizostachyum,* were monophyletic (99%BS, 1.00PP) within Melocannineae. However, the relationships among these genera were unclear. Soderstrom and Ellis (1987) placed *Neohouzeaua* under *Schizostachyum*. Clayton and Renvoize (1986) treated *Neohouzeaua* and *Cephalostachyum* as synonymous with *Schizostachyum,* whereas Dransfield and Widjaja (1995), Clark (1995), Li (1998), and Ohrnberger (1999) treated them as separate genera. Our results are more consistent with the *Schizostachyum* s.l. hypothesis of Clayton and Renvoize (1986). The recently established genus *Temburongia*, from Ulu Temburong National Park, Brunei (Dransfield and Wong 1996), was the only representative of the subtribe Hickeliinae according to Ohrnberger (1999) in our analyses. *Temburongia* was treated as *incertae sedis* by Clark et al. (2007). In our study it was sister to the remaining palaeotropical and Austral woody bamboos (99%BS, 1.00PP).

Subtribe Bambusinae

None of our analyses supported the monophyly of subtribe Bambusinae according to Ohrnberger (1999), because *Vietnamosasa*, which he positioned in Racemobambosinae, was embedded within it. All analyses also showed that Bambusinae according to Clayton and Renvoize (1986) are polyphyletic, because its taxa were distributed across the phylogenetic tree of Bambuseae. Like *Racemobambos*, *Vietnamosasa* has a determinate inflorescence (Dransfield 2000a) instead of an indeterminate inflorescence as found in Bambusinae. However, if *Vietnamosasa* (and *Temochloa*, also possessing a determinate inflorescence, Dransfield 2000b) could be included within Bambusinae, then we show no evidence against the monophyly of Bambusinae sensu Ohrnberger (1999).

There are four recently established genera (*Mullerochloa*, *Neololeba, Phuphanochloa*, and *Temochloa*) that can be included within Bambusinae according to Ohrnberger (1999). *Phuphanochloa* is a new genus from northeastern Thailand, composed of a single species, *P. speciosa* (Sungkaew et al., in press). It is morphologically similar to, and phylogenetically related to, *Bambusa*, *Dendrocalamus*, and *Gigantochloa* (Bambusinae). The results showed that *Phuphanochloa* is sister to a group consisting of *Bambusa beecheyana, B. malingensis, B. oldhamii*, and

Neosinocalamus affinis (= *B. affinis*). The other newly established genera, *Mullerochloa, Neololeba*, and *Temochloa*, may be best interpreted as phylogenetically outlying within a broadly circumscribed Bambusinae or they could merit subtribe status (Fig. 1). *Temochloa* is a monotypic and endemic genus (*T. liliana*) from limestone regions of southern Thailand, which has no subtribe applied to it (Dransfield 2000b). *Mullerochloa* is a monotypic and endemic genus (*M. moreheadiana*) from Queensland, Australia (Wong 2005). *Neololeba* (represented by *N. atra*, see Table 2) is a relatively new genus established to accommodate five bamboos known from South Mindanao in the Philippines, North Sulawesi, Moluccas, New Guinea, Solomon Islands, and Queensland, Australia (Widjaja 1997). Ohrnberger (1999), without referring to Widjaja (1997), treated this species under *Bambusa* as *B. atra* Lindley and placed it in Bambusinae. A *Neololeba* and *Dinochloa* group was sister to the remaining palaeotropical and Austral woody bamboos including *Temochloa* and *Mullerochloa* (61%BS, 0.97PP). *Dinochloa* is mainly distributed on the Malay Peninsula, in Borneo, Indonesia, and Philippines, extending to southern Thailand and the Andaman and Nicobar Islands (Dransfield 1981).

Dinochloa and *Melocalamus* are consistently classified in Bambusinae (Clayton and Renvoize 1986; Soderstrom and Ellis 1987; Dransfield and Widjaja 1995; Clark 1995; Li 1998; Ohrnberger 1999). Surprisingly, *Dinochloa malayana* was sister to *Neololeba atra* (100%BS, 1.00PP). On the basis of morphology we might expect *Dinochloa* to group either with *Melocalamus* or *Mullerochloa,* rather than with *Neololeba* (McClure 1966; Dransfield 1981; Wong 1995; Li and Stapleton 2006). This suggests that one reproductive character (berrylike caryopsis with thick and fleshy pericarp) and two vegetative characters (presence of an abrupt swelling of the very basal part of the culm internodes and presence of a rugose basal zone of the culm sheath) are homoplasious because they have evolved independently among these three genera.

Apart from the most outlying taxon, *Temburongia*, the biogeographical range of the remaining outlying lineages (*Neololeba/Dinochloa* and *Mullerochloa*) is outside that of the core Bambusinae. This suggests that the ancestors of Bambusinae were from somewhere in mainland Asia, possibly south China, India, or even mainland Southeast Asia and two outlying lineages evolved separately from southern Thailand and Malaysia to northern Australia. The paucity of bamboo species in Australia would also support this hypothesis. If the origin of these bamboos had been near Australia we would expect much higher diversification in this area.

Oreobambos and *Oxytenanthera* were sister taxa (100%BS, 1.00PP). Both *Oreobambos* and *Oxytenanthera* are monotypic genera, naturally distributed in tropical Africa (Ohrnberger 1999). Clayton and Renvoize (1986) treated these two genera in different subtribes (Fig. 1). However, it is geographically (Ohrnberger 1999) and phylogenetically (this study) clear that they should be grouped together and included in Bambusinae.

Morphologically, the genus *Dendrocalamus* is similar to several other genera including *Bambusa*, *Dendrocalamopsis, Gigantochloa*, *Houzeaubambus*, *Klemachloa*, *Oreobambos*, *Oxytenanthera*, *Sinocalamus*, and *Neosinocalamus* (Holttum 1958; McClure 1966; Clayton and Renvoize 1986; Soderstrom and Ellis 1987; Dransfield and Widjaja 1995; Wong 1995; Stapleton and Xia 1997; Li 1997; Li and Xue 1997; Li 1998; Ohrnberger 1999; Li and Stapleton 2006) and our results confirmed that *Dendrocalamus* is closely related to *Bambusa, Dendrocalamopsis* (= *Bambusa*), and *Gigantochloa.* Our results also showed that *Melocalamus* grouped with *Dendrocalamus* (100%BS, 1.00PP, Fig. 1). This relationship has never been reported before. However, it could be because of hybridization (S. Sungkaew et al., in preparation). *Melocalamus* was expected to group with *Dinochloa* or *Mullerochloa* on the basis of morphology. The results also showed that *Bambusa oldhamii* groups with *Bambusa beecheyana, Bambusa malingensis*, and *Neosinocalamus affinis* (= *Bambusa emeiensis*), with 80%BS (1.00PP). This would be consistent with the placement of *Bambusa oldhamii* in *Bambusa*. The delimitation of *Sinocalamus* has proven extremely difficult (McClure 1940; Raizada 1948; Chia and Fung 1980; Xia and Stapleton 1997). Our results do not support the recognition of *Sinocalamus* (represented by *S. oldhamii*, see Table 2) and indicate that its species are better placed in *Bambusa* than *Dendrocalamus*.

To conclude, we have conducted one of the most comprehensive multi-gene region phylogenetic studies on Bambusoideae s.s. by including over 60 taxa representing all the subtribes of the traditionally recognized tribe Bambuseae and a representative sample of related taxa including Olyreae. The results have resolved a number of patterns, summarized in Fig. 2, that were previously unrecognized or poorly supported, for example:

1. the sister-group status of Pooideae to Bambusoideae s.s.;
2. the non-monophyly of Bambuseae;
3. the sister-group status of Olyreae to the tropical Bambuseae;
4. the sister-group status of temperate Bambuseae to a tropical Bambuseae/Olyreae clade;
5. the sister-group status of Melocanninae to the remaining palaeotropical and Austral Bambuseae; and
6. the division of Bambuseae s.s. into Neotropical and Palaeotropical/Austral groups.

The results indicate a need to revise the classification of Bambuseae and we recommend use of the tribal name

Arundinarieae to accommodate the temperate woody bamboos.

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