# CHAPTER 1

# INTRODUCTION

## 1.1 The woody bamboos, a taxonomically difficult group

The woody bamboos refer to those grasses (Poaceae) that have woody vegetative axes (made up of heavily sclerenchymatous and lignified tissue); segmented and typically hollow culms; a complex system of branching on the culms; leaf blades with a basally narrowed stalk-like connection ('pseudopetiole') at its junction with the leaf sheath, and arm and fusoid cells in the chlorenchyma; as well as flowers typically with three perianth-like structures (lodicules) and six stamens (Soderstrom and Ellis, 1987). Woody bamboos are difficult to study or document because they are little collected (their often large and complex plant body makes collection difficult, putting off many collectors from careful field documentation) (Holttum, 1956; McClure, 1966; Soderstrom and Young, 1983), and flowering material (important for conventional classification) and key vegetative materials (important for identification using such available features as culm shoots) are not always present together (Holttum, 1958). Furthermore, flowering occurs rarely in many groups (Janzen, 1976; Wong, 1995a). Frequently, only few morphological characters are selected as criteria for recognising taxonomic groups, so delimitations are often contentious (Holttum, 1946, 1956; Dransfield and Wong, 2004).

Woody bamboos have a wide geographical and altitudinal distribution. They are native to all continents except Europe. There is an estimated 81–98 genera and ca. 1300 species of woody bamboos (Bamboo Phylogeny Group, 2005).

### 1.2 New perspectives from molecular evidence

The advent of molecular techniques has brought new perspectives into understanding the systematic relationship among the major bamboo groups. The Bambusoideae subfamily, including a number of herbaceous bambusoid grasses, was shown to be monophyletic (Clark et al., 1995; GPWG, 2001, Bouchenak-Khelladi et al., 2008). Although woody bamboos had been considered as a single tribe, Bambuseae (Soderstrom and Ellis, 1987), molecular phylogenetic studies now support their classification as two tribes, Bambuseae *sensu stricto* (accommodating the Palaeotropical, Austral and Neotropical bamboos) and the Arundinarieae (for the temperate woody bamboos) (Bouchenak-Khelladi et al., 2008; Sungkaew et al., 2009). The woody bamboos did not form a monophyletic group, as the herbaceous bamboo group (the tribe Olyreae) was found embedded between the temperate and tropical bamboo clades in phylogenetic analyses using larger taxon samplings (Bouchenak-Khelladi et al. 2008; Sungkeaw et al. 2009).

Within the Palaeotropical Bambuseae, only two major subtribes could be clearly demonsrated thus far: Melocanninae and Bambusinae. Yang et al. (2007) showed that Melocanninae is a monophyletic subtribe consisting of *Cephalostachyum*, *Schizostachyum*, *Melocanna* and *Pseudostachyum*. It was consistently placed as the

sister group to the Bambusinae and all other climbing-scrambling bamboo genera of uncertain subtribal designation, based on both nuclear DNA and chloroplast DNA (cpDNA) markers (Yang et al., 2007; Yang et al., 2008; Sungkaew et al., 2009). On the other hand, Bambusinae is a poorly revised subtribe, where subtribal and generic limits are still contentious.

Despite varied and long flowering intervals (Janzen, 1976) that make it less likely for the reproductive phases of two different taxa to coincide, natural hybridization among woody bamboos has been suggested by morphological and experimental perspectives (Holttum, 1956; Muller, 1998; Zhang and Chen, 1980; Muramatsu, 1981; Takahashi et al., 1994; Maruyama et al., 1979; Clark et al., 1989). The possibility of hybridization is perhaps not unexpected among woody bamboos that were mostly hexaploids or tetraploids based on basic chromosome numbers of $x$ = 10 or 12. However, only recently did molecular studies (using AFLP and cpDNA sequence data) demonstrate that natural hybridization among *Arundinaria* taxa accounted for some previous taxonomic difficulty (Triplett et al., 2010).

### 1.3 Scope of the present work

More comprehensive morphology-based taxonomic revisions of SE Asian bamboos have been made since the 1980s (Dransfield and Widjaja, 1995). New genera such as *Kinabaluchloa*, *Maclurochloa*, *Phuphanochloa*, *Soejatmia* and *Sphaerobambos*, were considered part of the Bambusinae by their authors, while *Holttumochloa*, *Mullerochloa*, *Neololeba* and *Temburongia* were of uncertain taxonomic placement.

Recent molecular studies have also indicated contentious relationships between the climbing bamboo genera and the Bambusinae. For example, inclusion of *Dinochloa* in Bambusinae was supported by cpDNA but not nuclear DNA markers (Yang et al., 2007; Yang et al., 2008). *Mullerochloa*, *Neololeba* and *Temochloa,* were recovered as outlying groups near the core Bambusinae (Sungkaew et al., 2009). Other SE Asian climbing bamboo genera, i.e., *Holttumochloa*, *Kinabaluchloa*, *Maclurochloa, Soejatmia*, *Sphaerobambos* and *Temburongia*, were not included in any molecular phylogenetic analysis prior to the present study.

The nuclear *GBSS*I markers employed by Yang et al. (2008) and Yang et al. (2010) are highly variable, advantageous for generic- and specific-level comparisons. A number of cpDNA non-coding regions were also recommended by the Bamboo Phylogeny Group (2005) for establishing low taxonomic level phylogenetic analyses but thus far no publications have reported their use for Palaeotropical bamboo taxa. It is, therefore, expected that the combined sequence data of the *GBSS*I region and at least four cpDNA intergenic regions (as suggested by Zeng et al., 2010) would be helpful in resolving major lineages among and within the subtribes.

While inclusion of additional DNA markers and increased taxon sampling for potentially better phylogenetic resolution are prompted by the results of previous studies, it is also equally important to scrutinize the taxonomic problems of bamboos through other perspectives. Prior to the start of this research, a hybrid between *Dendrocalamus pendulus* and *Gigantochloa scortechinii* was discovered based on its

morphological intermediacy and localities where both parental taxa and their putative hybrid offspring were found. A genetic parentage analysis will not only confirm the relationship of these taxa, but also the occurrence of natural hybridization among SE Asian bamboos. At the same time, it is also of much interest to further investigate the phylogenetic structure of the well-known taxonomic complexes in Bambusinae, especially those formed by the speciose genera *Bambusa*, *Dendrocalamus* and *Gigantochloa*, in which some named species were difficult to clearly assign. There is circumstantial evidence to suggest hybridization and introgression could have been involved in the origin of various taxa, especially among *Bambusa vulgaris* and *Gigantochloa* spp. that are known only in cultivation, as some observed flowering events of individual clumps (implying selfing) have resulted in poor seed set (high levels of apparent sterility), poor seedling survival, and morphologically aberrant seedlings that do not resemble one another or the parent (Koshy and Jee, 2001; Muller, 1998; Ramanayake and Yakandawala, 1998; Wong, 2004). However, attempts to explore phylogenetic relationships in the *Bambusa-Dendrocalamus-Gigantochloa* complex using a molecular approach with more than just one or two gene regions and a wide taxon sampling have not yet appeared.

Based on this background, the present study incorporated three objectives as its key components:

1. to investigate the relationships of the morphologically distinctive subset of Southeast Asian bamboos with climbing-scrambling culms with erect-suberect bamboos hitherto placed in or near *Bambusa*;

2. to investigate a putatively natural intergeneric hybrid between *Dendrocalamus pendulus* and *Gigantochloa scortechinii* in order to establish if evidence for such an intergeneric hybridization could be adduced with molecular techniques; and

3. to utilize both nuclear and plastid gene regions in a study of the phylogenetic relationships among members of the Bambusinae, especially the *Bambusa-Dendrocalamus-Gigantochloa* complex.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Problematic classification of the Bambusinae: a review

Classification of woody bamboos at the subtribal level, and even tribal level, has been problematic on different fronts. Early classification by Munro (1868) used the concept of "sections" (that could be taken as roughly equivalent to subtribes), in which *Bambusa*, *Gigantochloa* and *Oxytenantera* were placed in the section "Bambuseae verae" together with *Nastus* and *Guadua*; and *Dendrocalamus* and *Dinochloa* were placed in a different section "Bacciferae" together with *Cephalostachyum*, *Melocanna*, *Schizostachyum*, and other genera. This influenced others, such as Bentham (1883) and Gamble (1896), in grouping the alliances of the various genera known. Holttum (1956) discussed the significance of ovary, fruit and spikelet structure, as well as rhizome branching, as a basis for refining the classification into the following alliances (Holttum did not use subtribal or other ranks and just grouped the genera informally):

A. (*Schizostachyum* type of ovary): *Melocanna, Ochlandra, Schizostachyum*;

B. (*Oxytenanthera* type of ovary): *Oxytenanthera*;

C. (*Bambusa-Dendrocalamus* type of ovary): *Melocalamus, Dinochloa*, *Thyrsostachys*, *Bambusa*, *Guadua, Dendrocalamus, Gigantochloa*; *Racemobambos*.

D. (*Arundinaria* type of ovary): *Arundinaria*, *Chusquea, Perrierbambos*.

These alliances (Holttum, 1956) are equivalent to the subtribes of later ranking.

Dransfield (1983) disagreed with Holttum (1958) regarding the placement of *Racemobambos* with *Bambusa*, because *Bambusa* and its allies have indeterminate inflorescences whereas *Racemobambos* has determinate inflorescence development. The unit of an indeterminate inflorescence is the pseudospikelet, characterized by having two or more basal prophyllated buds that could develop into secondary pseudospikelets that proliferate in the same manner to form a cluster of pseudospikelets. On the other hand, the determinate inflorescence has true spikelets without basal prophyllated buds. Instead, Dransfield (1983) suggested that *Racemobambos* was allied to *Arundinaria* and its allies. Later, Stapleton (1994) established the subtribe Racemobambosinae to accommodate *Racemobambos* and *Neomicrocalamus.*

Other long-standing confusion centred on the problematic delimitation of genera, especially of *Bambusa* and *Dendrocalamus*. For instance, Soderstrom and Ellis (1987) emphasized that although many genera have been described in this alliance, some of the genera could be congeneric to *Bambusa* while others may not be different from *Dendrocalamus*, although the limits between *Bambusa* and *Dendrocalamus* are not yet satisfactorily clarified. A number of genera were newly described in the past three decades. Some of them have been placed in Bambusinae, e.g., *Kinabaluchloa* (Wong, 1993a), *Maclurochloa* (Wong, 1993a), *Phuphanochloa* (Sungkaew et al., 2008), *Soejatmia* (Wong, 1993a) and *Sphaerobambos* (Dransfield, 1989), while others are of uncertain taxonomic placement, e.g., *Holttumochloa* (Wong, 1993a), *Mullerochloa*

(Wong, 2005), *Neololeba* (Widjaja, 1997) and *Temburongia* (Dransfield and Wong, 1996). *Sellulocalamus* was segregated from *Dendrocalamus* (Lin, 1989).

Problems in rank designation have also arisen. In the Flora of China (Chinese version: Keng and Wang, 1996), *Melocalamus* and *Thyrsostachys* were placed in the tribe Melocanneae; *Bambusa* in the tribe Bambuseae; and *Dendrocalamus* and *Gigantochloa* in the tribe Dendrocalameae. This classification into such distinct ranks as tribes has been discussed and regarded as unrepresentative by Stapleton et al. (2009), after the advent of molecular studies began to reveal how these genera are closely related (see further discussion in section **2.2.1**).

Bambusinae was defined by Clayton and Renvoize (1986) to include mainly tropical bamboo genera with a precocious growth of the pericarp at the ovary apex soon after fertilization, when it forms a broadly conical cap. Soderstrom and Ellis (1987) summarized the morphological characteristics of the Bambusinae as including sympodial rhizomes, solitary primary branch buds, iterauctant inflorescences and 6 stamens, and with the most common chromosome numbers 2n = 48 and 2n = 72. This subtribe is mainly distributed in the Old World, with its centre of diversification in Southeast Asia and South China, extending to the west to India, Africa, and to the south east to North Australia (Soderstrom and Ellis, 1987; Li, 1997). The morphological circumscription by Soderstrom and Ellis (1987) was in agreement with Holttum's (1958) treatment on Malayan bamboos, based on characters of spikelet and flower structure, which placed *Melocalamus*, *Dinochloa*, *Thyrsostachys*, *Bambusa*,

*Dendrocalamus* and *Gigantochloa* into a group equivalent to Bambusinae (Wong, 1995b). Different recent classifications of Bambusinae are summarized in **Table 2.1**.

**Table 2.1.** List of genera assigned to Bambusinae by recent workers.

| | |
|---|---|
| Clayton and Renvoize (1986) | *Bambusa*, *Dendrocalamus*, *Gigantochloa*, *Melocalamus*, *Thyrsostachys*, *Oreobambos*, *Dinochloa*, *Arthrostachyum*, *Rhipidocladum*, *Guadua*, *Phyllostachys*, *Pseudosasa*, *Oligostachyum*, *Chimonocalamus*. |
| Soderstrom and Ellis (1987) | *Bambusa* (sensu lato, including *Bonia*, *Dendrocalamopsis*, *Leleba*, *Lingnania*, *Neosinocalamus*, *Sinocalamus*), *Dendrocalamus* (sensu lato, including *Gigantochloa*, *Houzeaubambus*, *Oreobambos*, *Oxytenanthera*), *Dinochloa*, *Klemachloa*, *Melocalamus*, *Thyrsostachys*. |
| Stapleton et al. (in Dransfield and Widjaja, 1995) | For SE Asian genera only: *Bambusa*, *Dendrocalamus*, *Dinochloa*, *Gigantochloa*, *Holttumochloa*, *Kinabaluchloa*, *Maclurochloa*, *Melocalamus*, *Oreobambos*, *Oxytenanthera*, *Soejatmia*, *Sphaerobambos*, *Thyrsostachys*. |
| Ohrnberger (1999) | *Bambusa*, *Bonia*, *Dendrocalamus*, *Dinochloa*, *Gigantochloa*, *Holttumochloa*, *Kinabaluchloa*, *Klemachloa*, *Maclurochloa*, *Melocalamus*, *Oreobambos*, *Oxytenanthera*, *Pseudobambusa*, *Pseudoxytenanthera*, *Soejatmia*, *Sphaerobambos*, *Thyrsostachys*. |

### 2.1.1 Taxonomic problems relating to *Bambusa*

*Bambusa* is the largest genus of the Bambusinae, and also among woody bamboos in general, and comprises 40–50 species in tropical and subtropical Asia (Wong, 1995a). *Bambusa* once included such vegetatively diverse taxa that Holttum (1958) described it as not having ‘any vegetative character as distinctive’. For example, *B. blumeana* and *B. arundinacea* bear thorny branches at the lower culm nodes, the ‘mountain

species of *Bambusa*', such as *B. klossii*, *B. wrayi*, *B. pauciflora*, *B. montana* and *B. magica*, have a slender or climbing culm habit, and a few species have slightly zig-zag culms. Even then, he was doubtful that these montane species belonged with *Bambusa*.

In revising *Bambusa* sensu lato in Peninsular Malaysia, Wong (1993a, b) has justified the removal of these so-called aberrant species by comparing them with the type species (*B. bambos*) and other species with many shared features that together form the "type alliance". He established four new genera, i.e., *Holttumochloa*, *Kinabaluchloa*, *Maclurochloa* and *Soejatmia*, to accommodate such aberrant species, leaving a core group of *Bambusa* spp. in which only *B. farinacea* was indigenous to Peninsular Malaysia (Wong, 1993a, b). These four segregate genera, as well as *Neololeba* (Widjaja, 1997) and *Mullerochloa* (Wong, 2005), which are also genera described to accommodate taxa formerly misplaced in *Bambusa*, are listed in **Table 2.2** together with their previous placements. *Bambusa* was distinguished from its closely related genera, *Dendrocalamus* and *Gigantochloa*, by its elongated rachilla internodes of the spikelets (Wong, 1995a).

*Holttumochloa* is distinguished from the type alliance of *Bambusa* (Wong, 1993a, 1995a) based on the presence of multiple branch buds at each culm node, branch bud prophylls that are completely divided into two unequal portions, linear reflexed culm-sheath blades, and typically non-rebranching primary culm branches. The inflorescence of *Holttumochloa* consists of pseudospikelet clusters arranged along an axis developing from such a primary culm branch. (These primary culm branches

typically do not develop any vegetative side-branches, hence their description as 'non-rebranching', although in a strict morphological sense, they do branch if they eventually develop pseudospikelets.) This mode of branch development was unique among Southeast Asian bamboos. Thus far, this genus of three known species is endemic to the Malay Peninsula (Wong, 1993b). *Kinabaluchloa* (two known species) differs from the type alliance of *Bambusa* in having a reduced number of flowers in the pseudospikelet, branch bud prophylls with free margins and a branch complement formed of subequal branch axes. *Maclurochloa* (with a single known species in Peninsular Malaysia) differs from *Bambusa* in having a reduced number of (1–2) perfect flowers, more transitional glumes, and free margins in the branch bud prophyll. *Soejatmia* (also one species known) differs from typical *Bambusa* in having a transversely wrinkled thickened basal zone of the culm sheath, free margins in the branch bud prophyll, and a bifid palea in the flower. Wong (2005) transferred the Northern Australian species, *Bambusa moreheadiana*, into a new genus, *Mullerochloa* (**Table 2.2**). *Mullerochloa* differs from typical *Bambusa* in having clambering-scrambling culms, culm sheaths with a transversely wrinkled basal zone, reflexed culm-sheath blades, inconspicuous culm-sheath auricles, four stamens with fused filaments and a glabrous ovary (Wong, 2005). **Table 2.3** summarizes the vegetative and inflorescence characters of the type alliance of *Bambusa* versus these six segregate genera.

**Table 2.2:** A summary of taxonomic changes to various *Bambusa* species in the SE Asian and Australasian regions by Wong (1993a, 2005) and Widjaja (1997). There were no changes to *B. blumeana*, *B. heterostachya* and *B. vulgaris*, all known in most parts of Southeast Asia and Australasia only from cultivated material. The authorities of the scientific names are also shown in the table.

| **Species enumerated under *Bambusa*** | **Status of names** | **Accepted identities** |
|---|---|---|
| *B. atra* Lindl. | basionym of another genus | *Neololeba atra* (Lindl.) Widjaja |
| *B. forbesii* (Ridl.) Holttum | synonym | *Neololeba atra* |
| *B. burmanica* Holttum | incorrectly applied | *B. farinacea* K.M.Wong (newly named species) |
| *B. klossii* | | |
| (a) *B. klossii* Ridl. *sensu stricto* | synonym | *Dendrocalamus hirtellus* Ridl. (Holttum) |
| (b) *B. klossii*, *pro parte*, *sensu* Holttum | synonym | *Maclurochloa montana* (Ridl.) K.M.Wong |
| *B. magica* Ridley | basionym of another genus | *Holttumochloa magica* (Ridl.) K.M.Wong |
| *B. montana* (Ridl.) Holttum | basionym of another genus | *Maclurochloa montana* (Ridl.) K.M.Wong |
| *B. moreheadiana* F.M.Bailey | basionym of another genus | *Mullerochloa moreheadiana* (F. M. Bailey) K.M.Wong |
| *B. pauciflora* Ridley | synonym | *Maclurochloa montana* (Ridl.) K.M.Wong |
| *B. ridleyi* Gamble | basionym of another genus | *Soejatmia ridleyi* (Gamble) K.M.Wong |
| *B. wrayi* Stapf | basionym of another genus | *Kinabaluchloa wrayi* (Stapf) K.M.Wong |

**Table 2.3**: Summary of morphological characters of *Bambusa sensu stricto* (as represented by the type alliance) and two recently established genera, *Mullerochloa* and *Neololeba*: **(a)** vegetative characters; and **(b)** characters of pseudospikelets and flowers. Adapted from Wong (1993a; 2005) and Widjaja (1997), and augmented for the primary-branch bud prophyll character-state for *Neololeba* using *Eyma 1794* (SING) for *Neololeba amahussana* and *Nedi 487* (SING) for *N. atra*. Character-states distinct from *Bambusa sensu stricto* are italicized.

**(a)** vegetative characters

| | ***Bambusa sensu stricto*** | ***Holttumochloa*** | ***Kinabaluchloa*** | ***Maclurochloa*** | ***Mullerochloa*** | ***Neololeba*** | ***Soejatmia*** |
|---|---|---|---|---|---|---|---|
| Culm habit | erect, rigid | *scrambling, clambering* | *scrambling, clambering* | *scrambling / clambering* | *scrambling / clambering* | *scrambling / clambering* | *scrambling / clambering* |
| Culm internode, basal part | not swollen | not swollen | not swollen | not swollen | *swollen* | not swollen | not swollen |
| Branch complement | derived from solitary bud, with a dominant primary branch and lesser axes | *derived from multiple primary buds, developing into a cluster of subequal branchlets* | *derived from solitary bud, developing into a cluster of subequal branchlets* | derived from solitary bud, with a dominant primary branch and lesser axes | derived from solitary bud, with a dominant primary branch and lesser axes | derived from solitary bud, with a dominant primary branch and lesser axes | derived from solitary bud, with a dominant primary branch and lesser axes |
| Primary-branch bud prophyll | with fused margins | *completely divided into two unequal portions* | *with free margins* | *with free margins* | *with free margins* | with fused margins | *with free margins* |
| Culm sheath, basal part | smooth | smooth | smooth | smooth | *transversely wrinkled* | *transversely wrinkled* | *transversely wrinkled* |
| Culm sheath blade | blade often broad-domelike, erect (at least initially) to patent | *blade linear, reflexed* | *blade narrowly linear, reflexed* | *blade broad-lanceolate, patent- reflexed* | *blade narrow-lanceolate, patent-reflexed* | *blade broad- to narrow-lanceolate, erect-patent* | blade often broad-domelike, erect |
| Culm sheath auricles | conspicuous, ear-like lobes; bristly | *low; bristly* | *low, inconspicuous; glabrous* | *low rims; mostly glabrous* | *inconspicuous; glabrous* | conspicuous, ear-like lobes; bristly or glabrous | conspicuous, ear-like lobes; bristly |

**(b)** pseudospikelet and flower characters

| | ***Bambusa sensu stricto*** | ***Holttumochloa*** | ***Kinabaluchloa*** | ***Maclurochloa*** | ***Mullerochloa*** | ***Neololeba*** | ***Soejatmia*** |
|---|---|---|---|---|---|---|---|
| Number of perfect flowers in spikelet | 3–10 | 2–5 | *1– 2* | *2* | 4– 9 | 3–12 | *1–2* |
| Transitional (empty) glumes below flowers | 0–3 | 1– 2 | 1 | 3–5 | 1–2 | 0 | 1 |
| Rudimentary flowers between transitional glumes and perfect flowers | 0 | 0 | 0 | 0 | 0 | 0 | *1– 2* |
| Palea apex | rounded to acute, truncate or slightly cleft | rounded | rounded | rounded | acute to rounded | acute to rounded | *with 2 hooked tips* |
| Inner palea surface between keels | glabrous | glabrous | glabrous | glabrous | *densely flexuous-hairy* | *glabrous or sparsely short-hairy* | *sparsely long-hairy* |
| Ovary | obovoid-cylindric; apex thickened, hairy | ovoid-cylindric; apex thickened, hairy | slender cylindric, tapering apically; apex not thickened, glabrous to hairy | slender cylindric, tapering apically; apex not thickened, glabrous to hairy | narrowly ovoid to cylindric; apex not thickened, glabrous | gradually tapering upwards; apex not thickened, hairy | ovoid-cylindric; apex thickened, hairy |

Widjaja (1997) reduced *Bambusa forbesii*, an Australian species, and *Bambusa atra*, a species from New Guinea through to Sulawesi and the Philippines, to the synonymy of *Neololeba atra* (**Table 2.2**), one of five species she enumerated. *Neololeba* is different from typical *Bambusa* by its simpler branching system, smaller pseudospikelets, shorter rachilla internodes and lack of lodicules (Widjaja, 1997; Franklin, 2008).

The majority of taxonomic problems relating to *Bambusa* in South China is with subgeneric-level classification. For example, in the Flora of China account by Xia et al. (2007), the four subgenera (subgen. *Bambusa*, subgen. *Dendrocalamopsis*, subgen. *Leleba* and subgen. *Lingnania*) were distinguished in a simple key using a limited number of morphological characters, such as culm sheath blade width, culm internode length, culm wall thickness, prominence of culm sheath auricles, and presence of thorny branches. This is recognized as inadequate, as at the outset the authors state that "The taxonomy of China's bamboos still remains in a largely unrevised state. The majority of species has been described...frequently without knowledge of the flowers...Generic delimitation has often been highly speculative and remains controversial."

*Sinocalamus* McClure was established to accommodate taxa that appeared to McClure (1940) to vary too much from the *Bambusa* or *Dendrocalamus* species known to him. One of the main features of *Sinocalamus* according to McClure was the presence of 3 lodicules in the flower, in spite of these being lacking in the type species itself. However, McClure (1966) subsequently admitted that he could no longer uphold

*Sinocalamus* as a distinct genus, as its species could probably be accommodated in either *Bambusa* or *Dendrocalamus* (Sun et al., 2006).

Chia and Fung (1980) transferred those *Bambusa* taxa that were once in *Sinocalamus* into their *Bambusa* subgen. *Dendrocalamopsis*. Keng (1983) upgraded *Dendrocalamopsis* to genus rank and established a new genus, *Neosinocalamus,* with the type species *N. affinis*. *Neosinocalamus affinis* was described as having vegetative parts of typical *Lingnania* but the spikelet characteristics of *Dendrocalamopsis* and was placed in *Bambusa* subgen. *Lingnania* by Xia et al. (2007). Another subgenus, *Leleba,* was created to accommodate the *Bambusa* species that did not develop thorny basal branches. All thorny species of *Bambusa* were classified as *Bambusa* subgen. *Bambusa* (Xia et al., 2007). **Table 2.4** shows the few (and sometimes variable) morphological characteristics used for defining the four subgenera as elucidated in the Flora of China (Xia et al., 2007).

Wong (1993a, 2005) considered a suite of morphological characters he identified for the type alliance of *Bambusa* as potentially synapomorphic for the delimitation of *Bambusa*, i.e., which would be useful in distinguishing *Bambusa* from even the closely related *Dendrocalamus* and *Gigantochloa*. Although the type and its close alliance is fundamentally important from a nomenclatural perspective, a great many species placed in *Bambusa* and *Dendrocalamus* in subtropical and tropical Asia (especially in both China and India) still need to be taxonomically revised. In other words, character

variation in both type-alliance taxa and others still need to be better understood, and until then, morphological synapomorphies could be hard to assess.

In summary, the key genera in the Bambusinae, especially *Bambusa*, require taxonomic appraisal for their refinement. This situation is compounded by the large number of taxa, e.g., more than a hundred species in *Bambusa* alone (Xia et al., 2007) and 40 species of *Dendrocalamus* (Li and Stapleton, 2007), that should be further revised. The existing subgeneric classification seems unsatisfactory, and so this also needs to be investigated further. It is, therefore, vital to look into this problem through a broader, regional perspective. The entire realm of problems requires a more fundamental approach for its potential solution.

**Table 2.4:** Key morphological characteristics, geographical distribution and type species of the four subgenera of *Bambusa* according to the Flora of China (Xia et al., 2007).

| **Subgenus** | ***Bambusa*** | ***Dendrocalamopsis*** L.C.Chia & H.L.Fung | ***Leleba*** (Rumphius ex Nakai) P.C.Keng ex L.C.Chia & X.L.Feng | ***Lingnania*** (McClure) L.C.Chia & H.L.Fung) |
|---|---|---|---|---|
| Culm internode length | shorter than 30 cm | 30–110 cm | shorter than 30 cm | 30–110 cm |
| Wall thickness | to 2 cm | less than 8 mm, sometimes to 2 cm | to 2 cm | less than 8 mm, sometimes to 2 cm |
| Culm branching | arise at basal, mid-culm and distal nodes; usually 3 co-dominant | absent towards basal nodes; usually subequal | absent towards basal nodes; usually 3 co-dominant | absent towards basal nodes; usually subequal |
| Presence of thorns | weak or tough thorns | (nil) | (nil) | (nil) |
| Culm sheath | "thickly leathery"; blade persistent, "broad"; auricles nil to large | "thickly papery"; blade deciduous, "narrow"; auricles nil or small | "thickly papery"; blade deciduous, "broad"; auricles nil to large | "thickly papery"; blade deciduous,"narrow"; auricles nil or small |
| Number of species | more than 35 | 10 | more than 35 | about 14 |
| Geographical distribution | tropical and subtropical Asia | China and Myanmar | tropical and subtropical Asia | China and Vietnam |
| Type species | *B. bambos* | *B. oldhami* | *B. multiplex* | *B. chungii* |

### 2.1.2 Taxonomic problems in the genus *Dendrocalamus*

The delimitation of the genus *Dendrocalamus* has been contentious. This genus includes different groups of taxa with distinctive vegetative and floral characteristics (Wong, 1995a; Dransfield and Wong, 2004). *Dendrocalamus* appears to be most speciose in the region of SW China and Burma (Li and Hsueh, 1989) but extends to S and SE Asia (Ohrnberger, 1999).

*Dendrocalamus* consisted of eight species in Munro's monograph (Munro, 1868) but this number increased to 16 in Gamble's treatment in 1896. By that time, it was clear that some taxa could be difficult to assign between *Bambusa* and *Dendrocalamus*. Gamble listed several of such species, including *Bambusa balcooa*, *B. griffithiana* and *B. tulda* that had been transferred to *Dendrocalamus* but were considered by Gamble to belong better in *Bambusa*, where he maintained them. Still later, McClure (1940) segregated *D. latiflorus* and three *Bambusa* species to form the basis of his *Sinocalamus*, a move he apparently later regretted (see preceding section **2.1.1**).

Subsequently, Li and Hsueh (1988a, 1988b, 1989) supported a broad view of *Dendrocalamus* and adopted *Sinocalamus* as one of two subgenera in *Dendrocalamus*. Li and Stapleton (2006) have distinguished *Dendrocalamus* subgen. *Sinocalamus* as having "apically pendulous" culms without branching at the basal nodes; and subgenus *Dendrocalamus* as including species with culms "apically nodding" and branching at basal nodes. They further divided these two subgenera into five sections, i.e., Sect. *Dendrocalamus*, Sect. *Bambusoidetes*, Sect. *Draconicalamus*, Sect. *Sinocalamus* and

Sect. *Patellares*. Li and Stapleton (2006) have also transferred a few taxa they considered aberrant from Sect. *Patellares* to *Ampelocalamus*. The major morphological differences between the two subgenera are shown in **Table 2.5**.

**Table 2.5**. Key morphological characters, geographical distribution and type species of the two subgenera of *Dendrocalamus* according to Li and Stapleton (2006).

| **Subgenus** | ***Dendrocalamus*** | ***Sinocalamus*** |
|---|---|---|
| Culm habit | "apically nodding" | "apically pendulous" |
| Branches | arising from basal nodes; unequal with 1 – 3 dominant | absent at basal nodes; subequal |
| Culm sheath | "thickly papery" | "thickly leathery" |
| Pseudospikelet | "in spiny globose mass"; "light yellow-green" | "in soft globose mass"; "yellow brown" |
| Number of florets in pseudospikelet | (1 or) 2–4 | 2–8 |
| Geographical distribution | tropical Asia | SE Asia and China |
| Type species | *D. strictus* (Roxburgh) Nees | *D. latiflorus* Munro |

Among the Peninsular Malaysian species placed in *Dendrocalamus* by Wong (1995a), two groups could be discerned, distinguished by floral and pseudospikelet characters (Wong, 2004). *Dendrocalamus* sensu stricto (represented by the type species, *D. strictus* and its close allies) typically has 1–2 (rarely 3) perfect flowers, no terminal vestigial flower and the sole or uppermost palea not keeled or slightly keeled. This group appears to include *D. hirtellus* and *D. pendulus* from Peninsular Malaysia (Wong, 1995a), and several others such as *D. longispathus* and *D. membranaceus* (Wong, 2004). The other group of *Dendrocalamus* has 3–5 perfect flowers, a terminal vestigial flower in the pseudospikelet and the uppermost palea keeled. Wong (2004)

considers this second group as including *D. latiflorus* (the type species of *Sinocalamus),* as well as *D. asper* and *D. giganteus.*

As such, there is no real consensus on how *Dendrocalamus* should be defined. For nomenclatural reasons, the genus has to be delimited around the type species, but there is sufficient variation in pseudospikelet and even vegetative characters to create uncertainty in the assignment of a number of taxa.

## 2.2 Molecular phylogenetic studies of the Bambusinae

### 2.2.1 Delimitation and taxonomic position of the Bambusinae

Prior to the emergence of molecular phylogenetic analyses of the grass family, including the Bambusoideae sensu lato, Soderstrom and Ellis (1987) had proposed a classification where they grouped the "core" Bambusoideae (including a woody tribe, Bambuseae, and four other herbaceous tribes) using 10 morphological and anatomical character states that were differently and less encountered in groups considered less closely related. In their treatment, Bambuseae consisted of nine subtribes, one of which was Bambusinae. Bambusinae comprised 16 genera which they considered could probably be reduced to just six, i.e., *Bambusa*, *Dendrocalamus*, *Dinochloa, Klemochloa, Melocalamus* and *Thyrsostachys* (see **Table 2.1**). They did, however, acknowledge difficulties in distinguishing bamboo subtribes.

Subsequently, several molecular phylogenetic studies (Clark et al., 1995; Zhang, 2000; Grass Phylogeny Working Group, 2001) have revealed the non-monophyly of the

"core" Bambusoideae of Soderstrom and Ellis (1987). Only the woody bamboos, Bambuseae, and the herbaceous bamboos, Olyreae, could be interpreted as a monophyletic bambusoid group (Clark et al., 1995).

This bambusoid group (Bambusoideae sensu stricto) was also supported by recent studies using more robust molecular analyses (Bouchenak-Khelladi et al., 2008; Sungkaew et al., 2009; Grass Phylogeny Working Group II, 2012). The woody bamboos, however, are not a monophyletic group, as the temperate woody bamboo clade is sister to the Olyreae-tropical woody bamboo clade (Bouchenak-Khelladi et al., 2008; Sungkaew et al., 2009). This led to a proposal to reclassify Bambusoideae sensu stricto into three tribes, (1) Bambuseae sensu stricto (accommodating the paleotropical, Austral and neotropical bamboos), (2) Arundinarieae (temperate woody bamboos), and (3) Olyreae (the herbaceous bamboos) (Sungkaew et al., 2009).

Within the tropical woody Bambuseae, molecular phylogenetic studies have revealed distinct lineages representing the neotropical bamboos and the paleotropical bamboos (Clark et al., 1995; Kelchner and Clark, 1997; Sungkaew et al., 2009). Among the paleotropical bamboos, four subtribes are currently recognized, viz., Bambusinae, Hickeliinae, Melocanninae and Racemobambosinae (Bamboo Phylogeny Group, 2012).

Melocanninae was well defined by the likely morphological synapomorphies of a glabrous ovary with a long, slender, hollow style and an S-shaped keel in the foliage

leaf blade (Bamboo Phylogeny Group, 2012). It has been shown to be a monophyletic subtribe consisting minimally of *Cephalostachyum*, *Schizostachyum*, *Melocanna* and *Pseudostachyum* (Yang et al., 2007). It was consistently placed as the sister group of the core members of Bambusinae (i.e., *Bambusa*, *Dendrocalamus* and *Gigantochloa*) and all other climbing-scrambling bamboo genera of uncertain subtribe based on both nuclear DNA and cpDNA markers (Yang et al., 2007; Yang et al., 2008; Sungkaew et al., 2009).

On the other hand, as has also been acknowledged in Bamboo Phylogeny Group (2012), the current concept of Bambusinae may need further improvement because morphological synapomorphies are yet to be clearly identified. This working concept is largely based on the provisional inclusion of a number of genera around the core group comprising *Bambusa*, *Dendrocalamus* and *Gigantochloa* by Soderstrom and Ellis (1987). No actual circumscriptions of the Bambusinae have been made according to defining morphological characters, although generally the acceptance of genera making up the Bambusinae was influenced by various past groupings around *Bambusa* and *Dendrocalamus*, especially the basis of classifying paleotropical genera according to ovary and fruit characters proposed by Holttum (1956). This situation has been compounded by the more recent circumscription of smaller genera that could be defined more specifically by morphological characters, and which various authors (Wong, 1993; Dransfield and Widjaja, 1995; Sungkaew et al., 2008) have suggested could still be accommodated within the Bambusinae. There is, however, some recent evidence that suggests that this provisional placement may require reconsideration.

For example, molecular phylogenetic analyses have indicated some distinction between the core Bambusinae and various climbing genera once considered to be part of *Bambusa* itself. Inclusion of *Dinochloa* Büse in the Bambusinae was supported by the cpDNA marker but not the nuclear DNA marker (Yang et al., 2008). *Mullerochloa, Neololeba* and *Temochloa* were associated with the core Bambusinae to form a clade, sister to the Melocanninae, but they were not included in the well-supported core Bambusinae (Sungkaew et al., 2009).

### 2.2.2 Relationships among genera in Bambusinae

The long-standing problems of taxonomic delimitation among *Bambusa, Dendrocalamus* and *Gigantochloa* have hitherto not been satisfactorily addressed. Clearly, experimental design influences results that affect interpretation. Yang et al. (2008), using combined *trnT-L+GBSS*I+ITS data, recovered a topology in which *Bambusa* was apparently monophyletic, with *Dendrocalamus, Gigantochloa, Oxytenanthera* Munro and *Neosinocalamus* Keng f. forming a complex distinct from the *Bambusa* clade. However, this conclusion was only based on the Bayesian inference and a small number (seven) of *Bambusa* taxa. Later, the same research group indicated that only *Bambusa* sensu stricto is strongly supported as monophyletic in analyses that used the more informative cpDNA markers and the *GBSS*I gene (Yang et al., 2010).

In fact, non-monophyly of *Bambusa* was already suggested in the phylogenetic analysis based on ITS sequence for a small taxon sample by Sun et al. (2005), as

*Bambusa* taxa were intermixing with *Dendrocalamus strictus*, *D. latiflorus* and *D. membranaceus*. Monophyletic *Bambusa*, *Dendrocalamus* and *Gigantochloa* clades were also not recovered in the analyses of the combined cpDNA data *trnL-F* + *atpB-rbcL* + *rps*16 + *matK* by Sungkaew et al. (2009). Lineage complexity in the last mentioned work was also compounded by the strong affinity of species from these three genera with *Melocalamus* Benth., *Vietnamosasa* Nguyen, *Oreobambos* K.Schum, *Oxytenanthera*, *Neosinocalamus* and *Phuphanochloa* Sungkaew and Teerawat (Sungkaew et al., 2009). Thus, complex relationships have been consistently reported for *Dendrocalamus*, *Gigantochloa*, *Oxytenanthera* and *Neosinocalamus* (Yang et al., 2008; Yang et al., 2010).

The current subgeneric classification of *Bambusa* has been contentious (see section **2.1.1** above), although it was still employed in the recent account by Xia et al. (2007), by their clarification purely because there was no better system. An earlier investigation with DNA fingerprinting (Sun et al., 2006) revealed that the four subgenera of *Bambusa* had their sampled representatives mostly intermixed, i.e., not forming distinct clades, so that only species of subgenus *Bambusa* (albeit without the type species) clustered convincingly. At least with the small taxon sampling in that investigation, the subgeneric system appeared not to be tenable.

### 2.2.3 Molecular methods for bamboo phylogenetic studies

Most molecular phylogenetic studies on bamboos have utilized nuclear DNA and/or plastid DNA sequence data. Single-locus DNA regions were employed by the earlier phylogenetic studies, e.g., those using the cpDNA *rpl*16 intron for *Chusquea* (Kelchner and Clark, 1997) and for the Bambusoid group in Poaceae (Zhang, 2000), and those using the nrITS region for Alpine woody bamboos (Guo et al., 2001) and for *Bambusa* (Sun et al., 2005). More recently, investigations have utilized combined DNA datasets from multiple DNA regions. This includes potentially more informative characters for resolving phylogenetic relationships at lower taxonomic levels, because different DNA regions evolve at different rates and could be helpful in resolving different parts of the phylogeny. A number of studies have also shown that multi-gene analysis provides a better resolving power in phylogenetic studies of woody bamboos (Guo et al., 2004; Yang et al., 2007; Yang et al., 2008; Fisher et al., 2009; Sungkaew et al., 2009; Yang et al., 2010; Triplett and Clark, 2010; Zeng et al., 2010).

The Random Amplified Polymorphic DNA (RAPD) approach utilizes a number of universal primers to amplify random DNA fragments from genomic DNA and the DNA fragments of different sizes are separated in gel electrophoresis to produce a DNA fingerprint. This technique has been used by Hsiao and Rieseberg (1994) for assessing population genetic structure in *Yushania niitakayamensis* populations, Nayak et al. (2003) and Ramanayake et al. (2007) for investigating phylogenetic relationships among relatively small numbers (12 and nine) of tropical bamboo taxa, and Sun et al. (2006) for testing the validity of subgeneric classification within

*Bambusa*. RAPD analysis was able to infer a high genetic diversity among the *Y. niitakayamensis* populations in Taiwan (Hsiao and Rieseberg, 1994). However, phylogenetic inferences from RAPD analyses were confusing, e.g., the temperate bamboo, *Sasa* sp., was not distinguished from all other tropical bamboo genera (*Bambusa, Dendrocalamus* and *Cephalostachyum*) in Nayak et al. (2003), while an *Arundinaria* sp. was associated with *Dendrocalamus longispathus* and *Neololeba atra* to form a sister clade to the *Bambusa-Dendrocalamus-Gigantochloa* clade (Ramanayake et al., 2007). Furthermore, RAPD methods are relatively more predisposed to erroneous results because the nonstringent PCR condition required by RAPD could allow mismatch priming in RAPD and thus lead to artefactual DNA bands (Mueller and Wolfenbarger, 1999).

DNA fingerprinting using the Amplified Fragment Length Polymorphism (AFLP) approach, on the other hand, is more robust, highly reproducible and able to detect more polymorphisms (i.e., informative characters) compared to the RAPD approach. It was used by Loh et al. (2000) and Pattanaik and Hall (2011) in their phylogenetic studies of the major genera within Bambusinae. Several interesting clustering patterns were observed in the phylogenetic trees. *Bambusa lako* (Timor Giant Black), a taxon which has been confused with *G. atroviolacea* in having black-purplish culms (Widjaja, 1997), was found in the *Gigantochloa* clade (Loh et al., 2000). *Dinochloa macclellandii*, a taxon which does not possess a vining and climbing habit like other *Dinochloa* taxa do (Koshy, 2010), was intermixed with *Bambusa* and *Dendrocalamus* taxa (Pattanaik and Hall, 2011). There is, however, controversy in using AFLP for

phylogenetic studies because the DNA bands obtained for different samples could be non-homologous (Mueller and Wolfenbarger, 1999). Hodkinson et al. (2000) have shown that AFLP is more useful than nrITS sequence in distinguishing sections and sub-sections of *Phyllostachys*, whereas Suyama et al. (2000) have depicted the clonal structure of a large (i.e., 10 ha) population of *Sasa senanensis* based on the AFLP fingerprints. Triplett et al. (2010), using the AFLP approach, demonstrated a complex relationship among the closely related *Arundinaria appalachiana, A. gigantea* and *A. tecta,* each of them represented by a number of individuals across their geographical ranges. This suggests that AFLP could be more suitable for analyses at lower taxonomic levels.

The inter-simple sequence repeat (ISSR) marker was shown to be useful in understanding the genetic structure of *Dendrocalamus giganteus* populations in China (Tian et al., 2011) but not informative in resolving phylogenetic relationships within Bambusinae (Mukherjee et al., 2010). Microsatellite markers were established for *Bambusa arnhemica* in North Australia and the microsatellite DNA variation was used to indicate the pattern of gene flow among the *B. arnhemica* populations (Kaneko et al., 2008).

## 2.3 Hybridization in bamboos

### 2.3.1 Hybrid swarms and ancient enduring clones (AECs)

Bamboos have long been cultivated by the peoples of India, China, Japan and Southeast Asian countries because of their economic and cultural importance. In a wider context, bamboos may also have been transported along the ancient maritime spice routes between China, Indonesia, Sri Lanka and India (Soderstrom and Calderón, 1979). Holttum (1958) used the label "village bamboos" to refer to the cultivated bamboos in SE Asia that did not seem to occur in the wild state where they are grown. In this connection, he suggested that a number of cultivated *Gigantochloa* taxa could have been brought to Java and Malaya with past human migration through the region, from the Lower Burma region where the genus appears most diverse. Holttum (1958) also deduced the existence of hybrid swarms among closely related *Gigantochloa* taxa, based on the bewildering morphological variation among the wild *Gigantochloa* bamboos (especially in the *G. latifolia-G. ligulata* complex) in the northern Malay Peninsula, suggesting also that selected clones could have been maintained in cultivation. Increased variation has been described as a major consequence of introgressive hybridization (Anderson and Hubricht, 1938; Anderson, 1948). From the repeated backcrosses to one or to both parents, the introgressants would resemble the parental species to some degree and form a hybrid swarm. They could have been treated as 'varieties' or 'aberrant individuals' of the parental species (Anderson, 1948).

Holttum's suggestion, however, did not find immediate agreement with the morphology-based numerical analysis by Widjaja and Lester (1987), although they

acknowledged that more studies of Thai and Burmese taxa were needed. Although their combined analyses based on morphology, anatomy, phenolic compounds and protein electrophoresis suggested the distinctiveness of the 18 *Gigantochoa* taxa they analyzed, Widjaja and Lester (1987) nevertheless recognized that some taxa did have special affinities, i.e., *G. atter* and *G. atroviolacea* were closely related, and *G. achmadii, G. hasskarliana, G. latifolia, G. manggong, G. nigrocilliata, G. pruriens* and *G. rostrata* probably formed a closely related group.

Muller (1996) noted that there are many more *Gigantochloa* clones which do not easily fall within the 18 species circumscribed by Widjaja (1987). Subsequently, he also presented arguments for at least some of this variation and "strange reproductive behaviour" to be due to hybrid derivation (Muller 1998, 2003). Among a few *G. ridleyi* clumps that Muller brought to Mount Mirinjo Farm, Australia, one flowered and what must have been self-fertilization produced an extremely low seed set. Half of the seedlings that germinated were albinos that subsequently also perished, while the remaining seedlings later displayed vegetative morphological characteristics that were highly dissimilar among themselves and even from the parent clumps. Muller (1998, 2003) compared such variation in the offspring morphology to that found among the F2 progeny in the selfing of a hybrid F1 as was surmised by Holttum (in McClure 1966: 178).

Muller (1999) suggested that the bamboo clones that existed only in cultivation in Indonesia and Malaysia were "ancient enduring clones" (heretoafter referred to as

AECs). These would have included selections from hybrid swarms described above, that could have originated much farther north (such as in the Burmese region) and were brought to where they are by the ancient migration of human communities, as suggested by Holttum (1958).

More examples of bamboo taxa showing characteristics of AECs were given by Wong (2004). Both Wong (2004) and Muller (1999) regarded relative infertility (maintenance of a long vegetative phase, limited rather than whole-clump flowering that did not cause death of the clone, poor seed set) as a general characteristic that could have been selected for in AECs because these characteristics made the clones more durable in cultivation and utilization. This selection has not been always successful in this way, for instance, death following flowering of Thai *Dendrocalamus asper* raised in plantations led to severe economic loss for their farmers (Thammincha et al. 1995; Muller 1996).

Muller (2003) also described how roughly half of all seedlings from the extreme low seed set of a selfing event produced albino seedlings (which died soon). This phenomenon is, again, probably an indication of the hybrid origin of the selected *Gigantochloa* clones. In fact, albinism in the interspecific hybrids has been reported for various plant groups, i.e., *Impatiens* (Arisumi, 1985), *Trifolium* (Panday et al., 1987), *Zantedeschia* (Yao et al. 1994; Yao et al., 1995), *Hibiscus* (van Laere et al., 2007) and *Rhododendron* (Eeckhaut et al., 2007). Gene deletion and incompatibility between the nuclear and chloroplast genome were suggested to have resulted in such a

pigment defect (Kirk and Tilney-Bassett, 1978; Yao et al., 1994; Yao et al., 1995). Albinism is a lethal recessive trait controlled by one or more gene loci (reviewed by Kumari et al., 2009), implying that the parents of the albino *G. ridleyi* had a degree of heterozygosity for the chlorophyllous (green) condition, and the surviving (green) seedlings would still retain this variation.

There are, however, insufficient observations to say if green or striped culm forms may be specially linked to flowering habits that affect, for example, clonal longevity. In support of the ease with which potential genetic traits could be recognized from simple clonal characteristics, Wong (2004) described how wholly green and pale-striped culm forms of *G. balui* found in two forest localities in Peninsular Thailand apparently flowered separately at different times, and how both green and pale-striped culm forms are frequently found in natural *G. ligulata* populations in the north of Peninsular Malaysia.

In the same way, *Dendrocalamus strictus* in India is a well-known polymorphic species. Seedling-derived populations of this species vary widely in overall size, culm habit, culm wall thickness, texture and pubescence of the culm leaf, relative congestion of culms in a clump, and drought resistance levels (McClure, 1966). Indeed, a number of extreme forms of *D. strictus* have been recognized as varieties, including a variegated form (McClure, 1966), so clearly a variable natural population provides the different clones that could be selected for various purposes, and it is not surprising if linked traits, such as clonal longevity, also became the basis for selection.

### 2.3.2 Other possible indications of hybridization among woody bamboos

Wide infraspecific variation, morphological intermediacy and unclear species boundaries

*Bambusa* is highly diverse—the Flora of China alone has recorded 90 species in four subgenera and listed 14 affiliated taxa as *incertae sedis* (of uncertain subgeneric position) in this genus (Xia et al., 2007). Seven *Bambusa* taxa were reported to have 2–4 varieties. Most of these varieties were found only in cultivation (Xia et al., 2007). Morphological intermediacy has also been noted between and among some taxa and taken as a possible indication of hybridity. Thus, *Bambusa pervariabilis* was suggested as a hybrid between *B. tuldoides* and *B. eutuldoides* (McClure, 1940). Another taxon, *Bambusa affinis* was formerly considered as representing a distinct genus, *Neosinocalamus*, because Keng (1983), in naming that genus, regarded it as having vegetative parts typical of *Lingnania* (now subsumed in *Bambusa* as one of its subgenera) but the spikelet characteristics of *Dendrocalamopsis* (or subgen. *Dendrocalamopsis*). *Bambusa intermedia* was described as "intermediate between *Bambusa* subgen. *Leleba* and *B.* subgen. *Lingnania*" (Xia et al., 2007). It is apparent that the taxonomy of *Bambusa* confounded by such morphological intergradation as observed could benefit from critical assessment based on a hybridization hypothesis.

Sterility

Because meiotic anomalies are common in hybrids and lead to comparative sterility, the latter could be an indication of hybrid origin. Sterility in bamboos is expressed as very long vegetative phases and relative infrequency of flowering or seeding, or even

lack of seed viability. **Table 2.6** lists documented incidences of seeding among the bamboos in Peninsular Malaysia (thus a sample from a discrete geographical unit where the inventory and taxonomy of bamboos are reasonably well understood) for identifying those that have never been known to set seed.

**Table 2.6.** Indications of seed formation in Peninsular Malaysian bamboos as documented in Wong (1995a) or other sources (specially indicated).

Annotations–[1]Seeding not yet documented in Peninsular Malaysia, but known in wild Bangladesh populations (Banik, 1980); [2]K.M. Wong, pers. comm.; [3]Muller (1998); [4]Seethalakshmi and Muktesh Kumar (1998); [5]Xia et al. (2007); [6]Li and Stapleton (2007); [7]Widjaja and Dransfield (1995); [8]Bhanwra et al. (2008).

| Taxon | Documented seeding |
|---|---|
| (**A**) *Indigenous taxa (wild populations in Peninsular Malaysia)* | |
| *Bambusa farinacea* | Yes |
| *Dendrocalamus dumosus* | Yes |
| *D. elegans* | Unknown |
| *D. hirtellus* | Yes |
| *D. longispathus* | Unknown[1] |
| *D. pendulus* | Yes |
| *D. sinuatus* | Yes |
| *Gigantochloa albovestita* | Unknown |
| *G. holttumiana* | Unknown |
| *G. latifolia* | Yes |
| *G. ligulata* | Yes |
| *G. rostrata* | Yes |
| *G. scortechinii* | Yes |
| *G. wrayi* | Yes |
| *Holttumochloa korbuensis* | Yes |
| *H. magica* | Yes |
| *H. pubescens* | Unknown |
| *Kinabaluchloa wrayi* | Unknown |
| *Maclurochloa montana* | Yes |
| *Racemobambos setifera* | Yes |
| *Schizostachyum aciculare* | Unknown |
| *S. gracile* | Yes |
| *S. grande* | Yes |
| *S. latifolium* | Yes[2] |
| *S. lengguanii* | Yes[2] |
| *S. terminale* | Yes |
| *S. zollingeri* | Yes |
| *Soejatmia ridleyi* | Yes |

**Table 2.6 (cont'...)**

| Taxon | Documented seeding |
|---|---|
| **(B)** *Cultivated taxa not known in the wild* | |
| *Bambusa blumeana* | Unknown |
| *B. heterostachya* | Yes |
| *B. laxa* | Unknown |
| *B. multiplex var. rivereorum* | Unknown |
| *B. vulgaris* | Unknown |
| *B. vulgaris cv. vittata* | Unknown |
| *B. vulgaris cv. wamin* | Unknown |
| *Dendrocalamus asper* | Unknown |
| *Gigantochloa albopilosa* | Unknown |
| *G. ridleyi* | Unknown (but seeding reported elsewhere[3]) |
| *G. thoii* K.M.Wong | Unknown |
| *Schizostachyum brachycladum* Kurz | Unknown |
| *S. jaculans* Holttum | Unknown |
| **(C)** *Cultivated exotic taxa (observed for clones in Peninsular Malaysia)* | |
| *Bambusa bambos* (Indian subcontinent, Burma, Thailand) | Unknown (seeds in native area[4]) |
| *B. multiplex* (S China) | Unknown (Also unknown elsewhere[5]) |
| *B. ventricosa* (as 'B. tuldoides' in Wong 1995a) (S China) | Unknown (Also unknown elsewhere[5]) |
| *Chimonobambusa quadrangularis* (S China) | Unknown (Also unknown elsewhere[6]) |
| *Dendrocalamus giganteus* (Lower Burma, S China) | Yes (seeds in native area[6]) |
| *D. strictus* (India to Burma and west Thailand) | Unknown (seeds in native area[4]) |
| *Gigantochloa hasskarliana* (Sumatra, Java, Bali) | Unknown (seeds in native area[7]) |
| *Melocanna baccifera* (East Bengal region of India and Bangladesh) | Yes (seeds in native area[4]) |
| *Schizostachyum iraten* (Java, Sumatra, Bali) | Unknown (Also unknown elsewhere[7]) |
| *Thyrsostachys siamensis* (Burma and Thailand) | Unknown (seeds in native area[8]) |

Seeding has been reported for most (i.e., 21 out of 28 in the present sample) of the indigenous bamboo taxa in Peninsular Malaysia or other native areas (Banik, 1980; Wong, 1995a; K.M. Wong, pers. comm.; **Table 2.6(A)**). On the contrary, almost all taxa that were known only in cultivation (i.e., not known anywhere in the wild condition) (11 out of 13 in the present sample) were apparently unable to set seed or did so only to a limited extent (**Table 2.6(B)**). Among them, the widely cultivated *B. vulgaris* is well-known for its sterility (McClure, 1966) and considered most unnatural for a species (Muller, 1999). Specific reasons for the absence of seed set in *B. vulgaris* have been identified as low pollen viability, meiotic irregularities in pollen mother cells and failure of pollen tube elongation on the stigma (Koshy and Jee, 2001). Similarly, *B. multiplex*, *B. ventricosa*, *Chimonobambusa quadrangularis* and *Schizostachyum iraten* have not been known to produce seed even in their native areas (**Table 2.6(C)**; Dransfield and Widjaja, 1995; Li and Stapleton, 2006; Xia et al., 2007).

Given the scenario of hybrid swarms being a possible source of cultivated clones maintained by human societies, and the apparent sterility associated with most or all of such clones, there is thus some justification to more carefully investigate the role of hybridity in relation to such sterility. Koshy and Jee (2001) further showed that the root-tip cells of *B. vulgaris* frequently demonstrated mosaicism (inconstancy of the somatic diploid chromosomal count). Mosaicism is common in vegetatively propagated monocots (Sharma, 1956) and much associated with hybridity, polyploidy or chemical treatment (Koshy and Jee, 2001).

Self-incompatibility could also contribute to relative sterility (Janzen, 1976) so not every case of sterility should simply imply a background of hybridity. *Bambusa bambos*, *Dendrocalamus strictus*, *Gigantochloa hasskarliana* and *Thyrsostachys siamensis* do not set seed in Peninsular Malaysia, where they were introduced, but seed in their native areas (**Table 2.6(C)**). McClure (1973) had already observed that wild bamboos had higher incidences of seed set compared to cultivated clones, and there are also records of seed set being absent for about 40 species of introduced bamboos in Taiwan (see Janzen 1976). Moreover, cross pollination seems important to seed set in bamboos (Janzen, 1976); for instance, *Dendrocalamus strictus* did not set seed when its inflorescences were shielded from wind pollination (Nadguada et al., 1993), and *G. scortechinii* did not appear to produce seed when flowering as solitary clumps (Wong, 1995a).

However, seeding records for isolated clumps of bamboo, such as *B. bambos* (Indira, 1988; Adarshkumar et al., 1995), *G. ridleyi* (Muller, 1998) and *G. rostrata*, (Wong, 1995a) suggest that some bamboo taxa are not completely self-incompatible. In fact, natural populations of most angiosperms have a wide range of self-fertilization rates, which are correlated with a number of factors of reproductive and vegetative ecology (Lloyd, 1979). Selfing is advantageous when new populations are founded as a single individual, when the environmental conditions (such as drought and cold) prohibit pollen dispersal, and when the individuals are sparsely distributed across a landscape (Baker, 1955; reviewed by Wells, 1979; Pannell and Barnett, 1998). Many plant

species show higher rates of self-fertilization in the disturbed habitats compared to the undisturbed habitats (Eckert et al., 2010).

Albino seedlings

Besides the incidence of albino seedlings discussed above in the context of a selfing episode in *G. ridleyi* (Muller 1998, 2003), there are also other reports on similar aberration in bamboo seedlings. Some seedlings with variegated leaves were found under flowering clumps of *Dendrocalamus giganteus* in the Kandy District of Sri Lanka, while a number of *in vitro* germinated seedlings were albino (Ramanayake and Yakandawala, 1998). Each of two flowering clumps of *B. bambos* in Kerala, India, were reported to produce albino seedlings at 1:3 (green : albino) ratio, suggesting that albinism is a homozygous recessive trait produced by selfing of the heterozygous parent (Indira and Koshy, 1986; Indira, 1988; Adarshkumar et al., 1995). Albinism in seedlings has also been documented for *Dendrocalamus strictus* (Babeley and Kandya, 1984; Yadav et al., 1987; Kumar et al., 1993; Rane et al., 2010) and *Ochlandra travancorica* (Abdul Kader et al., 2001). Seedlings that were albino or had other abnormalities such as stunted radicle, leafless plumule or upwardly growing radicles have also been observed during incidences of seeding in *Melocanna baccifera* (Dakshinadas, 1995; Seethalakshmi and Muktesh Kumar, 1998).

### 2.3.3 Detection of hybridization in bamboos

Hybridization in bamboos is often easiest to hypothesize based on morphological intermediacy and flowering synchrony between putative parents. *Sasaella* was suggested to be an intergeneric hybrid between *Sasa* and *Pleioblastus* as *Sasaella* shows intermediate morphological characteristics of the two parental genera, such as the number of stamens in a floret which varies from 3 to 6 (Suzuki, 1987; Watanabe et al., 1991), the length of the first glume and the length of anthers (Watanabe et al., 1991). Furthermore, *Sasaella* spp. had lower pollen fertility, i.e., less than 50%, compared to either *Sasa* spp. or *Pleioblastus chino* Makino (Watanabe et al., 1991). Likewise, the genus *Hibanobambusa* was established to accommodate a natural hybrid between a *Semiarundinaria* species and *Sasa veitachii* var. *hirsuta*, inferred from the morphological intermediacy and low fertility of the putative hybrid, as well as the coincidence of the flowering period of both parental species (Murayama et al., 1979). Three natural hybrids in *Chusquea* sect. *Swallenochloa*, *Chusquea subtessellata* × *C. amistadensis*, *C. subtessellata* × *C. vulcanalis* and *C. spencei* × *C. tessellata*, were also detected based on intermediate morphological and anatomical characteristics, and the flowering coincidence of their respective parental species (Clark et al., 1989).

Experimental crosses have also provided insight for detecting natural hybrids. Four successful experimental hybrids were produced by Zhang and Chen (1980), namely Hybrid No. 1 (*Bambusa pervariabilis* × *Dendrocalamus latiflorus*), Hybrid No. 4 and No. 11 (*B. textilis* × *D. latiflorus*), Hybrid No. 5 (*D. minor* × *D. latiflorus*) and Hybrid No. 25 (*B. pervariabilis* × *D. latiflorus*). These crosses produced bamboo hybrids with

desirable traits including fast growth, cold resistance, high asexual productivity and somewhat ornamental light yellow stripes along the lower internodes. The morphology and chromosome counts of the bamboo hybrids were verified as intermediate between the parental species. On this basis, as well as the resemblance of Hybrid No.11 to *B. stenoaurita*, Zhang (1985) suspected that the latter was a natural F1 hybrid between *B. textilis* and *D. latiflorus*. Indeed, *B. stenoaurita* has proven difficult to classify and has been transferred from one genus to another without clear resolution (Xia et al. 2007, Yang et al. 2010). The successful artificial crosses of *Pleioblastus* × *Phyllostachys* and *Pleioblastus* × *Sasa* suggested that weak crossing barriers may also exist among temperate woody bamboos, which would theoretically enable both inter-specific and inter-generic hybridization (Muramatsu, 1981).

Molecular techniques have now shown to be a promising tool in demonstrating hybridization in the bamboos. Hybridization has been suggested as a cause of taxonomic difficulties in the *Arundinaria* complex (McClure, 1973) but this has only been investigated genetically when Triplett et al. (2010) demonstrated an F1 natural hybrid between *A. gigantea* and *A. tecta* using the Amplified Fragment Length Polymorphisms (AFLP) technique and cpDNA phylogenetic analysis. Furthermore, multiple, reciprocal hybridization and introgression events were implicated based on the complex mosaic pattern of the genetic composition in the three hybrid individuals. Their complex origin involves not only *A. gigantea* and *A. tecta*, but also *A. appalachiana* (Triplett et al., 2010).

### 2.3.4 High ploidy level and hybridity: inter-related?

Polyploidy among the woody bamboos seems to be a remarkably constant feature with few exceptions of documented diploid woody bamboos (Pohl and Clark, 1992). It has been suggested that this increase in ploidy level accompanied the evolution of woodiness in bamboos from a largely diploid herbaceous ancestor (Soderstrom, 1981), although this could be said to be a very simplistic model.

Chromosome records of woody bamboos (Soderstrom, 1981; Hunzinker et al., 1982; Zhang, 1985; Clark et al., 1989; Pohl and Clark, 1992; Koshy and Jee, 2001; Chen et al., 2004) show that apparently consistent ploidy levels characterize each woody bamboo clade: paleotropical Bambuseae are mainly hexaploid; neotropical Bambuseae, tetraploid; and the Arundinarieae (temperate woody bamboos), also tetraploid. Woody bamboo genera have a typical basic number of $x = 12$, with the exception of *Chusquea* ($x = 10$; Clark et al., 1989).

However, woody bamboos are not a single major lineage. The herbaceous Olyreae is a sister group to tropical woody bamboos (Bambuseae), both arising from the same lineage that is in turn sister to the temperate bamboos (Arundinarieae) (Bouchenak-Khelladi et al., 2008; Sungkaew et al., 2009). The Olyreae consists of both diploid and polyploid members (mainly $x = 10$, 11 or 12, or occasionally other lower basic numbers as a result of aneuploidy (Hilu, 2004). The co-existence of the woody bamboos and herbaceous Olyreae within the Bambusoid clade thus raises the question of whether the Bambusoideae ancestor as a whole was diploid or polyploid, although

the woody sub-lineages themselves (such as the Arundinarieae or the Bambuseae as now recognized) are likely to have developed from polyploid most recent common ancestors.

Ploidy increases and past introgression and reticulate evolution has been suggested to be significant among grasses. Polyploidy is common in Ehrhartoideae and Pooideae, and the diploid ancestors are known in many cases (Bowden, 1959, 1960a, 1960b, 1961; Cotton and Stace, 1976; Stebbins, 1981; Armstrong, 1991; Kellogg et al., 1996; Kellogg, 2009). In the Pooideae, widespread hybridization has been demonstrated within the Triticeae (Mason-Gamer, 2004; Mason-Gamer et al., 2010) and the Airinea subtribe (Soreng et al., 2007). There is evidence for many natural hybrids (e.g., those between *Poeae* and *Stipeae*, *Calamagrostis* and *Ammophila*, *Calamagrostis* and *Agrostis*, *Agrostis* and *Polypogon*, *Festuca* and *Vulpia*) (Soreng and Davis, 2000; Döring et al., 2007). In the Chloridoideae, many polyploid complexes have been detected, suggesting extensive hybridization within this subfamily (Roodt and Spies, 2003). Among the woody bambos, natural hybridizations have been reported for the neotropical *Chusquea* (Clark et al., 1989) and introgressive hybridization is demonstrated in the temperate Arundinarieae (Triplett and Clark, 2010; Triplett et al., 2010). Thus it seems probable that among many groups of grasses including the woody bamboos, ploidy increases and other chromosomal rearrangements have paralleled the occurrence of hybridization and reticulate evolution.

What is the source of this polyploid development for the various lineages? In the most simplistic model, as explained by Harlan and deWet (1975), polyploidy could be most commonly generated as triploids via (2n+n) reproduction following production of unreduced gametes on one parental side. Such triploids would then be able to yield 4*x* plants on backcrossing, and 6*x* plants on selfing. They also point out that wide crosses by way of (2n+n) reproduction are probably a common and successful pathway, as already documented by various workers (Stebbins, 1983; Hilu, 1985). Overall, Harlan and deWet (1975) conclude that the spontaneous generation of polyploids may be a very frequent incidence, but whether or not such polyploids persist would depend on their vigor and competitive ability, in other words, successful adaptation to the particular environment at hand.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Field collection of voucher specimens and materials for molecular work

Voucher specimens were collected following the guidelines by Soderstrom and Young (1983) for collecting bamboos, i.e., shoots, culm leaf, culms, branch complements, leafy branches and inflorescence (whenever available) were collected, pressed and dried in the oven at 65 $^{o}$C for two weeks. The dried materials were mounted and stitched on the herbarium sheets. On the labels, general data such as date of collection, locality and habitat, and other characteristics were stated. Voucher specimen reference numbers, the herbaria where deposited and collection localities for each sample were listed in **Table 3.1**. Young leaves (which are still rolled up) were preferred for molecular studies. The leaves collected for each species were preserved with silica gel and kept at room temperature.

**Table 3.1:** List of bamboo taxa collected for this study, their voucher specimen reference numbers and collection localities.

| Taxa | No. of accession | Collector(s), Voucher No. (Herbarium) | Localities |
|---|---|---|---|
| *Bambusa arnhemica* | 1 | Franklin et al., 112 | Daly River, Australia S 13°56.700' E 131°11.633' |
| *Bambusa bambos* | 1 | Goh, Univ. of Malaya Bambusetum Acc. 78 | Rimba Ilmu Botanic Garden, Malaysia |
| *Bambusa bambos* | 2 | K.C.Koshy, 61223 (TBGT) | TBGRI, Kerala, India |
| *Bambusa blumeana* | 1 | Goh, Univ. of Malaya Bambusetum Acc. 11 | Rimba Ilmu Botanic Garden, Malaysia |
| *Bambusa boniopsis* | 1 | Zheng, Zheng C.H.37 (IBSC) | South China Botanical Garden, China |
| *Bambusa burmanica* | 1 | Zheng, Zheng C.H.185 (IBSC) | South China Botanical Garden, China |
| *Bambusa distegia* | 1 | Zheng, Zheng C.H.70 (IBSC) | South China Botanical Garden, China |
| *Bambusa eutuldoides var. viridivittatta* | 1 | Yang, YHQ08153 (SWFC) | Kunming World Expo Grounds, China |
| *Bambusa farinacea* | 1 | Goh, Univ. of Malaya Bambusetum Acc. 57 | Rimba Ilmu Botanic Garden, Malaysia |
| *Bambusa farinacea* | 2 | Wong, WKM2897 (KLU) | Johor, Malaysia |
| *Bambusa flexuosa* | 1 | Zheng, Zheng C.H.3 (IBSC) | South China Botanical Garden, China |
| *Bambusa gibba* | 1 | Zheng, Zheng C.H.16 (IBSC) | South China Botanical Garden, China |
| *Bambusa grandis* | 1 | Zheng, Zheng C.H.85 (IBSC) | South China Botanical Garden, China |
| *Bambusa intermedia* | 1 | Yang, YHQ08154 (SWFC) | Kunming World Expo Grounds, China |
| *Bambusa multiplex* | 1 | Zheng, Zheng C.H.51 (IBSC) | South China Botanical Garden, China |
| *Bambusa oldhamii* | 1 | Zheng, Zheng C.H.80 (IBSC) | South China Botanical Garden, China |

**Table 3.1 (cont'...)**

| Taxa | No. of accession | Collector(s), Voucher No. (Herbarium) | Localities |
|---|---|---|---|
| *Bambusa polymorpha* | 1 | K.C.Koshy, 61645 (TBGT) | TBGRI, Kerala, India |
| *Bambusa sinospinosa* | 1 | Sunye, Sunye15 (IBSC) | Xishuangbanna Tropical Botanical Garden, China |
| *Bambusa textilis* | 1 | Zheng, Zheng C.H. 47 (IBSC) | South China Botanical Garden, China |
| *Bambusa tuldoides* | 1 | Goh, Univ. of Malaya Bambusetum Acc. 58 | Rimba Ilmu Botanic Garden, Malaysia |
| *Bambusa valida* | 1 | Zheng, Zheng C.H. 81 (IBSC) | South China Botanical Garden, China |
| *Dendrocalamus elegans* | 1 | Goh, Univ. of Malaya Bambusetum Acc. 73 | Rimba Ilmu Botanic Garden, Malaysia |
| *Dendrocalamus hirtellus* | 1 | Goh & Wong, GWL3 (KLU) | Hulu Langat, Selangor, Malaysia |
| *Dendrocalamus khoonmengii* | 1 | Sungkaew, SS&AT257 (KUFF, TCD) | Peninsular Thailand |
| *Dendrocalamus pendulus* | 1 | Goh & Wong, GWL6 (KLU) | Rimba Ilmu Botanic Garden, Malaysia |
| *Dendrocalamus pendulus* | 2 | Goh et al., s.n. | Gombak Road, Selangor, Malaysia |
| *Dendrocalamus pendulus* | 3 | Goh et al., s.n. | Gombak Road, Selangor, Malaysia |
| *Dendrocalamus strictus* | 1 | Zheng, Zheng C.H.90 (IBSC) | South China Botanical Garden, China |
| *Dendrocalamus strictus* | 2 | K.C.Koshy, 64501 (TBGT) | TBGRI, Kerala, India |
| *Dinochloa malayana* | 1 | Goh, Univ. of Malaya Bambusetum Acc. 59 | Rimba Ilmu Botanic Garden, Malaysia |
| *Dinochloa scabrida* | 1 | Dransfield, JD5134 (KEP) | Rimba Ilmu Botanic Garden, Malaysia |
| *Dinochloa* sp. | 1 | Goh, Univ. of Malaya Bambusetum Acc. 13 | Rimba Ilmu Botanic Garden, Malaysia |
| *Dinochloa trichogona* | 1 | Jumian, SAN152334 (SAN) | Sepilok, Sandakan, Sabah |

**Table 3.1 (cont'...)**

| Taxa | No. of accession | Collector(s), Voucher No. (Herbarium) | Localities |
|---|---|---|---|
| ×*Gigantocalamus malpenensis* | 1 | Goh et al., GWL13 (KLU) | Hulu Gombak valley, Selangor, Malaysia N 03°19.816'; E 101°45.570' |
| ×*Gigantocalamus malpenensis* | 2 | Goh et al., GWL14 (KLU) | Hulu Gombak valley, Selangor, Malaysia N 03°19.829'; E 101°45.574' |
| ×*Gigantocalamus malpenensis* | 3 | Wong et al., WKM2895 (KLU, SING, ISC) | Tapah-Cameron Highlands Road, Peninsular Malaysia |
| *Gigantochloa apus* | 1 | Sussman, 149 (KLU) | 4769 Live Oak Canyon Rd, La Verne, CA USA |
| *Gigantochloa atter* | 1 | Sussman, s.n. 2010 (KLU) | 4769 Live Oak Canyon Rd, La Verne, CA, USA |
| *Gigantochloa balui* | 1 | Goh & Wong, GWL6 (KLU) | Rimba Ilmu Botanic Garden, Malaysia |
| *Gigantochloa latifolia* | 1 | Goh & Lee, GWL8 (KLU) | Rimba Ilmu Botanic Garden, Malaysia |
| *Gigantochloa ligulata* | 1 | Goh & Lee, GWL7 (KLU) | Rimba Ilmu Botanic Garden, Malaysia |
| *Gigantochloa scortechinii* | 1 | Goh & Wong, GWL2 (KLU) | Hulu Langat, Selangor, Malaysia |
| *Gigantochloa scortechinii* | 2 | Goh et al., s.n. | Hulu Gombak valley, Selangor, Malaysia N03°19.667' E 101°45.283' |
| *Gigantochloa scortechinii* | 3 | Goh & Low, GWL9 (KLU) | Road from Kuala Kubu Baru to Fraser Hill, Selangor, Malaysia |
| *Gigantochloa scortechinii* | 4 | Low et al., s.n. | Chebar, Kedah, Malaysia |
| *Gigantochloa scortechinii* | 5 | Goh, Univ. of Malaya Bambusetum Acc. 52 | Rimba Ilmu Botanic Garden, Malaysia |
| *Gigantochloa wrayi* | 1 | Goh & Wong, GWL4 (KLU) | Rimba Ilmu Botanic Garden, Malaysia |
| *Guadua cf. chacoensis* | 1 | Zheng, Zheng C.H.76 (IBSC) | Xishuangbanna Tropical Botanical Garden, China |
| *Holttumochloa magica* | 1 | Low, LYW136 (KLU) | Cameron Highlands, Malaysia |

**Table 3.1 (cont'...)**

| Taxa | No. of accession | Collector(s), Voucher No. (Herbarium) | Localities |
|---|---|---|---|
| *Kinabaluchloa nebulosa* | 1 | Wong et al. WKM2892 (KLU) | Sabah, Malaysia |
| *Kinabaluchloa wrayi* | 1 | Nong Van Duy, s.n. (SING) | Yang Ly, Bidoup Mountain, Lac Duong District, Lam Dong Province, Vietnam. N12$^{o}$10.467' E108$^{o}$41.967' |
| *Maclurochloa montana* | 1 | Wong & Sugumaran, WKM2890 (KLU) | Fraser's Hill, Pahang |
| *Melocanna baccifera* | 1 | Goh, Univ. of Malaya Bambusetum Acc. 75 | Rimba Ilmu Botanic Garden, Malaysia |
| *Mullerochloa moreheadiana* | 1 | Sussman, s.n. 2008 (KLU) | Queensland, Australia |
| *Neololeba atra* | 1 | ex J. Nilsson, s.n. Nov 2009 (KLU) | Queensland, Australia |
| *Phuphanochloa speciosa* | 1 | Sungkeaw, SS&AT191 (KUFF, TCD) | Peninsular Thailand |
| *Racemobambos gibbsiae* | 1 | Dawat et al., SAN152024 (SAN) | The summit trail in Kinabalu National Park. Altitude 2455 m. |
| *Racemobambos hepburnii* | 1 | Wong et al., WKM2891 (KLU) | 1500 m, Mt. Kinabalu, Sabah |
| *Racemobambos hepburnii* | 2 | Wijadesa, s.n. (KLU) | 2000 m, Mt. Kinabalu, Sabah |
| *Schizostachyum gracile* | 1 | Goh, Univ. of Malaya Bambusetum Acc. 29 | Rimba Ilmu Botanic Garden, Malaysia |
| *Schizostachyum zollingeri* | 1 | Goh, Univ. of Malaya Bambusetum Acc. 16 | Rimba Ilmu Botanic Garden, Malaysia |
| *Soejatmia ridleyi* | 1 | Wong et al., LYW135 (KLU) | Bukit Rengit, Pahang, Malaysia |
| *Sphaerobambos hirsuta* | 1 | Wong et al., WKM2894 (KLU) | Lohan River, Sabah, Malaysia |
| *Temburongia simplex* | 1 | Wong, WKM3129 (BRUN) | Temburong, Brunei |
| *Thyrsostachys siamensis* | 1 | Sungkeaw, SS&AT020704-3 (THNHM, KUFF) | Peninsular Thailand |
| *Yushania tessellata* | 1 | Wong, WKM2893 (KLU) | Mt. Kinabalu, Sabah, Malaysia |

## 3.2 Molecular Methods

### 3.2.1 Total DNA extraction

Silica gel-dried leaves were examined visually so that only those that are free from fungal-infection selected for DNA extraction. Only the portion near the base of the leaf blade was used because for the grasses, the tissues further from the base of the leaf blade will have a higher degree of DNA degradation (Rogers and Bendich, 1994). The leaves were homogenized with liquid nitrogen using sterilized mortar and pestle. The DNA extractions were done using Qiagen DNeasy Extraction kits following the manufacturer's instructions or the conventional method developed by Fulton et al. (1995), depending on the availability of the chemicals. The protocols were provided in **Appendix A**.

### 3.2.2 Polymerase Chain Reaction (PCR) and DNA sequencing

Previous phylogenetic studies for the Bambusinae have utilized the nuclear ribosomal ITS region (Sun et al., 2005; Yang et al., 2008) and *GBSS*I (Yang et al., 2008), as well as chloroplast *trnL-F* (Sungkaew et al., 2009; Yang et al., 2008), *trnL* intron, *atpB-rbcL*, *rps*16 intron and *matK* (Sungkaew et al., 2009). Yang et al. (2008) suggested a higher resolving power of the ITS and *GBSS*I regions compared to the *trnL-F* region while Sungkaew et al. (2009) showed that members of the *Bambusa-Dendrocalamus-Gigantochloa* complex formed a few well supported clusters based on the combined cpDNA regions. In this study, considering availability of primer sequences and the level of sequence variability that could sufficiently address our problem, and cost- and time-effectiveness, four cpDNA and a single nuclear DNA regions were employed.

Among the chloroplast DNA markers, *rps16-trnQ*, *trnC-rpoB* and *trnD-T* intergenic spacers were selected under the recommendation by the Bamboo Phylogeny Group (Bamboo Phylogeny Group; L.G. Clark, pers. comm.), as these markers show high variability among bamboos at lower taxonomic level. Chloroplast *trnH-psbA* intergenic spacer was selected as it has also been suggested to be an informative chloroplast marker in angiosperms the DNA barcoding project (Kress et al., 2005). Attempt to amplify the ITS region was unsuccessful. A forward primer for the partial *GBSS*I region was designed (**Table 3.2**) based on the DNA sequences for woody bamboos available in the GenBank, so that the amplified DNA fragments are in a suitable length (~ 750 bp) for direct sequencing.

Preliminary study has indicated the potential usefulness of these markers in addressing taxonomic problems among *Bambusa* complex and the allied climbing bamboo genera, as well as the evolutionary process within Bambusinae (Goh et al., 2010). As the chloroplast *trnH-psbA* marker appears to provide the least informative characters among the four chloroplast markers (Goh et al., 2010), it was not included in the subsequent DNA analysis using a broader taxon sampling.

**Table 3.2.** Sequences of the PCR primers used in this study.

| DNA region | Primer | Forward/ Reverse | Sequence (5' - 3') | References |
|---|---|---|---|---|
| Partial nuclear *GBSS*I gene | Gin (F) | Forward | AAGTTTGAGCGCATGTTCCAGAGC | This contribution |
| | GBSS(R) | Reverse | GGCGAGCGGCGCGATCCCTCGCC | Mason-Gamer et al., 1998 |
| cpDNA *rps16-trnQ* | 16Q1(F) | Forward | GCACGTTGCTTTCTACCACA | Bamboo Phylogeny Group, 2005 |
| (partial, 1–700 bp) | 16Q1(R) | Reverse | CTTTTGGTATTCKAGTCGAAG | Bamboo Phylogeny Group, 2005 |
| cpDNA *rps16-trnQ* | 16Q2(F) | Forward | CGAGATGGTCAATCCTGAAATG | Bamboo Phylogeny Group, 2005 |
| (partial, 350–1100 bp) | 16Q2(R) | Reverse | ATCCTTCCGTCCCAGATTTT | Bamboo Phylogeny Group, 2005 |
| cpDNA *trnC-rpoB* | CB1(F) | Forward | TGGGGATAAAGGATTTGCAG | Bamboo Phylogeny Group, 2005 |
| (partial, 1–700 bp) | CB1(R) | Reverse | CGTAGTAGTAGAATTGCTAG | Bamboo Phylogeny Group, 2005 |
| cpDNA *trnC-rpoB* | CB2(F) | Forward | CAGGTCCGAACAGCATTA | Bamboo Phylogeny Group, 2005 |
| (partial, 450–1200 bp) | CB2(R) | Reverse | ATTGTGGACATTCCCTCRTT | Bamboo Phylogeny Group, 2005 |
| cpDNA *trnD-T* | DY(F) | Forward | ACCAATTGAACTACAATCCC | Bamboo Phylogeny Group, 2005 |
| (partial 1–800 bp) | DY(R) | Reverse | CTCTTTGCTTTGGATCTAG | Bamboo Phylogeny Group, 2005 |
| cpDNA *trnD-T* | ET(F) | Forward | GCCTCCTTGAAAGAGAGATG | Bamboo Phylogeny Group, 2005 |
| (partial, 400–1100 bp) | ET(R) | Reverse | CCCTTTTAACTCAGTGGTA | Bamboo Phylogeny Group, 2005 |
| cpDNA *trnH-psbA* | HA(F) | Forward | CGCGCATGGTGGATTCACAATCC | Kress et al. 2005 |
| (full range) | HA(R) | Reverse | GTWATGCAYGAACGTAATGCTC | Kress et al. 2005 |

The PCR reaction mixture contains 1.5 mM $MgCl_2$, 0.5 µM forward and reverse primers each, 0.2 mM of dNTPs, 1× PCR buffer and ~10 ng of DNA samples. The reaction was run using a Perkin Elmer GeneAmp 9600 Thermocycler with the programme set at 2 min at 95.0 $^{o}$C; 30 cycles of 30 s at 94.0 $^{o}$C, 45 s at annealing temperature, 1 min at 72.0 $^{o}$C; 5 min at 72.0 $^{o}$C; hold at 4.0 $^{o}$C. Annealing temperature was 59.0 $^{o}$C for the *GBSS*I primers and 55.0 $^{o}$C for primers for cpDNA, *rps16-trnQ*, *trnC-rpoB*, *trnH-psbA* and *trnD-T*. PCR product purification was carried out using Promega PCR Clean-up System kits following instructions by the manufacturer. Direct sequencing of purified PCR products was commercially done by FirstBase Laboratory Sdn. Bhd. (Malaysia). All sequences obtained were deposited in Genbank (**Appendix B**).

### 3.2.3 PCR cloning, haplotype-specific primer design and DNA sequencing for the hybrid taxa

Purified PCR products for the partial *GBSS*I gene of the putative hybrid individuals were ligated into *pDrive* vectors and transformed into EZ competent cells following the instructions of the Qiagen PCR Cloning Plus kit. White colonies were picked to perform colony-PCR using the primers Gin (forward) and GBSS (reverse). Nine to fifteen clones for each hybrid individual were successfully amplified and sequenced. The sequences of all clones were aligned. Three indel regions and a number of variable sites were observed in the DNA sequences of the clones. As some of the unique nucleotide substitutions could be possibly due to PCR or cloning errors, two sets of internal primers were specifically designed for each *GBSS*I haplotype in order

to obtain unambiguous DNA sequence for each allele. The location of haplotype-specific primers are shown in **Figure 3.1**. Internal primers Gin336/1 (forward) and Gin336/2 (forward) were designed for the indel region, and Gin396/1 (reverse) and Gin396/2 (reverse) were designed for the region containing three variable sites. Primer sequences are shown in **Table 3.3**. PCR was performed using the following primer-pairs: (i) Gin–Gin396/1, (ii) Gin–Gin396/2, (iii) Gin336/1–GBSS, and (iv) Gin336/2–GBSS for each putative hybrid individual. Direct sequencing of the purified PCR products was commercially done by FirstBase Laboratory Sdn. Bhd. (Malaysia).

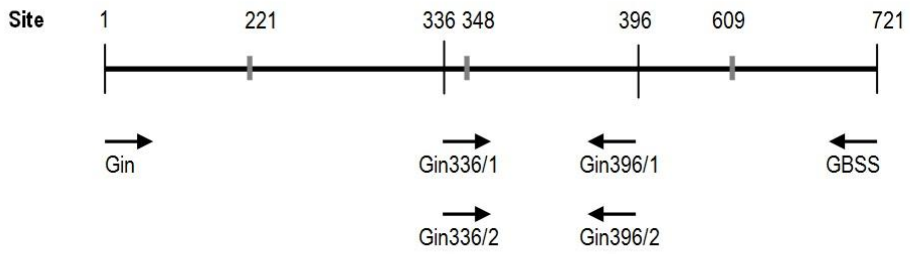



**Figure 3.1**. Schematic diagram showing the position of homoeolog-specific primers (site numbers) and indel regions (grey bars) in the partial *GBSS*I gene. Arrows indicate directions of primers.

**Table 3.3.** Haplotype-specific PCR primers designed for the partial *GBSS*I gene of the hybrid *Dendrocalamus pendulus* × *Gigantochloa scortechinii*.

| Primer | Forward/ Reverse | Sequence (5' - 3') |
|---|---|---|
| Gin336/1 | Forward | GTC TTA GTC TTC TCC TTG CAG C |
| Gin336/2 | Forward | GTC CTA GTC TTC TTG CAG CTC |
| Gin396/1 | Reverse | CAA GAG TAA CGC CAT ATA TG |
| Gin396/2 | Reverse | CAA GAG TAA CAC CAT GTA CG |

## 3.3 DNA sequence analysis

### 3.3.1 DNA sequence alignment and character coding

All DNA sequences were inspected carefully to avoid any ambiguity due to sequencing error. Duplicate accessions were sequenced to verify whenever there were ambiguities from the DNA chromatogram or unexpected placements in the phylogenetic analyses.

For the *GBSS*I data, the unambiguous signals from forward sequencing (towards the 3'-end) and those from reverse sequencing (towards the 5'-end) were combined, leaving noisy signals in the middle coded as missing data. The DNA sequences of each region were aligned using ClustalX (Thompson, 1997) and examined manually so that matrix blocks containing missing data and ambiguous bases were eliminated using Bioedit v7.0.9 (Hall, 1999).

The potentially informative indels were scored following the Simple Indel Coding (SIC) method (Simmons and Ochoterena, 2000) using FastGap 1.1 (Borchsenius, 2009). As minute inversions in the non-coding cpDNA regions have been reported for

some grass and bamboo taxa (Kelchner and Wendel, 1996; Kelchner and Clark, 1997; Yamane and Kawahara, 2005; Zeng et al., 2010), the cpDNA matrix was carefully examined. Cases of minute inversions identified in this study were treated as single mutation events by binary scoring as suggested by Kelchner and Wendel (1996) because such inversions occur when the loops become inverted by a single-step recombination (Kelchner, 2000). Structures of the inverted repeats were verified using 'The mfold Web Server' (Zuker, 2003). Indels resulted from varying lengths of mononucleotide repeats (in cpDNA data) or dinucleotide repeats (in nuclear DNA data) were not coded as informative characters because such length variation could be due to polymerase error in PCR and DNA sequencing. DNA sequence alignment is available from the author.

### 3.3.2 Partition homogeneity (PH) test

Partition Homogeneity (PH) tests were performed with 1000 bootstrap replicates using PAUP4.0b10 (Swofford, 2002) before the cpDNA and *GBSS*I datasets were combined. As the large disparity in the size of datasets being compared could lead to a significant incongruence in the PH test (Levin et al., 2005), individual PH tests were conducted for the nuclear *GBSS*I dataset and each cpDNA region.

### 3.3.3 Maximum parsimony (MP) analysis

Maximum parsimony analysis was performed using PAUP 4.0 b10 (Swofford, 2002). A strict consensus tree was reconstructed using heuristic search with 1000 random sequence additions and tree bisection reconnection (TBR) branch swapping.

Bootstrapping the MP trees was done using 1000 replicates but with the 'MulTrees' option switched off for TBR searching (i.e., saving only one tree at a time). This strategy was employed to avoid excessive computational time without inflating the bootstrap proportion values (DeBry and Olmstead, 2000). Dimorphic sites detected in the *GBSS*I sequences were coded as "polymorph" (polymorphic) in the MP analyses.

The bootstrap proportion (BP) was interpreted as a measure of accuracy or an estimate of $1 - \alpha$, where $\alpha$ is the probability of mistakenly concluding the group is a clade when it is not (Sanderson and Shaffer, 2002). Bootstrap proportion (BP) values > 70 % for MP analyses indicate a strong support as suggested by Hillis and Bull (1993).

### 3.3.4 Bayesian inference (BI) analysis

The best-fit model for the BI analysis was tested using MrModeltest 2.2 (Nylander, 2004). BI analyses were performed in MrBayes 3.1 (Huelsenback and Ronquist, 2001), using 2 runs of 4 chains each, and run for 1000000 generations with trees sampled every 100 generations. The first 2500 trees were discarded as burn-in. Posterior probabilities (PP) > 0.95 was considered a strong support based on the commonly adopted cutoff value (Taylor and Piel, 2004) in this study.

### 3.3.5 Shimodaira-Hasegawa (SH) Test

The SH tests (Shimodaira and Hasegawa, 1999) were used to assess the monophyly of the major climbing bamboo lineages. These tests were performed by individually forcing the lineages of: (1) DMNS, (2) *Racemobambos,* (3) DMNS + *Racemobambos,*

(4) DMNS + *T. simplex*, (5) *Racemobambos* + *T. simplex*, and (6) DMNS + *Racemobambos* + *T. simplex*, to be a monophyletic group in the maximum likelihood analyses and assessing the confident levels of the difference between the constrained and unconstrained maximum likelihood topologies (MLTs). Constrained and unconstrained MLTs were prepared using PAUP4.0 b10 (Swofford, 2002) under the model selected by MrModeltest 2.2 (Nylander, 2004). The tree searching strategy used was ASIS and branch swapping method employed was NNI. Resampling in the SH test was estimated by log-likelihood (RELL) optimisation with 1000 bootstrap replicates.

The SH tests were also performed for assessing the incongruence between the subclades in the BDG complex by cpDNA and *GBSS*I phylogenetic analyses. The alternative hypotheses were defined as: (1) constraining the members of Subclade D (of the *GBSS*I topology) to be monophyletic in the cpDNA topology, (2) constraining the members of Subclade G (of the *GBSS*I topology) to be monophyletic in the cpDNA topology, (3) constraining the members of Subclade BDG1 (of the cpDNA topology) to be monophyletic, and (4) constraining the members of Subclade BDG2 (of cpDNA topology) to be monophyletic.

# CHAPTER 4

# PHYLOGENETIC RELATIONSHIPS AMONG SE ASIAN CLIMBING BAMBOOS AND THE *BAMBUSA* COMPLEX

## 4.1 Introduction and scope of the analysis

In the original scheme of Soderstrom and Ellis (1987), Bambusinae consisted of *Bambusa* (sensu lato, including *Bonia*, *Dendrocalamopsis*, *Leleba*, *Lingnania*, *Neosinocalamus*, *Sinocalamus*), *Dendrocalamus* (sensu lato, including *Gigantochloa*, *Houzeaubambos*, *Oreobambos*, *Oxytenanthera*), *Dinochloa*, *Klemachloa*, *Melocalamus* and *Thyrsostachys*. Newly recognized genera in this alliance, including some based on species first placed with the above, viz., *Sphaerobambos* (Dransfield, 1989), *Kinabaluchloa* (Wong, 1993a), *Maclurochloa* (Wong, 1993a) and *Soejatmia* (Wong, 1993a), were also considered as part of this subtribe (Dransfield & Widjaja, 1995), as is the recently discovered *Phuphanochloa* that has demonstrated phylogenetic relatedness (Sungkaew et al., 2009). Among these genera, *Dinochloa*, *Kinabaluchloa*, *Maclurochloa*, *Soejatmia* and *Sphaerobambos* are remarkably distinct from the others (i.e., mainly *Bambusa* and *Dendrocalamus*) in having a climbing-scrambling habit.

In this chapter, special attention is given to the molecular phylogenetic relationships among these climbing-scrambling bamboo genera and the key Bambusinae genera. Also included were those SE Asian climbing bamboo genera whose relationship to the Bambusinae have been contentious (see **Literature Review**, section **2.1**), viz.,

*Holttumochloa* (Wong, 1993a), *Mullerochloa* (Wong, 2005), *Neololeba* (Widjaja, 1997), *Racemobambos* (Holttum, 1958; Dransfield, 1983, 1992; Stapleton, 1994) and *Temburongia* (Dransfield & Wong, 1996).

The bamboos with erect-suberect culms which are typical of most of the Bambusinae were represented in this analysis by its key genera, *Bambusa, Dendrocalamus* and *Gigantochloa. Bambusa* was represented by its type species, *B. bambos*, and closely related species forming the type alliance, as well as a few representatives from each of the three other subgenera (i.e., *Leleba, Lingnania*, and *Dendrocalamopsis*). *Dendrocalamus* was represented by its type species, *D. strictus,* and also *D. elegans, D. khoonmengii, D. hirtellus* and *D. pendulus*, which are Malay Peninsula species sharing a number of type characters including few flowers in the pseudospikelet. *Gigantochloa* was represented by its type species, *G. atter*, as well as *G. apus, G. balui, G. latifolia, G. ligulata, G. scortechinii* and *G. wrayi*. *Phuphanochloa* and *Thyrsostachys* were also included as they have been shown to be closely related to the Bambusa complex in previous phylogenetic studies (Yang et al., 2008; Sungkaew et al., 2009).

As Melocanninae has been consistently recovered as a sister subtribe of Bambusinae *s. l.* in previous phylogenetic studies (Yang et al., 2007; Yang et al., 2008; Sungkaew et al., 2009; Goh et al., 2010), it was also included as an ingroup element in the present study. It was represented by *Melocanna baccifera*, *Schizostachyum gracile* and *S. zollingeri*. *Guadua cf. chacoensis,* representing the neotropical woody bamboo clade

(which is sister to the paleotropical woody bamboos; Sungkaew et al. 2009), and *Yushania tessellata*, representing the temperate bamboo lineage (which is sister to the tropical woody bamboos; Sungkaew et al. 2009), were used as the outgroup. Information for voucher specimens employed in this study is provided in **Table 3.1** (see **Chapter 3**).

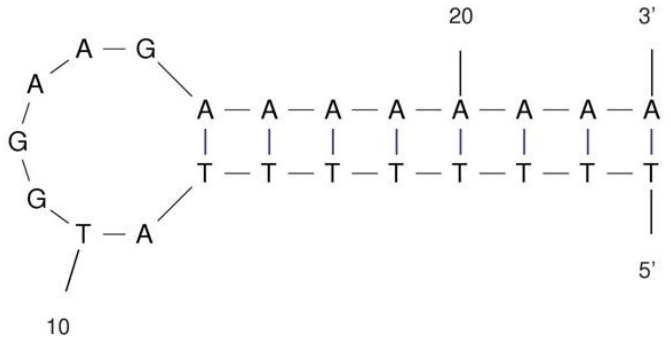
### 4.2 Analyses based on individual and combined DNA markers

In the cpDNA matrix, two cases of hairpin inversions were observed in the *trnD-T* dataset (**Fig. 4.1**). They were treated as single mutation events by binary scoring, as suggested by Kelchner & Wendel (1996). Minute inversions in the non-coding cpDNA regions have also been reported for some grass and bamboos taxa (Kelchner & Wendel, 1996; Kelchner & Clark, 1997; Yamane & Kawahara, 2005; Zeng et al., 2010). They are often associated with a small stem-loop secondary structure, or 'hairpin', which consists of a loop of 4–6 nucleotides and a stem made of inverted repeats flanking the nucleotides of the loop. The loop becomes inverted by recombination and thus should be treated as single mutation event, instead of multiple independent base substitutions (Kelchner & Wendel, 1996; Kelchner, 2000).

Direct sequencing of the partial *GBSS*I gene fragment produced unambiguous signals for 37 taxa, 1–8 dimorphic sites and / or noisy signals after a particular site (due to length polymorphism among alleles) for 22 taxa (**Table 4.1; Appendix C**). This suggests that the *GBSS*I gene is a homozygous locus for some taxa but heterozygous in the others.

Hairpin 1

5′-TTTTTTTT**ATGGAAG**AAAAAAAA-3′
3′-TTTTTTTT**CTTCCAT**AAAAAAAA-5′




Hairpin 2

5′-TATACTC**GAA**GAGTATA-3′
3′-TATACTC**TTC**GAGTATA-5′

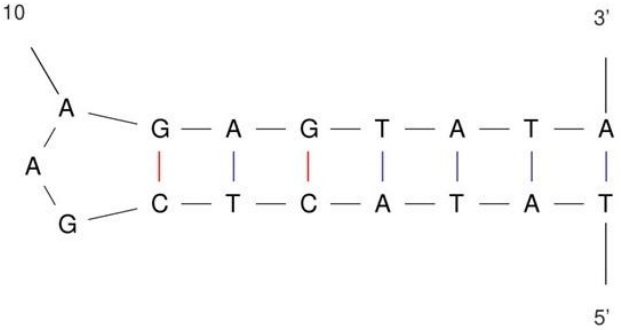



**Fig. 4.1**: Minute inversion associated with hairpin and stem-loop structure in the cpDNA data matrix. Nucleotides which form the loops were bold-faced. Folding structures were constructed using The mfold Web Server (Zuker, 2003).

**Table 4.1**: List of taxa which show heterozygosity in the partial *GBSS*I sequence.

| Pattern of heterozygosity | Taxa |
|---|---|
| Showing dimorphic site(s) | *Bambusa gibba*, *B. multiplex*, *B. textilis*, *B. tuldoides*, *B. valida*, *Dendrocalamus elegans*, *D. hirtellus*, *Gigantochloa balui*, *G. scortechinii* (1), *G. wrayi* |
| Showing length polymorphism | *Bambusa bambos* (1) & (2), *B. blumeana*, *Holttumochloa magica*, *Racemobambos gibbsiae* , *Schizostachyum zollingeri*, *Temburongia simplex* |
| Showing dimorphic sites and length polymorphism | *B. grandis*, *B. intermedia*, *B. oldhamii*, *Gigantochloa atter*, *Schizostachyum gracile* |

Tree lengths, consistency indices (CI) and retention indices (RI) for the MP analysis for individual cpDNA region, combined cpDNA regions (*rps16-trnQ* + *trnC-rpoB* + *trnD-T*), *GBSS*I region and combined cpDNA + *GBSS*I regions are shown in **Table 4.2**. MP analyses on the individual cpDNA marker (**Appendix D**) did not result in well resolved phylogenetic trees, thus all three regions were combined for the cpDNA-based phylogenetic analysis. Chloroplast DNA is a circular molecule that does not undergo recombination and it is inherited uniparentally, therefore incongruence among the different cpDNA regions is unlikely and hence combining the cpDNA regions in phylogenetic analyses is generally acceptable.

**Table 4.2**: Tree statistics for MP analyses of separate and combined data partitions.

| Dataset | DNA characters | Indel characters | Total characters | Parsimony-informative characters, PIC (number / %) | MP tree length | Consistency index, CI | Retention index, RI |
|---|---|---|---|---|---|---|---|
| cpDNA | | | | | | | |
| (i) *rps16-trnQ* | 1561 | 21 | 1582 | 34 / 2.15 | 102 | 0.8235 | 0.9086 |
| (ii) *trnC-rpoB* | 1464 | 20 | 1484 | 41 / 2.76 | 134 | 0.7985 | 0.8402 |
| (iv) *trnD-T* | 1165 | 17 | 1182 | 42 / 3.55 | 135 | 0.8593 | 0.8797 |
| (v) *rps16-trnQ+trnC-rpoB+ trnD-T* | 4190 | 58 | 4248 | 117 / 2.75 | 380 | 0.8079 | 0.8607 |
| Nuclear DNA | | | | | | | |
| (i) *GBSS*I | 694 | 25 | 718 | 99 / 13.79 | 267 | 0.7678 | 0.8558 |
| Combined cpDNA+*GBSS*I | 4884 | 83 | 4966 | 216 / 4.35 | 700 | 0.7314 | 0.8029 |

MP analyses using the cpDNA and *GBSS*I datasets resulted in 67 and 35 equally most-parsimonious trees, respectively. Strict consensus trees for the cpDNA and *GBSS*I datasets are shown in **Fig. 4.2**. Bootstrap proportion (BP) values higher than 70% are shown below the branches (**Fig. 4.2; Appendix E**). The best-fit models for the BI analyses chosen using MrModeltest 2.2 (Nylander, 2004) were GTR + I + G and K80 + G for the cpDNA and *GBSS*I datasets, respectively. Bayesian topologies are largely consistent with those from the MP analyses for both datasets (**Appendix F**). Posterior probabilities (PP) higher than 0.95 were mapped on the MP topologies (above the branches; **Fig. 4.2**).

Five major clades were recovered in the cpDNA topology (**Fig. 4.2; left**): Clade 1 (1.00 PP/ 100 BP), consisting of members of Melocanninae; Clade 2 (0.95 PP/ 75% BP), consisting of two *Racemobambos* taxa; Clade 3 (0.95 PP/ 73% BP), consisting of *Dinochloa*, *Mullerochloa*, *Neololeba* and *Sphaerobambos*; Clade 4 (1.00 PP/ 100% BP), consisting of *Holttumochloa* and *Kinabaluchloa*; and Clade 5 (0.95 PP/ 86% BP), consisting of *Bambusa, Dendrocalamus, Gigantochloa, Maclurochloa, Phuphanochloa*, *Soejatmia* and *Thyrsostachys*. Similarly, the nuclear *GBSS*I analyses yielded five major clades **(Fig. 4.2; right)**, corresponding to the major clustering pattern in the cpDNA topology, except that *Mullerochloa moreheadiana* was not recovered in Clade 3 in the *GBSS*I topology. *Temburongia simplex* was unresolved among Clade 2 and Clade 3 in both cpDNA and *GBSS*I topologies.

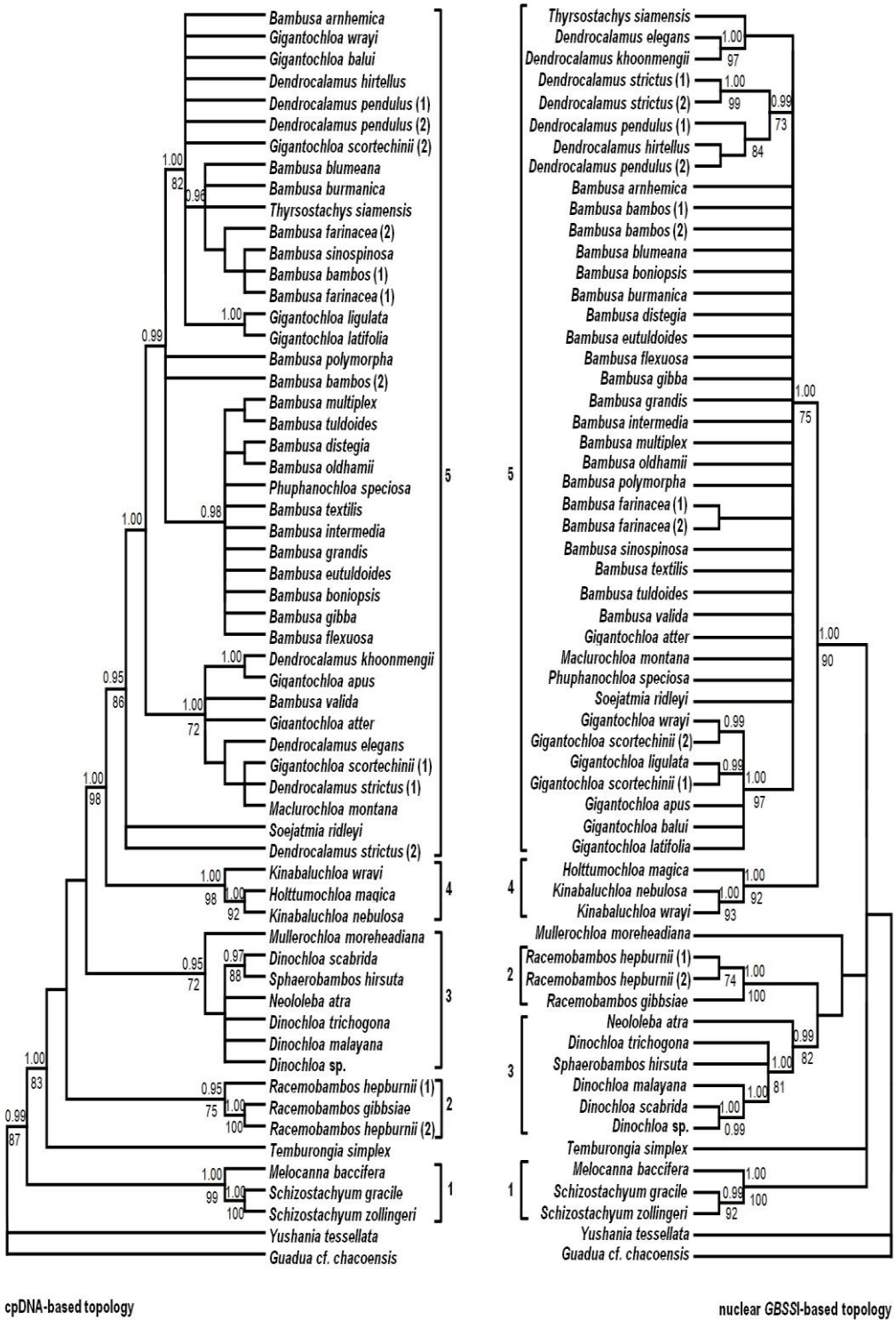



**Fig. 4.2**: Strict consensus of the most parsimonious trees based on three cpDNA data (left) and the partial nuclear *GBSS*I data (right). Posterior probabilities >0.90 are shown above the nodes, bootstrap support values >70% below the nodes. Parentheses next to the taxon name indicate the number of accession whenever there is more than one individual included.

The Clade 5 of both topologies characterize a complex group of taxa with unresolved inter-generic relationships, which is an expanded representation of what was defined as the BDG (*Bambusa-Dendrocalamus-Gigantochloa*) complex in the preliminary study (Goh et al., 2010), where only a smaller taxon sampling was used. With this increased taxon sampling, the BDG complex now comprises all genera placed in Clade 5: *Bambusa*, *Dendrocalamus*, *Gigantochloa*, *Maclurochloa*, *Phuphanochloa*, *Soejatmia*, and *Thyrsostachys*.

The PH tests confirmed the incongruence between each cpDNA marker and the *GBSS*I marker used (**Table 4.3**). However, when the DNA sequences of the BDG complex were eliminated from the DNA dataset, more cases of congruence between the individual cpDNA markers and the *GBSS*I marker were observed (**Table 4.3**). This indicates that incongruence between the two datasets very likely centred on the BDG complex. As such, the cpDNA and *GBSS*I datasets were combined in the subsequent MP and BI analyses for inferring relationships only among major clades. The relationships among the genera within the BDG complex, especially in aspects of incongruence between datasets, are dealt with in more detail in **Chapter 6**.

**Table 4.3**: Results of partition homogeneity (PH) tests for assessing congruency between cpDNA and *GBSS*I datasets.

| Nuclear *GBSS*I vs. | *p* value | | |
|---|---|---|---|
| | All taxa | Only taxa of BDG complex | Excluding taxa of BDG complex |
| *rps16-trnQ* | 0.001* | 0.001* | 0.026 |
| *trnC-rpoB* | 0.001* | 0.001* | 0.023 |
| *trnD-T* | 0.001* | 0.002* | 0.001* |
| Combined cpDNA regions | 0.001* | 0.001* | 0.001* |

* indicates rejection at the 95% confidence level.

The strict consensus tree and one of the 33 most parsimonious trees based on the combined cpDNA+*GBSS*I dataset are shown in **Fig. 4.3** and **Fig. 4.4**, respectively. The GTR + I + G model was selected for the BI analysis. Considering that MrBayes does not treat the multistate characters as polymorphic characters and the analysis using the cpDNA+*GBSS*I data is only for inferring the major clades, the dimorphic sites within the BDG complex were manually and randomly coded as one of the dimorphic bases to permit a more robust phylogenetic approximation. The 50% majority-rule consensus tree recovered in the Bayesian analysis (**Appendix F**) has a similar topology to that of the strict consensus tree in the MP analysis.

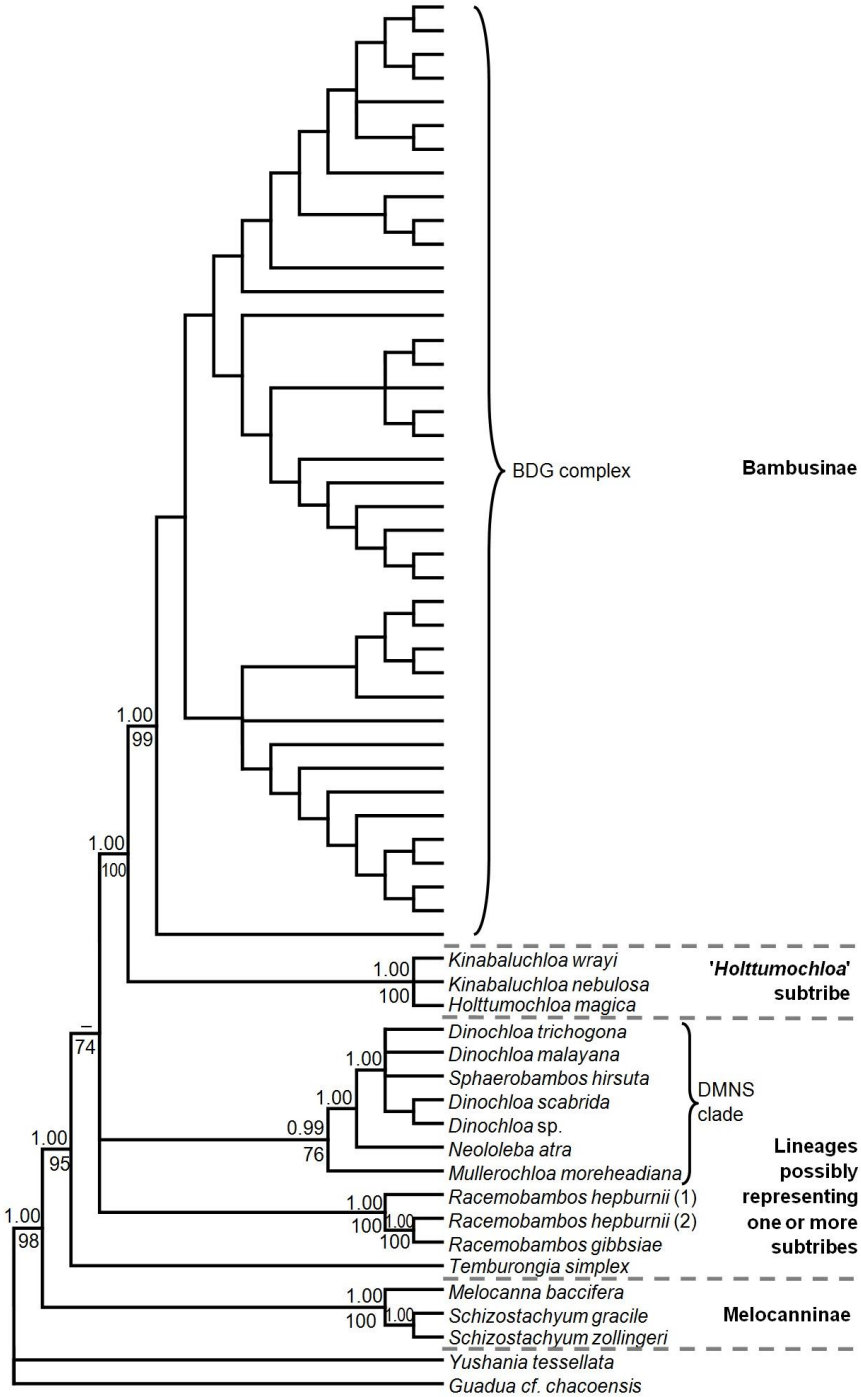



**Fig.4.3:** Strict consensus of the 33 most parsimonious trees from the analysis based on the combined datasets of three cpDNA regions (*rps16-trnQ+trnC-rpoB+trnD-T*) and the partial *GBSSI* gene. Bayesian posterior probabilities higher than 0.95 are shown above branches, bootstrap values higher than 70 % below branches. Parentheses next to the taxon name indicate the number of accession whenever there is more than one individual included.

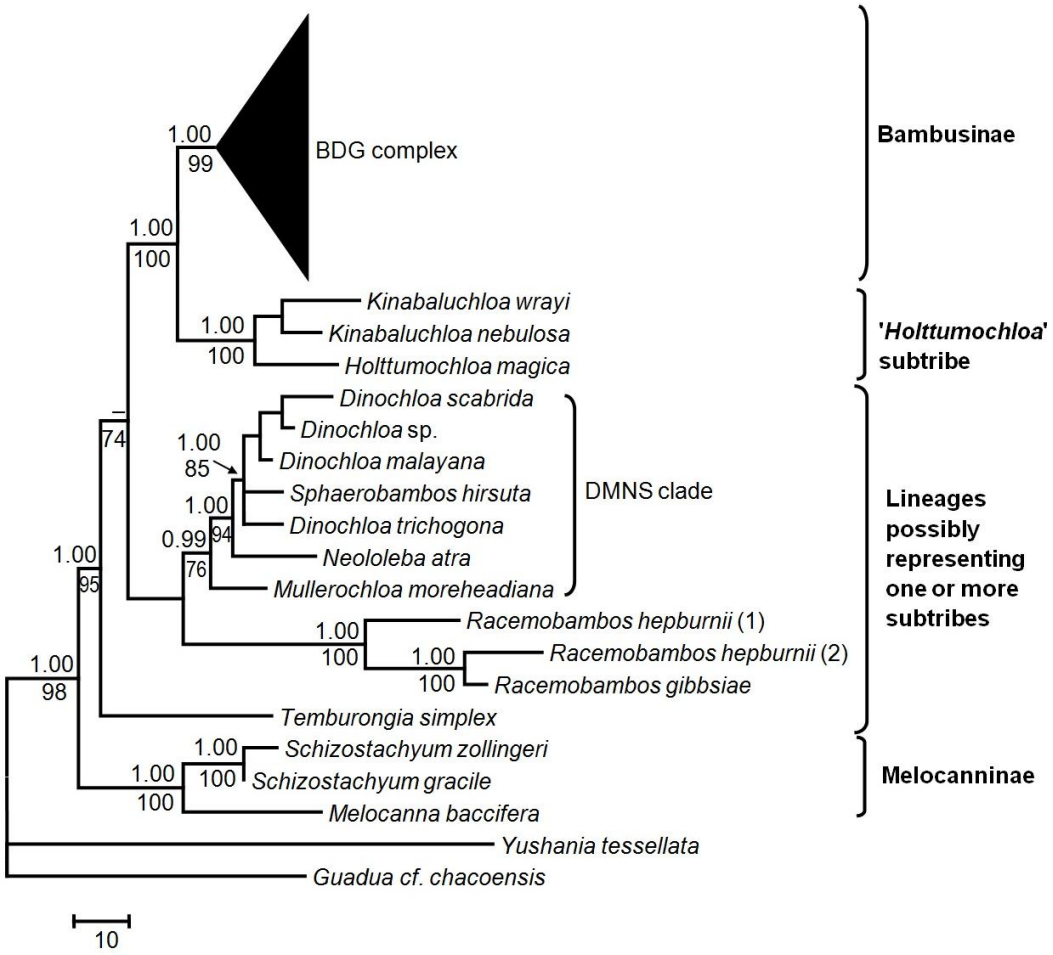



**Fig. 4.4:** One of the 33 most parsimonious trees from the analysis based on the combined datasets of the three cpDNA regions (*rps16-trnQ+trnC-rpoB+trnD-T*) and the partial *GBSS*I gene. Bayesian posterior probabilities higher than 0.95 are shown above branches, bootstrap values higher than 70 % below branches. Parentheses next to the taxon name indicate the number of accession whenever there is more than one individual included.

In the phylogenetic tree based on the combined cpDNA+*GBSS*I datasets (**Fig. 4.3** and **Fig. 4.4**), the Melocanninae clade (1.00 PP/ 100% BP) was sister to the remainder of the ingroup taxa. Among the non-Melocanninae ingroup genera, the BDG complex (1.00 PP/ 99% BP) was sister to the *Holttumochloa-Kinabaluchloa* clade (1.00 PP/ 100% BP). Other well-supported clusters included the *Dinochloa-Mullerochloa-Neololeba-Sphaerobambos* clade (hereafter, DMNS clade; 0.99 PP/ 76% BP), the *Racemobambos* clade (1.00 PP/ 100% BP) and the branch representing *Temburongia simplex* (**Fig. 4.3** and **4.4)**.

A battery of SH tests based on the combined data of cpDNA and *GBSS*I were performed for testing the relationship among the climbing bamboo clades (**Table 4.4; Appendix G)**. The tests could not reject the hypothesis that each of the DMNS clade, the *Racemobambos* clade and *T. simplex* was individually monophyletic (i.e., sister to the other clades in the topology). However, possible monophyletic relationships between DMNS clade + *Racemobambos* clade, DMNS clade + *T. simplex* and *Racemobambos* clade + *T. simplex* could not be rejected as well (**Table 4.4**). This suggests that the relationship among these lineages is uncertain.

**Table 4.4:** Results of Shimodaira-Hasegawa (SH) tests for assessing the relationships among the climbing bamboo clades recovered in the combined cpDNA-*GBSSI* analysis.

| Alternative hypothesis | Constraint | *p* value* |
|---|---|---|
| DMNS clade is monophyletic | *Dinochloa* sp., *D. malayana, D. scabrida, D. trichogona, M. moreheadiana, N. atra, S. hirsuta* | 0.387 |
| *Racemobambos* clade is monophyletic | *R. gibbsiae, R. heburnii* (1), *R. hepburnii* (2) | 0.180 |
| DMNS and *Racemobambos* clades form a monophyletic group | *Dinochloa* sp., *D. malayana, D. scabrida, D. trichogona, M. moreheadiana, N. atra, S. hirsuta, R. gibbsiae, R. heburnii* (1), *R. hepburnii* (2) | 0.180 |
| DMNS clade and *T. simplex* form a monophyletic group | *Dinochloa* sp., *D. malayana, D. scabrida, D. trichogona, M. moreheadiana, N. atra, S. hirsuta, T. simplex* | 0.239 |
| *Racemobambos* clade and *T. simplex* form a monophyletic group | *R. gibbsiae, R. heburnii* (1), *R. hepburnii* (2), *T. simplex* | 0.121 |
| DMNS clade, *Racemobambos* clade and *T. simplex* form a monophyletic group | *Dinochloa* sp., *D. malayana, D. scabrida, D. trichogona, M. moreheadiana, N. atra, S. hirsuta, R. gibbsiae, R. heburnii* (1), *R. hepburnii* (2), *T. simplex* | 0.189 |

* all values not significant at the 95% confidence level.

### 4.3 Phylogenetic relationships among the climbing bamboo genera and the BDG complex

Most of the climbing bamboo genera (*Dinochloa*, *Holttumochloa*, *Kinabaluchloa*, *Mullerochloa*, *Neololeba*, *Racemobambos*, *Sphaerobambos* and *Temburongia*) in this study were found to be distinct from the BDG complex (**Fig. 4.3** and **4.4**). However, climbing taxa such as *Maclurochloa* and *Soejatmia* remain with the BDG complex. The distinction between the *Holttumochloa-Kinabaluchloa* clade and the BDG complex, as have already been reported in the preliminary study (Goh et al., 2010), supports the removal of *Holttumochloa* and *Kinabaluchloa* from *Bambusa s. str.*, which was proposed by Wong (1993a) on morphological grounds.

The present study also clearly supports the removal of *N. atra* and *M. moreheadiana* from *Bambusa* sensu stricto by Widjaja (1997) and Wong (2005), respectively. *Neololeba atra* includes *Bambusa forbesii* (applied to the species in Australia) and *Bambusa atra* (applied to the species in New Guinea, Sulawesi and the Philippines). It is different from *Bambusa* in having a simpler branching system, smaller pseudospikelets, shorter rachilla internodes and no lodicules (Widjaja, 1997). *Mullerochloa* is a monotypic genus established to accommodate the northern Australian species, *Bambusa moreheadiana*. It differs from *Bambusa* sensu stricto in having clambering-scrambling culms, culm sheaths with a transversely wrinkled basal zone, reflexed culm-sheath blades, inconspicuous culm-sheath auricles, four stamens with fused filaments and a glabrous ovary (Wong, 2005).

Fruits with thick and fleshy pericarp such as found in *Dinochloa* also occur in other genera, such as *Melocanna, Ochlandra* and *Schizostachyum*, and the taxonomic significance of this has been the subject of ponderance for over a hundred years (Munro, 1868; Dransfield, 1981). Holttum (1958) placed *Dinochloa* near to *Bambusa* based on the ovary structure and his conclusion appeared to have been reinforced by some recent molecular studies (Yang, et al., 2007; 2008; Sungkaew et al., 2009). Holttum's impression was based on the general difference between two very different ovary structures represented by the *Bambusa*-type (thickened ovary apex with a solid style, which the *Dinochloa* ovary resembles), contrasted with the *Schizostachyum*-type (not specially thickened ovary apex with a hollow style enclosing a central stylar strand) (Holttum, 1956). In this study, *Dinochloa* was shown to associate with neither the BDG complex nor the Melocanninae (**Fig. 4.3** and **Fig. 4.4**).

*Racemobambos* was once suggested as closely related to *Bambusa* based on the ovary structure (Holttum, 1958) and later considered to be allied to the temperate *Arundinaria,* based on its determinate inflorescence (having true spikelets, rather than pseudospikelets that proliferate to add further similar units from their base; Dransfield, 1983, 1992) and culm anatomical similarities (Wong, 1995b). In the present study, it was unambiguously resolved as one of the ingroup clades, when *Yushania* (representing the temperate lineage) was used as the outgroup, suggesting that *Racemobambos* is only distantly related to *Arundinaria* (**Fig. 4.3** and **Fig. 4.4**). Its phylogenetic placement also suggests that it is distinct from the BDG complex.

*Temburongia simplex* has determinate inflorescences with short, paniculate branches bearing 1–3 spikelets. Unlike typical determinate inflorescences, the inflorescence of *Temburongia* possesses bracts and prophylls at the base of the inflorescence branches and its main inflorescence axes are clearly segmented. As such, *Temburongia* is difficult to place with other Malesian genera. A similar type of inflorescence is only found in a few genera, viz., *Glaziophyton* from South America, *Greslania* from New Caledonia and *Hickelia* from Madagascar (Dransfield & Wong, 1996). Although more comparisons including these genera will need to be made, it is not surprising that *Temburongia* was somewhat isolated from the other groups identified in this study (**Fig. 4.3** and **4.4**).

### 4.4 Taxonomic implications: subtribal relationships and classification

The present study depicted some clustering patterns in the phylogenetic tree which are similar to that of the phylogenetic tree presented by Sungkaew et al. (2009). However, increased representation of climbing bamboos in this study provides more insight into possible subtribal-level relationships among the Bambusinae and allied taxa. In this study, Melocanninae was recovered as a well distinct clade, consistent with previous studies (Yang et al., 2007; 2008; Sungkaew et al., 2009), but the broadly categorized Bambusinae (Bamboo Phylogeny Group, 2012) included four lineages (**Fig. 2** and **3**).

The *Holttumochloa-Kinabaluchloa* clade was consistently recovered as a sister clade to the BDG complex with strong support (cpDNA, *GBSS*I and combined cpDNA-*GBSS*I topologies in Goh et al., 2010 and the present study). Despite their common

ancestry, these two montane genera are morphologically rather different: *Holttumochloa*, for example, has multiple primary branch buds at each culm node, whereas *Kinabaluchloa* has a solitary primary branch bud. They superficially resemble each other in having a branch complement that comprises subequal branches (i.e., without a clearly dominant branch) and linear culm leaf blades (which is also found in *Racemobambos*). Wong (1995a) had suggested *Kinabaluchloa* should be classified in the Bambusinae but *Holttumochloa* occupied an isolated position in or near to the Bambusinae based on the branch complement, primary branch bud and inflorescence structure. It is feasible to consider these two clades as independent subtribes, i.e., the '*Holttumochloa'* subtribe and the Bambusinae subtribe formed by the BDG complex given the unequivocal phylogenetic distinction between them (**Fig. 4.3** and **4.4**).

Besides the Melocanninae (which is a distinct clade and well-recognized subtribe), there is, however, less certainty about the subtribal position of the remaining clades that are separated from the BDG complex / *Holttumochloa-Kinabaluchloa* clade (**Fig. 4.3** and **4.4**). The DMNS clade and the *Racemobambos* clade effectively form a polytomy with the BDG complex-*Holttumochloa-Kinabaluchloa* clade. *Temburongia* was sister to the polytomy but the sister relationship was only equivocally supported (by MP but not BI; **Fig. 4.3** and **4.4**). Therefore, from the present results, it is not sufficiently clear if they formed one or more subtribes.

If the *Racemobambos* clade (which is represented by its type species, *R. gibbsiae,* and a member of the type alliance, *R. hepburnii,* in this study) could merit subtribal status,

it is less likely to include *Neomicrocalamus* and *Vietnamosasa* although Stapleton (1994) had defined the original Racemobambosinae as including all three genera. Dransfield (1992) already noted that the indeterminate inflorescences of both *R. prainii* (previously also placed in *Neomicrocalamus*) and *R. cilliata* (later transferred to the genus *Vietnamosasa*) precluded their placement with true *Racemobambos*. These differences, as well as the phylogenetic placement of *Neomicrocalamus* and *Vietnamosasa* within the core Bambusinae (Yang et al., 2008; Sungkaew et al., 2009), the present study indicates that *Neomicrocalamus* and *Vietnamosasa* are unlikely to belong to the same subtribe as *Racemobambos*, which is well distinct from the BDG complex as shown in the present study (**Fig. 4.3** and **Fig. 4.4**).

This study, however, does not attempt to formally revise the subtribal limits of tropical Bambuseae because the relationships, especially among the climbing bamboo genera, could be better resolved and possible morphological synapomorphies for the putative subtribes are yet to be assessed based on the full membership for each of them. The wider analyses of the Bamboo Phylogeny Group, now being organized, are also expected to reveal more relationships.

## 4.5 Biogeographic implications

### 4.5.1 The biogeographic signatures of Sunda lineages

The present study has also shown an inconsistency in the clustering among *H. magica*, *K. nebulosa* and *K. wrayi* in the cpDNA and *GBSS*I topologies (**Fig. 4.2**). While the incongruence between the cpDNA and nuclear DNA data could be a result of a number of evolutionary processes, such as hybridization and incomplete lineage sorting (will be discussed in **Chapter 6**) and the current taxon sampling density of the *Holttumochloa-Kinabaluchloa* group could be insufficient to address such observation, the separation of the two *Kinabaluchloa* taxa in the cpDNA analysis (**Fig. 4.2**) appears to correspond to the landscape evolution of SE Asia. The genetic divergence between *Kinabaluchloa wrayi* (endemic to Indochinese and Malayan highlands; Wong, 1993a; Tran et al., 2012) and *K. nebulosa* (endemic to Kinabalu and NW Borneo mountains; Wong, 1993a) is likely to imply the vicariance events resulting from the development of the South China Sea, which has fluctuated in extent over the ice ages (Heaney, 1991; Voris, 2000) and in its more expansive state would have segregated ancestral populations of organisms (Wong, 2011). Similar genetic divergence between the populations of mainland SE Asia / Malay Peninsula and Borneo has also been revealed by several cpDNA phylogeography analyses on other plant groups, e.g., *Lithocarpus* (Cannon and Manos, 2003), *Macaranga* (Bänfer et al., 2006) and *Shorea curtisii* (Kamiya, Nanami et al. 2011).

The presently patchy distribution of the closely related genera *Kinabaluchloa* and *Holttumochloa* on mountains along the SE fringe of mainland Asia (Wong, 1993a;

Tran et al., 2012), and their distinction as a separate lineage compared to the highly diversified BDG complex (this study) are interesting aspects. These are consistent with the idea that these genera or their ancestors could have been part of a more extensive, ancient Indochinese tropical montane flora that survived the ice ages in such places as the Annamite Mountains east of the Mekong River, where higher mammal richness and endemism, compared to regions farther west in SE Asia, probably indicate the existence of a past Indochinese refugium (Meijaard and Groves, 2006). Over the greater Sundaland area, when tropical and subtropical vegetation fluctuated in extent significantly over the Pliocene-Pleistocene (Morley, 2000), the spread of such bamboos could have been encouraged during past episodes of suitable climate and vegetation development, and interrupted during unsuitable intervals.

### 4.5.2 The DMNS clade: a possible Australasian clade in the Bambuseae

The Australian woody bamboo flora, comprising two endemic taxa and one species shared with islands to the north (i.e., New Guinae and Luzon), has been something of a biogeographical enigma. Their marked morphological dissimilarities could represent rather different lineages, so *Mullerochloa moreheadiana, Neololeba atra* and *Bambusa arnhemica* could have colonized Australia separately (Franklin, 2003; 2008). There was also an alternative possibility that a residual woody bamboo lineage on the Australasian plate could have persisted and contributed to the current biogeographical distribution (Wong, 2005). Bouchenak-Khelladi et al. (2010) have produced a dated phylogenetic tree using a large dataset of combined plastid DNA sequences of the Poaceae, employing a likelihood-based method for the estimation of ancestral

polymorphism and calibrated with four independent fossils of grasses. Their results corroborate a Gondwanan (either African or S American) origin for Poaceae (as discussed by Bremer, 2002) and molecular dating of the Bambusoideae at around 30 MYA (Bouchenak-Khelladi et al., 2010). Hodkinson et al. (2010) infer the minimum age of the tropical Bambuseae as 17.5 MY. In this scenario, it is unlikely that (1) an out-of-India dispersal (McKenna, 1973), in which plants of Gondwanan origin have proliferated to Asia via the rafting of the Indian plate towards collision with Asia, operated for woody bambusoid forms; and (2) woody bambusoid forms had been retained early enough on the Australasian portion of a disintegrating Gondwana. This is because the Indian / S Asia collision and the separation of Australasian portion both considerably predate estimates for the origin of Bambusoideae (Morley, 2000). This implies the spread of the Bambusoideae through Asia into SE Asia and Australia. Thus, the recovery of *B. arnhemica* within the BDG complex suggests a possible range expansion of *Bambusa* from Asia towards N Australia. The center of diversity of *Bambusa* is S China but *B. arnhemica* is the only *Bambusa* species in Australia.

However, it is possible that the DMNS clade could be an Australasian innovation. The Australasian-SE Asia plate collision that initiated about 25 MYA precipitated a complex series of island paleogeographical scenarios between Australasian region and Sundaland (Hall, 2009). The arrival of the DMNS clade ancestor in the Australasian region and the divergence and diversification of the clade were possibly enabled from 20–15 MYA (Miocene), and again during Late Miocene and later Pleistocene glaciation events (Batchelor, 1979; Voris, 2000), when intermittent shallow-sea and

island-chain connectivity or land bridges variously facilitated floristic transfer or exchange among Sundaland, Sulawesi, the Philippines, and Australia-New Guinea (Morley, 2003; Hall, 2009). It is, however, not yet possible to determine if *Mullerochloa* had a wider distribution historically with significant extinction elsewhere.

The area relationships of the genera in the DMNS clade are illustrated in **Fig. 4.5.** *Mullerochloa moreheadiana* is endemic to NE Australia while *Neololeba atra* is more widely distributed from NE Australia and New Guinea to Sulawesi and Luzon (Widjaja, 1997). *Sphaerobambos* consists of three species, *S. hirsuta*, *S. philippinensis* and *S. subtilis*, endemic to Sabah, Mindanao and Sulawesi, respectively (Dransfield, 1989). *Dinochloa*, consisting of about 20 species, is distributed mainly in the Borneo-Sulawesi-Philippines area with outliers in Palawan, Hainan, Indochina, Myanmar, the Andamans, Malay Peninsula, Sumatra, Java, and the Lesser Sunda Islands (Dransfield, 1981; 1992; Dransfield & Widjaja, 1995). Such a geographical distribution of the DMNS clade could imply a dispersal pathway between the Australia and mainland Asia via New Guinea, Sulawesi, Borneo and the Philippines. In fact, the similar dispersal route has also been postulated for many plant groups derived from Asia and dispersed towards Australia / New Zealand to Asia, or *vice versa* (reviewed by Raven, 1973 and Wong, 2011). Nevertheless, these ideas need to be further tested, as the DMNS clade has only moderate support in our analysis and its relationship to even the main BDG complex-*Holttumochloa-Kinabaluchloa* clade is not clear. Also, additional

taxon sampling to include more bamboo taxa from the region around the Australasian region may help to provide increased insight.


**Fig. 4.5:** Phylogeny of the genera in the DMNS clade implied from combined cpDNA-*GBSS*I data, and their corresponding geographic distribution in the Australasian-SE Asian region.

# CHAPTER 5

# CHARACTERIZATION AND SIGNIFICANCE OF HYBRIDIZATION

## 5.1 An intergeneric hybrid

A putative natural hybrid was collected among the clumps of *Dendrocalamus pendulus* and *Gigantochloa scortechinii* along the Tapah-Cameron Highlands road, Peninsular Malaysia, on 28 November 2001 (this is hereafter referred to as 'Hybrid Tapah'). Material raised from a rhizome offset was planted in the Bambusetum of the Rimba Ilmu Botanical Garden, University of Malaya, in Kuala Lumpur, Peninsular Malaysia. This clone (**Plate 1A-D**) flowered in April 2007, i.e., quite soon after it grew to mature size and then died completely in July 2008. Voucher material was deposited with the Herbarium of the University of Malaya (KLU), the Singapore Herbarium (SING) and the Iowa State University Herbarium (ISC), and leaf material was collected and preserved in silica gel for molecular studies.

A population of the same putative hybrid was also encountered in 2009 along the Old Gombak Road, Selangor, Peninsular Malaysia, again sympatric with *D. pendulus* and *G. scortechinii* clumps (**Plates 2A** and **2B**). Voucher material of two individuals (Hybrid Gombak-1 and Hybrid Gombak-2) was collected and deposited with KLU (listed as × *Gigantocalamus malpenensis* (accessions 1 and 2) in **Table 3.1**), detailed morphological observations were made, and leaf material dried in silica gel was also obtained.


**Plate 1.** The putative hybrid *Dendrocalamus pendulus* × *Gigantochloa scortechinii*: clump habit (A), culm shoot (B), culm internode characteristics (C), and seudospikelet cluster (D). From Goh et al. (2011).

The positions of the two hybrid clumps are shown in **Fig. 5.1**. Besides these three hybrid accessions, three accessions of leaf material of *D. pendulus* and five accessions of *G. scortechinii* were likewise obtained.

A comparison of the morphological characters of the hybrid and its parental species is shown in **Table 5.1**. As has been noted for many hybrids and hybrid derivatives (Rieseberg, 1995), the morphology of the hybrid is a mixture of qualitative characters that match one or the other parental species, e.g., culm leaf auricles with bristles is observed in the hybrid and *D. pendulus* (**Plates 2B** and **2C**), and fused staminal filaments in the hybrid and *G. scortechinii*, or are intermediate between the parents, e.g., the length of the pseudospikelets.

During the entire flowering period of Hybrid Tapah and up to a month afterwards, no caryopses were found in spite of careful searches. Similarly, in a parallel study on the hybrid population in the Gombak valley (where Hybrid Gombak-1 and Hybrid Gombak-2 were collected), no caryopses were found even though 10 hybrid individuals had flowered or were flowering when the study was carried out (Wong and Low, 2011).

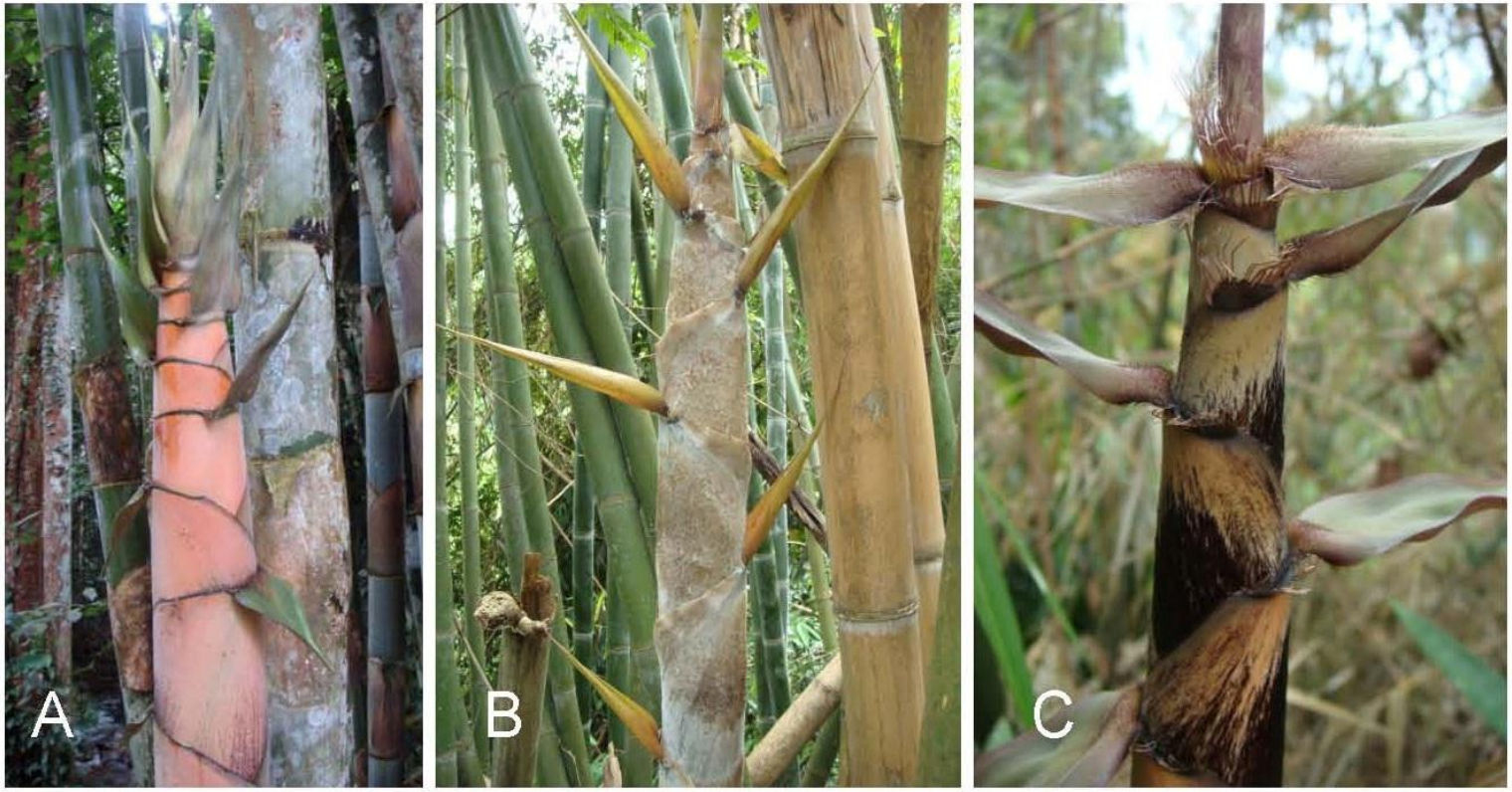



**Plate 2.** Culm shoots of the two parental species, *Gigantochloa scortechinii* (A) and *Dendrocalamus pendulus* (B), and their hybrid (C), from clumps within the hybrid zone along the Gombak road, Selangor, Peninsular Malaysia. From Goh et al. (2011)

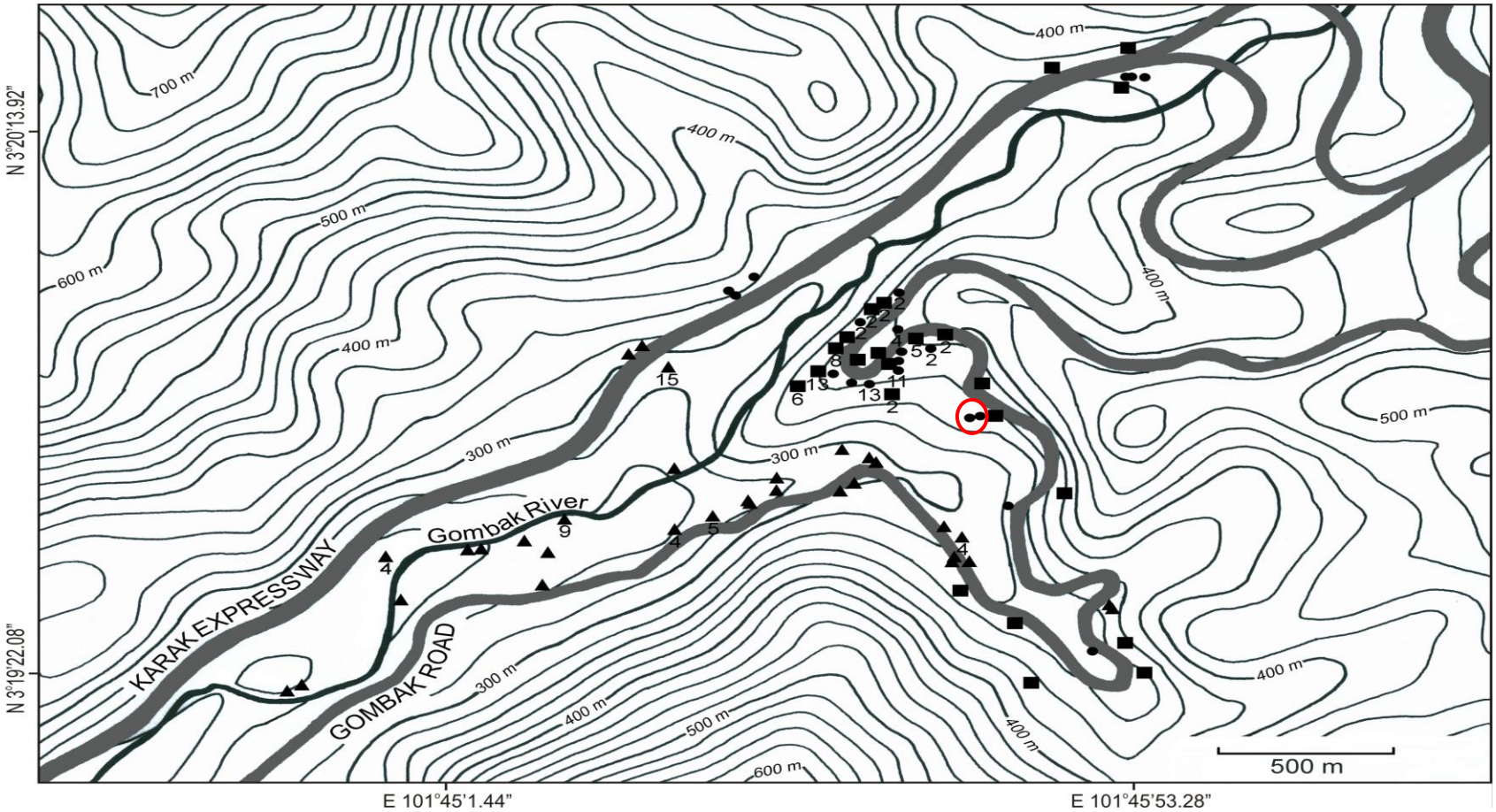



**Fig. 5.1**: Map showing the hybrid zone in the Gombak valley (Low and Wong, 2011). The Gombak River, Gombak Road and Karak Expressway are shown as thick lines. Locations of hybrid clumps are indicated by dots, *D. pendulus* by squares, and *G. scortechinii* by triangles. Numerals represent the number of clumps whenever there were more than a single clump at one site. The positions of the two hybrid clumps employed in the DNA analyses are circled in red.

**Table 5.1**: Some character states of the putative bamboo hybrid. Those intermediate between *Gigantochloa scortechinii* and *Dendrocalamus pendulus*, or resembling one of them, are given in bold.

| *Character* | *Gigantochloa scortechinii* | Hybrid | *Dendrocalamus pendulus* |
|---|---|---|---|
| *Culm: habit* | Erect with nodding tips | **Erect, with finely drawn out, pendulous apical parts** | Flexuose, leaning on neighbouring plants, with apical parts finely drawn out and whiplike |
| *Culm: internode waxiness* | Copiously white-waxy | Not to only slightly white-waxy | Copiously white-waxy |
| *Culm: internode hairiness* | Glabrous generally except for bands of silvery brown hairs flanking each node; sparsely covered with pale hairs in juvenile clumps | Generally covered with scattered dark-brown hairs, with bands of silvery brown hairs flanking each node | Glabrous generally with bands of silvery brown hairs flanking each node |
| *Culm leaf: sheath colour* | Green at base, flushed intense orange towards the top | **Pale yellow-orange with slight tint of pink or dark purple brown** | Greenish to yellowish pink-orange near apex |
| *Cum leaf: sheath hairs* | **Dark brown to black hairs** | **Dark brown hairs** | Loose pale brown hairs |
| *Culm leaf: sheath waxiness* | Very slight waxiness | **Slight to moderately white waxy on the back** | Copious loose white wax mixed with the hairs |
| *Culm leaf: sheath margins* | Firm, not drying faster than the rest of the sheath | **Papery, drying as a thin marginal zone compared to the rest of the sheath** | **Papery, drying as a thin marginal zone compared to the rest of the sheath** |
| *Culm leaf: auricle form* | Low plane rim, 0.5 – 1.5 mm high, glabrous | **Rounded lobes to about 5 mm high with marginal bristles** | **Small rounded lobes, 1.5 – 3.0 mm high, sometimes crisped, with marginal bristles** |

**Table 5.1 (continued...)**

| *Character* | *Gigantochloa scortechinii* | Hybrid | *Dendrocalamus pendulus* |
|---|---|---|---|
| *Culm leaf: blade colour* | **Medium green and leaf-like with pink flush** | **Medium green and leaflike with pink flush** | Yellowish green to brown often with pink flush |
| *Midculm dominant branch: habit* | Dominant primary branch rigid-ascending, | **Dominant primary branch rigid ascending, tending to extend and droop at its tips** | Dominant primary branch long-flexuous, becoming pendulous-whiplike |
| *Pseudospikelet: length* | 12-24 mm | **7-11 mm** | 5-8 mm |
| *Empty glumes: number* | 3-5 | **2-3** | **2-3** |
| *Florets: number* | 4-5 | **2 (rarely 3)** | 1-2 |
| *Terminal empty lemma: presence* | Present | **Present (but absent when there is a 3rd floret formed)** | Absent |
| *Lemmas: hairiness* | Pale-brown long-hairy all over | **Glabrous** | **Glabrous** |
| *Staminal filaments* | **Fused into a tube** | **Fused into a tube** | Free |
| *Anther: colour* | Yellow | **Pink to pale lilac** | Maroon |

## 5.2 Molecular evidence for hybridization

Considering that allelic heterozygosity is a strong indication of an F1 hybrid status, the partial *GBSS*I gene of the putative hybrid individuals and their parental species was sequenced to obtain possible indication. This approach is suitable for the current sampling scale in terms of cost- and time-effectiveness.

Chloroplast DNA sequences (*rps16-trnQ, trnC-rpoB, trnH-psbA* and *trnD-T* intergenic spacers) were also obtained for the hybrid and their parental species for the phylogenetic analyses. *Holttumochloa magica* and *Kinabaluchloa nebulosa* (**Table 3.1**) were used to form the outgroup because of their sister relationship to the *Bambusa-Dendrocalamus-Gigantochloa* complex (BDG complex) as demonstrated in **Chapter 4**. *Dinochloa malayana* (**Table 3.1**), a member of the DMNS clade (which is sister to the BDG complex-*H. magica-K. nebulosa* alliance; see **Chapter 4**), was also included in the outgroup.

### 5.2.1 Sequence characteristics and phylogenetic analyses

PCR-cloning on the *GBSS*I region has successfully extracted haplotypes of the different alleles in the hybrid individuals. Two *GBSS*I haplotypes were identified for each hybrid individual, called Haplotype D and Haplotype G, respectively. Haplotypes D and G are 705–706 bp in length. Multiple DNA sequence alignment for the *GBSS*I haplotypes of the hybrid, *D. pendulus* and *G. scortechinii* revealed that 26 out of 35 variable / indel sites are indicative of the parentage of the hybrid (**Table 5.2**).

**Table 5.2:** The 28 variable sites and 11 indel sites of the partial *GBSSI* gene (722 bp) of the hybrid and its parental species. Dots indicate identical nucleotides compared to those in the first line. Dashes indicate the alignment gaps. Twenty-six sites characterizing the hybrid origin of the hybrid individuals are highlighted in this table.

| Site / Taxon | 1 8 | 2 8 | 6 8 | 1 2 4 | 1 3 9 | 1 5 1 | 1 8 2 | 2 1 6 | 2 2 2 | 2 3 3 | 3 4 0 | 3 5 0 | 3 5 1 | 3 5 2 | 3 6 9 | 3 7 9 | 3 8 0 | 3 9 2 | 3 0 8 | 4 1 1 | 4 4 0 | 4 6 9 | 4 6 2 | 4 6 5 | 4 6 6 | 5 2 4 | 5 7 8 | 6 1 0 | 6 1 1 | 6 1 2 | 6 1 3 | 6 1 5 | 6 3 8 | 6 4 4 | 6 6 3 | 6 6 7 | 6 7 9 | 7 1 1 | 7 2 1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *D. pendulus* (1) | A | A | G | C | T | A | A | T | A | A | C | - | - | - | T | G | C | G | G | C | T | A | C | G | G | C | G | T | A | T | A | T | C | T | T | G | A | C | C |
| *D. pendulus* (2) | . | . | . | . | . | T | . | . | . | . | . | - | - | - | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| *D. pendulus* (3) | - | . | . | . | . | . | . | . | . | . | . | - | - | - | C | . | . | . | . | . | A | . | . | . | . | G | . | . | . | . | . | . | T | . | . | . | . | . | . | . |
| Hybrid Gombak-1 (haplotype D) | . | . | . | . | . | . | . | . | . | . | . | - | - | - | . | . | . | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Hybrid Gombak-2 (haplotype D) | . | . | . | . | . | . | . | . | . | . | . | - | - | - | . | . | . | . | . | . | . | - | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | . | . | . |
| Hybrid Tapah (haplotype D) | . | . | . | . | . | T | . | . | . | . | . | - | - | - | . | . | . | . | . | . | . | - | . | . | . | G | . | . | . | . | . | . | T | . | . | . | . | . | . | . |
| Hybrid Gombak-1 (haplotype G) | . | G | . | T | . | . | T | C | - | - | T | C | C | T | A | A | A | A | T | T | C | - | . | . | A | G | A | - | - | - | - | G | . | C | C | T | T | T | A |
| Hybrid Gombak-2 (haplotype G) | . | G | . | T | . | . | T | C | - | - | T | C | C | T | A | A | A | A | T | T | C | . | . | . | A | G | A | - | - | - | - | G | . | C | C | T | T | T | A |
| Hybrid Tapah (haplotype G) | . | G | A | . | . | . | . | C | - | - | T | C | C | T | A | A | A | A | T | T | C | . | . | A | A | G | A | - | - | - | - | G | . | C | C | T | T | T | A |
| *G. scortechinii* (1) | . | G | . | . | . | . | T | C | - | - | T | C | C | T | A | A | A | A | T | T | C | . | T | . | A | G | A | - | - | - | - | G | . | C | C | T | T | T | A |
| *G. scortechinii* (2) | . | G | . | T | . | . | T | C | - | - | T | C | C | T | A | A | A | A | T | T | C | . | . | . | A | G | A | - | - | - | - | G | . | C | C | T | T | T | A |
| *G. scortechinii* (3) | . | G | . | T | . | . | T | C | - | - | T | C | C | T | A | A | A | A | T | T | C | . | . | . | A | G | A | - | - | - | - | G | . | C | C | T | T | T | A |
| *G. scortechinii* (4) | . | G | . | . | C | . | T | C | - | - | T | C | C | T | A | A | A | A | T | T | C | . | . | . | A | G | A | - | - | - | - | G | . | C | C | T | T | T | A |
| *G. scortechinii* (5) | . | G | . | . | . | . | T | C | - | - | T | C | C | T | A | A | A | A | T | T | C | . | T | . | A | G | A | - | - | - | - | G | . | C | C | T | T | T | A |

The aligned data matrix of the partial *GBSS*I gene for the ingroup consists of 707 characters, of which 26 are parsimony-informative. MP analysis resulted in four equally most parsimonious trees (**Fig. 5.2**). Bayesian analysis using Model K80 has generated a similar topology. All five *G. scortechinii* accessions form a clade with G haplotypes of the hybrid accessions, whereas all three *D. pendulus* accessions form a clade with the D haplotypes of the hybrid accessions (**Fig. 5.2**).

The aligned data matrix of the combined cpDNA (*rps16-trnQ* + *trnC-rpoB* + *trnH-psbA* + *trnD-T*) dataset for the ingroup consists of 3889 characters, of which 26 are parsimony-informative. MP analysis resulted in 4 equally most parsimonious trees (**Fig. 5.3**). Bayesian analysis using Model HKY + I generated a similar topology. One of the major clades was formed by all three *D. pendulus* accessions, all three hybrid accessions, as well as three of the *G. scortechinii* accessions. The remaining two accessions of *G. scortechinii* form another clade.

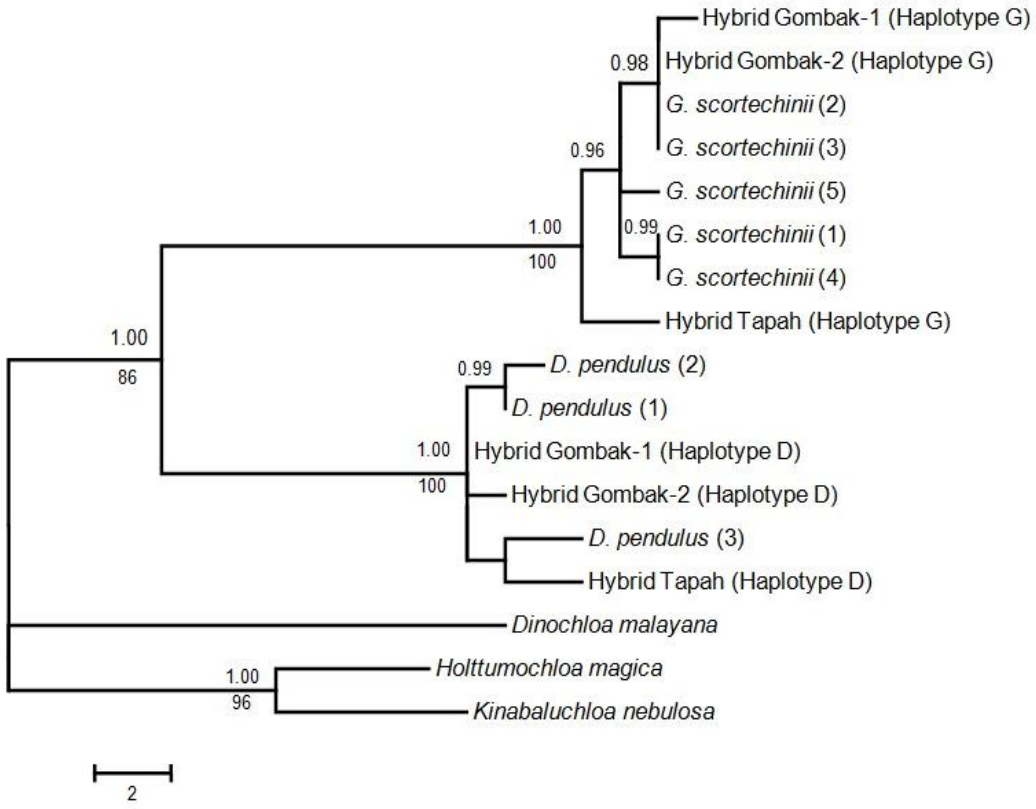



**Figure 5.2**: One of the four most parsimonious trees from the maximum parsimony analysis based on the partial *GBSSI* region (Tree length = 67, CI = 0.9254, RI = 0.9655). Posterior probabilities >0.90 are shown above the nodes, bootstrap support values >70% below the nodes. The tree is drawn to scale, with branch lengths indicating evolutionary distances as number of base substitutions per site.

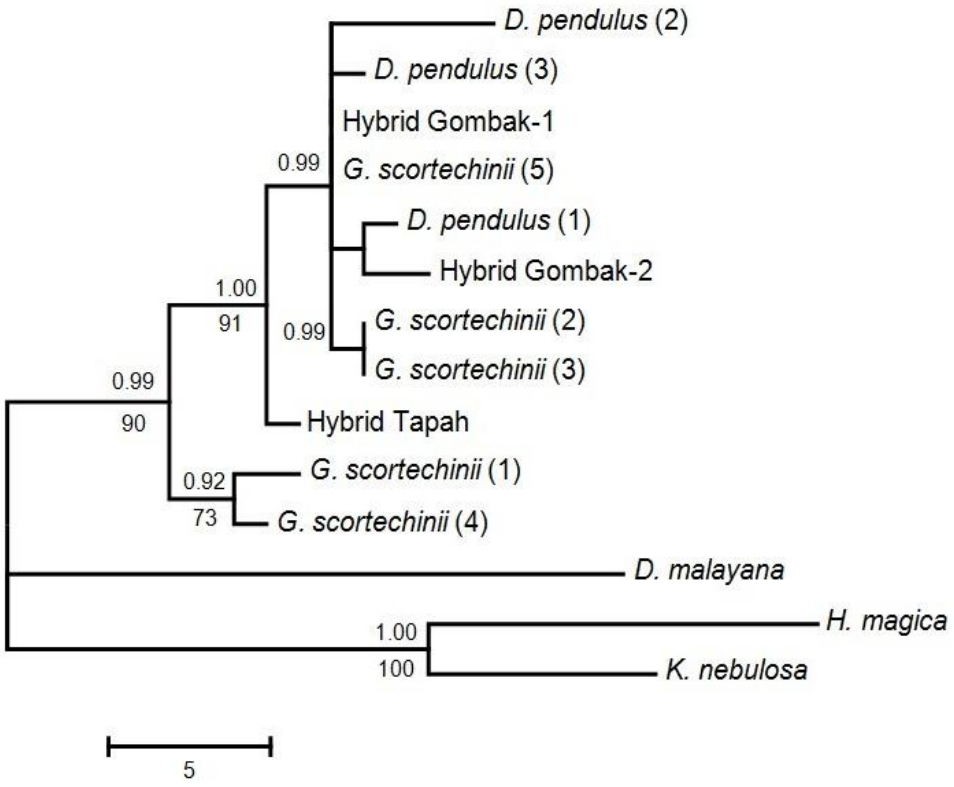



**Figure 5.3**: One of the four most parsimonious trees from the analysis using maximum parsimony analysis based on 4 cpDNA intergenic spacers, *rps16-trnQ, trnC-rpoB, trnH-psbA*, and *trnD-T* (Tree length = 78, CI = 0.9231, RI = 0.8868). Posterior probabilities >0.90 are shown above the nodes, bootstrap support values >70% below the nodes. The tree is drawn to scale, with branch lengths indicating evolutionary distances as number of base substitutions per site.

### 5.2.2 Parentage of the hybrid

Indels and nucleotide substitutions observed in the partial *GBSS*I gene sequences of Hybrid Gombak-1, Hybrid Gombak-2 and Hybrid Tapah (**Table 5.1**) suggest that haplotype D is derived from *D. pendulus*, and haplotype G, from *G. scortechinii*, as would be expected. This hypothesis was also supported by the placement of haplotypes D and G in the *GBSS*I topology, where haplotypes D form a single clade with *D. pendulus* and haplotypes G form a single clade with *G. scortechinii* (**Fig. 5.2**). From the genotypes of the hybrid and its parental species, the hybrid is reasonably interpreted as a relatively recent F1 offspring.

Inference of the seed parent of the hybrid was intended in the analysis based on the cpDNA data (**Fig. 5.3**), assuming that cpDNA is maternally inherited in the bamboos, as reported for many angiosperm taxa (Corriveau and Coleman, 1988). However, *D. pendulus* and *G. scortechinii* did not form distinct clades in the cpDNA topology. Rather, one of the two clades consists of *D. pendulus*, *G. scortechinii* and the hybrids, and another clade consists of only *G. scortechinii* (**Fig. 5.3**). Although this clustering pattern suggests a possibility that *D. pendulus* could be always grouped with the hybrids, the small sample size used in this study does not permit any further inference. A careful examination on the aligned cpDNA data matrix also fails to detect any trace on the maternal origin of the hybrids because the cpDNA sequences of both parental species are highly similar to that of the hybrids. The variable sites extracted from the cpDNA data is provided in **Appendix H**. It is, therefore, not feasible to identify the seed parent of the hybrid taxa from the limited phylogenetic analysis here.

### 5.3 Ecological aspects of the hybridization

A concurrent study of a 4-km stretch of the hybrid zone in the Gombak valley recorded 48 hybrid clumps (individuals), 55 clumps of *D. pendulus* and 67 clumps of *G. scortechinii* (Wong and Low, 2011). Most hybrid individuals were found to have established near the *D. pendulus* clumps, indicating that *D. pendulus* is the seed parent of the hybrid because caryopses are expected to have poorer dispersal ability compared to pollen and mostly fall around the seed parent clump (Wong, 1995a, 1995b).

From the assumed demographic structure of the hybrid population, of 35 clumps (73%) ranked as mature (indicated by presence of culms exceeding 3.5 cm diameter) and only 7 young clumps considered young (defined as clumps without mature-size culms present), this hybrid population was likely derived from at most 1–2 parental seeding events. Of all the hybrid individuals, only seven had flowered (with signs of recent flowering but no further fresh flowers during the census) and three were flowering at the time of the survey, suggesting a variation in flowering age; this, as well as the fact that hybrid progeny were distributed among many maternal (parental) clumps, suggested that the hybrid population was likely to contain a degree of heterozygosity. This is likely to result in variation in vegetative longevity as well, suggesting that the hybrid is likely to persist and become stabilized in time (Wong and Low, 2011).

It was also suggested that this hybrid population was established only relatively recently, and probably related to the development of the Karak Expressway that runs parallel to the Gombak Valley where the hybridization was studied, just before 1980. Greater openness in the valley probably allowed an increase in abundance of both *D. pendulus* and *G. scortechinii* in the intervening period. Land clearing removed forest trees which could have been a natural impediment to gene flow among bamboo clumps, thus also increasing the opportunity for cross pollination among *G. scortechinii* and *D. pendulus* clumps (Wong and Low, 2011). Cross-fertilization of course required that both *D. pendulus* and *G. scortechinii* flowered coincided in their flowering.

Demonstration of the hybrid between *D. pendulus* and *G. scortechinii* in the present study, its recurrence in different geographical localities, and likelihood of persistence, have been the precursor to the naming of the hybrid as × *Gigantocalamus malpenensis* K.M.Wong (Goh et al., 2011).

### 5.4 Implications for possible introgressive hybridization

Molecular evidence for the natural hybrid between *D. pendulus* and *G. scortechinii*, the two common bamboos in Peninsular Malaysia, also provides support for the various existing hypotheses on hybridization among SE Asian bamboos, e.g., hybrid swarms of the Malayan-Javan *Gigantochloa* taxa (Holttum, 1958) and the hybrid origin of *G. ridleyi* that had given rise to disparate variants among its probable F2 progeny (Muller, 1998, 2003). The non-seeding behaviour observed in the hybrid

between *D. pendulus* and *G. scortechinii* suggests that sterility or low fertility in a number of SE Asian bamboo taxa, especially those never found in the wild (e.g., *B. vulgaris*; Koshy and Jee, 2001), could be an indication of hybrid origin.

The present study also showed that there are two cpDNA haplotypes among the *G. scortechinii* individuals. Similar intra-specific cpDNA variations have been reported for *Quercus* (Whittemore and Schaal, 1991; Petit et al., 1997; Bordács et al., 2000; Petit et al., 2008) and such patterns were attributed to the interspecific gene flow resulting from introgression (Lexer et al., 2006) or shared polymorphism (Muir and Schlötterer, 2005, 2006). Other studies inferring chloroplast capture based on the cpDNA haplotype sharing patterns include those in Saxifragaceae (Soltis et al., 1991; Okuyama et al., 2005), Pinaceae (Watano et al., 1996; Senjo et al., 1999; Ito et al., 2008), *Phlox* (Ferguson et al., 2002), *Salix* (Hardig et al., 2000), *Nothofagus* (Acosta and Premoli, 2010) and *Gossypium* (Álvarez and Wendel, 2006; Wendel et al., 2010). Two of these studies demonstrated that the chloroplast introgression has a strong association to geographic distributions rather than taxonomic relationships (Whittermore and Schaal, 1991; Acosta and Premoli, 2010). It is noteworthy that sharing of the chloroplast DNA haplotype was also observed in the two well-defined North American bamboos, *Arundinaria tecta* and *A. appalachiana* (Triplett et al., 2010). The present study implies that past chloroplast introgression had been possible between *D. pendulus* and *G. scortechinii*. Thus, the cpDNA phylogenetic tree topology represents the result of reciprocal crosses followed by introgression. Extensive study of more populations of *D. pendulus* and *G. scortechinii*, and perhaps

more of their congeners in Peninsular Malaysia are much needed to further clarify this situation.

# CHAPTER 6

# THE POSSIBILITY OF RETICULATE EVOLUTION IN THE BAMBUSINAE

## 6.1 Implications of possible hybridization in previous phylogenetic analyses

Recent molecular studies addressing individual aspects of Bambusinae phylogeny were based on combined cpDNA and nuclear DNA markers (Yang et al., 2008; Yang et al., 2010) and multi-locus cpDNA data (*trnL-F* intergenic spacer, *atpB-rbcL* intergenic spacer, *rps*16 intron and *mat*K; Sungkaew et al., 2009). Results from Yang et al. (2008) and Yang et al. (2010) were, however, largely influenced by the *GBSS*I gene data because of the relatively low variability of cpDNA markers compared to that of the *GBSS*I gene marker (4.6 % vs. 18.0% in Yang et al., 2008; 1.5–2.2 % vs. 12.4% in Yang et al., 2010), and the largely unresolved phylogenetic trees based on individual cpDNA markers (Yang et al., 2008; Yang et al., 2010). Although there is no attempt to explore the possible incidences of hybridization in Bambusinae by studying the incongruence between the nuclear and cpDNA topologies, some inconsistencies in clustering pattern within the Bambusinae are already noticeable from the previous studies. For instance, Sungkaew et al. (2009) obtained three clusters within the core Bambusinae, each formed by a mixture of *Bambusa* taxa and taxa from a few other genera. Yang et al. (2008) and Yang et al. (2010), on the other hand, indicated a well-supported group of *Bambusa* sensu lato and suggested a close relationship within a *Dendrocalamus* complex (which includes *Dendrocalamus, Gigantochloa, Oxytenanthera* and *Neosinocalamus affinis*).

A phylogenetic tree was reconstructed in the present study, using sequences of all 3 cpDNA regions published by Yang et al. (2010) (MP tree length = 67, CI = 0.7015, RI = 0.9177). Three cases of minute inversions were observed in the aligned data matrix and treated as single characters to avoid overwieghting these mutational changes in the data analysis (Kelchner, 2000; **Appendix G**). Although partition homogeneity (PH) tests indicated that the *GBSS*I and the individual cpDNA data were congruent (Yang et al., 2010), the *GBSS*I topology and the combined cpDNA topology did contain a degree of inconsistency (**Fig. 6.1**). For example, in the cpDNA phylogeny, *D. farinosus, D. jianshuiensis, D. peculiaris, D. ovatus, D. semiscandens, D. tsiangii,* and *D. tomentosus* formed a clade (1.00 PP) but in the *GBSS*I phylogeny (Fig. S1 in Yang et al., 2010), they were placed in different clades, i.e., Clade 2E and Clade 2D (**Fig. 6.1**). Disparity in the clustering pattern was also observed among a few *Bambusa* taxa in Yang et al. (2010)'s study. *Bambusa cerossissima, B. chungii, B. remotiflora, B. surrecta* and *B. yunnanensis* together formed a clade (72 BP / 0.97 PP) in the *GBSS*I topology (Fig. S1 in Yang et al., 2010), but only *B. cerossissima* and *B. surrecta* formed a well-supported group (0.99 PP / 75 BP) in the cpDNA topology (**Fig. 6.1**).

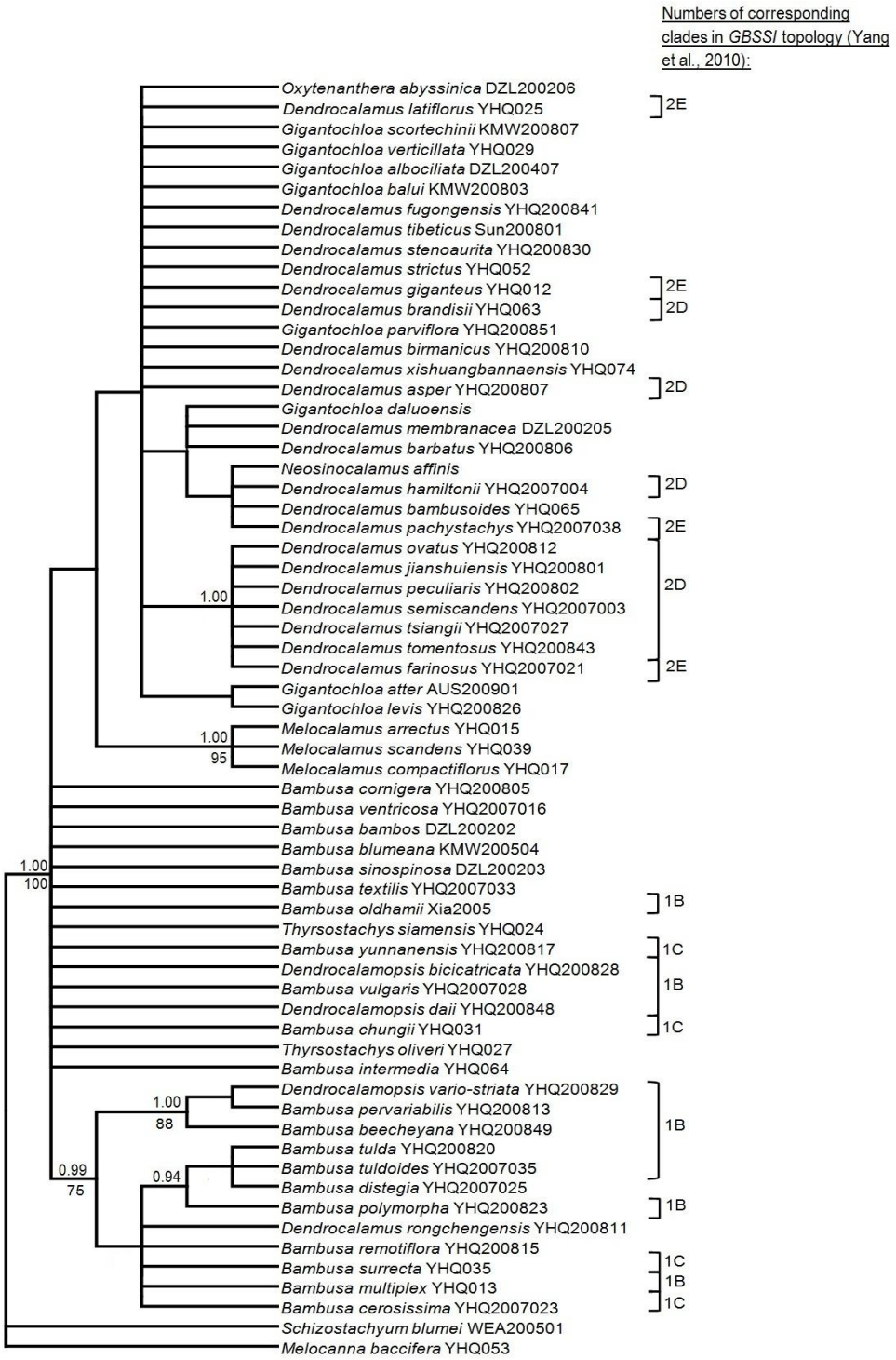



**Fig. 6.1**: Strict consensus tree of 475 most parsimonious trees reconstructed based on the cpDNA *psb*A-*trn*H intergenic spacer, *rps*16 intron and *rpl*32-*trn*L intergenic spacer (Yang et al., 2010). Posterior probabilities (≥ 0.90) of Bayesian analysis were shown above the branches, bootstrap support values (≥ 70 %) of maximum parimony analysis were shown below the branches.

Other molecular phylogenetic studies for the Bambusinae have used relatively smaller taxon samplings, in some cases less informative DNA markers, or tackled more specific problems, e.g., the possible inclusion of *Racemobambos* in the Bambusinae (Stapleton et al., 2009), or the subgeneric classification of Chinese *Bambusa* (Sun et al., 2005; Sun et al., 2006). These studies are not readily comparable, and the taxonomic placement of the major genera in Bambusinae has yet to find general agreement. In spite of this, the source of such phylogenetic confusion has not been addressed.

A robust multi-locus cpDNA study on the temperate bamboo tribe, undertaken by Triplett and Clark (2010), has also recovered much inconsistency between the current morphology-based classification and the phylogenetic relationship. Some associations in their analyses supported existing hypotheses of the intergeneric hybridization. *Hibanobambusa* was established as a hybrid genus between *Phyllostachys* and *Sasa* (Maruyama et al., 1979) and this was supported by *H. tranquillans* sharing a common cpDNA sequence with *Sasa veitchii* (Triplett and Clark, 2010). The hybrid origin of *Sasaella*, i.e., between *Pleioblastus* and *Sasa*, has long been speculated and later supported by various morphological examinations (Suzuki, 1987; Watanabe et al., 1991). Identical cpDNA sequences of *Sasaella ramosa*, *Sasaella bitchuensis* and *Sasa veitchii*, as well as the association between *Sasaella masamuneana* and *Pleioblastus* sect. *Nezasa*, appeared to suggest a reticulate relationship among *Sasaella*, *Sasa* and *Pleioblastus* (Triplett and Clark, 2010). *Brachystachyum* and *Semiarundinaria* were both morphologically intermediate between *Phyllostachys* and *Pleioblastus* and the

hybrid origin of the latter was supported by Muramatsu (1981). Their placement in two different major clades, one with *Phyllostachys* and the other with *Pleioblastus*, suggest that they represent reciprocal crosses between members of these two parental genera (Triplett and Clark, 2010).

## 6.2 Possibility of reticulate evolution in the BDG complex and Bambusinae

### 6.2.1 Incongruences between nuclear and plastid topologies in the present study

For the cpDNA- and *GBSS*I-based phylogenetic trees recovered from the analysis in **Chapter 4**, incongruence between the two topologies was assessed visually (**Fig. 6.2**). Subclades identified as BDG1 (1.00 PP / 79% BP) and BDG2 (1.00 PP / 70% BP) in the cpDNA topology (**Fig. 6.2; left**) were not recovered in the *GBSS*I topology. Conversely, Subclades D (0.99 PP / 70 % BP) and G (1.00 PP / 96% BP) in the *GBSS*I topology (**Fig. 6.2; right**) were not recovered in the cpDNA topology.

All four alternative hypotheses, (1) constraining the members of Subclade D (of the *GBSS*I topology) to be monophyletic in the cpDNA topology, (2) constraining the members of Subclade G (of the *GBSS*I topology) to be monophyletic in the cpDNA topology, (3) constraining the members of Subclade BDG1 (of the cpDNA topology) to be monophyletic, and (4) constraining the members of Subclade BDG2 (of cpDNA topology) to be monophyletic, were rejected by the SH tests (**Table 6.1; Appendix I**). This indicated that these subclades were responsible for significant incongruence between the cpDNA and *GBSS*I topologies.

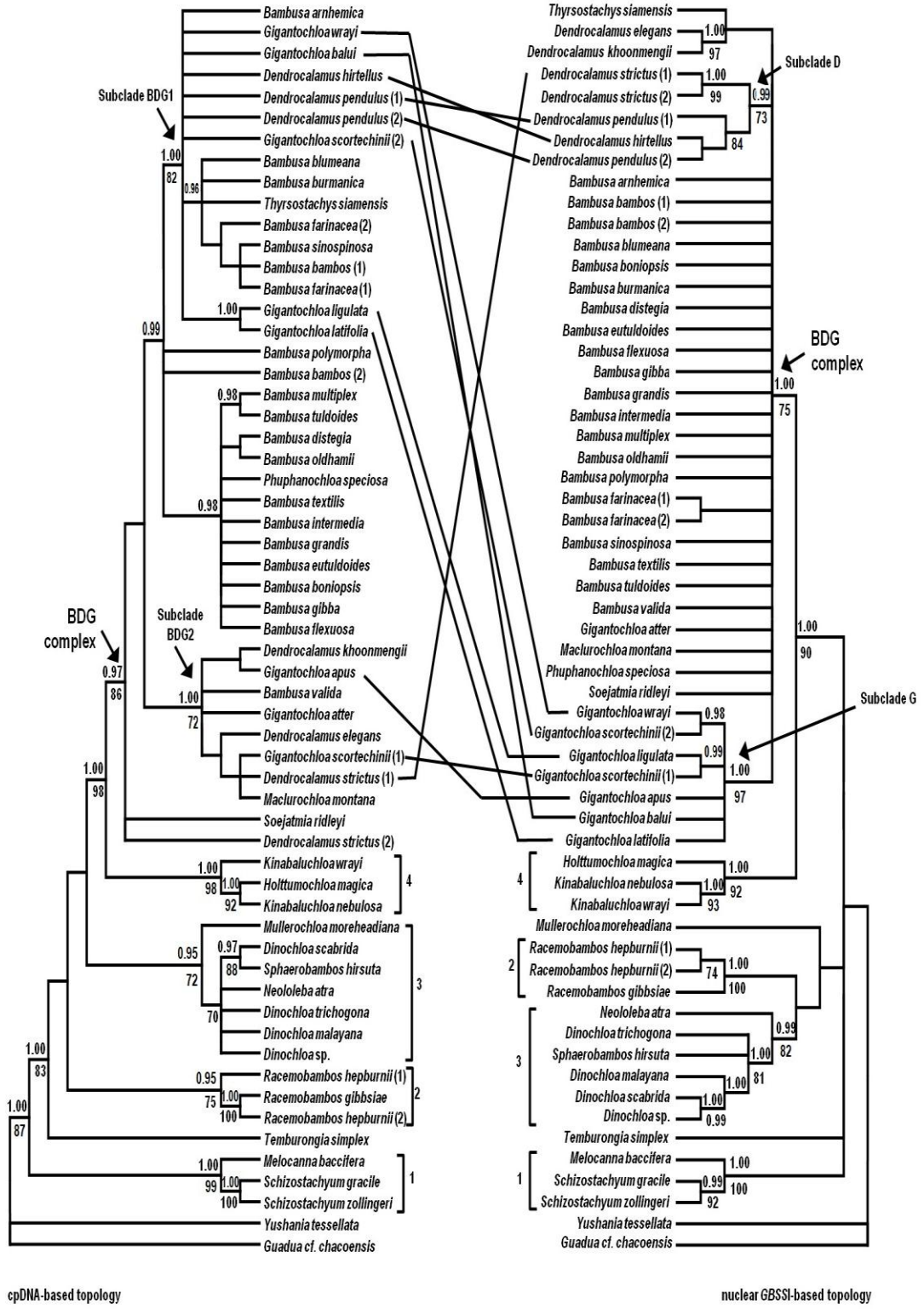



**Fig. 6.2:** Strict consensus of the most parsimonious trees based on three cpDNA data (left) and the partial nuclear *GBSS*I data (right). Taxa involved in the incongruence between the two topologies are connected by lines between the two topologies shown.

**Table 6.1:** Results of Shimodairo-Hasegawa (SH) tests for assessing incongruences within the BDG complex.

| Alternative hypothesis | Constraint | Constrained topology | *p* value |
|---|---|---|---|
| Members of Subclade D in the *GBSS*I topology are also monophyletic in the cpDNA topology | *Dendrocalamus hirtellus, D. pendulus* (1) & (2), *D. strictus* (1) & (2) form a clade in the cpDNA analysis | cpDNA | 0.008* |
| Members of Subclade G in the *GBSS*I topology are also monophyletic in the cpDNA topology | *Gigantochloa apus, G. balui, G. latifolia, G. ligulata, G. scortechinii* and *G. wrayi* form a clade in the cpDNA analysis | cpDNA | 0.015* |
| Members of Subclade BDG1 in the cpDNA topology are also monophyletic in the *GBSS*I topology | *Bambusa arnhemica, B. bambos* (1), *B. blumeana, B. burmanica, B. farinacea* (1) & (2), *B. sinospinosa, Dendrocalamus hirtellus, D. pendulus* (1) & (2), *Gigantochloa balui, G. latifolia, G. ligulata, G. scortechinii* (2), *G. wrayi* and *Thyrsostachys siamensis* form a clade in the *GBSS*I analysis | *GBSS*I | 0.001* |
| Members of Subclade BDG2 of the cpDNA topology are also monophyletic in the *GBSS*I topology | *Bambusa valida, Dendrocalamus elegans, D. khoonmengii, D. strictus, Gigantochloa apus, G. atter, G. scortechinii* and *Maclurochloa montana* form a clade in the *GBSS*I analysis | *GBSS*I | 0.000* |

* indicates rejection at the 95% confidence level.

The taxa which were recovered in different associations in the cpDNA- and *GBSS*I-based topologies are as follows:

(a) *Gigantochloa*

In the cpDNA topology (**Fig. 6.2; left**), two groups of *Gigantochloa* spp. are differently associated. *Gigantochloa balui, G. ligulata, G. latifolia, G. scortechinii* (Accession 2), *G. wrayi* and other non-*Gigantochloa* taxa form Subclade BDG1. *Gigantochloa apus*, *G. atter*, *G. scortechinii* (Accession 1) and other non-*Gigantochloa* taxa form Clade BDG2. The two accessions of *G. scortechinii* are not closely associated. However, in the *GBSS*I topology (**Fig. 6.2; right**), all *Gigantochloa* taxa except for *G. atter* (unresolved) form a single group, Subclade G.

(b) *Dendrocalamus*

In the *GBSS*I topology (**Fig. 6.2; right**), *Dendrocalamus hirtellus* Ridley, *D. pendulus* and *D. strictus* formed a well-supported subclade (Subclade D), whereas in the cpDNA topology (**Fig. 6.2; left**), *D. hirtellus* and *D. pendulus* joined Subclade BDG1 and *D. strictus* (Accession 1) joined subclade BDG2.

(c) *Bambusa*, *Maclurochloa*, *Phuphanochloa, Thyrsostachys*

*Bambusa*, *Maclurochloa* and *Thyrsostachys* were resolved into clades BDG1 and BDG2 in the cpDNA topology together with *Dendrocalamus* and *Gigantochloa,* but largely unresolved in the *GBSS*I topology (**Fig. 6.2**). A cluster in the cpDNA topology was made up of the 11 species of *Bambusa* sampled (i.e., the bulk of the

genus) and *Phuphanochloa* with equivocal support (i.e., supported by PP but not BP). Such clustering was not observed in the *GBSS*I topology.

### 6.2.2 Possible widespread reticulate evolution in the BDG complex

The present study has demonstrated strong incongruence within the BDG complex between the cpDNA and *GBSS*I topologies. The type species and type alliance of *Dendrocalamus* (Subclade D), as well as nearly all *Gigantochloa* taxa in this study (Subclade G) formed distinct monophyletic groups in the *GBSS*I topology recovered. On the other hand, in the cpDNA analyses, these *Dendrocalamus* and *Gigantochloa* taxa were recovered in Subclades BDG1 and BDG2 that contained an assortment of species regardless of their generic designation.

The *GBSS*I topology would appear to correspond more with the morphology-based distinction between the type alliance of *Dendrocalamus* and *Gigantochloa* as represented in the Malay Peninsula (Holttum, 1958; Wong, 1995a), based on such character states as small spikelets with typically 1-2 flowers and free staminal filaments (*Dendrocalamus*) versus larger spikelets with more flowers and fused, tube-forming filaments (*Gigantochloa*). The exception among *Gigantochloa* taxa was *G. atter*, which did not cluster with the others in the *Gigantochloa* subclade G. Unlike the culm-leaf auricles of Subclade G members, which are low rim-like and largely glabrous, those of *G. atter* are raised, lobe-like and densely long-bristly (Widjaja, 1987). This species is thus somewhat aberrant and has been considered doubtfully

wild, perhaps a selected clone carried to Indonesia as ancient peoples migrated (Holttum, 1958; Wong, 1995a).

Incongruence between the biparentally derived *GBSS*I and the the maternally derived cpDNA topologies is consistent with the possibility of past introgressive hybridization (which produces stabilized introgressants and, eventually, new hybrid species; Rieseberg and Brunsfield, 1992; Rieseberg and Wendel, 1993), with or without the contribution of incomplete lineage sorting (due to retention of ancestral polymorphisms; Avise et al., 1987; Pamilo and Nei, 1988). While relative recency of origin and possible incomplete lineage sorting for the BDG complex cannot be dismissed, other evidence for past introgression includes the intergeneric hybrid between *Dendrocalamus* and *Gigantochloa* documented in the present work, as summarized in Goh et al. (2011), as well as the observations of Holttum (1958) and Muller (1998) regarding the possible existence of hybrid swarms. Past hybridization has also been inferred in explaining the chloroplast genealogy of *Macaranga* (Euphorbiaceae) in SE Asia (Bänfer et al., 2006), nucleotide polymorphism in nuclear gene regions of *Shorea* species (Dipterocarpaceae) (Ishiyama et al., 2008), and incongruence between the nuclear and chloroplast phylogenies of *Shorea*. In the latter case, capture of foreign cpDNA by *S. curtisii* was demonstrated (Kamiya, Nanami et al., 2011) and morphological and molecular evidence exists for hybridization between *S. curtisii* and *S. leprosula* (Kamiya, Gan et al., 2011). Such introgression could also explain the morphological variability and distinct forms of *Dendrocalamus strictus*, a widespread Indian bamboo (Bahadur and Jain, 1981), as the cpDNA topology in the

present study shows only one of two accessions recovered in the well-supported Subclade BDG2.

In the *GBSS*I analyses, *Bambusa* taxa were largely unresolved within the BDG complex, perhaps due to insufficiently informative variability of the DNA marker employed. Some of them were resolved as part of Subclade BDG1 in the cpDNA analyses, indicating that they share a common maternal ancestor with some of the *Dendrocalamus* and *Gigantochloa* taxa, and are probably involved in past introgressive hybridization within the group. There is also a possibility that some hybrid taxa are not readily detectable in the BDG complex. Morphologically, introgressants are often overlooked because they resemble the parental species with which they have been backcrossed, and may be taxonomically placed as varieties of the parental species; over generations, introgressed components could be genetically diluted or changed because of mutations (Rieseberg and Wendel, 1993).

**Table 6.2** shows the dimorphic sites in the *GBSS*I data of the BDG complex (see **Chapter 4**, **Table 4.1**). For most of the heterozygous taxa (11 out of 14; asterisked in **Table 6.2**), both bases of their dimorphic sites are present in the other homogyzous taxa. This suggests that the heterozygosity in these taxa are probably derived from the introgression among some other members in the BDG complex. *Bambusa grandis* and *B. oldhamii* are likely to have acquired one of their heterozygous alleles from *Gigantochloa* as they share the common bases at the sites specific to the *Gigantochloa*

clade (i.e., 369, 370 and 372). The origins of the other heterozygous taxa are not readily detectable because their dimorphism do not occur at the genus-specific sites.

The present study indicates that widespread reticulate evolution and introgression events within the BDG complex are probable events that have contributed to blurring the morphological boundaries among genera in this group, which is acknowledged to be taxonomically difficult (e.g., Soderstrom and Ellis, 1987). The taxa involved in the genealogical incongruence identified in the present study are therefore specific cases that deserve further study at the population level for obtaining a better understanding of the mechanisms involved, and of the relative importance of introgressive hybridization.

**Table 6.2:** The dimorphic sites in the aligned *GBSS*I sequence among the BDG complex. Dots indicate identical nucleotides compared to those in the first line. The highlighted sites indicate the sites where both bases of the dimorphic sites are present in the other homozygous taxa. The taxa possessing such dimorphic sites are indicated by asterisks. (Dimorphic sites: R = A / G; Y = C / T; M = A / C; K = G / T; S = C / G; W = A / T)

| Taxon \ Site | 1 2 | 5 3 | 9 2 | 1 1 5 | 1 8 5 | 1 8 7 | 1 9 4 | 2 0 2 | 2 5 2 | 2 6 4 | 3 3 0 | 3 3 2 | 3 3 4 | 3 3 9 | 3 6 0 | 3 7 2 | 3 8 2 | 3 9 4 | 4 4 7 | 4 4 5 | 4 4 3 | 4 7 6 | 4 8 5 | 5 0 7 | 5 5 6 | 5 5 8 | 5 6 3 | 6 6 3 | 6 6 5 | 6 8 9 | 6 8 1 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *B. arnhemica* | T | C | C | C | A | T | T | T | G | T | T | A | T | G | C | G | T | G | A | G | A | G | A | C | G | A | G | C | T | A | A |
| *B. bambos* (1) | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | A | T | G | . | . |
| *B. bambos* (2) | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | A | . | A | A | T | G | T | . |
| *B. blumeana* | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | A | . | A | A | T | G | . | . |
| *B. boniopsis* | . | . | . | . | . | C | . | . | . | . | C | . | C | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . |
| *B. burmanica* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | A | . | A | A | T | G | . | . |
| *B. distegia* | . | T | . | . | . | C | . | . | . | C | . | . | . | . | . | . | . | A | . | . | . | A | . | T | A | . | A | T | G | . | . |
| *B. eutuldoides* | . | . | . | . | . | . | . | . | . | C | C | . | . | . | . | . | . | . | . | . | . | . | . | A | . | A | A | T | G | . | . |
| *B. farinacea* (1) | . | . | . | . | . | C | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | A | . | A | A | T | G | . | . |
| *B. farinacea* (2) | . | . | . | . | . | C | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | A | . | A | A | T | G | . | . |
| *B. flexuosa* | . | . | T | . | . | . | . | T | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | A | . | A | A | T | . | . | . |
| **B. gibba* | . | . | Y | . | . | Y | . | . | K | . | Y | . | C | . | . | . | . | . | . | . | . | . | . | R | . | R | . | . | . | . | . |
| **B. grandis* | . | . | . | . | . | . | . | . | . | . | . | . | . | R | M | R | C | . | M | . | R | . | . | A | . | . | Y | . | W | R | |
| **B. intermedia* | . | Y | Y | . | . | Y | . | . | K | Y | . | . | . | . | . | . | . | R | M | . | . | R | . | Y | A | . | A | T | G | . | . |
| **B. multiplex* | . | . | . | . | . | C | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | G | . | R | . | R | Y | Y | . | . | |
| **B. oldhamii* | . | . | . | . | . | Y | . | . | . | . | C | . | C | R | M | R | . | . | . | . | . | G | . | R | . | . | . | . | . | R | |
| *B. polymorpha* | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | G | . | G | |
| *B. sinospinosa* | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | . | . | A | . | A | T | G | . | . | |
| **B. textilis* | . | . | . | . | . | C | . | . | . | Y | Y | . | Y | . | . | . | . | R | . | . | R | R | Y | . | . | A | . | G | . | . | |
| **B. tuldoides* | . | . | Y | . | . | Y | . | Y | . | . | Y | . | . | . | . | . | . | . | M | . | . | . | R | . | R | . | R | . | . | . | |
| **B. valida* | . | . | Y | Y | . | . | . | . | K | . | . | M | . | . | . | . | . | . | M | . | R | . | . | A | . | R | Y | . | W | R | |
| **D. elegans* | . | . | . | . | R | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | G | . | . | A | . | A | T | G | . | G | |
| *D. hirtellus* | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | . | . | S | . | . | . | W | A | T | G | . | . | |
| *D. khoonmengii* | . | . | . | . | G | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | G | . | . | A | . | A | T | G | . | G | |
| *D. pendulus* (1) | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | A | . | . | . | G | . | . | . | . | A | T | G | . | . | |
| *D. pendulus* (2) | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | . | . | G | . | . | . | . | A | T | G | . | . | |
| *D. strictus* (1) | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | . | . | G | . | . | . | . | A | T | . | . | G | |
| *D. strictus* (2) | . | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | . | . | G | . | . | . | . | A | T | . | . | G | |
| *G. apus* | . | . | . | . | T | . | . | . | . | . | . | . | A | A | A | C | . | . | . | . | . | . | . | A | . | A | . | . | T | . | |
| **G. atter* | . | . | . | Y | . | . | . | . | . | . | . | C | . | . | . | . | Y | . | . | . | R | . | . | A | . | . | Y | G | W | . | |
| *G. balui* | . | . | . | . | . | . | Y | . | . | . | . | . | A | A | A | C | . | . | R | . | . | . | . | A | . | A | . | . | T | . | |
| *G. latifolia* | . | . | . | . | C | . | . | . | . | . | . | . | A | A | A | C | . | . | . | . | . | . | . | A | . | A | . | . | T | . | |
| *G. ligulata* | . | . | . | . | T | . | . | . | . | . | . | . | A | A | A | C | . | . | . | . | . | . | . | A | . | A | . | . | T | . | |
| *G. scortechinii* (1) | . | . | . | . | T | . | . | . | . | . | . | . | A | A | A | C | . | . | . | . | . | . | . | A | . | A | . | . | T | . | |
| **G. scortechinii* (2) | . | . | . | . | W | . | . | . | . | . | . | . | A | A | A | C | . | . | . | . | . | . | . | A | . | A | . | . | T | . | |
| *G. wrayi* | W | . | . | . | T | . | . | . | . | . | . | . | A | A | A | C | . | . | . | . | . | . | . | A | . | A | . | . | T | . | |
| *P. speciosa* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | A | T | G | . | . | |
| *T. siamensis* | . | . | . | . | . | . | . | . | . | C | . | . | . | . | . | . | . | . | . | . | G | . | . | A | . | . | . | G | . | . | |
| *M. montana* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | A | . | A | T | G | . | . | |
| *S. ridleyi* | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | A | . | . | . | . | . | . | . | A | T | . | . | . | |

# CHAPTER 7

# CONCLUSIONS

Among Paleotropical bamboos, the delimitation and classification of genera and subtribes has been problematic. In particular, the relationships of *Bambusa*, *Dendrocalamus* and *Gigantochloa*, which are most diverse in mainland SE Asia (and here taken to form the bulk of the BDG complex), with other genera in the insular SE Asian bamboo flora, notably a number of morphologically distinctive climbing forest bamboos, have not been satisfactorily resolved.

**General phylogenetic relationships of SE Asian bamboos**

The present work addresses the phylogenetic relationships among the Bambusinae and climbing bamboo genera using a molecular approach (employing data from three cpDNA and a nuclear regions). A total of 15 genera in the Bambusinae s.l. (a sister clade to the Melocanninae) were sampled for the present study. The major lineages recovered for the Bambusinae s.l. are: (1) the BDG complex, (2) the *Holttumochloa-Kinabaluchloa* clade, (3) the DMNS (*Dinochloa*, *Mullerochloa*, *Neololeba*, *Sphaerobambos*) clade, (4) the *Racemobambos* clade and (5) *Temburongia simplex*. Within the BDG complex, there are three species-rich genera, *Bambusa*, *Dendrocalamus* and *Gigantochloa*, as well as the smaller *Thyrsostachys* (only one of two known species used here) and three monotypic genera, viz., *Maclurochloa*, *Phuphanochloa* and *Soejatmia*. The *Holttumochloa-Kinabaluchloa* clade is sister to

the BDG complex; these together form a clade that is distinct from the DMNS clade, *Racemobambos* and *T. simplex*.

While the BDG complex is essentially a clade of erect-suberect bamboos (with only two exceptions, *Maclurochloa* and *Soejatmia* that have a climbing habit), the other major lineages are formed only by climbing bamboo genera. The present study highlighted two noticeable distinctions between the BDG complex and the other clades of climbing bamboo genera:

(a) ***Reticulate evolution is prevalent in the BDG complex but not among the climbing bamboo lineages***

The BDG complex consists of closely related genera in which past introgressive hybridization could have occurred. This is shown by significant incongruences between the biparentally inherited nuclear DNA and maternally inherited cpDNA datasets. The nuclear DNA topology is more consistent with the morphology-based classification of *Dendrocalamus* and *Gigantochloa*, while the cpDNA topology would appear to reflect the chloroplast introgression in this complex.

Existing hybridization between *Dendrocalamus pendulus* and *Gigantochloa scortechinii*, as demonstrated in this study, serves as a vivid demonstration of the extent of hybridization that could occur among members of the BDG complex as suggested by the topological incongruences shown among cpDNA and nuclear DNA datasets. The molecular evidence for hybridization obtained in the present study also

lend support to suggestions by Holttum (1958) and Muller (1998) that bamboo hybrid swarms exist, and probably include the Javan-Malayan cultivated *Gigantochloa* clones. Introgressive hybridization is also likely to account for the ambiguous generic or species boundaries (on both morphological and genetic bases) and lack of successful seed-set in many taxa (especially the cultivated ones that have also been referred to as 'Ancient Enduring Clones') in the BDG complex.

The climbing bamboo lineages, however, do not appear to have participated in the kind of introgressive genetic exchange detected within the BDG complex.

(b) ***The maximum development of the two groups are centered in different ecogeographical regions***

*Bambusa* and *Dendrocalamus* are most speciose in S China (Li and Hsueh, 1989; Xia et al., 2007) and *Gigantochloa* has many species both in the Burmese-Malayan area as well as in Java (Widjaja, 1987), implying that the center of diversity of the BDG complex is likely to be in the mainland SE Asia and Sundaland region. These are essentially subtropical areas with markedly seasonal climates. On the other hand, the climbing bamboo lineages outside the BDG complex are mainly distributed in the Malay Archipelago (Dransfield, 1981, 1989, 1992; Dransfield and Widjaja 1995; Widjaja 1997; Dransfield and Wong, 1996; Wong, 2005), i.e., in the insular region between mainland SE Asia and Australia. Thus the development of these climbing bamboo lineages apparently correspond with a largely everwet tropical climatic regime, although short periods of lower rainfall (not amounting to pronounced

seasonality) (Richards, 1952) may occur in the eastern and central parts of the archipelago. Moreover, there is some suggestion that the DMNS clade could be of Australasian origin and that subsequent diversification of this clade could have occurred with dispersal towards SE Asia. While clear ecological correlates may be difficult to pin down at this stage, it is clear that these clades had their maximal diversification in different geographical regions.

**Implications for subtribal classification**

The present results suggest that the Bambusinae should be delimited to contain only the BDG complex, and the *Holttumochloa-Kinabaluchloa* clade could merit subtribal status. However, the relationship among three other climbing bamboo lineages (the DMNS clade, *Racemobambos* clade and *T. simplex*) has only equivocal support which does not permit a clear interpretation of their status. Perhaps a meaningful taxonomic revision at subtribal-level could be forthcoming with better sampling of *Racemobambos* and other SW Pacific genera, as well as including genera such as *Cyrtochloa*, *Greslania* and *Fimbribambusa* that have not be possible to include presently. For these reasons, it could be said that, apart from good support for the BDG complex as a cohesive phylogenetic grouping that practically defines the Bambusinae (its reticulate internal relationships apparently restricted to members), it is best that no other conclusions be reached about other subtribal recognition at this stage.

## Untangling reticulate evolution: more research required

*The nature of introgressive complexes*

It is clear that the taxonomic confusion within the BDG complex cannot be resolved without more complete untangling of the reticulate relationships among *Bambusa*, *Dendrocalamus*, *Gigantochloa*, *Maclurochloa*, *Phuphanochloa*, *Soejatmia* and *Thyrsostachys*. The present work has merely adduced evidence that reticulate evolution could be a significant factor in the diversification of this subtribe. Increased taxon sampling and utilization of more cpDNA and nuclear DNA markers could be expected to reveal more cases of past hybridization as well as indicate clearer relationships among taxa. These would in turn provide views of genetic relatedness or distinction against which to better assess the validity of the present morphology-based classification.

Different accessions of some "species" within this complex (e.g., *B. bambos*, *D. strictus* and *G. scortechinii*) are shown to have arisen from different maternal stocks. It is unclear if the cpDNA variation within a single such species represented random introgressive events or if they could be considered as more consistent lineage characteristics. Only increased investigations of such aspects, for more taxa within this complex, can provide increased insight. Even studies of population genetic structure could reveal pertinent aspects about introgression.

*Reticulate evolution and ploidy increases*

This evolutionary development of increased ploidy levels is a correlate of past hybridization and lineage mixing that has resulted in reticulate evolution among the woody bamboo lineages above. In addition to the widespread reticulate evolution in the Paleotropical Bambusinae (present study), natural hybrids of the Neotropical *Chusquea* have also been reported (Clark et al., 1989), and introgressive hybridization has been found to be significant in the temperate Arundinarieae (Triplett and Clark, 2010; Triplett et al., 2010).

We may ask what might have stimulated or permitted an accumulation (i.e., successful adaptation and persistence) of such polyploids historically? Hilu (1985: 112) says, "If hybridization is still effective and common at the present and after millions of years of evolution and divergence, the frequency and ease of this phenomenon ought to be exceedingly higher among the ancestral lines." It is, however, premature to form such a hypothesis because of the complicated chromosomal evolution in the grass family (see Hilu, 2004; Devos, 2010), and the lack of demonstrated dependable historical timeframes. We simply require much more insight into not only chromosomal and lineage characteristics of the various groups of grasses, but also better evolutionary time estimates and an increased understanding of biogeographical perspectives.

♦ ♦ ♦

There is a general increase in interest in how the different alliances of woody bamboos may be related, especially following classic work in demonstrating the key tribes of bambusoid grasses (Clayton and Renvoize, 1986; Soderstrom and Ellis, 1987), recent advances in uncovering complex relationships (Kelchner and Clark, 1997; Zhang, 2000; Guo and Li., 2004; Yang et al., 2007; Bouchenak-Khelladi et al., 2008; Yang et al., 2008; Fisher et al., 2009; Sungkaew et al., 2009; Yang et al., 2010), and the coming together of various perspectives that show the potential significance of past introgressive hybridization (Triplett and Clark, 2010; Triplett et al., 2010). The insights presented here suggest that the Bambusinae and other (largely climbing) bamboo lineages are important in the SE Asian flora and landscape not only have interesting biological attributes but also exciting aspects of evolutionary development that we are only beginning to understand. Only with an increased effort in better understanding reproductive biology and genetics, and phylogenetic development, will a clearer picture emerge of how the various groups are (inter-)related and best classified.

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# APPENDICES

## Appendix A: Protocols for total DNA extraction

Procedures of the total DNA extraction using Qiagen DNeasy Extraction kit

1. Grind 5 mg of silica gel-dried leaf tissue in liquid nitrogen.
2. Transfer the tissue powder into a 1.5 ml microcentrifuge tube filled with 400 µl of Buffer AP1 and 4 µl of RNase A (100 mg/ml), mix well.
3. Incubate the mixture at 65 $^{o}$C for 30 minutes. Mix 2 or 3 times during incubation by inverting tube.
4. Add 130 µl Buffer AP2 to the lysate, mix, and incubate for 5 minutes on ice.
5. Centrifuge the lysate for 5 minutes at 14000 rpm.
6. Pipet the lysate into the QIAshredder Mini spin column placed in a 2 ml collection tube, and centrifuge for 2 minutes at 14000 rpm.
7. Transfer the flow-through fraction from the previous step into a new tube without disturbing the cell-debris pellet.
8. Add 1.5 volume of Buffer AP3/E to the cleared lysate, and mix by pipetting.
9. Pipet 650 µl of the mixture from the previous step into the DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 minute at > 8000 rpm and discard the flow-through.
10. Repeat the previous step with remaining sample. Discard flow-through and collection tube.
11. Place the DNeasy Mini spin column into a new 2 ml collection tube, add 500 µl of Buffer AW, and centrifuge for 1 minute at > 8000 rpm. Discard the flow through.
12. Add 500 µl of Buffer AW to the DNeasy Mini spin column, and centrifuge for 2 minutes at 14000 rpm to dry the membrane.
13. Transfer the DNeasy Mini spin column to a 1.5 ml microcentrifuge tube and pipet 100 µl of Buffer AE directly onto the DNeasy membrane. Incubate for 5 minutes at room temperature and then centrifuge for 1 minute at 8000 rpm to elute.

Procedures of the total DNA extraction method modified from Fulton et al. (1995)

1. Prepare the following buffers:
2. DNA extraction buffer: 0.35 M sorbitol, 0.1 M Tris-HCl, 50mM EDTA
3. Nuclei lysis buffer: 0.2 M Tris-HCl, 50 mM EDTA, 2 M NaCl, 2 % w/v CTAB
4. DNA isolation buffer: 5 volume of DNA isolation buffer: 5 volume of nuclei lysis buffer: 2 volume of Sodium Laurate Sulphate 5%.
5. Grind 0.5 g of silica gel-dried leaf tissue in liquid nitrogen.
6. Transfer the tissue powder into a 1.5 ml microcentrifuge tube filled with 700 µl of DNA isolation buffer and 4 µl of RNase A (100 mg/ml), mix well.
7. Incubate the mixture at 65 $^{o}$C for 45 minutes. Mix 2 or 3 times during incubation by inverting tube.
8. Add 600 µl of chloroform: isoamyl (24:1), shake vigorously. Centrifuge the tube at maximum speed for 5 minutes. Carefully pipet the top layer and transfer into a new tube. Repeat this step.
9. Add equal volume of ice-cold absolute isopropanol. Invert the tube a few times.
10. Incubate on ice for 10 minutes.
11. Centrifuge at 12000 rpm for 5 minutes.
12. Discard the supernatant.
13. Add 700 µl of ice-cold ethanol 70 %. Centrifuge at 12000 rpm for 2 minutes. Discard the supernatant. Repeat this step.
14. Dry the pellet by placing the tube (inverted) on a piece of clean hand towel for about 15 minutes.
15. Resuspend the pellet with sterilised distilled water.

**Appendix B: GenBank accession numbers for the DNA sequences**

| Taxa | GenBank accession | | | | |
|---|---|---|---|---|---|
| | ***rps16-trnQ*** | ***trnC-rpoB*** | ***trnH-psbA*** | ***trnD-T*** | ***GBSSI*** |
| *Bambusa arnhemica* | JN033886 | JN033913 | JN033969 | JN033941 | JN033997 |
| *Bambusa bambos* (1) | FJ416342 | GU390912 | GU390993 | GU390939 | GU390987 |
| *Bambusa bambos* (2) | JN033887 | JN033914 | JN033970 | JN033942 | JN033998 |
| *Bambusa blumeana* | GU390903 | GU390913 | GU390994 | GU390940 | GU390988 |
| *Bambusa boniopsis* | JN033888 | JN033915 | JN033971 | JN033943 | JN033999 |
| *Bambusa burmanica* | JN033889 | JN033916 | JN033972 | JN033944 | JN034000 |
| *Bambusa distegia* | GU390904 | GU390914 | GU390995 | GU390941 | GU390966 |
| *Bambusa eutuldoides var. viridivittatta* | JN033890 | JN033917 | JN033973 | JN033945 | JN034001 |
| *Bambusa farinacea* (1) | FJ416341 | GU390915 | GU390996 | GU390942 | GU390967 |
| *Bambusa farinacea* (2) | JN033891 | JN033918 | JN033974 | JN033946 | JN034002 |
| *Bambusa flexuosa* | GU390905 | GU390916 | GU390997 | GU390943 | GU390968 |
| *Bambusa gibba* | GU390906 | GU390917 | GU390998 | GU390944 | GU390986 |
| *Bambusa grandis* | JN033892 | JN033919 | JN033975 | JN033947 | JN034003 |
| *Bambusa intermedia* | JN033893 | JN033920 | JN033976 | JN033948 | JN034004 |
| *Bambusa multiplex* | FJ416351 | GU390918 | GU390999 | GU390945 | GU390969 |
| *Bambusa oldhamii* | JN033894 | JN033921 | JN033977 | JN033949 | JN034005 |
| *Bambusa polymorpha* | JN033895 | JN033922 | JN033978 | JN033950 | JN034006 |
| *Bambusa sinospinosa* | FJ416352 | GU390919 | GU391000 | GU390946 | GU390970 |
| *Bambusa textilis* | JN033896 | JN033923 | JN033979 | JN033951 | JN034007 |
| *Bambusa tuldoides* | GU390907 | GU390920 | GU391001 | GU390947 | GU390989 |

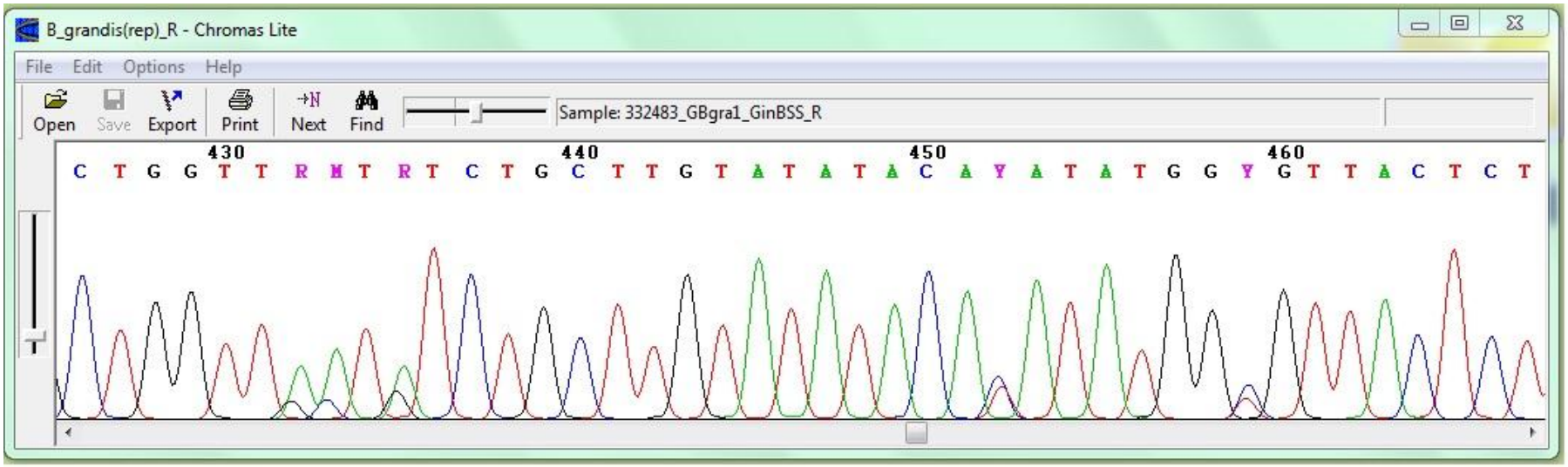
**Appendix B (cont'...)**

| Taxa | GenBank accession | | | | |
|---|---|---|---|---|---|
| | ***rps16-trnQ*** | ***trnC-rpoB*** | ***trnH-psbA*** | ***trnD-T*** | ***GBSSI*** |
| *Bambusa valida* | GU390908 | GU390921 | GU391002 | GU390948 | GU390990 |
| *Dendrocalamus elegans* | FJ416344 | GU390922 | GU391003 | GU390949 | GU390971 |
| *Dendrocalamus hirtellus* | FJ416362 | JN033924 | JN033980 | JN033952 | JN034008 |
| *Dendrocalamus khoonmengii* | JN033897 | JN033925 | JN033981 | JN033953 | JN034009 |
| *Dendrocalamus pendulus* (1) | HQ697855 | HQ697866 | HQ697902 | HQ697877 | HQ697890 |
| *Dendrocalamus pendulus* (2) | HQ697856 | HQ697867 | HQ697903 | HQ697878 | HQ697889 |
| *Dendrocalamus strictus* (1) | FJ416364 | GU390923 | GU391004 | GU390950 | GU390972 |
| *Dendrocalamus strictus* (2) | JN033898 | JN033926 | JN033982 | JN033954 | JN034010 |
| *Dinochloa malayana* | FJ416343 | GU390924 | GU391005 | GU390951 | GU390973 |
| *Dinochloa scabrida* | GU390910 | GU390926 | GU391007 | GU390953 | GU390975 |
| *Dinochloa* sp. | GU390909 | GU390925 | GU391006 | GU390952 | GU390974 |
| *Dinochloa trichogona* | JN033899 | JN033927 | JN033983 | JN033955 | JN034011 |
| *Gigantochloa apus* | JN033900 | JN033928 | JN033984 | JN033956 | JN034012 |
| *Gigantochloa atter* | JN033901 | JN033929 | JN033985 | JN033957 | JN034013 |
| *Gigantochloa balui* | FJ416359 | GU390927 | GU391008 | GU390954 | GU390976 |
| *Gigantochloa latifolia* | FJ416361 | GU390929 | GU391010 | GU390956 | GU390978 |
| *Gigantochloa ligulata* | FJ416360 | GU390928 | GU391009 | GU390955 | GU390977 |
| *Gigantochloa scortechinii* | HQ697861 | HQ697872 | HQ697908 | HQ697883 | HQ697897 |
| *Gigantochloa scortechinii* | HQ697864 | HQ697875 | HQ697911 | HQ697886 | HQ697899 |
| *Gigantochloa wrayi* | FJ416365 | GU390930 | GU391011 | GU390957 | GU390979 |

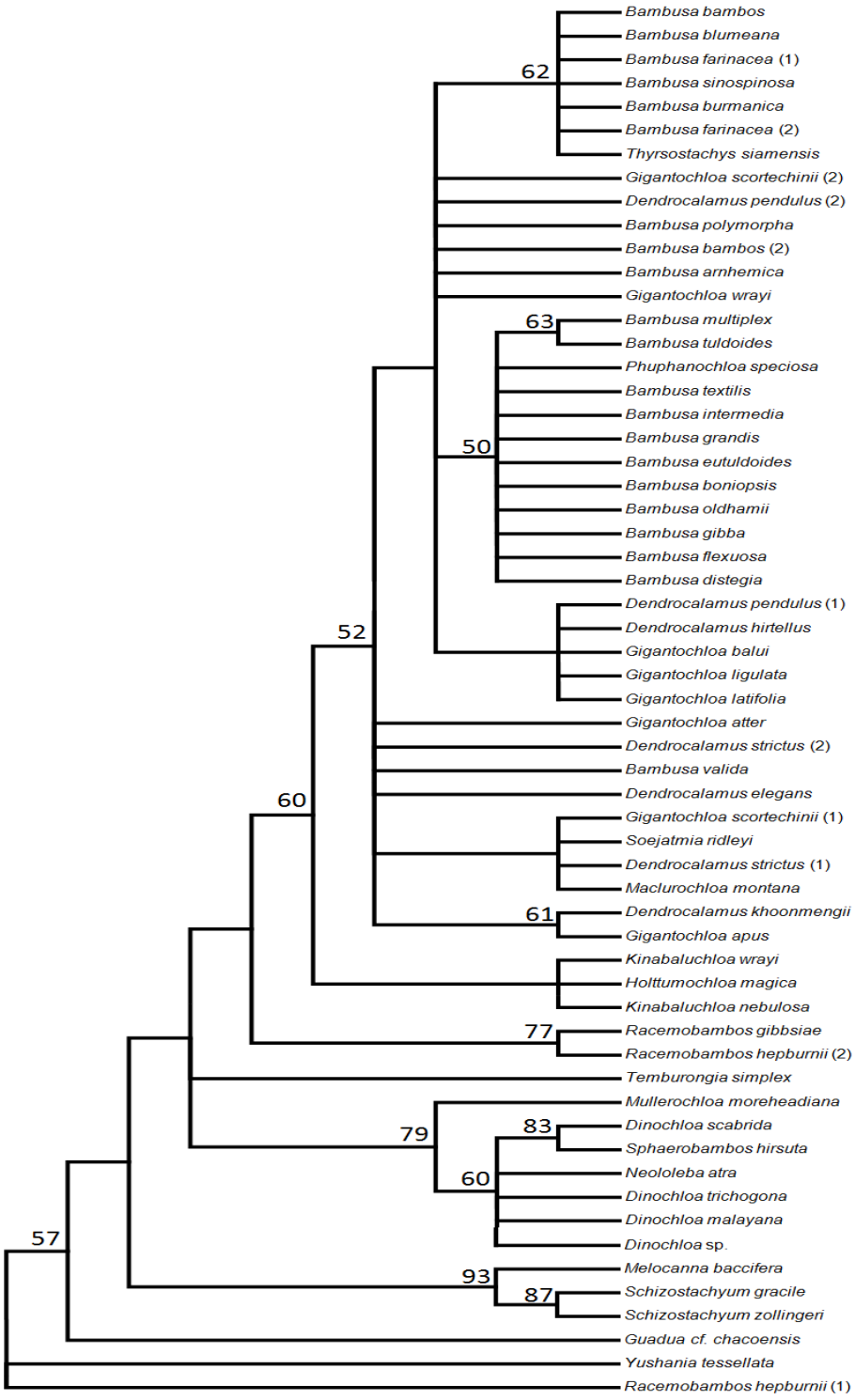
**Appendix B (cont'...)**

| Taxa | GenBank accession | | | | |
|---|---|---|---|---|---|
| | ***rps16-trnQ*** | ***trnC-rpoB*** | ***trnH-psbA*** | ***trnD-T*** | ***GBSSI*** |
| *Guadua cf. chacoensis* | JN033902 | JN033930 | JN033986 | JN033958 | JN034014 |
| *Holttumochloa magica* | FJ416348 | GU390931 | GU391012 | GU390958 | GU390980 |
| *Kinabaluchloa nebulosa* | FJ416356 | GU390932 | GU391013 | GU390959 | GU390981 |
| *Kinabaluchloa wrayi* | JN033903 | JN033931 | JN033987 | JN033959 | JN034015 |
| *Maclurochloa montana* | FJ416349 | GU390933 | GU391014 | GU390960 | GU390982 |
| *Melocanna baccifera* | FJ416347 | GU390934 | GU391015 | GU390961 | GU390983 |
| *Mullerochloa moreheadiana* | JN033904 | JN033932 | JN033988 | JN033960 | JN034016 |
| *Neololeba atra* | JN033905 | JN033933 | JN033989 | JN033961 | JN034017 |
| *Phuphanochloa speciosa* | JN033906 | JN033934 | JN033990 | JN033962 | JN034018 |
| *Racemobambos gibbsiae* | JN033907 | JN033935 | JN033991 | JN033963 | JN034019 |
| *Racemobambos hepburnii* (1) | JN033908 | JN033936 | JN033992 | JN033964 | JN034020 |
| *Racemobambos hepburnii* (2) | JN033909 | JN033937 | JN033993 | JN033965 | JN034021 |
| *Schizostachyum gracile* | FJ416353 | GU390937 | GU391018 | GU390964 | GU390991 |
| *Schizostachyum zollingeri* | FJ416354 | GU390938 | GU391019 | GU390965 | GU390992 |
| *Soejatmia ridleyi* | FJ416355 | GU390936 | GU391017 | GU390963 | GU390984 |
| *Sphaerobambos hirsuta* | GU390911 | GU390935 | GU391018 | GU390962 | GU390985 |
| *Temburongia simplex* | JN033910 | JN033938 | JN033995 | JN033966 | JN034022 |
| *Thyrsostachys siamensis* | JN033911 | JN033939 | JN033994 | JN033967 | JN034023 |
| *Yushania tessellata* | JN033912 | JN033940 | JN033996 | JN033968 | JN034024 |

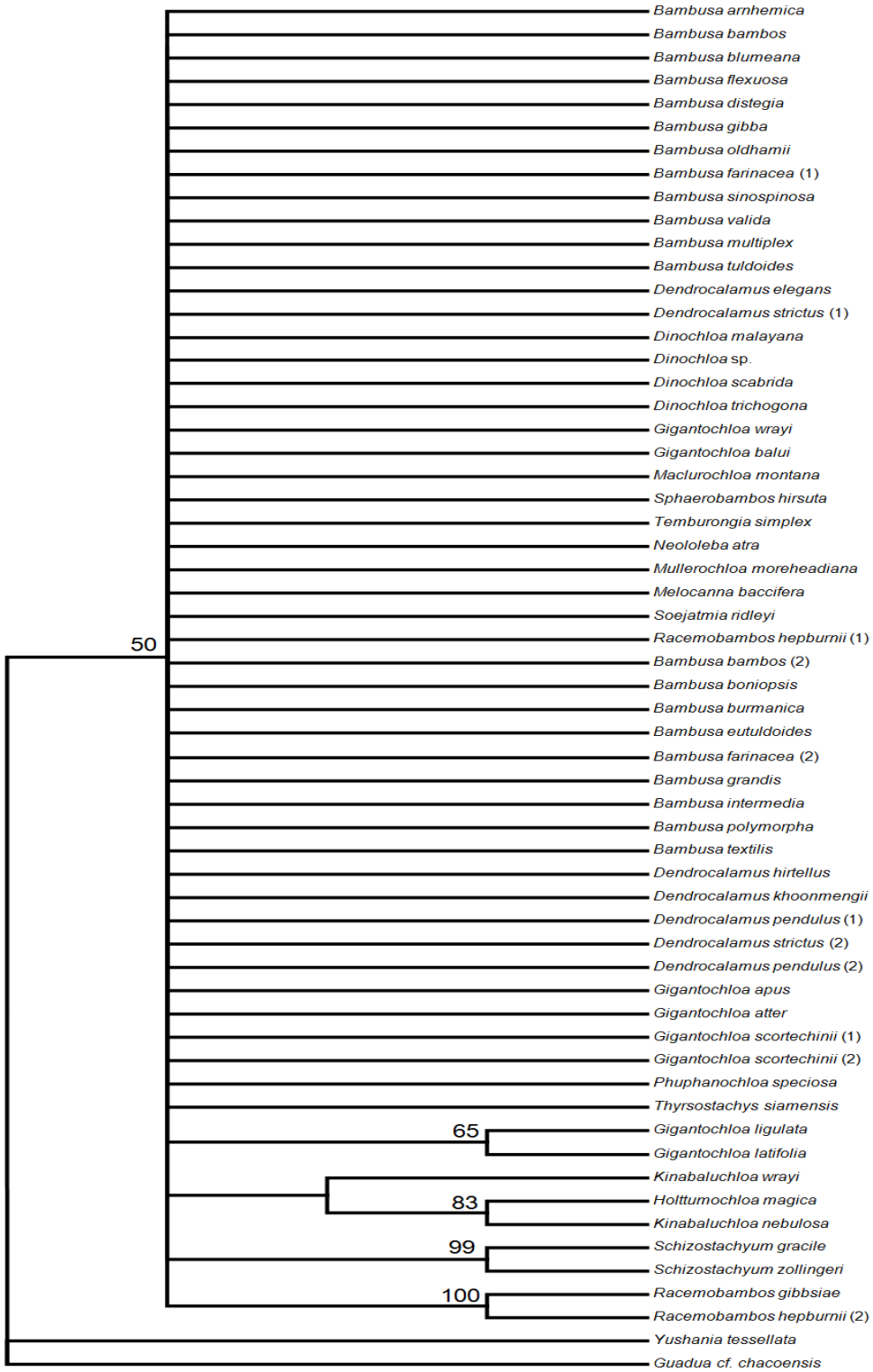
**Appendix C: Double peaks in the DNA chromatogram showing five dimorphic sites (the *GBSSI* sequence of *Bambusa grandis* as an example)**



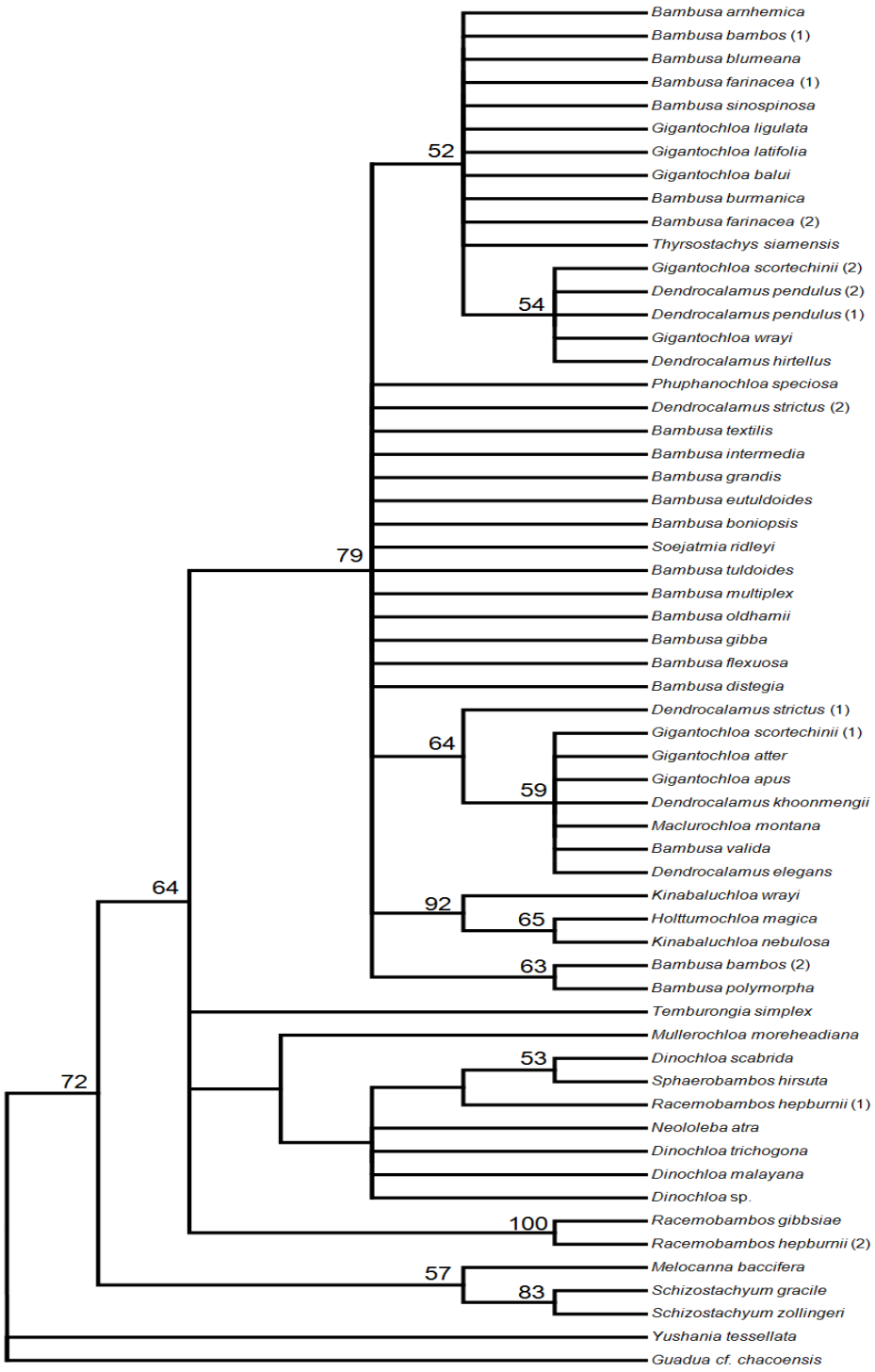
**Appendix D: MP analyses of individual cpDNA region**




(i) Strict consensus of the 63 most parsimonious rees based on chloroplast *rps16-trnQ* spacer.

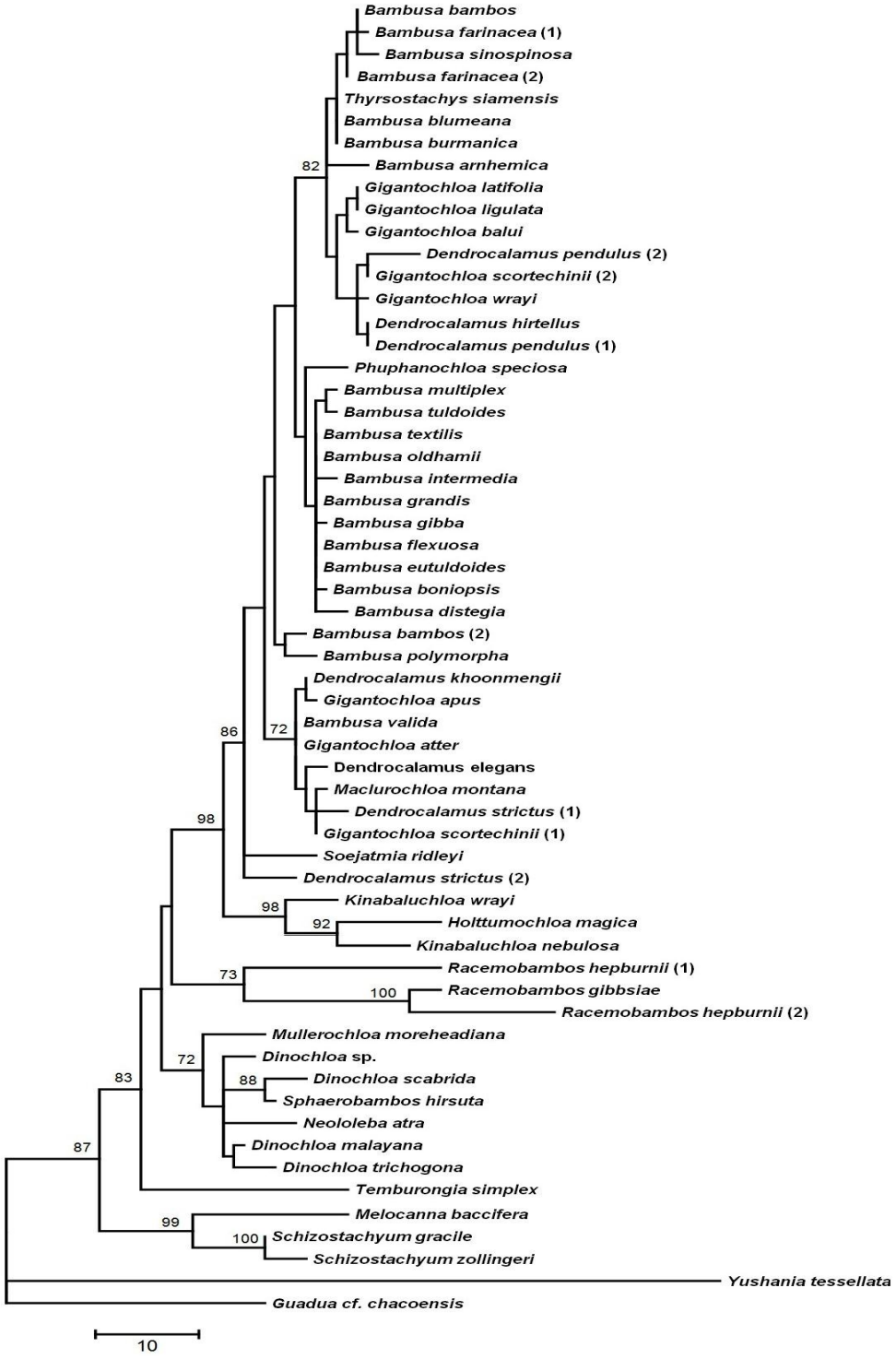



(ii) Strict consensus of the 75 most parsimonious trees based on chloroplast *trnC-rpoB* spacer.

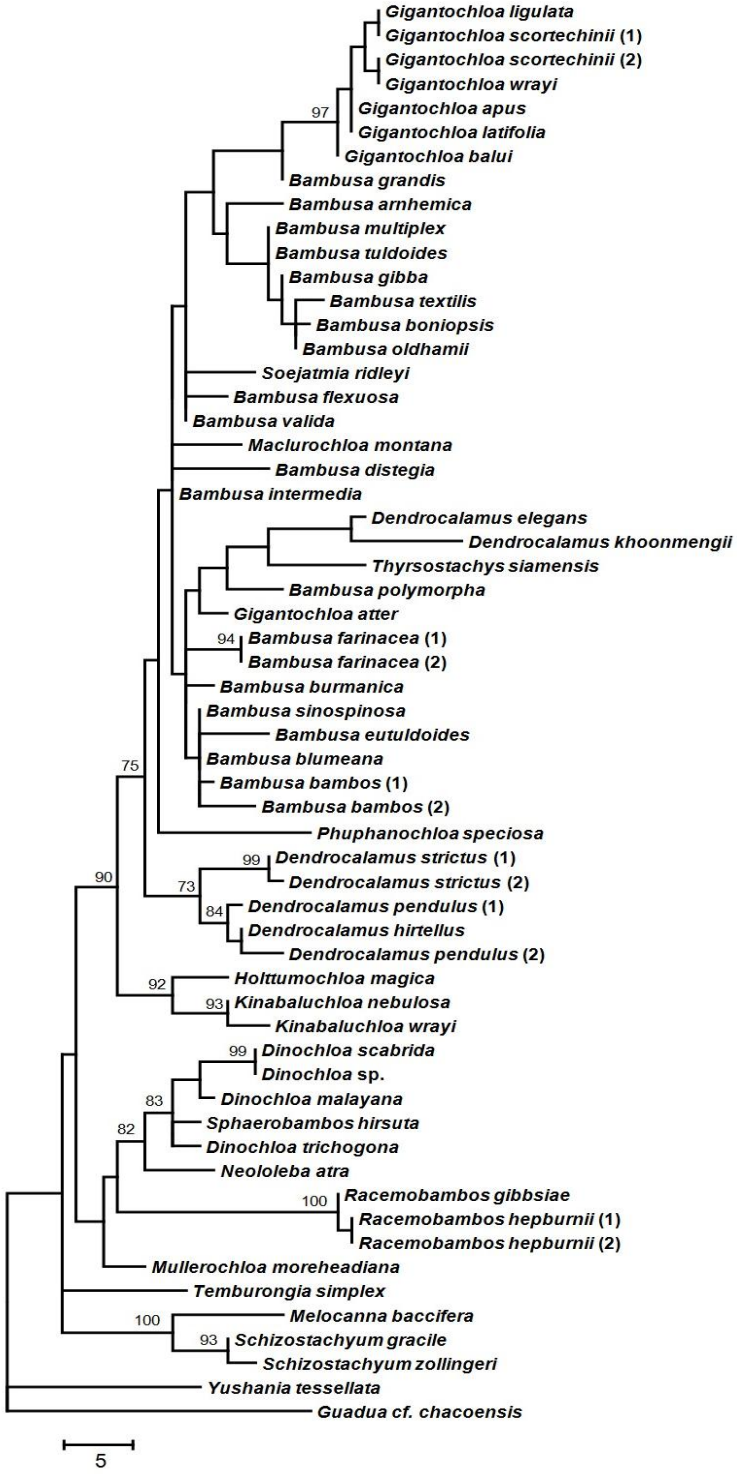



(iii) Strict consensus of the 88 most parsimonious trees based on chloroplast *trnD-T* spacer.

**Appendix E: Phylogenetic trees reconstructed using maximum parsimony analysis**

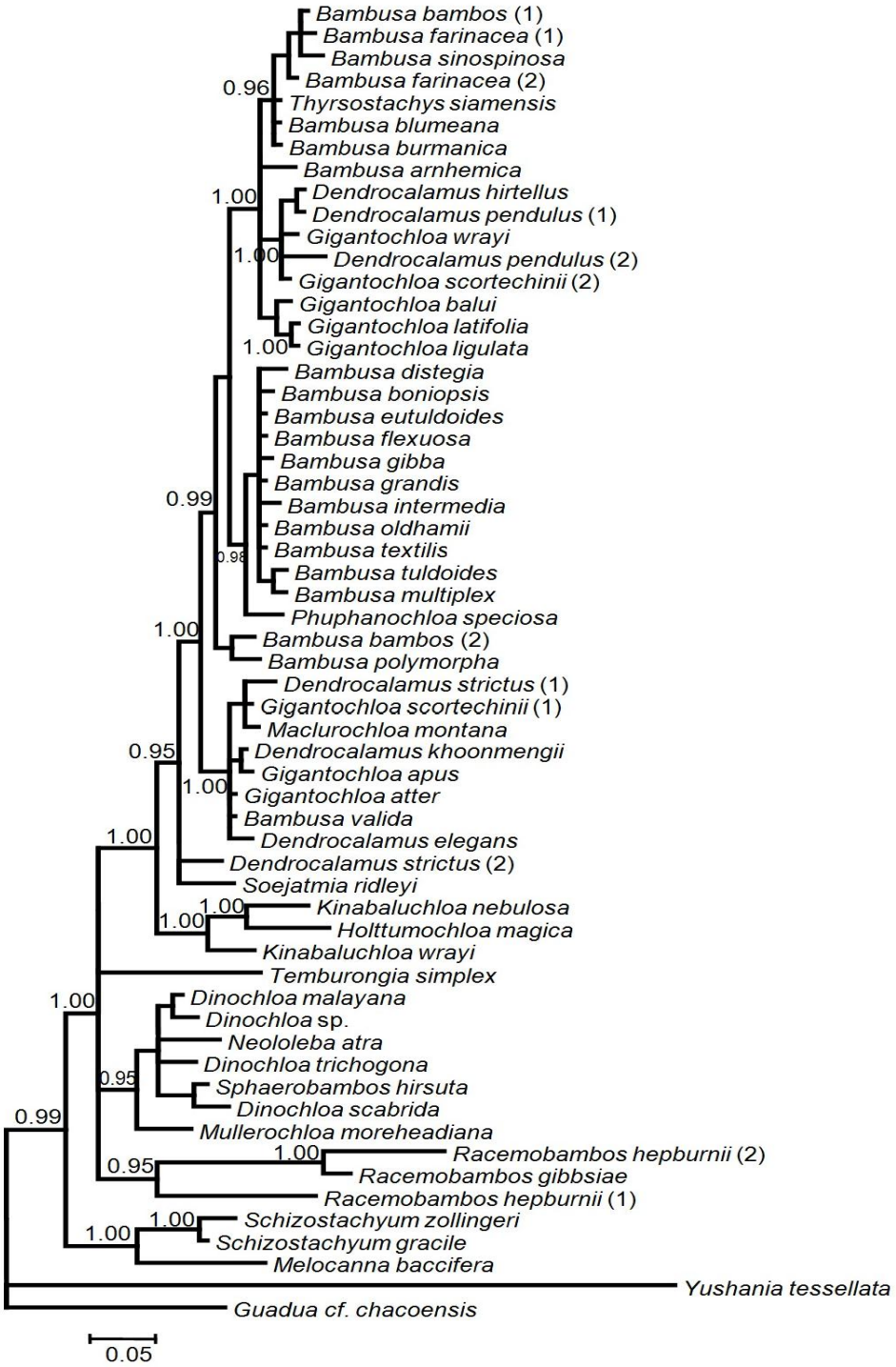



(i) One of the 429 equally most parsimonious trees based on the cpDNA data (*rps16-trnQ* + *trnC-rpoB* + *trnD-T*). Bootstrap proportion values > 70 % are shown. The tree is drawn down to scale, with branch lengths indicating evolutionary distances as number of base substitution per site.

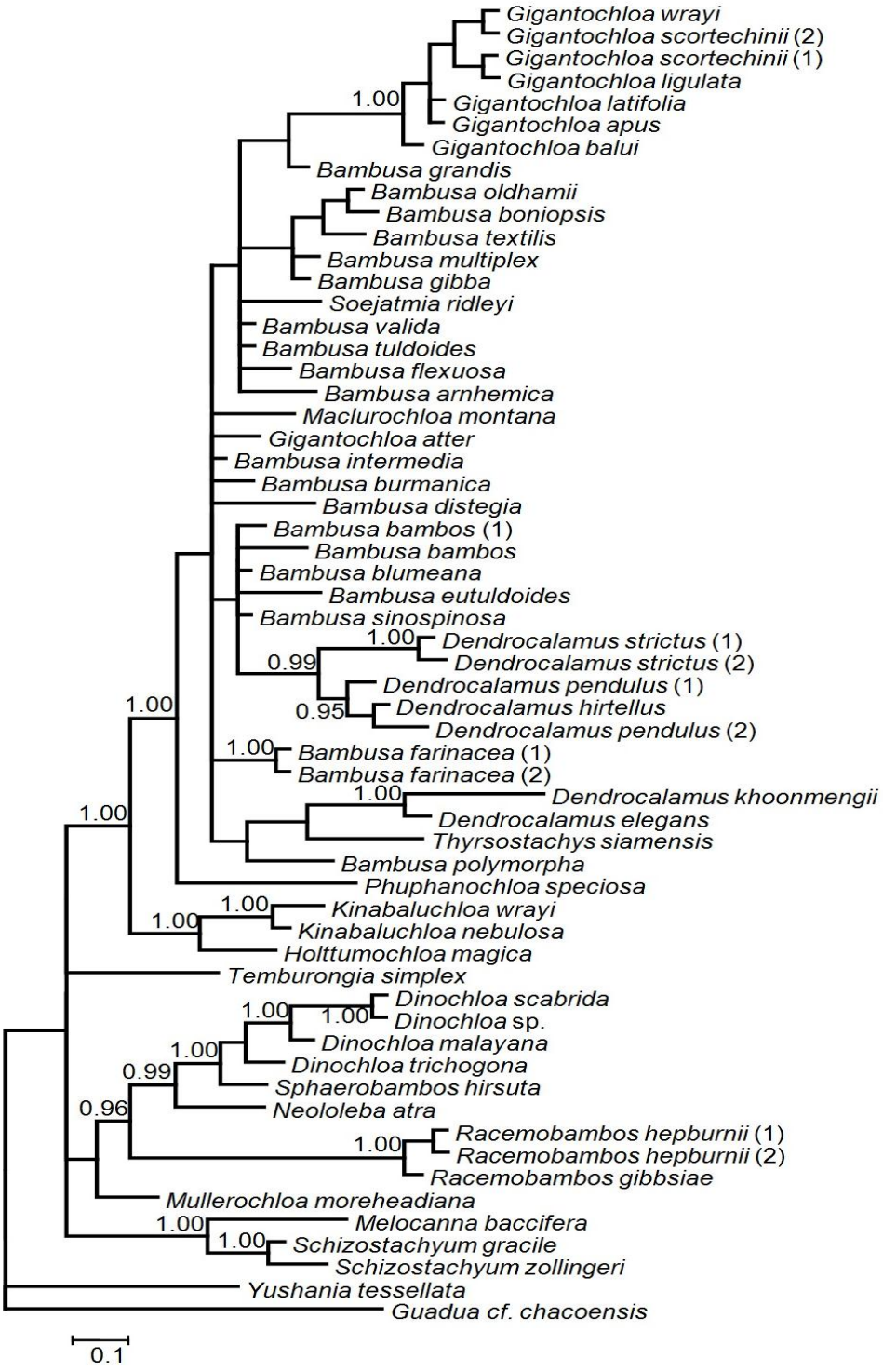



(ii) One of the 461 equally most parsimonious trees based on the partial *GBSSI* gene data. Bootstrap proportion values > 70 % are shown. The tree is drawn down to scale, with branch lengths indicating evolutionary distances as number of base substitution per site.

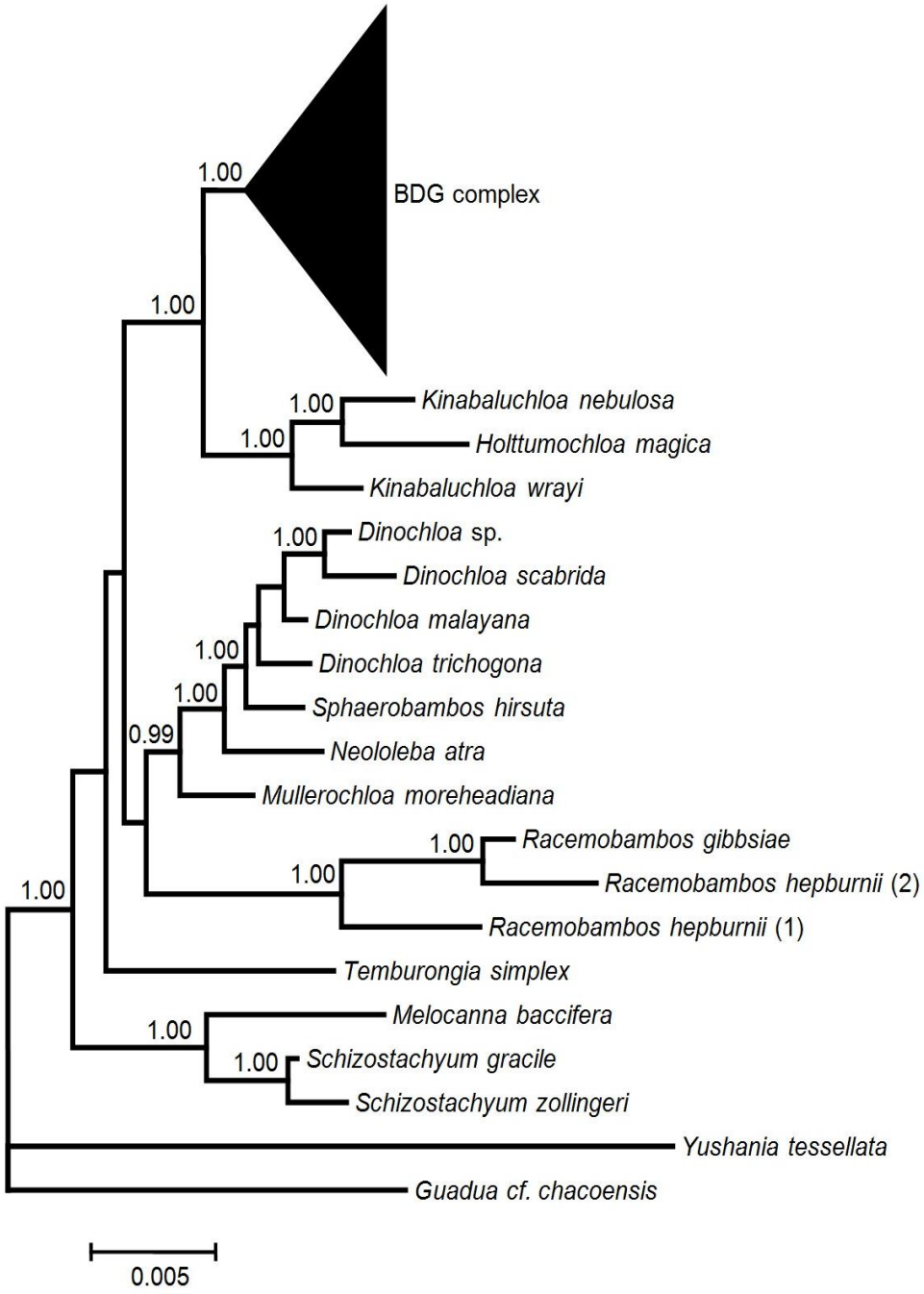
**Appendix F: Phylogenetic trees reconstructed using Bayesian Inference (BI)**




(i) Phylogram of the 50% majority-rule consensus tree from the Bayesian analysis of the *rps*16-*trn*Q + *trn*C-*rpo*B + *trn*D-T data. Posterior probabilities (PP) values > 0.95 are shown at the nodes.




(ii) Phylogram of the 50% majority-rule consensus tree from the Bayesian analysis of the *GBSSI* data. Posterior probabilities (PP) values > 0.95 are shown at the nodes.

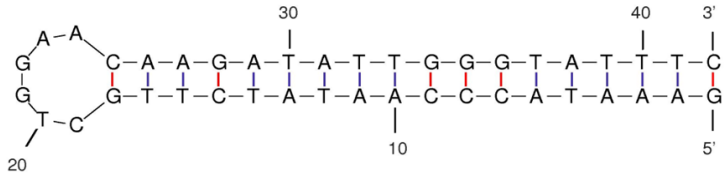



(iii) Phylogram of the 50% majority-rule consensus tree from the Bayesian analysis of the combined cpDNA + *GBSS*I data. Posterior probabilities (PP) values > 0.95 are shown at the nodes.

**Appendix G:** Hairpin inversions in the cpDNA data matrix aligned using sequences published by Yang et al. (2010) (Nucleotides which form the loops were in bold face and underlined).

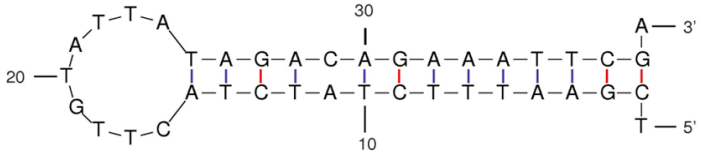
Hairpin 1:
alignment positions 67-108
inversion: 85-90

GAAATACCCAATATCTTG**CTGGAA**CAAGATATTGGGTATTTC
GAAATACCCAATATCTTG**TTCCAG**CAAGATATTGGGTATTTC



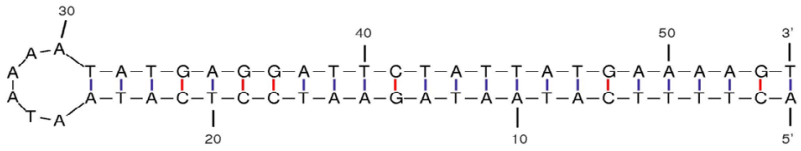

Hairpin 2:
alignment positions 675-714
inversion: 685-704

TCGAATTTCT**ATCTACTTGTATTATAGAC**AGAAATTCGA
TCGAATTTCT**GTCTATAATACAAGTAGAT**AGAAATTCGA




Hairpin 3:
alignment postions 1914-1957
inversion: 1938-1943

ACTTTTCATAATAGAATCCTCATA**ATAAAA**TATGAGGATTCTATTATGAAAAGT
ACTTTTCATAATAGAATCCTCATA**TTTTAT**TATGAGGATTCTATTATGAAAAGT



**Appendix H: The variable sites and indel sites of the cpDNA sequence of the hybrid and its parental species**
Dots indicate identical nucleotides compared to those in the first line. Dashes indicate the alignment gaps.

| Site / Taxon | 111112222222222222222222222233333333333333333333333333333333333<br>333333333356013360000000000000000001111674555555555577777777777777777777<br>668999999904611961111111222222222233313676663333333338125566666666667777777<br>947934567896899018345678901234567890125304981012345678129890123456789012345 67 |
|---|---|
| D. pendulus (1) | --GAATTAGAAATCCCA-ATTGTCTCTATTCATATGTAT--TC-T---------TAC---------- |
| D. pendulus (2) | A-.............G-.....................--..-.---------...---------- |
| D. pendulus (3) | A-............A.G-.....................--..-.---------...---------- |
| Hybrid Gombak-1 | A-.............G-.....................--..-.---------...---------- |
| Hybrid Gombak-2 | --.........T.-..G-.....................--G.-.---------...---------- |
| Hybrid Tapah | A-.............-.....................--..-.ATAAGAATA..G---------- |
| G. scortechinii (1) | -AT.-------.G..A.A--------------------CGG.---ATAAGAATAAG.TAAGGTGAATAAATAATAAA |
| G. scortechinii (2) | A-.............G-.....................--..A.---------...---------- |
| G. scortechinii (3) | A-.............G-.....................--..A.---------...---------- |
| G. scortechinii (4) | A-.............G-.....................--..-.---------...---------- |
| G. scortechinii (5) | A-TT-------.G....--------------------C--.---ATAAGAATAAG.TAAGGTGAATAAATAATAAA |

**Appendix I: Shimodaira-Hasegawa (SH) tests**

(i) Results of SH tests on the cpDNA + *GBSS*I topology for assessing the monophyly of the climbing bamboo lineages.

| Monophyly | - *ln L* | Diff. ( - *ln* L) | *p** |
|---|---|---|---|
| (Unconstrained MLT) | 11895.10291 | – | – |
| MNDS | 11894.23291 | 0.87000 | 0.387 |
| MNDS + *Racemobambos* | 11891.92679 | 3.17612 | 0.180 |
| MNDS + *Racemobambos* + *T. simplex* | 11892.05427 | 3.04864 | 0.189 |
| MNDS + *T. simplex* | 11901.39587 | 6.29296 | 0.239 |
| *Racemobambos* | 11891.92679 | 3.17612 | 0.180 |
| *Racemobambos* + *T. simplex* | 11906.80399 | 11.70107 | 0.121 |

* all values not significant at $p = 0.05$

(ii) Results of SH tests on the cpDNA topology for assessing the incongruence between the cpDNA and *GBSS*I topologies.

| Constraint | - *ln L* | Diff. ( - *ln* L) | *p* |
|---|---|---|---|
| (Unconstrained MLT) | 7874.73654 | – | – |
| *Dendrocalamus strictus* (1), *D. strictus* (2), *D. hirtellus*, *D. pendulus* (1), *D. pendulus* (2) | 7957.29281 | 82.55628 | 0.008* |
| *Gigantochloa apus*, *G. balui*, *G. latifolia*, *G. ligulata*, *G. scortechinii* (1), *G. scortechinii* (2), *G. wrayi* | 7948.50666 | 73.77013 | 0.015* |

* indicates rejection at $p = 0.05$

(iii) Results of SH tests on the *GBSS*I topology for assessing the incongruence between the cpDNA and *GBSS*I topologies.

| Monophyly | - *ln L* | Diff. (- *ln* L) | *p* |
|---|---|---|---|
| (Unconstrained MLT) | 2540.18225 | – | – |
| *Bambusa arnhemica*, *B. bambos* (1), *B. blumeana*, *B. burmanica*, *B. farinacea* (1) & (2), *B. sinospinosa*, *Dendrocalamus hirtellus*, *D. pendulus* (1) & (2), *Gigantochloa balui*, *G. latifolia*, *G. ligulata*, *G. scortechinii* (2), *G. wrayi* and *Thyrsostachys siamensis* | 2650.09790 | 109.91566 | 0.001* |
| *Bambusa valida*, *Dendrocalamus elegans*, *D. khoonmengii*, *D. strictus*, *Gigantochloa apus*, *G. atter*, *G. scortechinii* and *Maclurochloa montana* | 2660.25214 | 120.06989 | 0.000* |

* indicates rejection at $p = 0.05$

## Appendix J: Abstract of presentations resulting from the present work

(i) Poster presentation at the13th Biological Sciences Graduate Congress, National University of Singapore, 15–17 December 2008.

### Phylogenetic relationships of the Bambusa complex (Poaceae: Bambusoideae: Bambusinae)

W.L. Goh, S. Chandran, K.M. Wong

The classification of recognised genera and their relationships in the Bambusinae, a subtribe of woody bamboos important in Southeast Asia, has been contentious in a number of areas. The clear delimitation of *Bambusa*, a large genus, appears to have been better achieved with recent morphological work but requires to be further confirmed, especially with molecular approaches. In particular, the recent establishment of the genera *Holttumochloa, Kinabaluchloa, Maclurochloa* and *Soejatmia* from taxa previously classified as *Bambusa* can be tested in a phylogenetic analysis. This is the main subject of inquiry in a recently begun project, which incorporates the type species of *Bambusa*, its type alliance (the suite of species most closely related to the type), representative species from various portions of the generic geographical range, together with these newly circumscribed genera, in a cladistic analysis based on molecular characteristics and another morphology-based analysis. Here we outline the approach adopted and preliminary results obtained with two chloroplast DNA regions and a nuclear gene region. Just three months into the present project, these results indicate the further work required that would be prospective in confirming the relationships of the *Bambusa* complex.

(ii) Poster presentation at the 8th International Flora Malesiana Symposium, Singapore Botanic Gardens, 23–28 August 2010.

**Grass encounters of the third kind: an intergeneric bamboo hybrid from Peninsular Malaysia**

K.M. Wong, Y.W. Low, W.L. Goh, K. Koichi

A previously undetected bamboo with morphological characteristics intermediate between *Gigantochloa scortechinii* Gamble and *Dendrocalamus pendulus* Ridl. was collected from two different localities (Perak and Selangor states) in Peninsular Malaysia. Its molecular characteristics were also intermediate, as shown by the presence of two nuclear *GBSSI* haplotypes in the hybrid with sequences identical to each parent (i.e., all parent-specific sites are dimorphic in the hybrid). Population studies at one of the hybrid localities in the Gombak Valley (Selangor) using GPS-coordinates mapped onto a Googlemap scene of the area demonstrated that hybrid individuals occurred together with *D. pendulus* clumps, suggesting the latter was the maternal parent (seed grains are expected to have poorer dispersability compared to pollen). The respective sites of the parental species were interesting: *G. scortechinii* occupied flatter, streamside places along the Gombak river, whereas *D. pendulus* typically occurred on steeper hillsides along the same valley. Why were these hybrids not detected earlier, although this part of the Gombak valley has been a routinely well-botanized locality? We suggest that the "corridor of change" brought about by development (i.e., opening up) of the Karak Highway (parallel to this part of the Gombak valley) just before 1980 was a key factor. This enormous cleared corridor caused greater openness, spreading and an increased commonness of both species, as well as the removal of forest tree cover that probably served as a natural impediment to genetic exchange between the bamboo species. Greater exposure along this corridor had probably increased chances for air currents carrying pollen from *G. scortechinii* clumps in flatter terrain at one end (southwest) to find hillside patches occupied by *D. pendulus* near the other end (northeast). The tendency for *D. pendulus* to flower gregariously from time to time probably increased the possibility of fertilization. When named, this will be SE Asia's first natural intergeneric bamboo hybrid. Continuing research examines the question of generic boundaries among this group of bamboos, including comparative studies of both nuclear and plastid gene topologies in this group, and dealing with the difficulties of classifying a mystifying complex of bamboos that have defied simple lineage studies so far.

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**The plot thickens:**
**Unravelling molecular evidence for widespread hybridization in the Bambusinae**

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Holttum had hypothesized that many bamboo taxa in Bambusinae were likely the clonal materials selected and transported by human migration in the past. Muller (1999) coined the term "ancient enduring clone" (AEC) and suggested possible hybrid ancestry in AECs. Indications of hybridization would include unclear species boundaries owing to unusually large extents of infra-specific morphological variation, low fertility and aberrant seedlings. Although many other bamboo taxa also show these features, hardly any studies have attempted to recognize hybridization as a potential cause. While most hypotheses of natural hybridization in bamboos were supported mainly by morphometric analyses, we progress toward obtaining a molecular perspective of possible hybrid origins in the Bambusinae. Molecular phylogenetic analyses have shown a degree of incongruence between the chloroplast DNA (which is maternally inherited) and the nuclear GBSSI (biparentally inherited) data among the *Bambusa-Dendrocalamus-Gigantochloa* (BDG) complex. The parentage of a putative hybrid between *Dendrocalamus pendulus* and *Gigantochloa scortechinii* in Peninsular Malaysia was demonstrated by the *GBSS*I gene sequence. Phylogenetic analyses using increased taxon sampling of the BDG complex is expected to shed more light on natural hybridization in the Bambusinae.

**Appendix K: Copy of publications resulting from the present work**

(i) Biochemical Systematics and Ecology 38 (2010): 764–773 (ISI-cited).

(ii) Gardens' Bulletin Singapore 62(2) (2011): 223–238.

(ii) Plant Systematics and Evolution (2012) (DOI: 10.1007/s00606-012-0718-1)