GENETIC RESOURCES OF *GIGANTOCHLOA* (POACEAE: BAMBUSOIDEAE: BAMBUSEAE) IN PENINSULAR MALAYSIA

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By

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Specially dedicated to
my beloved family

ABSTRACT

GENETIC RESOURCES OF *GIGANTOCHLOA* (POACEAE: BAMBUSOIDEAE: BAMBUSEAE) IN PENINSULAR MALAYSIA

Dhanendiren A/L Narayanasamy

Gigantochloa is a genus of paleotropical woody bamboo genus that has been widely cultivated in Southeast Asia because of its traditional and commercial usefulness. However, the species boundaries between *Gigantochloa* species are sometimes ambiguous because of a bewildering range of variation in morphology. Recent studies have also shown that species of this genus enter an introgression complex with other genera of the same subtribe Bambusinae. Therefore, this study aims to assess the phylogenetic relationships, population structures and the possible hybridization events among the three common indigenous *Gigantochloa* species of Peninsular Malaysia, i.e., *Gigantochloa ligulata*, *G. scortechinii* and *G. wrayi* based on the PCR-based restriction fragment length polymorphism (PCR-RFLP) profiling method and the cpDNA-nuclear DNA sequence data. The PCR-RFLP marker that distinguished the two chloroplast DNA (cpDNA) lineages, the Gombak- and Langat-type within *G. scortechinii* was developed for a rapid screening among the specimens collected. The results showed that the Gombak-type was the dominant cpDNA genotype for *G. scortechinii* in Peninsular Malaysia. The phylogenetic relationships of *Gigantochloa ligulata*, *G. scortechinii* and *G. wrayi* and other related species were investigated using two chloroplast DNA markers, *rps16-trnQ* and *trnD-T* intergenic spacers, and two nuclear

DNA markers, *GBSS*I (granule-bound starch synthase I) and *PabpI* (poly-A binding protein1). Bayesian Inference (BI) and Maximum Parsimony (MP) analyses based on the cpDNA data recognized two major clades: Clade 1 (0.82 PP/ - BP), consisting of members of Gombak-type haplotype and Clade 2 (0.93 PP/ 54 BP) consisting of Langat–type haplotype. Meanwhile the nuclear DNA topologies recovered three major clades: Clade 1 (0.97 PP/ -BP) consisting of members of *Dendracalamus pendulus, D. strictus* and the putative hybrid DS120 clone B; Clade 2 (1.00 PP/ 95 BP) consisting of *Mullerochloa montana* and the putative hybrid DS117 clone B; and Clade 3 (1.00 PP/ 100 BP) consisting of all *Gigantochloa* species (*G. balui*, *G. latifolia*, *G. manggang*, the two putative hybrids, DS117 clone A and DS120 clone A, as well as the Gombak- and Langat-type *Gigantochloa* species) except *G. atter*. The incongruence between the cpDNA- and nuclear DNA-topologies suggests that there is chloroplast introgression in some *G. scortechinii* and *G. ligulata*. The putative hybrid DS117 is likely to have the maternal origin from *Maclurochloa montana*, while the putative hybrid DS120 is probably a hybrid between *Gigantochloa* and *Dendrocalamus*. While the inter-specific relationships among the *Gigantochloa* species are unclear in the phylogenetic trees, the AMOVA and the pairwise F_{ST} based on the cpDNA support the differentiation among the three *Gigantochloa* species. Population structure analysis displayed that among group and among populations within groups fixation index (F_{ST} and F_{SC}) of *Gigantochloa* populations for both hypothesized structures (a) species boundaries and (b) geographical distribution are significant, but the within populations fixation index (F_{CT}) is not significant.

G. scortechinii at Janda Baik was shown to be significantly different from all other *Gigantochloa* populations. In summary, this study suggests that, for woody bamboos, nuclear DNA could be more useful than cpDNA in providing taxonomic implication. Phylogenetic relationships among the *Gigantochloa* species of Peninsular Malaysia appear to be complex. Introgressive hybridization and incomplete lineage sorting are possible underlying causes for this complexity.

Key words: Gigantochloa, population genetics, cpDNA differentiation, incomplete lineage sorting, introgressive hybridization, Southeast Asia

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APPROVAL SHEET

This dissertation/thesis entitled **"GENETIC RESOURCES OF *GIGANTOCHLOA* (POACEAE: BAMBUSOIDEAE: BAMBUSEAE) IN PENINSULAR MALAYSIA"** was prepared by **DHANENDIREN A/L NARAYANASAMY** and submitted as partial fulfillment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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SUBMISSION OF DISSERTATION

It is hereby certified that **DHANENDIREN A/L NARAYANASAMY** (ID No: **15ADM01410**) has completed this dissertation entitled "**GENETIC RESOURCES OF *GIGANTOCHLOA* (POACEAE: BAMBUSOIDEAE: BAMBUSEAE) IN PENINSULAR MALAYSIA"** under the supervision of Dr. Goh Wei Lim (Supervisor) from the Department of Biological Science, Faculty of Science, and Dr. Gideon Khoo (Co-Supervisor) from the Department of Biological Science, Faculty of Science.

I understand that University will upload softcopy of my dissertation in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and public.

Yours truly,

_____

(Dhanendiren A/L Narayanasamy)

DECLARATION

I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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(DHANENDIREN A/L NARAYANASAMY)

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of molecular variance
*Apo*I	*Arthrobacter protophormiae*
BI	Bayesian Inference
BP	Bootstrap Proportion
BDG	*Bambusa-Dendrocalamus-Gigantochloa*
bp	Base pair
cp	Chloroplast
df	Degree of freedom
DNA	Deoxyribonucleic Acid
F_{CT}	Fixation indices within populations
F_{ST}	Fixation indices among groups
F_{SC}	Fixation indices among populations within group
GPS	Global Positioning System
Hap	Haplotype
kb	kilobase
ng	Nanogram
µM	Micromolar
µg	Microgram
µL	Microliter
ml	Milliliter
MP	Maximum parsimony

nm	Nanometer
PCR	Polymerase Chain Reaction
PP	Posterior Probability
RAPD	Random Amplification Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphisms
SNP	Single-nucleotide Polymorphisms
SDS	Sodium dodecyl sulfate
TAE	Tris-Acetate-EDTA
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

CHAPTER 1

INTRODUCTION

1.1 *Gigantochloa* of Peninsular Malaysia

Gigantochloa Kurz ex Munro is one of the paleotropical woody bamboo genus that belongs to the Bambuseae tribe (Bamboo Phylogeny Group, 2012). *Gigantochloa* is also a part of the *Bambusa* Schreber-*Dendrocalamus* Nees-*Gigantochloa* (BDG) complex, the main core of the Bambusinae subtribe (Goh, et al., 2010). This genus is so far one of the most useful bamboo species in Peninsular Malaysia (Wong, 1995a) which thrives naturally in the foothills and valleys of prominent mountain ranges. They also inhabit lowland forests (Wong, 2004).

A typical member of *Gigantochloa* is distinguished from other closely related genera by several characters (Wong, 1995a):

(i) Spikelets of sterile terminal floret with lemma, sessile, lodicules absent;

(ii) Stamen filaments joined to form a firm tube;

(iii) Ovary with hairs at top.

Furthermore, few other morphological key features can also be used to recognize this genus, e.g., the culm-sheath blades are erect, patent or reflexed, lanceolate to narrowly triangular, and always green and leaf-like when fresh; culm-sheath auricles are low, firm and distinct rim-like structures or rounded lobes and rachilla internodes not joined below the lemma attachment (Wong, 1995a).

Bamboos of Southeast Asia are classified as village or wild bamboos (Holttum, 1958), and are referred to as the cultivated or native bamboos. *Gigantochloa* also comprises wild species (*G. albovestita* Holttum, *G. holttumiana* Wong, *G. latifolia* Ridley, *G. ligulata* Gamble, *G. rostrata* Wong, *G. scortechinii* Gamble, *G. wrayi* Gamble) and cultivated species (*G. albopilosa* Wong, *G. hasskarliana* Kurz, *G. levis* Merr, *G. ridleyi* Holttum, *G. thoii* Wong) (Holttum, 1958; Widjaja, 1987; Wong, 1995a; Goh, et al., 2013). Among these species, *G. latifolia*, *G. ligulata*, *G. scortechinii* and *G. wrayi* are common while *G. rostrata* and *G. hasskarliana* are rare in Peninsular Malaysia (Wong, 1995a).

Gigantochloa is a very well-known and valuable bamboo genus in Peninsular Malaysia (Holttum, 1958; Widjaja, 1987; Wong, 1995a; Wong, 2004). The traditional application of *Gigantochloa* varies from their use in handicrafts (Azmy and Razak, 1991), ornaments (Wong, 1995a; Wong, 2004), the use of young shoots for cuisines (Holttum, 1958; Widjaja, 1987; Azmy and Razak, 1991; Wong, 2004), to their use as construction materials such as water pipes and bridges (Azmy and Razak, 1991; Wong, 1995a; Wong, 2004).

Furthermore, *Gigantochloa* is also recognized in the vegetable basket and poultry coop-making industry (Holttum, 1958; Wong, 2004), skewer and chopstick industry (Azmy and Razak, 1991; Wong, 2004). *Gigantochloa* is also useful in providing structural support when used as scaffolding for building constructions (Wong, 2004) and as walls of houses (Wong, 2004). *Gigantochloa* bamboos hold enormous potential (Hisham, et al., 2006; Mustafa, et al., 2011; Wahab, et al., 2013) to be a wood substitute because of their fast-growing rate, long and straight culm-internodes, durability (Rassiah, et al., 2014; Chaturbhuj, et al., 2016) as well as insects and fungal infection resistance. *Gigantochloa* can be produced in large-scale plantations and the raw materials can be used by the furniture, paper and pulp industries (Bystriakova, et al., 2003), whereas engineered or processed bamboo "board" can be used as structural plywood (Anwar, et al., 2004) and urea-formaldehyde particleboards (Kasim, et al., 2001).

1.2 Problem Statement and Possible Causes of Taxonomic Complications of *Gigantochloa*

The botanical and taxonomic classifications of bamboos are generally complicated and poorly understood due to the lack of documentation as most bamboo collectors found difficulties in compiling good quality bamboo specimens (Holttum, 1958; McClure, 1966). Furthermore, understanding the morphology and physiology properties of a bamboo species is taxing due to insufficient reference materials for identification, e.g., poor representation of flowering specimens and main vegetative structures in the herbaria (Wong, 2004).

Furthermore, morphological-based taxonomic classifications do not provide clear-cut resolutions because of the absence of synapomorphic characteristics in the individual genus of Bambusinae subtribe (Holttum, 1958; McClure, 1966; Wong, 1995a). Many characteristics of the *Gigantochloa* genera in the Bambusinae subtribe can be explained based on a combined character states. For instance, culm sheath blade pattern that is found to be erect in some *Gigantochloa* species is also present in almost all *Bambusa* species and in some *Dinochloa* Buse; the auricles are commonly low and rim-like in *Gigantochloa* species and *Maclurochloa* Wong but in some *G. thoii* and few other species of *Gigantochloa* they have a bristly lobe; the fused filament tube which is present in *Gigantochloa* also appears in *Schizostachyum* Nees and in *D. sinuatus* Gamble (Holttum, 1958; Widjaja, 1987; Wong, 1995a and 1995b; Wong, 2004). Moreover, the stamen filament tube which is a peculiar character that describes *Gigantochloa* cannot be evaluated when a species does not undergo flowering phase. This is particularly evident in the introduced species (Holttum, 1958; Widjaja, 1987; Wong, 1995a; Wong, 2004; Goh, et al., 2013).

On the other hand, the taxonomic problems in *Gigantochloa* are also due to the possible hybridization among closely related species. Holttum (1958) highlighted that the bewildering morphological variation among wild *Gigantochloa* bamboos (especially in the *G. latifolia-G. ligulata* complex) in northern Malay Peninsula may be due to the occurrence of hybrid swarms among closely-related *Gigantochloa* taxa. This suggested the possibility that only chosen *Gigantochloa* clones were cultivated. Hybrid swarm is defined as a population of individuals that are all hybrids by varying numbers of

generations of backcrossing with parental types, and by mating among hybrids (Anderson, 1949). According to Anderson and Hubricht (1938) and Anderson (1948), elevated variation has been referred to as a major significance of introgressive hybridization. The introgressants would resemble the parental species to a certain level and form a hybrid swarm after repeated backcrosses to one or to both parents for few generations. Usually in taxonomic assessments, hybrid swarm elements are considered as ‘diversities’ or 'anomalous characters' of the related parental species (Anderson, 1948).

Recent molecular systematics and phylogenetic studies also revealed the inconsistency in the evolutionary pathway of *Gigantochloa*. These complications include:

(i) *G. scortechinii* includes two distinct chloroplast DNA (cpDNA) haplotypes (Goh, et al., 2013)

(ii) Inconsistencies between maternally derived cpDNA and the biparentally derived nuclear DNA, *GBSS*I gene trees (Goh, et al., 2010; Goh, et al., 2013) that emphasized possible events of chloroplast capture/introgression in *Gigantochloa* genus (Goh, et al., 2013)

(iii) The extent of past introgressive hybridization (Rieseberg and Brunsfield, 1992; Rieseberg and Wendel, 1993), with or without the contribution of incomplete lineage sorting (Avise, et al., 1987; Pamilo and Nei, 1988).

Chloroplast capture is known as the introgression of chloroplasts genes from one species into another after intra-generic and inter-generic hybridization (Wolfe and Elisens, 1995; Van Raamsdonek, et al., 1997; Jackson, et al., 1999; Kornkven, et al., 1999). Furthermore, the natural intergeneric hybrid bamboo, × *Gigantocalamus malpenensis* and its parental species, *Dendrocalamus pendulus* and *Gigantochloa scortechinii* in Peninsular Malaysia further verified the extent of hybridization in *Gigantochloa* taxa (Goh, et al., 2011).

On the other hand, recent molecular phylogenetic studies utilizing chloroplast DNA and nuclear DNA markers at generic level have never resolved classification of *Gigantochloa* species with other related genera species into a monophyletic group (Yang, et al., 2008; Sungkaew, et al., 2009; Goh, et al., 2010; Yang, et al., 2010; Bamboo Phylogeny Group, 2012; Goh, et al., 2013; Chokthaweepanich, 2014). It was implied that these previous phylogenetic and systematic investigations sampled too few species of *Gigantochloa* to satisfactorily address the intra- and inter-generic boundary delimitations and the underlying causes of taxonomic complexity of the genus. The species boundaries among *Gigantochloa* in Malaysia were evaluated by Widjaja (1987) and Widjaja and Lester (1987) but no other molecular study has assessed *Gigantochloa* at specific and population levels with wide taxon sampling.

1.3 Objectives of the Study

In the present study, two selected chloroplast DNA (cpDNA) and two nuclear DNA markers were utilized to investigate the phylogenetic relationship, hybrid origin and population structure of selected *Gigantochloa* species. In addition, PCR-based RFLP analysis was employed to further investigate the cpDNA differentiation in *G. scortechinii.*

The specific objectives are:

1. To assess the chloroplast DNA differentiation among *Gigantochloa scortechinii* using restriction fragment length polymorphisms (PCR-RFLP);
2. To evaluate the population structure of the three-common indigenous *Gigantochloa* species in Peninsular Malaysia, i.e., *Gigantochloa ligulata*, *G. scortechinii* and *G. wrayi*;
3. To investigate the phylogenetic relationships of the *Gigantochloa* species and its closely related genera; and
4. To examine the hybrid origin of the *Gigantochloa* hybrids in Peninsular Malaysia.

CHAPTER 2

LITERATURE REVIEW

2.1 *Gigantochloa*: Taxonomy and Distribution

Gigantochloa is a paleotropical woody bamboo which belongs to the Bambuseae tribe and Bambusinae subtribe of the grass family (Poaceae) (Wong, 1995a). The initial taxonomic classification of *Gigantochloa* was attempted by Kurz (1864) when he listed four species into the newly created genus, i.e., *G. atter*, *G. maxima*, *G. apus* and *G. nigrociliata* which had been reported by earlier taxonomists and botanists under *Bambusa* (Hassakarl, 1848; Miquel, 1855). In 1868, Munro considered three species, *G. atter*, *G. heterostachya* and *G. verticillata* into *Gigantochloa* genus in his monograph about Bambusaceae, and he distinguished this genus from *Bambusa* by referring the filaments that joined together to form a firm tube. He also reviewed the correct terminology of *G. maxima* and termed it as *G. verticillata* (Munro, 1868). A few years later, Kurz (1876) assessed Munro's descriptions and proposed six species, i.e., *G. apus*, *G. atter*, *G. heterostachya*, *G. maxima*, *G. nigrociliata* and *G. robusta*, most of which can only be observed in cultivation. He defined the *Gigantochloa* genus with more diagnostic characters by referring to the membranous pericarp of fruits, deciduous styles and 2-keeled plea (Kurz, 1876).

Subsequently, Gamble (1896) who investigated the Bambuseae of British India incorporated nine *Gigantochloa* species into the genus, eight of the species (*G. heterostachya*, *G. kurzii*, *G. latispiculata*, *G. ligulata*, *G. verticillata* and *G. wrayi*) recognized from Malaya and Burma and one species from Chittagong and Assam (*G. macrostachya*). Furthermore, two species, i.e., *G. atter* and *G. robusta*, which were found in Java and other islands Indonesia were also described by Gamble (1896) in his monograph. Later, in 1956 and 1958, Holttum evaluated the bamboos of Malay Peninsula (Peninsular Malaysia and the southernmost tips of Myanmar and Thailand) and signified this genus based on ovary, fruit, spikelet structure and rhizome branching characters. Holttum explained about *G. atter* (Holttum, 1956) and nine species of *Gigantochloa* in Malay Peninsula, i.e., *G. apus*, *G. hasskarliana*, *G. latifolia*, *G. levis*, *G. ligulata*, *G. maxima*, *G. ridleyi*, *G. scortechinii* and *G. wrayi* (Holttum, 1958) which was established in the wild and in cultivation.

The taxonomic classifications of *Gigantochloa* were further reviewed and included in the subtribe and genera studies investigated by Clayton and Renvoize (1986) based on ovary appendage, inflorescence and culm sheath characters and by Soderstrom and Ellis (1987) based on sympodial rhizomes, primary branching buds, floral structures and chromosome numbers. Subsequent work on *Gigantochloa* at species boundary level in Malesia (a floristic eco-region that includes Malay Peninsular and Malay Archipelago) was carried out by Widjaja (1987) who provided detailed information on 18 species of *Gigantochloa*, i.e., *G. achmadii*, *G. apus*, *G. atroviolacea*, *G.* atter,

G. hasskarliana, *G. holttumiana*, *G. latifolia*, *G. levis*, *G. ligulata*, *G. manggong*, *G. nigrocilliata*, *G. pseudoarundinacea*, *G. pruriens*, *G. ridleyi*, *G. rostrata*, *G. robusta*, *G. scortechinii* and *G. wrayi*. Following the investigations, Widjaja and Lester (1987) acknowledged the distinctiveness of 18 *Gigantochoa* taxa according to a combined analysis conducted based on morphology, anatomy, phenolic compounds and protein electrophoresis. At the end of the 19th century, further classification of *Gigantochloa* was provided through phylogenetics and systematics studies at the subtribe and genera levels, e.g., inflorescences and leaf anatomical characters-based classification (Dransfiled and Widjaja, 1995); botanical monograph explanation on morphology, anatomy, biology and classifications of Peninsular Malaysia bamboos (Wong 1995(a) and 1995(b)); rhizome structure, inflorescence morphology and ovary appendage characters-based classification (Ohrnberger, 1999).

Gigantochloa is differentiated from the other genera of the subtribe *Bambusineae* by its spikelets of sterile terminal floret with lemma, sessile, lodicules absent, filaments joined to form a tube and ovary with hairs at the top (Holttum, 1958; Widjaja, 1987; Wong 1995(a) and 1995(b). Furthermore, *Gigantochloa* can be recognized based on a few unique morphological features (Figure 2.1), as follows (Munro, 1896; Kurz, 1876; Gamble, 1896; Holttum, 1956; Holttum, 1958; Widjaja, 1987; Wong, 1995(a); Wong, 1995(b); Wong, 2004):

i) The culm-sheath blades erect, patent or reflexed, lanceolate to narrowly triangular, and always green and leaf-like when fresh.

ii) Culm-sheath auricles low, firm and distinct rim-like structures or rounded lobes.

iii) Flower with stamen filaments fused to form a firm tube.

iv) The rachilla internodes not joined below the lemma attachment.

The vegetative parts of member of this genus resemble those of *Bambusa* and *Dendrocalamus* in having one dominant lateral branch, but their culms are straight with aerial roots and mostly without white wax (Kurz, 1876).

(a)

(b)

(c)

(d)

Figure 2.1: *Gigantochloa* species used in the present study. (a) *Gigantochloa ligulata* with conspicuous long leaf-sheath ligules, (b) *Gigantochloa ligulata* with 1 main dominant branch without subdominants at its base, (c) *Gigantochloa scortechinii* with culm sheaths green at the base, flushed intense orange towards the top and densely tufted, appear whitish because of waxy powder on the young culms, (d) *Gigantochloa wrayi* with culm sheath green, streaked with paler green, covered with dark brown hairs and glabrous culm internodes.

Gigantochloa genus appears to be native to Indo China, e.g., Lower Burma and Peninsular Thailand (Holttum, 1958) and are broadly cultivated in different regions of Southeast Asia, e.g., Philippines, Northern Borneo, Java and at the southern end of Main Range in Peninsular Malaysia (Gamble, 1896; Holttum, 1958; Widjaja, 1987; Dransfield, 1992; Dransfield and Widjaja, 1995; Wong, 1995a; Shouliang, et al., 2007). Table 2.1 illustrates the documented *Gigantochloa* species in Southeast Asia including China and India:

Table 2.1: Documented *Gigantochloa* species in Southeast Asia, China and India.

Peninsular Malaysia	Borneo	Indo China and Burma (Myanmar)	Thailand	India	China	Indonesia (includes Bali, Java and Sumatera)	Philippines	Singapore
G. albopilosa (C)[8,11] *G. albovestita* (C)[8,11] *G. hasskarliana* (C)[8,11] *G. heterostachya* (C)[2] *G. holttumiana* (C)[3,8,11] *G. kurzii* (C)[2] *G. latifolia* (C)[1,3,8,11] *G. latispiculata* (C)[2] *G. levis* (C)[1,3,9,10] *G. ligulata* (C)[1,2,3,8,9,11] *G. verticilliata* (C)[2,10] *G. scortechinii* (C)[1,2,8,9,11] *G. ridleyi* (C)[8,11] *G. rostrata* (C)[3,8,11] *G. thoii* (C)[8,9,11] *G. wrayi* (C)[1,2,3,8,9,11]	*G. balui* (C)[4] *G. levis* (C)[1,3,4] *G. hasskarliana* (C)[8] *G. verticilliata* (C)[2]	*G. albociliata* (C)[9] *G. apus* (N)[3] *G. hasskarliana* (N)[1] *G. kurzii* (C)[2] *G. levis* (C)[1,3] *G. macrostachya* (C)[2] *G. nigrocilliata* (C)[10] *G. rostrata* (C)[8] *G. verticilliata* (C)[10]	*G. albociliata* (C)[10] *G. auriculata* (C) *G. atroviolacea* (C)[3] *G. balui* (C)[9] *G. latifolia* (C)[8] *G. ligulata* (N)[3,8] *G. nigrocilliata* (C)[10] *G. scortechinii* (C)[8] *G. rostrata* (C)[8] *G. verticilliata* (C)[10] *G. wrayi* (C)[8]	*G. albociliata* (C)[7,10] *G. apus* (C)[7] *G. atter* (C)[7] *G. atroviolacea* (C)[3,7] *G. levis* (C)[7] *G. macrostachya* (C)[2,7] *G. manggong* (C)[3] *G. nigrocilliata* (C)[10] *G. pseudoarundinacea* (C)[3,7] *G. rostrata* (C)[3,7] *G. verticilliata* (C)[2,10]	*G. albociliata* (C)[10] *G. felix* (C)[10] *G. levis* (C)[10] *G. nigrocilliata* (C)[10] *G. parviflora* (C)[10] *G. verticilliata* (C)[10]	*G. achmadii* (C)[3] *G. apus* (C)[1,3] *G. atter* (C)[3] *G. atroviolacea* (N)[3] *G. levis* (C)[3,4] *G. hasskarliana* (N)[1,3,8,11] *G. manggong* (C)[3] *G. maxima* (C)[1] *G. nigrocilliata* (C)[3,10] *G. pseudoarundinacea* (N)[3] *G. pruriens* (C)[3] *G. ridleyi* (C)[9] *G. robusta* (N)[3,9] *G. verticilliata* (C)[2,10] *G. wrayi* (C)[3]	*G. levis* (C)[1,3.10] *G. verticilliata* (C)[2]	*G. levis* (C)[1] *G. ligulata* (C)[1,8] *G. hasskarliana* (C)[1,8] *G. ridleyi* (C)[1,3,8] *G. verticilliata* (C)[2]

*Annotations: Native; Nil (species not present); [1]Holttum, 1958; [2]Gamble, 1896; [3]Widjaja, 1987;[4]Dransfield, 1992; [5]Widjaja and Dransfield, 1995; [6]Muller, 1998; [7]Seethalaksmi and Kumar, 1998; [8]Wong, 1995a; [9]Wong, 2004; [10]Shouliang, et al., 2007; [11]Goh, et al., 2013

Earlier studies suggested that *Gigantochloa* is not native to Borneo, Java and Philippines (Holttum, 1958) and is known only in cultivation in Java (Wong, 2004). The current distribution of *Gigantochloa* shows that these plants have their diversity-rich relatives in the Indo China and their occurrence in the further south part of the Southeast Asia islands, i.e., Malaysia Peninsula, Borneo, Peninsular Thailand, Java and Sumatera are possibly due to the historical migration of peoples from Indo China (Gamble, 1896; Holttum, 1958; Widjaja, 1987; Dransfield, 1992; Dransfield and Widjaja, 1995; Wong, 1995a). Based on Holttum's (1958) observation, there are natural populations of *G. ligulata* in the southern part of Johor state whereas cultivated species are found around the southern end of the Main Range in Peninsular Malaysia (Wong, 1987). Most of the *Gigantochloa* species (*G. latifolia*, *G. scortechinii* and *G. wrayi*) occur at the foothills and mountain range valleys. They also colonize disturbed forest sites in lowlands (Holttum, 1958; Widjaja, 1987; Wong, 2004). Only two species of *Gigantochloa* (*G. balui* and *G. levis*) have been recorded in Sabah. The possible existence of other species requires further clarification (Dransfield, 1992).

2.2 Hybridization in *Gigantochloa*

Bamboos are routinely used by people in Southeast Asia, China, Japan and India (Wong, 2004). Holttum (1958) described bamboos from Peninsular Malaysia as native or forest bamboos and village or cultivated bamboos. He also suggested that some species of *Gigantochloa* are known only in cultivation and were possibly brought to Peninsular Malaysia and Java by historical migrations of people from Southern Myanmar where the wild

Gigantochloas were originally established. Holttum (1958) further clarified that the confusing morphological variation among the wild *Gigantochloa* bamboos (especially in the *G. latifolia-G. ligulata* complex) found in the northern Malay Peninsula was likely possibly due to the occurrence of hybrid swarms among closely related *Gigantochloa* which proved that only selected *Gigantochloa* clones have been used for cultivation. According to Anderson and Hubricht (1938) and Anderson (1948), the major significance of introgressive hybridization referred as elevated variation among the introgressants where the introgressants would display intermediate characteristics of the parental species to a certain level and form a hybrid swarm after repeated backcrosses to one or to both parents for few generations. Usually in taxonomic assessments, hybrid swarm elements could be recognized as ‘diversities’ or 'anomalous individuals' of the related species (Anderson, 1948). This is because hybrid swarms can progress rapidly and overcome parental species through genetic homogenization or competitive exclusion in as few as five generations causing the erosion of species boundary (Rhymer and Simberloff, 1996; Mooney and Cleland, 2001; Wolf, et al., 2001; Perry, et al., 2002; Hall, et al., 2006).

Subsequently, the outcome of the morphology-based numerical analysis conducted by Widjaja and Lester (1987) was not consistent with Holttum’s (1958) initial postulate on the morphological variation among the wild *Gigantochloa* bamboos found in the northern Malay Peninsula and the presence of hybrid swarms. Although their research on morphology, anatomy, phenolic compounds and protein electrophoresis exhibited uniqueness among

the 18 *Gigantochoa* taxa, they also found out that some of the taxa did have special co-relation, e.g., *G. atter* and *G. atroviolacea* were closely related, and *G. achmadii, G. hasskarliana, G. latifolia, G. manggong, G. nigrocilliata, G. pruriens* and *G. rostrata* possibly cluster into same group.

A more recent investigation by Muller (1996) did not correspond with the morphology-based analysis presented by Widjaja and Lester (1987). Muller identified *Gigantochloa* clones which were not included within the 18 species and justified that the anomalous reproduction behavior and progressive morphological variation of *Gigantochloa* clones were due to hybrid derivation (Muller, 1998; Muller, 2003). The self-fertilization of one of the single parent clump of *G. ridleyi* (introduced from Bali) that Muller had brought to Mount Mirinjo Farm, Australia, generated limited seed set and some seedlings that sprouted were albinos and not viable, while the other half exhibited vegetative morphological traits that were mostly distinct among themselves and from the parent species. Muller (1998, 2003) also differentiated the morphology variation among F2 offsprings, i.e., the selfing outcome of hybrid F1 hybrid as concluded by Holttum (1958). Furthermore, Muller (1999) proposed that the bamboo clones that were cultivated only in Indonesia and Malaysia were "Ancient Enduring Clones" and these clones comprised the hybrid swarms as suggested by Holttum (1958). This is also supports the distribution of the cultivated *Gigantochloa* species by historical migration of people from Southern Myanmar which was likely the centre of diversity of *Gigantochloa* (Holttum, 1958).

Muller (2003) further gathered the genetic factors behind the occurrence of albinism from the self-fertilization event of the low seed set seedlings groups which also showed low mortality rate. Pigment defect in albinism was attributed to inconsistency between nuclear and chloroplast genomes and gene deletion (Kirk and Tilney-Bassett, 1978). According to Kumari, et al. (2009), albinism has lethal recessive features that are dominated by one or more gene loci and this could explain the heterozygosity for the chlorophyllous (green) trait, which was retained by the parent species of the albino (*G. ridleyi* in Muller's case study) to a certain level and the viable (green) seedlings that would still preserve the genotype trait. Albinism appears to be a possible factor of the hybrid origin of the chosen *Gigantochloa* clones (Muller, 2003) as there were records on the existence of albinism in interspecific hybrids in different plant studies 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). Meanwhile, a recent molecular study on natural hybrid, × *Gigantocalamus malpenensis* K.M. Wong, the intermediate of *D. pendulus* and *G. scortechinii* (Goh, et al., 2011), further proved the existence of past hybridization among *Gigantochloa* bamboos, i.e., the occurrence of hybrid swarms (Holttum, 1958; Muller, 1998).

Furthermore, it was suggested that the cultivated bamboo could be associated to hybrid origin as indicated by their sterility (Holttum, 1958; Wong, 1995b; Muller, 1998; Muller, 1999; Wong, 2004; Goh, et al., 2011; Goh, et al., 2013). Muller (1999) and Wong (2004) stated that infertility traits (such as: the continuation of a long vegetative period, minimal flowering state that prolong

the clone's survival phase and low seed set) have been introduced for Ancient Enduring Clones (AECs) and practiced in cultivation as this selected characteristic guaranteed the durability of AECs in cultivation and utilization (Muller, 1999; Wong, 2004). For example, *G. robusta* clumps that were cultivated in Bogor Botanical Garden in 1844 during the time of the botanist Hasskarl have stayed alive for 150 years without flowering. This further supported the hypothesis that infertility have been chosen as AEC's traits during cultivation (Wong, 2004). In addition, low fertility behavior was found in the intergeneric hybrid between *D. pendulus* and *G. scortechinii* which further suggested that sterility signified the occurrence of hybridization within the *Gigantochloa* bamboo taxa (Goh, et al., 2011). Table 2.2 summarizes the fertility (represented by flowering and fruiting incidents) of the *Gigantochloa* species within and outside their native areas.

Table 2.2: Flowering and fruiting incidents among the *Gigantochloa* species within and outside their native areas.

***Gigantochloa* taxa**	**Peninsular Malaysia**	**Borneo**	**Indo China**	**Burma (Myanmar)**	**Thailand**	**China**	**India**	**Indonesia (includes Bali, Java and Sumatera)**	**Philippines**	**Singapore Botanical Garden**
G. achmadii	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Documented flowering and fruiting unknown (C)[3]	Nil	Nil
G. albociliata	Nil	Nil	Nil	Documented flowering and fruiting unknown (C)[10]	Documented flowering and fruiting unknown(C)[10]	Documented flowering and fruiting unknown(C)[10]	Documented flowering and fruiting unknown(C)[7,10]	Nil	Nil	Nil
G. albopilosa	Unknown(C)[8,11]	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
G. albovestita	Unknown(C)[8,11]	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
G. auriculata	Nil	Nil	Nil	Nil	Unknown	Nil	Nil	Nil	Nil	Nil
G. apus	Nil	Nil	Nil	Yes[3]	Nil	Nil	Yes[7]	Yes (C)[1,3]	Nil	Nil
G. atroviolacea	Nil	Nil	Nil	Nil	Unknown[3]	Nil	Documented flowering and fruiting unknown(C)[7]	Documented flowering and fruiting unknown(N)[3]	Nil	Nil
G. atter	Nil	Nil	Nil	Nil	Nil	Nil	Documented flowering and fruiting unknown(C)[7]	Documented flowering and fruiting unknown(C)[3]	Nil	
G. balui	Unknown	Documented flowering and fruiting unknown (C)[4]	Nil	Nil	Unknown	Nil	Nil	Nil	Nil	Nil
G. felix	Nil	Nil	Nil	Nil	Nil	Documented flowering and fruiting unknown(C)[9]	Nil	Nil	Nil	Nil
G. hasskarliana	Documented flowering and fruiting unknown[1,8](C) (Seeds in native area[5])	Yes[1,8]	Nil	Nil	Nil	Nil	Nil	Yes[1,3,8](N) (Seeds in native area[5])	Nil	Yes[1,8]

*Annotations: Native (N); Cultivated (C); Nil (species not present); [1]Holttum, 1958; [2]Gamble, 1896; [3]Widjaja, 1987;[4]Dransfield, 1992; [5]Widjaja and Dransfield, 1995; [6]Muller, 1998; [7]Seethalaksmi and Kumar, 1998; [8]Wong, 1995a; [9]Wong, 2004; [10]Shouliang, et al., 2007; [11]Goh, et al., 2013

Table 2.2 (Cont'd):

Gigantochloa taxa	Peninsular Malaysia	Borneo	Indo China	Burma (Myanmar)	Thailand	China	India	Indonesia (includes Bali, Java and Sumatera)	Philippines	Singapore Botanical Garden
G. heterostachya	Documented flowering and fruiting unknown(C)[2]	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
G. holttumiana	Documented flowering and fruiting unknown(C)[3,8,11]	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
G. kurzii	Yes(C)[2]	Nil	Nil	Yes(C)[2]	Nil	Nil	Nil	Nil	Nil	Nil
G. latifolia	Documented flowering and fruiting unknown(C)[1,3,8,11]	Nil	Nil	Nil	Documented flowering and fruiting unknown[8]	Nil	Nil	Nil	Nil	Nil
G. latispiculata	Documented flowering and fruiting unknown(C)[2]	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil
G. levis	Yes[1](C)[3,10]	Yes(C)[1,3,4]	Yes(C)[3]	Nil	Nil	Documented flowering and fruiting unknown(C)[1,10]	Nil	Documented flowering and fruiting unknown(C)[4]	Yes(C)[1,3,10]	Documented flowering and fruiting unknown(C)[1]
G. ligulata	Yes(C)[1,2,3,8,11]	Nil	Nil	Nil	Yes(C)[1,8]	Nil	Nil	Nil	Nil	Nil
G. manggong	Nil	Nil	Nil	Nil	Nil	Nil	Unknown(C)[3]	Documented flowering and fruiting unknown(C)[3]	Nil	Nil
G. maxima	Unknown(C)[1]	Nil	Nil	Nil	Nil	Nil	Nil	Documented flowering and fruiting unknown(C)[1]	Nil	Nil
G. macrostachya	Nil	Nil	Nil	Yes(C)[2]	Nil	Nil	Yes(C)[2]	Nil	Nil	Nil

*Annotations: Native (N); Cultivated (C); Nil (species not present); [1]Holttum, 1958; [2]Gamble, 1896; [3]Widjaja, 1987;[4]Dransfield, 1992; [5]Widjaja and Dransfield, 1995; [6]Muller, 1998; [7]Seethalaksmi and Kumar, 1998; [8]Wong, 1995a; [9]Wong, 2004; [10]Shouliang, et al., 2007; [11]Goh, et al., 2013

Table 2.2 (Cont'd):

Gigantochloa taxa	Peninsular Malaysia	Borneo	Indo China	Burma (Myanmar)	Thailand	China	India	Indonesia	Philippines	Singapore Botanical Garden
G. nigrocilliata	Nil	Nil	Nil	Documented flowering and fruiting unknown(C)[10]	Documented flowering and fruiting unknown(C)[10]	Documented flowering and fruiting unknown(C)[10]	Documented flowering and fruiting unknown(C)[10]	Yes(C)[3,10]	Nil	Nil
G. parviflora	Nil	Nil	Nil	Nil	Nil	Documented flowering and fruiting unknown(C)[10]	Nil	Nil	Nil	Nil
G. pseudoarundinacea	Nil	Nil	Nil	Documented flowering and fruiting unknown(C)[3,7]	Nil	Nil	Yes[3,7]	Documented flowering and fruiting unknown(N)[3]	Nil	Nil
G. pruriens	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Documented flowering and fruiting unknown(C)[3]	Nil	Nil
G. ridleyi	Unknown(C)[8,1 1] (But seeding reported elsewhere[6])	Nil	Nil	Nil	Nil	Nil	Nil	Documented flowering and fruiting unknown(C)[9]	Nil	Unknown[1]

*Annotations: Native (N); Cultivated (C); Nil (species not present); [1]Holttum, 1958; [2]Gamble, 1896; [3]Widjaja, 1987;[4]Dransfield, 1992; [5]Widjaja and Dransfield, 1995; [6]Muller, 1998; [7]Seethalaksmi and Kumar, 1998; [8]Wong, 1995a; [9]Wong, 2004; [10]Shouliang, et al., 2007; [11]Goh, et al., 2013

Table 2.2 (Cont'd):

Gigantochloa taxa	Peninsular Malaysia	Borneo	Indo China	Burma (Myanmar)	Thailand	China	India	Indonesia	Philippines	Singapore Botanical Garden
G. robusta	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Documented flowering and fruiting unknown(N)[3]	Nil	Nil
G. rostrata	Yes(C)[3,8,11]	Nil	Nil	Yes(C)[8]	Yes(C)[8]	Nil	Yes(C)[3,7]	Nil	Nil	Nil
G. scortechinii	Yes(C)[1,2,3,8,11]	Nil	Nil	Nil	Documented flowering and fruiting unknown(C)[8]	Nil	Nil	Nil	Nil	Nil
G. thoii	Documented flowering and fruiting unknown(C)[1,8,11]	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Nil	Documented flowering and fruiting unknown(C)[1,8]
G. verticilliata	Documented flowering and fruiting unknown(C)[2]	Documented flowering and fruiting unknown(C)[2]	Unknown	Documented flowering and fruiting unknown(C)[2]	Unknown[10]	Unknown[10]	Documented flowering and fruiting unknown(C)[2]	Documented flowering and fruiting unknown(C)[2]	Documented flowering and fruiting unknown(C)[2]	Nil
G. wrayi	Yes(C)[1,2,3,8,11]	Nil	Nil	Nil	Yes(C)[3,8]	Nil	Nil	Yes(C)[3]	Nil	Nil

*Annotations: Native (N); Cultivated (C); Nil (species not present); [1]Holttum, 1958; [2]Gamble, 1896; [3]Widjaja, 1987;[4]Dransfield, 1992; [5]Widjaja and Dransfield, 1995; [6]Muller, 1998; [7]Seethalaksmi and Kumar, 1998; [8]Wong, 1995a; [9]Wong, 2004; [10]Shouliang, et al., 2007; [11]Goh, et al., 2013

Based on the flowering and fruiting occurrence list in *Gigantochloa* (Table 2.2), native or forest *Gigantochloa* species in Peninsular Malaysia, Peninsular Thailand, India, China, Burma and Indonesia produced flower and fruits, i.e., *G. apus*, *G. hasskarliana*, *G. latifolia*, *G. ligulata*, *G. macrostachya*, *G. nigrociliata*, *G. parviflora*, *G. rostrata*, *G. scortechinii* and *G. wrayi*. Some *Gigantochloa* species have been recorded to flower and fruit in their native regions but reported to only flower at the introduced regions, e.g., *G. hasskarliana*, *G. nigrocilliata* and *G. ridleyi*. Meanwhile, some of the remaining cultivated *Gigantochloa* species are known only for their flowering event while some are considered as "unknown" since no records were available. Since the hybrid swarms were hypothesized to be selected for cultivation (Holttum, 1958; Muller, 1999) and that most of the cultivated *Gigantochloa* species (Table 2.2) are unable to flower and set seed to yield fruit, more studies are needed to determine the significance of hybridity in relation to such sterility.

2.3 Other Molecular Systematic Studies on *Gigantochloa*

Prior systematic studies of *Gigantochloa* were mostly focused on taxonomic placement based on morphological and vegetative characters (Munro, 1868; Kurz, 1876; Gamble, 1896; Holttum, 1956; Holttum, 1958; Clayton and Renvoize, 1986; Soderstrom and Ellis, 1987; Widjaja, 1987; Widjaja and Lester, 1987; Dransfield, 1992: Dransfiled and Widjaja, 1995; Wong, 1995b; Ohrnberger, 1999) (Section 2.1). The advancement of molecular markers in phylogenetics and systematics has paved the way for a better resolution of the existing complexity in bamboos. Numerous molecular phylogenetic studies of

selected *Gigantochloa* taxa at subtribal and generic level have been conducted utilizing molecular DNA-fingerprinting based methods such as PCR-RFLP (Arnab and Goyal., 2014), AFLP (Loh, et al., 2000), RAPD (Das, et al., 2007; Ramanayake, et al., 2007), transposons (Zhong, et al., 2010), microsatellites markers (Mukherjee, et al., 2010) and DNA-sequence methods such as organellar genes, cpDNA and nuclear DNA (Watanable, et al., 1994; Sungkaew, et al., 2009; Yang, et al., 2008; Yang, et al., 2010; Goh, et al., 2010; Triplett, et al., 2010; Bamboo Phylogeny Group, 2012; Goh, et al., 2013; Chokthaweepanich, 2014).

Even though molecular-based investigations have been documented, the established taxonomic limitation of the *Gigantochloa* genus has still been contentious due to the long-standing complications of the genus with its associated genera group, *Bambusa and Dendrocalamus.* Alliance of these three genera as *Bambusa-Dendrocalamus-Gigantochloa* (BDG) complex (Goh, et al., 2011; Goh, et al., 2013) which is significantly acknowledged as the core of Bambusinae, further magnify the taxonomic problems that involved *Gigantochloa* at the inter- and intra-generic boundary level. *Gigantochloa* has never been confirmed as a monophyletic group in the previous studies based on multi-locus cpDNA (Sungkaew, et al. 2009; Yang, et al., 2010) and a combined cpDNA and nuclear DNA (Yang, et al., 2008; Goh, et al., 2010; Goh, et al., 2013). Moreover, there are strong associations between *Gigantochloa* and *Melocalamus*, *Oreobambos*, *Oxytenanthera*, *Neosinocalamus*, *Phuphanochloa* and *Vietnamosasa* (Sungkaew, et al., 2009), and *Maclurochloa* and *Soejatmia* (Goh, et al., 2010). Additionally, there are

complex associations exhibited among *Gigantochloa* and other genera, e.g., *Dendrocalamus*, *Oxytenanthera* and *Neosinocalamus* (Yang, et al., 2008; Yang, et al., 2010). The existence of incongruence between chloroplast DNA and nuclear gene topologies in a study by Goh, et al. (2013), highlighted the tangled evolutionary history of the BDG (*Bambusa-Dendrocalamus-Gigantochloa*) complex. The potential factors of these complications, e.g., extensive incomplete linage sorting and introgressive hybridization events, further distorted the morphological boundaries among these genera (Goh, et al., 2013), especially *Gigantochloa*, thus making the genus taxonomically problematic.

2.4 Economic Importance and Potential of *Gigantochloa*

Gigantochloa is one of the useful bamboo genus in Peninsular Malaysia (Holttum, 1958; Widjaja, 1987; Wong, 1995a; Wong, 2004). Traditionally *Gigantochloa* have been utilized as bamboo cannons during festive seasons (Wong, 2004) and some *Gigantochloa* produce good quality edible young shoots, e.g., *G. levis* (Holttum, 1958; Widjaja, 1987), *G. latifolia* (Wong, 2004), *G. ligulata* (Holttum, 1958; Wong, 2004), *G. thoii* (Wong, 2004) and *G. wrayi* (Azmy and Razak, 1991).

Gigantochloa has also been used commercially in the poultry cage-making industry, vegetable basket industry, skewer and chopstick industry, and for the manufacturer of other handicrafts (Wong, 2004). The bigger *G. ligulata* and *G. scortechinii* culms are useful for general structural purposes (Holttum, 1958) including as scaffolding of modern building constructions (Wong, 2004).

Meanwhile *Gigantochloa* culms with less thick walls are utilized for constructing walls of houses through a process of splitting and flattening (Holttum, 1958).

Furthermore, *Gigantochloa* with medium-size culm walls that are not too thick (Holttum, 1958) are used for the poultry cage and vegetable basket making industry, e.g., *G. scortechinii* and *G. wrayi* in the state of Kedah and Perak (Wong, 2004). In stick-producing industries, *G. scortechinii* have been employed for the Chinese incense-stick manufacturing, *G. levis* for chopsticks manufacturing and *G. wrayi* used for toothpicks and skewer sticks production (Azmy and Razak, 1991; Wong, 2004). In Kelantan and Kedah, *Gigantochloa* bamboos appears in the handicraft industries, e.g., *G. scortechinii* (Azmy and Razak, 1991; Wong, 2004) and *G. wrayi* (Azmy and Razak, 1991).

Recently there is a growing recognition of *Gigantochloa* in industrial usages as they are very fast-growing (compared to trees), more resistant to insects and fungal infection, considerably durable and rigid (Holttum, 1958; Wong, 1995a; Wong, 2004). It is also an economically important bamboo genus because of certain properties such as large-diameter, long and straight culm portions, variously thick- to medium-walled culms, uniformity in size between the nodes and internodes, and ease of cultivation. All these features make this bamboo genus suitable for industrial and commercial applications (Holttum, 1958; Wong, 1995a; Wong, 2004).

Numerous studies have been conducted on the properties of selected *Gigantochloa species*. The studies on the chemical composition (Wahab, et al., 2013), anatomical properties and microstructures features (Mustafa, et al., 2011) of four cultivated tropical bamboo in *Gigantochloa* genus (*G. brang*, *G. levis*, *G. scortechinii* and *G. wrayi*) showed that the existence of different chemical compositions in the extractives and the ultra-structures of *Gigantochloa* have diverse characteristics compared to wood. The anatomical, physical and chemical properties of *G. scortechinii* from different ages also demonstrated its potential as chewing sticks (Hisham, et al., 2006). Additional investigation on the mechanical characteristics of *G. scortechinii* culm fiber from the Bukit Larang village in Melaka which consists of different thicknesses highlighted that the incorporation of unsaturated polyesters enhances bamboo strip thickness and increases the properties of the middle part of the bamboo strips. These imply that bamboo strips are a viable alternative to composite-based reinforcing fibers and produce excellent mechanical properties (Rassiah, et al., 2014). The thermal stability of *G. scortechinii* was identified by isolation and characterization of cellulose nanofibers (CNF) and it was found that CNF showed reliable and smooth morphological structures, with a higher percentage of crystalinity from raw fibers to cellulose nanofibers and major expansion in thermal stability (Chaturbhuj, et al., 2016).

Furthermore, the moisture content that contributes to the shrinkage of *G. scortechinii* at different heights of culm demonstrated that the nodes shrink faster than internodes and these shrinkage patterns affect the dimensional

strength of bamboo (Anokye, et al., 2014). The other potential use of *G. scortechinii* in the production of structural plywood and urea-formaldehyde particleboards was evaluated based on strength values (strength/density) and mechanical properties (Anwar, et al., 2004). The study recorded stronger strength values for bamboo plywood as compared to commercial plywood (Anwar, et al., 2004) and that the elements of *G. scortechinii* are appropriate for the urea-formaldehyde particleboards production (Kasim, et al., 2001). The bond properties of *G. scortechinii* have also been evaluated and it reveals that various parts of the bamboo culm significantly affect the superiority of the resulting glue bond. Firmer laminated products are made from peripheral strips than those made either from the inner strips or from the combination of both (Zaidon, et al., 2004).

CHAPTER 3

MATERIALS AND METHODS

3.1 Field Collection of Voucher Specimens and Materials for Molecular Work

For phylogenetic relationship and population structure analysis, six to 10 specimens from each population of *G. ligulata, G. scortechinii* and *G. wrayi* were collected across Peninsular Malaysia to represent the range of their natural distribution. Figure 3.1 shows the localities of *Gigantochloa* specimens collected for this study.

Figure 3.1: Localities of *Gigantochloa* specimens collected for this study (Peninsularclipart, 2016).

The specimens were identified based on the morphological descriptions by Wong (1995a). Global Positioning System (GPS) coordinates, name of the sampling locations and the dates of sample collection are listed in Table 3.1.

Table 3.1: Details of *Gigantochloa* specimens collected in the present study for phylogenetic and population structure analyses.

Population	Species	Individual Voucher Number	Collection Localities	Coordinate Range
Kuala Kubu Bharu	*G. scortechinii*	DS5, DS6, DS7, DS8, DS9, DS12, DS13, DS14	Fraser Hill, Kuala Kubu Bharu, Selangor	N 03˚34.101', E 101˚41.073'-N 03˚36.560', E 101˚44.451'
Janda Baik	*G. scortechinii*	DS38, DS39, DS40, DS41, DS42, DS43, DS44, DS45	Bentong Highway, Hutan Lipur Konifer, Janda Baik Roadside Pahang	N 03˚19.824', E 101˚45.574'-N 03˚20.535', E 101˚49.352'
Serendah	*G. scortechinii*	DS15, DS16, DS17, DS18, DS21, DS22, DS23	Kampung Orang Asli and Sekeping Serendah Retreat, Serendah, Selangor	N 03˚21.771', E 101˚37.615'-N 03˚22.178', E 101˚37.853'
Gabai	*G. scortechinii*	DS24, DS25, DS26, DS27, DS28, DS29, DS31, DS33	Gabai Waterfall, Hulu Langat	N 03˚09.770', E 101˚53.804'-N 03˚18.307', E 101˚44.302'
Kelantan	*G. scortechinii*	DS121, DS122, DS123, DS126, DS127, DS128	Gua Musang and Rantau Panjang, Kelantan	N 04˚52.761', E 101˚55.085'-N 05˚59.428', E 101˚57.511'
Kinjang	*G. wrayi*	DS60, DS61, DS62, DS63, DS64, DS65, DS66, DS67 DS68	Hutan Lipur Lata Kinjang, Lata Kinjang, Perak	N 04˚17.068', E 101˚15.275'-N 04˚18.100', E 101˚15.275'
Taiping	*G. wrayi*	DS89, DS90, DS91, DS92, DS93, DS94, DS95, DS96, DS97, DS98	Hutan Lipur Kaki Bukit Larut, Taiping, Perak	N 04˚51.773', E 100˚45.694'-N 04˚51.900', E 100˚45.716'
Sintok	*G. ligulata*	DS102, DS103, DS104, DS105, DS107, DS108, DS109, DS110, DS111, DS112	Sintok, Kedah	N 06˚26.379' , E 100˚26.960'-N 06˚29.414' , E 100˚28.823'
Kinta	*G. ligulata*	DS79, DS80, DS81, DS82, DS83, DS84, DS85, DS86, DS87, DS88	Hutan Lipur Ulu Kinta, Ulu Kinta, Perak	N 04˚40.300', E 101˚11.863'-N 04˚40.356', E 101˚11.847'

Young leaves were preferred for molecular studies. The leaves collected for each species were preserved with silica gel and kept at room temperature in the laboratory for future use. Voucher samples were collected whenever possible following the guidelines by Soderstrom and Young (1983) for the collection of bamboos, i.e., shoots, culm leaf, culms, branch complements, leafy branches and inflorescence. Below are the list of *Gigantochloa* taxa and allied genera used for the phylogenetic analyses, i.e., some were collected and sequenced (Table 3.2) while others were retrieved from GenBank (Table 3.3).

Table 3.2: List of *Gigantochloa* taxa which are collected for phylogenetic analyses.

Taxa	**Voucher number**	**Collectors**	**Localities**	**GPS Coordinates**
Gigantochloa ligulata	GWL30	KM Wong, Prof NH Xia, WL Goh, N. Dhanendiren and Khairul	Western Hill, Penang	N 05˚25.006' E 100˚ 15.513'
Possible hybrid: *G. ligulata* × *G.latifolia*	GWL31	KM Wong, Prof NH Xia, WL Goh, N. Dhanendiren and Khairul	Western Hill, Penang	-
Gigantochloa wrayi	GWL33	KM Wong, Prof NH Xia, WL Goh, N. Dhanendiren and Khairul	Western Hill, Penang	-
Gigantochloa wrayi	GWL34	KM Wong, Prof NH Xia, WL Goh, N. Dhanendiren and Khairul	Western Hill, Penang	-
Gigantochloa ligulata	GWL41	KM Wong, Prof NH Xia, WL Goh, N. Dhanendiren and Khairul	Sintok, Kedah	N 06˚28.550' E 100˚29.291'
Possible hybrid: *Gigantochloa latifolia* × *G. ligulata*	GWL42	KM Wong, Prof NH Xia, WL Goh, N. Dhanendiren and Khairul	Sintok, Kedah	N 06˚24.014' E 100˚19.857'

Table 3.3: DNA sequences retrieved from GenBank.

Taxa	GenBank accession		
	rps16-trnQ	*trnD-T*	*GBBS*I
Bambusa bambos	JN033887 (Goh, et al., 2013)	JN033942 (Goh, et al., 2013)	GU390987 (Goh, et al., 2010)
Dendrocalamus pendulus	HQ697855 (Goh, et al., 2011)	HQ697877 (Goh, et al., 2011)	HQ697890 (Goh, et al., 2011)
Gigantochloa apus	JN033900 (Goh, et al., 2013)	JN033956 (Goh, et al., 2013)	JN034012 (Goh, et al., 2013)
Gigantochloa atter	JN033901 (Goh, et al., 2013)	JN033957 (Goh, et al., 2013)	JN034013 (Goh, et al., 2013)
Gigantochloa balui	FJ416359 (Goh, et al., 2010)	GU390954 (Goh, et al., 2010)	GU390976 (Goh, et al., 2010)
Gigantochloa latifolia	FJ416346 (Goh, et al., 2010)	GU390956 (Goh, et al., 2010)	GU390977 (Goh, et al., 2010)
Holttumochloa magica	FJ416348 (Goh, et al., 2010)	GU390958 (Goh, et al., 2010)	GU390980 (Goh, et al., 2010)
Kinabaluchloa wrayi	JN033903 (Goh, et al., 2013)	JN033959 (Goh, et al., 2013)	JN034015 (Goh, et al., 2013)
Maclurochloa montana	FJ416349 (Goh, et al., 2010)	GU390960 (Goh, et al., 2010)	GU390982 (Goh, et al., 2010)

3.2 Molecular Methods

3.2.1 Total DNA Extraction

Fungus-free, silica gel-dried leaves were used for DNA extraction. Since progressive DNA degradation occurs at the tissues further from the base of leaf blade (Rogers and Bendich, 1994), only the part at the base of the leaf blade was utilized for DNA extraction. The leaves were powdered using a sterilized mortar and pestle. The DNA extractions were done using the conventional protocol from Fulton, et al. (1995) with some modifications. Approximately 0.05 g of dried leaf tissue was ground in a mortar with pestle and the homogenate was incubated at 65 °C for 1 hour and 30 min after which were added 116 µl SDS (Bio Basic, Canada), 5 µl ß-mercaptoethanol (Bio-Rad Laboratories, US), 5 µl proteinase K (Bio Basic Inc, Canada), 300 µl DNA extraction (made-manually) and 300 µl nuclei lysis buffer (made-manually). The DNA pellet was re-suspended in 200–300 µl of distilled water prior to treatment with 600 µl chloroform-isoamyl alcohol (24:1) (Sigma-Aldrich,

USA), 100 µl RNase (Merck KGaA, Germany), 500 µl isopropanol (Bendosen, Malaysia) and 700 µl 70 % ethanol (Scharlab S.L., Spain). DNA quantification was performed by visualizing under Ultraviolet light transilluminator (Syngene, India), after electrophoresis on a 1.0 % agarose gel (First Base Laboratories Sdn. Bhd). Extracted DNA purity and concentrations were recorded by using a nano spectrophotometer, 2000 (Thermo Fisher Scientific, US). The DNA samples were stored at –20 °C for future use.

3.2.2 Polymerase Chain Reaction (PCR) and DNA Sequencing

The primers for the investigation were chosen based on the efficiency of the molecular markers that have been employed in earlier phylogenetic studies. The earlier investigations for Bambusinae utilized chloroplast DNA markers such as *trnL* intron, *atpB-rbcL, rps16* intron and *matK* (Sungkaew et al., 2009), *trnL-F* (Sungkaew et al., 2009; Yang et al., 2008) as well as nuclear markers, for instance ribosomal ITS region (Sun, et al., 2005; Yang, et al., 2008) and *GBSS*I (Yang, et al., 2008). The *Bambusa-Dendrocalamus-Gigantochloa* complex formed few well supported clades in the combined cpDNA-based phylogenetic analysis (*trnL-F*+ *atpB-rbcL*+ *rps16*+ *matK*; Sungkaew, et al., 2009) and combined cpDNA and nuclear DNA phylogenetic analysis (*rps16-trnQ*+ *trnC-rpoB*+ *trnH-psbA* + *trnD-T*+ *GBSS*I; Goh, et al., 2010) while Yang, et al. (2008) showed a higher resolving power of the ITS and *GBSS*I regions compared to the *trnL-F* region. Furthermore, the application of low-copy nuclear DNA sequences such poly-A binding protein1 (*Pabp1*) can provide more informative phylogenetic data, untangle the complications of chloroplast and nuclear genomes and can be used to evaluate the evolutionary

processes of plant speciation (Soltis and Soltis, 1998; Sang, 2002; Wendel and Cronn, 2003). Low-copy nuclear DNA sequences are also have been employed to determine the occurrence of allopolyploidy in plants (Mason-Gamer et al., 1998; Sang, 2002; Spooner, et al., 2008) and resolving the phylogenetic relationships among the Poaceae at inter- and intra-generic levels (Mason-Gamer, et al., 1998; Gorgoni and Gray, 2004; Guo and Ge, 2005; Sun, et al., 2009; Triplett et al. 2010; Estep, et al., 2012; Chokthaweepanich, 2014).

In this study, two cpDNA and two nuclear DNA regions were utilized after considering the cost- and duration-efficiency, the availability of primers sequences and variability level of sequences that potentially provide solutions to complications. The intergenic spacers, *rps16-trnQ* and *trnD-T*, were chosen among the chloroplast DNA markers, based on the suggestion by the Bamboo Phylogeny Group (L.G. Clark, pers. comm.), since these informative markers exhibited high levels of variability among bamboos in lower taxonomic studies. For the nuclear DNA part, *GBBS*I region and *Pabp1* were selected as they have proven valuable for phylogenetic investigations (Mason-Gamer, et al., 1998; Gorgoni and Gray, 2004; Guo and Ge, 2005; Yang, et al., 2007; Yang, et al., 2008; Sun, et al., 2009; Yang, 2010; Goh, et al., 2010; Triplett, et al. 2012; Estep, et al., 2012; Goh, et al., 2013; Chokthaweepanich, 2014). The PCR primers used in this study are shown in Table 3.4.

Table 3.4: PCR primers used in this study.

DNA region	Primer	Forward/ Reverse	Sequence (5' - 3')	References
rps16-trnQ (partial 1-800bp)	16Q1_F	Forward	GCA CGT TGC TTT CTA CCA CA	Bamboo Phylogeny Group, 2005
	16Q2_R	Reverse	ATC CTT CCG TCC CAG ATT TT	Bamboo Phylogeny Group, 2005
trnD-T (partial 1-800bp)	DT1_F	Forward	ACC AAT TGA ACT ACA ATC CC	Bamboo Phylogeny Group, 2005
	DT2_R	Reverse	CCC TTT TAA CTC AGT GGT A	Bamboo Phylogeny Group, 2005
Partial nuclear *Pabp1* gene (partial 1-600bp)	*Pabp1*_all	Forward	TTG TGC AGG CTA HRW AAG TTG C	Chokthaweepanich, 2014
	*Pabp1*_R	Reverse	GTG TTA GCA AAG GGT CTG GAT TT	Chokthaweepanich, 2014
*Partial nuclear GBSS*1 gene	*GIN*_F	Forward	AAG TTT GAG CGC ATG TTC CAG AGC	Goh, et al., 2010
	*GBSS*_R	Reverse	GGC GAG CGG CGC GAT CCC TCG CC	Mason-Gamer, et al., 1998

For each sampled individual, the chosen non-coding cpDNA intergenic spacer's (IGS) (*rps16-trnQ, trnD-T*) and selected nuclear DNA regions (*GBBS*I and *PabpI*) were amplified using the selected primers (Table 3.4). The isolated DNA was amplified in a reaction solution containing approximately 50 ng total DNA, 25 µl of Promega 2X GoTaq Green Master Mix-2X (Thermo Fisher Scientific), 0.5 µM of both forward and reverse primers and topped up to 50 µl using nuclease-free distilled water. Amplification was achieved in a MyCycler thermal cycler (BioRad) programmed for a preliminary 2 min denaturation step at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 secs, annealing at 55 °C for 45 secs and extension at 72 °C for 1 min 30 sec, with a final extension step at 72 °C for 2 min. The annealing temperature was obtained using the average melting temperature of both the forward and reverse primers. Amplification products were separated alongside a molecular weight marker (GeneRuler 1 kb DNA Ladder) by electrophoresis on 1.2 % agarose gels run in 0.5× TAE (Tris Acetate EDTA) buffer, stained with ethidium bromide and visualized under UV light. Gel

photographs were scanned through GeneSnap (SynGene). Selected PCR products were purified using the Geneaid Gel/PCR DNA Fragments Extraction Kit (Axon Scientific Sdn. Bhd.) following the manufacturer's protocol (Appendix A) and were sequenced by First Base Laboratories Sdn. Bhd. (Malaysia). All the outcome sequences from the DNA sequencing were deposited in GenBank.

3.2.3 PCR-RFLP based on Chloroplast DNA

The amplification of the multiple regions of chloroplast DNA in the previous phylogenetic analysis of *Bambusinae* subtribe revealed the existence of two different haplotypes among the *Gigantochloa scortechinii* samples which fall into the BDG1 and BDG2 subclades, while all the *Gigantochloa* taxa form homogeneous clade (Subclade G) for nuclear DNA data (Goh, et al., 2010; Goh, et al., 2013). The study postulates the possibility of introgressive hybridization contributing to the discordance to the conflict between the chloroplast DNA and nuclear DNA topologies (Goh, et al., 2010; Goh, et al., 2013). Therefore, it is necessary for the chloroplast DNA differentiation in *Gigantochloa scortechinii* to be further investigated to determine the possible causes of the existence of two haplotypes and the significance of introgressive hybridization in the chloroplast DNA evolution. For the present study, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) was utilized as it is rapid and yet effective for large scale screening in detecting single nucleotide polymorphisms (SNPs). The cpDNA *rps16-trnQ* of two individuals from GenBank (accession numbers HQ697864.1 and HQ697861.1, hereafter termed as Gombak and Langat,

respectively), representing subclades BDG1 and BDG2 that employed in the previous studies (Goh, et al., 2010; Goh, et al., 2013), were retrieved from GenBank and aligned to recognize their SNPs. The NEB cutter 1.0 (Vincze, et al., 2003) was used to search for the restriction enzymes that cut at the SNPs. The expected virtual digested band profiles using the selected restriction enzymes were screened for their usefulness in differentiating the two subclades, BDG 1 and BDG 2. The *Apo*I restriction enzyme were chosen for the present RFLP screening as could distinguish the two subclades of the amplified *rps16-trnQ* chloroplast regions. The samples from four selected geographical locations (Table 4.2) were subjected for restriction enzyme analysis using the *Apo*I restriction enzyme following the manufacturer's protocols. Gel electrophoresis was conducted at 100 V for 25 min to view the RFLP profile in a 2 % agarose gel. The chloroplast *rps16-trnQ* and *trnD-T* (Bamboo Phylogeny Group (BPG), 2005) regions of DS24, DS32 and DS36 were commercially sequenced for evaluation purposes with the reported sequences of three other *Gigantochloa scortechinii* specimens, namely Gombak, Langat and Acc.52, (HQ697864, HQ697861, HQ697862, HQ697886, HQ697883 and HQ697884; Goh, et al., 2011), the last from the living collection in Rimba Ilmu Botanic Gardens, University of Malaya and which was found to be of the Langat-type (Goh, et al., 2011). The aligned data matrix was imported into PAUP 4.0 (Swofford, 2001) for UPGMA phylogram reconstruction with *Kinabaluchloa nebulosa* (FJ416360 and GU390959) and *Holttumochloa magica* (FJ416348 and GU390958) as outgroups.

3.3 DNA Data Analysis

3.3.1 DNA Sequence Alignment and Character Coding

The commercially obtained DNA sequences were examined carefully with Chromas 2.4.4 (Technelysium, 1998) for the identification of noisy signals and overlapping peaks. The hybrid origins and heterogeneity in the chosen nuclear DNA regions (*GBSS*I and *Pabp1* gene) were identified based on the noisy signals and overlapping peaks in the DNA chromatogram (Appendix B). The overlapping peaks or noisy signals were termed as dimorphic characters, whereas the mono- and di-nucleotide repeats of undefined length were not included in the data matrix as stated in Goh, et al. (2013). Some of the chloroplast and nuclear DNA regions data involved merging the forward and reverse sequencing and excluding the noisy signals in the middle as ambiguous data. The sequences were then completely aligned using Clustal X v2.1 (Larkin, et al., 2007). BioEdit Sequence Alignment Editor v7.2.5 software (Hall, 1999) was utilized for manual edition of the DNA data matrix to remove missing and ambiguous data caused by sequencing errors. The gap in the DNA sequences were coded as "missing" data whereas the indels coded as additional informative characters.

3.3.2 Haplotype Analysis of Chloroplast DNA

The corrected combined chloroplast DNA regions (*rps16-trnQ* + *trnD-T*) data matrix were used for the population haplotypes diversity data generation using DnaSP v5 (Librado and Rozas, 2009) where alignment gaps were not considered and the invariable sites were removed. A haplotype table (Table

4.4) was build based on the *Gigantochloa* species and location of the specimens.

3.3.3 Sequence Characteristics of Chloroplast DNA and Nuclear DNA

MEGA v5.2 (Tamura, et al., 2011) was used to obtained parsimony informative characters (PIC) and to construct the indels and variable table for chloroplast and nuclear DNA data matrix. Indel and variable sites outcomes were arranged according to the clades in phylogenetic tree topologies. The indels and variable sites were useful information to distinguish the chloroplast DNA haplotypes and to determine the parents of *Gigantochloa* hybrids by arranging the aligned sequences. For the nuclear DNA dataset, the overlapping or dimorphic sites that was initially stated in International Union of Pure and Applied Chemistry (IUPAC) nucleotide code were replaced with its respective bases: R = A / G; Y = C / T; S = G / C; W = A / T; K = T / G; M = A / C (Excoffier and Lischer, 2010).

3.3.4 PCR Molecular Cloning for Nuclear DNA

Purified PCR products for the partial *GBSS*I and *PabpI* gene of the suspected hybrid individuals were ligated into *pDrive* vectors and transformed into EZ competent cells following the instructions of the Qiagen PCR Cloning Plus kit. The protocols were provided in Appendices part (Appendix C). White colonies were picked to perform colony-PCR using specific primers of previous studies (Mason-Gamer, et al., 1998; Goh, et al., 2010; Chokthaweepanich, 2014), *Gin* (forward) and *GBSS* (reverse); *Pabp*_F1 (Forward) and *Pabp*_all (Reverse). Four to six clones of each hybrid colony

were effectively amplified and sequenced. Direct sequencing of the purified PCR products was commercially done by First Base Laboratory Sdn. Bhd. (Malaysia). The sequences of all clones were aligned.

3.3.5 Phylogenetic Analysis

The generated haplotypes of chloroplast DNA and nuclear DNA data sequences were rechecked for gap code errors and reformatted as input files of MrBayes and PAUP by using FastGap v1.2 (Borchsenius, 2009). The outcome data matrix file was re-edited with command orders as required by MrBayes v3.1.2 (Huelsenback and Ronquist, 2001) and PAUP 4.0 b10 (Swofford, 2002) for the phylogenetic analysis. Bayesian Inference (BI) analyses were performed in MrBayes v3.1.2 (Huelsenback and Ronquist, 2001), using 2 runs of 4 chains each, and run for 10 million generations with trees sampled every 1000 generations for the combined chloroplast DNA dataset (Appendix D). The first 25 % of the sampled trees were discarded as burn-in. Based on the previous empirical studies, posterior probabilities (PP) more than 0.95 is indicated as a strongly-supported and preferred values (Taylor and Piel, 2004) but in the present study, posterior probabilities (PP) more than 80 % was chosen as the support measure value. Maximum parsimony (MP) analysis was executed using PAUP 4.0 b10 (Swofford, 2002) for combined chloroplast DNA and nuclear DNA data matrix (Appendix E). A strict consensus tree was reconstructed using heuristic search with 100 random sequence additions and tree bisection reconnection (TBR) branch swapping. ‘MulTrees’ was limited to 10,000 trees and dimorphic sites identified in the *GBSS*I and *Pabp1* sequences were coded as "polymorph" (polymorphic) in the MP analyses.

Bootstrap proportion (BP) more than 50 % was considered as preferred values in this study. The suitable outgroups were recognized based on the in-group's genetic proximity, phylogenetic proximity and strong association of base constitutions (Rota-Stabelli and Telford, 2008). Thus, chloroplast and nuclear DNA topologies were rooted using *Kinabaluchloa wrayi* and *Holttumochloa magica* as outgroups because these species were constantly resolved 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; Goh, et al., 2013).

3.3.6 Population Genetic Structure Analysis based on Chloroplast DNA

The genetic variation within and among *Gigantochloa* populations were determined by analysis of molecular variance (AMOVA) using ARLEQUIN 3.5.1 (Excoffier and Lischer, 2010). MEGA v5.2 (Tamura, et al., 2011) was employed to convert the haplotypes of chloroplast DNA dataset files to the input file mode as required by the ARLEQUIN v3.5.2 (Excoffier and Lischer, 2010) software. Two hypothesis of populations structures based on *Gigantochloa* species and geographical distribution were assessed for the AMOVA analysis. ARLEQUIN v3.5.2 (Excoffier and Lischer, 2010) was also used to assess the pairwise fixation indices (F_{ST}) for genetic distance. F_{ST} is the proportion of the total genetic variance comprised in a subpopulation relative to the total genetic variance (Wrights, 1965). The values can range from 0.0 (presence of shared allelic constitution in a pair populations) to 1.0 (fixed single distinctive allele in each population) (Wrights, 1965). High F_{ST} indicates a significant differentiation level among groups (Wrights, 1965). Parameters such as population comparisons, population differentiation,

linkage disequilibrium (pairwise linkage) and molecular diversities indices were included throughout the AMOVA and F_{ST} evaluations.

3.3.7 Neighbor Network Analysis

Reticulate evolution cannot be reconstructed linearly through distance- or parsimony-based tree-building approaches (Vriesendorp and Bakker, 2005). Thus, alternative neighbor-net analysis was conducted in SplitsTree4 v4.14.4 (Huson and Bryant, 2006) using the haplotypes of chloroplast DNA and nuclear DNA sequences to explore the relationships among and within the three species including the possible hybrids. Neighbor Network Analysis is a set of phylogenetic network techniques that produced networks directly from distance matrices.

CHAPTER 4

RESULTS

4.1 RFLP Profiling for Selected *Gigantochloa scortechinii* Populations

4.1.1 RFLP Marker Selection

NEB cutter 1.0 was used to screen suitable restriction enzymes and exhibited that *Mluc*I, *Apo*I, *Ags*I and *Mse*I were able to differentiate the two chloroplast DNA (cpDNA) haplotypes of *Gigantochloa scortechinii* shown in Figure 4.1 and Table 4.1. From the virtual restriction digestion, *Mluc*I and *Mse*I were identified to yield too much unnecessary bands that may lead to ambiguity in band scoring. Virtual digestion of *Apo*I restriction enzyme exhibited banding profiles of 100 bp, 127 bp, 156 bp, 261 bp, and 370 bp for Langat, while 9 bp, 156 bp, 233 bp, 253 bp and 370 bp were detected for Gombak. These banding models were utilized to represent the two cpDNA genotypes of *G. scortechinii*.

Table 4.1: Summary of the restriction enzymes and their respective cut sites that can produce different RFLP profiles for Langat and Gombak.

Restriction enzyme	**Langat**		**Gombak**	
	Number of cut site	Expected band size, bp	Number of cut site	Expected band size, bp
***Mluc*I**	17	4, 5, 8, 14, 25, 27, 36, 41, 62, 63, 67, 76, 78, 89, 90, 100, 225	18	4, 5, 6, 9, 15, 25, 26, 36, 44, 62, 63, 67, 76, 78, 89, 90, 99, 225
***Apo*I**	4	100, 127, 156, 261, 370	4	9, 156, 233, 253, 370
***Ags*I**	4	100, 130, 161, 206, 417	5	100, 104, 108, 133, 162, 417
***Mse*I**	10	4, 5, 7, 31, 54, 63, 123, 134, 152, 179, 262	9	5, 7, 31, 61, 63, 126, 134, 152, 180, 262

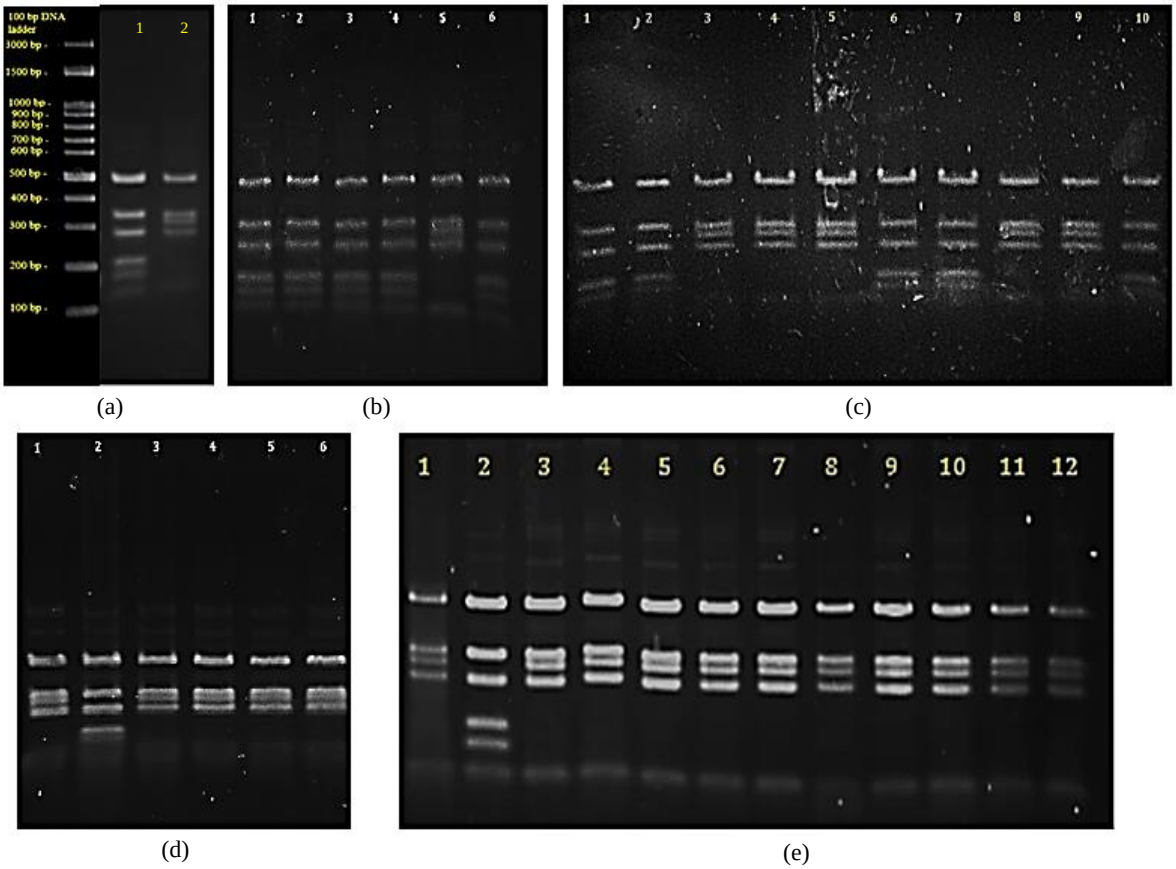

Figure 4.1: PCR-RFLP profiles of **(a)** Langat (lane 1) and Gombak (lane 2), **(b)** DS4 – DS10 (lanes 1 – 6), **(c)** DS11 – DS20 (lanes 1 – 10), **(d)** DS21 – DS26 (lanes 1 – 6), **(e)** DS29, DS32, DS33, DS34, DS36, DS38, DS39, DS40, DS41, DS42, DS43 and DS44 (lanes 1 – 12). Lane L indicates 100 bp DNA ladder (GeneDireX® H3 RTU).

4.1.2 RFLP Profiling Using *Apo*I based on the Chloroplast *rps16-trnQ* Region

The PCR amplification of the chloroplast *rps16-trnQ* region produced a visible band of 1.2 kb. Two different banding patterns were shown by Langat individuals referring to Langat-type, and those by the Gombak individuals referring to Gombak-type, when the *Apo*I restriction enzyme digested the chloroplast *rps16-trnQ* region. The Gombak-type revealed three bands between 200–300 bp, and none between 100–200 bp. Meanwhile, the Langat-type revealed two bands between 200–300 bp, and two bands between 100–200 bp (Figure 4.1). The band sizes appeared to be slightly different from those expected using the NEB cutter 1.0 because the virtual restriction digestion was executed using trimmed DNA dataset. Eight out of 11 individuals (72.7 %) from Kuala Kubu Bharu and four out of nine individuals (44.4 %) from Serendah were recognized to be of the Langat-type. Among the six individuals collected from Sungai Gabai Waterfall zones (within the district of Hulu Langat in Selangor), only DS32 shows the Langat-type banding pattern. All the individuals collected from Janda Baik village are of the Gombak-type (Table 4.2 and Figure 4.1). All the resulting bands were different and precise.

Table 4.2: *Gigantochloa scortechinii* specimens collected for this study and their chloroplast DNA types. The grey-shaded specimens are Langat-type while the non-shaded ones are Gombak-type.

Population	Collection Locality (Date)	Collection Number	GPS Coordinates
1	Road from Kuala Kubu Baru to Fraser Hill, Selangor (24 Dec 2014)	DS4	N 03˚34.101'; E 101˚41.073'
		DS5	N 03˚37.242'; E 101˚38.133'
		DS6	N 03˚34.145'; E 101˚41.163'
		DS7	N 03˚34.148'; E 101˚41.169'
		DS8	N 03˚35.561'; E 101˚44.098'
		DS9	N 03˚35.579'; E 101˚44.126'
		DS10	N 03˚36.047'; E 101˚44.269'
		DS11	N 03˚36.048'; E 101˚44.263'
		DS12	N 03˚36.399'; E 101˚44.416'
		DS13	N 03˚36.562'; E 101˚44.446'
		DS14	N 03˚36.560'; E 101˚44.451'
2	Serendah, Selangor (24 Dec 2014)	DS15	N 03˚21.967'; E 101˚37.710'
		DS16	N 03˚21.999'; E 101˚37.734'
		DS17	N 03˚21.990'; E 101˚37.716'
		DS18	N 03˚21.971'; E 101˚37.715'
		DS19	N 03˚21.871'; E 101˚37.671'
		DS20	N 03˚21.771'; E 101˚37.615'
		DS21	N 03˚22.177'; E 101˚37.862'
		DS22	N 03˚22.178'; E 101˚37.853'
		DS23	N 03˚21.994'; E 101˚36.743'
3	Gabai Waterfall, Selangor (1 Jan 2015)	DS24	N 03˚10.003'; E 101˚54.581'
		DS25	N 03˚09.996'; E 101˚54.570'
		DS26	N 03˚10.000'; E 101˚54.543'
		DS29	N 03˚09.982'; E 101˚54.509'
		DS32	N 03˚10.186'; E 101˚52.417'
		DS33	N 03˚18.307'; E 101˚44.302'
4	Janda Baik, Pahang (2 Jan 2015)	DS34	N 03˚19.873'; E 101˚45.604'
		DS36	N 03˚20.512'; E 101˚49.369'
		DS38	N 03˚20.411'; E 101˚49.461'
		DS39	N 03˚20.346'; E 101˚49.535'
		DS40	N 03˚20.132'; E 101˚49.692'
		DS41	N 03˚20.116'; E 101˚49.697'
		DS42	N 03˚20.209'; E 101˚49.648'
		DS43	N 03˚20.176'; E 101˚49.639'
		DS44	N 03˚20.206'; E 101˚49.645'

4.1.3 SNPs and Indels

The aligned DNA sequence of *rps16-trnQ* and *trnD-T* comprises 999 and 1,101 characters, respectively. The uniqueness between the Langat- and Gombak types was observed at the variable sites of 12, 356, 637, 799 and 930, as well as the 7 bp-indels (382–388) and the 20-bp indels (977–996) (Table 4.3). However, the 9 bp-insertion (at sites 748–756) in DS24 was identified to correspond to the Langat-type individuals (Table 4.3). The DNA sequence data of the *trnD-T* region and the plotted UPGMA phylogram based on the combined DNA data matrices further reinforced the distinctiveness of the Langat- and Gombak-types (Figure 4.2).

Table 4.3: Variable sites and the indels extracted from the *rps16-trnQ* and the *trnD-T* data matrices which comprise 999 characters and 1,101 characters, respectively. Dash indicates gap. The individuals of Gombak-type were boldfaced.

Accession	**Site number**									
	rps16-trnQ						*trnD-T*			
	12	353	356	382 - 388	637	679	748 - 756	799	930	977 – 996
DS24	–	–	G	A T T A G A A	A	T	A T A A G A A T A	T	A	– – – – – – – – – – – – – – – – – – – –
DS33	–	–	G	A T T A G A A	A	T	– – – – – – – – –	T	A	– – – – – – – – – – – – – – – – – – – –
DS36	–	–	G	A T T A G A A	A	T	– – – – – – – – –	T	A	– – – – – – – – – – – – – – – – – – – –
Gombak	–	–	G	A T T A G A A	A	T	– – – – – – – – –	T	A	– – – – – – – – – – – – – – – – – – – –
DS32	A	A	T	– – – – – – –	G	–	A T A A G A A T A	A	G	A A G G T G A A T A A A T A A T A A A T
Acc.52	A	–	T	– – – – – – –	G	–	A T A A G A A T A	A	G	A A G G T G A A T A A A T A A T A A A T
Langat	A	A	T	– – – – – – –	G	–	A T A A G A A T A	A	G	A A G G T G A A T A A A T A A T A A A T

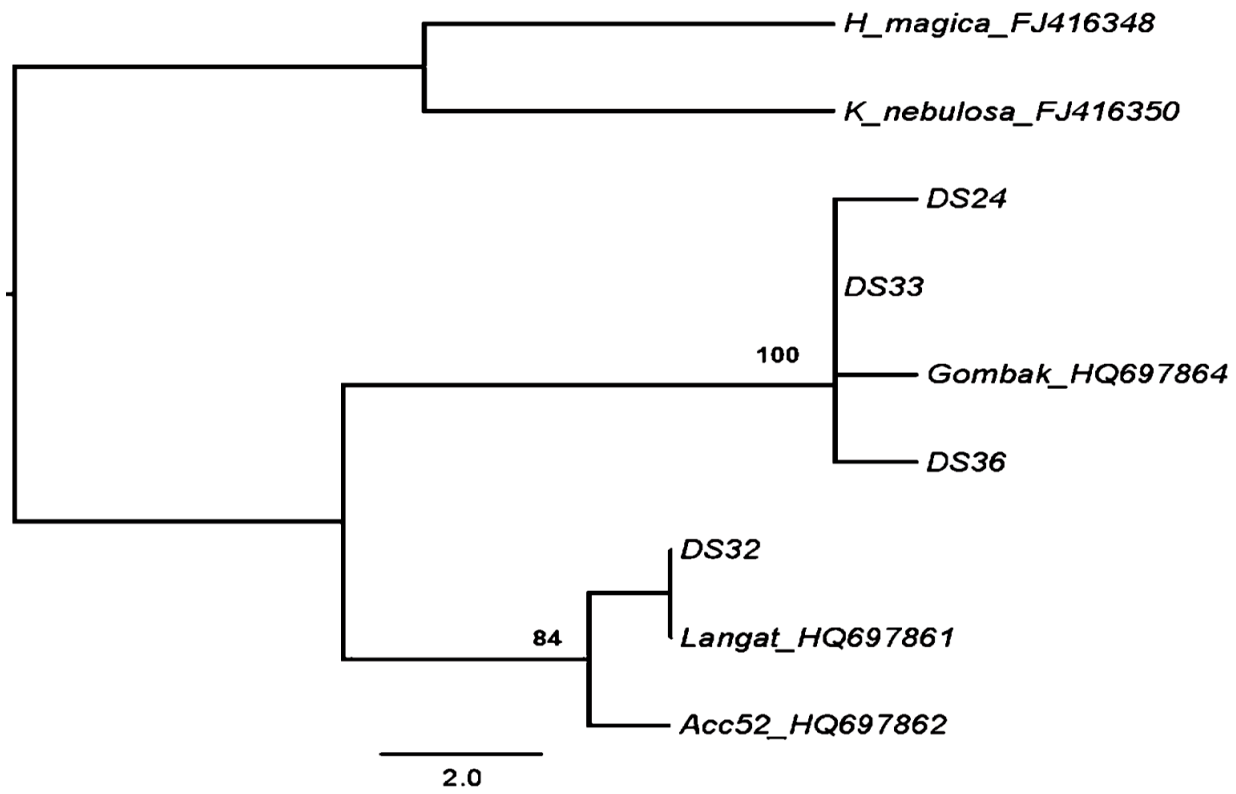

Figure 4.2: UPGMA phylogram reconstructed for DS24, DS32, DS33, DS36, Acc.5, Gombak and Langat based on the combined *rps16-trnQ + trnD-T* region. *Kinabaluchloa nebulosa* and *Holttumochloa magica* were used as outgroups.

4.2 Chloroplast DNA Haplotypes

Table 4.4 displays the chloroplast haplotypes yielded from the combined DNA sequences data of chloroplast intergenic spacers, *rps16-trnQ* and *trnD-T* (1548 bp) of the *Gigantochloa* species. In this study, DnaSP.v5 allowed the identification of 32 distinct haplotypes from the 82 individuals of the combined cpDNA regions. Out of 32 haplotypes, seven haplotypes (Hap1, Hap2, Hap3, Hap4, Hap7, Hap10 and Hap12) were found to be of the Langat-type while the remaining ones were identified to be of the Gombak-type (Table 4.4). Based on the haplotypes list (Table 4.4), it was detected that different haplotypes have different frequencies. Haplotypes 5, which includes 34 individuals (39.5 % out of all investigated individuals), are present in all three *Gigantochloa* species over most of the geographical regions. Haplotype 15 (12.2 %) and haplotype 18 (2.4 %) were found to be present in both *Gigantochloa ligulata* and *Gigantochloa wrayi* individuals. Meanwhile, all the other haplotypes were geographically restricted to only one of the *Gigantochloa* species.

Table 4.4: Haplotypes of the cpDNA intergenic spacer, (1,548 bp) [*rps16-trnQ* (1- 470 bp) + *trnD-T* (471-1,548 bp)] of the *Gigantochloa* species. Haplotype labels in boldfaced.

cpDNA Haplotype	Species	KKB	Janda Baik	Serendah	Gabai	Kelantan	Kinjang	Taiping	Sintok	Kinta	Total
Hap1	*G. scortechinii*	DS5	-	-	-	-	-	-	-	-	**1**
Hap2	*G. scortechinii*	DS6	-	-	-	-	-	-	-	-	**1**
Hap3	*G. scortechinii*	**(DS7)**	-	-	-	-	-	-	-	-	**1**
Hap4	*G. scortechinii*	DS8	-	-	-	-	-	-	-	-	**1**
Hap5	*G. scortechinii*	DS9 DS13 DS14	DS39	DS15 DS18 (**DS21)** DS23	DS24 **(DS25)** DS26 DS27 DS28 DS31 DS33	DS122 DS123 DS126 DS127	-	-	-	-	**32**
	G. wrayi	-	-	-	-	-	DS62 DS64	-	-	-	
	G. ligulata	-	-	-	-	-	-	-	DS104 DS111	DS79 DS81 DS82 DS83 DS84 DS85 DS86 DS87 **(DS88)**	
Hap6	*G. scortechinii*	DS12	-	-	-	-	-	-	-	-	**1**
Hap7	*G. scortechinii*	-	-	**(DS16**) DS17 DS22	-	-	-	-	-	-	**3**
Hap13		-	-		-	-		DS89	-	-	**1**
Hap14	*G. wrayi*	-	-		-	-	DS60 DS67	DS90 **(DS92)**	-	-	**4**

*****Grey-shaded labels (Hap1, Hap2, Hap3, Hap4, Hap7, Hap10 and Hap12) indicate the Langat-type while the dark blue-shaded ones indicate the Gombak-type. Specimens selected for nuclear DNA analyses were in parentheses.

Table 4.4 (Cont'd):

cpDNA Haplotype	Species	KKB	Janda Baik	Serendah	Gabai	Kelantan	Kinjang	Taiping	Sintok	Kinta	Total
Hap15	*G. wrayi*	-	-	-	-	-	DS61 DS65 DS68	**(DS91**) DS93 DS94 (**DS95)** DS98	-	-	**10**
	G. ligulata	-	-	-	-	-	-	-	DS102 DS103	-	
Hap16	*G. wrayi*	-	-	-	-	-	-	DS96	-	-	**1**
Hap17	*G. wrayi*	-	-	-	-	-	-	DS97	-	-	**1**
Hap18	*G. wrayi*	-	-	-	-	-	DS63	-	-	-	**2**
	G. ligulata	-	-	-	-	-		-	-	DS80	
Hap19	*G. wrayi*	-	-	-	-	-	DS66	-	-	-	**1**
Hap20	*G. ligulata*	-	-	-	-	-	-	-	DS105	-	**1**
Hap21	*G. ligulata*	-	-	-	-	-	-	-	DS107	-	**1**
Hap22	*G. ligulata*	-	-	-	-	-	-	-	DS108 DS109	-	**2**
Hap23	*G. ligulata*	-	-	-	-	-	-	-	DS110	-	**1**
Hap24	*G. ligulata*	-	-	-	-	-	-	-	**(DS112)**	-	**1**
Hap26	*G. scortechinii*	-	DS38	-	-	-	-	-	-	-	**1**
Hap27	*G. scortechinii*	-	DS40	-	-	-	-	-	-	-	**1**

*Grey-shaded labels (Hap1, Hap2, Hap3, Hap4, Hap7, Hap10 and Hap12) indicate the Langat-type while the dark blue-shaded ones indicate the Gombak-type. Specimens selected for nuclear DNA analyses were in parentheses.

Table 4.4 (Cont'd):

cpDNA Haplotype	Species	KKB	Janda Baik	Serendah	Gabai	Kelantan	Kinjang	Taiping	Sintok	Kinta	Total
Hap28	*G. scortechinii*	-	DS41	-	-	-	-	-	-	-	**1**
Hap29	*G. scortechinii*	-	DS42 DS43 DS44 DS45	-	-	-	-	-	-	-	**4**
Hap30	*G. scortechinii*	-	-	-	DS29	-	-	-	-	-	**1**
Hap31	*G. scortechinii*	-	-	-	-	DS121	-	-	-	-	**1**
Hap32	*G. scortechinii*	-	-	-	-	DS128	-	-	-	-	**1**
TOTAL		**8**	**8**	**7**	**8**	**6**	**9**	**10**	**10**	**10**	**76**

*Grey-shaded labels (Hap1, Hap2, Hap3, Hap4, Hap7, Hap10 and Hap12) indicate the Langat-type while the dark blue-shaded ones indicate the Gombak-type. Specimens selected for nuclear DNA analyses were in parentheses.

*cpDNA haplotypes for the specimens included in the cpDNA phylogenetic analyses but excluded from the population structure analyses:

Hap8 (Penang) – (DS114) *G. wrayi*
Hap9 (Penang) – (DS115) *G. wrayi*
Hap10 (Penang) **– (**DS117) Possible Hybrid No. 1 (possible *G. ligulata* collected at highest elevation in the record, floppy culms)
Hap11 (Penang Botanical Garden) – DS118 *G. wrayi*
Hap12 (Penang Botanical Garden) - DS119 *G. ligulata*
Hap25 (Sintok) – (DS120) Possible Hybrid No. 2 (possible *G. latifolia* × *G. ligulata* with culms broadly arched over and with lacerate ligules)

4.3 Phylogenetic Analyses

Table 4.5 shows the tree statistics for Maximum Parsimony analyses among the ingroups based on individual and combined data of chloroplast and nuclear DNA. The *rps16-trnQ* cpDNA data matrices (527 bp) were trimmed to 470 characters after adding *trnD-T* data matrix (1,079 bp) and other several related genera sequences from GenBank. The number of indels, variable characters and parsimony informative characters of the Maximum Parsimony (MP) analysis for combined cpDNA regions (*rps16-trnQ* + *trnD-T*) are shown in Table 4.5. MP analysis of the combined cpDNA dataset (1,548 bp) generated 26 parsimonious trees. Of these, 46 characters were variable and 19 of them were parsimony-informative.

Meanwhile, the *PabpI* data matrix (480 bp) was trimmed to 401 characters after the addition of the *GBBS*I data matrix (681 bp) and other several related genera sequences from GenBank. The number of indels, variable characters and parsimony informative characters of the MP analysis for combined nuclear DNA regions (*GBSS*I + *PabpI*) are shown in Table 4.5. Of these, 76 characters were variable and 39 of them were parsimony-informative.

Since the Maximum Parsimony (MP) parsimonious tree topologies were largely consistent with those of the Bayesian Inference (BI) analyses, bootstrap support (BS) values higher than 50 % were mapped onto the phylogenetic trees. Posterior probabilities (PP) of BI higher than 0.80 were also included in the phylogenetic trees. *Holttumochloa magica* and *Kinabaluchloa wrayi* were used as outgroups in the phylogenetic analysis partly due to their sister relationship to the *Bambusa-Dendrocalamus-Gigantochloa* complex (BDG complex) as reported by Goh, et al. (2013).

Table 4.5: Tree statistics for Maximum Parsimony analyses among the ingroups based on individual and combined data.

Dataset	DNA characters	Indel characters	Total characters	Variable characters	Parsimony-informative characters, PIC (number/%)	MP tree length
cpDNA: *rps16-trnQ + trnD-T*	1470	78	1548	46	19/1.23	56
Nuclear DNA: (i) *GBSS*I	640	41	681	49	27/3.96	31
(ii) *PabpI*	476	4	480	39	13/2.70	35
(iii) *GBBS*I *+PabpI*	1041	42	1083	76	39/3.60	71

4.3.1 Chloroplast DNA (*rps16-trnQ + trnD-T*)

The phylogenetic analysis based on the combined cpDNA regions dataset resolved cpDNA sequences into two major clades (Figure 4.3), i.e., Clade 1 (0.82 PP/ - BP), consisting of members of Gombak-type haplotype with three subgroups (Hap 6 and 11; Hap 16 and 19; Hap 21, 22 and 23), *Bambusa bambos, Dendrocalamus pendulus, G. balui, G. latifolia* ; and Clade 2 (0.93 PP/54 BP), consisting of Langat-type haplotype minor subgroup (Hap 1 and Hap 4), *G. apus* , *G. atter* , *G. manggang, Maclurochloa montana*. *Dendrocalamus strictus* was unresolved in cpDNA topologies.

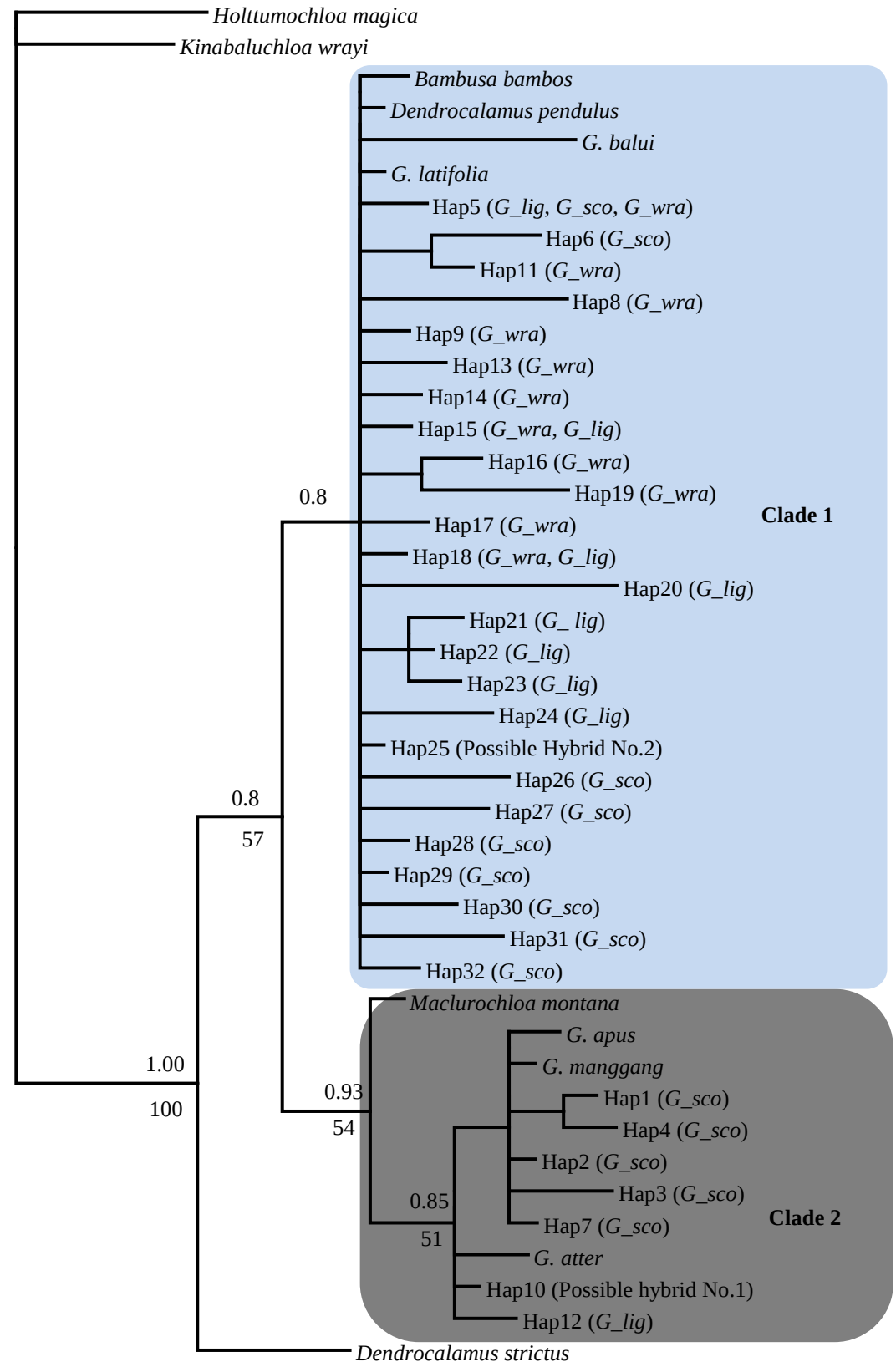

Figure 4.3: Bayesian tree based on the combined chloroplast *rps16-trnQ* and *trnD-T* dataset, rooted with *Holttumochloa magica* and *Kinabaluchloa wrayi* as outgroups. Upper nodal figures represent support values for Bayesian inference posterior probability (0.80 and above) and lower nodal figures shows are the bootstrap values (50 % and above) in maximum parsimony analysis. Parsimony-informative sites among the ingroups are 19/1548.The grey-shaded haplotypes are Langat-type (Hap1, Hap2, Hap3, Hap4, Hap7, Hap10 and Hap12) while the dark blue-shaded ones are Gombak-type.

4.3.2 Nuclear DNA (*GBSSI* + *PabpI*)

The phylogenetic analysis based on the combined regions of the nuclear DNA dataset resolved nuclear sequences into three major clades (Figure 4.4), i.e., Clade 1 (0.97 PP/ - BP) consisting of members of *Dendracalamus pendulus, Dendrocalamus strictus* and a possible hybrid clone, DS120 clone B; Clade 2 (1.00 PP/ 95 BP), comprising *Mullerochloa montana* and a possible hybrid clone, DS117 clone B ; and Clade 3 with high support (1.00 PP/ 100 BP), consisting of *G. balui*, *G. latifolia*, *G. manggang*, and two possible hybrid clones (DS117 clone A and DS120 clone A) and Gombak- and Langat-type *Gigantochloa* species. Consistent with previous studies (Goh, et al., 2013), the type species of *Gigantochloa*, *G. atter*, was not resolved into the clade where other *Gigantochloa* reside (i.e., Clade 3).

Within Clade 3, there was no clear species delination except for *Gigantochloa scortechinii* (0.91 / - BP). One of the clones of DS120 was clustered with the typical *Gigantochloa* clade and another clone clustered with the *Dendrocalamus* clade (with moderate support). The unresolved *Dendrocalamus strictus* in the cpDNA phylogenetic tree, was well recovered in the nuclear DNA phylogenetic tree.

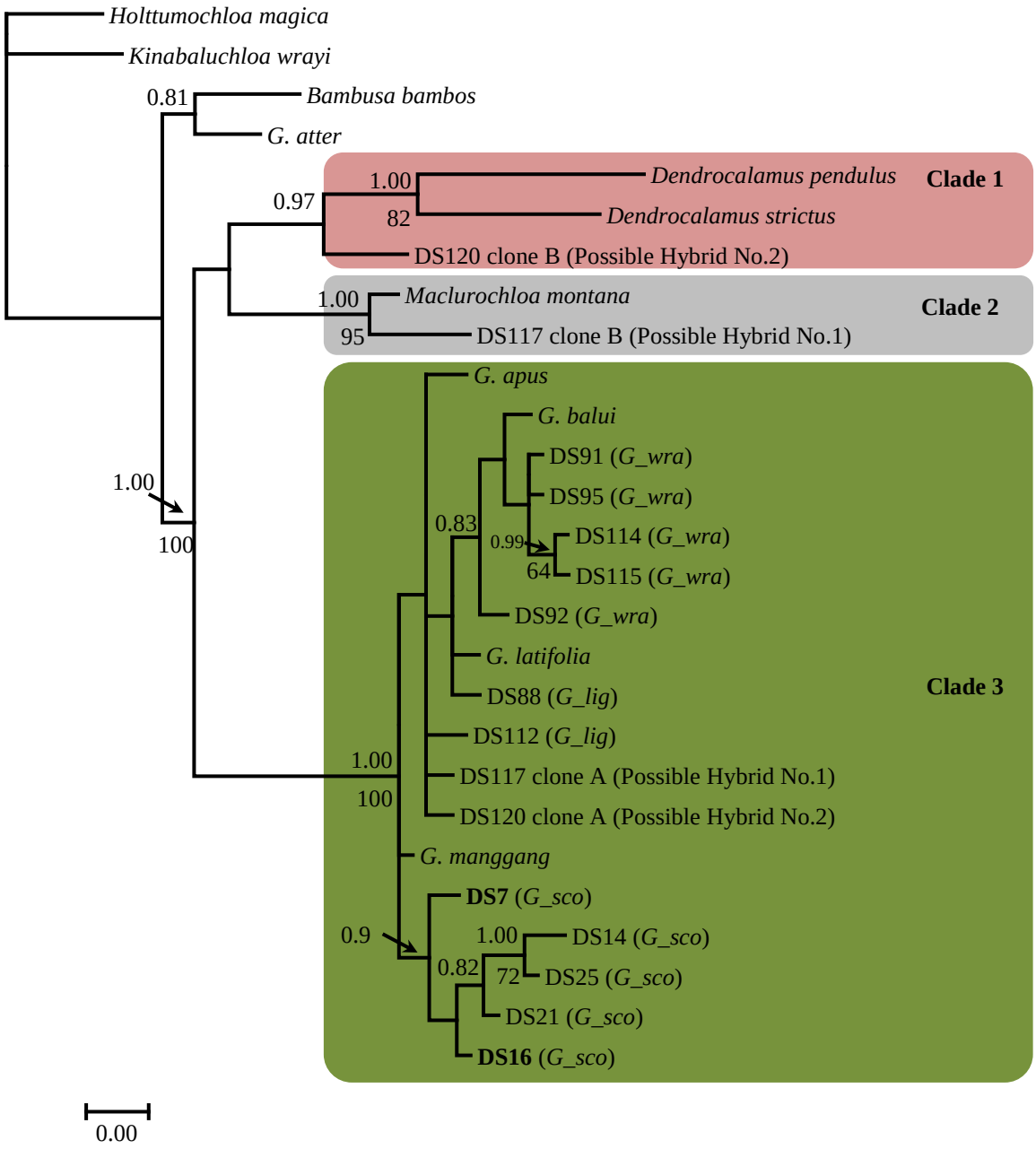

Figure 4.4: Bayesian tree based on the combined partial nuclear *GBSS*1 gene and *Pabp*1 gene dataset of the representatives from both Gombak-type and Langat-type specimens. The tree was rooted with *Holttumochloa magica* and *Kinabaluchloa wrayi* as outgroups. Upper nodal figures represent support values for Bayesian inference posterior probability and the lower nodal figures shows the bootstrap values for maximum parsimony analysis. Bootstrap values were obtained with 1000 replicates. Parsimony-informative sites among the ingroups are 39/1083. The red-shaded specimens indicate Clade 1 and the grey-shaded specimens indicate Clade 2, while the green-shaded specimens show Clade 3.

4.4 Variable Sites and Indel Sites

4.4.1 Chloroplast DNA (*rps16-trnQ & trnD-T*)

The variable sites and indel sites of all the haplotypes of cpDNA and other related genera are shown in Table 4.6 (a) and (b). The aligned data matrices of *rps16-trnQ* and *trnD-T* comprises 469 and 1,079 characters, respectively. Variable sites at 1,271 (A) and 1,402 (G) as well as the 7 bp-indels (at sites 392-398) and 1 bp-indel (at site 1,148) distinguishes the Langat-type from the Gombak- type haplotypes (Table 4.6 (a) and (b)). It is, however, identified that the same 7 bp-indels (at sites 392-398) are present in haplotype 6 and haplotype 8 of the Gombak-type.

Table 4.6: The variable sites (a) and indels (b) (i) and (ii) of the intergenic spacer, *rps16-trnQ* and *trnD-T* (1,548 bp) of the *Gigantochloa* species and the ingroups. The dots indicate identical nucleotide compared to those in the first row. Dashes indicate the alignment gaps. The specimens are separated by the clades of the Bayesian tree (Figure 4.3). The grey-shaded specimens are Langat-type while the dark blue-shaded ones are Gombak-type.

(a)

Site / Taxa	1 2 6	3 2	3 6 7	3 8 9	3 9 9	4 1 5	4 7 5	4 8 2	5 1 3	6 2 3	8 8 4	8 9	1 1 6	1 1 6	1 2 3	1 2 7	1 3 3	1 3 5	1 4 7	1 4 0	1 4 0	1 4 0	1 4 2	1 4 3	1 4 4	1 4 4	1 4 5	1 4 6	1 4 7	1 4 7	1 4 7	1 4 7	1 4 8	1 4 9	1 4 9	1 4 9	1 4 9	1 5 0	1 5 0	1 5 1	1 5 3	1 5 3	1 5 4	1 5 4	1 5 4
B_bambos	T	C	G	A	A	A	C	T	A	C	G	A	T	T	A	T	T	G	A	A	A	G	G	–	T	–	–	A	T	A	G	A	A	–	G	A	T	G	A	T	T	A	A	A	T	A
D_pendulus	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	–	–	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
D_strictus	.	T	T	.	T	.	A	.	.	.	T	.	.	.	.	A	.	.	G	.	.	.	.	–	.	–	–	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
M_montana	.	.	T	.	–	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	–	.	–	–	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
G_apus	.	.	T	T	–	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	–	.	T	A	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
G_atter	.	.	T	.	–	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	–	.	T	A	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
G_balui	A	.	T	.	–	.	.	.	.	.	.	.	G	A	.	.	.	.	.	.	.	.	.	–	.	–	–	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
G_latifolia	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	–	–	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
G_manggang	.	.	T	T	–	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	–	.	–	–	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
Hap1	.	.	T	T	–	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	A	.	–	.	–	–	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
Hap2	.	.	T	T	–	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	–	.	–	–	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
Hap3	.	.	T	T	–	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	A	.	–	–	.	.	.	.	.	.	–	.	.	.	.	C	.	.	.	.	.	.	.
Hap4	.	.	T	T	–	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	A	.	–	.	–	–	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
Hap7	.	.	T	T	–	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	–	.	T	A	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
Hap10	.	.	T	.	–	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	–	.	T	A	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
Hap12	.	.	T	.	–	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	–	.	G	A	.	.	.	A	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
Hap5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	–	T	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
Hap6	.	.	T	T	–	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	–	.	–	–	.	.	.	A	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
Hap8	.	.	T	.	–	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	G	G	–	–	T	G	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
Hap9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	–	.	–	–	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
Hap11	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	–	.	–	–	.	.	.	A	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
Hap13	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	–	–	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	.	.	.	.
Hap14	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	–	–	.	.	.	A	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
Hap15	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	–	–	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
Hap16	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	.	–	–	.	.	.	.	.	.	–	A	.	.	.	.	.	.	.	.	.	.	.
Hap17	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	–	–	.	.	.	.	.	.	–	.	G	A	.	.	.	.	.	.	.	.	.
Hap18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	–	–	.	.	.	A	.	.	–	.	.	.	.	.	.	.	.	.	.	.	.
Hap19	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	.	–	T	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
Hap20	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	–	–	T	G	T	A	G	.	–	.	.	.	.	.	.	T	.	.	.	T
Hap21	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	–	–	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	T
Hap22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	–	–	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	T
Hap23	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	–	.	–	–	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	T
Hap24	.	.	.	.	.	.	.	.	T	A	.	.	.	.	.	.	.	.	.	.	.	.	.	–	.	–	–	.	.	.	.	.	.	–	.	.	.	.	.	.	.	.	.	.	.

Table 4.6 (a) (Cont'd):

Taxa \ Site	126	322	367	389	399	415	475	482	513	623	884	899	1166	1167	1234	1271	1333	1351	1372	1400	1402	1405	1423	1431	1434	1454	1468	1472	1474	1475	1476	1477	1489	1492	1494	1495	1496	1499	1501	1506	1511	1535	1538	1543	1545	1546
Hap25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	-	-	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap26	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	A	.	-	T	.	.	.	.	.	.	-	.	G	A	.	.	.	.	.	.	.	.	.	.
Hap27	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	-	.	-	-	.	.	.	.	.	.	C	.	.	.	C	.	.	.	.	T	.	.	.	.
Hap28	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	-	.	-	-	.	.	.	A	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap29	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	-	.	-	-	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap30	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	-	-	.	.	.	.	.	T	C	.	.	.	.	.	.	A	.	.	.	.	.
Hap31	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	-	-	.	.	.	.	.	.	-	.	.	.	C	.	G	.	.	.	C	C	.
Hap32	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	-	.	-	-	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.

Table 4.6 (b) (i):

Site / Taxa	11	23	392-398	721	747	1148	1149	1159	1170-1201
B_bambos	A	A	A T T A G A A	T	T	T	A	T	C G G A T C A T C T C C T A C T T T T T T A G G G A A A T T C A
D_pendulus	-	.	. . . .	.	.	.	.	.	. . . .
D_strictus	.	.	A T T A G A T	.	.	.	.	.	. . . .
M_montana	-	.	- - - - - - -	.	.	.	.	.	. . . .
G_apus	.	.	- - - - - - -	.	.	-	.	.	. . . .
G_atter	.	.	A - - - - - -	.	.	-	.	.	. . . .
G_balui	.	.	A - - - - - -	.	.	.	.	-	- -
G_latifolia	-	.	. . . .	.	.	.	.	.	. . . .
G_manggang	-	.	- - - - - - -	.	.	-	.	.	. . . .
Hap1	-	.	- - - - - - -	.	.	-	.	.	. . . .
Hap2	-	.	- - - - - - -	.	.	-	.	.	. . . .
Hap3	-	-	- - - - - - -	.	.	-	.	.	. . . .
Hap4	-	-	- - - - - - -	.	.	-	.	.	. . . .
Hap7	-	.	- - - - - - -	.	.	-	.	.	. . . .
Hap10	-	.	- - - - - - -	.	.	-	.	.	. . . .
Hap12	-	.	- - - - - - -	.	.	-	.	.	. . . .
Hap5	-	.	. . . .	.	.	.	.	.	. . . .
Hap6	-	.	- - - - - - -	.	.	.	.	.	. . . .
Hap8	-	.	- - - - - - -	.	.	.	.	.	. . . .
Hap9	-	.	. . . .	.	.	.	.	.	. . . .
Hap11	-	.	. . . .	.	.	.	.	.	. . . .
Hap13	.	.	. . . .	.	.	.	.	.	. . . .
Hap14	.	.	. . . .	.	.	.	.	.	. . . .
Hap15	-	.	. . . .	.	.	.	.	.	. . . .
Hap16	-	.	. . . .	.	.	.	.	.	. . . .
Hap17	-	.	. . . .	.	.	.	.	.	. . . .
Hap18	-	.	. . . .	.	.	.	.	.	. . . .
Hap19	-	.	. . . .	.	-	.	.	.	. . . .
Hap20	-	.	. . . .	.	.	.	.	.	. . . .
Hap21	-	.	. . . .	.	.	.	.	.	. . . .
Hap22	-	.	. . . .	.	.	.	.	.	. . . .
Hap23	-	.	. . . .	.	.	.	.	.	. . . .
Hap24	-	.	. . . .	.	.	.	-	.	. . . .
Hap25	-	.	. . . .	.	.	.	.	.	. . . .
Hap26	-	.	. . . .	.	.	.	.	.	. . . .
Hap27	-	.	. . . .	.	.	.	.	.	. . . .

Table 4.6 (b) (i) (Cont'd):

Site / Taxa	11	23	392-398	721	747	1148	1149	1159	1170-1201
Hap28	-	.	. . . . .	.	.	.	.	.	. . . . .
Hap29	-	.	. . . . .	.	.	.	.	.	. . . . .
Hap30	-	.	. . . . .	.	.	.	.	.	. . . . .
Hap31	-	.	. . . . .	.	.	.	.	.	. . . . .
Hap32	-	.	. . . . .	.	.	.	.	.	. . . . .

Table 4.6 (b) (ii):

Site / Taxa	1217-1225	1428	1446-1465	1478	1489
B_bambos	A T A A G A A T A	-	- -	-	-
D_pendulus	. . . . .	-	- -	-	-
D_strictus	. . . . .	-	- -	-	-
M_montana	. . . . .	-	- -	-	-
G_apus	. . . . .	-	T A A G G T G A A T A A A T A A T A A A	-	-
G_atter	. . . . .	-	T A A G G T G A A T A A A T A A T A A A	-	-
G_balui	. . . . .	-	- -	-	-
G_latifolia	. . . . .	-	- -	-	-
G_manggang	. . . . .	-	- -	-	-
Hap1	. . . . .	-	- -	-	-
Hap2	. . . . .	-	- -	-	-
Hap3	. . . . .	A	- -	-	-
Hap4	. . . . .	-	- -	-	-
Hap7	. . . . .	-	T A A G G T G A A T A A A T A A T A A A	-	-
Hap10	. . . . .	-	T A A G G T G A A T A A A T A A T A A A	-	-
Hap12	. . . . .	-	T A A G G T G A A T A A A T A A T A A A	-	-
Hap5	. . . . .	-	- -	-	C
Hap6	. . . . .	-	- -	-	-
Hap8	. . . . .	G	- -	-	-
Hap9	. . . . .	-	- -	-	-
Hap11	. . . . .	-	- -	-	-
Hap13	. . . . .	A	- -	-	C
Hap14	. . . . .	A	- -	-	-
Hap15	- - - - - - - - -	-	- -	-	-

Table 4.6 (b) (ii) (Cont'd):

Taxa \ Site	1217-1225	1428	1446-1465	1478	1489
Hap16	. . . . .	A	- -	-	-
Hap17	. . . . .	A	- -	-	-
Hap18	- - - - - - - - -	-	- -	-	-
Hap19	. . . . .	A	- T	A	G
Hap20	. . . . .	-	- -	A	-
Hap21	. . . . .	-	- -	-	-
Hap22	. . . . .	-	- -	-	-
Hap23	. . . . .	-	- -	-	-
Hap24	. . . . .	-	- -	-	-
Hap25	. . . . .	-	- -	-	-
Hap26	- - - - - - - - -	A	- T	A	-
Hap27	- - - - - - - - -	-	- -	-	C
Hap28	- - - - - - - - -	-	- -	-	-
Hap29	- - - - - - - - -	-	- -	-	-
Hap30	. . . . .	-	- -	-	C
Hap31	- - - - - - - - -	-	- -	-	-
Hap32	- - - - - - - - -	-	- -	-	-

4.4.2 Nuclear DNA (*GBBS*I and *PabpI*)

The variable sites and indel sites of all the 16 *Gigantochloa* samples selected for nuclear DNA sequencing and the other related genera are shown in Table 4.7 (a) and (b). The combined data matrices of partial nuclear *GBSS*I and *PabpI* gene consists 681 and 402 characters, respectively. Meanwhile, the aligned data matrix of individual nuclear DNA of *GBSS*I and *Pabp*I consists of 681 and 480 characters, respectively. As the individual nuclear DNA sequence dataset provide less information, the combined nuclear DNA sequence datasets were considered for further phylogenetic and population study analysis. The overlapping or dimorphic sites in the sequences were replaced with the degenerative nucleotide codes as stated in International Union of Pure and Applied Chemistry (IUPAC). The variable site at 561 (T specific to Clade 1; - specific to Clade 2; C specific to Clade 3) and 3 bp-indels at the site 561-563 (TAT specific to Clade 1; - specific to Clade 2; CGA specific to Clade 3) found to be specific to each clade of nuclear DNA matrices. However, it was noted that the Clade 1 Bayesian tree putative hybrid DS120 clone B resembled the variable site and indel uniqueness of Clade 2 Bayesian tree individuals (*M. montana* and DS120 clone B).

Table 4.7: The variable sites (a) and indels (b) of the partial nuclear *GBBS*I (1–681 bp) and *PabpI* gene (682–1,083 bp) of the *Gigantochloa* species and other ingroups. The dots indicate identical nucleotide compared to those in the first row. Dashes indicate the alignment gaps. The specimens are separated by the clades of the Bayesian tree (Figure 4.4). Species names of the Malaysian specimens sequenced in this study were indicated in parentheses using the abbreviations *G_lig = Gigantochloa ligulata*, *G_sco = G. scortechinii*, *G_wra = G. wrayi*. The red-shaded specimens indicate Clade 1 and grey-shaded specimens indicate Clade 2 while the green-shaded ones show Clade 3.

(a)

Clade (Fig.4.4)	Site / Taxa	19	75	133	147	162	164	166	169	173	178	228	288	291	293	301	313	320	326	330	331	333	349	350	357	359	416	417	455	529	560	561	562	563	576	579	581	589	595	614
	B. bambos	G	C	A	A	G	–	–	–	–	–	A	G	C	A	C	G	T	G	G	C	G	–	–	T	T	G	A	A	A	A	–	–	–	G	N	N	C	C	T
NIL	*G. atter*	.	.	.	G	.	A	–	C	T	G	.	.	T	C	.	.	.	.	.	.	.	A	T	Y	.	.	R	.	.	G	–	–	–	T	G	C	.	.	Y
1	*D. pendulus*	.	.	.	G	.	–	–	–	–	–	.	.	.	.	–	.	C	.	.	.	.	–	–	A	.	.	G	.	G	.	T	A	T	.	G	T	T	T	.
	D. strictus	.	.	.	G	T	–	–	–	–	–	.	.	.	.	–	.	.	T	.	.	.	–	–	.	C	.	G	.	G	.	T	A	T	.	G	T	.	T	.
	DS120_clone B (Hybrid No.2)	.	.	.	G	.	A	A	T	A	G	.	.	.	.	–	.	.	.	.	.	.	G	T	.	.	.	G	.	.	.	–	–	–	.	G	T	.	.	.
2	*M. montana*	.	.	.	G	.	A	–	C	T	C	G	A	T	.	–	.	.	.	.	.	.	A	T	.	.	.	.	G	.	.	–	–	–	.	T	T	.	.	.
	DS117_clone B (Hybrid No.1)	.	.	.	G	.	G	G	T	T	C	G	A	T	.	–	.	.	.	.	.	.	A	C	.	.	.	.	G	.	.	–	–	–	.	T	T	.	.	.
3	*G. apus*	A	.	T	G	.	A	–	C	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	.	.	.	.	.	C	G	A	.	G	T	.	.	C
	G. balui	A	.	.	G	.	A	–	Y	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	R	.	.	.	.	C	G	A	.	G	T	.	.	C
	G. latiflolia	A	.	T	G	.	A	–	C	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	.	.	.	.	.	C	G	A	.	G	T	.	.	C
	G. manggang	.	.	T	G	.	A	–	C	T	G	.	.	T	.	.	.	.	.	R	M	R	A	Y	Y	.	.	.	.	.	.	C	G	A	.	G	T	.	.	C
	DS7 (*G_sco*)	.	T	T	G	.	A	–	C	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	.	.	.	.	.	C	G	A	.	G	T	.	.	C
	DS14 (*G_sco*)	.	.	T	G	.	A	–	C	T	G	.	.	T	.	T	.	.	.	A	A	A	A	T	C	.	.	.	.	.	.	C	G	A	.	G	T	.	.	C
	DS16 (*G_sco*)	.	T	T	G	.	A	–	C	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	.	.	.	.	.	C	G	A	.	G	T	.	.	C
	DS21 (*G_sco*)	.	T	T	G	.	A	–	C	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	.	.	.	.	.	C	G	A	.	G	T	.	.	C
	DS25 (*G_sco*)	.	T	T	G	.	A	–	C	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	.	.	.	.	.	C	G	A	.	G	T	.	.	C
	DS88 (*G_lig*)	A	.	T	G	.	A	–	C	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	.	.	.	.	.	C	G	A	.	G	T	.	.	C
	DS91 (*G_wra*)	A	.	.	G	.	A	–	C	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	A	.	.	.	.	C	G	A	.	G	T	.	.	C
	DS92 (*G_wra*)	A	.	.	G	.	A	–	C	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	.	.	.	.	.	C	G	A	.	G	T	.	.	C
	DS95 (*G_wra*)	A	.	.	G	.	A	–	C	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	A	.	.	.	.	C	G	A	.	G	T	.	.	C
	DS112 (*G_lig*)	A	.	T	G	.	A	–	C	T	G	.	.	T	.	.	A	.	.	A	A	A	A	T	C	.	.	.	.	.	.	C	G	A	.	G	T	.	.	C
	DS114 (*G_wra*)	A	.	.	G	.	A	–	C	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	A	.	.	.	.	C	G	A	.	G	T	.	.	C
	DS115 (*G_wra*)	A	.	.	G	.	A	–	C	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	A	.	.	.	.	C	G	A	.	G	T	.	.	C
	DS117_clone A (Hybrid No.1)	A	.	T	G	.	A	–	C	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	.	.	.	.	.	C	G	A	.	G	C	.	.	C
	DS120_clone A (Hybrid No.2)	A	.	T	G	.	A	–	C	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	.	.	.	.	.	C	G	A	.	G	T	.	.	C

Table 4.7 (a) (cont'd):

Clade (Fig. 4.4)	Taxa \ Site	616	618	630	660	662	672	673	728	730	740	748	823	828	830	848	849	856	858	860	883	951	958	970	978	987	994	996	1006	1011	1013	1016	1017	1023	1025	1032	1039	1083
	B. bambos	G	G	A	A	C	C	T	A	A	G	C	G	C	A	A	A	G	T	C	G	C	G	C	A	C	T	T	G	T	A	C	A	G	C	T	A	C
NIL	*G. atter*	.	.	W	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
1	*D. pendulus*	.	.	.	.	.	.	.	.	.	.	A	A	.	G	.	.	.	.	.	A	.	.	.	.	.	G	G	A	A	C	A	T	A	A	.	.	.
	D. strictus	.	T	.	G	T	.	C	G	.	A	A	A	.	.	.	.	.	A	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.	.	.
	DS120_clone B (Hybrid No.2)	C	.	.	.	T	.	.	.	.	.	A	A	T	G	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
2	*M. montana*	.	.	.	.	T	.	.	.	.	.	A	A	.	.	.	.	A	.	.	.	A	T	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	T
	DS117_clone B (Hybrid No.1)	.	.	.	.	T	.	.	.	.	.	A	A	T	G	.	.	A	.	.	.	A	T	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
3	*G. apus*	.	T	T	.	T	A	.	.	.	.	A	A	.	.	.	.	.	.	T	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	T	.
	G. balui	.	T	T	.	T	A	.	.	.	.	A	A	T	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	T
	G. latiflolia	.	T	T	.	T	A	.	.	.	.	A	A	T	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	T
	G. manggang	.	T	T	.	T	A	.	.	.	.	A	A	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
	DS7 (*G_sco*)	.	T	T	.	T	A	.	.	.	.	A	A	.	.	T	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
	DS14 (*G_sco*)	.	T	T	.	T	A	.	.	G	.	A	A	T	.	T	T	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
	DS16 (*G_sco*)	.	T	T	.	T	A	.	.	.	.	A	A	T	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
	DS21 (*G_sco*)	.	T	T	.	T	A	.	.	G	.	A	A	T	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
	DS25 (*G_sco*)	.	T	T	.	T	A	.	.	G	.	A	A	T	.	T	T	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
	DS88 (*G_lig*)	.	T	T	.	T	A	.	.	.	.	A	A	T	.	.	.	.	.	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	A	.	.	.
	DS91 (*G_wra*)	.	T	T	.	T	A	.	.	.	.	A	A	T	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
	DS92 (*G_wra*)	.	T	T	.	T	A	.	.	.	.	A	A	T	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	C	.	.
	DS95 (*G_wra*)	.	T	T	.	T	A	.	.	.	.	A	A	T	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
	DS112 (*G_lig*)	.	T	T	.	T	A	.	.	.	.	A	A	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
	DS114 (*G_wra*)	.	T	T	.	T	A	.	.	.	.	A	A	T	.	.	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.
	DS115 (*G_wra*)	.	T	T	.	T	A	.	.	.	.	A	A	T	.	.	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	A	.	.	.
	DS117_clone A (Hybrid No.1)	.	T	T	.	T	A	.	.	.	.	A	A	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
	DS120_clone A (Hybrid No.2)	.	C	T	.	T	A	.	.	.	.	A	A	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.

Table 4.7 (b)

Clade (Fig.4.4)	Site / Taxa	163-178	301-303	341-354	561-567	646	989
	B. bambos	- - - - - - - - - - - - - - - -	C C T	- - - - - - - - - - - - - - - -	- - - - C T A	A	A
NIL	*G. atter*	T A T - - A C A T A T A T A T G	. . .	G T A T A T A C A T A T A T	- - - - C T A	.	.
	D. pendulus	- - - - - - - - - - - - - - - -	. . .	- - - - - - - - - - - - - - - -	T A T A C T A	.	-
	D. strictus	- - - - - - - - - - - - - - - -	- - -	- - - - - - - - - - - - - - - -	T A T A C T A	.	.
1	DS120_clone B (Hybrid No.2)	T A T A T A T A T A A A T A T G	- - -	G T A T A T A C G T A T A T	- - - - C T A	.	.
	M. montana	T A T - - A C A T A T A T A T C	- - -	G T A T A T A C A C A T A T	- - - - C T A	.	.
2	DS117_clone B (Hybrid No.1)	T G T G T A T A T A T A T A T C	- - -	G T A T A T A C A C A T A T	- - - - C T A	.	.
	G. apus	T A T - - A C A T A T A T A T G	- - -	G T A T A T A C A C A T A T	C G A - - - -	.	.
	G. balui	T A T - - A Y A T A T A T A T G	. . .	G T A T A T A C A C A T A T	C G A - - - -	.	.
3	*G. latiflolia*	T A T - - A C A T A T A T A T G	. . .	G T A T A T A C A C A T A T	C G A - - - -	.	.
	G. manggang	. . . - -	. . .	G T A T A T A C A Y A T A T	C G A - - - -	.	.
	DS7 (*G_sco*)	. . . - -	. . .	G T A T A T A C A C A T A T	C G A - - - -	.	.
	DS14 (*G_sco*)	. . . - -	T . .	G T A T A T A C A C A T A T	C G A - - - -	.	.
	DS16 (*G_sco*)	. . . - -	. . .	G T A T A T A C A C A T A T	C G A - - - -	.	.
	DS21 (*G_sco*)	. . . - -	. . .	G T A T A T A C A C A T A T	C G A - - - -	.	.
	DS25 (*G_sco*)	. . . - -	. . .	G T A T A T A C A C A T A T	C G A - - - -	.	.
	DS88 (*G_lig*)	. . . - -	. . .	G T A T A T A C A C A T A T	C G A - - - -	.	.
	DS91 (*G_wra*)	. . . - -	. . .	G T A T A T A C A C A T A T	C G A - - - -	.	.
	DS92 (*G_wra*)	. . . - -	. . .	G T A T A T A C A C A T A T	C G A - - - -	.	.
	DS95 (*G_wra*)	. . . - -	. . .	G T A T A T A C A C A T A T	C G A - - - -	.	.
	DS112 (*G_lig*)	. . . - -	. . .	G T A T A T A C A C A T A T	C G A - - - -	-	.
	DS114 (*G_wra*)	. . . - -	. . .	G T A T A T A C A C A T A T	C G A - - - -	.	.
	DS115 (*G_wra*)	. . . - -	. . .	G T A T A T A C A C A T A T	C G A - - - -	.	.
	DS117_clone A (Hybrid No.1)	. . . - -	. . .	G T A T A T A C A C A T A T	C G A - - - -	.	.
	DS120_clone A (Hybrid No.2)	. . . - -	. . .	G T A T A T A C A T A T A T	C G A - - - -	.	.

4.5 Morphological and Molecular Characteristics of Putative Hybrids

Two hybrids were detected along the Penang Western Hill roadside on 04/10/2015 (hereafter referred to as “DS117”) and Sintok, Kedah on 07/10/2015 (hereafter referred to as “DS120”) of Peninsular Malaysia. Morphological properties of the putative hybrids and their possible parent species were assessed and the characteristics were exhibited in Table 4.8. The morphology of the suspected hybrid is a combination of intermediate characters that correspond to one or the other parental species as has been indicated for many plant hybrids and hybrid derivatives (Rieseberg, 1995). The variable sites and indel sites of all the putative hybrids and their possible parent species are shown in Table 4.9.

Table 4.8: Morphological character states of the putative hybrid individuals (a) DS117 and (b) DS120. Those intermediate characteristics that are typical between possible parents are boldfaced; characteristics which are non-typical to possible parents are underlined.

(a) DS117

Character of the identified hybrids	*G. ligulata*	DS117 (Possible Hybrid no.1)	*M. montana*
Culm leaf: culm sheath blade	Culm sheath blades spreading at lower and mid culms (Wong, 1995a)	**Culm sheath blades spreading at lower** and mid culms.	-
Clump habit	Clumps densely tufted (Widjaja, 1987)	Slender **clumped bamboo**, culms c. 3-4 cm diam., **flopping over adjacent plants**; green with a few yellow stripes, scattered black hairs at upper part of internode.	Clump habit– floppy

(b) DS120

Character of the identified hybrids	*G. ligulata*	DS120 (Possible Hybrid no.2)	*G. latifolia*
Culm leaf: culm sheath blade	Culm sheaths with lacerate ligules (Wong, 1995a)	Culm sheaths with **erect blades** and **lacerate ligules**	Culm sheaths with erect blades (Wong, 1995a)
Clump habit	Clumps densely tufted (Widjaja, 1987)	Clumped bamboo, the culms broadly arched over nearly to the ground, to c. 5.5-6 cm.	Clumps densely tufted (Widjaja, 1987)

Table 4.9: The variable sites (a) and indels (b) of combined nuclear DNA dataset (partial nuclear *GBBS*I and *PabpI* gene) of the possible hybrids clones (DS117 and DS120) with the potential parental species separated by the four different colors (Red = DS117_clone B & *M. montana*; Olive green = DS117_clone A, DS88 (*G_lig*) & DS112 (*G_lig*); Tan = DS120_clone A, DS88 (*G_lig*) & DS112 (*G_lig*); Grey = DS120_clone B, *D. pendulus* & *D.stictus*). The dots indicate identical nucleotide compared to those in the first row. Dashes indicate the alignment gaps.

(a)

Site / Taxa	19	75	133	147	162	164	166	169	173	178	228	288	291	293	301	313	320	326	330	331	333	334	349	350	357	359	416	417	455	529	560	561	562	563	576	579	581	589	595	614
M. montana	G	C	A	G	G	A	-	C	T	C	G	A	T	A	-	G	T	G	G	C	G	A	T	T	T	G	A	G	A	A	-	-	-	G	T	T	C	C	T
DS117_clone B (Hybrid No.1)	.	.	.	G	.	G	G	T	T	C	G	A	T	.	-	.	.	.	.	.	.	A	C	.	.	.	.	G	.	.	-	-	-	.	T	T	.	.	.
DS88 (*G_lig*)	A	C	T	G	G	A	-	C	T	G	A	G	T	A	C	G	T	G	A	A	A	A	T	C	T	G	A	A	A	A	C	G	A	G	G	T	C	C	C
DS112 (*G_lig*)	A	.	T	G	.	A	-	C	T	G	.	.	T	.	.	A	.	.	A	A	A	A	T	C	.	.	.	.	.	.	C	G	A	.	G	T	.	.	C
DS117_clone A (Hybrid No.1)	A	.	T	G	.	A	-	C	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	.	.	.	.	.	C	G	A	.	G	C	.	.	C
DS88 (*G_lig*)	A	C	T	G	G	A	-	C	T	G	A	G	T	A	C	G	T	G	A	A	A	A	T	C	T	G	A	A	A	A	C	G	A	.	G	T	C	C	C
DS112 (*G_lig*)	A	.	T	G	.	A	-	C	T	G	.	.	T	.	.	A	.	.	A	A	A	A	T	C	.	.	.	.	.	.	C	G	A	.	G	T	.	.	C
DS120_clone A (Hybrid No.2)	A	.	T	G	.	A	-	C	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	.	.	.	.	.	C	G	A	.	G	T	.	.	C
G. latiflolia	A	.	T	G	.	A	-	C	T	G	.	.	T	.	.	.	.	.	A	A	A	A	T	C	.	.	.	.	.	.	C	G	A	.	G	T	.	.	C
D. pendulus	G	C	A	G	G	-	-	-	-	-	A	G	C	A	-	G	C	G	G	C	G	-	-	A	T	G	G	A	G	A	T	A	T	G	G	T	T	T	T
D. strictus	.	.	.	G	T	-	-	-	-	-	.	.	.	.	-	.	.	T	.	.	.	-	-	.	C	.	G	.	G	.	T	A	T	.	G	T	.	T	.
DS120_clone B (Hybrid No.2)	.	.	.	G	.	A	A	T	A	G	.	.	.	.	-	.	.	.	.	.	.	G	T	.	.	.	G	.	.	.	-	-	-	.	G	T	.	.	.

Table 4.9 (a) (cont'd):

Site / Taxa	616	618	630	660	662	672	673	728	730	740	748	823	828	830	848	849	856	858	860	883	951	958	970	978	987	994	996	1006	1011	1013	1016	1017	1023	1025	1032	1039	1083
M. montana	G	G	A	A	T	C	T	A	A	G	A	A	C	A	A	A	A	T	C	G	A	T	C	A	C	T	T	G	T	A	C	A	G	A	T	A	T
DS117_clone B (Hybrid No.1)	.	.	.	.	T	.	.	.	.	.	A	A	T	G	.	.	A	.	.	.	A	T	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
DS88 (*G_lig*)	G	T	T	A	T	A	T	A	A	G	A	A	T	A	A	A	G	T	C	A	C	G	C	A	T	T	T	G	T	A	C	A	G	A	T	A	C
DS112 (*G_lig*)	.	T	T	.	T	A	.	.	.	.	A	A	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
DS117_clone A (Hybrid No.1)	.	T	T	.	T	A	.	.	.	.	A	A	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
DS88 (*G_lig*)	G	T	T	A	T	A	T	A	A	G	A	A	T	A	A	A	G	T	C	A	C	G	C	A	T	T	T	G	T	A	C	A	G	A	T	A	C
DS112 (*G_lig*)	.	T	T	.	T	A	.	.	.	.	A	A	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
DS120_clone A (Hybrid No.2)	.	C	T	.	T	A	.	.	.	.	A	A	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
G. latiflolia	.	T	T	.	T	A	.	.	.	.	A	A	T	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	T
D. pendulus	G	G	A	A	C	C	T	A	A	G	A	A	C	G	A	A	G	T	C	A	C	G	C	A	C	G	G	A	A	C	A	T	A	A	T	A	C
D. strictus	.	T	.	G	T	.	C	G	.	A	A	A	.	.	.	.	.	A	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.	.	.
DS120_clone B (Hybrid No.2)	C	.	.	.	T	.	.	.	.	.	A	A	T	G	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.

Table 4.9 (b)

Taxa \ Site	163-178	301- 303	341-354	561-567	646	989
M. montana	T A T - - A C A T A T A T A T A T C	- - -	G T A T A T A C A C A T A T	- - - - C T A	A	A
DS117_clone B (Hybrid No.1)	T G T G T A T A T A T A T A T A T C	- - -	G T A T A T A C A C A T A T	- - - - C T A	.	.
DS88 (*G_lig*)	T A T - - A C A T A T A T A T A T G	C C T	G T A T A T A C A C A T A T	C G A - - - -	A	A
DS112 (*G_lig*)	. . . - -	. . .	G T A T A T A C A C A T A T	C G A - - - -	-	.
DS117_clone A (Hybrid No.1)	. . . - -	. . .	G T A T A T A C A C A T A T	C G A - - - -	.	.
DS88 (*G_lig*)	T A T - - A C A T A T A T A T A T G	C C T	G T A T A T A C A C A T A T	C G A - - - -	A	A
DS112 (*G_lig*)	. . . - -	. . .	G T A T A T A C A C A T A T	C G A - - - -	-	.
DS120_clone A (Hybrid No.2)	. . . - -	. . .	G T A T A T A C A T A T A T	C G A - - - -	.	.
G. latiflolia	T A T - - A C A T A T A T A T A T G	. . .	G T A T A T A C A C A T A T	C G A - - - -	.	.
D. pendulus	- - - - - - - - - - - - - - - - -	C C T	- - - - - - - - - - - - - - -	T A T A C T A	A	-
D. strictus	- - - - - - - - - - - - - - - - -	- - -	- - - - - - - - - - - - - - -	T A T A C T A	.	.
DS120_clone B (Hybrid No.2)	T A T A T A T A T A A A T A T G	- - -	G T A T A T A C G T A T A T	- - - - C T A	.	.

4.6 Phylogenetic Network Analysis

4.6.1 Neighbor Network Analysis

SplitTree was used to perform Neighbor Network Analysis based on the cpDNA haplotypes and nuclear DNA (outgroups were excluded). In this study, Neighbor Network Analysis shows a visual illustration of inconsistency in the data, thereby emphasizing the haplotype diversity for chloroplast DNA and genotypes of the possible hybrids. The analysis of cpDNA produced a split network pattern between Gombak-type and Langat-type haplotypes (Figure 4.5). However, the association within these groupings were not very clear. The association between haplotype 6, haplotype 8 and *G. balui* to the Gombak-type (Clade 1 in Figure 4.3) appears to be uncertain in the Neighbor Network Analysis based on cpDNA.

Meanwhile, the analysis of nuclear DNA produced a split network pattern of three groupings (Figure 4.6) which resembled the three clades in the phylogenetic analyses (Figure 4.4). Based on the network, the associations within these groupings were clearly similar to the cpDNA dataset. The network also shows the separation between Group III and the other two groups, Group I and II, at the end of long branch.

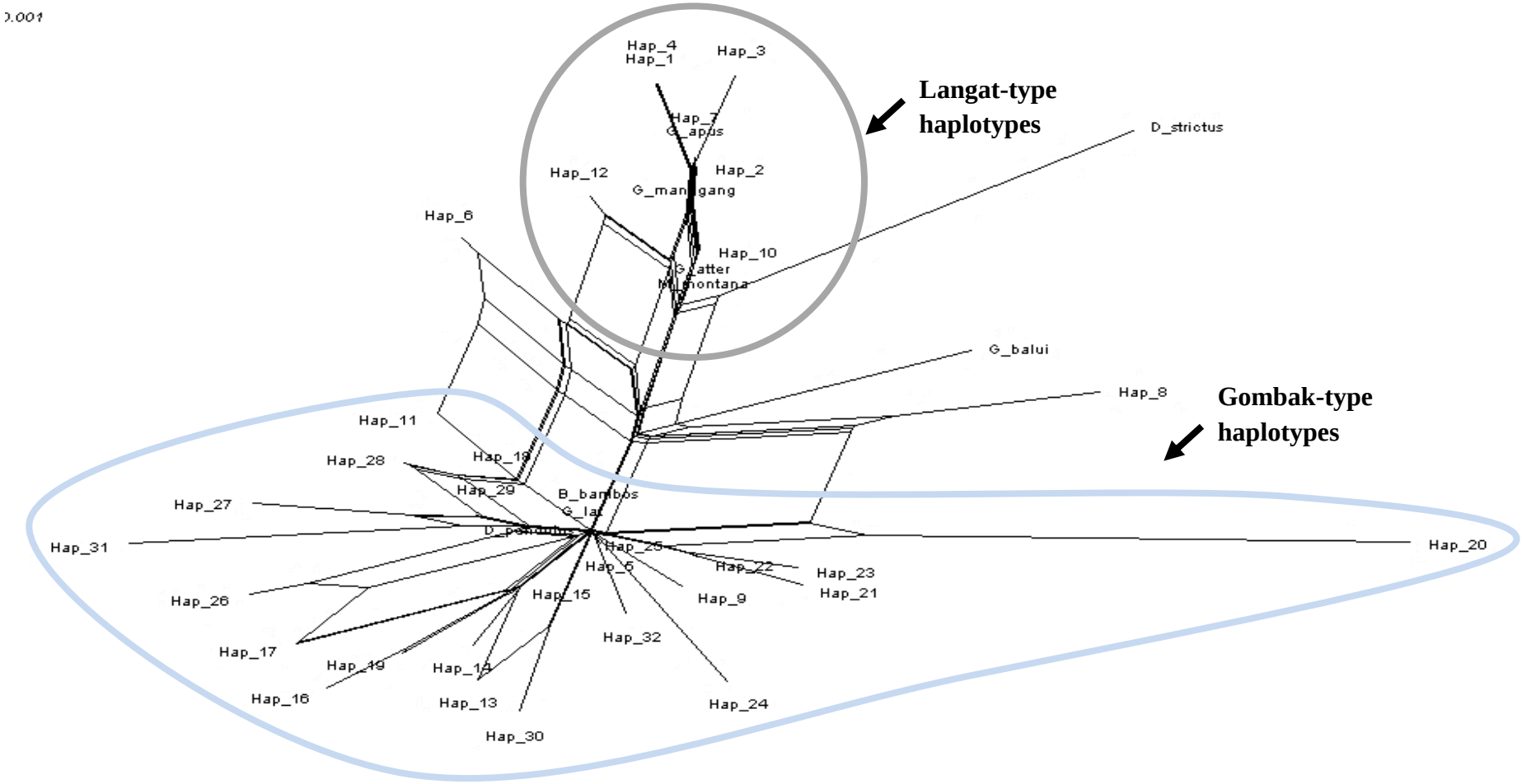

Figure 4.5: Neighbor Network Analysis of 32 *Gigantochloa* species haplotypes and ingroups (other related genera) based on the combined cpDNA regions dataset (*rps16-trnQ* + *trnD-T*). The Langat-type haplotypes are indicated by a grey-outlined circle whereas the Gombak-type haplotypes are indicated by the blue-outlined curve. *D. strictus* unresolved with cpDNA haplotypes and other genera present in the network. Outgroups were excluded.

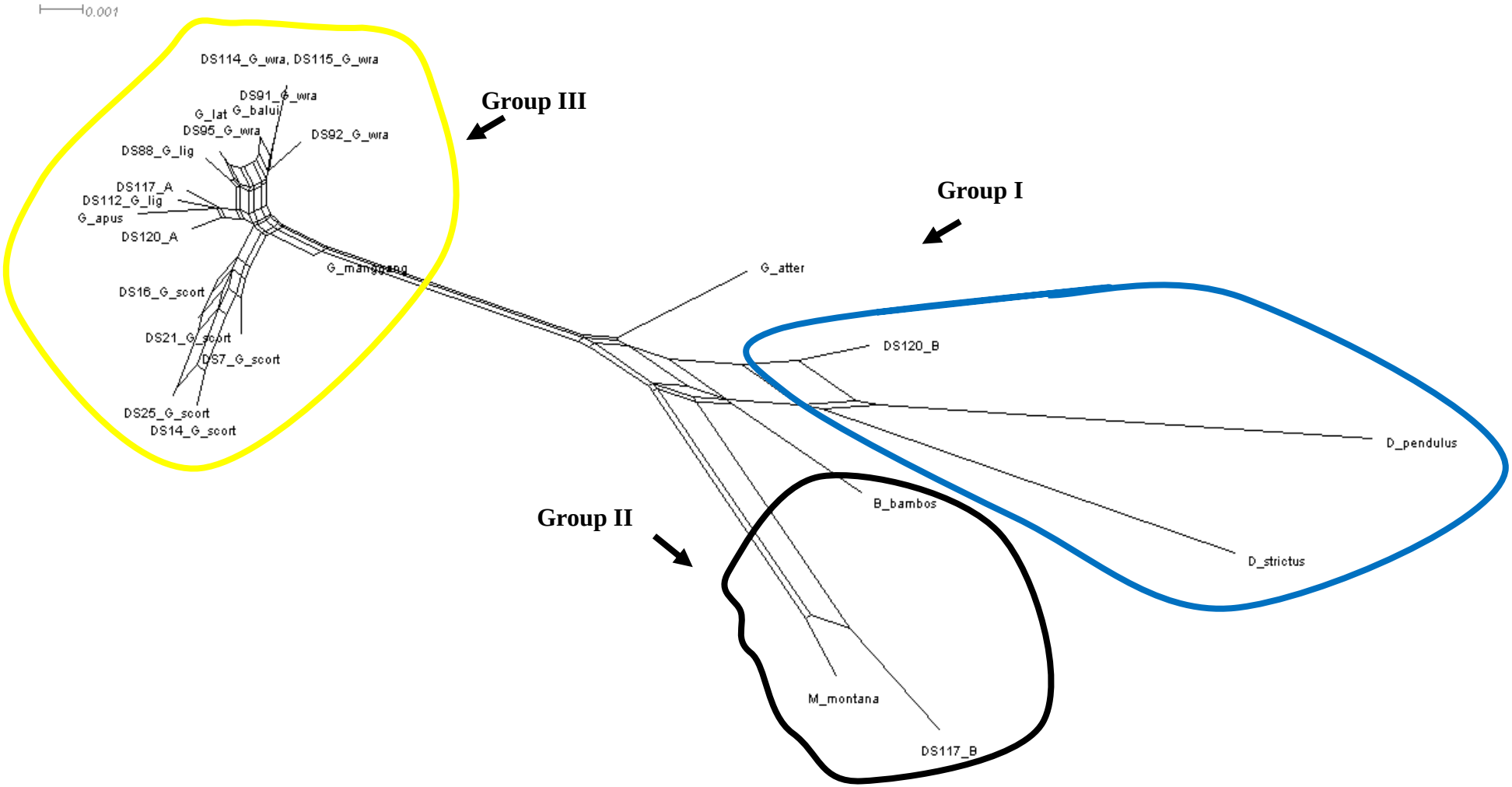

Figure 4.6: Neighbor Network Analysis of 16 selected *Gigantochloa* species and the ingroups (other related genera) based on the combined nuclear DNA dataset (*GBBS*I and *Pabp1*). The three main groupings (Group I, II and III resembled the clades retrieved from Bayesian Inference analysis) were highlighted with different colors (Dark blue = Group I; Black = Group II; Yellow = Group III). *G. atter* did not show any affinity to the other group. Outgroups were excluded.

4.7 Population Structure Analyses

While phylogenetic analyses did not show clear cut interspecific boundary relationship between the *Gigantochloa* species, the genetic differentiation among and within the species and the population structure were analyzed using Pairwise comparison F_{ST} (p = 0.05) and AMOVA based on the cpDNA data. Nine populations were included for the population structure analyses, and a summary of the population genetic diversity was presented in Table 4.10. Population pairwise comparison, F_{ST} for the population pairs based on species and geographical distribution was tested and the F_{ST} values which are significant at p = 0.05 are shown in Table 4.11.

Based on the pairwise F_{ST} analyses performed for the corrected data, sample specimens from Janda Baik was identified to be significantly different from all other populations. Assessment between the uncorrected and corrected data (i.e., with and without the Langat-type individuals, Table 4.11) demonstrated that the population genetic diversity (KKB and Serendah) and pairwise F_{ST} values (KKB-Gabai, KKB-Kelantan, KKB-Kedah, KKB-Kinta, Serendah-Kinjang, Serendah-Kedah, Serendah-Kinta) changed from significant to non-significant, highlighting the exclusion of the Langat-type individuals affected the population structure investigation.

Table 4.10: Summary of population genetic diversity based on chloroplast DNA. Populations with Langat-type were grey-shaded.

Population	All samples				Without Langat-type samples			
	No. of individuals (Sample Size)	No. of haplotype	Gene diversity	Nucleotide Diversity	No. of individuals (Sample Size)	No. of haplotype	Gene diversity	Nucleotide Diversity
KKB	8	6	0.893 +/- 0.111	0.007 +/- 0.004	4	2	0.500 +/- 0.265	0.005 +/- 0.003
Janda Baik	8	5	0.786 +/- 0.151	0.004 +/- 0.002	8	5	0.786 +/- 0.151	0.004 +/- 0.002
Serendah	7	2	0.571 +/- 0.120	0.0117 +/- 0.007	4	1	0.000 +/- 0.000	0.000 +/- 0.000
Gabai	8	2	0.250 +/- 0.180	0.001 +/- 0.001	8	2	0.250 +/- 0.180	0.001 +/- 0.001
Kelantan	6	3	0.600 +/- 0.215	0.005 +/- 0.003	6	3	0.600 +/- 0.215	0.005 +/- 0.003
Kinjang	9	5	0.861 +/- 0.087	0.006 +/- 0.003	9	5	0.861 +/- 0.087	0.006 +/- 0.003
Taiping	10	5	0.756 +/- 0.130	0.005 +/- 0.003	10	5	0.756 +/- 0.130	0.005 +/- 0.003
Kedah	11	8	0.946 +/- 0.054	0.005 +/- 0.003	11	8	0.946 +/- 0.054	0.005 +/- 0.003
Kinta	10	2	0.200 +/- 0.154	0.002 +/- 0.001	10	2	0.200 +/- 0.154	0.002 +/- 0.001

Table 4.11: Population Pairwise comparison, F_{ST} for the population pairs based on species and geographical distribution. F_{ST} values which are significant at $p = 0.05$ are boldfaced. Non-shaded rows = all samples; grey-shaded rows = populations without Langat-type samples.

Population	KKB	Janda Baik	Serendah	Gabai	Kelantan	Kinjang	Taiping	Kedah
Janda Baik	**0.575**							
	0.561							
Serendah	0.151	**0.523**						
	0.000	**0.683**						
Gabai	**0.427**	**0.724**	0.343					
	0.098	**0.724**	-0.109					
Kelantan	**0.307**	**0.320**	0.262	0.182				
	0.034	**0.320**	0.077	0.182				
Kinjang	**0.365**	**0.235**	**0.350**	**0.357**	0.008			
	0.186	**0.235**	0.272	**0.357**	0.008			
Taiping	**0.428**	**0.274**	**0.416**	**0.481**	0.152	-0.060		
	0.319	**0.274**	**0.416**	**0.481**	0.152	-0.060		
Kedah	**0.309**	**0.401**	**0.322**	**0.225**	0.023	0.054	**0.144**	
	0.081	**0.401**	0.153	**0.225**	0.023	0.054	**0.144**	
Kinta	**0.416**	**0.640**	**0.353**	-0.025	0.041	**0.260**	**0.409**	**0.163**
	0.049	**0.640**	-0.122	-0.025	0.041	**0.260**	**0.409**	**0.163**

AMOVA analysis was conducted based on the corrected cpDNA data (i.e., without Langat-type) to assess the population structures among *Gigantochloa* species based on the following groupings:

Structure (a): Based on species

Group 1 (*G. scortechinii*) = KKB, Janda Baik, Serendah, Gabai, Kelantan;
Group 2 (*G. wrayi*) = Kinjang, Taiping;
Group 3 (*G. ligulata*) = Kedah, Kinta

Structure (b): Based on geographical distribution
Group 1 (Selangor and Pahang) = KKB, Janda Baik, Serendah, Gabai; Group 2 (Kelantan) = Kelantan;
Group 3 (Perak) = Kinjang, Taiping, Kinta;
Group 4 (Kedah) = Kedah

Table 4.12 and 4.13 displays the outcome of AMOVA analysis. The F_{SC} (among populations within group) and F_{ST} (among groups) values were significant for both hypothesized structures. However, the F_{CT} (within populations) values for both hypothesized structures were not significant. Negative variance in F_{CT} (within populations) components have been detected in AMOVA analysis (Table 4.13) because what have been calculated for the AMOVA analyses were covariance. Negative variance indicates the presence of non-genetic structure.

Table 4.12: Analysis of molecular variance (AMOVA) based on the corrected (i.e., without Langat-type) cpDNA data. The grouping was hypothesized based on the *Gigantochloa* species (Group 1 = *G. scortechinii*; Group 2 = *G. wrayi*; Group 3 = *G. ligulata*).

Source of variation	**df**	**Sum of squares**	**Variance components**	**Percentage variation**	**Fixation indices*, *F***	**Significant test, *P***
Among groups (F_{ST})	2	31.729	0.148	3.516	0.284	0.000
Among populations within groups (F_{SC})	6	63.652	1.050	24.923	0.258	0.000
Within populations (F_{CT})	61	183.833	3.014	71.560	0.035	0.162
Total	69	279.214	4.211			

***Among groups** – F_{ST}; **Among populations within group** – F_{SC}; **Within populations** - F_{CT}

Table 4.13: Analysis of molecular variance (AMOVA) based on the corrected (i.e., without Langat-type) cpDNA data. The grouping was hypothesized based on the geographical distribution (Group 1 = populations located in central Peninsular Malaysia; Group 2 = populations located in Kelantan; Group 3 = populations located in Perak; Group 4 = population located in Kedah).

Source of variation	**df**	**Sum of squares**	**Variance components**	**Percentage variation**	**Fixation indices*, *F***	**Significant test, *P***
Among groups (F_{ST})	3	19.098	-0.66075	-16.422	0.251	0.000
Among populations within groups (F_{SC})	5	76.283	1.671	41.520	0.357	0.000
Within populations (F_{CT})	61	183.833	3.014	74.902	-0.164	1.000
Total	69	279.214	4.023			

***Among groups** – F_{ST}; **Among populations within group** – F_{SC}; **Within populations** - F_{CT}

CHAPTER 5

DISCUSSION

5.1 Chloroplast DNA Differentiation in *Gigantochloa scortechinii* based on PCR-RFLP

The existence of two distinct types of chloroplast DNA (cpDNA) haplotypes of *Gigantochloa scortechinii* in the present study were mentioned earlier in the phylogenetic and systematics studies of the Bambusinae subtribe (Goh, et al., 2011; Goh, et al., 2013). The phenotypic traits of *G. scortechinii* are uniform and easily distinguishable in the field (Figure 5.1). It displays a shade of bright orange near the top of the culm sheaths and covered by dark appressed hairs on the adaxial surface of white waxy young culm internodes (Holttum, 1958; Wong, 1995a).

Figure 5.1: *Gigantochloa scortechinii* - Culm sheaths green at the base and flushed intense orange towards the top.

Bamboos trees of the two different cpDNA haplotypes in Peninsular Malaysia were identical morphologically, which suggests the occurrence of chloroplast capture (Goh, et al., 2011). Chloroplast capture happens due to possible introgression, i.e., backcrossing of offspring to the parents through a parent's pollen grains following intra-generic and/or inter-generic hybridization (Riesberg and Soltis, 1991; Wolfe and Elisens, 1995; Van Raamsdonek, et al., 1997; Kornkven, et al., 1999). Chloroplast capture is not uncommon in plants as it has been reported for different plant groups, e.g., *Gossypium* (Wendel and Albert, 1992), *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) and *Nothofagus* (Acosta and Premoli, 2010), *Osmorhiza* (Yi, et al., 2015), as well as in the North American bamboos, *Arundinaria tecta* and *A. appalachiana* (Triplett, et al., 2010).

Normally, interspecific fertilization happens when the parental seed is low in frequency and permits the competition between the pollen of same species and pollen of invader species (Rieseberg, 1995). It is also estimated that the initial number of the invader species should be low. Among the three populations with both Gombak- and Langat-type cpDNA, the Serendah and Sungai Gabai Waterfall populations showed higher numbers of Gombak-type cpDNA haplotype compared to those of the Langat-type. Meanwhile, Kuala Kubu Bharu and Serendah sampling sites documented relatively higher percentage of the Langat-type cpDNA. The Langat-type cpDNA appears to be introduced from other species.

Overall, among the 33 specimens subjected to the preliminary screening of PCR-RFLP, the Gombak-type cpDNA is found to be great in number compared to that of the Langat-type cpDNA (60.7 % vs. 39.3 %), signifying that the Gombak-type is the dominant cpDNA genotype for *G. scortechinii*.

Anthropogenic factors could have possibly contributed to the cpDNA introgression in *G. scortechinii*. Compared to the specimens collected at Sungai Gabai Waterfall and Janda Baik regions, the sampling sites of both Kuala Kubu Bharu and Serendah appeared to be more disturbed due to current housing development and construction. The specimens from Serendah were gathered along an aboriginal settlement while the samples from Kuala Kubu Bharu were gathered along the roadside where Kuala Kubu Bharu dam is located.

5.2 Hybridization in *Gigantochloa*

Two putative hybrids were identified during the study, i.e., DS117 (possible intergeneric hybrid of *G. ligulata* and *M. montana*) found at the roadside of Penang Western Hill roadside on 04/10/2015 and DS120 (possible interspecific hybrid of *G. latifolia* and *G. ligulata*) found at Sintok, Kedah on 07/10/2015 based on the intermediate morphological characters (Table 4.8). As proposed previously by several authors (Anderson, 1949; Wilson, 1992; Riesberg, 1995; Rieseberg, et al., 2007), the hybrids can be recognized based on their morphological intermediate traits or "character coherence" which match to one or other parental

species. It is also stated that morphological evidence can be used as a reliable indicator of the hybridization patterns in plants (Cronn and Wendel, 2004). The hybrids in the present study are expected to be F1 offspring based on the allelic heterozygosity in the partial *GBSS*I and *PabpI* genes.

Based on the morphological observations (Table 4.8), possible hybrid DS117 shows intermediate traits, e.g., typical character states of parents, culm sheath blades that spread at the lower and middle of the culms (*G. ligulata*), culm habit of flopping over adjacent plants in hybrid (*M. montana*) and non-typical character states that are not found in both parents, i.e., green with a few yellow stripes and scattered black hairs at the upper part of the internodes in hybrids. The putative parental species were further supported by the molecular data of possible hybrids clones, i.e., the number of indels and variable characters (Table 4.9), MP trees and Bayesian trees (Figure 4.3 and 4.4). Furthermore, the hybrid DS117 was collected in Penang Hill, where *M. montana*, a lower montane species native to Peninsular Malaysia (Holttum, 1958; Wong, 1995a), was also recorded.

The cpDNA phylogenetic topology (Figure 4.3) recovered one major clade that comprises *D. pendulus*, the putative hybrid DS120 (Haplotype 25) with other *Gigantochloa* species (*G. balui*, *G. latifolia* and *G. scortechinii*) and another clade consisting of *M. montana*, the putative hybrid DS117 (Haplotype 10) with other *Gigantochloa* species (*G. apus*,

G. atter, *G. manggang* and *G. scortechinii*). This shows that *M. montana* could be the possible chloroplast donor for the putative hybrid DS117. Meanwhile, the possible hybrid DS120 also displayed intermediate characteristics (Table 4.8), i.e., culm sheaths with erect blades (typical to *G. latifolia*) along with lacerate ligules (typical to *G. ligulata*), and non-typical traits that are not found in both parents, such as culms that are broadly arched nearly to the ground. However, the morphological character states (Table 4.9) and molecular data in this study were unable to give a firm conclusion in identifying the parental species of the putative hybrid DS120. This is because the putative hybrid DS120 showed morphological characters of both *G. ligulata* and *G. latifolia*, but from the molecular study (Figure 4.3 and 4.4), one of its clones was clustered with the *Gigantochloa* clade (DS120 clone A) indicating that most *Gigantochloa* taxa used in this study are equally closely related to the DS120. Another clone (DS120 clone B) was associated with *Dendrocalamus* taxa but only with moderate support (0.97PP/ - BP).

Even though there are morphologically intermediate traits identified between the two possible hybrids, DS117 and DS120, a large data set of morphological characteristics is required for precise parental identification of hybrids and to investigate the phenotypic correlation of the putative hybrids with parental species (Anderson, 1949; Wilson, 1992). Morphological-based parental identification for hybrids can be difficult because some of the intermediacy traits occur due to several other reasons

such as the retention of ancestral polymorphisms and incomplete lineage sorting (Rieseberg, 1995; Judd, et al., 2002; Arnold, 2006).

The inferences on the possibility of hybridization in the present study support the findings in previous studies that suggested widespread introgressive hybridization within Bambusinae (Goh, et al., 2010; Goh, et al., 2013). In fact, other cpDNA studies (Yang, 2008; Sungkaew, et al., 2009; Yang, et al., 2010) could not resolve *Gigantochloa* into a single lineage. For instance, Sungkaew, et al. (2009), utilizing multi-locus chloroplast markers, could recover two *Gigantochloa* taxa in two clades together with the *Bambusa* and *Dendrocalamus* genera. It is possible that incongruences among cpDNA-based topologies in earlier studies of Southeast Asian woody bamboos (Yang, et al. 2008; Sungkaew, et al. 2009; Yang, et al., 2010; Goh, et al., 2013) could be partially due to chloroplast capture. Increasing the sampling size of the possible hybrids and their parental species in Peninsular Malaysia regions is expected to provide more useful insight of the introgressive hybridization complexity in *Gigantochloa* taxa. Prior to the advances of molecular techniques, hybridization in *Gigantochloa* was suggested by Holttum (1958), with reference to the hybrid swarms in the northern Malaya, and by Mueller (1998, 2003), with reference to *G. ridleyi* that produced anomalous F2 progenies. Overall, the result of the present study shows that the intraspecific cpDNA variation in *Gigantochloa* can be wide and incidences of hybridization could be more than what was thought

previously. All these need to be taken into consideration when assessing the phylogenetic relationships of *Gigantochloa* and its closely related taxa.

5.3 Population Structure of *Gigantochloa*

5.3.1 Species Boundaries and Geographical Structure

The AMOVA results (Table 4.12 and 4.13) explained that the among group and among populations within groups fixation index (F_{ST} and F_{SC}) for both hypothesized structures (a) species boundaries and (b) geographical distribution are significant, but the within populations fixation index (F_{CT}) is not significant and the variance values of within populations (F_{CT}) for structure (b) found to be negative (Table 4.13).

This shows that hypothesized structure (a) is more acceptable for *Gigantochloa* taxa. Structure (a) is the grouping based on the three species as identified from their morphological characters but are not distinctive in the phylogenetic trees (Figure 4.3 and 4.4). This study also shows that the three *Gigantochloa* species are closely related in which some of them share common haplotypes (Table 4.4) However, the population structure analysis still support the differentiation among the three *Gigantochloa* species.

One possible explanation for the differentiation among the three *Gigantochloa* species is possibly due to gene flow restriction. Most of the three *Gigantochloa* species are identified to occur in the foothills of mountains, e.g., Fraser Hill roadside, Kuala Kubu Bharu (*G. scortechinii*),

Gua Musang, Kelantan (*G. scortechinii*), Janda Baik, Pahang (*G. scortechinii*), Penang Western Hill, Penang (*G. wrayi*), and some are at forest reserves, beside streams and rivers areas, e.g., Gabai waterfall, Hulu Langat (*G. scortechinii*), Lata Kinjang, Perak (*G. wrayi*), Taiping, Perak (*G. wrayi*) and Ulu Kinta, Perak (*G. ligulata*), whereas some are found at low-land roadsides, e.g., Sekeping Serendah Retreat, Serendah (*G. scortechinii*), Rantau Panjang, Kelantan (*G. scortechinii*) and Sintok, Kedah (*G. ligulata*). Therefore, such geographical background probably restrict, but not completely prevent, gene flow and contribute to limited gene flow and high level differentiation among the three *Gigantochloa* species.

5.3.2 Uniqueness of Janda Baik Population

Based on the pairwise F_{ST} analyses, the *Gigantochloa* population in Janda Baik is found to be significantly different from all other populations (Table 4.11) after the Langat-type individuals are removed. The genetic differences between Janda Baik and other populations possibly occur due to their bio-geographical ranges and anthropogenic effects. Geographic range may play a partial role in the estimation of genetic variations among plant populations (Hamrick, et al., 1992).

Janda Baik, an ecological island with a cool, breezy climate, is located within a small valley adjacent to an untouched natural tropical rainforest that is bounded by waterfalls and streams. The bio-geographical isolation of Janda Baik from other continuously distributed sampling populations,

possibly allows Janda Baik to have significant genetic differentiation and increase the genetic diversity level among the *Gigantochloa* populations found there.

Janda Baik is a very well-known popular recreational destination for locals and this place has not fully undergone alterations in terms of structure, distribution and natural ecosystem functioning for tourism. Conversely, other *Gigantochloa* populations in the study are suspected to have undergone greater anthropogenic effects compared to Janda Baik as more human activities are observed, e.g, housing area construction, recreational parks establishment, deforestation, and land clearance, which transform the natural areas into anthropogenic landscapes. Since there has been no specific records or accurate data on the effects of past anthropogenic activities on the *Gigantochloa* taxa, the results of the pairwise Pairwise F_{ST} makes it interesting for extra investigations to be conducted particularly on the distribution mapping and modelling of bio-geographical aspects, that would involve a wider *Gigantochloa* taxon sampling in Peninsular Malaysia.

5.3.3 Chloroplast DNA versus Nuclear DNA

The population structure of *Gigantochloa* in this study was estimated based on cpDNA gene sequences. Chloroplast DNA (cpDNA) is broadly applied by plant evolutionary biologists for different reasons, including as a method to infer plant phylogenetics at various taxonomic level studies (Olmstead and Palmer, 1994; Kelchner, 2000; Wolfe and Randle, 2004)

and as a tool for population structure and phylogeography studies (Ennos, 1994; McCauley, 1995; Ouborg, et al., 1999; Provan, et al., 2001; Petit, et al., 2005).

The phylogenetic relationship study (Figure 4.3 and 4.4) was not sufficiently informative to distinguish the inter-specific relationship among the three *Gigantochloa* species. The occurrence of Haplotype 5 in the three *Gigantochloa* species (*G. ligulata, G. scortechinii* and *G. wrayi*) further showed that the species-specific delimitation was not well defined probably due to insufficient variability of the cpDNA marker. This is also possibly due to some drawbacks of cpDNA for molecular ecology assessments. Normally, the uniparental mode of inheritance for haploid markers like cpDNA might not be completely representative of populations. Furthermore, their relatively small effective sizes might cause the genetic diversity of chloroplasts to be lost more rapidly than nuclear diversity following either permanent or temporary reductions in population size (Vettori, et al., 2004). This loss of diversity in cpDNA might cause the oversimplification of the population history of an organism or underrate its genetic diversity (Vettori, et al., 2004).

Factors such as conservative evolution, introgressive hybridization, reticulate evolution and incomplete lineage sorting (Soltis and Soltis, 1998; Wendel and Doyle, 1998; Sang, 2002; Small, et al., 2004) also possibly decreased the effectiveness of cpDNA and potentially affect chloroplast-based phylogenetic inference. As a result of the suggested

cpDNA-inference contributing factors along with possible introgressive hybridization (Rieseberg and Brunsfield, 1992; Rieseberg and Wendel, 1993; Goh, et al., 2011) and reticulate evolution (Avise, et al., 1987; Pamilo and Nei, 1988) that is common in the BDG complex (Goh, et al., 2013), the *Gigantochloa* species in this study could not be clearly and completely distinguished. Hence chloroplast sequence data should always be combined with other sequences to achieve sufficient resolution in order to construct a robust phylogeny for plants.

The outcome of the study also demonstrates that nuclear *GBSS*I marker appears to be more useful than cpDNA in showing the generic boundary when compared to the closely related *Bambusa* and *Dendrocalamus* genera. Within Clade 3 (Figure 4.4), there was no clear species delination except for *Gigantochloa scortechinii* (0.91 / - BP). Earlier investigations conducted by Yang, et al. (2008) and Yang, et al. (2010) also noted that the *GBSS*I gene data contributed greatly to the results due to less informative 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).

These evidences further indicate that evaluations using nuclear DNA markers might to better reflect the larger distinctive phylogenetic characteristics and thus lay a significant foundation in understanding the systematics of the woody bamboos. Recent investigations on other bamboos based on nuclear markers have already exhibited such

systematic evidence and a more comprehensive employment of nuclear markers seems to be much better approach (Sun, et al., 2005; Yang, et al., 2007; Yang, et al., 2008; Goh, et al., 2010; Yang, et al., 2010; Goh, et al., 2013; Chokthaweepanich, 2014).

CHAPTER 6

CONCLUSIONS

6.1 Conclusions

In the present work, PCR-based restriction fragment length polymorphism (RFLP) marker that can produce different RFLP profiles for the two chloroplast DNA (cpDNA) lineages, the Gombak- and Langat-type within *G. scortechinii* was developed. The Gombak-type was the dominant cpDNA genotype for *G. scortechinii* in Peninsular Malaysia. The chloroplast haplotype analysis also highlighted the occurrence of two cpDNA clades, i.e., the Langat-type and Gombak-type, and the Haplotype 5 is found to be the most widespread which is shared among the three *Gigantochloa* species. PCR-RFLP still an applicable preliminary screening and cost-effective technique for population genetic screening or low-level systematic studies that usually involve a large number of samples. It is clear that PCR-based restriction fragment length polymorphism (RFLP) can be utilized to develop other RFLP markers for other plant studies in future.

The present work further addresses the phylogenetic relationships among the three *Gigantochloa* species by utilizing data from two chloroplast DNA markers, *rps16-trnQ* and *trnD-T* intergenic spacers, and two nuclear DNA markers, *GBSS*I and *PabpI,* along with some other related species. Bayesian Inference (BI) and Maximum Parsimony (MP) analyses

recovered two major clades based on cpDNA: Clade 1 consisting of members of Gombak-type haplotype and Clade 2 consisting of Langat–type haplotype. The phylogenetic relationship studies further support the existence of two different haplotypes in the *Gigantochloa* species chloroplast DNA as mentioned by Goh, et al. (2011). Based on the results, *M. montana* appears to be the possible chloroplast donor for the putative hybrid DS117.

The nuclear DNA topologies recognized three major clades: Clade 1 consisting of members of *Dendracalamus pendulus, Dendrocalamus strictus* and a putative hybrid DS120 clone B; Clade 2 comprising *Mullerochloa montana* and a putative hybrid DS117 clone B; and Clade 3 consisting of all *Gigantochloa* species except *G. atter* and two putative hybrids, DS117 clone A and DS120 clone A. The hybrids in the present study were identified based on the morphological characteristics and the allelic heterozygosity in the partial *GBSS*I and *PabpI* genes. The suspected hybrid DS117 displays the morphological characters of both *G. ligulata* and *M. montana* and is found in Penang Hill where both species were recorded. The suspected hybrid DS120 exhibited characteristics of *G. ligulata* and *G. latifolia*, but from the molecular study, one clone was clustered with the typical *Gigantochloa* clade and another clone was clustered with the *Dendrocalamus* clade (with moderate support). The possible existence of hybridization between *Gigantochloa* and *Maclurochloa* and the molecular outcome in the present study shows the widespread introgressive hybridization in the

Gigantochloa genus, and supports the recommendations by Holttum (1958) and Muller (1998) of the existence of bamboo hybrid swarms.

The population structure of *Gigantochloa* in the present work was determined based on cpDNA dataset. Population genetic structure analysis provides better resolution in distinguishing the intra-generic boundaries between the three *Gigantochloa* species. AMOVA analysis based on the cpDNA displayed significant fixation index (F_{ST}) value among *Gigantochloa* populations than within populations, thus supporting the differentiation among the three *Gigantochloa* species. Pairwise F_{ST} analysis suggest a strong genetic isolation of the *G. scortechinii* in the Janda Baik sampling site. The genetic isolation in Janda Baik could have occurred due to their bio-geographical ranges and anthropogenic factors.

In the present study, chloroplast DNA markers seemed to be unable to resolve the phylogenetic relationships and are not reliable for plant molecular and systematics studies due to some disadvantages of its own properties and widespread introgression events within the BDG (Goh, et al., 2013). Earlier investigations and the current study show that nuclear *GBSS*I marker appears to be more informative than cpDNA in delineating the generic boundary of *Gigantochloa* taxa when compared to the closely related genera.

6.2 Future Studies

The present work shows the complexity present among the three *Gigantochloa* species of Peninsular Malaysia and support the previous studies (Goh, et al., 2011; Goh, et al., 2013) that introgressive hybridization and incomplete lineage sorting are possible underlying causes for this complexity. More samples of *Gigantochloa* taxa, and more cpDNA and nuclear DNA markers are required to unravel the complexity of past hybridization as well as provide clearer relationships among the *Gigantochloa* species. Furthermore, different molecular techniques should be considered as it may help to provide increased insights into the population genetic complexity of *Gigantochloa*.

As the population structure analysis recognized strong genetic isolation for one of the *Gigantochloa* population, more distribution mapping and modeling bio-geographically investigations should be conducted to understand further the role and effects of biogeographical and anthropogenic factors in the population genetics of *Gigantochloa*.

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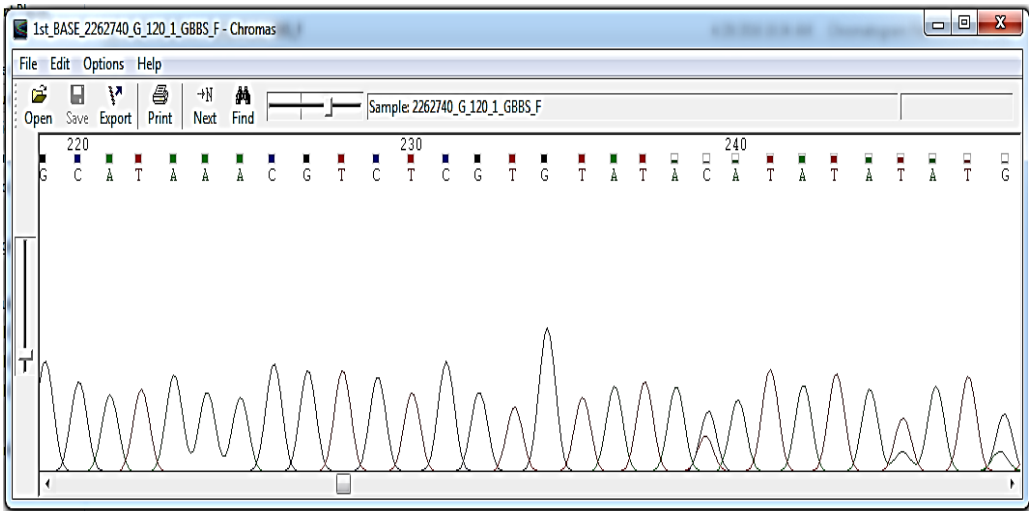
APPENDICES

APPENDIX A: Protocols of PCR DNA Fragments Purification using Geneaid Gel/PCR DNA Fragments Extraction Kit (Axon Scientific Sdn.Bhd., Malaysia).

1. Transfer up to 100 µl of reaction product to a 1.5 microcentrifuge tube.
2. Add 5 volumes of DF Buffer to 1 volume of the sample and mix by vortex.
3. Place a DF Column in a 2 ml Collection Tube.
4. Transfer the sample mixture to the DF Column.
5. Centrifuge at 14-16,000 x g for 30 seconds.
6. Discard the flow-through then place the DF Column back in the 2 ml Collection Tube.
7. Add 600 µl of Wash Buffer (make sure ethanol was added) into the center of the DF Column.
8. Let stand for 1 minute at room temperature.
9. Centrifuge at 14-16,000 x g for 30 seconds.
10. Discard the flow-through and place the DF Column back in the 2 ml Collection Tube.
11. Centrifuge for 3 minutes at 14-16,000 x g to dry the column matrix.
12. Transfer the dried DF Column to a new 1.5 ml microcentrifuge tube.
13. Add 20-50 µl of Elution Buffer or TE into the CENTER of the column matrix.
14. Let stand for at least 2 minutes to ensure the Elution Buffer is completely absorbed.
15. Centrifuge for 2 minutes at 14-16,000 x g to elute the purified DNA. NOTE: Using pre-heated Elution Buffer (60ºC) is recommended for eluting DNA fragments >5kb.

APPENDIX B: Double peaks in the DNA chromatogram showing three dimorphic sites (the *GBSSI* sequence of possible hybrid DS120 as an example).



APPENDIX C: Procedure of QIAGEN PCR Cloning Kit.

i) Ligation

1. Thaw 2x Ligation Master Mix, pDrive Cloning Vector DNA, and distilled water (provided). Place on ice after thawing. It is important to mix the solutions completely before use to avoid localized concentrations of salts. Keep 2x Ligation Master Mix on ice and immediately store at –15 to –30°C or –70°C after use.

2. Prepare a ligation-reaction mixture according to the following table:

Component Volume/reaction	Component Volume/reaction
pDrive Cloning Vector (50 ng/µl)	1 µl
PCR product	1–4 µl*
Distilled water	variable
Ligation Master Mix, 2x†	5 µl
Total volume	10 µl

* Purified PCR product. If using non-purified PCR product, do not add more than 2 µl PCR product.
† We recommend adding the Ligation Master Mix last.

3. Briefly mix the ligation-reaction mixture then incubate for 30 min at 4–16°C (e.g., in a refrigerator, water bath, or thermal cycling block). Mix gently, for example by pipetting the ligation-reaction mixture up and down a few times.
4. Proceed with the "Transformation Protocol" (page16) or store ligation reaction mixture at –15 to –30°C until use.

ii) Transformation

Important notes before starting:

- This protocol is for use with QIAGEN EZ Competent Cells. It is not for use with electro competent cells. If electro competent cells will be used, we strongly recommend inactivating the ligase in the ligation-reaction mixture prior to electroporation. See step 4 of the "Ligation Protocol" for details.
- Competent cells are extremely sensitive to temperature and mechanical stress. Do not allow QIAGEN EZ Competent Cells to thaw at any point prior to transformation. Keep thawed cells on ice.

Avoid excessive and/or rough handling, especially pipetting. Mix cells by gentle flicking.

- Thaw SOC medium and warm to room temperature. Store at –15 to –30°C or –70°C after use.
- Prepare fresh LB agar plates containing either ampicillin (100 µg/ml LB agar) or kanamycin (30 µg/ml LB agar) as a selection marker. Include IPTG (50 µM) and X-gal (80 µg/ml) for blue/white screening of recombinant colonies. Procedure

1. Thaw the appropriate number of tubes of QIAGEN EZ Competent Cells on ice. Thaw SOC medium and warm to room temperature.

   IMPORTANT: Competent cells should only be thawed on ice. Do not allow unused QIAGEN EZ Competent Cells to thaw. Test whether cells are thawed by gently flicking the tube. Proceed immediately to the transformation step once the cells have thawed.

2. Add 1–2 µl ligation-reaction mixture per tube of QIAGEN EZ Competent Cells, mix gently, and incubate on ice for 5 min. Mix gently, for example by flicking the transformation mixture a few times.
3. Heat the tube(s) in a 42°C water bath or heating block for 30 s without shaking.
4. Incubate the tube(s) on ice for 2 min.
5. Add 250 µl room temperature SOC medium per tube and directly plate 100 µl each transformation mixture onto LB agar plates containing ampicillin.

   Note: For kanamycin selection, incubate the cells at 37°C for 30 min with shaking prior to plating to allow recombinant outgrowth. The transformation mixture can be plated using a sterile bent glass rod or a specialized spreader. It is generally recommended to plate different amounts of each transformation mixture onto separate plates (e.g., 100 µl and 20 µl) to ensure good separation of colonies for subsequent single colony isolation. For more efficient plating of small volumes of transformation mixture (<50 µl) we recommend pipetting 100 µl LB medium onto the plate, and then pipetting the transformation mixture into the liquid LB.

6. Incubate the plate at room temperature until the transformation mixture has absorbed into the agar. Invert the plate and incubate at 37°C overnight (e.g., 15–18 h).

Note: For blue/white screening, we recommend a second incubation at 4°C (e.g., in a refrigerator) for a few hours. This “cold” incubation step enhances blue color development and thereby facilitates differentiation between blue colonies and white colonies.

APPENDIX D: Bayesian Inference (BI) analyses command files.

(a) Chloroplast DNA

```
BEGIN PAUP;
Set INCREASE=AUTO;
outgroup H_magica K_wrayi;
END;
begin mrbayes;
log start replace;
set autoclose = no nowarn=yes;
lset nst=2 rates=gamma;
unlink revmat=(all) shape=(all) pinvar=(all) statefreq=(all) tratio=(all);
mcmc ngen=1000000 printfreq=1000 samplefreq=100 nchains=4 temp=0.2 savebrlens=yes;
sumt burnin=2500 contype=halfcompat;
log stop;
end
```

(b) Nuclear DNA

```
BEGIN PAUP;
Set INCREASE=AUTO;
outgroup H_magica K_wrayi;
END;
begin mrbayes;
log start replace;
set autoclose = no nowarn=yes;
lset nst=2 rates=gamma;
unlink revmat=(all) shape=(all) pinvar=(all) statefreq=(all) tratio=(all);
mcmc ngen=1000000 printfreq=1000 samplefreq=100 nchains=4 temp=0.2 savebrlens=yes;
sumt burnin=2500 contype=halfcompat;
log stop;
end
```

APPENDIX E: Maximum Parsimony (MP) analyses log files.

(a) Chloroplast DNA

```
P A U P *
Version 4.0b10 for 32-bit Microsoft Windows
Sat Jan 07 13:18:37 2017

Processing of file "C:\Users\wlgoh\Desktop\PUB_Data_Analyses\16Q_DT.nex" begins...

Data read in DNA format

Data matrix has 43 taxa, 1571 characters
Valid character-state symbols: 01ACGT
Missing data identified by '?'
Gaps identified by '-'
"Equate" macros in effect:
  R,r ==> {AG}
  Y,y ==> {CT}
  M,m ==> {AC}
  K,k ==> {GT}
  S,s ==> {CG}
  W,w ==> {AT}
  H,h ==> {ACT}
  B,b ==> {CGT}
  V,v ==> {ACG}
  D,d ==> {AGT}
  N,n ==> {ACGT}

Outgroup status changed:
 2 taxa transferred to outgroup
 Total number of taxa now in outgroup = 2
 Number of ingroup taxa = 41

Processing of file "C:\Users\wlgoh\Desktop\PUB_Data_Analyses\16Q_DT.nex" completed.

Heuristic search settings:
 Optimality criterion = parsimony
```

Character-status summary:
Of 1571 total characters:
All characters are of type 'unord'
All characters have equal weight
494 characters are constant
42 variable characters are parsimony-uninformative
Number of parsimony-informative characters = 35
Gaps are treated as "missing"
Starting tree(s) obtained via stepwise addition
Addition sequence: random
Number of replicates = 100
Starting seed = 2008966313
Number of trees held at each step during stepwise addition = 1
Branch-swapping algorithm: tree-bisection-reconnection (TBR)
Steepest descent option not in effect
Initial 'MaxTrees' setting = 100 (will be auto-increased by 100)
Branches collapsed (creating polytomies) if maximum branch length is zero
'MulTrees' option not in effect; only 1 tree will be saved per replicate
Topological constraints not enforced
Trees are unrooted

Heuristic search completed
  Total number of rearrangements tried = 3607043
  Score of best tree(s) found = 115
  Number of trees retained = 22
  Time used = 0.58 sec

Tree-island profile:

Island	First Size*	Last tree	First tree	First Score	Times replicate	hit
1	1	1	1	115	5	1
2	1	2	2	115	9	1
3	1	3	3	115	19	1
4	1	4	4	115	22	1
5	1	5	5	115	25	1
6	1	6	6	115	27	1
7	1	7	7	115	35	1
8	1	8	8	115	48	1
9	1	9	9	115	50	1
10	1	10	10	115	58	1
11	1	11	11	115	60	1
12	1	12	12	115	66	1
13	1	13	13	115	69	1
14	1	14	14	115	75	1
15	1	15	15	115	76	1
16	1	16	16	115	77	1
17	1	17	17	115	80	1
18	1	18	18	115	83	1
19	1	19	19	115	85	1
20	1	20	20	115	92	1
21	1	21	21	115	95	1
22	1	22	22	115	97	1
23	1	-	-	116	1	62**
24	1	-	-	117	15	14**
25	1	-	-	118	17	2**

Note(s):

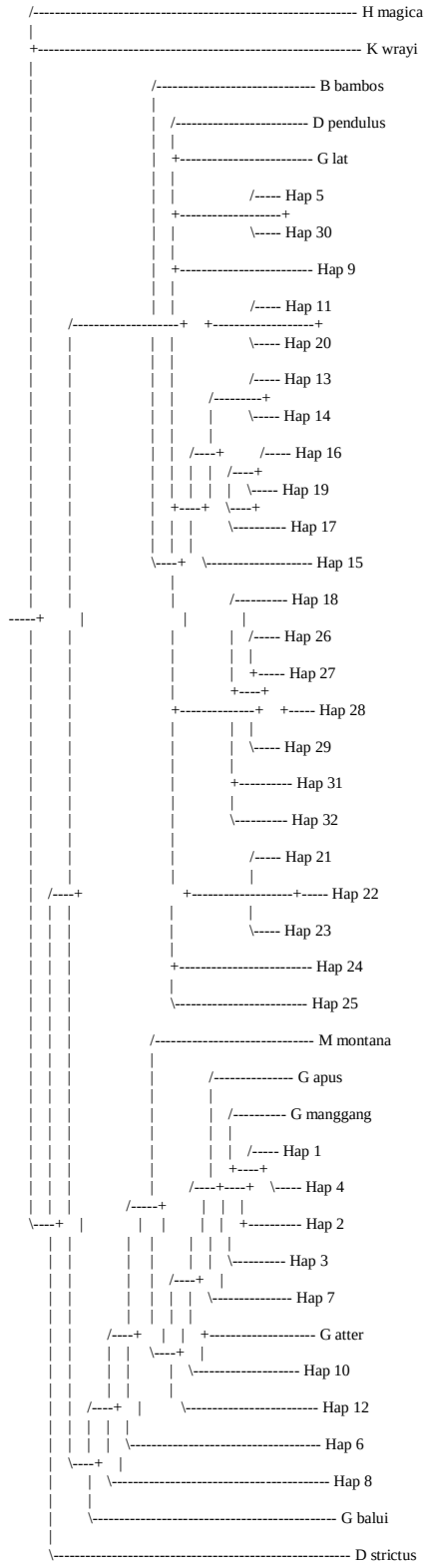
* Only one tree was saved per island; island structure is undetermined

** Multiple observations of the same score do not imply identity of the corresponding trees

22 trees converted from unrooted to rooted.

22 trees saved to file
"C:\Users\wlgoh\Desktop\PUB_Data_Analyses\16Q_DT.tre"

Tree number 1:

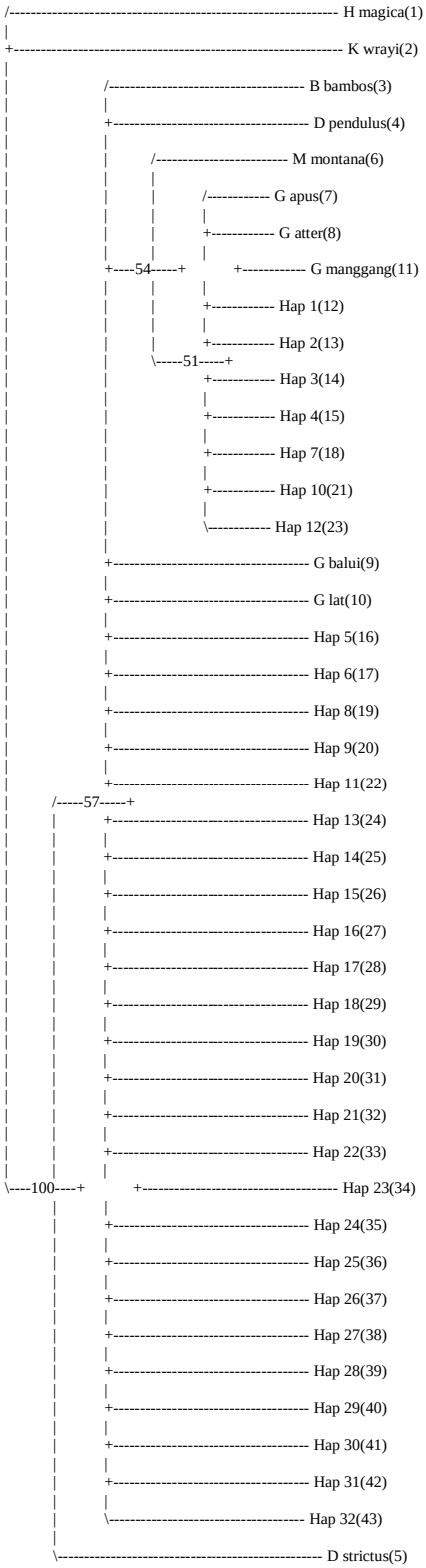


Bootstrap method with heuristic search:
Number of bootstrap replicates = 1000

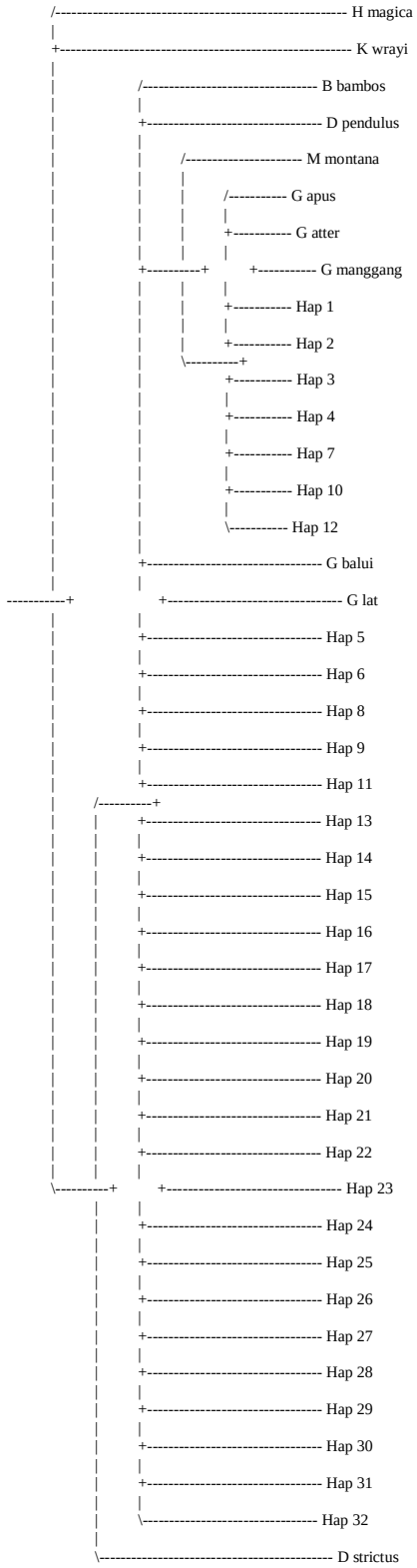
Starting seed = 1399370041
Optimality criterion = parsimony
Character-status summary:
Of 1571 total characters:
All characters are of type 'unord'
All characters have equal weight
1494 characters are constant
42 variable characters are parsimony-uninformative
Number of parsimony-informative characters = 35
Gaps are treated as "missing"
Starting tree(s) obtained via stepwise addition
Addition sequence: random
Number of replicates = 100
Starting seed = 1954681740
Number of trees held at each step during stepwise addition = 1
Branch-swapping algorithm: tree-bisection-reconnection (TBR)
Steepest descent option not in effect
Initial 'MaxTrees' setting = 100 (will be auto-increased by 100)
Branches collapsed (creating polytomies) if maximum branch length is zero
'MulTrees' option not in effect; only 1 tree will be saved per replicate
Topological constraints not enforced
Trees are unrooted

1000 bootstrap replicates completed
Time used = 00:11:31.4

Bootstrap 50% majority-rule consensus tree



Strict consensus of 22 trees:



(c) Nuclear DNA

```
P A U P *
Version 4.0b10 for 32-bit Microsoft Windows
Sat Jan 07 13:35:51 2017

      ----------------------------NOTICE---------------------------
        This is a beta-test version.  Please report any crashes,
        apparent calculation errors, or other anomalous results.
        There are no restrictions on publication of results obtained
        with this version, but you should check the WWW site
        frequently for bug announcements and/or updated versions.
        See the README file on the distribution media for details.
      -------------------------------------------------------------

Processing of file "C:\Users\wlgoh\Desktop\PUB_Data_Analyses\GBSS_Pabp.nex"
begins...

Data read in DNA format

Data matrix has 27 taxa, 1095 characters
Valid character-state symbols: 01ACGT
Missing data identified by '?'
Gaps identified by '-'
"Equate" macros in effect:
  R,r ==> {AG}
  Y,y ==> {CT}
  M,m ==> {AC}
  K,k ==> {GT}
  S,s ==> {CG}
  W,w ==> {AT}
  H,h ==> {ACT}
  B,b ==> {CGT}
  V,v ==> {ACG}
  D,d ==> {AGT}
  N,n ==> {ACGT}

Outgroup status changed:
  2 taxa transferred to outgroup
  Total number of taxa now in outgroup = 2
  Number of ingroup taxa = 25

Processing of file "C:\Users\wlgoh\Desktop\PUB_Data_Analyses\GBSS_Pabp.nex"
completed.
```

Heuristic search settings:
Optimality criterion = parsimony
Character-status summary:
Of 1095 total characters:
All characters are of type 'unord'
All characters have equal weight
992 characters are constant
50 variable characters are parsimony-uninformative
Number of parsimony-informative characters = 53
Gaps are treated as "missing"
Multistate taxa interpreted as uncertainty
Starting tree(s) obtained via stepwise addition
Addition sequence: random
Number of replicates = 100
Starting seed = 839574727
Number of trees held at each step during stepwise addition = 1
Branch-swapping algorithm: tree-bisection-reconnection (TBR)
Steepest descent option not in effect
Initial 'MaxTrees' setting = 100 (will be auto-increased by 100)
Branches collapsed (creating polytomies) if maximum branch length is zero
'MulTrees' option not in effect; only 1 tree will be saved per replicate
Topological constraints not enforced
Trees are unrooted

Heuristic search completed
Total number of rearrangements tried = 777731
Score of best tree(s) found = 130
Number of trees retained = 30
Time used = 0.22 sec

Tree-island profile:

Island	First Size*	Last tree	tree	First Score	Times replicate	hit
1	1	1	1	130	2	1
2	1	2	2	130	4	2
3	1	3	3	130	6	1
4	1	4	4	130	10	2
5	1	5	5	130	13	3
6	1	6	6	130	14	3
7	1	7	7	130	15	2
8	1	8	8	130	19	1
9	1	9	9	130	25	1
10	1	10	10	130	29	3
11	1	11	11	130	35	2
12	1	12	12	130	36	2
13	1	13	13	130	37	2
14	1	14	14	130	40	1
15	1	15	15	130	42	2
16	1	16	16	130	43	1
17	1	17	17	130	46	2
18	1	18	18	130	56	1
19	1	19	19	130	58	1
20	1	20	20	130	59	1
21	1	21	21	130	60	1
22	1	22	22	130	61	1
23	1	23	23	130	62	1
24	1	24	24	130	63	1
25	1	25	25	130	69	1
26	1	26	26	130	71	1
27	1	27	27	130	77	1
28	1	28	28	130	85	1
29	1	29	29	130	87	1
30	1	30	30	130	93	1
31	1	-	-	131	1	42**
32	1	-	-	132	21	14**

Note(s):

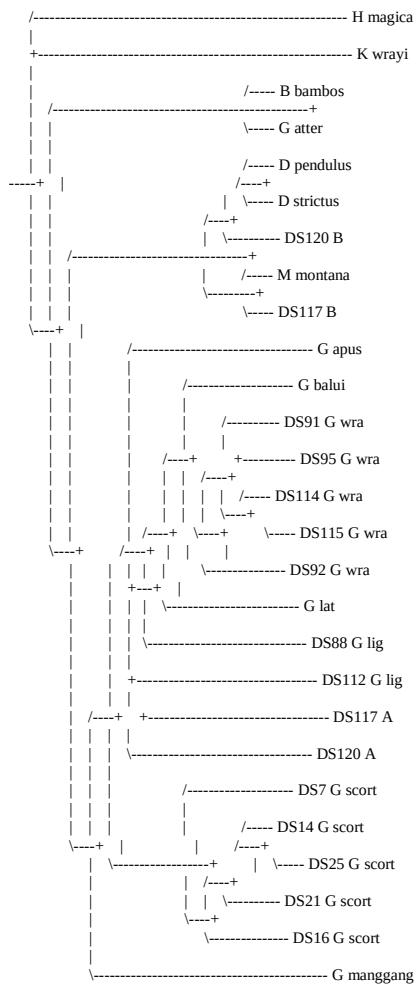
* Only one tree was saved per island; island structure is undetermined

** Multiple observations of the same score do not imply identity of the corresponding trees

30 trees converted from unrooted to rooted.

30 trees saved to file
"C:\Users\wlgoh\Desktop\PUB_Data_Analyses\GBSS_Pabp.tre"

Tree number 1:

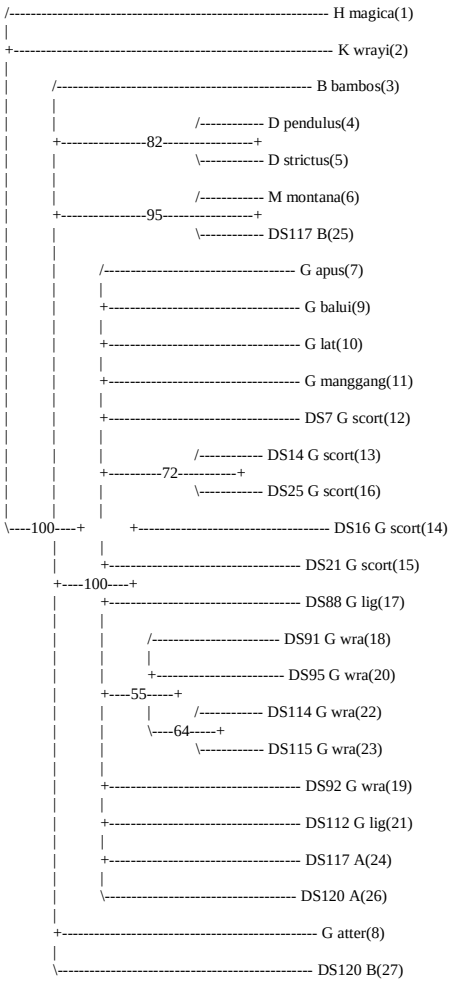


Bootstrap method with heuristic search:
Number of bootstrap replicates = 1000
Starting seed = 1431096063
Optimality criterion = parsimony
Character-status summary:
Of 1095 total characters:
All characters are of type 'unord'
All characters have equal weight
992 characters are constant
50 variable characters are parsimony-uninformative
Number of parsimony-informative characters = 53
Gaps are treated as "missing"
Multistate taxa interpreted as uncertainty
Starting tree(s) obtained via stepwise addition
Addition sequence: random
Number of replicates = 100
Starting seed = 2081105178
Number of trees held at each step during stepwise addition = 1
Branch-swapping algorithm: tree-bisection-reconnection (TBR)

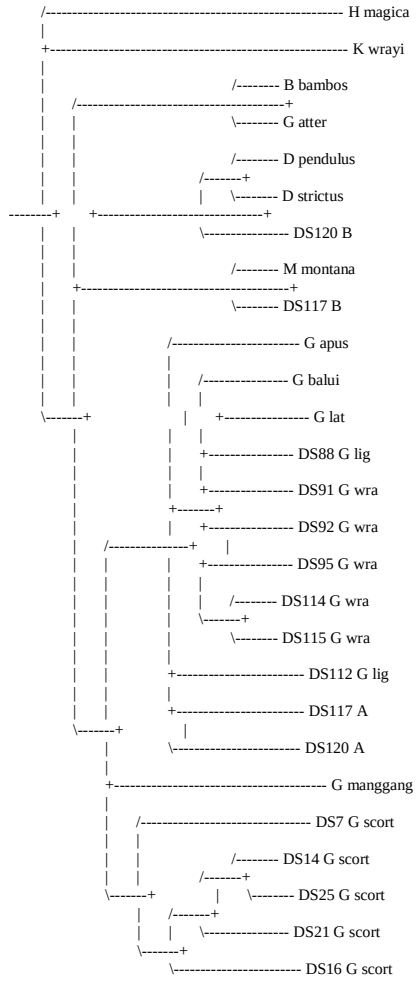
Steepest descent option not in effect
Initial 'MaxTrees' setting = 100 (will be auto-increased by 100)
Branches collapsed (creating polytomies) if maximum branch length is zero
'MulTrees' option not in effect; only 1 tree will be saved per replicate
Topological constraints not enforced
Trees are unrooted

1000 bootstrap replicates completed
Time used = 00:02:31.7

Bootstrap 50% majority-rule consensus tree



Strict consensus of 28 trees:



LIST OF PUBLICATION

1. Dhanendiren, N., Ong, H.Y., Khoo, G., Wong, K.M. and Goh, W.L., 2015. PCR-RFLP analysis of cpDNA in *Gigantochloa scortechinii* (Poaceae: Bambuseae) in Peninsular Malaysia and implications for the use of cpDNA markers in systematic studies. Silvae Genetica, J.D. Sauerlnders Publishing House, 4, pp. 194-200.