POPULATION GENETIC STRUCTURE OF A BAMBOO HYBRID (×*Gigantocalamus malpenensis*) IN PERAK, MALAYSIA, AND IMPLICATIONS FOR SILVICULTURE

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By

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ABSTRACT

POPULATION GENETIC STRUCTURE OF A BAMBOO HYBRID (×*Gigantocalamus malpenensis*) IN PERAK, MALAYSIA, AND IMPLICATIONS FOR SILVICULTURE

Kong Huei Huei

The most economically important wild bamboo species in Peninsular Malaysia, *Gigantochloa scortechinii* Gamble (*Buluh Semantan*), has lately been found to hybridise with another common bamboo species, *Dendrocalamus pendulus* Ridl. (*Buluh Akar*), in the locations where both species co-exist. Their hybrid species (×*Gigantocalamus malpenensis* K.M. Wong) appears to have large and straight culms like those of *G. scortechinii* and the flexural strength that is similar to *D. pendulus*. These characters make it a good candidate for various applications in the construction industry. In this study, a new locality (Sungai Siput, Perak) for ×*G. malpenensis* was reported. Molecular studies were based on the DNA sequence data of two nuclear DNA markers, Granule Bound Starch Synthase I (*GBSSI*) and cellulase-like protein 1 (*PvCel1*) genes; and four Inter-simple Sequence Repeats (ISSR) markers, Wolfe899, UBC 810, UBC 857, and UBC 864. Network analysis, Bayesian phylogenetic analysis, population STRUCTURE analysis, population pairwise F_{ST} analysis, and Principal Coordinate Analysis (PCoA) were used to infer the genetic differentiation and population structures of the hybrid and its parental populations. The hybrid population in Sungai Siput was suggested to consist of only F1 generation. No significant genetic differentiation was detected between the hybridising and non-hybridising populations of *G. scortechinii*, suggesting that the population

structure of the parental populations may not be adversely affected by the occurrence of the new hybrid population. The hybrid population demonstrated a considerably high degree of genetic heterozygosity, implying its non-uniformity in morphological and physiological properties among the hybrid individuals. Hence, there will be various choices for clone selection in silviculture. It was anticipated that more hybrid populations will emerge in the future as driven by environmental factors (e.g., anthropogenic disturbance). The promising side of it is that supply of this hybrid bamboo species from the wild would be continuous.

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APPROVAL SHEET

This dissertation/thesis entitled "**POPULATION GENETIC STRUCTURE OF A BAMBOO HYBRID (×*Gigantocalamus malpenensis*) IN PERAK, MALAYSIA, AND IMPLICATIONS FOR SILVICULTURE**" was prepared by Kong Huei Huei and submitted as partial fulfilment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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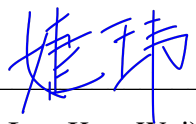

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SUBMISSION OF DISSERTATION

It is hereby certified that ***Kong Huei Huei*** *(*ID No: ***20ADM06236***) has completed this final year project/ dissertation/ thesis* entitled "***POPULATION GENETIC STRUCTURE OF A BAMBOO HYBRID (×Gigantocalamus malpenensis) IN PERAK, MALAYSIA, AND IMPLICATIONS FOR SILVICULTURE***" under the supervision of Dr Goh Wei Lim (Supervisor) from the Department of Biological Science, Faculty of Science, and Dr Loo Keat Wei (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.

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(KONG HUEI HUEI)

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.

Name Kong Huei Huei

(KONG HUEI HUEI)

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LIST OF ABBREVIATION

BDG	*Bambusa-Dendrocalamus-Gigantochloa*
bp	Base pair
CO_2	Carbon dioxide
PvCel1	Cellulase-like protein 1
°C	Celsius degree
cpDNA	Chloroplast DNA
CV	Coefficient of Variation
DNA	Deoxyribonucleic acid
He	Expected heterozygosity
EST-SSR	Expressed sequence tag-simple sequence repeats
F	Fixation indices
GARD	Genetic Algorithm for Recombination Detection
GenAlEx	Genetic Analysis in Excel
h	Genetic diversity
K	Genetic homologous cluster
GPS	Global Positioning System
GBSSI	Granule Bound Starch Synthase I
ITS	Internal Transcribed Spacer
INBAR	International Bamboo and Rattan Organisation
IUPAC	International Union of Pure and Applied Chemistry
ISSR	Inter-simple Sequence Repeats
MTIB	Malaysian Timber Industry Board

MCMC	Markov chain Monte Carlo
Mv	Median vector
Ho	Observed heterozygosity
PCR	Polymerase chain reaction
PP	Posterior probability
PCoA	Principal Coordinate Analysis
RAPD	Random Amplified Polymorphic DNA
RDP	Recombination Detection Program
SNPs	Single nucleotide polymorphisms
TAE	Tris-acetate-EDTA
uHe	Unbiased expected heterozygosity
uh	Unbiased genetic diversity
w/v	Weight by volume

LIST OF AUTHORITIES

Scientific names and the authorities of species mentioned in this dissertation.

Family Poaceae Barnhart
Subfamily Bambusoideae Luerss.
Tribe Bambuseae Kunth ex Dumort
Subtribes Bambusinae J. S. Presl
Bambusa Schreb
Bambusa arnhemica F. Muell.
Bambusa vulgaris Schrad. ex J. C. Wendl.
Bambusa vulgaris 'Striata' (Lodd. ex Lindl.) Gamble
Bambusa blumeana Schult.f.
Bambusa heterostachya (Munro) Holttum
Bambusa bambos (L.) Voss
Bambusa pervariabilis McClure
Bambusa multiplex (Lour.) Raeusch. ex Schult
Bambusa chungii McClure
Chusquea Kunth
Dendrocalamus Nees
Dendrocalamus pendulus Ridl.
Dendrocalamus asper (Schult.) Backer
Dendrocalamus strictus Nees
Dendrocalamus latiflorus Munro
Dendrocalamus hamiltonii Nees & Arn. ex Munro
Dendrocalamus membranaceus Munro
Dendrocalamus sinicus L.C. Chia & J.L. Sun
Dendrocalamus brandisii Kurz
Dendrocalamus giganteus Munro. Chia & J.L. Sun
Dinochloa Buse
Gigantochloa Kurz ex Munro
Gigantochloa scortechinii Gamble
Gigantochloa albociliata (Munro) Kurz
Gigantochloa atroviolacea Widjaja
Gigantochloa levis (Blanco) Merr
Gigantochloa ligulata Gamble
Gigantochloa wrayi Gamble
Gigantochloa ridley Holttum
×*Gigantocalamus* K.M. Wong
×*Gigantocalamus malpenensis* K.M. Wong
Guadua Kunth
Hibanobambusa Maruy. & H. Okamura
Hibanobambusa kamitegensis M. Kobay. & Wakasugi
Hibanobambusa tranquillans (Koidz.) Maruy. & H. Okamura
Holttumochloa K. M. Wong
Kinabaluchloa K. M. Wong
Kuruna Attigala, Kathr. & L.G. Clark
Kuruna debilis (Thwaites) Attigala, Kathr. & L.G. Clark
Maclurochloa K. M. Wong
Melocanna Trin
Melocanna baccifera Kurz
Racemobambos Holttum
Sasa Makino & Shibata
Schizostachyum Nees
Schizostachyum brachycladum Kurz
Schizostachyum zollingeri Steud.
Schizostachyum grande Ridl
Sinocalamus McClure
Sinocalamus mucclure (String bamboo)
Soejatmia K. M. Wong
×***Thyrsocalamus*** Sungkaew & W.L. Goh
×*Thyrsocalamus liangone* (*Phai Liang*) K.M. Wong & D. Ohrnberger

Thyrsostachys Gamble
Thyrsostachys siamensis Gamble

Tribe Arundinarieae Nees ex Asch. & Graebn.
Arundinaria
Arundinaria gigantea (Walter) Muhl.
Arundinaria appalachiana Triplett, Weakley & L. G. Clark
Arundinaria tecta (Walter) Muhl.
Chimonobambusa Makino
Phyllostachys Sieb. & Zucc.
Phyllostachys edulis J. Houz.
Arundinaria Michaux
Yushania Keng f.
Yushania niitakayamensis Keng f.
Tribe Oryzeae *Dumort.*
Oryza sativa L.

Family *Asteraceae* Bercht. & J. Presl.
Pericallis Webb & Berthel.
Helianthus L.
Senecio L.
Senecio squalidus Willd.

Family *Brassicaceae* Burnett.
Cardamine L.
Arabidopsis Heynh.

Family *Solanaceae* Juss.
Nicotiana L.

CHAPTER 1

INTRODUCTION

1.1 Bamboos in Southeast Asia

In Southeast Asia, bamboos are known as "*buluh*", "*bambu*" or "*aur*" in the Malay-speaking cultures. Bamboos are well known for their faster growth and relatively shorter harvest maturity compared to the timber trees. Bamboo clumps usually mature in 3 – 7 years, depending on the species, and can be harvested multiple times (Plantations International, n.d.). As an integral part of the traditional Asian lifestyle, bamboos are used to make vegetable baskets, poultry cages, fishing rods, cooking utensils, instrument for hunting, furniture, gates and even the walls, floorings, and pillars of their houses as well as making rafts for crossing the rivers (Rao, et al., 1996). Recently, bamboo is considered as a valuable emerging replenishable green resource because of its carbon sequestration capability and fast-growing characteristics. It is used as a wood and plastic substitute in the manufacturing and construction industries, such as thermoplastic composite, paper pulps, scaffolds of buildings, reinforcement of concrete, and further more (Bahari and Krause, 2016; Abdul Khalil, 2018; Yong, Ridzuan Ali and Mohd Ikmal Fazlan, 2019; Radzi, et al., 2022).

The most economically important bamboos in Southeast Asia are mainly the members of the Bambusinae subtribes (tropical woody bamboos), especially the species from *Bambusa*, *Dendrocalamus*, and *Gigantochloa*, which are notably used as building materials. According to Plant Resources of Southeast

Asia (PROSEA), 13 bamboo species are commonly used for construction. Many economically important species, such as *Bambusa vulgaris* (*Buluh Minyak*) and *Dendrocalamus asper* (*Buluh Betung*), are cultivar species that are never found in wild (Wong, 1995). These cultivated species are commonly cultivated in the villages or as small plantations owned by private enterprises (Rao and Rao, 1998). However, large scale plantations of bamboos are less desired in the Southeast Asian countries except for Thailand (Rao and Rao, 1998), likely due to the fear of potential economic loss resulting from synchronous mass flowering and whole clump death (Wong, 2004).

1.2 A Recently Discovered Hybrid Bamboo ×*Gigantocalamus malpenensis*

In Malaysia, the bamboo plantation is not widely practiced. Commercial supplies of bamboo culms are mainly reliant on direct harvesting from forests and natural sites. *Gigantochloa scortechinii* (locally known as *Buluh Semantan*), is the most sought-after native bamboo species for commercial uses in Peninsular Malaysia. It has straight and erected culms which are useful in the construction industry. This important species was found to have hybridised with another widespread native bamboo species, *Dendrocalamus pendulus* (locally known as *Buluh Akar*) to give rise to ×*Gigantocalamus malpenensis* (Goh, et al., 2011). Observations in the field found that the culms of this hybrid species were as straight as *G. scortechinii* and hopefully possessed the bending capability like *D. pendulus*, making it potentially valuable as a building material.

Currently, there are only two known distribution records for ×*G. malpenensis*: the forest remnants along the Tapah-Cameron Highland Road in Perak and the old Ulu Gombak Forest Reserve in Selangor, both of which had abundant populations of the parental species (Goh, et al., 2011). At least 48 clumps of ×*G. malpenensis* were documented in the hybrid zone of Ulu Gombak Forest Reserve, presumably deriving from rare coincidental parental flowering events (Goh, et al., 2011; Wong and Low, 2011). Among the hybrid individuals, only 27% were flowering or had flowered, but no seedlings were found, suggesting that ×*G. malpenensis* individuals were all sterile F1. The hybridisation events were hypothesised to be relatively recent, which could have been driven by anthropogenic disturbance such as the development of Karak Expressway in Ulu Gombak (Wong and Low, 2011). The establishment of Karak Expressway besides the Gombak River involved slope cuts, infills, removal of local forest trees and cross over the steep inclines. These events have increased the abundance of *D. pendulus* and *G. scortechinii* to replace the cleared areas and created greater openness and dryness for the airborne pollens to be airlifted for longer distances. Therefore, there would be higher chance for the two species to meet and interbreed.

1.3 Population Genetic Studies for Bamboo Silviculture

Harvesting bamboo culms directly from forests can be less time efficient, as compared to harvesting from plantations. Population genetic studies are expected to provide information for clone selection in silviculture and conservation. However, these studies are scarce and poorly understood,

especially the population genetics structures of paleotropical woody bamboos. There are molecular systematic studies at a higher taxonomic level for woody bamboos (Yang, et al., 2008; Goh, et al., 2010; Goh, et al., 2013; Yang, et al., 2016; Goh, et al., 2020; Liu, et al., 2020; Annisa, et al., 2021; Bels, Lomlek and Sompong, 2021), but those at the population studies level are lacking. Among the Asian bamboos, population structures of a few paleotropical woody bamboos species from the genus *Dendrocalamus* and *Melocanna* in China and India have been assessed using Inter-simple Sequence Repeats (ISSR) and microsatellite markers (Yang, et al., 2010; Yang, et al., 2012; Tian, et al., 2012; Nilkanta, et al., 2017). Genetic structures of a wild population of *Bambusa* species from Australia were studied based on the microsatellite DNA markers (Kaneko, et al., 2008). In Vietnam, *Sinocalamus* has a considerable amount of genetic variation among the population based on ISSR and random amplified polymorphic DNA (RAPD) markers (Hoang, De Filippis and Buckney, 2011). The genetic diversity assessed based on ISSR markers for the two village bamboos *Bambusa vulgaris* 'Striata' and ×*Thyrsocalamus liangone* was extremely low compared to the forest bamboo, *G. scortechinii* in Peninsular Malaysia, almost certainly due to the sterility of the village bamboos (Ooi, et al., 2022).

To date, the population structures of the wild *G. scortechinii* and *D. pendulus* in Peninsular Malaysia have not been investigated, not to mention the relatively new hybrid species, ×*G. malpenensis*. Hence, it was unclear if individuals of ×*G. malpenensis* are genetically uniform or diverse. If they have a high level of genetic variation, they will likely exhibit a wide range of varieties

in terms of the physicochemical properties (e.g., the diameter of culms, lignin, or cellulose content, and so) and the physiological properties (e.g., the timing of mass flowering). Variation in these characteristics provides choices for the propagation in plantations. Besides, the hybrid species has the potential risk of a narrow gene pool and limited supplies from the wild, so the conservation of ×*G. malpenensis* is somehow crucial to maintain the species.

Moreover, the bamboo plantation is often hindered by the potential risk of substantial economic loss resulting from whole clump death followed by unpredictable gregarious flowering activities. The gregarious flowering occurs when all individuals in a population flowered simultaneously, and then all die at the same time. This phenomenon had been observed in the parental species, *D. pendulus* in Kanching Valley, Selangor in 1974, Thailand in 1994, and Ulu Galas Forest Reserve, Kelantan in 1995 (Burgess, 1975; Thammincha, 1995; Azmy Hj. Mohamed, 2004; Wong, 2004). Besides, F1 inter-cross or advanced generation, if it occurred, would cause unpredictable degrees of variations in the physicochemical, mechanical, and physiological traits.

1.4 Objectives of Study

In this study, the newly located population of ×*G. malpenensis* in Sungai Siput, Perak was investigated. This population, as well as its parental populations, were assessed to provide more robust genetic information on the natural hybridisation event in woody bamboos. The objectives of this study are to (1) confirm if the ×*G. malpenensis* individuals are F1 or subsequent

generations; (2) assess the genetic diversity of the hybrid population in comparison with the parental populations. Additionally, tests were also conducted on the genetic differentiation between the hybridising and non-hybridising populations of *G. scortechinii* to (3) determine the impact of the hybrid population on the *G. scortechinii* population genotypes. The molecular studies were based on the inter-simple sequence repeat (ISSR) profiling markers, Wolfe899, UBC 810, UBC 857, and UBC 864; sequence data of the nuclear granule bound starch synthase (*GBSSI*) and cellulase-like protein1 (*PvCel1*, which targets Genome E in the CCDDEE genome system established for the hexaploidy tropical woody bamboos); as well as the chloroplast *rps16-trnQ* and *trnD-T* intergenic spacers. All the aforementioned markers had been demonstrated to be informative in depicting the phylogenetic relationships at the generic- and species-levels in Bambuseae tribes, including the species in genera such as *Bambusa*, *Dendrocalamus*, *Melocanna*, *Schizostachyum*, and more (Yang, et al., 2008; Goh, et al., 2010; Lin, et al., 2010; Goh, et al., 2013; Chokthaweepanich, 2014; Triplett, et al., 2014; Amom, et al., 2018; Amom, et al., 2020).

CHAPTER 2

LITERATURE REVIEW

2.1 Woody Bamboos in Peninsular Malaysia

There are at least 14 genera of woody bamboos, comprising more than 70 species, that grow naturally or are cultivated in Peninsular Malaysia (Wong, 1995; Goh, et al., 2011; Wong and Low, 2011; Goh, et al., 2018). The number of bamboo species is likely to be higher now as more species have been introduced into Peninsular Malaysia in recent decades (Ahmad Mazlan Othman, pers. comm.). All native genera are classified as paleotropical (i.e., African and Asian tropical) woody bamboos: *Bambusa*, *Dendrocalamus*, *Dinochloa*, *Gigantochloa*, *Thyrsostachys*, *Melocanna*, *Schizostachyum*, *Racemobambos*, *Maclurochloa*, *Soejatmia*, *Holttumochloa*, *Kinabaluchloa*, ×*Thyrsocalamus* (i.e., *Phai Liang* or *Bambusa nana*) and ×*Gigantocalamus*. A few neotropical (i.e., American tropical) and temperate woody bamboo genera, such as *Chimonobambusa, Guadua*, *Arundinarieae*, and *Phyllostachys* were also recorded in Peninsular Malaysia as introduced species (Wong, 1995; Ahmad Mazlan Othman, pers. comm.).

In Peninsular Malaysia, 16 bamboo species were reported to have been utilised commercially (Forestry Department of Peninsular Malaysia, n.d.; Holttum, 1958; Wong, 1989; Azmy Hj. Mohamed and Appanah, 1998; Mohd

Anim Hasnan, 2014; FRIM, 2020). These included *B. vulgaris* (*Buluh Minyak*), *B. blumeana* (*Buluh Duri*), *B. heterostachya* (*Buluh Galah*), *D. asper* (*Buluh Beting*), *D. pendulus* (*Buluh Akar*), *G. albociliata* (*Buluh Madu*), *G. atroviolacea* (*Buluh Betong Hitam*), *G. levis* (*Buluh Betong*)., *G. ligulata* (*Buluh Tikus/ Buluh Tilan*), *G. scortechinii* (*Buluh Semantan*), *G. wrayi* (*Buluh Beti*), *S. brachycladum* (*Buluh Lemang*), *S. zollingeri* (*Buluh Dinding*), and *S. grande* (Buluh Semenyeh).

B. vulgaris, *B. blumeana*, and *G. levis* are used in the construction industry to make buildings, bridges, and gates, while *B. blumeana*, *G. levis*, *G. scortechinii*, and *D. asper* with thick culm walls are split into strips for walls or flooring. *B. vulgaris*, *B. blumeana, B. heterostachya, D. asper*, *G. liguata* and *G. scortechinii* are widely used in the bamboo furniture industry. The strong culms of *B. heterostachya* are made into poles for harvesting fruits and pollinating oil palm flowers. *G. levis* has also been used for making cooking utensils. The culms of *G. atroviolacea* are used to make traditional musical instruments, such as *Angklung*, *Gambang*, and *Celempung*. *B. vulgaris* and *B. blumeana* are used in the paper pulp industry. *G. scortechinii* and *G. wrayi* are commonly used for making incensed prayer sticks by the local Chinese community. The young shoots of *G. levis*, *G. albociliata*, *G. atroviolacea*, *G. ligulate*, *B. blumeana,* and *D. asper* are edible. Locals in Indonesia, Malaysia, Singapore and Brunei use *S. brachycladum, S. zollingeri*, and *S. grande* to prepare a Malay traditional dish known as "*lemang*", where the culms are filled with glutinous rice seasoned with coconut milk and salt, while the leaves of *S. grande* are used as wrappers for Chinese glutinous rice dumplings during

festivals. *G. atroviolacea* with its outstanding black culms, as well as *G. ligulate*, *B. heterostachya, B. blumeana*, and *S. zollingeri* are planted as ornamental. (Wong, 1989; Mohd Anim Hasnan, 2014)

Alongside the international movement to promote the use of bamboo and bamboo products for the benefit of the economy and environment, the Malaysia government had drawn up the Bamboo Industry Development Action Plan 2011–2020 which would be carried out by the Malaysian Timber Industry Board (MTIB), and a new phase of the action plan would be implemented until 2030 (StarPicks, 2020). For example, a project of encouraging the locals to establish the bamboo plantation for five commercialised species (including *G. scortechinii*) was started at Kampung Teris, Kuala Berang in January 2021 (Norhaspida Yatim, 2020). Since the end of 2019, tissue culture facilities in Malaysia were able to export 100, 000 bamboo seedlings every month (Ahmad, 2019). The bamboo industry in Malaysia has grown significantly from 2015, when total exports were RM791,000, to RM9.16 million in 2019 (StarPicks, 2020). However, bamboos are still under-utilised in Malaysia. According to the International Bamboo and Rattan Organisation (INBAR), the entire global export of bamboo-based products was valued at US$68.8 billion (RM 287.2 billion) in 2018, but Malaysia only accounted for RM9.97 million (International Bamboo and Rattan Organisation, n.d.). There is no large-scale bamboo plantation in Malaysia currently, but there are a few small-scale plantations owned by private enterprises and planted by individual villages (International Bamboo and Rattan Organisation, n.d.; Nitiparna, n.d.; Abd. Razak Othman, 1994; Azmy Hj. Mohamed and Appanah, 1998; Asari and Suratman, 2010).

2.2 Reproduction and Propagation of Tropical Woody Bamboos

Like other plants, the woody bamboos are capable of reproducing sexually and asexually. Sexual reproduction in the tropical woody bamboos involves spikelets and pseudospikelets (i.e., the basic units of the inflorescence of the woody bamboos), which develop from the leafy branches during the flowering phase of the bamboo clumps. Pollination of the woody bamboos is usually assisted by wind but for some species, insect visitors, such as honeybees, Hemiptera, and syrphid fly facilitated the release of pollens into the air and promote the outcrossing events (Wong, 1995; Chen, et al., 2017; Ruiz-Sanchez, et al., 2017).

Six types of flowerings have been described for the woody bamboos: (1) sporadic flowering, in which the bamboo clump flowers in scattered or diffused form and arises irregularly; (2) gregarious flowering, where >50% of bamboo clumps in a population flower together (in some papers this is referred to as massive synchronised flowering when the whole population flowers simultaneously); (3) combined massive synchronised and sporadic flowering, where the flowering occurrs in sporadic and/or within small areas before or after a large area of the population undergoes flowering; (4) partial flowering, where bamboos flower in a state between the sporadic and gregarious, usually in patchy distribution; (5) continuous flowering mode, in which the flowering interval is particularly short (could be less than 1 year); and (6) annual flowering, where the bamboo flowers every year around the same period (Gamble, 1896; Brandis, 1906; Holttum, 1946; McClure, 1966; Campbell, 1985; Wong, 1995;

2004; Lin and Mao, 2007; Zheng, et al., 2020). In comparison to the polycarpic life cycle of the sporadic, continuous, and annual flowering mode, gregarious flowering bamboos are predominantly monocarpic. After gregarious flowering has taken place, whole bamboo culms and even rhizomes systems often die (Arun, Mukata and Ashesh, 2014). The flowering intervals of woody bamboos are long, which may take around 30 – 120 years depending on the species (Janzen, 1976; Zheng, et al., 2020).

It should be noted that a species may exhibit multiple flowering behaviours. The flowering mechanism in bamboos is still unclear but is expected to be triggered by climate stress (dry seasons or flooding); physiological conditions (lack or excess of certain nutrients); environmental issues (human disturbance); or genetic factors, such as variation in genes copy number, or malfunctions of certain genes (e.g., *SCO1*-like and *GA3*-like genes) (Wong, 1991; 1995; Azmy Hj. Mohamed, 2004; Guo, et al., 2019). The structure alterations in proteins and replicates of genomes may also have contributed to the long vegetative growth phase in woody bamboos (Guo, et al., 2019; Triplett, et al., 2014).

On the other hand, the vegetative spread is also common in tropical woody bamboo. Generally, new rhizomes serve as increasing the modular clone of the plant body. The rhizomes bear buds that can grow upwards to form a new culm to replace the old or dead culms. The rhizomes grow outward from the maturing clumps for more space and nutrients, hence seeming like the

"spreading" of the bamboo clumps. In some bamboos, "rhizomatous swellings" and "aerial rhizomes" can develop at the branch bases under specific conditions, such as when removal of emerging young shoots by local harvesters for foods (Banik, 1980; Hasan, 1980; Wong, 1995). The bamboos stretch invasively to the open habitat and maintain themselves as small and dispersed populations in the understory of forests. Some bamboos spread through new sprouting from the scattering rhizome fragments that may be dispersed by tractors during land clearance or logging, while some bamboo species may have developed longer rhizome necks, thus invading large open habitats from one single stand (Wong, 1991; 1995).

To maintain the bamboo stocks from the wild and for easy harvesting, individual villagers cultivated some useful bamboo species in their villages. Bamboo cultivation using seeds is usually unintended due to the unpredictable flowering and seeding events in bamboo. Therefore, some asexual propagation methods have been developed to raise the tropical woody bamboos, such as (1) branch cuttings; (2) partial culm cuttings; (3) whole culm cuttings; (4) culms with rhizomes attached; or (5) tissue culture (Xiao and Yang, 2001).

2.3 Wild and Cultivated Bamboos

Bamboos were grouped into native (or forest) bamboos and village (or cultivated) bamboos by Holttum (1958). The species were suggested as native in certain areas when they appeared to have developed genetic diversity *per se* compared to those phenotypically uniformed individuals that were known to be

cultivated. In some cases of native species, they were found to flower and set seeds (known as caryopses) more frequently and could have extended through the vegetative spreading, which play a part in their spreading and increase in abundance and made them "common" species in one region, such as *G. scortechinii*, *D. pendulus*, and *S. grande* in Peninsular Malaysia (Wong, 1998; 2004). On the other hand, the cultivated bamboos referred to the bamboos species planted in or near the villages that were either known as introduced species from their place of origins, such as *B. bambos* (origins from India to southern China, including Thailand) and *D. strictus* (native in India, Nepal, Bangladesh, Burma/Myanmar and Thailand) in Peninsular Malaysia, or those which could have endured since ancient times through the historical migration of humans, but not known for their places of origins, such as *B. heterostachya*, *B. vulgaris* and *D. asper* (Wong, 2004). These cultivated species were derived from limited genetic stocks of the important ancient enduring clone bamboo of unknown origin or certain selected clones based on the desired traits. These clones were mainly maintained and propagated vegetatively by villagers. These cultivated species were either less fertile or self-incompatible, thus having a lower genetic diversity than those outcrossing native and wild species in the forests (Goh and Wong, 2021; Ooi, et al., 2022).

2.4 Bamboo Hybridisation

2.4.1 Hybridisation in Plants

Hybridisation took place when there was a suitable source (parental species), habitat, and removal of reproduction barriers. Plant species produce hybrid zones when human (deforestation, railway or new road construction) or other agents (such as radical changes in climates and invasion of exotic species) had disturbed their habitats (Anderson and Stebbins, 1954; Guo, 2014; Grabenstein and Taylor, 2018). This was observed for a wide range of plant groups worldwide, e.g., interspecies hybrid formation in *Pericallis* (*Asteraceae*) caused by road construction in Tenerife, Canary Islands (Brochmann, Borgen and Stabbetorp, 2000; van Hengstum, et al., 2012); hybrid swarms of sunflowers (*Helianthus*) due to human invasive activities in California (Stebbins and Daly, 1961; Carney, Gardner and Rieseberg, 2000); hybrid daisies (*Senecio squalidus*) which arose from its parental species that were introduced in the late 17th centuries to Oxford, the United Kingdom along the railways (Abbott, et al., 2002; Harris, 2002); as well as the hybrid zones of *Cardamine* (*Brassicaceae*) which colonised the man-made habitats in Switzerland (Urbanska, et al., 1997).

2.4.2 Artificial Hybridisation of Woody Bamboos

Woody bamboos have been artificially hybridised to improve their quality and desired traits. A successful artificial bamboo hybrid between *D. latiflorus* and *B. pervariabilis* (Zhang and Chen, 1980) was resistant to cold in local cultivation during cultivation in Southern China. Later, Yuan, et al. (2019)

artificially hybridised *D. latiflorus* with *B. multiplex* and *B. chungii* with *B. multiplex*. These two hybridisations produced fast-growing hybrid species with better fibre quality and stalks with longer internodal lengths. The nutritional values and quality of edible bamboo shoots, including *D. latiflorus* × *D. hamiltonii*, *D. hamiltonii* × *D. latiflorus and D. latiflorus* × *D. asper* have also been improved through hybridisations (Zhang, Chen and Yang, 1993).

2.4.3 Natural Hybridisation of Woody Bamboos

Natural hybridisation of woody bamboos is expected to be rare, since the woody bamboos have long species-specific flowering intervals (Triplett, Oltrogge and Clark, 2010; Zheng, et al., 2020). However, the cross-pollination in woody bamboos is still possible for both the intra- and inter- generic hybridisation even the opportunities are infrequent. Natural hybridisations are necessary and have contributed to the complicated and high diversity of bamboos. Natural hybridisation potentially provides starting points for the new lineages in the evolution history, taxonomic impacts, genetic exchanges and diversification of woody bamboos (Triplett, Oltrogge and Clark, 2010). The natural intergeneric hybridisation in the bamboo plays an important role in the pass bamboo evolutionary history, causing the doubling and duplicating of genome ploidy, hence resulting in the long vegetative and flowering intervals in the woody bamboos (Triplett, et al., 2014; Guo, et al., 2019). Up to date, the natural hybridisations of woody bamboo in wild were less being studied and reported, as a putative hybrid would only be recognised based on the intermediate morphology of its known parental species (Clark, Davidse and

Ellis, 1989; Triplett, Oltrogge and Clark, 2010). The *Gigantochloa* genus in Peninsular Malaysia with great morphological variation was suggested could be due to hybrid swarms, as the variety of *Gigantochloa* species is found to increase from the southern to the northern of Peninsular Malaysia (Holttum, 1958; Muller, 1998; 2003). A few researchers described a putative intergeneric hybrid based on the intermediate morphology between *Phyllostachys* and *Sasa* in Japan and reported it as *Hibanobambusa,* where currently, there are two species reported under this hybrid genus (Maruyama, Okamura and Murata, 1979; Muramatsu, 1981; Kobayashi and Wakasugi, 2012). Three *Chusquea* intra-generic hybrids in America were reported by Clark, Davidse and Ellis (1989) based on the intermediate morphology. The hybridisation between three *Arundinaria* species in America, *A. gigantea* and *A. tecta* and *A. appalachiana,* suggested by McClure (1973) was confirmed by Triplett, Oltrogge and Clark (2010) based on Amplified Fragment Length Polymorphism (AFLP) profiling and chloroplast DNA data. The hybrid individuals were discovered in areas where distributions of either two *Arundinaria* species were overlapped. ×*Thyrsocalamus liang* (known as *Phai Liang* in its country of origin, Thailand), was demonstrated as an intergeneric hybrid species between *D. membranaceus* and *T. siamensis* based on morphological characteristics and the nuclear gene sequence data (Goh, et al., 2018).

2.5 *Dendrocalamus pendulus*, *Gigantochloa scortechinii* and ×*Gigantocalamus malpenensis*

Dendrocalamus pendulus and *G. scortechinii* are both classified under Bambuseae tribe, specifically under paleotropical woody bamboos as subtribes Bambusinae as both species have well-developed branching systems derived from a single bud per node with hollow and aerial culms (Bamboo Phylogeny Group, 2012). Both species were found spreading fast in the disturbed areas, including the logging area, secondary forests, and the newly constructed roads (Wong, 1995; Azmy Hj. Mohamed, 2004; Wong, 2004). Flower events for both species were observed in forests disturbed by human activities, such as new road construction and deforestation (Wong, 1995; 2004; Wong and Low, 2011). With the fulfilment of the criteria where parental species are available, and the reproduction barriers being removed from human activities, the natural hybridisation of *D. pendulus* and *G. scortechinii* was not a coincidence.

D. pendulus is commonly found to be naturally existing in foothills and valleys of mountains, as well as disturbed lowland forest areas. *D. pendulus* populations have an average height of 20–25 m and culm diameters of 6–9 cm. Their culms are dark green and coated in white wax. The bottom culm nodes of *D. pendulus* have visible verticils roots. The culms are unable to carry their own weight and thus arch over and rest on surrounding forest trees. The culm sheath is coated with white wax mixed with hairs. Additionally, the lower portion of the culm sheath is covered with rich dark brown hair, but the hair loosens, and the colour lightens as it reaches the top part. Low rims auricles are 1.5 to 3 mm

high, with the bristle on the margin measuring between 7 and 10 mm in length. The ligule is around 4–7 mm in height and is divided into coarse teeth. The sheath blades are lanceolate and spread to reflexed. The foliage leaf blades are glabrous on both sides. The pseudospikelets length is 6 – 9 mm, with one or two perfect flowers and 2 – 3 empty glumes. *D. pendulus* has no terminal vestigial flower. The lemmas and paleas are both around 8 mm long, with no hair observed. Its stamens have free filaments. Its anthers are purple in colour and 3.5 – 4 mm long.

G. scortechinii is found along the riversides. *G. scortechinii* clumps range in height from 10–20 m and have a culm diameter of 6–12 cm. The culms are dark green in colour, glabrous and waxy on the outside. Unlike *D. pendulus*, *G. scortechinii* has straight culms. The culm sheath is green at the base and flushed into bright orange at the top. Dark brown (almost black) hairs encrust the outer layer of the culm sheath. *G. scortechinii* has auricles with low rims barely 0.5–1.5 mm high that are glabrous or have extremely fine bristles. The ligule is deeply lacerated with bristles between 10–12 mm in length. The sheath blades are deltoid-like in form, green with a pink flush and spread to reflexed. The leaves on the branches are lanceolate, not fully green but with white stripes, and the bottom side is pale soft hairy. The pseudospikelets are 6 – 9 mm long, with 3 – 5 empty glumes and 4 – 5 perfect flowers. *G. scortechinii* has terminal vestigial flowers. The paleas margins are slightly shorter and edged with pale fine hairs, while the lemmas are 12 – 20 mm long with pale brown hairs. *G. scortechinii*'s filaments, like those of other *Gigantochloa* species, are fused into a tube. It has yellow anthers that are 9 – 11 mm long.

×*G. malpenensis* were recorded by Wong and Low (2011) to have grown closer to the *D. pendulus* clumps compared to the *G. scortechinii* clumps. Apart from the culm sheath, the exterior properties of ×*G. malpenensis* culms and leaves are akin to those of *G. scortechinii* (Goh, et al., 2011), as shown in Figure 2.1. The majority of the ×*G. malpenensis* culms are upright; though, some dangle loosely at the apical. The culm sheath is pale yellow to orange in colour with a hint of pink or purple-brown at the base. Dark brown hair covers the sheath, and the back of the sheath is slight to moderately white-waxy. The auricles feature rounded lobes that reach a height of 5 mm and wavy bristles up to over 10 mm long. The ligule is around 2 – 5 mm high and divided into coarse teeth. The sheath blades are leaf-like and spreading to reflexed, with a pink flush at the base. The pseudospikelets are 7 – 11 mm long, with two (or rarely three) perfect flowers, and two to three empty glumes. If there are only two perfect flowers, the terminal vestigial flowers are present; if there are three perfect flowers, they are absent. The lemmas are glabrous and hairless, similar to *D. pendulus*, and the filaments are fused into tubes like *G. scortechinii*. Its anthers range in colour from pink to pastel purple.

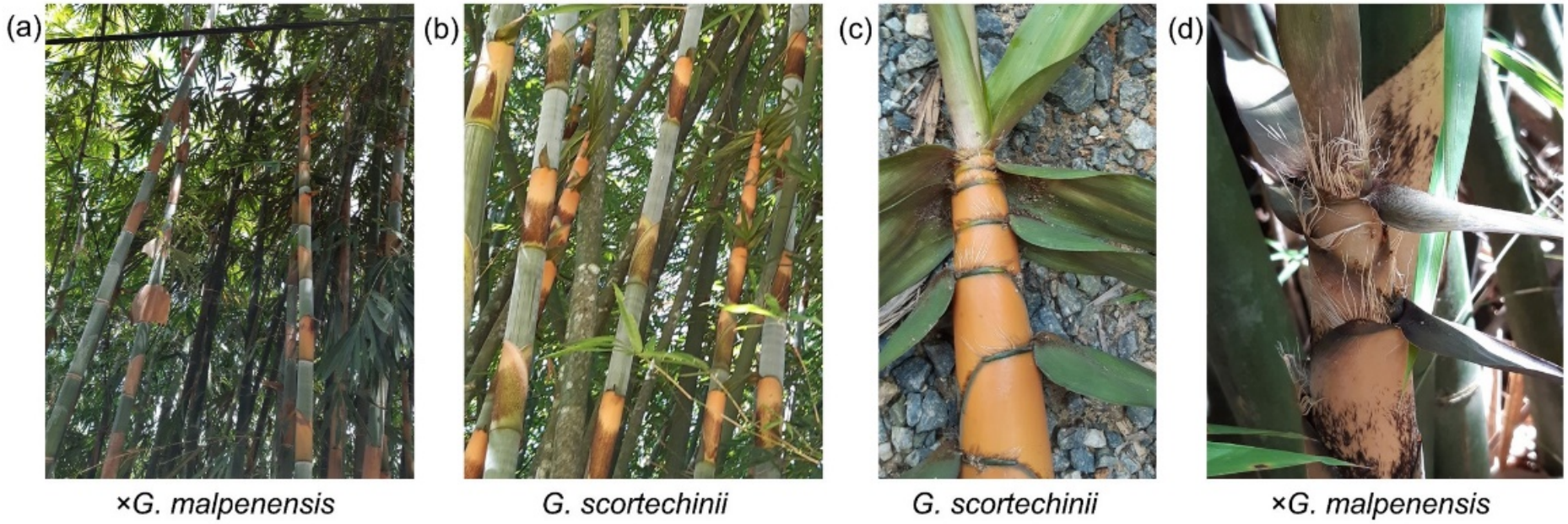


Figure 2.1: The culm shoots of ×*G. malpenensis* and *G. scortechinii.* The culm shoots of ×*G. malpenensis* (a) that resemble those of *G. scortechinii* (b); and the close-up image of the culm sheath characteristic for *G. scortechinii* (c) and ×*G. malpenensis* (d) that the auricles of latter have raised round lobes and wavy bristles

Currently, *D. pendulus*, *G. scortechinii* and ×*G. malpenensis* are not planted on a large-scale in plantations. Only records of *G. scortechinii* planted in FRIM at Kepong, Malaysia and in Pahang National Park (Abd. Razak Othman, 1994; Asari and Suratman, 2010), which were not planted for commercialised purposes, have been found.

D. pendulus produced gregarious flowering in 1974 and 1980 in Kanching Valley, Selangor (Burgess, 1975; Wong, 1995). In early April 1995, a mass gregarious flowering of this species was reported at the Ulu Galas Forest Reserve in Gua Musang, Kelantan (Azmy Hj. Mohamed, 2004). Meanwhile, diffuse sporadic flowering of entire clumps within a population had been reported previously in wild *D. pendulus* (Wong, 1995). Sporadic flowering was mainly observed in wild *G. scortechinii* (Holttum, 1958; Wong, 1995). However, gregarious flowering in the diffuse form of this species was reported in regions with intense disturbance (Burgess, 1975; Wong, 1991). Currently, a few clumps of ×*G. malpenensis* was reported to have flowered in patchy form during its first discovery in the Ulu Gombak reserve (Wong and Low, 2011). The flowering mode for this hybrid species is remain uncertain.

2.6 Bamboos as an Emerging Green Material

Bamboo is regarded as “old-man timber” in the society, as it is known to be made into traditional living tools and rural crafts (Rao, et al., 1996). Nowadays, bamboo is getting more attention in the construction and manufacturing industry because of its mechanical and chemical properties,

together with its rapid growing biomass. Bamboo species with straight, long and non-bulging culms have a great potential to be employed as scaffolding, pillar, and beam materials in the local construction industry as a substitute for steel, concrete, or wood along with their high mechanical and tensile strength in compression stress (Dinie Awalluddin, et al., 2017; Dinie Awalluddin, et al., 2019; Yong, Ridzuan Ali and Mohd Ikmal Fazlan, 2019). Besides, bamboo scaffolding could be upcycled. Once bamboo can no longer be used, it can be burned as a source of fuel (Neumann, 2020). Those species with moderate wall thickness that can be split and flattened easily and do not break transversely can be made into bamboo strips for various applications, such as handicrafts, walls, and flooring (Holttum, 1958; Dransfield, 1992). The bamboo species with high cellulose and low hemicellulose content could be used for making a variety of paper (Zhang and Ma, 2003), while those with high lignin could be extracted for their various unique characteristics, including biocompatibility, antioxidant, antimicrobial, and redox activity for the nano- and biomaterials (Verma, et al., 2021).

In addition, bamboo has emerged as a “green material” as it is a high carbon storage plant and can store significant of carbon amount proportionate to its rapid growth in biomass. As a result, using bamboo in construction can minimise or even neutralise carbon footprints. According to van der Lugt (2013), the net CO_2 credit of a final bamboo product, including from its life cycle to the product chain, was -0.38 to -0.57 CO2eq per kilogram. On the other hand, woody bamboos are a valuable resource for achieving the United Nations General Assembly’s 2021 Sustainable Development Goals, such as SDG 7

(affordable and clean energy), SGD 9 (Industry, Innovation and Infrastructure), and SDG 11 (Sustainable Cities and Communities). Those species with high lignocellulosic material and large non-food biomass have significant potential for the production of solid, liquid, or gaseous bioenergy fuels (Chin, et al., 2017). The woody bamboos are also a promising substitute for the wood-polymer composite industry in the production of thermoplastic composite or as a reinforcement for thermoplastic (Bahari and Krause, 2016; Radzi, et al., 2022), thereby reducing the use of fossil fuel-based materials. Besides that, bamboos could be planted on degraded land such as land slope to control erosion, restore the water table and in the phytoremediation of soils contaminated with heavy metal (Tardio, et al., 2018; Bian, et al., 2020).

G. scortechinii has been the most commercialised species in various cottage industries in Peninsular Malaysia, such as the manufacture of poultry cages, vegetable baskets, incense sticks, joss paper, skewers, chopsticks, and commercial handicrafts (Wong, 1995; Azmy Hj. Mohamed, 2004; Wong, 2004). *G. scortechinii* has also been researched to manufacture pulp and paper, bio-oil, as reinforcement of concretes, and thermoplastic particles (Kasim, 1999; Noridah, et al., 2014; Ainun, et al., 2018; Yong, Ridzuan Ali and Mohd Ikmal Fazlan, 2019). Meanwhile, *D. pendulus* is solely known for its use in making vegetable baskets and poles in previous decades. However, mechanical and chemical studies have been carried out on these two native species to access their potential uses. According to the findings of Zakikhani, et al. (2017), *D. pendulus* contains less holocellulose and lignin has a high mean strain to failure. These properties enable *D. pendulus* fibres to withstand the structural changes

without cracking. *D. pendulus* has lower thermal conductivity and so provides better thermal insulation (Noor Zuraida Jusoh, et al., 2013). On the other hand, *G. scortechinii* with a wider culm diameter (ca. 6–12 cm) and a thicker wall thickness (ca. 0.5–1.5 cm), exhibits a greater tensile strength, modulus of elasticity, and Young's modulus than *D. pendulus* (Razak Wahab, et al., 2012; Zakikhani, et al., 2017). Hence, both *D. pendulus* and *G. scortechinii* have a great potential to be used as as materials for construction and furniture in the contemporary era. ×*G. malpenensis*, the hybrid species between these two useful native bamboos, is expected to be a good candidate building material.

2.7 Population Genetic Studies in Woody Bamboos

So far, the population genetic studies conducted on bamboo in Southeast Asia based on molecular approaches are scarce. Most of the classification and diversity studies of woody bamboos are still based on morphological traits such as the culm anatomy, branching structure, inflorescence morphology, and flowering behaviour (Setiawati, et al., 2016; Utami and Pradnyawathi, 2017; Ervianti, Widjaja and Sedayu, 2018; Fimawati, et al., 2020; Thuy, et al., 2021). Morphological traits have also been used in assessing genetic diversity of *D. stocksii* at the population level (Rane, Sowmya and Viswanath, 2013). A total of 102 clumps of *D. stocksii* from 12 different populations in the Western Ghats, India were assessed through parameters such as culm diameter, internode length and diameter and the thickness of the culm wall. Results showed that the *D. stocksii* populations generally have narrow genetic variation, suggesting that

there is a lack of viable seed setting, as well as micropropagation maintained by local farmers over a period of time (Rane, Sowmya and Viswanath, 2013).

Low-copy nuclear sequencing markers had been developed and utilized in temperate woody bamboos (Triplett, et al., 2014; Zhang, et al., 2014; Zhang, et al., 2019), but lesser of them.have been developed for paleotropical woody bamboos, such as *GBSSI*, *LEAFY* gene, and the internal transcribed spacer (ITS) regions that were used for taxonomic-level studies (Yang, et al., 2008; Zhou, et al., 2017; Zhang, et al., 2019). There are limited population-level studies of paleotropical bamboos in Southeast Asia, while these studies are quite common in other Asian regions. Most of them are based on profiling markers, such as ISSR, microsatellites, or RAPD markers. The population structures of *D. membranaceus*, *D. sinicus*, *D. brandisii* and *D. giganteus* in Southwest China, Yunnan Province were assessed using ISSR and microsatellite markers (Yang, et al., 2010; Tian, et al., 2012; Yang, et al., 2012; Yang, et al., 2014). In overall, the *D. membranaceus* populations which have decline in number at local are found to possess high genetic diversity and lower genetic differentiation. Therefore, *D. membranaceus* are likely out-crossers and still represent the genetic variation inherited from their ancestors. On the other hand, *D. sinicus* and *D. brandisii* populations have higher genetic differentiation, are suggested due to habitat fragmentation. Seven *D. giganteus* populations have relatively low genetic diversity, which could be attributed to the clonal propagation. Besides that, genetic studies of seven populations of *Melocanna baccifera* from different geographical region in Northeast India, which were conducted using ISSR markers, showed a significant genetic differentiation within the

populations but low genetic diversity between populations (Nilkanta, et al., 2017).

Moreover, the endemic wild species, *B. arnhemica* in Northeast Australia did not show significant difference and linkage disequilibrium between populations from different sampling locations based on the microsatellite DNA markers, thus suggesting that the gregarious flowering of this species could be due to the low genetic diversity (Kaneko, et al., 2008). Besides that, 20 fluorescently labelled microsatellite markers were used to evaluate the genetic structure of *P. edulis* including 803 individuals from 34 representative populations in China (Jiang, et al., 2017). Overall, moderate genetic diversity was detected at the species level of *P. edulis* populations in China, while most of the genetic diversity occurred within the populations. Based on the results from the microsatellite markers, Jiang, et al. (2017) suggested that the *P. edulis* populations from west China had a high level of genetic diversity and differentiation compared to populations from east China, which had been cultivated intensively. Twelve microsatellite loci had also been used to assess the genetic diversity of six populations of the threatened species, *Kuruna debilis* in Sri Lanka and Southern India (Attigala, et al., 2017). Results showed that *K. debilis* exhibited a high genetic differentiation and strong isolation by distance grouping into three genetic clusters.

Furthermore, 17 Nuclear simple sequence repeats (nSSRs) were used to examine the genetic diversity and population genetic structure of 19 wild *D. hamiltonii* populations distributed across the northeast Himalayas (Meena, et al., 2019). These *D. hamiltonii* populations have maintained high genetic diversity at the species level but comparatively low at the population level. The researchers suggested that this species may have been derived vegetatively during evolution and propagated in different geographical regions since most of the populations were clustered following their geographical distribution. Another population study was carried out on 72 individuals from the three *D. hamiltonii* populations in the Northeast and Northwest Himalayas based on neutral Expressed sequence tag-Simple Sequence Repeats (EST-SSR) markers, which revealed a moderate to high-level genetic diversity with low gene flows within the populations (Bhandawat, et al., 2019). Three populations of *D. hamiltonii* were clustered into two major groups based on geographical separations similar to the outcomes of the previous study (Meena, et al., 2019).

RAPD markers were used to study the genetic diversity within 23 populations of *D. giganteus* in the Royal Botanic Gardens, Peradeniya in Sri Lanka (Ramanayake, Meemaduma and Weerawardene, 2007). This species was introduced to Sri Lanka in 1856 and appeared to have a relatively low genetic diversity. The results were consistent as the population was found to be phenotypically uniform, indicating that this species was maintained through clonal propagation. Besides, the population structure of a temperate woody bamboo, *Yushania niitakayamensis* in Taiwan was also accessed using RAPD markers (Hsiao and Rieseberg, 1994). Although this species was mostly spread

through vegetative spreading and rarely flowered, results showed that the population was genetically diverse which could probably be due to more frequent sexual reproduction in the history of the species, or high somatic mutation rates.

It appears that most of the studies on genetic diversity and genetic structures of woody bamboo have been conducted based on profiling markers instead of sequencing markers, as elaborated in Literature Review section 2.7. This is possibly because the profiling markers are relatively affordable and rapid methods (Yeasmin, et al., 2015). ISSR and microsatellite markers are more widely used for the study of genetic differentiation of woody bamboos than the RAPD markers (Hsiao and Rieseberg, 1994; Ramanayake, Meemaduma and Weerawardene, 2007; Kaneko, et al., 2008; Yang, et al., 2010; Tian, et al., 2012; Yang, et al., 2012; Yang, et al., 2014; Nilkanta, et al., 2017; Amom, et al., 2018; Amom, et al., 2020). Especially for ISSR markers where they do not require any sequence data for primer construction (Zietkiewicz, Rafalski and Labuda, 1994; Yeasmin, et al., 2015). Therefore, ISSR markers are useful as a preliminary evaluation of the genetic structures when lacking genetic information. Furthermore, ISSR is more reproducible, more reliable, and more stable than RAPD as ISSR markers have a longer primer sequence, greater annealing temperature that results in higher stringency and is able to generate a large yield of data (i.e., 10 – 20 bands per lane) from different sizes of intervening sequences (Zietkiewicz, Rafalski and Labuda, 1994; Hoang, De Filippis and Buckney, 2011; Yeasmin, et al., 2015; Amom, et al., 2020; Bhandari, et al., 2021).

CHAPTER 3

MATERIALS AND METHODS

3.1 Sampling Sites and Samples Collection

The sampling collection site of this study was located in Sungai Siput District, Perak. Samples were collected in Pos Piah and Pos Poi, and along the 13.6 km road that connects both villages. The map view of the sampling sites was shown in Figure 3.1 (Google map: https://www.google.com/maps). Heterogeneous landscape was observed in the collection site, which consists of secondary forests, less disturbed riparian vegetation along the river, indigenous communities, and the heavily disturbed areas resulted from extensive logging and plantation activities. solitary or extremely small patches of clumps of ×*G. malpenensis* were discovered in the massive disturbed areas.

Figure 3.1: Map view of the samples collection location in Google maps. Green, red, and yellow colour labels represent *D. pendulus*, *G. scortechinii* and ×*G. malpenensis*, respectively. Samples were collected within Sungai Siput district along Sungai Piah and the main road. Total sample collection area was around 38 km^2

The identification of the species in the field was based on the morphological traits of culm sheath described by Wong (1995) and Goh, et al. (2011). Since the external characteristics of leaves and culms of ×*G. malpenensis* and *G. scortechinii* are highly similar, the samples were only collected from the clumps when both the culm sheath and culm shoots were available. Young leaf samples were collected and kept in a bag with silica gel until future DNA extraction for molecular work. Global Positioning System (GPS) coordinates of the samples collected were listed in Table 3.1.

Table 3.1: List of specimens in this study and their corresponding GPS coordinates

Sample ID	GPS coordinates
×G. malpenensis	
HYB1	05˚05.647'N 101˚11.431'E
HYB2	05˚05.680'N 101˚11.419'E
HYB3	05˚05.540'N 101˚11.024'E
HYB4	05˚05.624'N 101˚11.445'E
HYB5	05˚05.636'N 101˚11.445'E
HYB6	05˚04.876'N 101˚16.226'E
HYB7	05˚04.787'N 101˚15.962'E
HYB8	05˚06.006'N 101˚11.271'E
HYB9	05˚05.761'N 101˚11.337'E
HYB10	05˚05.761'N 101˚11.337'E
HYB11	05˚05.763'N 101˚11.314'E
D. pendulus	
DEN1	05˚05.694'N 101˚11.418'E
DEN2	05˚05.720'N 101˚11.376'E
DEN3	05˚05.690'N 101˚11.183'E
DEN4	05˚05.692'N 101˚11.421'E
DEN5	05˚04.978'N 101˚15.710'E
DEN6	05˚04.398'N 101˚14.863'E
DEN7	-
DEN8	05˚05.763'N 101˚11.315'E
G. scortechinii	
GIG1	05˚05.206'N 101˚11.698'E
GIG2	05˚04.891'N 101˚16.192'E
GIG3	05˚04.785'N 101˚15.944'E
GIG4	05˚05.189'N 101˚13.022'E
GIG5	05˚06.297'N 101˚11.061'E
GIG6	05˚06.002'N 101˚11.279'E

3.2 Total DNA Extraction

The leaf samples were checked to ensure that they are free from fungus contamination. Total DNAs were extracted using EZ-10 Spin Column Plant Genomic DNA Miniprep Kit (BioBasic, Canada) following the manufacturer's protocols. First, 600 μL of Buffer PCB were added to 25 mg dry leaf sample and ground into pastes. The pastes were transferred to a 1.5 mL microcentrifuge tube and 12 μL of β-mercaptoethanol were added and vortexed thoroughly. The mixture was then incubated in a 65˚C water bath for 25 mins. Next, 0.6 mL of chloroform was added to the incubated mixture and mixed gently by inverting 10 times, followed by centrifugation at 12,000 × g for 2 mins. The supernatant was transferred to a new microcentrifuge tube with 200 μL of Buffer BD added and vortexed to mix well. Next, 200 μL of absolute ethanol was added and vortexed to mix well. All the liquid was then transferred to the EZ-10 column placed in the 2 mL collection tube and centrifuged at 9,000 × g for 1 min. Two washing phases, 500 μL of PW solution (isopropanol added) and 500 μL of Wash Solution (ethanol added) were added to the column, respectively, and centrifuged at 9,000 × g for 1 min. The column was spun to dry for 2 mins at 9,000 × g before 100 μL of distilled water was added and incubated at room temperature for 1 minute followed by the elution at 9,000 × g for 1 min. The extracted total DNA was quantified in a nano spectrophotometer, 2000 (Thermo Fisher Scientific, US). The DNA quality checks were done by visualizing with 1.5% agarose gel electrophoresis in 1×TAE buffer at 80 V for 50 mins. The DNA was stored at -20˚C for future use.

3.3 Primer Design for Genomic Specific Nuclear Gene Marker *Pvcel1*

Reverse primer for nuclear gene sequencing marker *PvCel1* which targeted Genome E was designed in the present project. Primer sequence candidates were designed by referring to genome-specific DNA sequences obtained from Fam (2016). The primers were assessed through OligoAnalyzer (Integrated DNA Technologies, Inc.) to ensure that they fulfil the four criteria: (1) 18 – 30 nucleotides; (2) 35 – 65% GC content; (3) avoiding long runs of G; and (4) preventing the primer dimers and secondary structures. The selected primers were then blasted with the DNA sequences from a similar class of bamboos deposited on the GenBank by Triplett, et al. (2014) using the online standard nucleotide basic local alignment search tools (blastn) platform (Altschul, et al., 1990) in National Centre for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/).

3.4 Polymerase Chain Reaction

All PCR reactions for sequencing markers were carried out with 300 ng DNA template in every 50 µL reaction with GoTaq® Green Master Mix (Promega) using the MiniMJ Thermal Cycler (Bio-Rad, California USA) or Eppendorf Mastercycler Nexus PCR Cycler (Eppendorf, Germany). In the preliminary test, ISSR profiles of three populations were obtained through PCR with 0.8 µM primers for all 4 ISSR markers (Wolfe899, UBC 810, UBC 857, and UBC 864). In the nuclear *GBSSI* gene amplification, 0.5 µM forward and reverse primers, Gin-F, an internal forward primer specifically designed for the woody bamboos (Goh, et al., 2010) and GBSS-R, a universal reverse primer for

Poaceae (Mason-Gamer, Weil and Kellogg, 1998) were used. The homeolog-specific *GBSSI* for ×*G. malpenensis* was performed using the primers pairs developed by Goh, et al., (2011) as shown in the schematic diagram in Figure 3.2: (i) Gin–Gin396/1, (ii) Gin–Gin396/2, (iii) Gin336/1–GBSS, and (iv) Gin336/2–GBSS to separate the *Gigantochloa* and *Dendrocalamus* specific haplotype for each putative hybrid individual. The nuclear *PvCel1* gene was amplified with 0.4 µM forward primers, PvCel1-F (Triplett, et al., 2014) and reverse primers (PvCel1-GER) designed in this study. PCR of the two cpDNA markers *rps16-trnQ* and *trnD-T* (Bamboo Phylogeny Group, 2005) were carried out with 0.4 µM forward and reverse primers. The primers' sequences and their respective PCR conditions were listed in Table 3.3.

Figure 3.2: Schematic diagram showing the position of haplotype-specific primers (site numbers). The arrows indicate directions of primers (Goh, et al., 2011)

Table 3.2: Primer list for the PCR reactions of sequencing markers in this study

	Primer Sequences (5'to 3')	PCR parameters (All end with 10˚C hold)	Expected amplicon size (bp)	References
ISSR markers				
Wolfe899	$(CA)_8RG$	94 ℃,1 min; 35× (94 ℃, 30 s; 50 ℃, 45 s; 72 ℃, 1 min); 72 ℃, 10 mins.	-	Wolfe, Xiang and Kephart (1998)
UBC 810	$(ATG)_6$		-	Biotechnology Laboratory, University of British Columbia
UBC 857	$(AC)_8YG$		-	
UBC 864	$(GA)_8$ T		-	
Nuclear sequencing marker				
GBSSI	Gin-F: AAG TTT GAG CGC ATG TTC CAG AGC	95℃, 2 mins; 30× (94℃, 30 s; 59℃, 45 s; 72℃, 1 min); 72℃, 5 mins.	550	Goh, et al. (2010)
	GBSS-R: GGC GAG CGG CGC GAT CCC TCG CC			Mason-Gamer, Weil and Kellogg (1998)
GBSSI G- and D-haplotype specific	Gin396/1: GTC TTA GTC TTC TCC TTG CAG C	95℃, 2 mins; 30× (94℃ for 30 s, 55℃ 45 s; 72℃, 1 min); 72℃, 5 mins.	400-500	Goh, et al. (2011)
	Gin396/2: CAA GAG TAA CGC CAT ATA TG			

Table 3.2 (cont.): Primer list for the PCR reactions of sequencing markers in this study

	Primer Sequences (5'to 3')	PCR parameters (All end with 10˚C hold)	Expected amplicon size (bp)	References
GBSSI G- and D-haplotype specific	Gin336/1: GTC CTA GTC TTC TTG CAG CTC Gin336/2: CAA GAG TAA CAC CAT GTA CG	95℃, 2 mins; 30× (94℃ for 30 s, 55℃ 45 s; 72℃, 1 min); 72℃, 5 mins.	400-500	Goh, et al. (2011)
PvCel1	PvCel1-F: GCC AAC ATG GTT CAG TTG G PvCel1-GER: CAG AAA TAT CGT CCA AAG TC	95℃ 5 min; 35x (95℃, 30 s; 49℃, 45 s; 72℃, 1 min 20 s); 72℃, 15 mins.	850-900	Triplett, et al. (2014) This study.
<u>cpDNA sequencing marker</u>				
rps16-trnQ	16Q2-F: CGA GAT GGT CAA TCC TGA AAT G 16Q2-R: ATC CTT CCG TCC CAG ATT TT	95℃, 2 mins; 35× (94℃, 30 s; 50℃, 45 s, 72℃, 1 min); 72℃, 5 mins.	750	Bamboo Phylogeny Group, 2005

Table 3.2 (cont.): Primer list for the PCR reactions of sequencing markers in this study

	Primer Sequences (5'to 3')	PCR parameters (All end with 10˚C hold)	Expected amplicon size (bp)	References
trnD-T	ET-F: ACC AAT TGA ACT ACA ATC CC ET-R: CCC TTT TAA CTC AGT GGT A	95℃, 2 mins; 35× (94℃, 30 s; 50℃, 45 s, 72℃, 1 min); 72℃, 5 mins.	650	Bamboo Phylogeny Group, 2005

Five µL of ISSR-PCR and two µL of sequencing-PCR products were run electrophoretically in 2% w/v and 1.5% w/v agarose gels, respectively, pre-stained with SYBR® Safe DNA gel stain (Invitrogen, Thermo Fisher Scientific) along with 100 bp DNA ladder (TransGen Biotech Co., LTD) as references at 80 volts for 50 mins in 1× Tris-acetate-EDTA (TAE) buffer. The gel was visualized using Molecular Imager® ChemiDoc™ XRS+ (Bio-Rad).

The ISSR markers amplicons were subject to fragment analysis by NeoScience Sdn. Bhd. (Malaysia) using Fragment Analyzer (Advanced Analytical Technologies, Inc) with DNF-910 dsDNA kits (35 bp – 1500 bp). On the other hand, the amplicons of the nuclear and cpDNA markers were purified using the Gel/PCR DNA Fragments Extraction Kits (Geneaid, Taiwan) following the manufacturer's gel purification protocol. The purified DNA fragments were then sequenced by First Base Laboratories Sdn. Bhd. (Malaysia)

and BioBasic Asia Pacific Pte Ltd (Singapore) through Sanger Sequencing methods with the 96-capillary system genetic analyser from Applied Biosystems®.

3.5 ISSR Profiling

Binary score matrices (1 for the presence of a band, 0 for the absence of the band) of each ISSR marker were constructed from the fragment analysis (Appendix A) report in Excel. Each locus was defined according to the band size CV (Appendix B). The binary score data was then analysed as dominant data through Genetic Analysis in Excel-GenAlEx 6.5 (Peakall and Smouse, 2012) for the genetic diversity, Nei's genetic distance, and Principal Coordinate Analysis (PCoA) graph.

3.6 DNA Sequence Data Analysis

3.6.1 Multiple Sequence Alignment

BioEdit 7.2.5 (Hall, 1999) was used to examine the sequencing results to identify any noise signals or overlapping peaks. The ambiguous peaks were manually replaced with the nucleotide IUPAC ambiguity code. The missing data was denoted by the character '?'. The heterozygous *PvCel1* gene detected in the hybrids was unfolded into two haplotypes using DnaSP6.12.03 software (Rozas, et al., 2017). This action was carried out based on the assumption that each hybrid individual possesses two haplotypes in Genome EE,. Multiple

sequence alignments were conducted using ClustalX V2.1 (Thompson, et al., 1997; Larkin, et al., 2007).

3.6.2 Network Analysis and Phylogenetic Trees

The haplotype(s) of the *GBSSI* and *PvCel1* gene were implemented with DnaSP v6.12.03 (Rozas, et al. 2017) and later analyzed with Network (1999) v10.2 (https://www.fluxus-engineering.com/index.htm) using Median-Joining algorithm (Bandelt, Forster and Röhl, 1999).

The combined sequences data of cpDNA markers (*rps16-trnQ* + *trnD-T*) and the haplotypes of nuclear genes were used in the reconstruction of phylogenetic trees. The trees were constructed based on the Bayesian Inferences using the Markov chain Monte Carlo (MCMC) algorithms (Larget and Simon, 1999) in MrBayes 3.2 software (Ronquist, et al., 2012). The gap-coding of aligned DNA matrices was performed using the software FastGap v1.2 (Borchsenius, 2009). The best-fit models were estimated in PAUP*4.0 software (Swofford, 2002) using the MrModeltest v2 extension (Nylander, 2004). Bayesian analyses were conducted separately for each nuclear and combined chloroplast marker. The K80+G model was implemented for *GBSSI* (nst set as 2 and rates set as gamma) and the GTR+I model for *PvCel1* (nst set as 6 and rates set as propinv). The F81+G model for concatenated cpDNA markers data (nst set as 1 and rates set as gamma). A total of 10×10^6 generations were run, applied with four chains, and the temperature was set at 0.2. The first 2500 trees served as burn-ins. Posterior probability (PP) values of 0.95 and above were

regarded as acceptable. The outgroup species in this study for the phylogenetic trees included *Kinabaluchloa nebulosa* and *Dinochloa malayana* (Goh, et al. 2013) for cpDNA and *GBSSI* trees, with *Bambusa vulgaris* (Triplett, et al., 2014) for PvCel1 trees.

3.6.3 Intragenic Recombination Detection

To screen for any possible intragenic recombination, tests were performed for both nuclear data sets using the Genetic Algorithm for Recombination Detection (GARD, Kosakovsky Pond, et al., 2006) through the Datamonkey server (Weaver, et al., 2018, http://www.datamonkey.org/GARD/). The best fit models of evolution were inferred using Akaike Information Criterion (Sugiura, 1978), where rate variation of *GBSSI* was set as general discrete, while *PvCel1* sequences had no site-to-site variation. Rate classes 2 – 9 were attempted, where all outcomes were similar and did not affect the conclusions. Additional recombinant detections were conducted to increase the chances of detecting the recombination events in the Recombination Detection Program Beta 5.23 version (RDP5, Martin, et al., 2021) using seven algorithms provided, including RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, and 3Seq (Smith, 1992; Padidam, Sawyer and Fauquet, 1999; Gibbs, Armstrong and Gibbs, 2000; Martin and Rybicki, 2000; Posada and Crandall, 2001; Martin, et al., 2005; Lam, Ratmann and Boni, 2018).

3.6.4 Genetic Diversity, Genetic Distance, Pairwise F_{ST}, and PCoA

The nuclear sequence data (*GBSSI* and *PvCel1* gene) was treated as codominant data. The matrix was converted from bases "A", "T", "C" and "G" to numerical numbers, 1 – 4, while the gaps were denoted as number 5 (Appendix C). The genetic diversity (*h*) was determined with the ISSR profiling matrix. The unbiased expected heterozygosity (*uHe*) and pairwise Nei's genetic distance, F_{ST} and Fixation indices (*F*) were estimated using GenAlEx 6.5 (Peakall and Smouse, 2006; 2017). The PCoA graph was plotted based on the tri-matrix of Nei's genetic distance in GenAlEx 6.5 as well.

3.6.5 Structure Analysis

The Structure analysis was performed using the profiling data of combined ISSR markers as well as the sequencing data of nuclear markers *GBSSI* and *PvCel1* genes. The single nucleotide polymorphisms (SNPs) and gaps data were extracted from the integrated data matrix of the nuclear markers and manually coded with numerical characters (Appendix D). The missing characters were denoted as '-9'. The genetic homologous cluster (*K*) for the populations was identified using the STRUCTURE V2.3.4 software (Pritchard, Stephens and Donnelly, 2000; Falush, Stephens and Pritchard, 2007) through the admixture model. The analysis was run using the parameter recommended by Porras-Hurtado, et al. (2013) with 20 iterations per *K* Value (*K*1–6) at a burn-in period of 200,000 followed by 200,000 MCMC replications. Structure Harvester Web v0.6.94 (Earl and vonHoldt, 2012,

http://taylor0.biology.ucla.edu/structureHarvester/) was used to find the optimal *K* value.

3.6.6 Genetic Differences Between the Hybridizing and Non-Hybridizing *G. scortechinii*

Based on nuclear GBSSI data, the population pairwise F_{ST} were calculated between the *G. scortechinii* individuals from Sungai Siput in the present study and other locations (here regarded as a panmictic population). The non-Sungai Siput *G. scortechinii* sequences used in this study were retrieved from GenBank was shown in Table 3.3. This analysis was performed using GenAlEx 6.5 (Peakall and Smouse, 2006; 2017). The G-statistic with 999 permutations was used to generate the pairwise F_{ST} matrix.

Table 3.3: GenBank accession numbers for the DNA sequences retrieved for non-Sungai Siput *G. scortechinii*

Sample ID	Localities	Genbank Accession	References
D7	Road from Kuala Kubu Baru to Fraser Hill, Selangor	ON357380	Dhanendiren, 2017
D14	Road from Kuala Kubu Baru to Fraser Hill, Selangor	ON357381	
D16	Serendah, Selangor	ON357382	
D21	Serendah, Selangor	ON357383	
D25	Gabai Waterfall, Selangor	ON357384	
Isolate 3	Gombak Road, Selangor, Peninsular Malaysia	HQ697899	Goh, et al., 2011
GWL 2	Hulu Langat, Selangor, Peninsular Malaysia	HQ697897	
GWL 9	Road from Kuala Kubu Baru to Fraser Hill, Peninsular Malaysia	HQ697900	
Bambusetum Acc. 52	Rimba Ilmu Botanical Garden, Univ. of Malaya, Peninsular Malaysia	HQ697901	
Isolate 2	Chebar, Kedah, Peninsular Malaysia	HQ697898	

CHAPTER 4

RESULTS

4.1 ISSR Polymorphism

The ISSR profiling was based on the samples collected before and on Oct 2020. The four ISSR primers generated a total of 64 scoreable loci, ranging in size from 200 to 1500 bp. UBC864 generated the most bands (20), whereas the rest of the primers generated 11 to 19 bands. In total, the ISSR primers yielded 56% of polymorphic loci in *G. scortechinii*, 48% in *D. pendulus* and 64% in ×*G. malpenensis*. Among the 64 scored bands, 20 were able to be used to distinguish the two parental species, i.e., ten loci are specific to *D. pendulus,* and ten loci are specific to *G. scortechinii*, meanwhile, the hybrids inherited both of their parental specific band patterns. The genetic diversity (*h*) estimated based on the ISSR was shown in Table 4.1. The unbiased genetic diversity (*uh*) of hybrids was similar to the outcrossing *G. scortechinii* and higher than the inbreeding *D. pendulus*.

Table 4.1: The genetic diversity (*h*) and unbiased genetic diversity (*uh*) estimated based on the partial samples from Sungai Siput

		ISSR	
Population	N	*h*	*uh*
D. pendulus	6	0.180 ± 0.025	0.216 ± 0.031
×*G. malpenensis*	7	0.218 ± 0.023	0.254 ± 0.027
G. scortechinii	6	0.206 ± 0.025	0.247 ± 0.029

4.2 DNA Sequence Data, Phylogenetic Relationships and Recombination Tests

The sequence characteristic for each chloroplast marker is shown in Table 4.2. The cpDNA tree topologies based on the combined cpDNA data (*rps16-trnQ* + *trnD-T*) showed that the three populations, *G. scortechinii*, *D. pendulus* and their hybrid were unresolved as they were intermixing with each other (Figure 4.1).

Table 4.2: Sequence characteristics of the two cpDNA markers for each population

	G. scortechinii (n=6)	×*G. malpenensis* (n=11)	*D. pendulus* (n=8)
rps16-trnQ			
Data matrix length (bp)	681	682	681
No. of variable sites	1	16	-
No. of parsimony-informative sites	1	-	-
No. of indels	-	1	-
trnD-T			
Data matrix length (bp)	595	595	596
No. of variable sites	1	2	1
No. of parsimony-informative sites	-	1	-
No. of indels	-	-	1

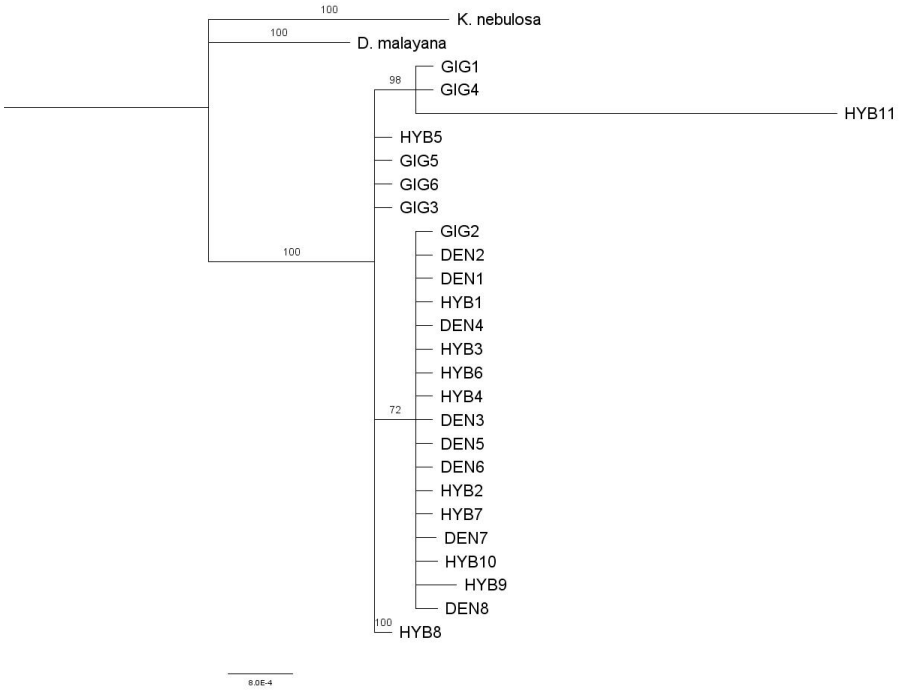


Figure 4.1: Bayesian Tree constructed based on the combined plastid marker *rps16-trnQ* and *trnD-T* sequence data using the F81+G model. *K. nebulosa* and *D. malayana* were included as the outgroup. All *D. pendulus*, *G. scortechinii* and hybrid populations were clustered under the same phylogenetic group. The code in the figures: DEN = *D. pendulus*; GIG = *G. scortechinii*; HYB = ×*G. malpenensis*

The sequence characteristic and the population genetic heterozygosity for each nuclear marker are shown in Table 4.3. Across the *GBSSI* and *PvCel1* markers, *D. pendulus* had a slightly higher genetic heterozygosity than *G. scortechinii* based on the consistent of *He* and *uHe*. ×*G. malpenensis* had the highest number of genetic heterozygosity comparing to its parental populations, with the *uHe* value of 0.268±0.024. The *D. pendulus* population has a higher inbreeding coefficient (F = 0.702±0.051) compared to *G. scortechinii*, while the hybrids obtained a negative fixation index value (F = -0.367±0.058) as shown in Table 4.3.

Table 4.3: Sequence characteristics of nuclear gene markers, observed heterozygosity (*Ho*), expected heterozygosity (*He*), unbiased expected heterozygosity (*uHe*), and fixation index (*F*) for each population

	G. scortechinii (n=6)	×*G. malpenensis* (n=11)	*D. pendulus* (n=8)
GBSSI			
Data matrix length (bp)	715	747	718
No. of variable sites	2	33	5
No. of parsimony-informative sites	-	23	3
No. of indels	-	6	-
PvCel1			
Data matrix length (bp)	426	427	426
No. of variable sites	4	6	5
No. of parsimony-informative sites	-	6	-
No. of indels	-	1	-
GBSSI + PvCel1			
Ho	0.016±0.007	0.411±0.047	0.004±0.002
He	0.024±0.009	0.256±0.023	0.029±0.010
uHe	0.026±0.009	0.268±0.024	0.031±0.011
F	0.280±0.058	-0.367±0.058	0.702±0.051

The individuals from the haplotype group in the network analysis for *GBSSI* and *PvCel1* markers could be referred to in Table 4.4 and the phylogenetic tree in Figure 4.2. At the *GBSSI* locus, the parental individuals exhibited homozygous allele, except for GIG5. *D. pendulus* had seven

haplotypes, and *G. scortechinii* had four haplotypes, respectively. ×*G. malpenensis* was the most variable, having 13 haplotypes. In between, two of the ×*G. malpenensis* haplotypes were shared with *D. pendulus*, and two were shared with *G. scortechinii*. The remaining nine haplotypes were only detected in the hybrid at low frequency. The haplotype GB_Hap14 (*D. pendulus*) and/or GB_Hap7 (*G. scortechinii*) were retrieved in most hybrid samples. All *D. pendulus* and *G. scortechinii* were homozygous in the *GBSSI* locus, except for GIG5 with two haplotypes. All ×*G. malpenensis* were heterozygous where two haplotypes were observed in every individuals. One haplotype of the hybrid is grouped under the *D. pendulus* clade, and another haplotype is grouped under*G. scortechinii* clade, supported by the phylogenetic trees with the PP value ≥ 95 (Figure 4.2a).

At the *PvCel1* locus, both *D. pendulus* and *G. scortechinii* had five haplotypes. Two *D. pendulus* and four *G. scortechinii* individuals were heterozygous, while the rest were homozygous. Most of the hybrids did not possess the present haplotypes of their parental populations. The hybrids had 17 haplotypes, in which only one was shared with *D. pendulus*, but none of them was shared with *G. scortechinii*. The remaining haplotypes detected in hybrids were at low frequency (one to two occurrences). PVE_Hap14 was the dominant haplotype in *D. pendulus* and was found to be homozygous in hybrid individual HYB1 (Table 4.4 and Figure 4.2b). The resolving power of the *PvCel1* markers was relatively low compared to the *GBSSI* markers, where the two parental species were not significantly divided into two clades, and the affinity of the

hybrid haplotypes to the parental species was ambiguous as well. All recombination tests performed in GARD as well as RDP5 showed no evidence of intragenic DNA recombination with no recombination signals and breakpoints detected in the *GBSSI* or *PvCel1* regions (Appendix E). The PHI tests did not find any significant evidence of recombination in both *GBSSI* (p-value = 0.464) and *PvCel1* (p-value = 0.261) datasets.

Table 4.4: Respective haplotype groups for *D. pendulus*, *G. scortechinii*, and ×*G. malpenensis*

Sample ID	*GBSSI* haplotypes	*PvCel1* haplotypes
D. pendulus		
DEN1	GB_Hap15	PVE_Hap 14
DEN2	GB_Hap13	PVE_Hap 16, PVE_Hap 17
DEN3	GB_Hap14	PVE_Hap 14
DEN4	GB_Hap15	PVE_Hap 14
DEN5	GB_Hap16	PVE_Hap 14, PVE_Hap 26
DEN6	GB_Hap12	PVE_Hap 14
DEN7	GB_Hap1	PVE_Hap 25
DEN8	GB_Hap2	PVE_Hap 14
G. scortechinii		
GIG1	GB_Hap7	PVE_Hap 3, PVE_Hap 4
GIG2	GB_Hap7	PVE_Hap 1, PVE_Hap 2
GIG3	GB_Hap7	PVE_Hap 1, PVE_Hap 2
GIG4	GB_Hap7	PVE_Hap 5
GIG5	GB_Hap8, GB_Hap9	PVE_Hap 1, PVE_Hap 2
GIG6	GB_Hap10	PVE_Hap 2
×*G. malpenensis*		
HYB1	GB_Hap7, GB_Hap17	PVE_Hap 14
HYB2	GB_Hap1, GB_Hap7	PVE_Hap 11, PVE_Hap 14
HYB3	GB_Hap7, GB_Hap14	PVE_Hap 10, PVE_Hap 19
HYB4	GB_Hap11, GB_Hap14	PVE_Hap 13, PVE_Hap 18
HYB5	GB_Hap10, GB_Hap18	PVE_Hap 14, PVE_Hap 20
HYB6	GB_Hap14, GB_Hap7	PVE_Hap 12, PVE_Hap 15
HYB7	GB_Hap11, GB_Hap14	PVE_Hap 10, PVE_Hap 19
HYB8	GB_Hap3, GB_Hap19	PVE_Hap 6, PVE_Hap 21
HYB9	GB_Hap6, GB_Hap22	PVE_Hap 9, PVE_Hap 24
HYB10	GB_Hap4, GB_Hap21	PVE_Hap 7, PVE_Hap 23
HYB11	GB_Hap5, GB_Hap20	PVE_Hap 8, PVE_Hap 22

Figure 4.2: Median Joining network analysis based on the (a) *GBSSI* and (b) *PvCel1* gene haplotype data. The pie-chart size is proportionate to the frequencies of each haplotype. *mv = median vector represents the hypothetical or unsampled haplotypes. The phylogenetic trees generated based on the haplotype data were shown beside their respective network diagram. *K. nebulosa* and *D. malayana* were included as the outgroup for the *GBSSI* tree, and *B. vulgaris* was included as the outgroup for the *PvCel1* tree

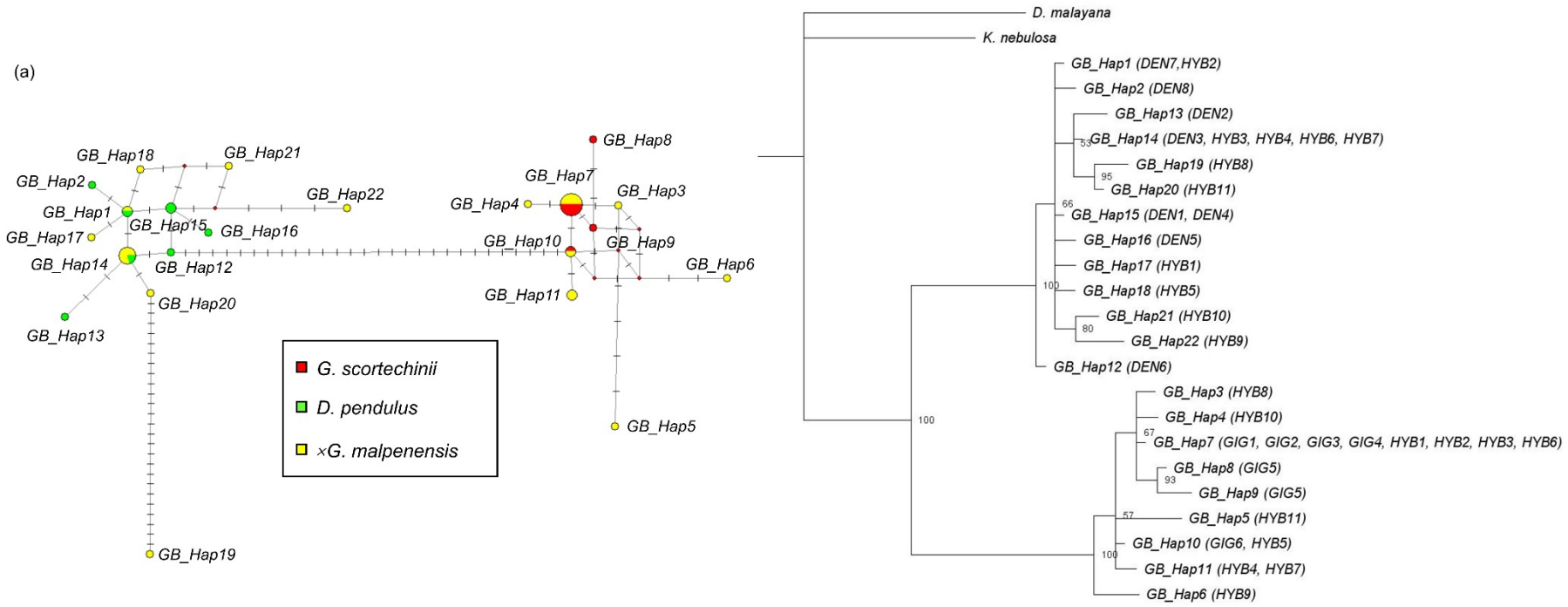
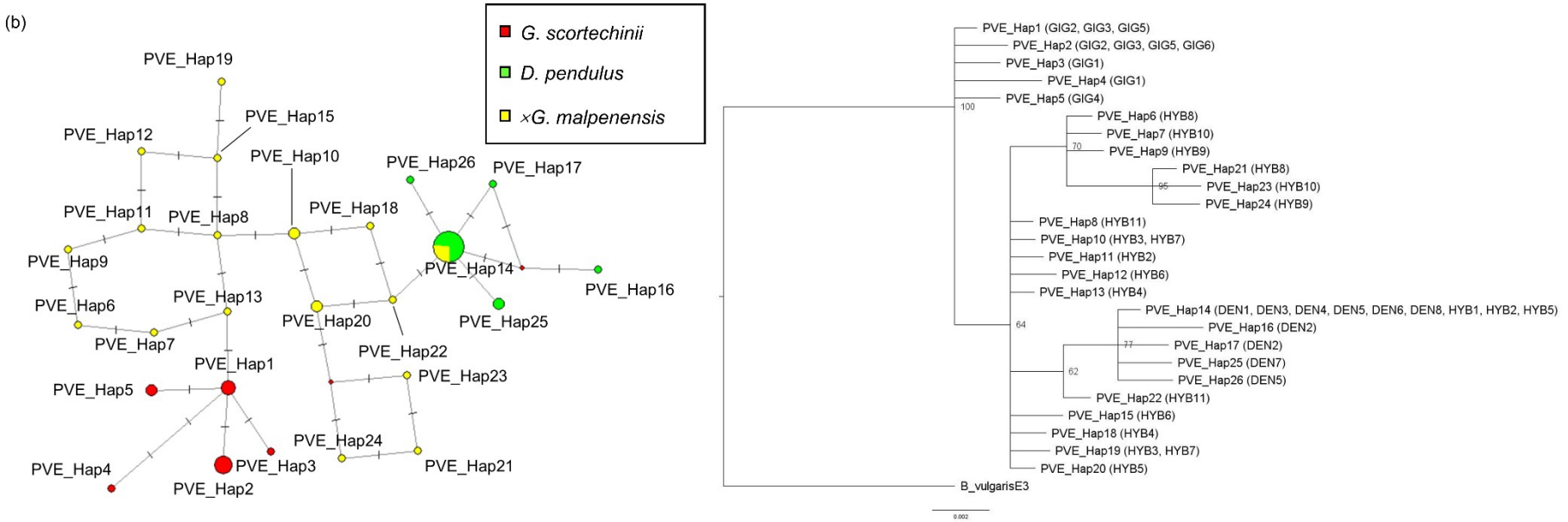


Figure 4.2 (cont.): Median Joining network analysis based on the (a) *GBSSI* and (b) *PvCel1* gene haplotype data. The pie-chart size is proportionate to the frequencies of each haplotype. *mv = median vector represents the hypothetical or unsampled haplotypes. The phylogenetic trees generated based on the haplotype data were shown beside their respective network diagram. *K. nebulosa* and *D. malayana* were included as the outgroup for the *GBSSI* tree, and *B. vulgaris* was included as the outgroup for the *PvCel1* tree



4.3 Pairwise Genetic Distance, F_{ST}, Mean Intrapopulation Genetic Distance and PCoA Graph of ×*G. malpenensis* and Its Parental Species

The pairwise genetic distance between *G. scortechinii* and *D. pendulus* was greater than the distance between ×*G. malpenensis* to both parental species in both ISSR (Table 4.5) and nuclear markers (Table 4.6). The F_{ST} values for each population pair were significant. The F_{ST} values of each population pair were significantly larger than 0.15, considering a great genetic differentiation between each population pair according to Sewall Wright's qualitative guidelines. The PCoA graph of both ISSR and nuclear markers showed that the hybrid individuals were located in between of two parental clusters and scattered across a relatively wider range compared to those of the parental populations (Figure 4.3).

Table 4.5: Pairwise population matrix of Nei's unbiased genetic distance of the three taxa collected from Sungai Siput based on the combined ISSR markers, UBC 810, UBC 857, UBC 864, and Wolfe899

	D. pendulus	×*G. malpenensis*	*G. scortechinii*
D. pendulus			
×*G. malpenensis*	0.089		
G. scortechinii	0.254	0.090	

Table 4.6: Pairwise population matrix of Nei's unbiased genetic distance (below the diagonal) and the pairwise population F_{ST} Values (above the diagonal) of the three taxa collected from Sungai Siput based on the combined nuclear markers, *GBSSI* and *PvCel1* gene sequence data

	D. pendulus	×*G. malpenensis*	*G. scortechinii*
D. pendulus		*0.271	*0.880
×*G. malpenensis*	0.123		*0.295
G. scortechinii	0.548	0.137	

*Significant at *P* value ≤ 0.05

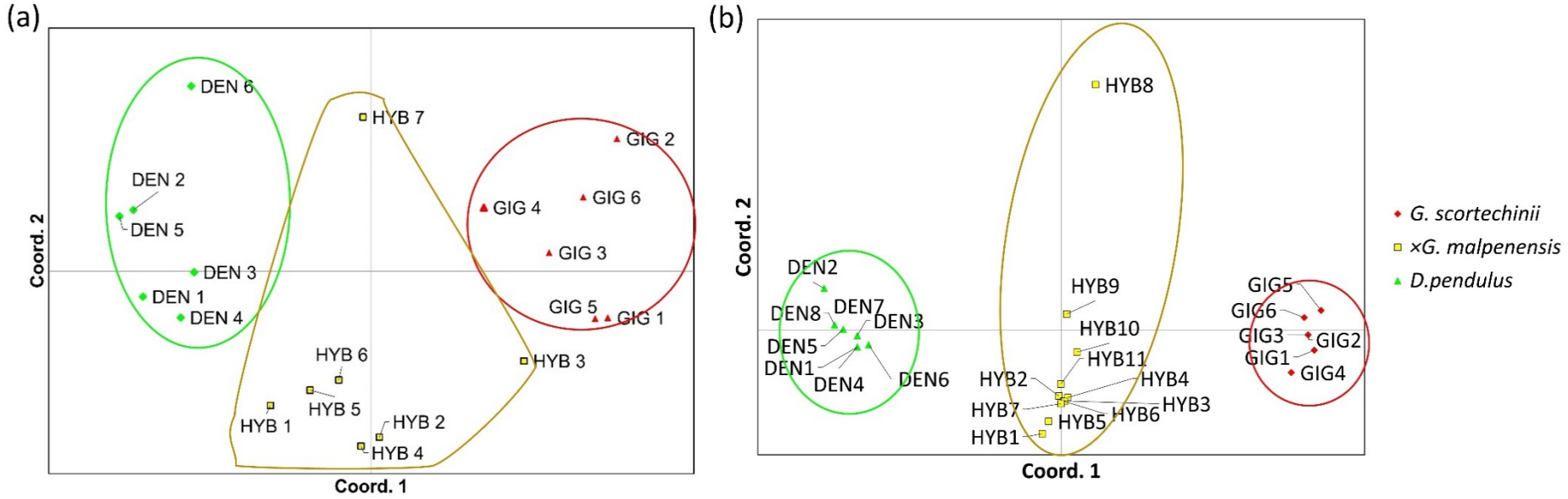


Figure 4.3: PCoA graphs based on the genetic distance of combined (a) ISSR markers and (b) nuclear markers, *GBSSI* + *PvCel1* gene from the three Sungai Siput populations.

4.4 STRUCTURE Analysis

From the Δ*K* graph and the maximum log-likelihood L graph (Appendix F), the samples from the three taxa were separated into two genetic clusters (*K*=2). Figure 4.4 showed that all the *G. scortechinii* were grouped into one genetic cluster. In contrast, all individuals of *D. pendulus* were grouped into another cluster. Both *G. scortechinii* and *D. pendulus* clusters showed a Q-value greater than 0.9 (90%), especially for the nuclear markers. In Figure 4.4a, some ×*G. malpenensis* individuals (HYB1, HYB3, HYB5 and HYB7) appeared to have greater proportion of either *G. scortechinii* or *D. pendulus* genetic composition, (Q-value at or greater than 0.75), while HYB2, HYB4 and HYB6 possessed nearly equal genetic proportion from each parent. The STRUCTURE result for ISSR markers is consistent with the distribution of hybrid individuals in the PCoA graph for ISSR markers (Figure 4.3a). On the other hand, the STRUCTURE result based on the nuclear markers showed that 11 ×*G. malpenensis* individuals displayed a mixture of the two genetic clusters in different proportions (Figure 4.4b) with the admixture coefficients ranging from 0.333 (± 5%) in HYB1 to 0.510 (± 5%) in HYB6 (Appendix G).

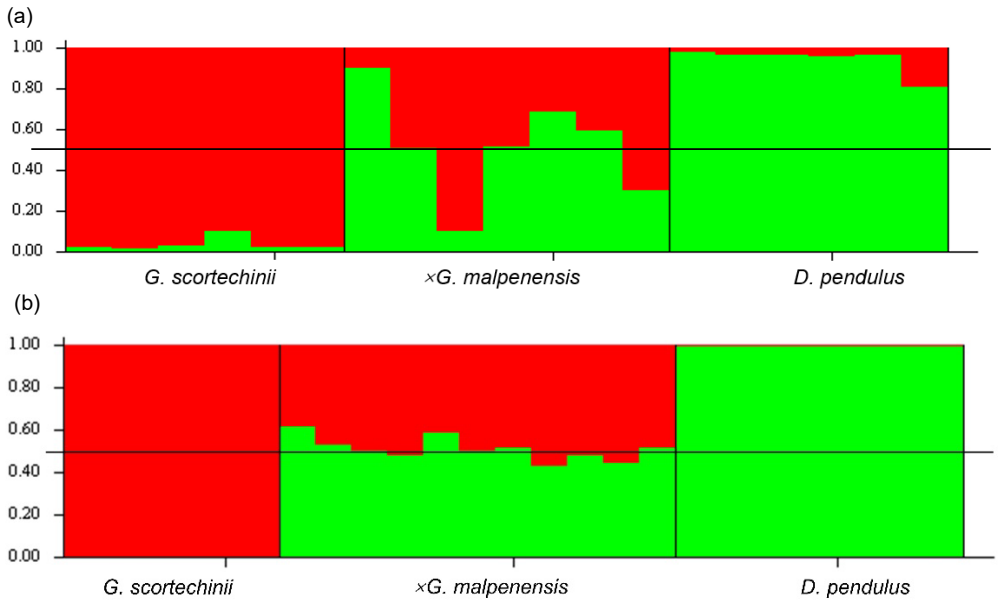


Figure 4.4: Structure analysis (*K*=2) based on the combined (a) ISSR markers and (b) nuclear markers *GBSSI* + *PvCel1* gene. The hybrid genetics obtained different proportions from *D. pendulus* and *G. scortechinii*.

4.5 Genetic Variation of *G. scortechinii* in Sungai Siput and Other Localities

The pairwise comparison between the *G. scortechinii* population in Sungai Siput (representing the hybridising population) and the individuals randomly collected elsewhere in Peninsular Malaysia (representing the non-hybridising population) generated the F_{ST} value of 0.026 ($p = 0.895$), suggesting that there is no significant genetic difference between the hybridising and non-hybridising populations of *G. scortechinii*.

CHAPTER 5

DISCUSSION

5.1 ×*G. malpenensis* as F1 Hybrid

×*G. malpenensis* samples collected in Sungai Siput were suggested to be F1 hybrids, as reflected in the pairwise F_{ST} and genetic distance of both ISSR and nuclear markers. Overall, ×*G. malpenensis* shared equivalent genetic distance and pairwise F_{ST} value to its parental species. This was further proven in the STRUCTURE analysis. Although, a few hybrid samples appeared to possess different proportions of parental genetic composition in the STRUCTURE analysis, which were contributed by the less accuracy of ISSR markers than the nuclear sequencing markers and variable of the confidence intervals of Q-value in the nuclear markers. Most of the ×*G. malpenensis* individuals received nearly equal influence from both parental species. Based on the haplotype analysis, all hybrids harboured one haplotype from *D. pendulus* and the other from *G. scortechinii* in the *GBSSI* locus. Although it was low resolution in the *PvCel1* phylogenetic tree and network, ten out of 11 hybrids received a haplotype similar to *D. pendulus* and another haplotype was similar to *G. scortechinii* haplotypes. These haplotype combinations are consistent with the pattern seen in first-generation hybrids. Given the recent occurrence of natural hybridisation between *D. pendulus* and *G. scortechinii* in Sungai Siput, and the flowering intervals of woody bamboos were quite long (Zheng, et al., 2020), it is reasonable to conclude that subsequent generations of

×*G. malpenensis* and/or backcrosses at much earlier generations were not detected. This finding corresponded to the ×*G. malpenensis* population in Ulu Gombak, where hybrid individuals were found and thought to be F1 hybrids (Wong and Low, 2011).

5.2 Genetic Attributes in *D. pendulus*, *G. scortechinii* and ×*G. malpenensis*

D. pendulus and *G. scortechinii* have different flowering modes. The former is known to display gregarious flowering and monocarpic, i.e. dies soon after flowering (Burgess, 1975; Wong, 1995; Azmy Hj. Mohamed, 2004). The latter exhibits diffuse sporadic flowering without whole-clump death (Holttum, 1958; Wong, 1995). From previous study, the clones or those population with low genetic diversity were reported to have massive synchronized or gregarious flowering (Kaneko, et al., 2008; Zheng, et al., 2020). However, the two parental populations with different flowering modes populations, have similar genetic diversity in this study, as showed in Table 4.1 and Table 4.3. Thus, no possible correlation between the flowering modes and the level of genetic diversity was discovered in this study. On the other hand, *D. pendulus* population in this hybrid zone has a higher inbreeding coefficient (F = 0.702) compared to *G. scortechinii* (F = 0.280). While *G. scortechinii* has been suggested to be self-incompatible (Wong, 1995), some other woody bamboo species, e.g. two *Dendrocalamus* species in Southwest China, *D. membranaceus* and *D. sinicus*, were reported to have some degree of self-compatibility although predominantly outcrossing (Chen, et al., 2017; Goh and Wong, 2021). The *D. pendulus* population was a like case in this study, whereby self-crosses could

have happened in response to population fragmentation in the disturbed areas and other kinds of ecological disturbances.

In ISSR profiling, hybrid populations (*uh* = 0.254) appeared as diverse as the out-crossing *G. scortechinii* populations (*uh* = 0.247) and had higher diversity than the *D. pendulus* population (*uh* = 0.216) as shown in Table 4.1. In between, each parental population was more genetically uniform than that of the hybrid population at the nuclear locus, as indicated by their relatively low *uHe* and their concentrated distributions in PCoA. Based on the nuclear markers, heterozygosity of ×*G. malpenensis* population was high (*uHe* = 0.268±0.024) compared to those of either parent (Table 4.3), as expected. It represents the homoeologous heterozygosity as the F1 hybrids naturally inherited one allele (or an equal number of alleles, in higher ploidy-level species) from each parent. Moreover, the distribution of hybrid individuals in both ISSR and nuclear PCoA graphs indicated that the hybrid population was not less diversified than the parental population or not totally uniform. There are multiple haplotypes present in the hybrid population: 13 haplotypes for the *GBSSI* marker and 17 haplotypes for the *PvCel1* marker. Although intragenic recombination appeared common in plants, such as *Arabidopsis*, *Nicotiana*, and *Oryza sativa* (Mourad, Haughn and King, 1994; Inukai, et al., 2000; Kelly, et al., 2010), there was no evidence of intragenic recombination detected in either *GBSSI* or *PvCel1* markers in the present study. Therefore, each nuclear DNA haplotype obtained in this study was suggested to represent a unique parental (i.e., non-recombined) allele. Considering higher numbers of the *GBSSI* and *PvCel1* haplotypes in the

F1 ×*G. malpenensis* population than those in the parental populations, our results indicated that the haplotypes in the bamboo hybrid population in Sungai Siput could have derived from multiple individuals of each parental species. This implies that the natural hybridisation events have involved a number of parental individuals.

×*G. malpenensis* population in Sungai Siput was similar to those in Ulu Gombak and Tapah-Cameron Road, which were assumed to possess a range of genetic variability as reflected in their various flowering onset ages and associated longevity (Wong and Low, 2011; Goh, et al., 2011). The negative F value of Sungai Siput's ×*G. malpenensis* also suggested that this species had undergone disassortative mating which resulted in increased heterozygosity and provided a particularly new and different phenotype from its parents.

5.3 Ecological Disturbance as A Driving Force Behind Bamboo Hybridisation

As suggested by Wong and Low (2011), environmental factors are more likely to be the driving force behind the hybridisation events, upon the existence of the bamboo hybrid zone in Ulu Gombak to the construction of the Karak Expressway. The widespread forest clearing for agriculture (oil palm plantation was observed in the field, as shown in Figure 5.1) and logging may have facilitated the natural hybridisation events in Sungai Siput. In this study, deforestation demolished the ecological barriers between *G. scortechinii* alongside Sungai Piah riverbanks and *D. pendulus* hillside along the logging

paths. Deforestation has also been a factor in the natural hybridisation of other plant species, such as the bittercresses in Switzerland; the oak tree species in Mexico; and the mangrove fern species in China (Urbanska, et al., 1997; Tovar-Sánchez and Oyama, 2004; Zhang, et al., 2013).

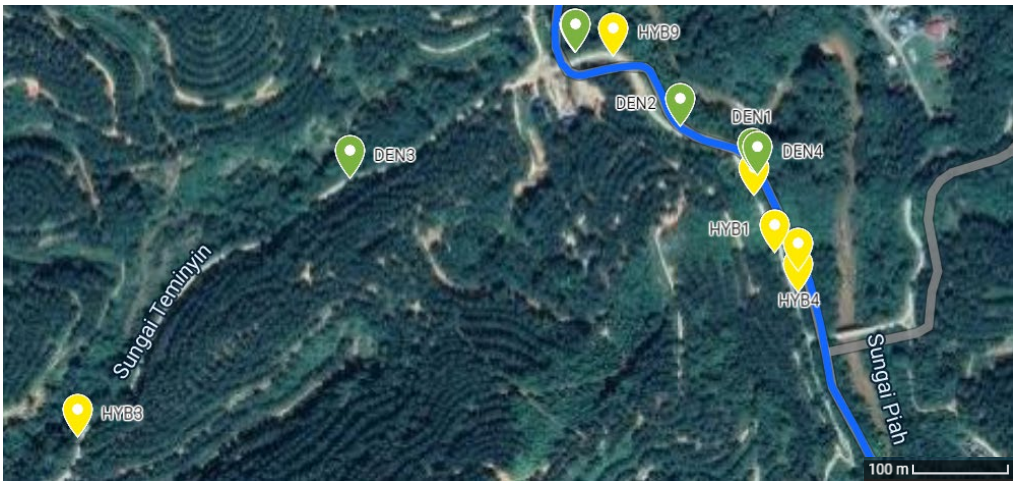


Figure 5.1: Oil palm plantation in the sample collection area.

The emergence of ×*G. malpenensis* created an ideal picture of how ecological disturbances might lead to hybrid speciation in a disturbance-tolerant plant community. Three natural populations of ×*G. malpenensis* have been identified so far (although one had been cleared for development), all of which were related to past ecological disturbances (Wong and Low, 2011). Indeed, the human-mediated ecological disturbance has created opportunities for the emergence of new species and hence new traits in bamboos, some of which may be useful or beneficial to humanity. Given Malaysia's rapid deforestation rate of approximately 90,000 ha per year between 1990 and 2010 (Rawshan Ara Begum, Asif Raihan and Mohd Nizam Mohd Said, 2020), and the possibility of

further forest disturbances due to industrialisation, urbanisation, mining, agricultural, and oil-palm plantation, the report of new localities for bamboo hybrids asides from ×*G. malpenensis* is predictable in the foreseeable future. The man-made ecological disturbance has opened opportunities for speciation in bamboo.

There is a high possibility that the parental population of *G. scortechinii* could endure without being adversely affected by ecological disturbances and/or by the emergence of the new hybrid population, as suggested by the non-significant genetic differentiation between the hybridising and non-hybridising *G. scortechinii* populations. Unfortunately, the genetic differentiation of the *D. pendulus* population could not be included in the present study as there was insufficient sequence data for non-hybridising *D. pendulus*.

D. pendulus populations were recorded on the northeast coast of Peninsular Malaysia (Kelantan and Terengganu) as well as in other countries such as Bangladesh, Burma, and Thailand (Wong, 1995; Rao and Rao, 1998). On the other hand, *G. scortechinii* were recorded in Northern and East Peninsular Malaysia, such as Kedah, Kelantan, Perak, Selangor and Terengganu, and in southern Thailand (Wong, 1995; Rao and Rao, 1998; Widjaja, 1998). Given that both parental species were reported to have undergone gregarious flowering when their habitats had been massively disturbed, a new population of ×*G. malpenensis* had a high probability of arising in the locality where both two parental populations occurred in northern Peninsular Malaysia and southern

Thailand. Since anthropogenic activities are likely to increase over the years and there was no reported evidence of this hybrid species being infertile as the hybrid had flowered before (Wong and Low, 2011), the hybrids could have affected their parental genetic compositions through possible gene flows, and thus, the formation of the hybrid zones requires more attention.

5.4 Limitation and Utility of The Genetic Markers in Present Study

Since the three populations were intermixing in the cpDNA Bayesian Inference tree, cpDNA data was suggested to be less informative in the present study. There was no genetic variation and heterozygosity detected between the two parental populations, not to mention the hybrid population. No genetic diversity information was obtained by using the chloroplast intergenic markers. The introgression of cpDNA had been reported in the Bambusinae species. Apart from *D. pendulus* and *G. scortechinii*, other *Dendrocalamus* and *Gigantochloa* species were found to be monophyletic in *Bambusa-Dendrocalamus-Gigantochloa* (BDG) complex as reported in previous studies (Goh, et al., 2013; Liu, et al., 2020; Chalopin, et al., 2021). The monophyletic BDG complex was presumably due to Incomplete Lineage Sorting, of which the ancestral polymorphisms in *Dendrocalamus* and *Gigantochloa* were conserved to the point that they cannot be proven as distinct lineages. On the other hand, the monophyletic relationship could be explained as chloroplast capture, in which certain *Gigantochloa* have *Dendrocalamus* chloroplasts as a result of historical introgression, and hence preserved the same chloroplast haplotypes, as suggested by Dhanendiren, et al. (2015) where two distinct

cpDNA haplotypes of *G. scortechinii* were discovered in Peninsular Malaysia. Consequently, ×*G. malpenensis* might have also retained similar cpDNA material as its parents. The seed parent of the hybrids remains uncertain. Nevertheless, the hybrid individuals sampled in this study were similar to those investigated by Wong and Low (2011), where 7 out of 11 hybrids were found growing near the *D. pendulus* populations. As seed dispersion is weaker than pollen, therefore, *D. pendulus* was suggested to be the female parent of ×*G. malpenensis*.

Overall, the ISSR and nuclear markers gave similar results in the present study. Both markers showed ×*G. malpenensis* as F1 hybrids and detected great genetic variation in this hybrid species. Even though the genetic diversity and genetic heterozygosity were inconsistent in parental species, where *D. pendulus* was less diversified than *G scortechinii* for ISSR markers, while the genetic heterozygosity of *D. pendulus* was higher than *G. scortechinii* based on the nuclear markers, both ISSR and nuclear markers estimated high diversity in ×*G. malpenensis*. ISSR markers were employed as preliminary testing for genetic diversity and population structure of the hybrid and parental populations. Similarly to the nuclear markers, ISSR were notably referred as somatic gene markers, since di- or tri- microsatellites were less likely in the cytoplasmic DNA of chloroplasts and mitochondria (Kuntal, Sharma and Daniell, 2012; Wang, et al., 2017), making them more informative than the cpDNA markers. However, the estimation may not be sufficiently accurate based on scorable bands generated by the ISSR markers, i.e., reproducibility of the bands may be

influenced by the condition of the template DNA or polymerase chain reaction (Yeasmin, et al., 2015), which may contribute to the inconsistent of the STRUCTURE result in ISSR and nuclear markers (Figure 4.4)

The *PvCel1* marker was suggested to have relatively low resolving power compared to the *GBSSI* marker, as reflected in their Bayesian Inference Trees. The PP values at the main divergence nodes of the *GBSSI* tree were acceptable at 95, while most of the PP values were lesser than 95 at the *PvCel1* tree. Furthermore, parental individuals were mainly homogeneous (except for GIG5 with one site of SNP) at the *GBSSI* locus, while many of the parental individuals have heterozygous alleles (at most 3 SNPs) at the *PvCel1* locus. The high variation of the confident interval of STRUCTURE analysis in the hybrid population appeared due to the low resolution of the *PvCel1* marker, especially where one hybrid (HYB1) possessed homozygous haplotypes in the *PvCel1* locus. Thus, it could be inferred that the *PvCel1* marker, though useful for taxonomic studies, might not have sufficient resolution for population-level studies (Chokthaweepanich, 2014; Triplett, et al., 2014). Even though, the sequence data of *PvCel1* marker is able to reveal the heterozygosity in the hybrid populations since all the hybrid individuals possess different haplotypes from its parental populations which are occurred at low frequency. Despite the limitations of each marker, the combination of multiple markers including ISSR, could distinguish the population structure of the hybrid population and its parental populations.

5.5 Silviculture Potential of ×*G. malpenensis*

Based on the morphology aspect, the hybrid samples collected from this study were found to have intermediate culm diameter (ca. 4 – 7 cm) between two parental species (6 – 12 cm for *G. scortechinii* and 2.5 – 3.5 cm for *D. pendulus*) and have similar wall thickness as *D. pendulus* (0.4 – 1.1 cm). The whole culm of hybrid samples with moderate culm diameter and wall thickness have the potential to be used for making ornament of buildings and walls, or split into bamboo splits for making furniture and flooring.

At the molecular level, the genetic attributes of ×*G. malpenensis* encompass a wide range of variations in the physicochemical (such as culm diameter, culm wall thickness, and lignin content), mechanical (such as tensile strength or young's of the modulus), and physiological (such as flowering interval and longevity and incidences of whole-clump death) characteristics in this hybrid population. This hybrid population could serve as a diverse genetic stock for silviculture as there will be choices for trait selection, and the desired ones can then be maintained in cultivation.

None of the hybrid clumps in Sungai Siput was found to flower during the collection period. In a previous study, a sole clump of hybrid collected from Tapah (WKM 2895) that was planted in Bambusetum, Rimba Ilmu Botanical Garden, University of Malaya, flowered soon after growing into mature size. However, no caryopses were found in this flowered hybrid, which died a year after blooming (Wong and Low, 2011). Flowering and seed sets of ×*G.*

malpenensis were not observed during the sample collections in the present study. However, it may be too early to conclude that these hybrid species are sterile. A hybrid bamboo, *G. ridley*, was reported to have seed sets, although they were less reproductive as the sprouts were albino and less viable (Muller, 1998; 2003). Even though the hybrid species were suspected to be sterile, this does not reduce their potential to persist and continue surviving in nature and cultivation as bamboos are generally capable of vegetative reproduction and regeneration (Goh and Wong, 2021).

5.6 Future Studies

First and foremost, the hybrid clumps in Sungai Siput may require time to time assessment to make sure the conservation of this species in wild. Although there will be promising supply of this hybrid from wild, cultivation of ×*G. malpenensis* and the development of plantations should be encouraged to maintain this species, since the natural hybridisation event of the two parental species in wild could be rare. Moreover, experimental cross pollination between *D. pendulus* and *G. scortechinii* could be carried out to assess their reproduction isolation and the effect of the environmental stress in promoting the hybridisation in between them. Furthermore, only the population genetic study of the hybridising and non-hybridising *G. scortechinii* was carried out in present study. Possible changes in the population genetic structure for *D. pendulus* should be further investigated. Finally, since ×*G. malpenensis* is suggested to have potential for its uses in construction and furniture making based the observation of its morphological traits, research in the other aspects, such as

chemical and mechanical properties of this hybrid species is necessary to evaluate its potential for development into a commercially viable natural resource.

CHAPTER 6

CONCLUSION

Based on the morphological observation during sample collection, ×*G. malpenensis* possesses intermediate morphological traits from its parental species, where it has moderate culm size which is as straight as *G. scortechinii* and appear to have good bending capability as *D. pendulus*. These characteristics make ×*G. malpenensis* potential as value materials in the construction industry. The genetic assessment based on the ISSR and nuclear markers, *GBSSI* and *PvCel1* showed that the ×*G. malpenensis* population in Sungai Siput is a suitable source for acquiring cultivation materials for commercial purposes since this population has high genetic diversity as well as heterozygosity. New hybrid populations, if they arise in the future as mediated by the environmental factors such as forest clearing, could further build up the genetic pool of this species. Although the hybrids were suspected to be sterile F1, further evaluations may still be necessary to ensure the sustainability of this hybrid species and its parental species.

Despite their great economic and ecological values, bamboos in Southeast Asia are lacking of population genetic structure research. Our present study demonstrated the usefulness of molecular genotyping in bamboo silvicultural management and clone selection for cultivation. However, the

PvCel1 marker developed in the present study does not having sufficient resolving power. Hence, it is hoped that more studies on the population genetics of bamboos, such as the development of other nuclear markers or implementation of the Next Generations Sequencing as well as integration with studies on their life history characteristics, will become available for proper management and conservation of the bamboo resources.

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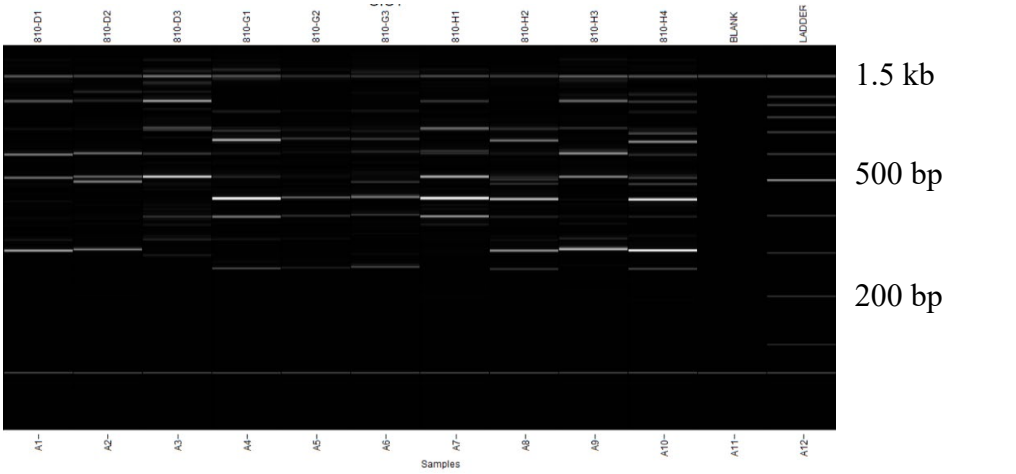
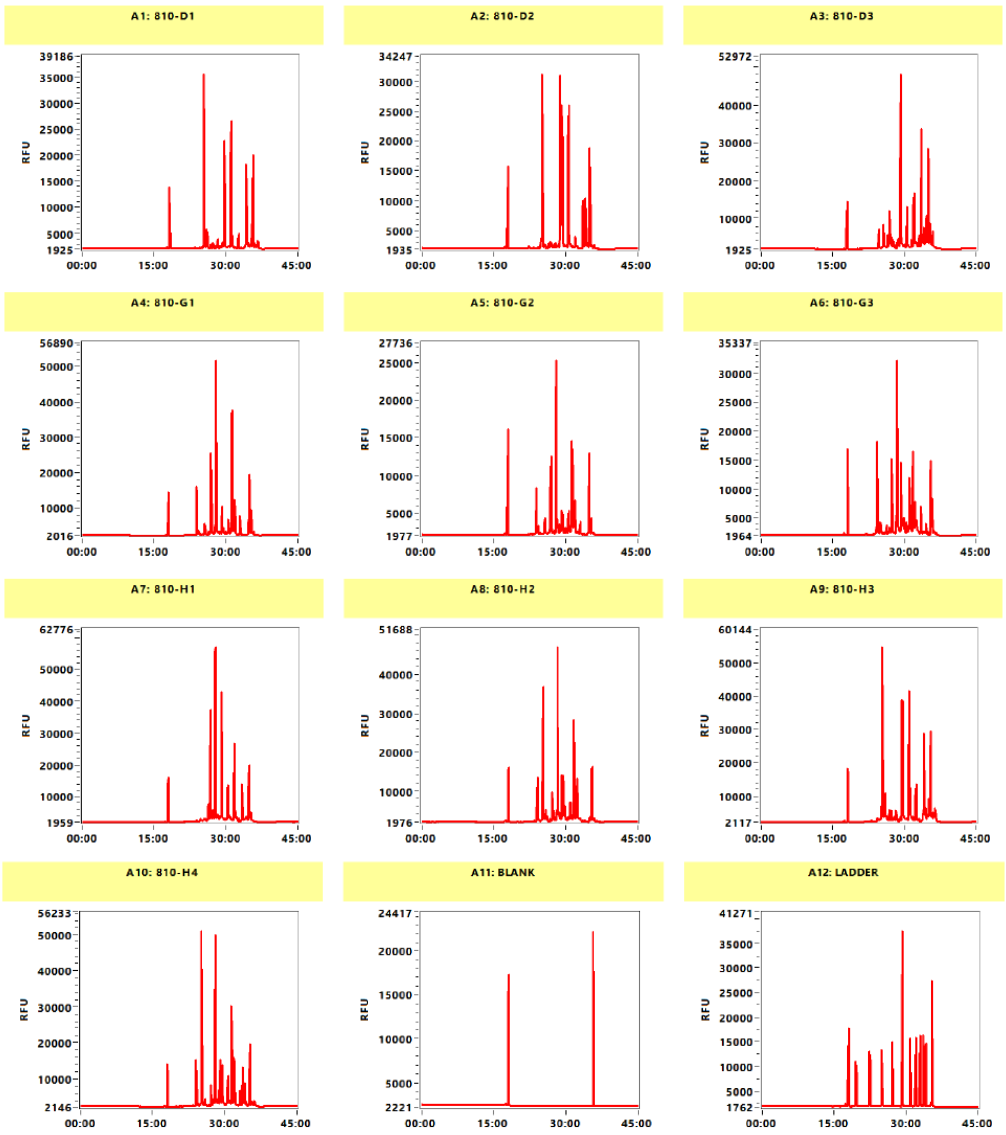
Appendix A

An example of the Fragment analysis report (the picture showing the report for marker UBC 810), where the bands of each marker were within the size range of 200 to 1500 bp.





Appendix B

The locus for ISSR binary scoring was defined according to the band size range.

Raw data from FA results							
Sample ID/size range (bp)	247-275	297-325	325 366	366-385	385 421	437 470	479 497
d1 (DEN1)	0	1	0	0	0	0	0
d2 (DEN2)	0	1	0	0	0	0	1
d3 (DEN3)	0	0	0	0	1	0	0
g1 (HYB4)	1	0	0	0	1	1	0
g2 (HYB5)	0	0	0	0	1	1	0
g3 (GIG1)	1	0	0	0	1	1	1
h1 (HYB1)	0	0	0	0	1	1	0
h2 (HYB2)	1	1	0	0	1	1	1
h3 (DEN4)	0	1	0	0	0	0	0
h4 (HYB3)	1	1	0	0	0	1	1
h01 (HYB6)	1	0	1	0	1	0	1
h02 (HYB7)	1	0	0	0	1	1	0
g01 (GIG2)	1	0	0	0	1	1	1
g02 (GIG3)	1	0	1	0	1	1	0
g03 (GIG4)	1	0	0	0	1	1	0
g04 (GIG5)	1	0	1	0	1	1	1
g05 (GIG6)	1	0	0	0	1	1	0
d02 (DEN5)	0	0	0	1	1	0	0
d03 (DEN6)	0	0	1	1	1	0	0

Appendix C

The Excel table shows data matrix of the codominant nuclear sequence data in GenAlEx 6.5.

	A	B	C	D	E	F	G	H	I	J
1	96	25	3	6	11	8				
2				G. scortec	×G. malpe	D.pendulus				
3		pop	locus1		locus2		locus3		locus4	
4	GIG1	G. scortech	5	5	4	4	4	4	1	1
5	GIG2	G. scortech	5	5	4	4	4	4	1	1
6	GIG3	G. scortech	5	5	4	4	4	4	1	1
7	GIG4	G. scortech	5	5	4	4	4	4	1	1
8	GIG5	G. scortech	5	5	4	4	1	1	1	1
9	GIG6	G. scortech	5	5	4	4	4	4	1	1
10	HYB1	×G. malpen	5	5	4	1	4	4	1	1
11	HYB2	×G. malpen	5	5	4	1	4	4	1	1
12	HYB3	×G. malpen	5	5	4	1	4	4	1	1
13	HYB4	×G. malpen	5	5	4	1	4	4	1	1
14	HYB5	×G. malpen	5	5	4	1	4	4	1	1
15	HYB6	×G. malpen	5	5	4	1	4	4	1	1
16	HYB7	×G. malpen	5	5	4	1	4	4	1	1
17	HYB8	×G. malpen	4	4	4	1	4	4	1	1
18	HYB9	×G. malpen	4	5	4	1	1	4	1	3
19	HYB10	×G. malpen	5	4	4	1	4	4	1	1
20	HYB11	×G. malpen	4	4	4	1	4	4	1	1
21	DEN1	D.pendulus	5	5	1	1	4	4	1	1
22	DEN2	D.pendulus	5	5	1	1	4	4	1	1
23	DEN3	D.pendulus	5	5	1	1	4	4	1	1
24	DEN4	D.pendulus	5	5	1	1	4	4	1	1
25	DEN5	D.pendulus	5	5	1	1	4	4	1	1
26	DEN6	D.pendulus	5	5	1	1	4	4	1	1
27	DEN7	D.pendulus	5	5	1	1	4	4	1	1
28	DEN8	D.pendulus	5	5	1	1	4	4	1	1

Appendix D

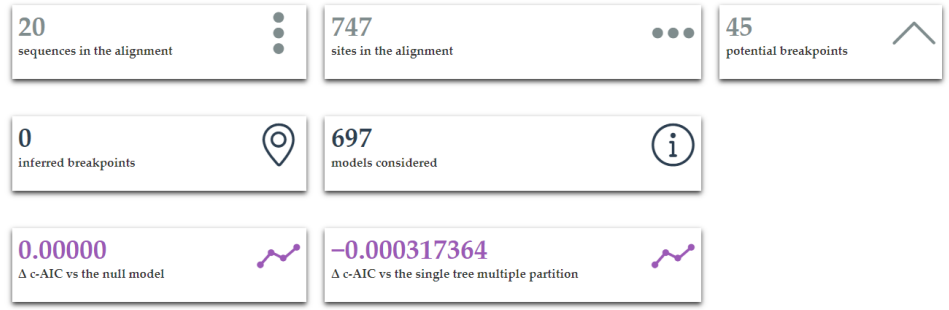
The Excel table shows the data matrix for the STRUCTURE analysis based on the *GBSSI* and *PvCel1* genes.

	A	B	C	D	E	F	G	H	I	J	K	L
1	GIG1	1	-9	3	3	4	1	4	1	2	4	-9
2	GIG1	1	-9	3	3	4	1	4	1	2	4	-9
3	GIG2	1	-9	3	3	4	1	4	1	2	4	-9
4	GIG2	1	-9	3	3	4	1	4	1	2	4	-9
5	GIG3	1	-9	3	3	4	1	4	1	2	4	-9
6	GIG3	1	-9	3	3	4	1	4	1	2	4	-9
7	GIG4	1	-9	3	3	4	1	4	1	2	4	-9
8	GIG4	1	-9	3	3	4	1	4	1	2	4	-9
9	GIG5	1	-9	3	1	4	1	4	1	2	4	-9
10	GIG5	1	-9	3	1	4	1	4	3	1	4	-9
11	GIG6	1	-9	3	3	2	1	4	1	2	4	-9
12	GIG6	1	-9	3	3	2	1	4	1	2	4	-9
13	HYB1	2	-9	3	3	4	1	4	1	2	4	-9
14	HYB1	2	-9	1	3	2	1	1	1	4	4	1
15	HYB2	2	-9	3	3	4	1	4	1	2	4	-9
16	HYB2	2	-9	1	3	2	1	1	1	4	4	1
17	HYB3	2	-9	3	3	4	1	4	1	2	4	-9
18	HYB3	2	-9	1	3	2	1	1	1	4	4	1
19	HYB4	2	-9	3	3	2	1	4	1	2	4	-9
20	HYB4	2	-9	1	3	2	1	1	1	4	4	1
21	HYB5	2	-9	3	3	2	1	4	1	2	4	-9
22	HYB5	2	-9	1	3	2	1	1	1	4	4	1
23	HYB6	2	-9	3	3	4	1	4	1	2	4	-9
24	HYB6	2	-9	1	3	2	1	1	1	4	4	1
25	HYB7	2	-9	3	3	2	1	4	1	2	4	-9
26	HYB7	2	-9	1	3	2	1	1	1	4	4	1
27	HYB8	2	3	3	3	4	1	4	1	2	4	-9
28	HYB8	2	3	1	3	2	1	1	1	4	4	1
29	HYB9	2	3	3	1	2	1	1	1	2	4	-9
30	HYB9	2	3	1	3	2	1	1	3	4	4	1
31	HYB10	2	-9	3	3	4	1	4	1	2	4	-9
32	HYB10	2	3	1	3	2	1	1	1	4	4	1
33	HYB11	2	3	3	3	2	1	4	1	2	4	-9
34	HYB11	2	3	1	3	2	1	1	1	4	4	1
35	DEN1	3	-9	1	3	2	1	1	1	4	4	1
36	DEN1	3	-9	1	3	2	1	1	1	4	4	1
37	DEN2	3	-9	1	3	2	4	1	1	4	2	1
38	DEN2	3	-9	1	3	2	4	1	1	4	2	1
39	DEN3	3	-9	1	3	2	1	1	1	4	4	1
40	DEN3	3	-9	1	3	2	1	1	1	4	4	1
41	DEN4	3	-9	1	3	2	1	1	1	4	4	1
42	DEN4	3	-9	1	3	2	1	1	1	4	4	1
43	DEN5	3	-9	1	3	2	4	1	1	4	4	1
44	DEN5	3	-9	1	3	2	4	1	1	4	4	1
45	DEN6	3	-9	1	3	2	1	1	1	4	4	1
46	DEN6	3	-9	1	3	2	1	1	1	4	4	1
47	DEN7	3	-9	1	3	2	1	1	1	4	4	1
48	DEN7	3	-9	1	3	2	1	1	1	4	4	1
49	DEN8	3	-9	1	3	2	1	1	1	4	4	1
50	DEN8	3	-9	1	3	2	1	1	1	4	4	1

Appendix E

The GARD (http://www.datamonkey.org/GARD/) analysis results for the recombination detection of (a) GBSSI and (b) PvCel1 gene.

(a)

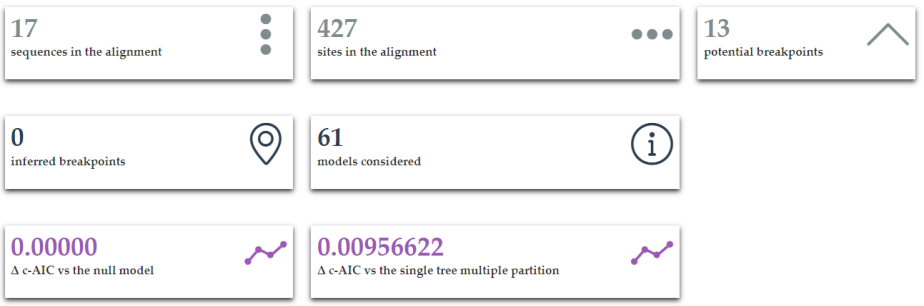


GARD **found no evidence** of recombination. GARD examined **697** models at a rate of **63.36** models per second. The alignment contained **45** potential breakpoints, translating into a search space of **45** models with up to **1** breakpoints, of which **1548.89%** was explored by the genetic algorithm.

See **here** for more information about this method.

Please cite **PMID 16818476** if you use this result in a publication, presentation, or other scientific work.

(b)



GARD **found no evidence** of recombination. GARD examined **61** models at a rate of **20.33** models per second. The alignment contained **13** potential breakpoints, translating into a search space of **13** models with up to **1** breakpoints, of which **469.23%** was explored by the genetic algorithm.

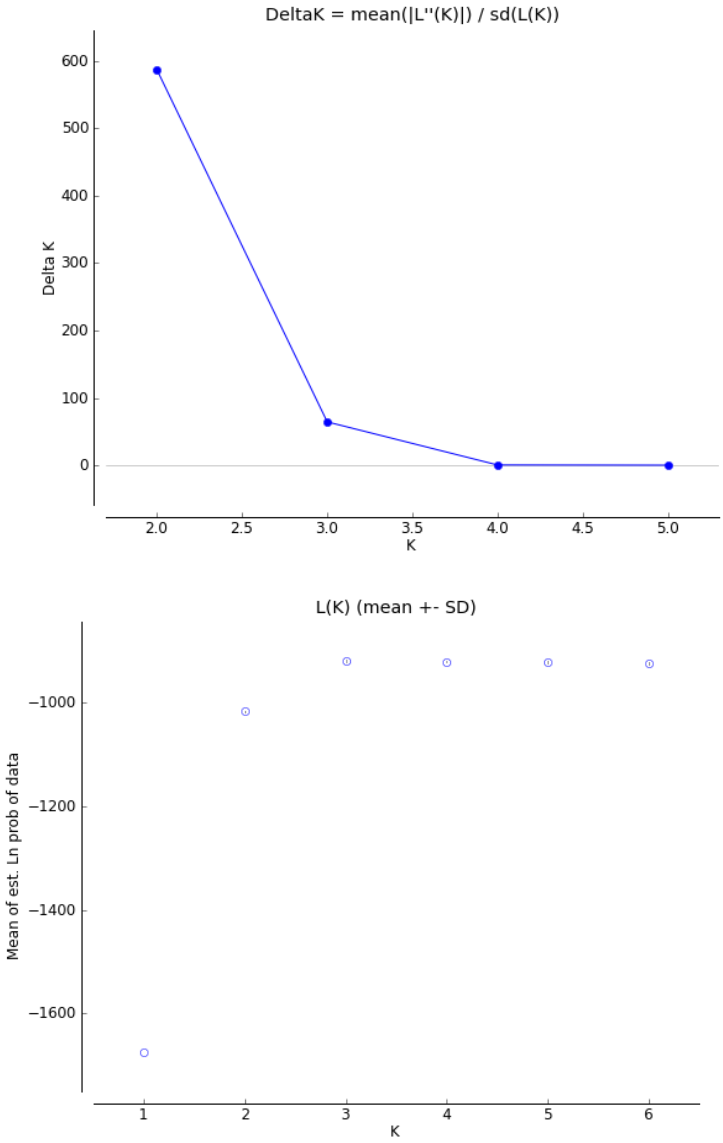
See **here** for more information about this method.

Please cite **PMID 16818476** if you use this result in a publication, presentation, or other scientific work.

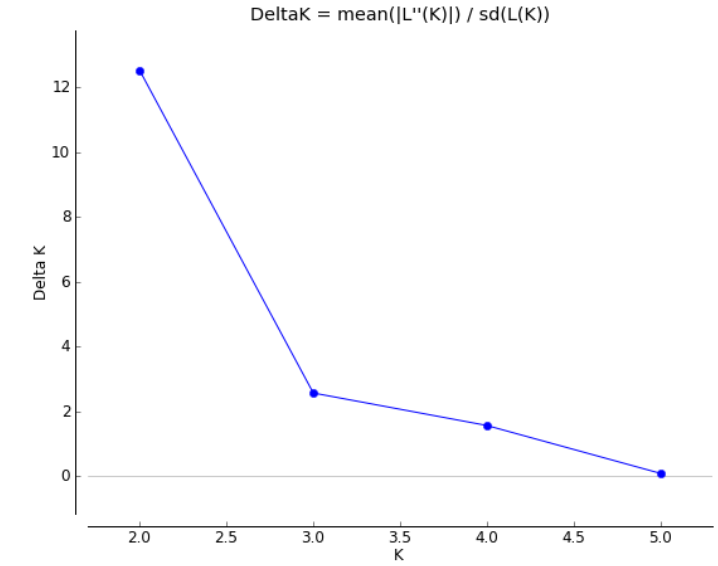
Appendix F

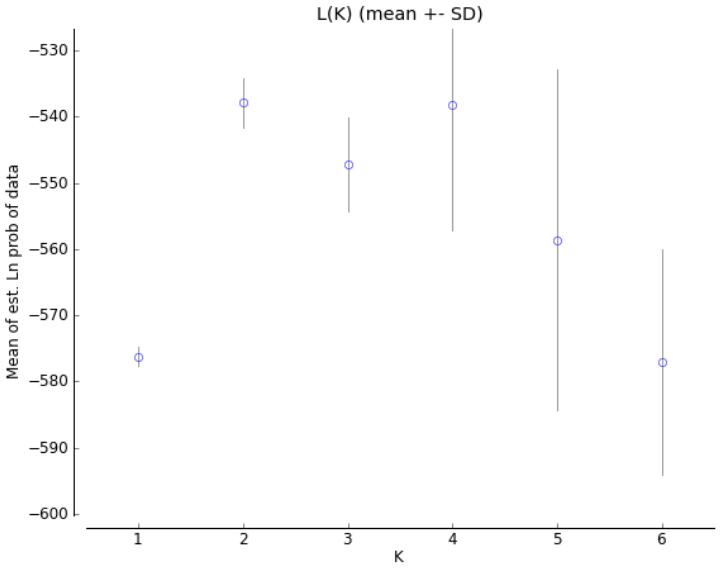
The Delta *K* (ΔK) graph and the maximum log-likelihood L graph for the STRUCTURE analysis based on the 20 iterations run using the admixture model. The best *K* value is 2 ($K = 2$) for both (a) *GBSSI* + *PvCel1* markers and (b) ISSR markers.

(a)



(b)


DeltaK = mean(|L''(K)|) / sd(L(K))
Delta K
K
12
10
8
6
4
2
0
2.0
2.5
3.0
3.5
4.0
4.5
5.0


L(K) (mean +- SD)
Mean of est. Ln prob of data
K
−530
−540
−550
−560
−570
−580
−590
−600
1
2
3
4
5
6

Appendix G

The table shows the 95% confidence interval of the Q-value for STRUCTURE analysis with the combined nuclear *GBSSI* and *PvCel1* gene data. The Q1 and Q2 thresholds were shown in the bracket behind the genetic composition.

	Genetic composition of *D. pendulus*		Genetic composition of *G. scortechinii*	
GIG1	0.007	(0.000,0.049)	0.993	(0.951,1.000)
GIG2	0.006	(0.000,0.048)	0.994	(0.952,1.000)
GIG3	0.016	(0.000,0.105)	0.984	(0.895,1.000)
GIG4	0.016	(0.000,0.105)	0.984	(0.895,1.000)
GIG5	0.006	(0.000,0.044)	0.994	(0.956,1.000)
GIG6	0.006	(0.000,0.046)	0.994	(0.954,1.000)
HYB1	0.667	(0.496,0.818)	0.333	(0.182,0.504)
HYB2	0.536	(0.364,0.701)	0.464	(0.299,0.636)
HYB3	0.522	(0.345,0.692)	0.478	(0.308,0.655)
HYB4	0.465	(0.293,0.637)	0.535	(0.363,0.707)
HYB5	0.616	(0.442,0.775)	0.384	(0.225,0.558)
HYB6	0.510	(0.340,0.676)	0.490	(0.324,0.660)
HYB7	0.523	(0.348,0.693)	0.477	(0.307,0.652)
HYB8	0.399	(0.238,0.569)	0.601	(0.431,0.762)
HYB9	0.383	(0.228,0.552)	0.617	(0.448,0.772)
HYB10	0.450	(0.275,0.626)	0.550	(0.374,0.725)
HYB11	0.519	(0.347,0.689)	0.481	(0.311,0.653)
DEN1	0.994	(0.953,1.000)	0.006	(0.000,0.047)
DEN2	0.993	(0.947,1.000)	0.007	(0.000,0.053)
DEN3	0.994	(0.953,1.000)	0.006	(0.000,0.047)
DEN4	0.994	(0.954,1.000)	0.006	(0.000,0.046)
DEN5	0.994	(0.953,1.000)	0.006	(0.000,0.047)
DEN6	0.994	(0.954,1.000)	0.006	(0.000,0.046)
DEN7	0.994	(0.955,1.000)	0.006	(0.000,0.045)
DEN8	0.994	(0.953,1.000)	0.006	(0.000,0.047)