POPULATION GENETICS AND PHYLOGENY OF THE MALESIAN PALM GENUS *JOHANNESTEIJSMANNIA* H.E.MOORE (PALMAE)

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Contents

Summary

Johannesteijsmannia is a palm genus of four tropical rain forest understorey species. Only *Jt. altifrons* is widespread, ranging from southern Thailand, Peninsular Malaysia, Sumatra to western Borneo while *Jt. lanceolata*, *Jt. magnifica* and *Jt. perakensis* are endemic to Peninsular Malaysia. Their increasing commercial exploitation as ornamental plants makes conservation a priority. To conserve effectively, more understanding is needed about their population genetics, systematics and phylogeny. Morphological and/or molecular data were employed to answer the following questions: 1) How well supported is the hypothesis of Dransfield (1972) that there are four congeners? 2) Is the morphological evidence sufficient to delimit the species? 3) How consistent is the morphological compared to molecular evidence? 4) Are there hybrids where *Jt. altifrons*, *Jt. lanceolata* and *Jt. magnifica* occur sympatrically? 5) Are the four *Johannesteijsmannia* species monophyletic? 6) What is the phylogenetic relationship of this genus with its sister group, *Licuala*? 7) What is the genetic variability within and between populations of each species? 8) Is the genetic variation correlated with geographical distance?

Twenty-two vegetative and reproductive characters compiled from 27 populations were employed to test Dransfield's hypothesis that there are four congeners using principal coordinates, principal component and cluster analyses. Results suggested that Dransfield's hypothesis is supported only when both vegetative and reproductive characters were employed and that *Jt. perakensis* is the most distinct species with an above ground stem. When the stem characters were excluded from the analysis, *Jt. perakensis* and *Jt. altifrons* formed a distinct cluster.

Amplified fragment length polymorphism (AFLP) profiles were generated from 209 accessions of *Johannesteijsmannia* collected from 27 localities to elucidate

the systematics of the genus. Results suggested that no hybridisation occurred between *Jt. altifrons, Jt. lanceolata* and *Jt. magnifica* despite their sympatric occurrence at Sungai Lalang Forest Reserve, Selangor, Peninsular Malaysia. *Jt. perakensis* operational taxonomic units (OTUs) overlapped or nested within *Jt. altifrons* OTUs in the principal coordinates scatter plots or phenograms suggesting that *Jt. perakensis* is a subspecies of *Jt. altifrons*. They are also more genetically and morphologically similar to each other than the other two species, indicating consistency between morphological and molecular evidence.

Sequences of the low-copy nuclear genes encoding for phosphoribulokinase (*PRK*) and the second largest subunit of RNA polymerase II (*RPB2*) were employed to examine the phylogenetic relationships of *Johannesteijsmannia* to *Licuala* and selected taxa from its subfamily, the Coryphoideae. Both *PRK* and *RPB*2 data sets resolved *Johannesteijsmannia* as monophyletic with high bootstrap support (99% and 100%) but without resolving the relationships of the four congeners because high allelic polymorphism was observed in the clone sequences of the four congeners in both data sets. Only the *PRK* and combined data set supported *Licuala* being a sister group to *Johannesteijsmannia*.

AFLP fingerprinting was generated using six primer combinations on DNA samples from 222 individuals collected from 27 populations throughout the distribution of *Johannesteijsmannia*. Populations of each species exhibited moderate genetic diversity (Nei's genetic diversity values ranged from 0.0861–0.1968 and the Shannon information index, from 0.0677–0.2355). Results of the Analysis of Molecular Variance (AMOVA) showed that all congeners partition higher genetic diversity within population (63%–88%) than between populations (12%–37%). The population differentiation measure, the F_{st} value, was highly significant ($P < 0.001$)

and ranged from 0.1235 (*Jt. magnifica*) to 0.3277 (*Jt. altifrons*) indicating that there is gene flow between populations of each species. AMOVA results were corroborated by Bayesian analysis with significant F_{st} values for all species. The best model selected was f = 0 (f being the inbreeding coefficient), suggesting all congeners are outcrossing species.

Results from Mantel test demonstrated there is a significant correlation between geographical and genetic distance in *Jt. altifrons* but insignificant for the others.

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List of Abbreviations

HCl	Acid hydrochloride
ATP	Adenosine Tri-phosphate
AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
bp	Base Pair
BLAST	Basic Local Alignment Search Tool
cm	Centimetres
cpDNA	Chloroplast DNA
cDNA	Complementary DNA
dNTPs	Deoxynucleotide-triphosphates
DNA	Deoxyribonucleic acid
EDTA	Diaminoethanetetra-acetic acid
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
fmol	Femtomole
g	Gram
HWE	Hardy-Weinberg Equilibrium
HEX	Hexachloro-fluorescein Phosphoramidite
CTAB	Hexadecyltrimethylammonium bromide
h	Hour
ITS	Internal Transcribed Spacer
IUCN	International Union for Conservation of Nature and Natural Resources
km	Kilometres
MCMC	Markov Chain Monte Carlo

MP	Maximum-parsimony
m	Metres
µl	Microliter
ml	Milliliter
mm	Milimetres
mM	Millimolar
min	Minute
MULTREES	Multiple Trees per Step
MVSP	Multi-Variate Statistical Analysis
ng	Nanogram
NCBI	National Centre fo Biotechnology Information
nrDNA	Nuclear Ribosomal DNA
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PCA	Principal Component Analysis
PCO	Principal Coordinates Analysis
PRK	Phosphoribulokinase
pmol	Picomole
PVP-40	Polyvinylpyrrolidone-40
pH	Potential of Hydrogen
RAPD	Random Amplified Polymorphic DNA
RBG	Royal Botanic Gardens
RFLP	Restriction Fragment Length Polymorphism
rpm	Revolutions per minute
RNA	Ribonucleic acid

RPB2	RNA Polymerase II
SSR	Simple Sequence Repeat
NaCl	Sodium chloride
TBR	Tree-bisection-reconection
TB	Transformation buffer
TET	Tetrachloro-6-carboxy-fluorescein
TE	Tris EDTA
U	Unit
UPGMA	Unweighted Pair Group Method
v/v	Volume per volume
w/v	Weight per volume
6FAM	6-carboxyfluorescein

CHAPTER 1

GENERAL INTRODUCTION

1.1 *Johannesteijsmannia* H.E.Moore

The genus *Johannesteijsmannia* is a small genus of the family Palmae or Arecaceae. The Palmae is one of the most economically important families in the monocotyledons (Stevens, 2001 onwards). Based on a more recent estimate, there are about 2,364 species in 190 genera in the family (Govaerts and Dransfield, 2005). *Johannesteijsmannia* is classified within the subfamily Coryphoideae, tribe Livistoneae and subtribe Livistoninae (Dransfield *et al*., 2005) (Figure 1.1). *Johannesteijsmannia* was transferred from the tribe Corypheae (see Figure 1.2 for former placement of *Johannesteijsmannia*) to a newly recognized tribe Livistoneae. The latest classification is based on phylogenetic analyses, mainly using DNA sequence data to validate the names of newly recognized tribes and subtribes as a precursor to a new edition of *Genera Palmarum* (Dransfield *et al*., in prep.). This new phylogenetic research not only strongly supported the groups recognized in the first edition of *Genera Palmarum* (Uhl and Dransfield, 1987) but also revealed that some of the groups are not monophyletic.

According to the latest classification (Dransfield *et al*., 2005), members of the subfamily Coryphoideae have expanded to eight tribes compared to the three tribes in the former (Uhl and Dransfield, 1987). The five new members included four newly recognized tribes (Chuniophoeniceae, Cryosophileae, Livistoneae and Sabaleae) and the placement of tribe Caryoteae after being removed from the subfamily Arecoideae. Members of the Coryphoideae are rather variable, and include about 451 species in 44 genera of subtropical and pantropical palms with palmate or costapalmate (pinnate) and induplicate leaves (Stevens, 2001 onwards; Dransfield *et al.*, 2005). The

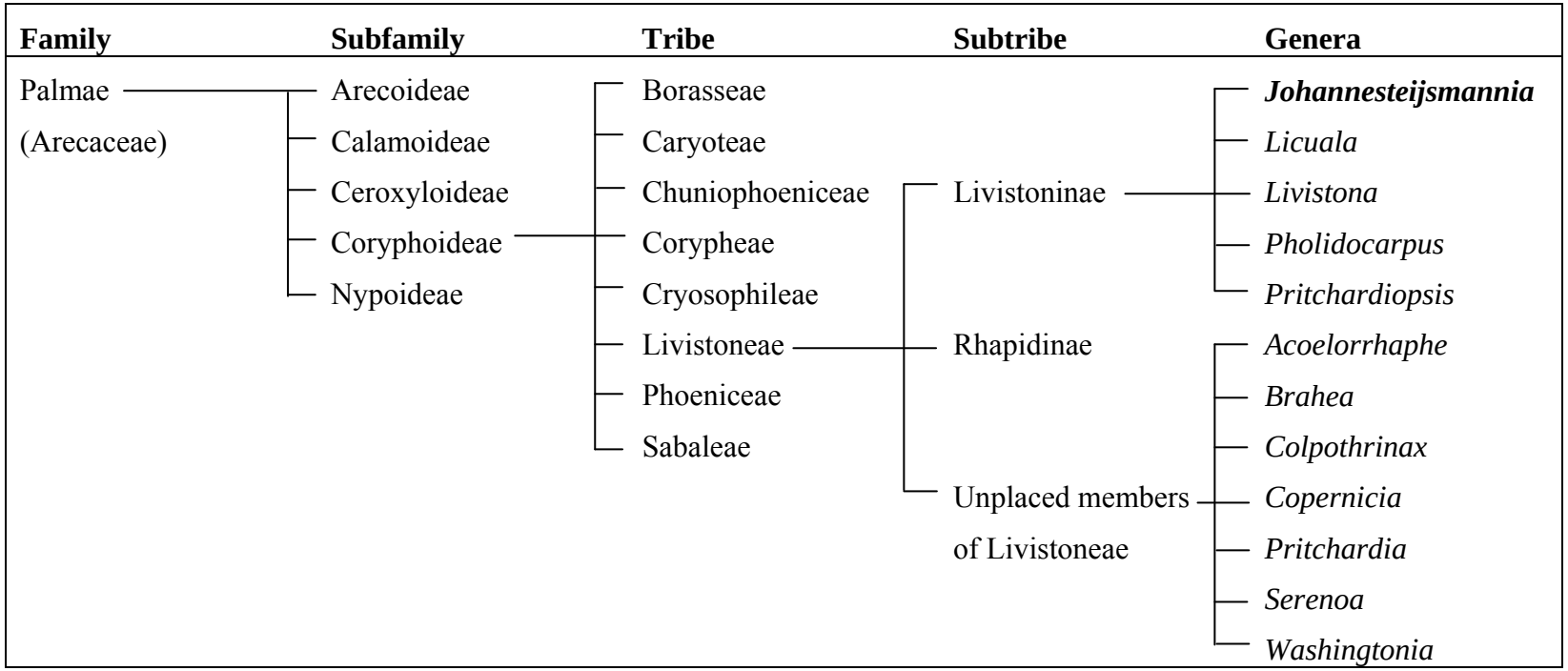

Figure 1.1. Position of *Johannesteijsmannia* according to the classification of the Palmae by Dransfield *et al*. (2005).

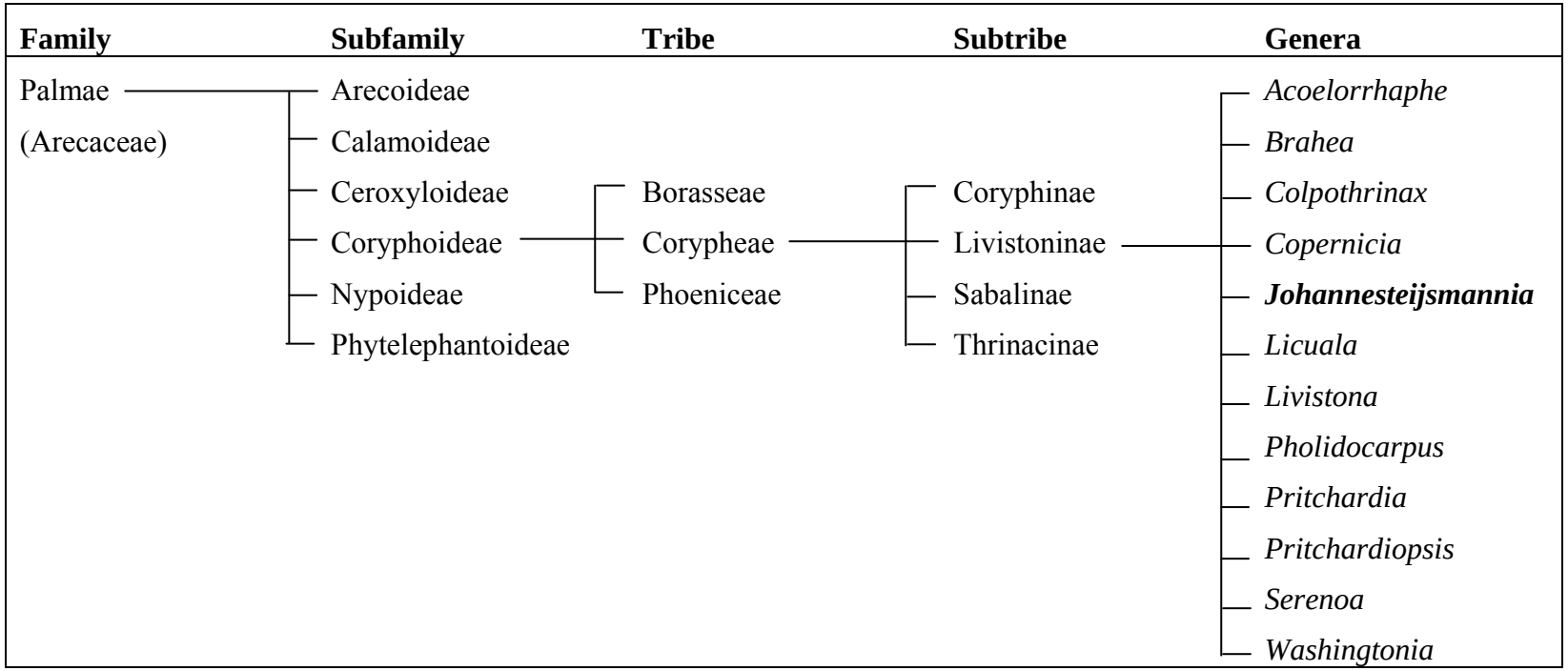

Figure 1.2. Position of *Johannesteijsmannia* according to the classification of the Palmae by Uhl and Dransfield (1987).

Livistoneae is the least well defined of the eight tribes and made up of 18 genera. It is composed of two recognized subtribes and the remaining genera are left unplaced. The subtribe Livistoninae includes five genera and has gynoecia of three carpels that are free at the base but joined throughout their styles.

1.1.1 *Species*

Johannesteijsmannia is more well known by its common name, Joey palm. It is known as 'daun payung' (umbrella leaf) in Malaysia and Indonesia. It consists of four species and its members are easily distinguished by the large, entire and diamond-shaped to broadly lanceolate leaf blades. The leaves are erect and range from 3.5 to 6 m in length and 0.3 to 2 m in width. *Johannesteijsmannia* is West Malesian, ranging from southern Thailand, Peninsular Malaysia, Sumatra to western Borneo. Only one species is widespread while the other three species are endemic to Peninsular Malaysia (Dransfield, 1972). The first species of *Johannesteijsmannia* was discovered by Johannes Elias Teijsmann (1808–1882, a Dutch botanist) during his expedition to the west coast of Sumatra from 1856–1857. This was described in the volume 28 of *Linnaea* as *Teysmannia altifrons* Reichb.f. et Zoll. in 1858 by Reichenbach and Zollinger. They suggested the palm should be placed next to *Salacca* and *Wallichia* and not far from *Nypa*. In 1868, Miquel made a more thorough description based on more materials and suggested that the palm was closer to the Coryphoideae rather than *Salacca*, *Wallichia* and *Nypa* (Dransfield, 1972).

The name *Johannesteijsmannia* was first published by Moore (1961) as substitute for *Teysmannia* because the latter had been assigned to a genus of the Apocynaceae in 1857. At the time, there was only a single species — *Jt. altifrons* (Reichb.f. et Zoll.) H.E. Moore. Dransfield (1972) later described three more species,

namely, *Jt. lanceolata* Dransfield, *Jt. magnifica* Dransfield and *Jt. perakensis* Dransfield in his revision of the genus. The four species were well defined by Dransfield with each species having its unique diagnostic characters.

Most *Johannesteijsmannia* species lack a discernable stem (the stem developes underground), except that *Jt. perakensis* developes an above-ground stem at maturity (Dransfield, 1972) (Figure 1.3A). *Jt. lanceolata* is easily distinguished by its lanceolate leaf blade whereas the rest exhibit broad diamond-shaped ones so given the specific epithet *lanceolata* (Figure 1.3B). *Jt. magnifica* is the most magnificent of the four species and thus was given the specific epithet *magnifica*. This striking palm is easily spotted in its natural habitat. It is very distinct from other species because of the presence of the grey-white indumentum on the lower surface of the lamina (Figure 1.3C). *Jt. altifrons* is the most common and well-known among the four species. It is the most similar to *Jt. perakensis*, except that it lacks a discernable trunk (Figure 1.3D).

Flowering behavior in four *Johannesteijsmannia* species is sporadic and occurs at irregular intervals throughout the year. Gregarious flowering was reported in *Jt. altifrons* in January 1968 and *Jt. magnifica* in May 1968 at Sungai Lalang Forest Reserve, Selangor, Peninsular Malaysia. During the year 1967/1968, it was reported to be very common to find one or two plants in flower within s given population at any given time in all *Johannesteijsmannia* species (Dransfield, 1972).

The flowers of each *Johannesteijsmannia* species have different scents (Dransfield, 1972). When the inflorescences are at anthesis, vast numbers of nitidulid beetle larvae and adults, staphylinid beetles, dipterous larvae, thrips, ants, termites and spiders were noted among the inflorescences. The flowers were also observed to have

signs of being chewed. To date, there is no study published on the flowering behavior of *Johannesteijsmannia* species.

1.1.2 *Distribution*

Jt. altifrons occurs in the east of southern Thailand, Peninsular Malaysia (the states of Kelantan, Terengganu, Pahang, Johor and Selangor), the east of northern Sumatra and western Borneo (from Kuching, Sarawak, East Malaysia westwards) (Figure 1.4). *Jt. altifrons* is more widespread but localized in distribution (Drandsfield, 1972). *Jt. lanceolata* has narrow distribution, and is only recorded in Ulu Sungai Tekal Besar, Temerloh, Pahang and Sungai Lalang Forest Reserve, Semenyih, Selangor (Dransfield, 1972) (Figure 1.5). The strikingly attractive *Jt. magnifica* only occurs in the Berembun Forest Reserve, Bukit Tangga, Negeri Sembilan and Sungai Lalang Forest Reserve, Semenyih, Selangor (Dransfield, 1972) (Figure 1.6A). *Jt. perakensis*, the rarest species, was recorded only in the Gunung Bubu Forest Reserve and Kledang Saiong Forest Reserve, Kuala Kangsar, all in the state of Perak (Figure 1.6B). Hence, it was given the specific epithet *perakensis* (Dransfield, 1972).

1.1.3 *Habitats*

Johannesteijsmannia is an understorey palm found in primary rain forests, never found in secondary forest and disturbed or open spaces (Dransfield, 1972). However, the plants can survive in selectively logged forest, but suffer from the damage caused by falling trees and searing when exposed to direct sunlight. They are ridge-top and hill slope plants, occasionally occurring near the banks of small rivers. They survive well on well-drained, humus-rich and podsolized soils.

Figure 1.3. *Johannesteijsmannia* species in their natural habitats. A. *Jt. perakensis*, the stick Joey. B. *Jt. lanceolata*, the slender Joey. C. *Jt. magnifica*, the grey Joey. D. *Jt. altifrons*, the Joey palm.

Most of the *Jt. altifrons* populations occur on hill slopes at more than 300 m above sea level (asl) (Dransfield, 1972). However, it was recorded occurring at 65 m asl on mild slopes and between fresh water swamps in Johor. In the Bako National Park, Sarawak, it occurs at 100 m asl in heath forest. The lowest record was in Sumatra, where the palm was found growing at 25 m asl (Palm and Jochems, 1924). Nevertheless, this palm can also occur as high as 1,200 m asl on Gunung Mandi Angin, the border of the states of Kelantan, Terengganu and Pahang (Dransfield, 1972).

Jt. lanceolata is found near the river or at steep banks of forest rivers in deep humid valleys and grows side by side with *Jt. magnifica* in Sungai Lalang Forest Reserve, Selangor (Dransfield, 1972). Although it grows in the proximity of rivers, it only grows on well-drained soils and not in swampy ground.

Jt. magnifica is a plant of ridge-tops and steep slopes but can occur at the side of a river in a humid valley bottom, in the Sungai Lalang Forest Reserve, Selangor, where it grows side by side with *Jt. lanceolata*. It ranges from valley bottom at 150 m asl to the ridge-top at 500 m asl. The population found in Bukit Tangga, Berembun Forest Reserve, Negeri Sembilan grows in a logged and much disturbed forest (Dransfield, 1972).

Jt. perakensis grows abundantly at hill slopes and ridge-tops from 175 to 850 m asl (Dransfield, 1972). It grows on well-drained soils derived from granite. Most of the *Jt. perakensis* populations were growing at selectively logged forest area and it has survived the logging.

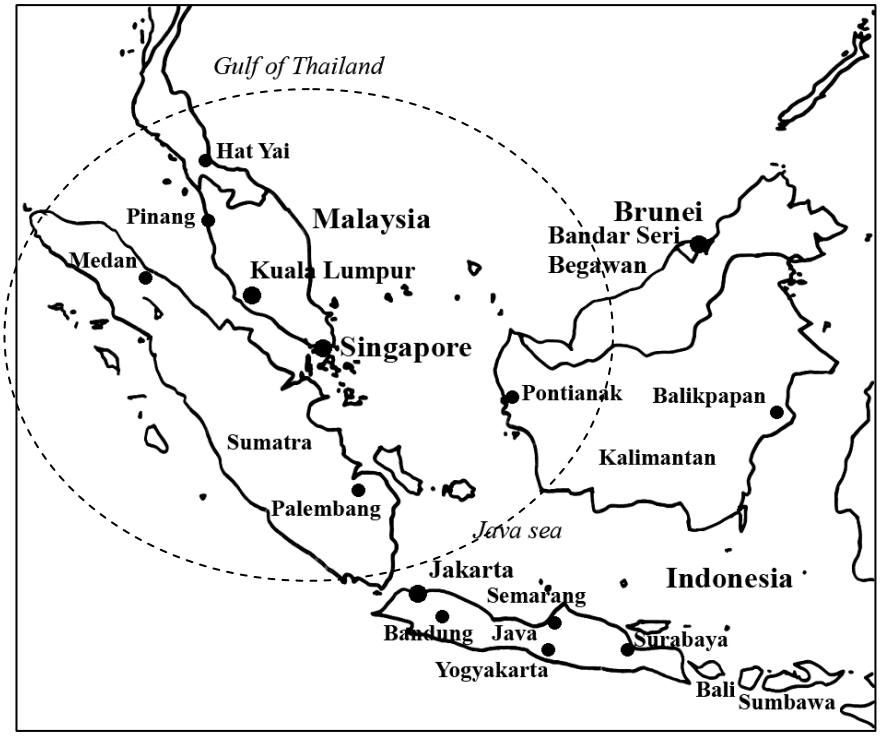

Figure 1.4. Distribution of *Jt. altifrons* delineated with dotted line. Map based on http://www.reisenett.no/map_collection/middle_east_and_asia/Southeast_Asia_pol97.jpg.

Figure 1.5. Distribution of *Jt. lanceolata*. Map based on http://www.malaysia-maps.com/malaysia-states-map.htm.

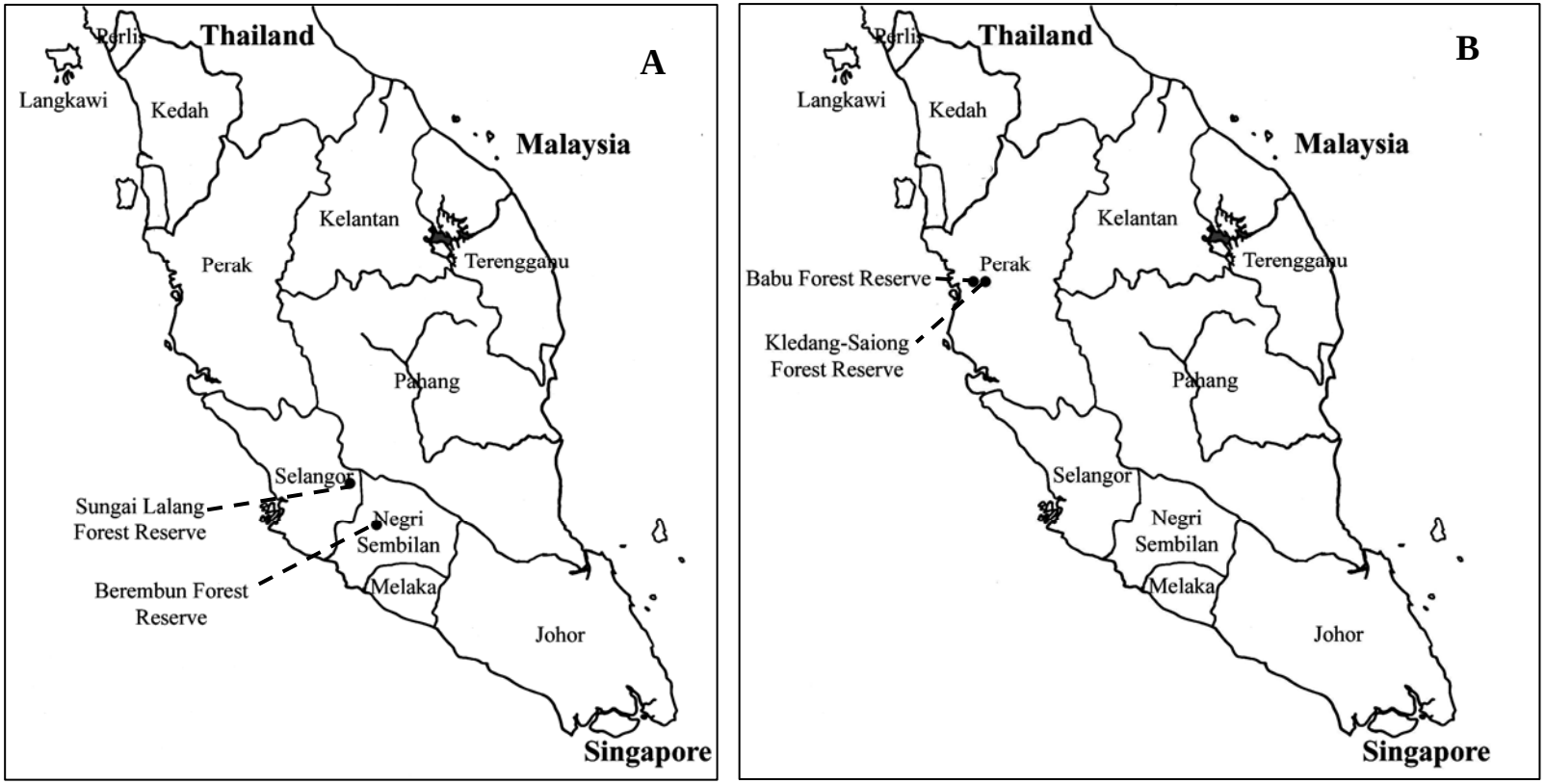

Figure 1.6. A. Distribution of *Jt. magnifica*. B. Distribution of *Jt. perakensis.* Map based on http://www.malaysia-maps.com/malaysia-states-map.htm.

1.1.4 *Uses*

Johannesteijsmannia is a highly prized and valued ornamental as all members are attractive plants. They have been used as indoor (e.g., lobby plants, Figure 1.7A) and outdoor (partially under shade or full shade) landscaping plants. Hence, they have been cultivated for the horticultural trade in their countries of origin and other places of the world by palm enthusiasts. The seeds, seedlings and plants are sold in nurseries in Australia, Germany, Malaysia, Singapore, Thailand (Figure 1.7B), the United Kingdom and the United States of America.

Jt. magnifica is more favoured in the horticultural trade because it is the most attractive of the four species (Figure 1.7A). The prices of the plants range from US$ 30 to 180 depending on the plant size. The price of *Jt. altifrons* is lower than that of *Jt. magnifica*, ranging from US$ 5 to 165. *Jt. lanceolata* is only available in nurseries in Malaysia and Singapore because it is relatively rare compared to both *Jt. magnifica* and *Jt. altifrons*. *Jt. perakensis* is not commercially available because it is the rarest among the four species (more information on pricing and distributors of the four species are listed in Table 1.1).

The leaves of *Johannesteijsmannia* also make excellent thatching materials because they are very broad, strong, durable, easy to arrange and waterproof. In Peninsular Malaysia, aborigine villagers in Endau-Rompin State Park, Johor (Figure 1.8A) and Sungai Lalang, Hulu Langat, Selangor (Figure 1.8B) have been reported to build their houses with the leaves of *Jt. altifrons* and *Jt. magnifica*, respectively. In the states of Johor and Pahang, Peninsular Malaysia, the leaves of *Jt. altifrons* are also used to thatch Chinese logging huts and wind-shelters for expedition camping (Dransfield, 1972). Palm and Jochems (1924) also reported the use of the leaves of *Jt. altifrons* in Tanjung Pura, the northern part of Sumatra for thatching. The thatch

Figure 1.7.A. *Jt. magnifica* in front of Hotel Swiss Garden, Kuala Lumpur, Malaysia. B. *Jt. altifrons* seedlings in a nursery in Narathiwat Province, southern Thailand.

Table 1.1. List of prices and distributors of four *Johannesteijsmannia* species.

Species	Price of Seeds	Price of Seedling/Plant	Distributor
Jt. altifrons	10 seeds at US$ 16	Not available	The Cloudforest Café (Thailand); http://www.cloudforest.com
	Not available	1 plant at US$ 44.90 (lowest bid)	Ebay (Southern California); http://home.listings.ebay.com/Seeds_Palm_
	15 gallon potted plant at US$ 350	Not available	JD Andersen Nursery (C.A.); http://www.jdandersen.com/
	Not available	1 plant at US$ 5 (Diameter of pot = 20 cm)	Floribunda Palms & Exotics (Hawaii); http://www.floribundapalms.com/
	Not available	1 plant at US$ 9 (8 inches root tube)	Tropical Gardens of Maui (Hawaii); http://www.tropicalgardensofmaui.com/
	No information	No information	The Palm Centre (Richmond, United Kingdom); http://www.thepalmcentre.co.uk/shape.htm
	Not available	1 plant at US$ 30 to 165	Palms for Brisbane (Brisbane, Australia); http://www.palmsforbrisbane.com.au
	Not available	1 plant at US$ 50 (Diameter of pot = 17.5 cm)	Utopia Palms & Cycads (Queensland, Australia); http://www.utopiapalmsandcycads.com/
	Not available	1 plant at US$ 19	Ang Mo Kio Floral & Landscape Pte Ltd (Singapore)
Jt. lanceolata	Not available	1 plant at US$ 25	Ang Mo Kio Floral & Landscape Pte Ltd (Singapore)
Jt. magnifica	100 seeds at US$ 309	Not available	The Palm Seed Centre (location not stated); http://palmseedcenter.net/
	10 seeds at US$ 50	Not available	The Cloudforest Café (Thailand); http://www.cloudforest.com
	100 seeds at US$ 325	Not available	Thai Palm Seeds (Thailand); http://thaipalmseeds.com/
	Not available	1 seedling at US$ 19.50	borneo-palm-seed.com (Kuching, Malaysia); http://www.borneo-palm-seed.com/html/home.htm
	Not available	1 plant at US$ 29.50 (Diameter of pot = 20 cm)	Palms for Brisbane (Brisbane, Australia); http://www.palmsforbrisbane.com.au
	Not available	1 plant at US$ 50 (Diameter of pot = 17.5 cm)	Utopia Palms & Cycads (Queensland, Australia); http://www.utopiapalmsandcycads.com/
	Not available	1 plant at US$ 176 (Diameter of pot = 30 cm, height = 100 cm)	Palmen-Bolschetz (Germany); http://www.palmen-bolschetz.de/
	Not available	1 plant at US$ 50	Ang Mo Kio Floral & Landscape Pte Ltd (Singapore)
Jt. perakensis	Not available	Not available	Not available

Figure 1.8.A. Roof made of leaves of *Jt altifrons* in the Endau-Rompin State Park, Johor, Peninsular Malaysia. B. The house of an aborigine family in Sungai Lalang, Hulu Langat, Selangor, Peninsular Malaysia, thatched with the leaves of *Jt. magnifica*.

made of *Johannesteijsmannia* can last three to four years depending on the thickness of the thatch.

1.1.5. *Conservation Status*

According to the 1997 IUCN red list of threatened plants: 1) *Jt. altifrons* is vulnerable in Thailand, Sumatra and Malaysia (Johor and Sarawak); 2) *Jt. lanceolata* is endangered in Peninsular Malaysia; 3) *Jt. magnifica* is endangered in Peninsular Malaysia and, 4) *Jt. perakensis* is vulnerable in Peninsular Malaysia.

Although *Jt. altifrons* is more widespread and common than the others, it should also be considered under threat. Field observations in Belum Forest, Perak over a ten-year period suggested that the native population of *Jt. altifrons* have decreased to approximately a quarter of the original population (Lim and Whitmore, 2000).

1.2 Questions That Need to be Addressed Regarding the Systematics and Population Genetics of *Johannesteijsmannia*

There are several specific questions to be addressed with respect to the systematics, population variability, population differentiation as well as phylogenetic relationship of *Johannesteijsmannia* species.

1. How well supported is the hypothesis of Dransfield (1972) that there are four species in *Johannesteijsmannia*? Is the morphological evidence sufficient to elucidate the systematics of the genus? How congruent is the morphological data compared to molecular evidence?
2. Are there hybrids formed in Sungai Lalang Forest Reserve, Hulu Langat, Selangor, Malaysia where three species (*Jt. altifrons*, *Jt. lanceolata* and *Jt. magnifica*) occur sympatrically at this locality?

3. Are the four *Johannesteijsmannia* species monophyletic as *Jt. perakensis* is only found in Perak, Malaysia and is the only species with a discernable trunk?
4. What is the phylogenetic relationship of this genus with its sister group, *Licuala*?
5. How high is the genetic diversity within a given population of each species and what kind of genetic variation exists among the populations of each species? Is the genetic variation correlated with the geographical distance of populations?

1.3 Aims

This study involved field sampling which covered almost the entire geographical range of the four *Johannesteijsmannia* species. The Sumatran populations where omitted in this study owing to the difficulties in accessing the populations because to the prevailing political unrest in the region at the time of sample collection. The overall objectives were to elucidate the systematics of the genus as well as to provide an overview of the extent of genetic variability within and among populations of each species and to obtain information needed for conservation, management and utilization of the species genetic resources. The specific aims have been defined in this study to address the questions mentioned above:

1. To test the hypothesis of Dransfield (1972) that there are four putative species as well as to elucidate the systematics of the genus using morphological and molecular data. This study covered a wider geographical range and included newly discovered localities compared to Dransfield (1972). Multivariate morphometrics and overall phenetic characters incorporating as many

qualitative and quantitative characters as possible were employed to delimit the species relationship. The molecular technique, amplified fragment length polymorphism (AFLP) fingerprinting was also employed to provide more robust data and to compare the congruence between morphological and molecular evidence (Chapter 2 and Chapter 3).

2. To study the phylogeny of *Johannesteijsmannia* species with its sister group, *Licuala* and selected taxa from the same subfamily, the Coryphoideae based on DNA sequence data and cladistic analysis. This is the pilot study to resolve the phylogenetic relationships within the genus. The work also attempted to clarify the phylogenetic relationship of the genus with *Licuala*. Sequence analysis of two low-copy nuclear genes encoding for phosphoribulokinase (*PRK*) and the second largest subunit of RNA polymerase II (*RPB*2) were adopted in this study. These two genes have proven to be informative for examining low-level relationships within the palm family. The merits of exploring the DNA sequence would be the homology assessment compared to the DNA fingerprinting methods, e.g., AFLP (Chapter 4).

3. To investigate the current genetic variability within populations and genetic differentiation among populations of each *Johannesteijsmannia* species at the DNA level using AFLP fingerprinting. AFLP analysis was reported to be useful to detect genetic variation in population genetic studies. Data generated would also allow assessment of gene flow among populations of each *Johannesteijsmannia* species. The correlation between genetic distance and geographical distance can be tested with Mantel test (Chapter 5).

4. To provide guidelines for the conservation, management and restoration of each *Johannesteijsmannia* species. Acquisition of sufficient information on the

pattern of genetic variation and population differentiation over geographical ranges is essential to establish guidelines for conservation, management, restoration and utilization of genetic resources of this genus (Chapter 6).

CHAPTER 2

MORPHOMETRIC ANALYSIS OF *JOHANNESTEIJSMANNIA* H.E. MOORE

2.1 Introduction

Johannesteijsmannia was devided into four species based on vegetative and reproductive characters by Dransfield (1972) (Table 2.1). The identification of the four species employed a conventional taxonomic approach, which involved an intuitive delimitation of groups of specimens and character complexes. Unfortunately, this approach often seems to fail (Dransfield, 1999). Therefore, techniques with greater discriminatory power are needed, such as multivariate morphometric analyses. This method has been widely used in assessing morphological variation in closely related species (e.g., Loo *et al*., 2001; Henderson, 2004; Lihova, 2004).

Morphometric analysis (multivariate analyses of morphological data) falls in the realm of phenetic analysis, which groups taxa by overall similarity, regardless of whether these similarities are symplesiomorphous or synapomorphous in a phylogenetic sense (Radford, 1986). It has the advantage that no specific hypotheses or *a priori* groupings of specimens are required and that the delimitation can be made explicit and repeatable. This approach has been demonstrated to be a powerful method to test species concepts in palms (e.g., Loo *et al*., 2001; Henderson, 2002; Henderson and Ferreira, 2002; Henderson, 2004). For instance, Henderson (2004) carried out multivariate statistical analysis of morphological data to delimit and test morphologically distinct subgroups in *Hyospathe* and applied the phylogenetic species concept of Davis and Nixon (1992) to the subgroups.

Table 2.1. Morphological character states of *Johannesteijsmannia* from Dransfield (1972).

Characters	***Jt. altifrons***	***Jt. perakensis***	***Jt. magnifica***	***Jt. lanceolata***
Habit	Solitary.	Solitary.	Solitary.	Solitary.
Stem	Acaulescent , underground, procumbent. 15 cm in diameter.	Robust ascending stem to 4 m tall, marked with annular scars. 15 cm in diameter.	Acaulescent, underground, procumbent. 15 cm in diameter.	Acaulescent, underground procumbent. 15 cm in diameter.
Leaves	Large, erect to 6 m long. Ca. 20–30 per plant.	Large, erect to 4 m long. Leaf base fibrous, withering to form a brown network of fibres.	Leaf base fibrous, to 25 cm long, withering to form a brown network of fibres.	Large, erect to 3.5 m long. Leaf sheath fibrous to 25 cm long, withering to form a brown network of fibres.
Petiole	Up to 2.5 m × 2 cm. Armed with short thorns to 1 mm long. Two lateral yellow lines at the abaxial surface.	Up to 1 m × 2 cm. Armed with short spines to 1 mm long. Marked with two yellow lines at the abaxial surface.	Armed with short spines to 1.5 mm long. Marked with two conspicuous yellow lines on the abaxial surface.	Armed with short spines to 1 mm long. Marked with two conspicuous yellow lines on the abaxial surface.
Lamina	Up to 3.5 × 1.8 m. Rhomboid. Scurfy brown scales along the costa and at the short marginal lobes. Plicae to 20 or more on either side of the costa with fine anastomosing veins between the main veins of the plicae.	Up to 3 × 1.6 m. Rhomboid. Scurfy brown scales along the underside of the costa and at the short marginal lobes. Plicae to 20 or more on either side of the costa with small anastomosing lateral nerves between the plicae. Lower lamina margins armed with	Up to 3 × 2 m. Rhomboid. Glabrous above, covered with white indumentum on the lower surface except along the costa and the marginal lobes. Plicae to 20 or more on either side of the costa, with small anastomosing lateral nerves between the plicae.	Up to 2.4 × 30 m. Lanceolate. Scurfy brown scales on the underside, along the midrib and the primary nerves. Plicae numerous to 25 along each side of the costa, with small anastomosing veins between the plicae.

Characters	***Jt. altifrons***	***Jt. perakensis***	***Jt. magnifica***	***Jt. lanceolata***
	Lower lamina margins armed with short spines.	short spines (to 1 mm long).	Lower lamina margin armed with short spines (to 1.5 mm long).	Lower lamina margin with short spines (to 1 mm long).
Inflorescence	Axillary, first erect then pendulous from the primary axis.	Axillary, acrhing out of the crown.	Axillary, arching out of the pile of dead leaves and other plant debris accumulating in the crown of leaves. With 5–6 orders of branching.	Axillary, buried between the petioles under a pile of dead leaves and other plant debris. With 3–6 thick branches.
Spathes	Covered in fugacious scurfy brown hairs. Cream initially, turning brown. 5–6 in number. 10–20 cm × 6–8 cm. Tubular at the base. Inflated above. Split on one side with acute apices.	Covered in fugacious scurfy scales. Cream initially, turning brown. 5–6 in number. 20 cm × 8 cm. Tubular at the base. Inflated above. Split on one side with acute apices.	Densely covered with a white indumentum. Cream initially, turning brown. 5–6 in number. 25 cm × 7 cm. Tubular at the base. Inflated above. Split on one side with acute apices.	Sparsely clothed with a brown indumentum. Cream initially, turning to brown. 3–4 in number. 20 cm × 2 cm. Tubular at the base. Not inflated. Split on one side with acute apices. Caducous.
Peduncle	30–50 cm long. Tomentose. 2 cm in diameter. 3 orders of branching.	To 50 × 2cm. Tomentose. 4 orders of divaricate branches.	To 80 cm long. Tomentose. 2 cm in diameter. 5–6 orders of branching.	To 25 cm × 10 mm. Tomentose. 1 order of branching.
Floriferous branches	1.5–2.5 mm in diameter. To 100 cm long. Ca. 20–100. Greenish. Covered in dense white tomentum. Densely covered in flowers	2.5 mm in diameter. To 10 cm long. 50–100. Greenish. Covered in white tomentum. Densely covered in flowers.	1.5 mm in diameter. To 7 cm long. 500–1000. White. Glabrous. Densely covered in flowers.	10 mm in diameter. To 16 cm long. 3–6. Pubescent. Curved or straight. Thick.
Flowers	Glabrous.	Glabrous.	Glabrous.	Papillate.

Characters	*Jt. altifrons*	*Jt. perakensis*	*Jt. magnifica*	*Jt. lanceolata*
				Sessile.
	White.	White.	White.	White.
	± acute in bud.	Rounded in bud.	Acute in bud.	Obtuse in bud.
	Solitary or grouped in 2's or 3's, rarely 4's.	Solitary or grouped in 2's or 3's.	Solitary or group in 2's and 3's.	Solitary or group in 2's and 3's.
				Arrange in a spiral on the branch.
	Borne on tubercles.	Borne on tubercles.	Borne on prominent tubercles.	
	Each subtended by a minute bracteole.	Each subtended by a minute bracteole.	Each subtended by a minute bract.	Each subtended by a minute bracteole.
	Smelling like sour milk.	Smelling sweet.	Smelling like flowers of *Tropaeolum majus.*	Smelling like Coumarin.
Calyx	To 2 mm long.	To 1 mm long.	To 0.5 mm long.	To 0.3 mm long.
	Fleshy.	Fleshy.	Fleshy.	Fleshy.
	Glabrous.	Glabrous.	Glabrous.	Glabrous.
	Shallowly 3-lobed.	Shallowly 3-lobed.	Shallowly 3-lobed.	Shallowly 3-lobed.
Petals	Fleshy.	Fleshy.	Fleshy.	Fleshy.
	White.	White.	White.	
				Thick.
	To 4 mm long.	To 3 mm long.	To 1.5 mm long.	To 1 mm long.
	Twice as long as broad, triangular.	Broadly triangular.	3 times as long as broad, narrowly triangular.	Broadly triangular.
	Connate basally to form a short tube c. 0.5 mm long.	Connate to form a short tube.	Connate basally to form a short tube.	Connate into a short tube.
			Rugose inside.	Papillose outside, somewhat verrucose inside towards the apex.
			Reflexed.	
Staminal ring	Minutely epipetalous, abruptly contracted above into 6 filaments.	Minutely epipetalous, abruptly contracted above into 6 filaments.	Minutely epipetalous, abruptly narrowed above into 6 equal filaments.	Minutely epipetalous, abruptly contracte into 6 equal filaments.
Anthers	Oval.	Oval.	Oval.	Oval.

Characters	***Jt. altifrons***	***Jt. perakensis***	***Jt. magnifica***	***Jt. lanceolata***
Pollen grains	White.	White.	White.	White.
Ovary	Glabrous. 3 carpels free at the base, but connate into a common style, 0.8 mm long at the apex.	Glabrous. 3 carpels free at the base, but connate into a common style, 0.8 mm long at the apex.	Glabrous. 3 carpels free at the base, but connate into a common style, 0.4 mm long at the apex.	Glabrous. 3 carpels free at the base, but connate into a common style, 0.3 mm long at the apex.
Fruits	3.9–4.6 cm in diameter. Usually developing from 1 carpel, rarely from 2–3 carpels. Covered with 60–80 brown, corky warts.	To 5 cm in diameter. Usually developing from 1 carpel, rarely from 2–3 carpels. Covered with c. 60 dirty brown, corky warts.	To 4 cm in diameter. Usually developing from 1 carpel, occasionally from 2–3 carpels. Covered with c. 150 short, reddish-brown, corky warts.	To 3.4 cm in diameter. Usually developing from 1 carpel, often from 2–3 carpels. Covered with c. 90 short, reddish-brown, corky warts.
Warts	6.2–8.2 mm long.	9 mm long.	To 2.5 mm long.	To 2.5 mm long.
Endocarp	Woody. To 1 mm thick.	Woody. To 1 mm thick.	Woody. To 1 mm thick.	Woody. To 1 mm thick.
Endosperm	Bony. 2.5 cm in diameter. Penetrated at the base by corky integumental tissue.	Bony. 2.5 cm in diameter. Penetrated at the base by corky integumental tissue.	Bony. 2.5 cm in diameter. Penetrated at the base by corky integumental tissue.	Bony. 2.5 cm in diameter. Penetrated at the base by corky integumental tissue.
Embryo	Lateral.	Lateral.	Lateral.	Lateral.

2.1.1 *Objectives*

This chapter aims to use multivariate analysis of morphological characters to test the hypothesis of Dransfield (1972) that there are four *Johannesteijsmannia* species. This is a pilot study of using multivariate analysis to address the morphological variation between *Johannesteijsmannia* species.

2.2 Materials and Methods

2.2.1 *Collection Localities*

Samplings were carried out throughout the distribution of the four *Johannesteijsmannia* species except for the Sumatran populations for the reasons as mentioned in Chapter 1 (Section 1.3). This study also attempted to search for new populations to cover a wider geographical sampling range. Six to 13 individuals were randomly selected from each natural population of each species (Tables 2.2, 2.3, 2.4, 2.5 and Figures 2.1, 2.2, 2.3, 2.4). Parts of one mature leaf were collected from a randomly selected plant of each population for making a voucher specimen.

Table 2.2. Collection localities of *Johannesteijsmannia altifrons* in Malaysia and southern Thailand.

S/No.	Population Code	Locality	State/Province	Country	Latitude (N)	Longitude (E)	Sample Size
1.	ASLA	Sungai Lalang Forest Reserve, Hulu Langat	Selangor	Malaysia	3° 06′ 01.6″	101° 54′ 52.9″	8
2.	BAK	Bukit Tambi, Bako National Park, Kuching	Sarawak	Malaysia	1° 43′ 22.5″	110° 27′ 05.2″	8
3.	BPA	Government Land, next to Compartment 58, Batu Papan Forest Reserve, Gua Musang	Kelantan	Malaysia	4° 47′ 03.4″	101° 52′ 52.9″	8
4.	GEE	Gunung Eedang, Kampung Tambun, Kawasan Sukhirin	Narathiwat	Thailand	5° 49′ 30.2″	101° 40′ 54.6″	8
5.	GJA	Gunung Janing, Endau-Rompin State Park	Johor	Malaysia	2° 31′ 52.2″	103° 24′ 50.8″	8
6.	KMR	48.5 km mark towards Jemaluang, along the Kluang-Mersing Road, Kluang	Johor	Malaysia	2° 18′ 35.2″	103° 41′ 06.2″	13
7.	LAH	Berkelah Forest Reserve, Jerantut	Pahang	Malaysia	4° 11′ 21.8″	102° 48′ 57.2″	8
8.	LEP	Gunung Lee Pa, Kampung Bukit Kuat, Kawasan Si Sakhon	Narathiwat	Thailand	6° 10′ 06.4″	101° 26′ 10.7″	9
9.	LUM	Belum Forest, Grik	Perak	Malaysia	4° 34′ 59.9″	101° 10′ 38.6″	8
10.	MAT	Matang, Kuching	Sarawak	Malaysia	1° 38′ 20.3″	110° 08′ 07.7″	8
11.	MUT	Gunung Belumut, Kluang	Johor	Malaysia	2° 03′ 58.6″	103° 31′ 34.1″	6
12.	SDU	Compartment 13, Sungai Durian Forest Reserve, Kuala Krai	Kelantan	Malaysia	5° 39′ 18.2″	102° 20′ 35.6″	8
13.	SNI	Compartment 72, Sungai Nipah Forest Reserve, Kemaman	Terengganu	Malaysia	4° 22′ 05.2″	103° 04′ 00.1″	8
14.	UGU	Upeh Guling, Endau-Rompin State Park	Johor	Malaysia	2° 31′ 37.9″	103° 21′ 49.5″	8

Table 2.3. Collection localities of *Johannesteijsmannia lanceolata* in Peninsular Malaysia.

S/No.	Population Code	Locality	State	Latitude (N)	Longitude (E)	Sample size
1.	ANG	Gunung Angsi Forest Reserve, Hulu Bendul, Kuala Pilah	Negeri Sembilan	1° 57′ 39.5″	99° 18′ 06.4″	8
2.	JBR	33 Mile Post Point along Jerantut-Benta Road, Jerantut	Pahang	3° 58′ 02.6″	102° 07′ 28.8″	8
3.	LSLA	Sungai Lalang Forest Reserve, Hulu Langat	Selangor	3° 03′ 29.1″	101° 52′ 22.4″	9
4.	RAU	Krau Wildlife Reserve, Kuala Krau, Temerloh	Pahang	3° 43′ 01.0″	102° 16′ 52.0″	8

Table 2.4. Collection localities of *Johannesteijsmannia magnifica* in Peninsular Malaysia.

S/No.	Population Code	Locality	State	Latitude (N)	Longitude (E)	Sample size
1.	BUN	Berembun Forest Reserve, Kuala Klawang, Jelebu	Negeri Sembilan	2° 51′ 37.7″	102° 01′ 00.0″	8
2.	GAT	26 Miles, Kampung Orang Asli, Sungai Lalang Forest Reserve, Hulu Langat	Selangor	3° 03′ 26.7″	101° 51′ 16.2″	8
3.	KIN	Compartment 78/79, Bukit Kinta Forest Reserve, Kinta	Perak	4° 34′ 59.9″	101° 10′ 38.4″	8
4.	MSLA	Compartment 14, Sungai Lalang Forest Reserve, Hulu Langat	Selangor	3° 04′ 54.4″	101° 51′ 46.6″	8
5.	SER	Compartment 12, Serendah Forest Reserve, Hulu Selangor	Selangor	3° 20′ 00.3″	101° 40′ 20.2″	8

Table 2.5. Collection localities of *Johannesteijsmannia perakensis* in Peninsular Malaysia.

S/No.	Population Code	Locality	State	Latitude (N)	Longitude (E)	Sample size
1.	BSU	Compartment 55, Gunung Bongsu Forest Reserve, Kulim	Kedah	5° 23′ 25.4″	100° 37′ 26.2″	8
2.	BUB	Compartment 44A, Gunung Bubu, Bubu Forest Reserve, Kuala Kangsar	Perak	4° 33′ 38.6″	100° 51′ 07.7″	8
3.	KSA	Kledang-Saiong Forest Reserve, Kuala Kangsar (A)	Perak	4° 42′ 57.2″	100° 58′ 00.0″	8
4.	KSB	Compartment 203, Kledang-Saiong Forest Reserve, Kuala Kangsar (B)	Perak	4° 42′ 49.1″	100° 58′ 19.1″	8

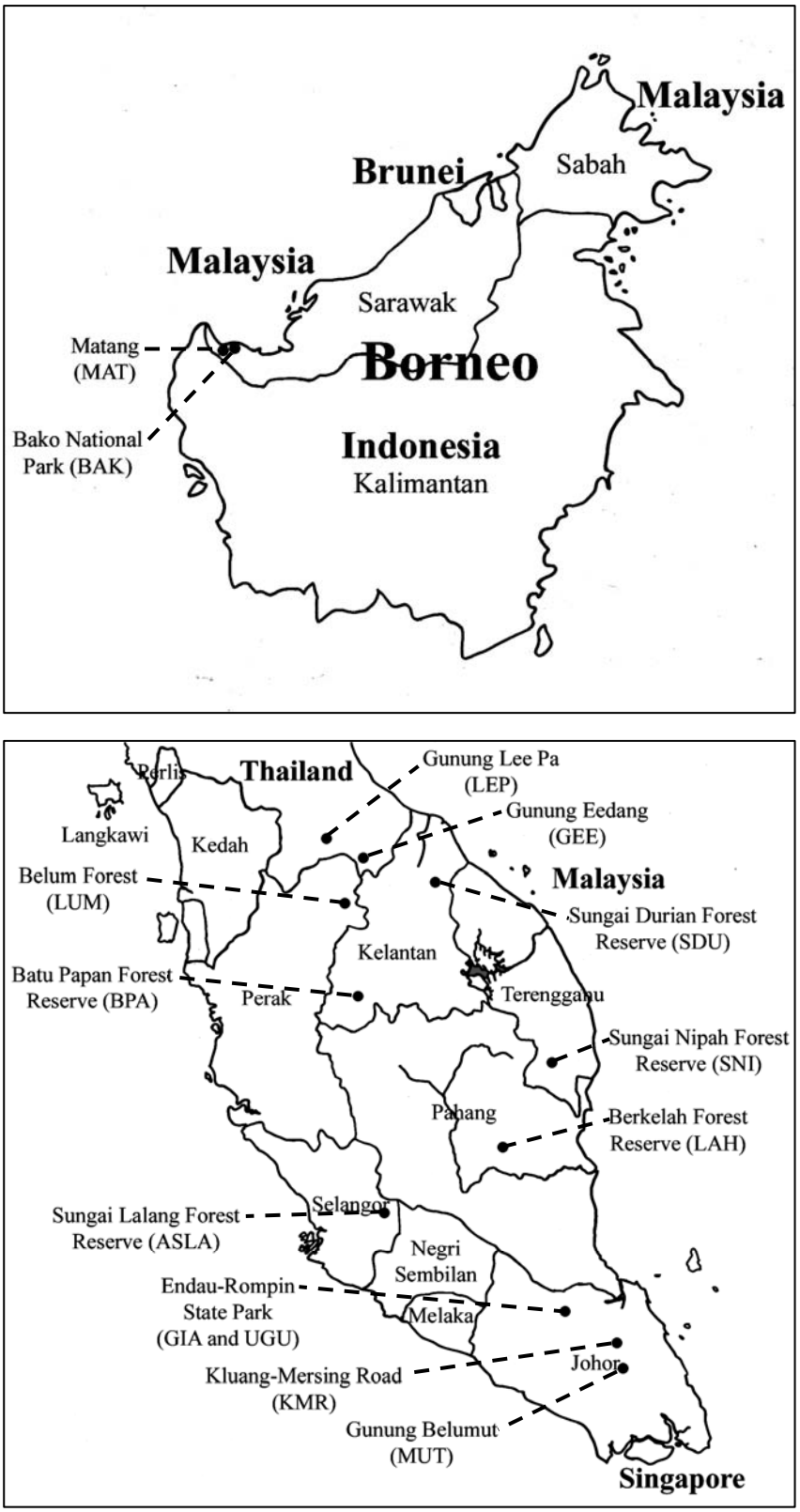

Figure 2.1. Collection localities of *Johannesteijsmannia altifrons*. Top and bottom maps based on http://www.malaysia-maps.com/malaysia-states-map.htm.

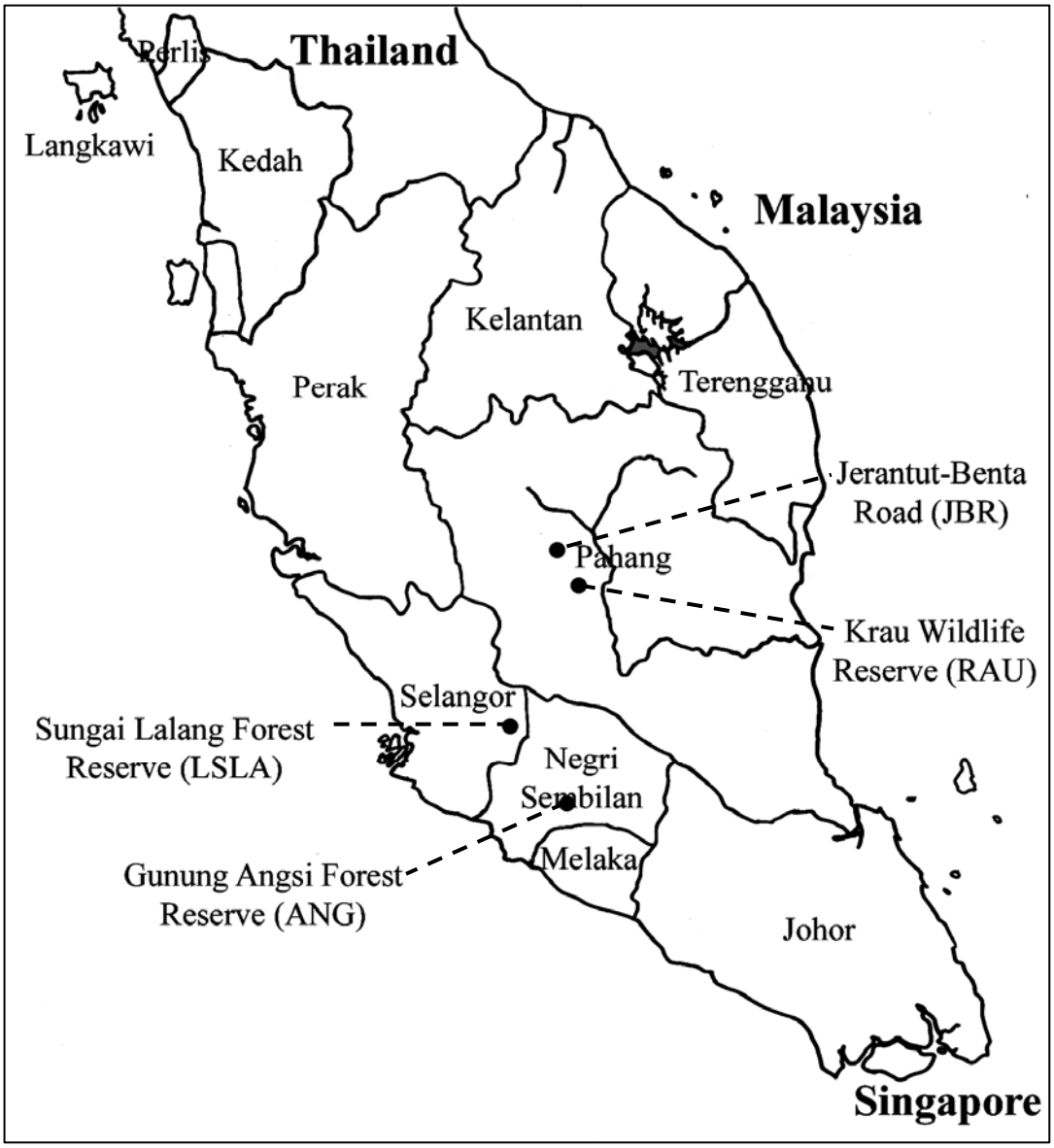

Figure 2.2. Collection localities of *Johannesteijsmannia lanceolata*. Map based on http://www.malaysia-maps.com/malaysia-states-map.htm.

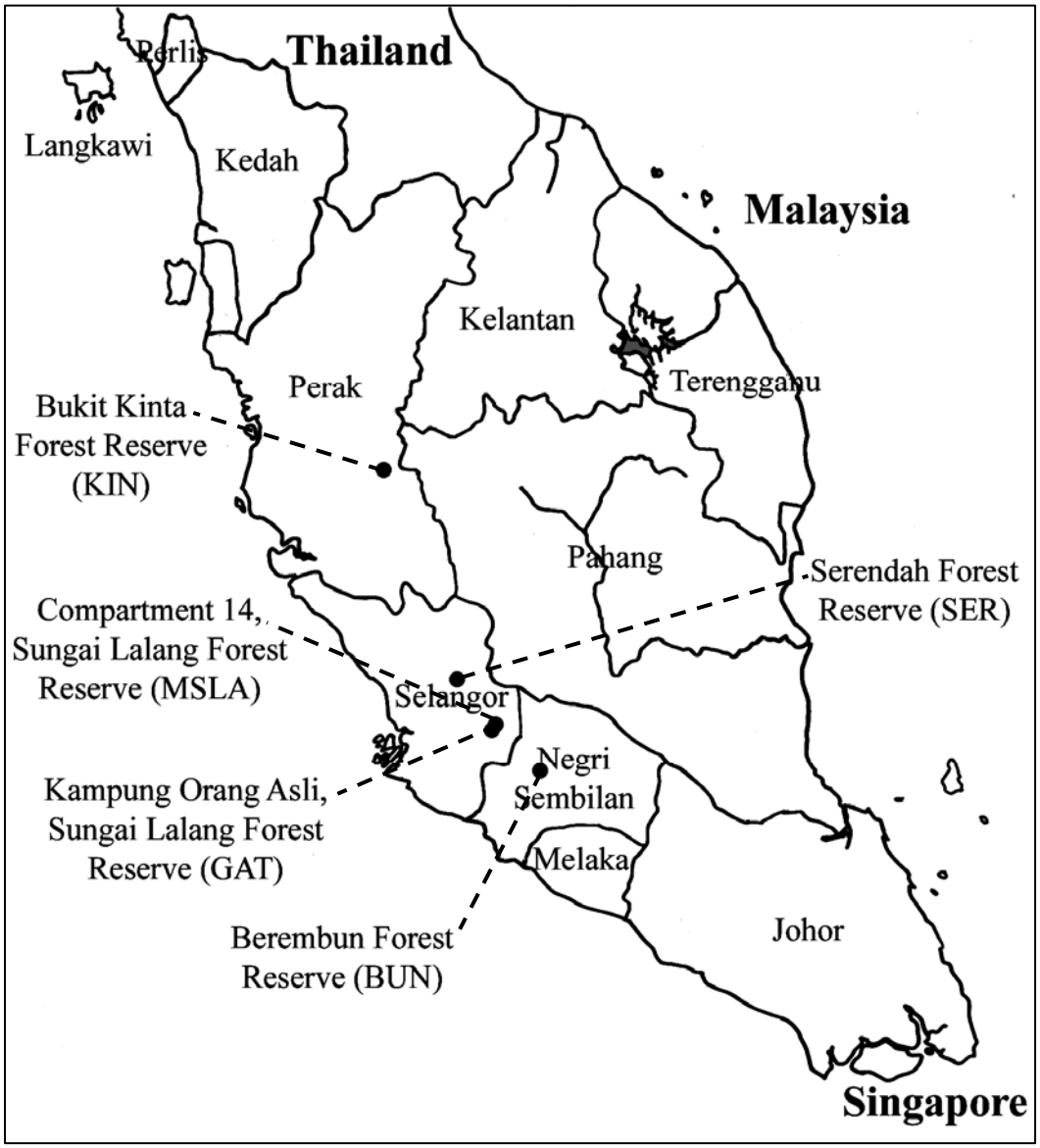

Figure 2.3. Collection localities of *Johannesteijsmannia magnifica*. Map based on http://www.malaysia-maps.com/malaysia-states-map.htm.

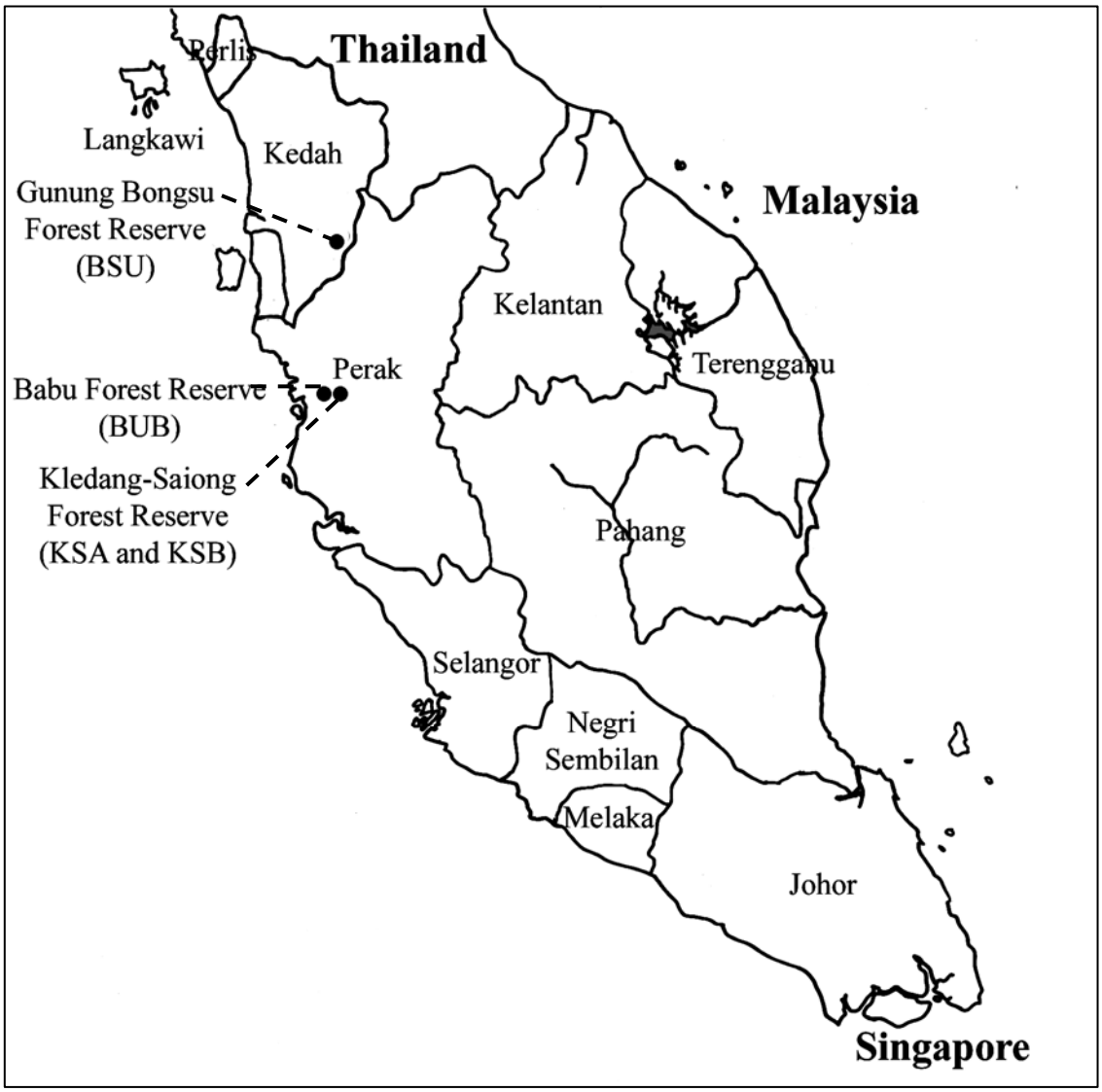

Figure 2.4. Collection localities of *Johannesteijsmannia perakensis*. Map based on http://www.malaysia-maps.com/malaysia-states-map.htm.

2.2.2 *Morphometric Characters*

This study aimed to use both vegetative and reproductive characters from fresh specimens. The leaf characters were measured or scored in the field for character states based on the fifth leaf from the baton. When plants were in flower and/or fruit, the inflorescence and/or infructescence was/were collected for measurement or scoring in the laboratory. Flower and fruit characters were not used in this study because flowers and fruits were not available for sufficient individuals throughout this study. Twenty-two character states (11 vegetative and 11 reproductive; Table 2.6) were selected and tabulated into a rectangular matric (Appendix 1).

Table 2.6. Morphometric characters, units of measurement or states for quantitative or qualitative characters and abbreviations of characters used.

S/No.	Vegetative Characters	Unit/States	Abbreviation
1.	Discernable stem	present (1) or absent (0)	Stem
2.	Discernable stem height	cm	Stemhei
3.	Discernable stem diameter at widest	cm	Stemdia
4.	Lamina of the fifth leaf underside with white indumentum	present (1) or absent (0)	Lamund
5.	Petiole length of the fifth leaf	cm	Petlen
6.	Petiole width of the fifth leaf	cm	Petwid
7.	Petiole depth of the fifth leaf	cm	Petdep
8.	Lamina length of the fifth leaf	cm	Lamlen
9.	Lamina width of the fifth leaf	cm	Lamwid
10.	The length of the widest part of lamina to the lamina base (of the fifth leaf)	cm	Lamwb
11.	Total number of pleats (plicae) per lamina of the fifth leaf	no.	Pleats
	Reproductive Characters	**Unit/States**	**Abbreviation**
12.	Peduncle length	cm	Pedlen
13.	Peduncle width	cm	Pedwid
14.	Peduncular bracts (total number)	no.	Pedbra
15.	The second peduncular bract length	cm	Pblen
16.	The second peduncular bract width	cm	Pbwid
17.	Maximum number of orders of branching	no.	Mxob1
18.	Total number of the branch with maximum number of orders of branching	no.	Brmxob
19.	Total number of rachillae on the first branch	no.	1Brano
20.	Total number of rachillae of whole inflorescence	1─25 (1), 26─100 (2), 101─200 (3), 201─500 (4), 501─1000 (5), >1001 (6)	Torano
21.	The longest rachilla length	cm	Racllen
22.	The longest rachilla width	cm	Raclwid

2.2.3 *Multivariate Analyses*

All samples collected were not *a priori* assigned to a specific group and each sample was treated as an individual Operational Taxonomic Unit (OTU) in composing the data matrix. The 22 characters consisted of a mix of 19 quantitative and three qualitative (two binary and one multistate) data types.

In this study, two multivariate methods of analysis were carried out, viz., ordination and cluster analysis. The ordination analyses employed were Principal Coordinates Analysis (PCO) and Principal Component Analysis (PCA). Ordination and cluster analyses are useful in diminishing the effective dimentionality of large data sets by generating combinations of variables showing common trends of variation. Their capability to identify relationships and minimize the effects of random variation gives rise to the recognition of meaningful patterns in the data (Radford, 1986; Peres-Neto *et al*., 2003). For mixed data sets of qualitative and quantitative characters, PCO is the preferred option over PCA (Lagendre and Lagendre, 1983). The main advantage of PCO is that many different kinds of similarity or distance measures can be used. For instance, if one is working with mixed data, in which some variables are measurements whereas others are binary or multistate, Gower's General Similarity Coefficient (Gower, 1971) can be used to combine these data. These coefficients can then be analyzed using PCO, whereas this data matrix would not be able to be analyzed by other ordination methods without recoding the data so that they are all in the same form. In contrast, the use of the covariance and correlation matrix in PCA is implicit. Nevertheless, PCA provides component scores associated with each variable, which may consider a measure of the relative importance of each variable in the extracted PCA axis. Unlike in PCO, no results are given for the variables (Thorpe, 1976). Cluster analysis produces a

hierarchical classification of entities (taxa) based on the similarity matrix (Radford, 1986). The results are displayed as distinct clusters but do not indicate clinal variation (Thorpe, 1983). The cluster analysis conducted in this study was to resolve phenetic relationships among the four *Johannesteijsmannia* species but not to estimate variation.

Four separate sets of character matrices were used in the analyses: 1) All characters (PCO and cluster analysis); 2) Inclusion of only vegetative characters (PCO and cluster analysis); 3) Inclusion of only reproductive characters (PCO and cluster analysis); 4) Inclusion of only quantitative characters (PCA and cluster analysis). Data were standardized and $\log_{10}$ transformed before analysis. PCO was constructed using the Gower's General Similarity Coefficient (Gower, 1971). The Nearest Neighbour clustering method and Gower's General Similarity Coefficient were used in the cluster analysis. The PCO, PCA and cluster analysis were carried out using the computer programme Multi-Variate Statistical Package (MVSP) version 3.10b (Kovach Computing Services, 1999).

2.3 Results and Discussion

2.3.1 *Observations*

Field collections were carried out at 27 localities of all *Johannesteijsmannia* species. New localities were recorded for *Jt. lanceolata*, *Jt. magnifica* and *Jt. perakensis*, i.e., populations ANG (*Jt. lanceolata*; personal communication, L.G. Saw), JBR (*Jt. lanceolata*; personal communication, R. Kiew), RAU (*Jt. lanceolata*; personal observation), KIN (*Jt. magnifica*; personal communication, J. Dransfield) and BSU (*Jt. perakensis*; personal observation). It should be highlighted that population RAU is possibly the population adjacent to that of Ulu Sungai Tekal Besar

(collection made by M.R. Henderson on March, 1923) since both localities are in the district of Temerloh, Pahang. Relocating the Ulu Sungai Tekal Besar population was unsuccessful as the locals and Peninsular Malaysian Forestry Department staff members were unaware of this species at that site.

The specimens of *Jt. magnifica* and *Jt. perakensis* collected from new localities were found to be similar to those of the holotypes. In contrast, morphological variation was found in *Jt. lanceolata* specimens collected from the newly discovered localities. Samples collected from population JBR exhibited two orders of branching at the infructescences, broader lamina dimensions and the wart lengths of the fruits were greater than those of the holotypes (Figures 2.9A, B, E and F). Two orders of branching were also observed for some of the samples collected from populations ANG (Figure 2.9C) and RAU (Figure 2.9D). Nevertheless, only two infructescences out of six collected from population ANG and four out of six inflorescences or infructecences from population RAU showed such a pattern.

The morphological variations observed in *Jt. lanceolata* populations ANG, JBR and RAU suggested the taxa in those populations exhibited intermediate characters between *Jt. altifrons* and *Jt. lanceolata*, and the possibility of hybridization having occurred. The nearest known *Jt. altifrons* population from JBR is population LAH, both of which are about 81 km apart. For populations ANG and RAU, the nearest known *Jt. altifrons* population is ASLA, about 316 and 63 km apart, respectively. As *Jt. altifrons* is a more widespread species, there may be populations that were not discovered in the present study but located near the above named *Jt. lanceolata* populations. Owing to the lack of knowledge on dispersal and mating systems of these two species, it is not possible to postulate if hybridization occurred. To date, there is neither experimental evidence of interspecific hybridization nor any

molecular evidence to support these claims. It is difficult at this stage of knowledge to determine if the intermediate individuals are hybrids, morphological variants or ecotypes.

Samples collected for each species in their natural habitats are shown in Figures 2.5 to 2.12. Based on field observation of the morphological characters, it was evident that variations at the interspecific and intraspecific levels exist in the genus.

Figure 2.5. *Johannesteijsmannia altifrons*. A. Habitat. B. Habit. C. Seedling growing next to a mature plant. D. Inflorescence not fully developed, covered with creamy white peduncular bracts E. Inflorescence with floriferous branches densely bearing flowers.

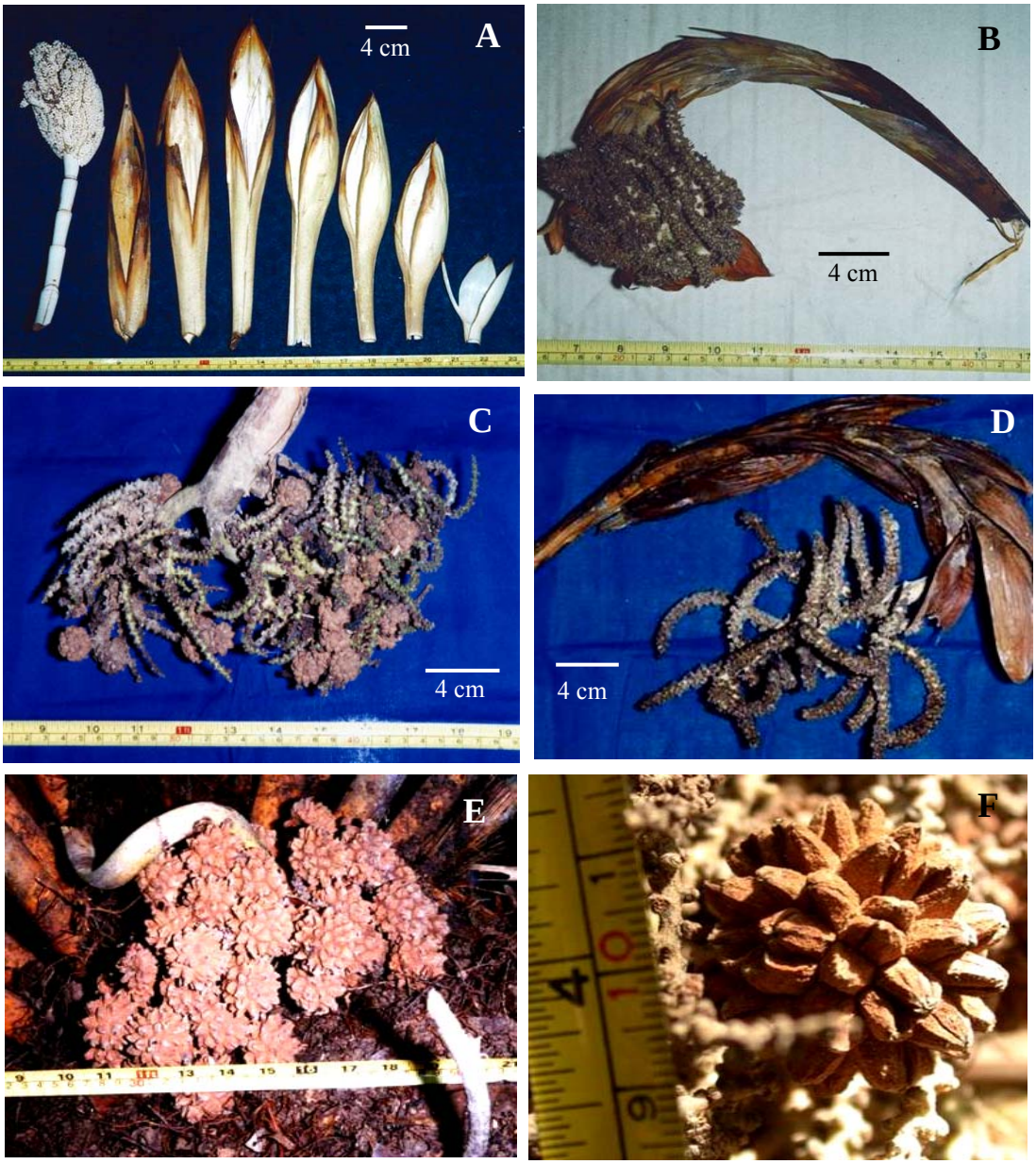

Figure 2.6. *Johannesteijsmannia altifrons*. A. Young inflorescences, flowers not fully developed and peduncular bracts creamy white colour. From left to right: inflorescence stalk with peduncular bracts removed, prophyl, the first to the sixth peduncular bracts. B. Old inflorescence, with only one order of branching was observed on this specimen. C. Infructescence with divaricate rachillae. D. Inflorescence with relatively fewer rachillae. E. Infructescence densely bearing fruits. F. Fruit covered with brown corky warts.

Figure 2.7. *Johannesteijsmannia altifrons* in Sarawak, East Malaysia. A. Plants growing in the heath forest. B. Habit. C. Habitat. D. Inflorescence not fully developed, covered with creamy white peduncular bracts and a brown prophyl. E. Old inflorescence. F. Infructescence bearing dull brown young fruits.

Figure 2.8. *Johannesteijsmannia lanceolata*. A. Habitat with plants growing in the forest canopy understorey. B. Inflorescence with flowers still developing. C. Habit. Plant with oblanceolate laminas. D. Infructescence bearing newly developed fruits. Only four rachillae were observed on the infructescence. E. Five fruits were found on one rachilla.

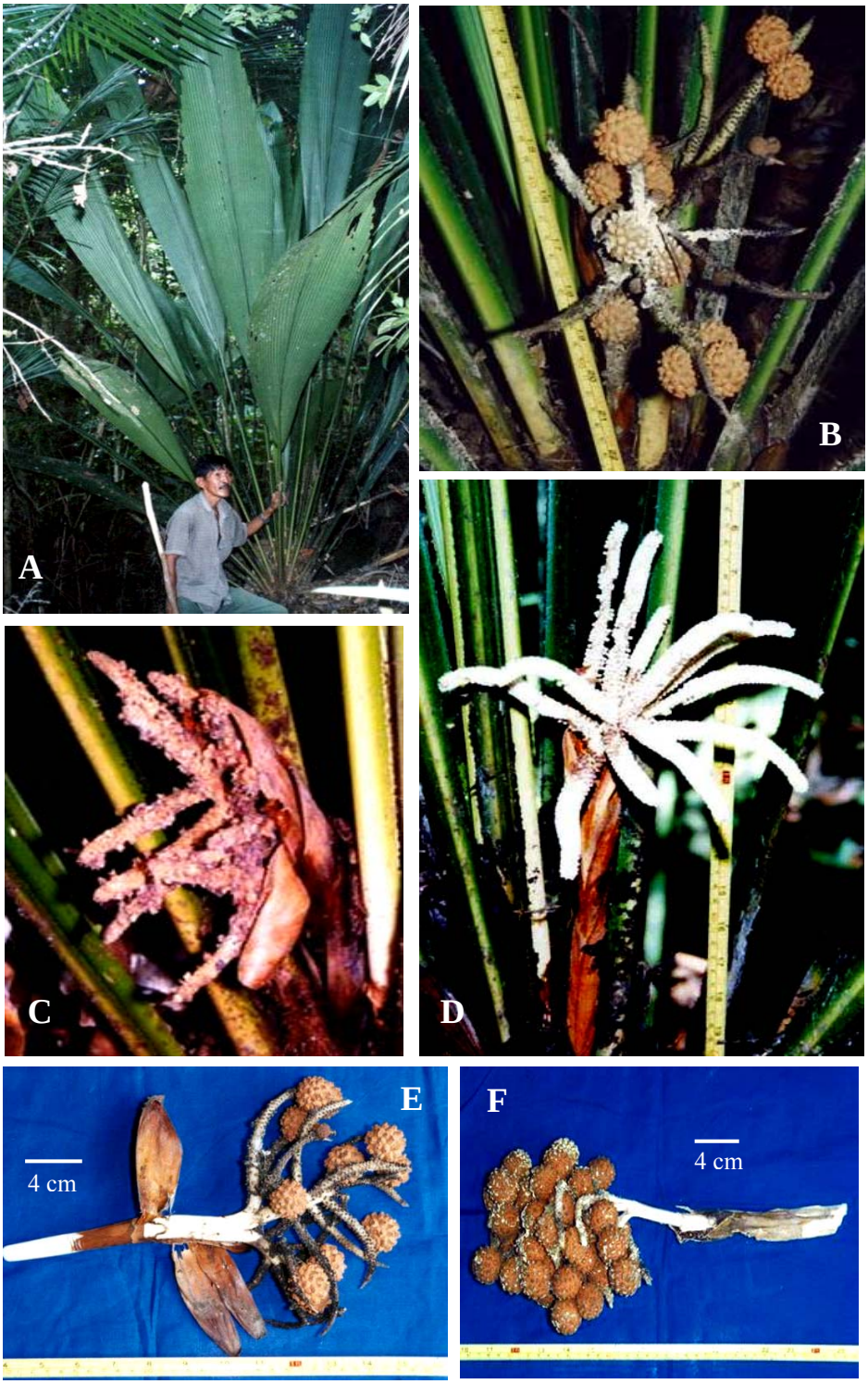

Figure 2.9. *Johannesteijsmannia lanceolata*. A, B, E and F from Population JBR; C from Population ANG; D from Population RAU. A. Broader laminas were observed on this plant. B. Infructescence bearing dull brown fruits. For C, D and E, more than one order of branching was observed in the inflorescences or infructescences; F. Infructescence densely bearing mature fruits.

Figure 2.10. *Johannesteijsmannia magnifica*. A. Habit. Plants are very large and always found on slopes. B. Infructescence bearing young reddish brown fruits. The undersides of the peduncular bracts are covered with a white indumentum. C. Habitat. Plants growing in the understorey of the forest canopy. D. Inflorescence with densely floriferous branches.

Figure 2.11. *Johannesteijsmannia perakensis*. A Habitat. Plants found growing near the bank of a small stream. B. Infructescence bearing newly developed fruits. C. Habit, with stem height more than 1 m tall. D. Inflorescence with densely floriferous branches and divaricate branching.

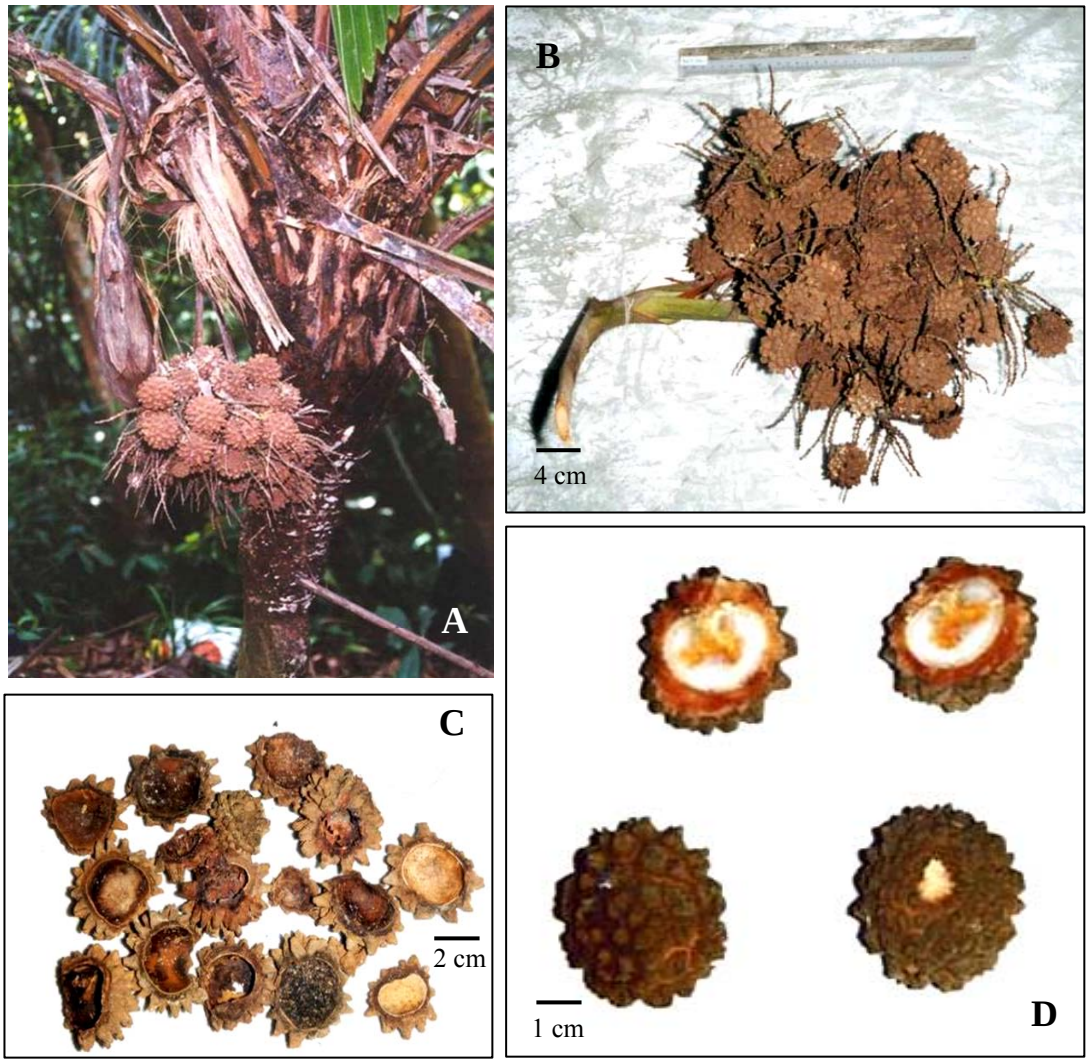

Figure 2.12. *Johannesteijsmannia perakensis*. A. Plant with a short discernable stem with an infructescence arching out of the pile of dead leaves and plant debris accumulating in the crown. B. Infructescence densely bearing immature fruits. C. Fruits showed the signs of being chewed, perhaps by rodents. D. Fruits.

2.3.2 *Vegetative and Reproductive Characters*

The matrix used in the PCO and cluster analysis consisted of only 83 accessions (56 accessions of *Jt. altifrons*; 16 accessions of *Jt. lanceolata*; five accessions of *Jt. magnifica* and six accessions of *Jt. perakensis*) owing to the limited reproductive characters in all accessions collected in this study. Using all 22 characters, the PCO scatterplot of the first and second coordinates revealed four clear groupings depicting four species (Figure 2.13). OTUs belonging to a particular species grouped together without overlapping with OTUs from other species The first coordinate measures 21.5% of the total variation while the second, 13.4%, the third, 6.9%, the fourth, 6.1% and the fifth, 5.4%. The first to the fifth coordinates together explain 53.2% of the variation in the data matrix (Table 2.7).

The phenogram of the cluster analysis of the OTUs using both reproductive and vegetative characters revealed a strong agreement between morphological similarities and species groups (Figure 2.14). Accessions belonging to a particular species clustered together without overlapping with accessions from other species. *Jt. altifrons* and *Jt. lanceolata* clustered closer to each other and *Jt. perakensis* furthest away from the other three species. The morphometric classification of the OTUs within the four groups shown in the PCO plot was further resolved when using cluster analysis to show the relationships among the OTUs.

Table 2.7. Summary of Principal Coordinate Analysis (PCO) of reproductive and vegetative characters of 83 accessions for four *Johannesteijsmannia* species.

	Axis 1	**Axis 2**	**Axis 3**	**Axis 4**	**Axis 5**
Eigenvalues	4.476	2.796	1.442	1.276	1.117
Percentage	21.455	13.403	6.913	6.114	5.353
Cumulative Percentage	21.455	13.403	6.913	6.114	5.353

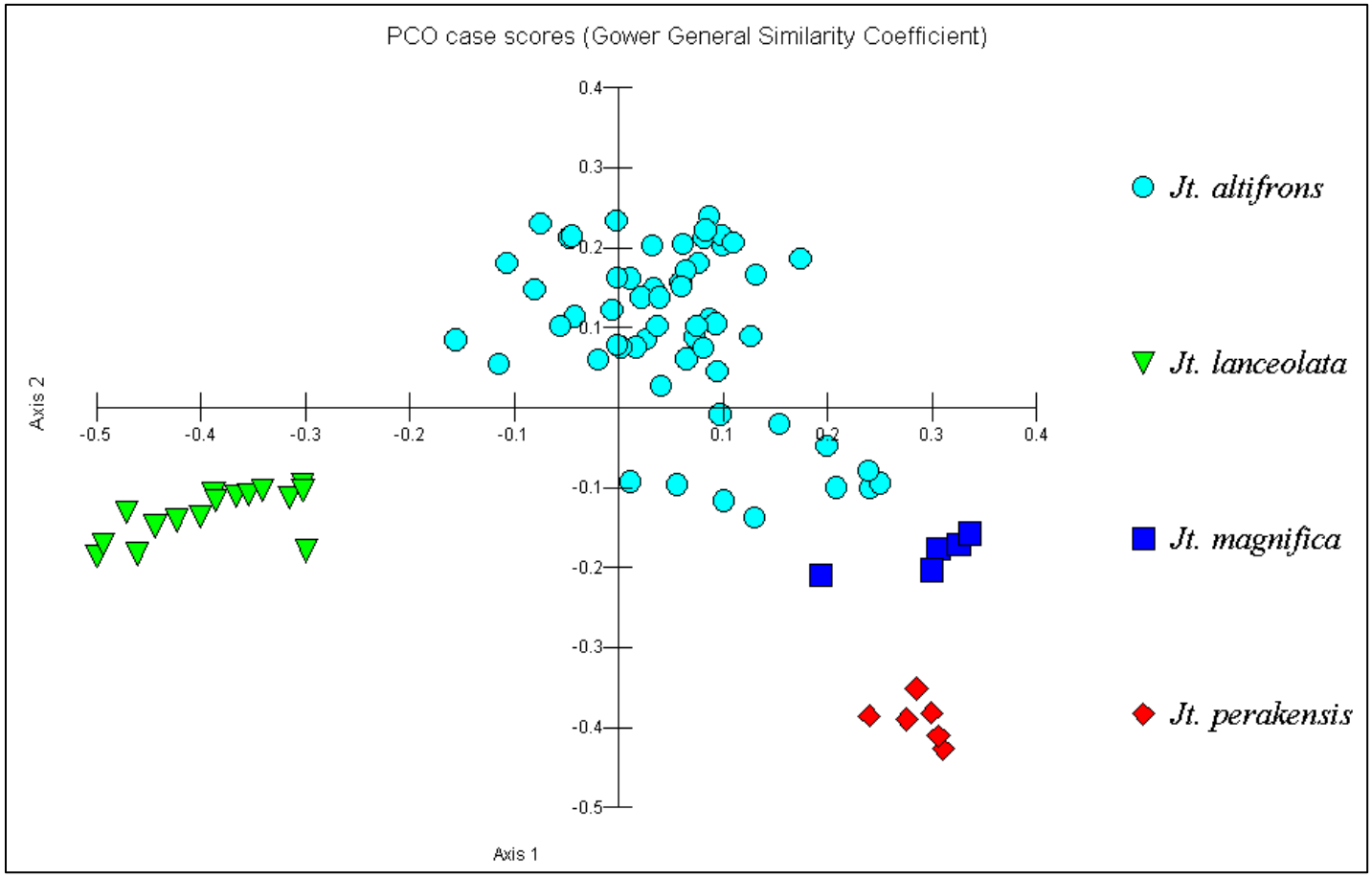

Figure 2.13. Differentiation of OTUs along the first and second principal coordinates when both reproductive and vegetative characters were used. Four clear groupings formed depicting four species. The first coordinate measures 21.5% of the total variation while the second, 13.4%, and the third 6.9%.

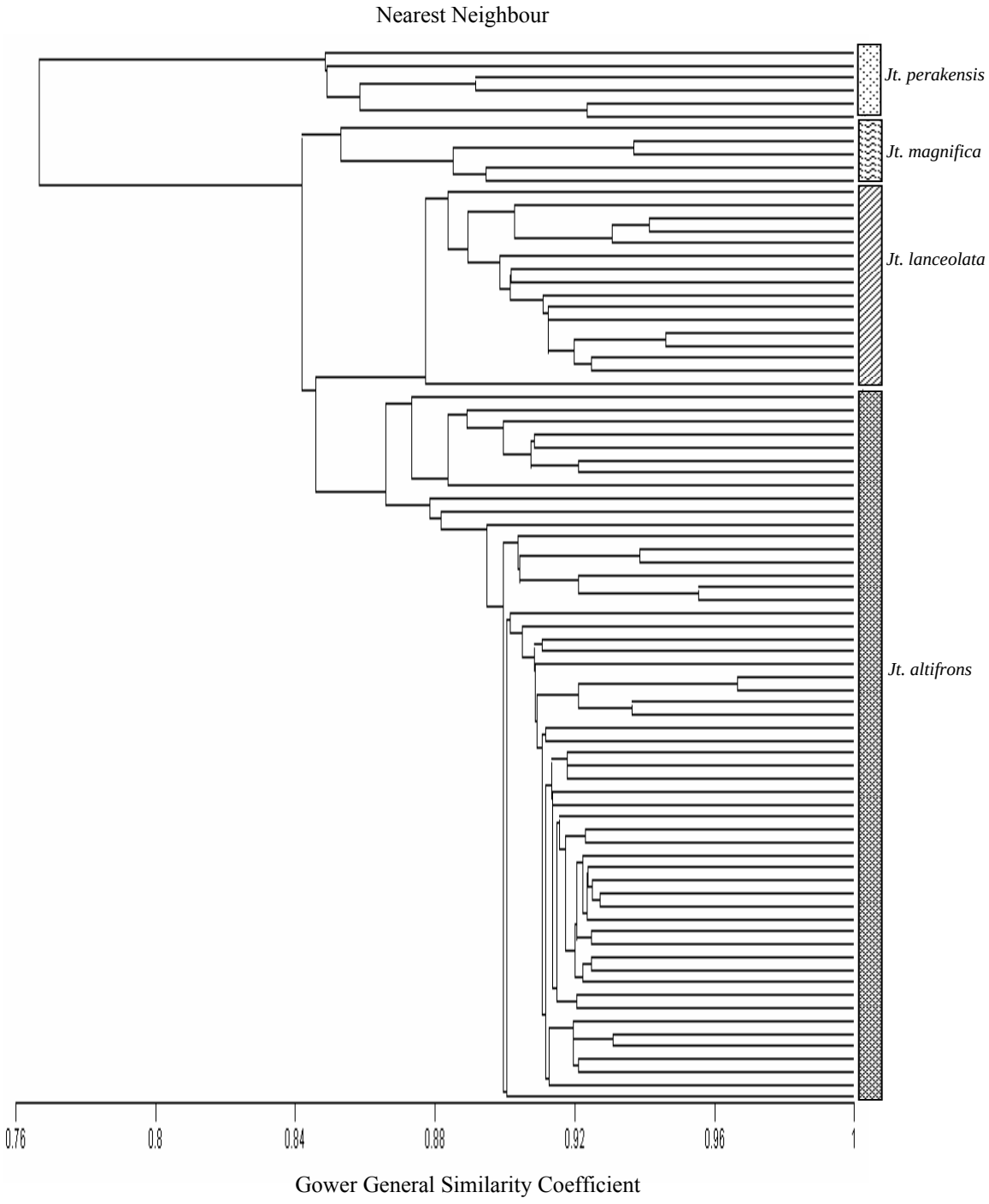

Figure 2.14. Phenogram of cluster analysis using reproductive and vegetative characters. The analysis was carried out using Gower's General Similarity Coefficient and the Nearest Neighbour clustering method. Four distinct clusters formed and each cluster represents a different species.

It is very likely that the presence of above ground stem resulted *Jt. perakensis* considered the most distinct from the rest. Earlier results suggested it always clustered furthest away from any of the other three groups and did not overlap with them. An additional PCO and cluster analysis has been carried out, where stem characters (presence of discernable stem, discernable stem height and discerble stem diameter at widest) were excluded in the data set. PCO scatterplot and phenogram when stem characters were excluded are shown in Figures 2.15 and 2.16.

The PCO scatterplot of the first and second coordinates did not fully reveal clear groupings corresponding to each species (Figure 2.15). Only *Jt. lanceolata* resolved as the most distinct group, OTUs of *Jt. altifrons*, *Jt. magnifica* and *Jt. perakensis* grouped close to each other with *Jt. altifrons* OTUs overlapped with *Jt. perakensis* OTUs. The first coordinate measures 22.5% of the total variation while the second, 12.4%, the third, 6.8%, the fourth, 6.3% and the fifth, 4.6%. The first to the fifth coordinates together explain 52.6% of the variation in the data matrix (Table 2.8). The phenogram of the cluster analysis showed that only *Jt. lanceolata* and *Jt. magnifica* formed a distinct cluster but *Jt. perakensis* OTUs nestested within *Jt. altifrons* cluster (Figure 2.16).

Table 2.8. Summary of Principal Coordinate Analysis (PCO) of reproductive and vegetative characters of 83 accessions for four *Johannesteijsmannia* species when stem characters were excluded in the analysis.

	Axis 1	**Axis 2**	**Axis 3**	**Axis 4**	**Axis 5**
Eigenvalues	4.862	2.692	1.466	1.356	1.003
Percentage	22.456	12.432	6.773	6.261	4.631
Cumulative Percentage	22.456	34.888	41.661	47.922	52.553

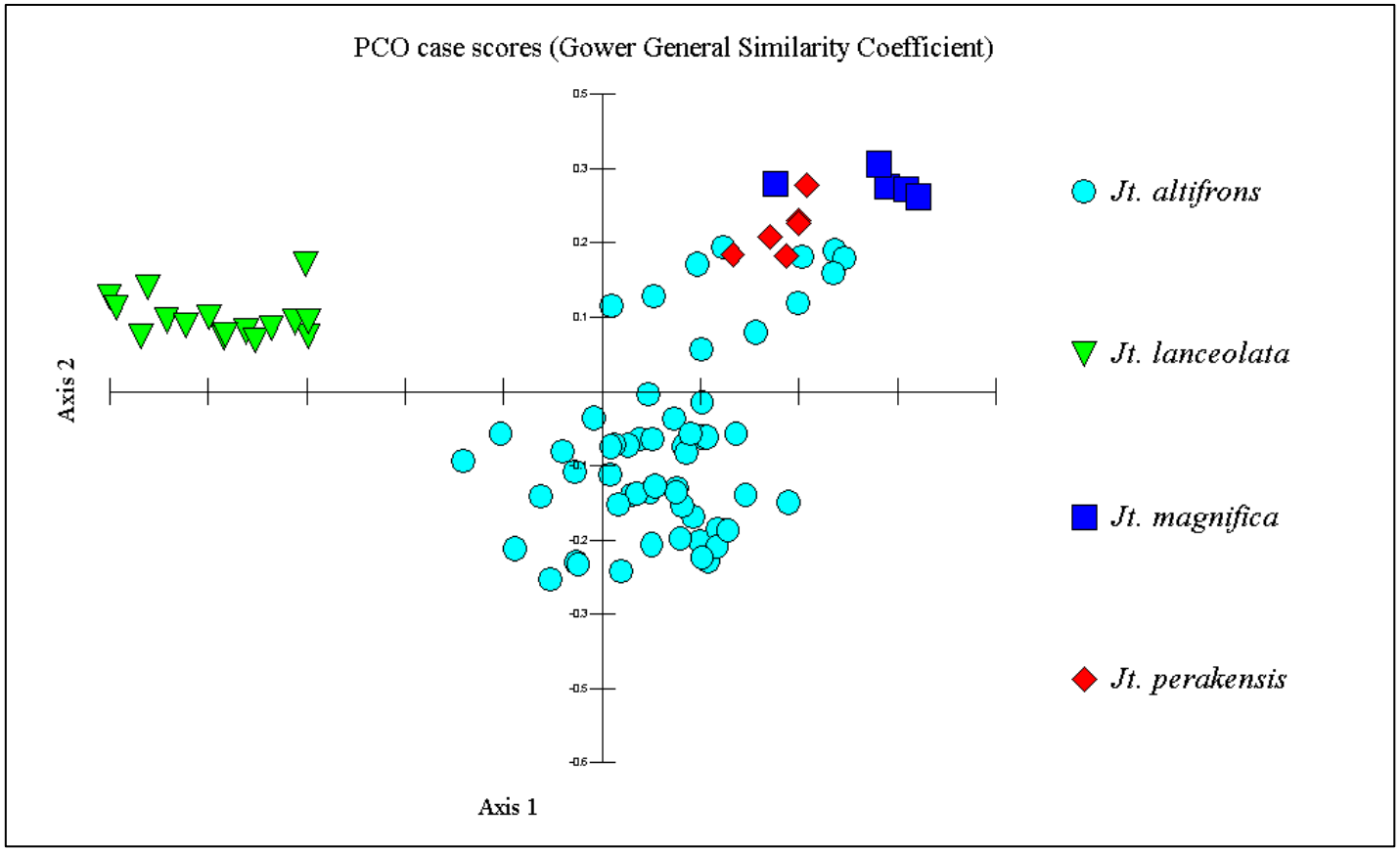

Figure 2.15. Differentiation of OTUs along the first and second principal coordinates when stem characters were excluded from the data matrix. The first coordinate measures 22.5% of the total variation while the second, 12.4%, and the third 6.8%. *Jt. altifrons* OTUs overlapped with *Jt. perakensis* OTUs.

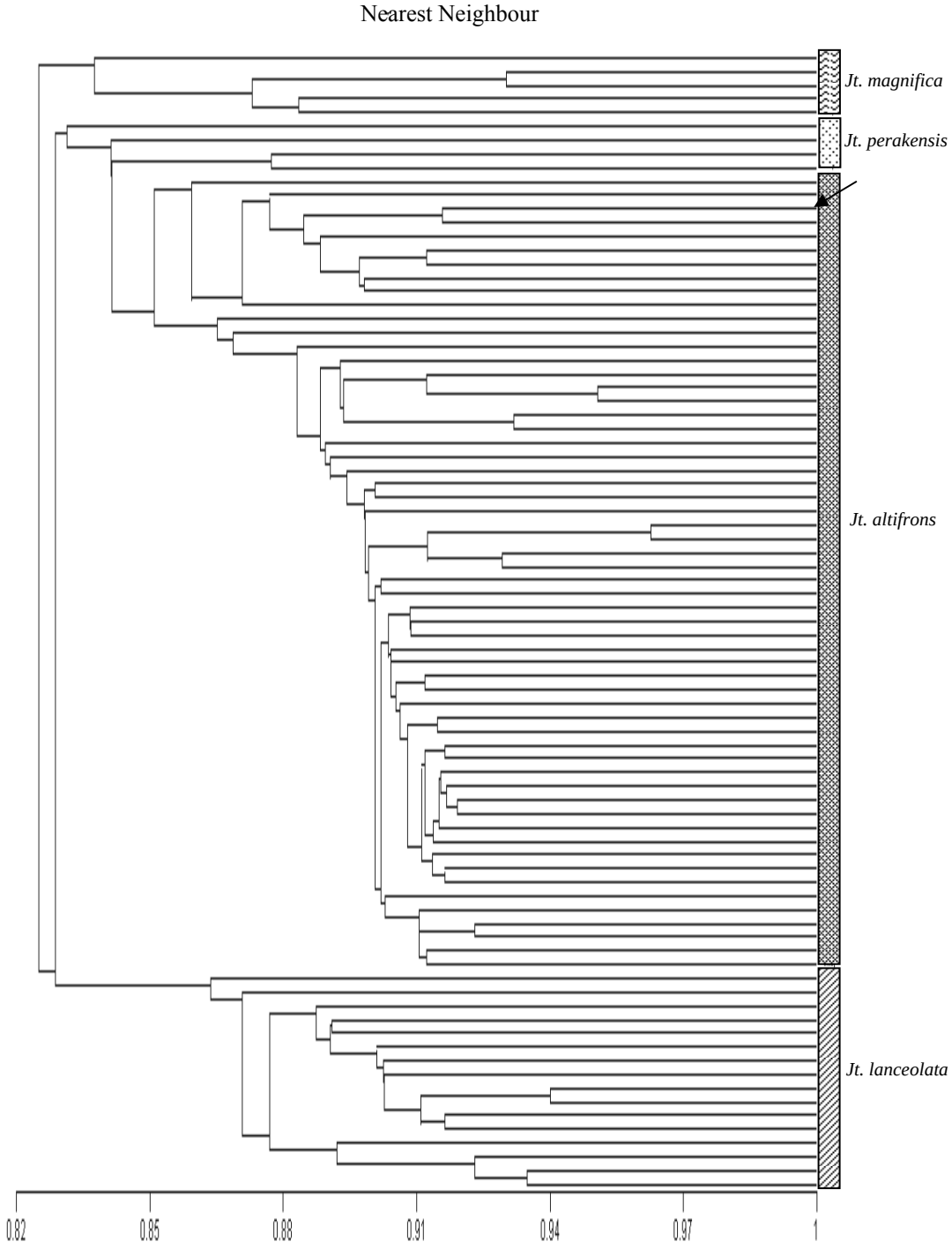

Figure 2.16. Phenogram of cluster analysis using reproductive and vegetative characters, with stem characters removed from data matrix. The analysis was carried out using Gower's General Similarity Coefficient and the Nearest Neighbour clustering method. Only *Jt lanceolata* and *Jt. magnifica* formed distinct clusters but *Jt. perakensis* OTUs nested within *Jt. altifrons* cluster. Arrow indicated *Jt. perakensis* OTU.

2.3.3 *Vegetative Characters*

More accessions (a total of 202 accessions; 105 accessions of *Jt. altifrons*; 25 accessions of *Jt. lanceolata*; 40 accessions of *Jt. magnifica* and 32 accessions of *Jt. perakensis*) were used in the analysis of vegetative characters because they were more likely to be encountered in the field. A total of 12 vegetative characters were used in the analysis and the phenogram generated showed that *Jt. perakensis* and *Jt. magnifica* accessions retained their cohesiveness and formed distinct clusters (Figure 2.17). The presence of an ascending stem on *Jt. perakensis* and the presence of white indumentum at the lamina underside on *Jt. magnifica* were the key characters to separate these two groups from the remaining OTUs. However, *Jt. altifrons* and *Jt. lanceolata* accessions did not separate and their OTUs formed one cluster. None of the vegetative characters were useful to separate these two groups, notwithstanding the lamina dimension was the diagnostic character of *Jt. lanceolata* (hence lanceolate form) to distinguish this species with other OTUs.

It was also observed that three *Jt. perakensis* accessions (indicated with arrows in Figure 2.17) were nested within the *Jt. altifrons* and *Jt. lanceolata* cluster. These accessions were sampled from population KSB; and observed to have no above ground stem. Population KSB was found in disturbed forest, with many open spaces created by falling trees. The *Jt. perakensis* plants at this site were not healthy, perhaps from environmental stress. This may lead to the absence of the ascending stem in the above accessions. More ecological and molecular studies should be carried out to clarify this phenomenon before any conclusions could be drawn. Hence, when using vegetative characters alone, at least two *Johannesteijsmannia* species do not resolve well.

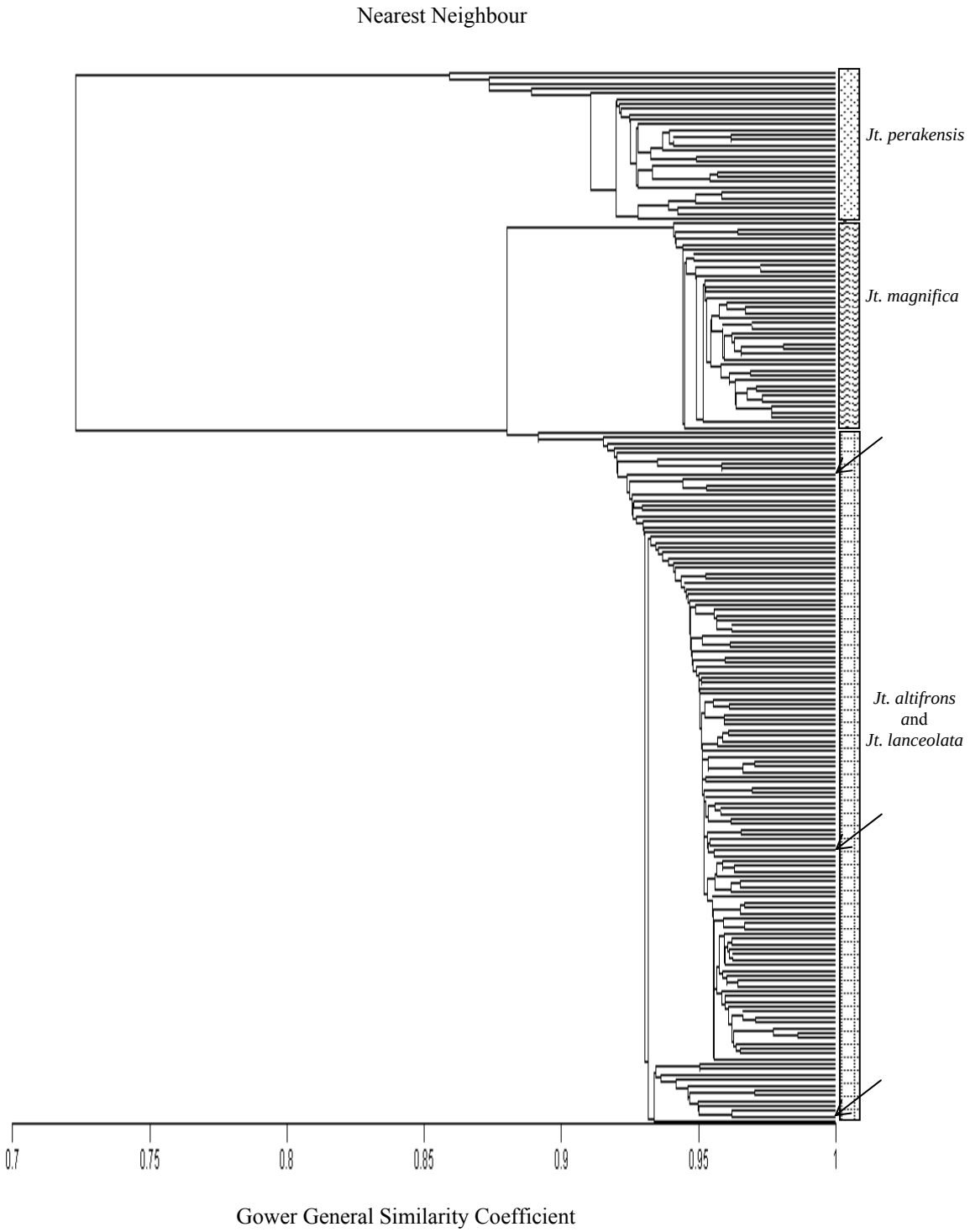

Figure 2.17. Phenogram of cluster analysis using only vegetative characters. The analysis was carried out using Gower's General Similarity Coefficient and the Nearest Neighbour clustering method. Arrows indicate three *Jt. perakensis* accessions nested within the *Jt. altifrons* and *Jt. lanceolata* clusters.

2.3.4 *Reproductive Characters*

Only 92 accessions (56 accessions of *Jt. altifrons*; 25 accessions of *Jt. lanceolata*; five accessions of *Jt. magnifica* and six accessions of *Jt. perakensis*) were used in the analysis of 11 reproductive characters. Cluster analysis of OTUs using only reproductive characters showed that *Jt. altifrons* and *Jt. lanceolata* formed a distinct cluster (Figure 2.18). The OTUs of *Jt. magnifica* and *Jt. perakensis* lost their group cohesiveness and nested within the *Jt. altifrons* cluster. When using reproductive characters alone, the OTUs could not be fully resolved into distinct species groups.

Jt. lanceolata is the most distinct when using reproductive characters alone because its OTUs did not nest within the other groups. The key characters to separate this group from the other groups are the lesser maximum number of orders of branching (one to two orders only), the total number of the branch with maximum number of orders branching, the total number of rachillae on the first branch, the rachilla width (usually thicker) and the total number of rahicllae (less than 25 rachillae).

It was also noted that *Jt. altifrons* formed another cluster but with several OTUs of *Jt. magnifica* and *Jt. perakensis* nested within it. Both *Jt. magnifica* and *Jt. perakensis* did not form a distint group, possibly because of some overlapping of the reproductive characters among species *Jt. altifrons*, *Jt. magnifica* and *Jt. perakensis*. It should be highlighted that the numbers of specimens of *Jt. magnifica* and *Jt. perakensis* used in the analysis were relatively small, therefore this analysis had limited power.

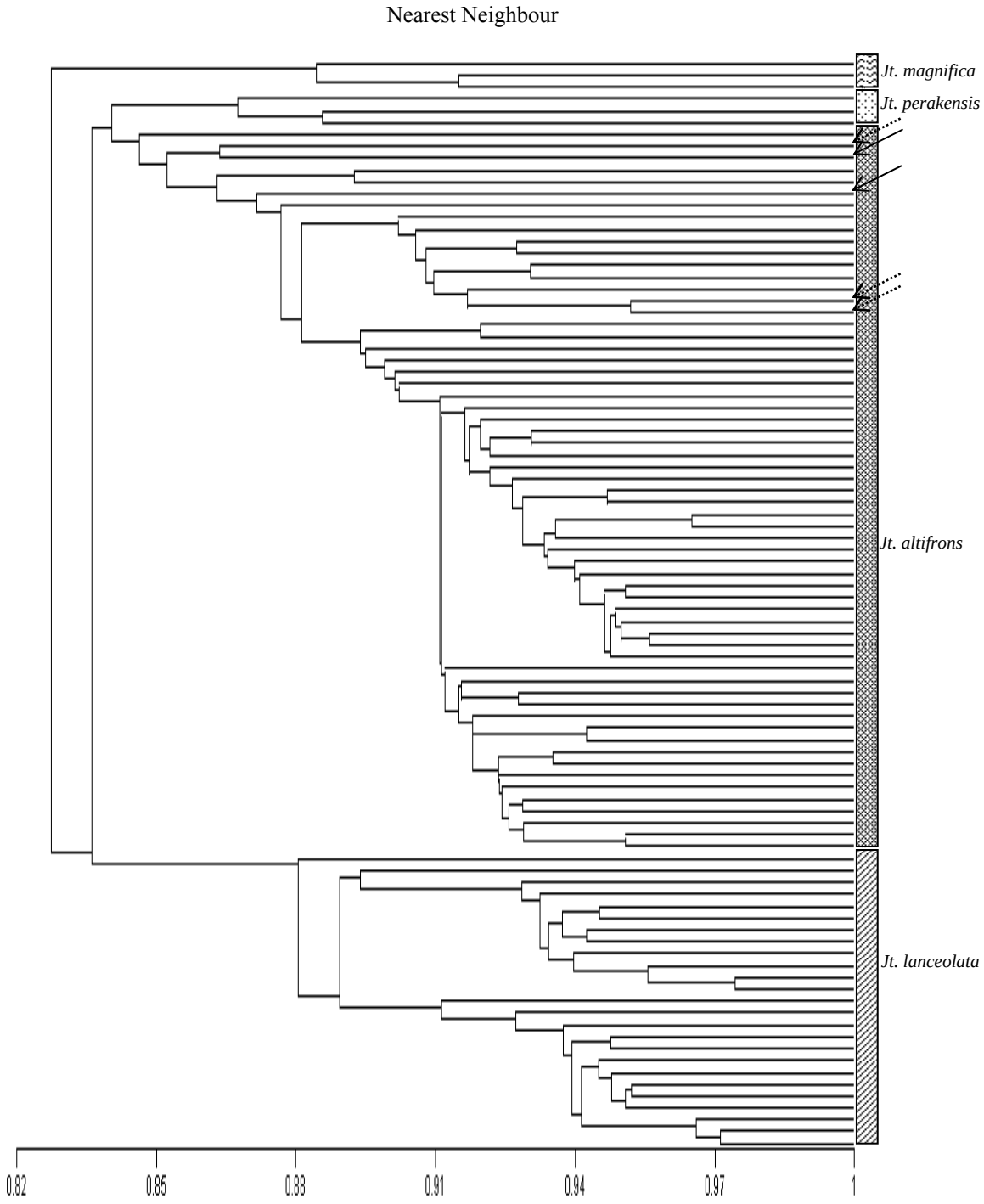

Figure 2.18. Phenogram of cluster analysis using only reproductive characters. The analysis was carried out using Gower's General Similarity Coefficient and the Nearest Neighbour clustering method. Solid line arrows indicate *Jt. magnifica* individuals and dotted line arrows indicate *Jt. perakensis* individuals.

2.3.5 *Quantitative Characters*

Both cluster analysis and PCA were carried out using only quantitative characters to analyze the same set of OTUs as shown in PCO when using both vegetative and reproductive characters. In the phenogram, OTUs of same species tended to cluster together. This was especially prominent in *Jt. perakensis* because the most distinct cluster was formed (Figure 2.19). Most of the *Jt. altifrons* OTUs clustered together except one individual nested within *Jt. lanceolata* cluster and two individuals closer to both *Jt. lanceolata* and *Jt. perakensis* clusters (indicated with arrows in Figure 2.19). This suggested that the possibility of overlapping or intermediate characters exhibited by the above *Jt. altifrons* OTUs with the other *Johannesteijsmannia* species. In addition, it should be also highlighted that regardless the relatively fewer number of specimens of *Jt. perakensis*, the OTUs of this species retained its cohesiveness.

PCA biplot of the first and second axis revealed clearer groupings of *Johannesteijsmannia* species except that one *Jt. lanceolata* OTU overlapped with *Jt. altifrons* OTU (Figure 2.20). *Jt. perakensis* formed the most distinct group because the OTUs grouped furthest away from any of the other three groups and did not overlap with them. OTUs of *Jt. lanceolata* and *Jt. magnifica* also formed their own groups but there was overlapping between *Jt. lanceolata* and *Jt. altifrons* OTUs as mentioned earlier. *Jt. magnifica* OTUs did not overlap with other species but were very close to *Jt. altifrons* OTUs. The first component measured 38.3% of the variation while the second componenet measured 14.1% and the third component measured 10.9%. The first to the fifth together accounted for 76.8% of the variation (Table 2.9).

The grouping of *Jt. perakensis* OTUs was correlated with loadings for the components from the characters of stem diameter (Stemdia) and stem height

(Stemhei), maximum number of orders of branching (Mxob1) and the total number of rachillae at the first branch (1Brano). *Jt. perakensis* is the only decribed species with presence of ascending stem, hence, the stem diameter and stem height give great influence for the grouping. For *Jt. lanceolata*, the main loadings influencing the clustering were observed to be rachilla length (Racllen) and rachilla width (Raclwid). This is because this species has less rachillae and the size of the rachilla (hence length and width) is generally larger in comparison with the other *Johannesteijsmannia* species. There was a degree of separation between *Jt. magnifica* and *Jt. altifrons* — in the field, the main character used to distinguish the two is the white indumentum on the abaxial surface of the *Jt. magnifica* lamina as well as the smaller rachillae in *Jt. magnifica*. The PCA does not show a large proportion of characters in the direction of the *Jt. magnifica* and *Jt. altifrons* clusters, perhaps the second peduncular bracts width (Pbwid), peduncle width (Pedwid) and the total number of pleats (plicae) per lamina (Pleats) separate the both using the PCA biplot (Figure 2.20).

Table 2.9. Summary of principal component loadings on the first five axes of quantitative characters for four *Johannesteijsmannia* species with the cumulative variance for five components.

S/No.	Characters	Axis 1	Axis 2	Axis 3	Axis 4	Axis 5
1	Pedlen	0.230	0.183	-0.319	0.172	-0.191
2	Pedwid	0.266	-0.001	-0.013	0.156	0.145
3	Pedbra	0.113	0.360	-0.229	-0.166	0.130
4	Pblen	0.264	0.085	-0.011	0.105	-0.508
5	Pbwid	0.304	-0.080	0.039	0.047	0.095
6	Racllen	-0.104	0.152	0.052	0.346	-0.586
7	Raclwid	-0.170	0.299	-0.071	0.413	0.307
8	Mxob1	0.276	-0.233	0.026	-0.297	-0.172
9	Brmxob	0.161	0.302	-0.132	-0.015	0.231
10	1Brano	0.296	-0.222	0.019	-0.283	-0.105
11	Lamlen	0.096	0.056	0.605	0.117	0.089
12	Lamwid	0.330	0.121	-0.142	-0.080	0.055
13	Lamwb	0.139	0.132	0.538	0.080	0.164
14	Petlen	0.167	0.308	-0.030	0.223	-0.148
15	Petwid	0.295	0.098	0.117	0.015	0.123
16	Petdep	0.263	0.161	0.270	0.009	0.132
17	Pleats	0.319	0.043	-0.187	-0.018	0.025
18	Stemhei	0.158	-0.419	-0.103	0.426	0.129
19	Stemdia	0.159	-0.416	-0.096	0.434	0.131
	Eigenvalues	7.272	2.681	2.064	1.455	1.124
	Percentage	38.274	14.110	10.866	7.659	5.914
	Cumulative Percentage	38.274	52.384	63.250	70.909	76.823

Figure 2.19. Phenogram of cluster analysis using only quantitative characters. The analysis was carried out using Gower's General Similarity Coefficient and the Nearest Neighbour clustering method. Arrows indicate *Jt. altifrons* individuals.

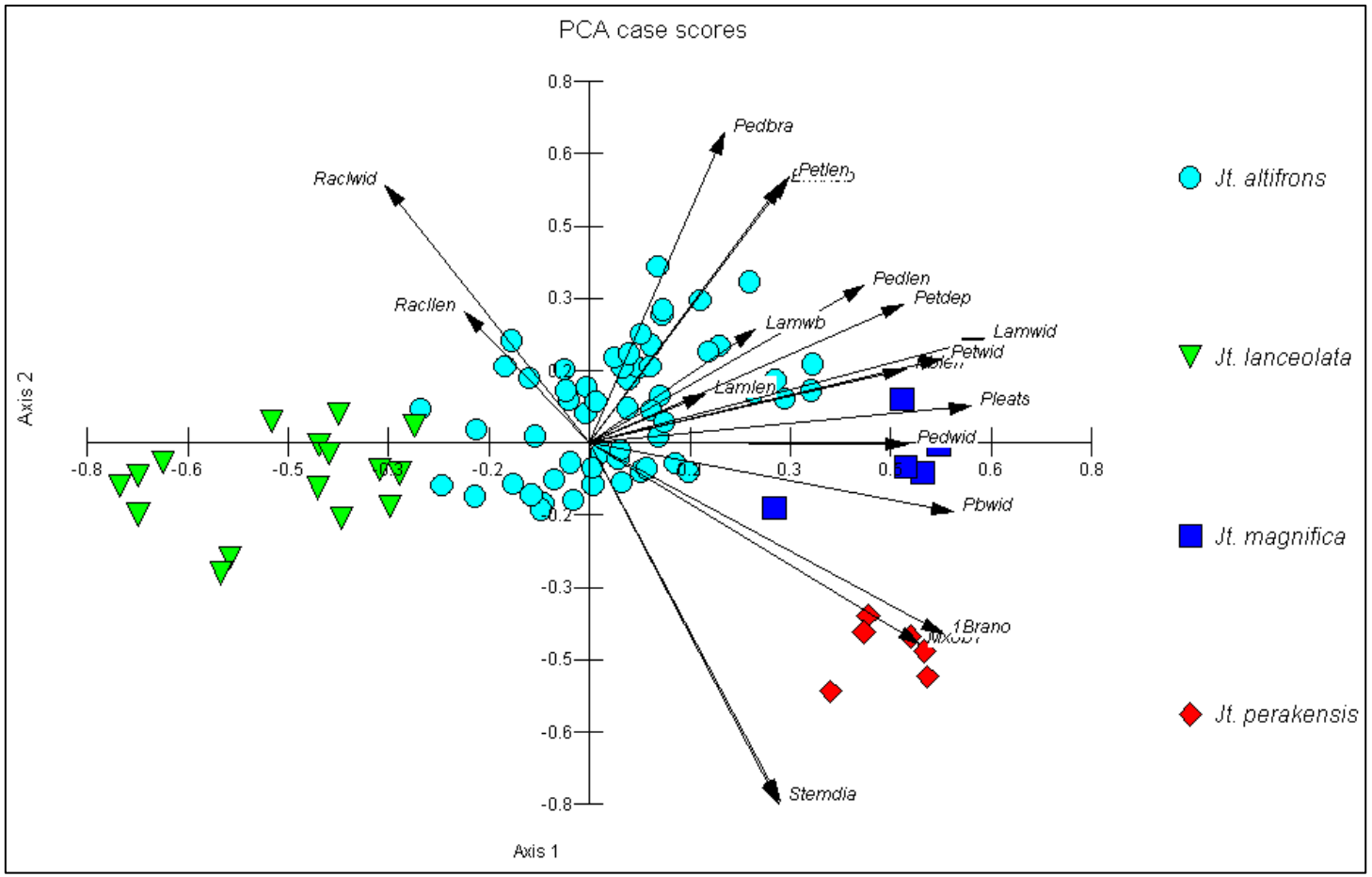

Figure 2.20. Correlation of characters with component loadings indicated by the direction and length of vectors. The first component measures 38.3% of the total variation while the second, 14.1% and the third, 10.9%.

2.4 Conclusions

Based on field observations, morphological variation was detected among the four *Johannesteijsmannia* species. In addition, intermediate morphological variation was observed in *Jt. lanceolata* populations (JBR, ANG and RAU). Specimens collected from population JBR demonstrated two orders of branching at the infructescences, broader lamina dimensions and the wart lengths observed on the fruits were greater than those of the holotype (Figures 2.9A, B, E and F). Two orders of branching were also observed on some of the samples collected from populations ANG (Figure 2.9C) and RAU (Figure 2.9D). The presence of these intermediate characters between *Jt. lanceolata* and *Jt. altifrons* suggested the following possibilities: 1) Hybridization occurred; 2) Spatial isolation among the populations that formed barriers to gene flow; 3) Populations undergo environmental stress. More studies are needed to clarify these findings before any conclusions can be drawn.

When combinations of both reproductive and vegetative characters were used in morphometric analysis, four species groups could be identified within the genus. The most distinct species group belongs to *Jt. perakensis*, as both PCO (Figure 2.13) and cluster (Figure 2.14) analyses demonstrated no overlapping of OTUs with any of the three other species groups. In addition, this finding was supported when vegetative (Figure 2.17) or quantitative (Figures 2.19 and 2.20) characters alone were used in the analysis. However, when additioanal analyses carried out with stem characters removed from the anaysis, *Jt. altifrons* OTUs were overlapping with *Jt perakensis*.in PCO (Figure 2.15) and phenogram (2.16). This suggested *Jt. altifrons* and *Jt. perakensis* are more closely related. The reproductive characters (Figure 2.18) were shown to be useful to distinguish *Jt. lanceolata* from the other three species. The most widely distributed species, *Jt. altifrons* exhibited a degree of morphological similarity

with the remaining three species because the OTUs were either nested (Figures 2.17 and 2.18) or overlapped (Figures 2.15 and 2.20) with the other species groups.

Based on the results of the morphometric analysis, the hypothesis of Dransfield (1972) was supported only when both vegetative and reproductive characters were employed in the analysis as would be expected. *Jt. perakensis* is the most distinct species with an above ground stem while the others are all acaulescent. When the stem characters were excluded in the analysis, *Jt. altifrons* OTUs and *Jt. perakensis* OTUs were either overlapping (Figure 2.15) or nested in a same cluster (Figure 2.16). Despite not having assigned OTUs into groups *a priori*, individuals of each species generally formed their own cluster or group, indicating consistency of the methods in distinguishing species.

CHAPTER 3

MOLECULAR SYSTEMATICS OF *JOHANNESTEIJSMANNIA* H.E. MOORE BASED ON AFLP FINGERPRINTING

3.1 Introduction

Results of morphometric analysis in Chapter 2 supported the hyphothesis of Dransfield (1972) when combinations of vegetative and reproductive characters were used in the analysis. Nonetheless, when either vegetative or reproductive characters alone were used, the results revealed limited power to delimit the species groups — with overlapping of OTUs from different species in PCO; and OTUs nested within other clusters of different species in phenograms. This suggested that morphological data has its limitations in generating stable classification of *Johannesteijsmannia*, because of the morphological variation observed was continuous. In addition, another question underlying the basis of the morphological variation observed — that is, was it due to environmental factors or was it part of the genetic variation? Hence, to answer the question above as well as to further resolve *Johannesteijsmannia* species, generating molecular evidence is much desirable

The greatest advantage of molecular data perhaps is the extent of the data set. All heritable information of an organism is encoded in the DNA. Therefore, the set of morphological data with a genetic basis represents only a small subset of molecular information (Hillis, 1987). Molecular markers have been widely used to delimit plant species (e.g., Koopman *et al*., 2001; van de Wouw *et al*., 2001; González-Pérez *et al*., 2004; Lihova *et al*., 2004). The use of this method, based on phylogenetic species concept (Davis and Nixon, 1992; Wiens and Servedio, 2000), is especially useful when specific taxonomic hypotheses are available, such as distinct morphological subgroups. This method seems much more objective than other methods because it involves robust statistical analyses and criteria for the rejection of hypotheses. In

palms, many studies using molecular markers have successfully resolved species complexes (e.g., Loo *et al*., 1999; González-Pérez *et al*., 2004; Kjaer *et al*., 2004; Bacon and Bailey, 2006). For instance, Bacon and Bailey (2006) used AFLP fingerprinting to resolve the relationships of two taxonomically controversial species, *Chamaedorea alternans* and *C. tepejilote* and delimited them as two separate species: *C. alternans* and *C. tepejilote*.

A series of molecular genetic markers have been developed to study systematics, e.g., analyses by amplified fragment length polymorphism (AFLP), allozymes, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), simple sequence repeat (SSR) or microsatellites and DNA sequencing (Table 3.1). It is important to recognize that there is no perfect genetic marker as each has its advantages and limitations. An ideal molecular marker should meet the following criteria (Muller and Wolfenbarger, 1999):

1. Cheap and time-efficient.
2. Able to generate multiple yet independent markers.
3. Provide sufficient resolution of genetic differences.
4. Reliable and reproducible.
5. Can be used on small samples of tissue or even partially degraded DNA samples.
6. Little molecular expertise is required.
7. No prior knowledge of organism's genome is required.

Table 3.1 Comparison of molecular genetic markers (adapted from Rafalski and Tingey, 1993; Muller and Wolfenbarger, 1999; O'Hanlon *et al*., 2000).

Marker Characteristics	**AFLP**	**Allozymes**	**DNA sequencing**	**Microsatellites**	**RAPD**	**RFLP**
Principle of variation	Selectively amplified fragments to identify point mutations	Differences in charge and size	Differences in base substitution	Differences of tandem repeats	Point mutation in priming site or length mutation between priming sites	Differences in restriction site or length mutation
Quantity of information	High	Low	Moderate	High	High	Low
Dominance	Dominant	Codominant	Codominant	Codominant	Dominant	Codominant
Reproducibility	High	High	High	High	Variable	High
Resolution of genetic differences	Very high	Moderate	Moderate	Very high	High	High
Requirement for sequence information	No	No	Yes	Yes	No	No
Ease of use and development	Moderate	Easy	Difficult	Difficult	Easy	Difficult
Development time	Short	Short	Long	Long	Short	Long
Cost	Medium	Low	Very high	Very high	Low	Medium

3.1.1 *Principles of AFLP Analysis*

Amplified fragment length polymorphism (AFLP) analysis is a Polymerase Chain Reaction (PCR)-based fingerprinting method, which selectively amplifies restriction fragments from a total digested genomic DNA (Vos *et al.*, 1995). This method was developed by Zabeau and Vos (1993), patented by Keygene N.V. (Wageningen, The Netherlands) and first published by Vos *et al.* (1995).

AFLP analysis is a dominant marker technique as genetic polymorphisms are identified by the presence or absence of DNA fragments. There are four major steps involved in this method: 1) Digestion of genomic DNA using two restriction enzymes (one rare six-base cutter and one frequent four-base cutter) to generate sticky ends; 2) Ligation of double-stranded oligonucleotide adapters (one homologous to the 5′- end and the other to the 3′-end) to the restricted DNA fragments; 3) PCR amplification of ligated fragments using primers complementary to the adapter and restriction site sequences followed by selective amplification with an additional selective nucleotide at the 3′-end (the use of selective primers is optional and mainly used for organisms with complex genomes); 4) Detection of DNA polymorphism by fractionating the amplified fragments on a denaturing polyacrylamide gel.

3.1.2 *Advantages of AFLP Analysis*

AFLP analysis is a robust and reliable technique because it is performed under stringent reaction conditions for primer annealing as it combines the merits of the RFLP technique together with the power of the PCR. The advantages of AFLP analysis include the following (Vos *et al*., 1995; Jones *et al*., 1997; Rouppe Van Der Voort *et al*., 1997 and Blears *et al*., 1998):

1. It can be used for DNA samples of any origin and complexity without prior sequence knowledge.

2. Only a small quantity of genomic DNA (0.05–0.5 µg) is needed to detect even minor sequence variations.

3. Insensitivity to the template DNA concentration.

4. Very efficient because many polymorphic bands may be revealed in one lane and many bands in a gel can thus be analyzed simultaneously.

5. Reliable and highly reproducible within and between laboratories.

6. Relatively easy and inexpensive.

7. Unlimited number of markers can be produced by changing the restriction enzymes, the nature and the number of selective nucleotides.

8. Virtually free of artifacts.

9. Comigration of nonallelic fragments occurs at extremely low levels.

3.1.3 *Limitations of AFLP Analysis*

The major limitation of AFLP analysis is the difficulty in identifying homologous markers (alleles), making AFLP analysis less valuable for studies where accurate assignment of allelic states is needed, e.g., heterozygosity analyses (Muller and Wolfenbarger, 1999).

3.1.4 *Factors Affecting the Reproducibility of AFLP Markers*

One of the great advantages of AFLP analysis is its reproducibility. Nevertheless, reproducibility can be affected by the purity of the genomic DNA. High purity genomic DNA is required to ensure complete digestion by the restriction endonucleases. Incomplete digestion of DNA generates partially restricted fragments that are mostly of high molecular weight. The amplification of partially digested DNA producing altered banding patterns can ultimately lead to identification of false polymorphisms (Blears *et al*., 1998).

3.1.5 *Applications of AFLP Analysis*

Since the development of AFLP analysis, it has been widely used in DNA fingerprinting of both eukaryotes and prokaryotes. Its practical applications include analyses of genetic diversity in plants (e.g., Hartl and Seefelder, 1998; Mitchell and Heenan, 2002; Ude *et al*. 2002; Odat *et al*., 2004), systematic analyses of different taxa of various ranks (e.g., Koopman *et al*., 2001; van de Wouw *et al*., 2001; Lihova *et al*., 2004), population and conservation genetic studies (e.g., Miller *et al*., 2000; Rottenberg and Parker, 2003; Juan *et al*., 2004; Nielsen, 2004; Cardoso *et al*. 2005), generation of high-resolution genetic maps in plants (e.g., Waugh *et al*., 1997), genotypic analyses of bacteria (e.g., Huys *et al*., 1996; Janssen *et al*., 1996), epidemiological typings of bacteria (e.g., Dijkshoorn *et al*., 1996; Janssen *et al*., 1996) and genotypic classifications of fungi (Mueller et al, 1996).

3.1.6 *Objectives*

Our results from the use of AFLP fingerprinting to verify the relationships among *Johannesteijsmannia* species as well as attempts to answer some of the

questions that arose from Chapter 2 are presented in this chapter. Hence, the main objectives are:

1. To determine if the individuals exhibiting intermediate morphological characters of *Jt. altifrons* and *Jt. lanceolata* are hybrids.
2. To determine if hybridization occurred among *Jt. altifrons*, *Jt. lanceolata* and *Jt. magnifica* because they were found sympatrically in Sungai Lalang Forest Reserve, Hulu Langat, Selangor, Malaysia.
3. To determine any congruence between morphological and genetic data to further resolve the systematics of *Johannesteijsmannia*.

Genetic data using AFLP fingerprinting in this chapter would complement the morphological evidence discussed in Chapter 2. Combination of both morphological and molecular approaches can maximize both information content and could shed light on the systematics of *Johannesteijsmannia*.

3.2 Materials and Methods

3.2.1 *Sampling and Collection Localities*

Samplings and collection localities were as described in Chapter 2 (section 2.2.1; Tables 2.2, 2.3, 2.4 and 2.5). The plant materials for DNA extraction were collected from part of the unfurled sword leaves (also known as the baton) to ensure that they are relatively unexposed to the environment and no epiphyllous growth or necrotic spots were present. The plant materials were then wrapped in damp tissue paper, kept in a resealable polythene bag before transportation back to the laboratory (Department of Biological Sciences, National University of Singapore, Singapore or Forest Research Institute Malaysia, Kepong, Selangor, Malaysia) for further processing and DNA extraction. The plant materials were then surface-sterilized by

washing with 10% Clorox™ (v/v) for 10 min, followed by rinsing three times with autoclaved deionized water, frozen in liquid nitrogen after wrapping portions of known fresh weight in aluminium foil and storing in a −80°C freezer until DNA extraction.

3.2.2 *DNA Extraction*

The total genomic DNA was extracted according to the method described by Murray and Thompson (1980) with modifications. Twenty ml of 2× CTAB extraction buffer [2% (w/v) CTAB, 20 mM EDTA, 1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), 0.2% β-mercaptoethanol and 1% PVP-40] was preheated to 60 °C in a water bath. Approximately 3–5 g of the frozen leaf tissue was ground into a fine powder in a mortar and pestle with liquid nitrogen. The preheated CTAB extraction buffer was then added into the finely ground leaf material, followed by inverting the tube to homogenize the slurry. The mixture was incubated at 60 °C for 30 min with occasional mixing and then allowed to cool to room temperature before an equal volume of chloroform-isoamyl alcohol (24:1) was added. The mixture was then mixed gently for 15 min followed by centrifugation at 2700 rpm for 10 min. The aqueous (upper) phase was gently removed and transferred into a new tube and re-extracted with equal volume of chloroform-isoamyl alcohol (24:1). Two-third volume of cold (−20°C) isopropanol was added and mixed gently to precipitate the nucleic acid in the aqueous phase of the second extraction. The tube was then kept in −20°C for 20 min or overnight for complete precipitation, followed by centrifugation at 2700 rpm (Eppendorf Centrifuge 5414C) for 10 min. The nucleic acid (white visible pellet) was collected and transferred into 1 ml of wash buffer (76% ethanol and 10 mM ammonium acetate) and the tube was inverted 10 times to wash. After at least an hour

in the wash buffer, the nucleic acid was centrifuged at 3000 rpm (Eppendorf Centrifuge 5417C) for 10 min. The supernatant was carefully discarded and the pellet was allowed to air dry at room temperature. The DNA pellet was then dissolved in 400 µl TE buffer (50 mM Tris-HCl, pH 8.0 and 20 mM EDTA). The DNA concentration was quantified using a spectrophotometer as well as estimated using agarose gel electrophoresis together with the intact DNA ladder of known concentrations.

3.2.3 *Protocol for AFLP Fingerprinting Reactions*

The AFLP fingerprinting method used was according to the procedure described by Vos *et al*. (1995) with minor modifications. The *Eco*RI primer was labelled with three fluorescent dyes (PE Applied Biosystems, www.appliedbiosystems.com), i.e., 6-carboxyfluorescein (6FAM, blue colour), tetrachloro-6-carboxy-fluorescein (TET, green colour) and hexachloro-fluorescein phosphoramidite (HEX, yellow colour) instead of radioactive labelling.

Approximately 250 ng of total genomic DNA in a 15 µl volume was digested with *Eco*RI and *Mse*I in 10 µl restriction digestion mixture [1× Buffer 4 (New England Biolabs, www.neb.com) 1× BSA, 5 mM DTT, 2.5 U *Eco*RI (Amersham Pharmacia Biotech., www.apbiotech.com), 2.5U *Mse*I (New England Biolabs) and autoclaved deionized water]. The mixture was incubated at 37^{o}C for 1 h. One µl of each *EcoR*I adapters [2.5 pmol for each strand and Tris-HCl (pH 8.0)] and *Mse*I adapters [25 pmol for each strand and Tris-HCl (pH 8.0)] were prepared by heating the mixture in 95^{o}C for 10 min followed by decreasing the temperature to 4^{o}C in 1 h. To the digested DNA was added 5 µl of ligation mix [1× Buffer 4 (New England Biolabs), 1× BSA, 5 mM DTT, 1mM ATP, *EcoR*I adapters, *Mse*I adapters, 0.5 U T4

DNA ligase (New England Biolabs) and autoclaved deionized water]. The ligation reaction was incubated at 16^{o}C for 16 h.

PCR reactions were performed after diluting the ligated DNA 10-fold with autoclaved deionized water. The 20 µl pre-amplification reaction mixture contained 1× PCR buffer with $(NH_4)_2SO_4$ (Fermentas, www.fermentas.com), 2 mM dNTPs, 4 mM $MgCl_2$ (Fermentas), 0.25 µM pre-amplification *Eco*RI primer (with single selective nucleotide at 3′ end), 0.25 µM pre-amplification *Mse*I primer (with single selective nucleotide at 3′ end), 0.5 U *Taq* DNA polymerase (Fermentas) and autoclaved deionized water. The fragments were pre-amplified by 20 PCR cycles (a 30s DNA denaturation at 94^{o}C, a 1 min annealing step at 56^{o}C and 1 min extension at 72^{o}C). PCR products from pre-amplification were diluted 10-fold and used as templates for the selective amplification using *Eco*RI and *Mse*I primers, both of which have three selective nucleotides at the 3′ end. The *Eco*RI primers were labelled with three fluorescent dyes. The amplification reactions were performed for one cycle at 94 ^{o}C for 30 s, 65 ^{o}C for 30 s and 72 ^{o}C for 60 s, followed by reduction of the annealing temperature at each cycle by 0.7 ^{o}C for 12 cycles; the annealing temperature was maintained at 56 ^{o}C for the remaining 23 cycles. All the PCR reactions were carried out using the GeneAmp PCR System 9700 (PE Applied Biosystems). The sequences of *Eco*RI and *Mse*I adapters, pre-amplification *Eco*RI primer, pre-amplification *Mse*I primer, selective *Eco*RI primes and selective *Mse*I primers are listed in Table 3.2.

Table 3.2. Adapter and primer sequences used in the AFLP reactions.

Adapter or Primer	**Sequence (5′→ 3′)**	**Length**
*Eco*RI adapter	CTCGTAGACTGCGTACC AATTGGTACGCAGTCTAC	17 mer 18 mer
*Mse*I adapter	GACGATGAGTCCTGAG TACTCAGGACTCAT	16 mer 14 mer
Pre-amplification *Eco*RI primer (1 selective nucleotide)	GACTGCGTACCAATTCA	17 mer
Pre-amplification *Mse*I primer (1 selective nucleotide)	GATGAGTCCTGAGTAAC	17 mer
Fluorescent labeled selective *Eco*RI primer (3 selective nucleotides)	6FAM-GACTGCGTACCAATTCAAC TET-GACTGCGTACCAATTCAAG HEX-GACTGCGTACCAATTCACA 6FAM-GACTGCGTACCAATTCACT TET-GACTGCGTACCAATTCACC HEX-GACTGCGTACCAATTCACG 6FAM-GACTGCGTACCAATTCAGC TET-GACTGCGTACCAATTCAGG	19 mer
Selective *Mse*I Primer (3 selective nucleotides)	GATGAGTCCTGAGTAACAA GATGAGTCCTGAGTAACAC GATGAGTCCTGAGTAACAG GATGAGTCCTGAGTAACAT GATGAGTCCTGAGTAACTA GATGAGTCCTGAGTAACTC GATGAGTCCTGAGTAACTG GATGAGTCCTGAGTAACTT	19 mer

One and a half microliter selective amplification products were then mixed with 2.5 µl deionized formamide (BDH, Poole, UK), 1 µl loading buffer (50 mg ml^{-1} blue dextran and 25 mM EDTA, pH 8.0) and 0.5 µl (equivalent to 2 fmol, as recommended by the manufacturer) GeneScan-500 TAMRA (PE Applied Biosystems) internal size standard. The GeneScan-500 TAMRA internal size standard was used to accurately size the amplified fragments. It is designed for achieving high precision in sizing DNA fragments in the 35–500 base pair (bp) range. The mixture

was then denatured at 92^{o}C for 3 min and chilled on ice immediately. The AFLP products were fractionated in 6% denatured polyacrylamide gel with an automated DNA sequencer (ABI Prism 377, PE Applied Biosystems). The electropherograms generated by the sequencer were collected and interpreted with GeneScan 3.1 software (PE Applied Biosystems). Genotyper 2.0 software (PE Applied Biosystems) was then used to create a list of fragments detected in each lane by fragment size.

3.2.4 *Scoring AFLP Band*

Amplified fragments were transformed into peaks in the electropherograms in which peak height reflects the fluorescent signal and peak width reflects band intensity. The fragments (peaks) were scored as discrete character states, 1 for band presence and 0 for absence. Only fragments with molecular weights that ranged between 50–400 bp with peak heights more than 50 arbitrary fluorescence units in the electropherogram were retained for subsequent analyses. The peaks less than 50 arbitrary fluorescence units were left out as background noise as most of the peaks are several hundred to several thousand units high. Fragments that differ by 0.5 relative migration units (bp) between samples were identified as different. The band profiles were then used to form a binary data matrix for each primer combination. There was no bias in scoring monomorphic fragments versus polymorphic fragments.

3.2.5 *AFLP Data Analysis*

AFLP bands were analyzed using the computer programme Multi-Variate Statistical Package (MVSP) version 3.10b (Kovach Computing Services, 1999). Two phenetic analyses were carried out, viz., principal coordinate analysis and cluster analysis to analyze and visualize genetic distance and similarity among species.

Firstly, an unweighted pair group method (UPGMA; Sneath and Sokal, 1973) phenogram was generated using Simple Matching Coefficient (Sokal and Michener, 1958) because it gives weightage to both presense and absence of a band and is relatively easy to interpret. Secondly, PCO using the Euclidean distance was also carried out to visualize potential intermediate genotypes that might be hybrids or incomplete differentiation. Such a pattern is easier to detect in a PCO scatterplot rather than in a strictly divergent UPGMA phenogram.

Two separate sets of binary character matrices were used in the analyses to determine the objectives of this chapter: 1) Inclusion of *Jt. altifrons* and *Jt. lanceolata* OTUs. This is to clarify if the individuals exhibited intermediate morphological characters of *Jt. altifrons* and *Jt. lanceolata* are real hybrids. Several *Jt. magnifica* and *Jt. perakensis* OTUs were used as outgroup; 2) OTUs of all species collected from this study to test any congruence between morphological and genetic data.

3.3 Results and Discussion

Twelve primer combinations were screened, and of these six yielded excellent results and hence were selected for further analyses. AFLP fingerprinting revealed 288 unambiguous bands when using six selected primer combinations. The numbers of scorable bands of each primer combination are listed in Table 3.3. The use of AFLP fingerprinting to assess genetic relationships among species is promising because it can produce many polymorphic loci fairly easily in a relatively short time.

Table 3.3. Primer combinations screened, primer combinations selected for analyses and total scorable bands.

Primer Combinations Tested	Results	Total Scorable Bands
*Eco*RI AAG + / *Mse*I + CAC	Retained for analyses	103
*Eco*RI ACA + / *Mse*I + CAG	Retained for analyses	48
*Eco*RI ACT + / *Mse*I + CAT	Retained for analyses	41
*Eco*RI AGC + / *Mse*I + CTG	Retained for analyses	39
*Eco*RI AGG + / *Mse*I + CAA	Retained for analyses	32
*Eco*RI AGC + / *Mse*I + CAC	Retained for analyses	25
*Eco*RI ACA + / *Mse*I + CTC	Not selected for analyses	Not applicable
*Eco*RI AAC + / *Mse*I + CAA	Not selected for analyses	Not applicable
*Eco*RI ACC + / *Mse*I + CTA	Not selected for analyses	Not applicable
*Eco*RI ACG + / *Mse*I + CTC	Not selected for analyses	Not applicable
*Eco*RI AGG + / *Mse*I + CTT	Not selected for analyses	Not applicable
*Eco*RI ACG + / *Mse*I + CAG	Not selected for analyses	Not applicable

3.3.1 *Jt. altifrons and Jt. lanceolata*

The matrix used in both PCO and cluster analyses composed of *Jt. altifrons* and *Jt. lanceolata* OTUs from all localities to test if hybridization occurred on individuals showing intermediate morphological characters of these two species. A total of 146 OTUs (113 accessions of *Jt. altifrons*; 24 accessions of *Jt. lanceolata*; five accessions of *Jt. magnifica* and four accessions of *Jt. perakensis*) were used, of which *Jt. magnifica* and *Jt. perakensis* OTUs were outgroup.

PCO scatterplot of the first and third coordinates revealed clear groupings of *Jt. altifrons* and *Jt. lanceolata*, no overlapping found on OTUs of these two species (Figure 3.1). Three *Jt. altifrons* groups were observed on the PCO scatterplot; and *Jt. perakensis* OTUs nested within one of the *Jt. altifrons* groups. In contrast, *Jt. magnifica* OTUs formed a distinct group. The first coordinate measures 18.8% of the total variation while the second, 9.5%, the third, 5.5%, the fouth, 3.9% and the fifth, 3.0%. The first to the fifth coordinates together explain 40.7% of the variation in the data matrix (Table 3.4).

The phenogram of the cluster analysis revealed no mixing of *Jt. altifrons* and *Jt. lanceolata* OTUs within a cluster (Figure 3.2). Accessions belonging to a particular species clustered together except *Jt. perakensis* OTUs nested within *Jt. altifrons* cluster. The three grouping of *Jt. altifrons* shown in PCO scatterplot was further resolved when using cluster analysis — all *Jt. altifrons* OTUs grouped together and formed a distinct cluster. It should be highlighted that East Malaysian populations (BAK and MAT in Sarawak) formed a subset nested within *Jt. altifrons* cluster. The similarity coefficient calculated from this analysis ranged from 0.817 to 0.965.

Results from PCO and cluster analyses implied that no hybridization occurred between the two species tested. The intermediate morphological characters observed perhaps resulted from environmental factors. Many studies supported that phenotypes of plants are determined by interactions between genotype and environment. To a given set of environmental conditions, different genotypes react differently; and the same plant genotypes produce different phenotypes under contrasting environmental conditions (Briggs and Walters, 1993).

Table 3.4. Summary of Principal Coordinate Analysis on the first five axes of *Jt. altifrons* and *Jt. lanceolata* with *Jt. magnifica* and *Jt. perakensis* as outgroup.

	Axis 1	**Axis 2**	**Axis 3**	**Axis 4**	**Axis 5**
Eigenvalues	575.684	289.285	166.977	120.622	91.580
Percentage	18.825	9.460	5.460	3.944	2.995
Cumulative Percentage	18.825	28.285	33.745	37.689	40.684

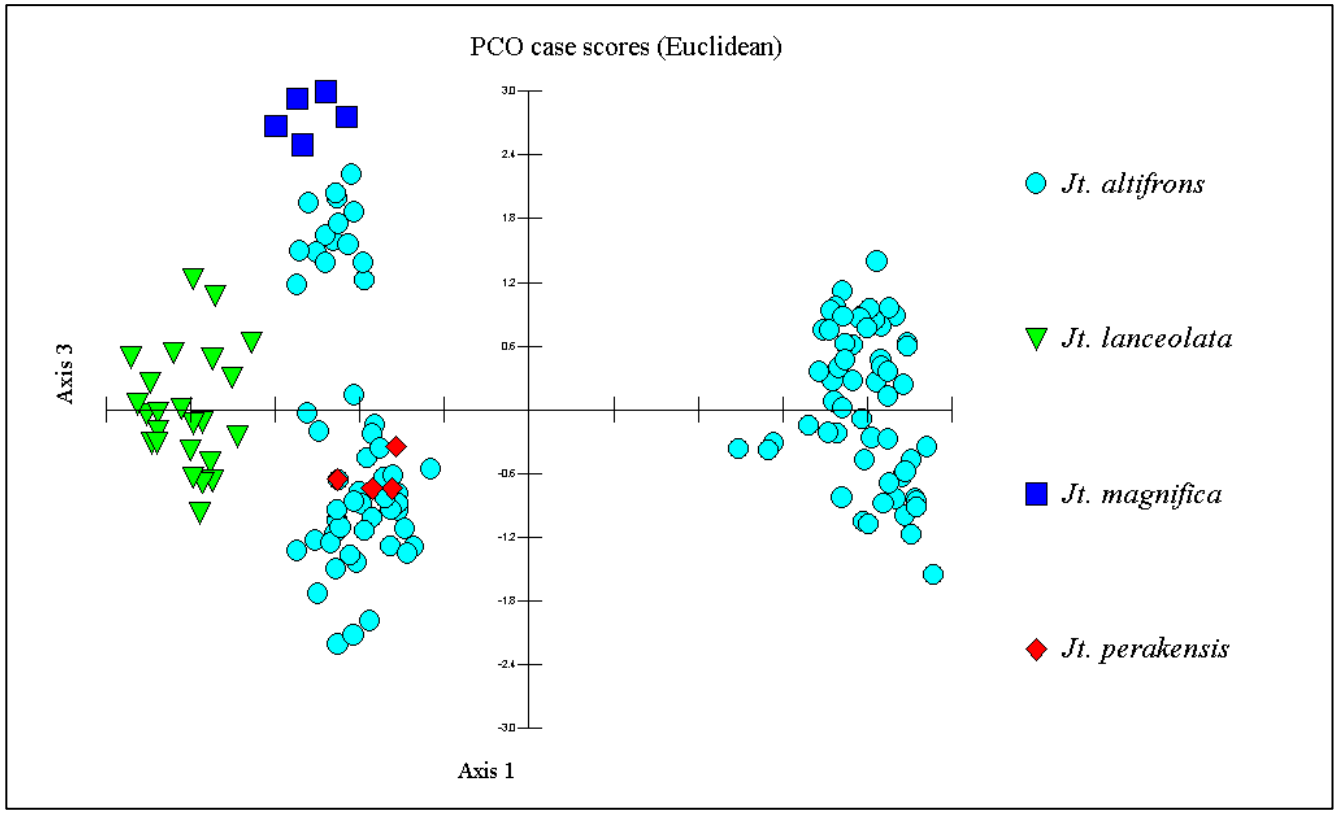

Figure 3.1. Differentiation of *Jt. altifrons* and *Jt. lanceolata* OTUs along the first and third principal coordinates. The first coordinate measured 18.8% of the total variation while the second, 9.5% and the third, 5.5%. *Jt. altifrons* and *Jt. lanceolata* formed distinct species groups; no overlapping observed among the OTUs of these two species. *Jt. magnifica* formed another distinct group but *Jt. perakensis* OTUs overlapped with *Jt. altifrons* group.

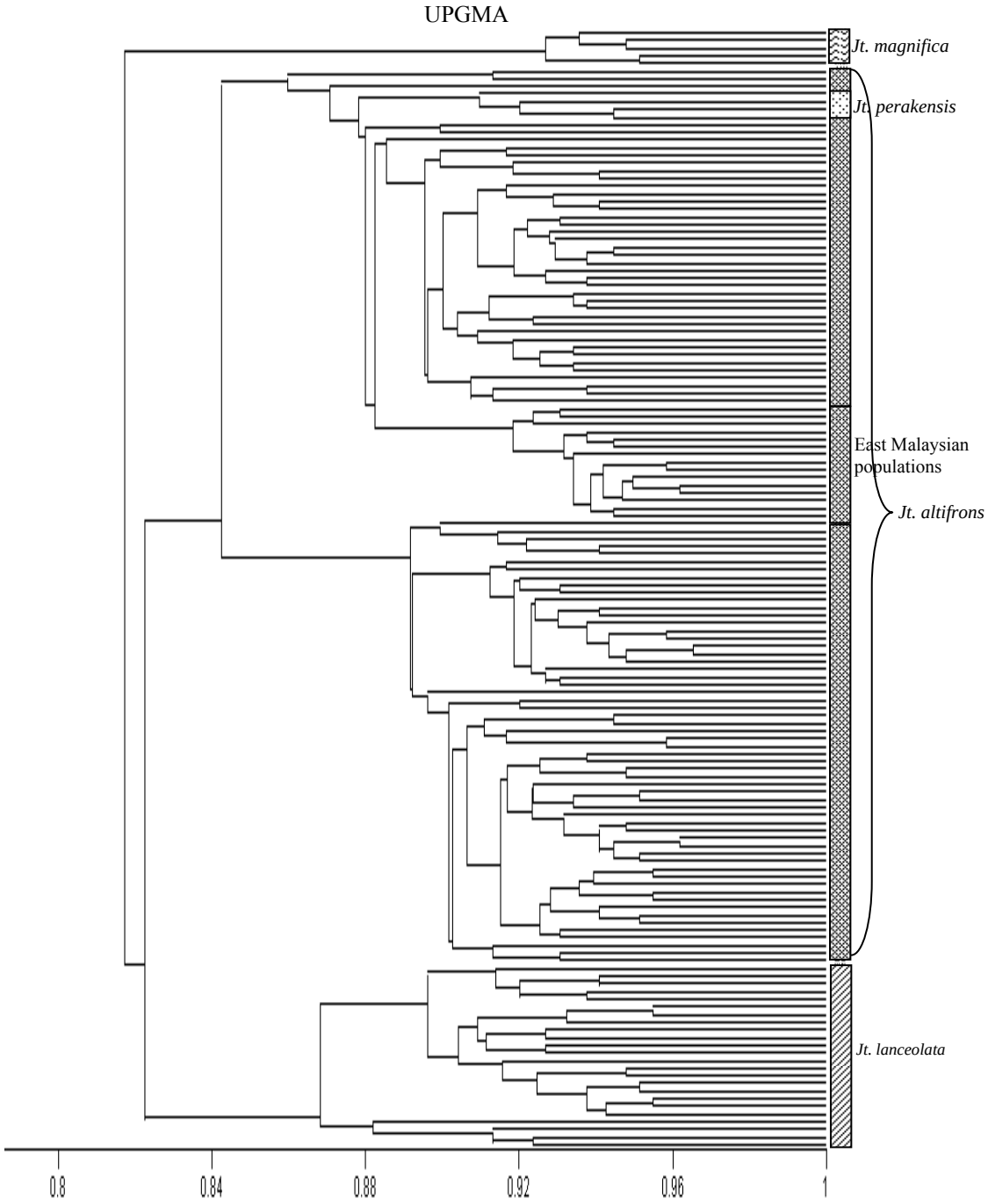

Figure 3.2. Phenogram of cluster analysis of *Jt. altifrons* and *Jt. lanceolata* OTUs. The analysis was carried out using Simple Matching Coefficient and the UPGMA clustering method. *Jt. altifrons*, *Jt. lanceolata* and *Jt. magnifica* formed a distinct cluster whereas *Jt. perakensis* OTUs were nested within the *Jt. altifrons* cluster. East Malaysian OTUs also formed a distinct cluster nested within the *Jt. altifrons* cluster.

3.3.2 *Inclusion of All Johannesteijsmannia OTUs*

In this section, all accessions of *Johannesteijsmannia* species collected in the present study were included in both PCO and cluster analyses except 13 accessions that were missing AFLP data. A total of 209 OTUs (115 accessions of *Jt. altifrons*; 24 accessions of *Jt. lanceolata*; 40 accessions of *Jt. magnifica* and 30 accessions of *Jt. perakensis*) were used to compose a binary character matrix.

PCO scatterplot using AFLP data showed clear groupings of *Jt. altifrons*, *Jt. lanceolata* and *Jt. magnifica*, no overlapping was found among OTUs of these species (Figure 3.3). There were two *Jt. altifrons* groups observed on the PCO scatterplot; and *Jt. perakensis* OTUs overlapped with one of the *Jt. altifrons* groups. The first coordinate measures 18.3% of the total variation while the second, 11.2%, the third, 6.7%, the fourth, 4.9% and the fifth, 3.2%. The first to the fifth coordinates together explain 44.4% of the variation in the data matrix (Table 3.5).

The cluster analysis also revealed clear grouping of all species except that *Jt. perakensis* group nested within *Jt. altifrons* cluster (Figure 3.4). The two grouping of *Jt. altifrons* observed in PCO scatterplot was further resolved when using cluster analysis — all *Jt. altifrons* accessions grouped together and formed a distinct cluster. The East Malaysian populations (BAK and MAT in Sarawak) formed a subset nested within *Jt. altifrons* cluster. This suggested that the East Malaysian populations are evolving and more genetically distinct from the remaining *Jt. altifrons* populations due to geographical separation. It should be highlighted that no morphological variation was observed between East Malaysian populations (Figures 2.7B, C, D, E and F) compared to other *Jt. altifrons* populations. The similarity coefficient calculated between the nodes resulted from this analysis ranged from 0.822 to 0.976, the highest similarity was shared by two *Jt. magnifica* accessions.

Based on the herbarium specimens of *Jt. altifrons* in Sumatra, there are no significant morphological differences from *Jt. altifrons* growing in other localities. However, AFLP data showed that *Jt. altifrons* populations from East Malaysia formed a distinct subcluster nested within *Jt. altifrons* cluster as mentioned earlier. This suggests that geographical isolation of the species leads to the emergence of distinct genotypes. Therefore, the possibilities that the Sumatran population may form its own subcluster within *Jt. altifrons* cluster cannot be ruled out until AFLP analysis is carried out on the Sumatran specimens.

Results from PCO and cluster analyses indicated no hybridization occurred among *Jt. altifrons*, *Jt. lanceolata* and *Jt. magnifica* even though they were growing sympatrically at Sungai Lalang Forest Reserve, Hulu Langat, Selangor, Malaysia. Results from this chapter clearly show that they are distinct species and are reproductively isolated.

The results based on AFLP data are largely in agreement with the morphometric survey (combinations of reproductive and vegetative characters — 11 reproductive and 11 vegetative) except that *Jt. perakensis* was nested within *Jt. altifrons*. Despite the observation that *Jt. perakensis* was nested within *Jt. altifrons*, they formed a discrete cluster between clusters of *Jt. altifrons*. Thus, there is a possibility that *Jt. perakensis* may be a subspecies that evolved from a population of *Jt. altifrons*. *Jt. perakensis* is found only in the Bintang and Kledang-Saiong mountain ranges (100 to 300m above sea level). This may have served as a form of vicariance to reproductively isolate the *Jt. perakensis* populations from the rest of the *Jt. altifrons* populations. The discrete clustering of *Jt. perakensis* based on the AFLP analysis corroborates this hypothesis.

Table 3.5. Summary of Principal Coordinate Analysis on the first five axes of *Johannesteijsmannia* OTUs.

	Axis 1	**Axis 2**	**Axis 3**	**Axis 4**	**Axis 5**
Eigenvalues	824.319	506.447	303.048	222.851	142.367
Percentage	18.295	11.240	6.726	4.946	3.160
Cumulative Percentage	18.295	29.535	36.261	41.207	44.367

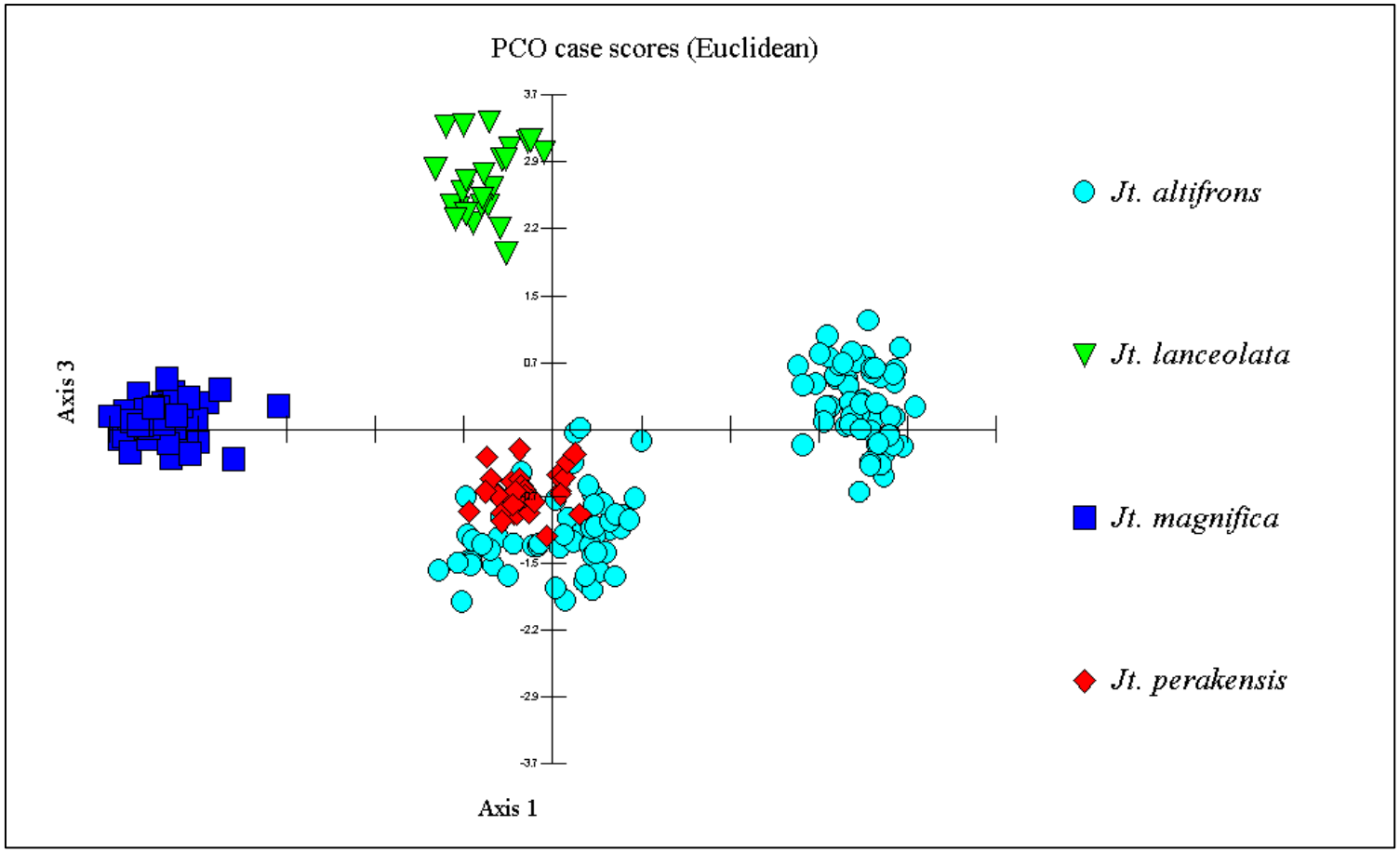

Figure 3.3. Differentiation of *Johannesteijsmannia* OTUs along the first and third principal coordinates. The first coordinate measured 18.3% of the total variation while the second, 11.2% and the third, 6.7%. *Jt. altifrons*, *Jt. lanceolata* and *Jt. magnifica* formed distinct species groups except *Jt. perakensis* OTUs overlapped with *Jt. altifrons* group.

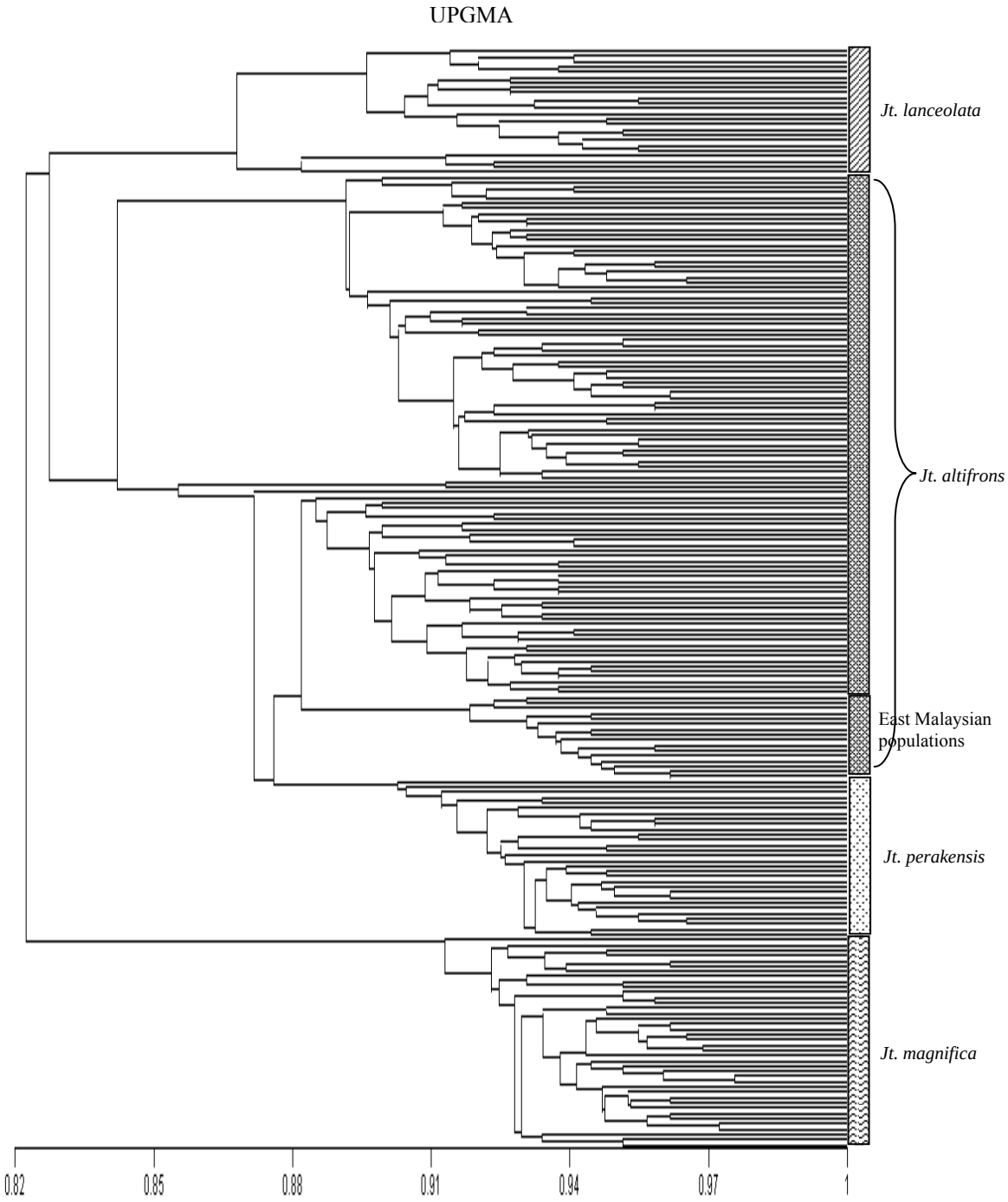

Figure 3.4. Phenogram of cluster analysis of all *Johannesteijsmannia* OTUs. The analysis was carried out using Simple Matching Coefficient and the UPGMA clustering method. *Jt. altifrons*, *Jt. lanceolata* and *Jt. magnifica* formed a distinct cluster whereas *Jt. perakensis* OTUs were nested within the *Jt. altifrons* cluster. East Malaysian OTUs also formed a distinct cluster nested within the *Jt. altifrons* cluster.

3.4 Conclusions

Based on the results of the AFLP analysis, no evidence of hybridization occurs among *Johannesteijsmannia* species and they are reproductively isolated. The morphological evidence was in agreement with genetic data except that *Jt. perakensis* nested within *Jt. altifrons* based on AFLP data. It is very likely that there may be only three *Johannesteijsmannia* species, i.e., *Jt. altifrons, Jt. lanceolata* and *Jt. magnifica*, with *Jt. perakensis* as a subspecies of *Jt. altifrons*, rather than four as originally erected by Dransfield (1972). In addition, when stem characters were removed, *Jt. altifrons* and *Jt. perakensis* were nested within the same clade (Figure 2.16). This morphological data is congruent with molecular evidence presented in this chapter. *Jt. perakensis* is the only described taxon with an above ground stem while the others are all acaulescent. Although the unique morphology of *Jt. perakensis* is highly supported by the genetic data, it forms a distinct group nested within the *Jt. altifrons* OTUs. Thus, based on the AFLP data, *Jt. perakensis* cannot be considered an independent species, but more appropriately, a subspecies within *Jt. altifrons*.

CHAPTER 4

MOLECULAR PHYLOGENY OF *JOHANNESTEIJSMANNIA*

4.1 Introduction

Phylogenetics has become the main focus of systematic botany over the past 15 years particularly through the study of several well-characterized DNA loci. Both coding and non-coding regions of chloroplast (cpDNA) and nuclear ribosomal DNA (nrDNA) have been useful in elucidating the phylogenetic relationships in plants (e.g., Chase *et al*., 1993; Soltis and Soltis, 1998; Soltis *et al*., 1999; Soltis and Soltis, 2004). Work on the phylogeny of angiosperms has confirmed that the family Arecaceae (Palmae) is monophyletic and its placement among the basal commelinids (APG II, 2003). Additionally, all higher-level molecular studies of the monocotyledons (e.g., Chase *et al*., 2000; Asmussen and Chase, 2001) have supported that view that the Arecaceae is monophyletic.

4.1.1 *Molecular Phylogeny of the Arecaceae*

For the past 15 years, substantial progress in the study of the molecular phylogeny of the Arecaceae and the relationships among subfamilies, tribes, subtribes and genera have been elucidated (e.g., Baker *et al*., 1999; Asmussen *et al*. 2000; Baker *et al*. 2000a; Baker *et a*l., 2000b; Lewis and Doyle, 2001; Hahn, 2002a; Hanh, 2002b; Lewis and Doyle, 2002; Gunn, 2004; Dransfield *et a*l. 2005; Roncal *et al*., 2005; Thomas *et al*., 2006).

The starting point to investigate the relationships within the Arecaceae was the classification scheme of Uhl and Dransfield (1987), in which 200 genera were recognized and placed into six subfamilies, 14 tribes and 37 subtribes. A range of molecular data types have been used to conduct the family-wide phylogenetic studies,

such as cpDNA restriction site variation (e.g., Wilson *et al*., 1990; Uhl *et al*., 1995), coding and noncoding sequence data of cpDNA (e.g., Baker *et al*., 1999; Asmussen *et al*., 2000; Asmussen and Chase, 2001; Hahn, 2002b), and nrDNA sequence data (e.g., Baker *et al.*, 2000b; Lewis and Doyle, 2001; Hahn, 2002a). Nevertheless, molecular phylogenetic studies of the Arecaceae using conventional markers yielded insufficient informative sites owing to the slow rate of molecular evolution. Studies conducted by Wilson *et al*. (1990) indicated that there was a potential 5–13-fold decrease in substitution rates within the palms compared to the rates estimated for annual plants. Furthermore, the commonly used cpDNA genes for constructing plant phylogenies, e.g., *atp*B, *rbc*L, *rps*16 intron and *trn*L-*trn*F, are highly conserved in palms, leading to low average nodal support and restricted resolution within phylogenies (e.g., Asmussen, 1999; Baker *et al*., 1999; Asmussen and Chase, 2001; Hahn, 2002b). Regardless of the slow rate of evolution, these commonly used markers have contributed much in understanding the relationships of tribe to subfamily.

4.1.2 *Relationship between* Johannesteijsmannia *and other Coryphoid Palms*

The subfamily Coryphoideae is a diverse group of subtropical and pantropical palms, consisting of 40 genera in three tribes and six subtribes. Almost all of Coryphoideae members are palmate- or costapalmate-leaved palms with induplicately folded leaves (Uhl and Dransfield, 1987; 1999). There are minor differences in leaf form within the subfamily: 1) *Johannesteijsmannia* and a few *Licuala* species exhibiting entire leaves (undivided lamina form); 2) Only one pinnate-leaved genus, *Phoenix*, was placed into this subfamily, whose lamina is divided into induplicately-folded segments; 3) Only *Guihaia* spp. possess palmate and reduplicate leaves; and 4)

Licuala, *Rhapidophyllum* and *Rhapis* possess laminas with anomalous splitting superimposed on a basic induplicate structure.

For most of the phylogenetic analyses of the palm family based on DNA sequences, the subfamily Coryphoideae was deduced to be polyphyletic (e.g., Baker *et al*., 1999; Asmussen *et al*., 2000; Asmussen & Chase, 2001; Hahn, 2002a). However, the plastid restriction fragment length polymorphism (RFLP) fingerprinting phylogeny conducted by Uhl *et al*. (1995) resolved the Coryphoideae (including the tribe Caryoteae from the subfamily Arecoideae) as a monophyletic group, despite the fact that taxonomic sampling provided more weightage towards the coryphoids. Studies of Lewis & Doyle (2001), based on DNA sequences of malate synthase (a nuclear gene), and that of Hahn (2002a), based on a combined and reduced data set, resolved the Coryphoideae as monophyletic. It was noteworthy that the sample size was small in both studies. The tribe Caryoteae of the subfamily Arecoideae was grouped together with members of the subfamily Coryphoideae in many data sets, often with close relationships to the subtribe Coryphinae or the tribe Borasseae (Uhl *et al*., 1995; Asmussen *et al*., 2000; Asmussen & Chase, 2001; Hahn, 2002a).

The subtribe Livistoninae and the Old World members of subtribe Thrinacinae together with the New World *Rhapidophyllum* were resolved as monophyletic by the plastid DNA restriction site analysis of Uhl *et al*. (1995) but were unresolved in the study of *trn*L-*trn*F chloroplast DNA sequences of Baker *et al*. (1999). In the study of Asmussen *et al*. (2000) using the *rps*16 intron and *trn*L-*trn*F plastid DNA sequences, subtribes Livistoninae and *Rhapidophyllum* were resolved as a monophyletic group and highly supported in the analysis of successive weighted characters but were unresolved in the analysis of equally weighted characters. Hence more data are required to confirm the monophyly of the Livistoninae.

Together with 11 other genera, *Johannesteijsmannia* was placed within the subtribe Livistoninae and tribe Corypheae in the classification scheme of Uhl and Dransfield (1987; Chapter 1, Figure 1.2) mentioned above. Phylogenetic studies of the Arecaceae showed that *Licuala* is a sister group to *Johannesteijsmannia* (e.g., Uhl *et al*., 1995; Baker *et al*., 1999; Asmussen *et al*., 2000). The phylogeny of the Arecaceae based on a chloroplast DNA restriction site by Uhl *et al*. (1995), chloroplast DNA sequences from *trn*L-*trn*F region by Baker *et al*. (1999) and combined *rps*16 intron and *trn*L-*trn*F plastid DNA sequences by Asmussen *et al*. (2000) resolved *Licuala* and *Johannesteijsmannia* to belong to a well-supported monophyletic clade.

4.1.3 *Low Copy Number Nuclear Genes*

Systematists working on palms started exploring other categories of molecular markers owing to the restricted variation found in plastid DNA. More rapidly evolving non-coding regions of highly repeated nuclear ribosomal DNA cistrons, e.g., the internal transcribed spacer (ITS) (Baldwin *et al*., 1995) and 5S spacer regions (Sastri *et al*., 1992) have been frequently used for phylogenetic studies in plants. The use of the non-transcribed spacer of the 5S nrDNA in *Phoenix* was reported to be informative but highly conserved (Barrow, 1999). In another study of *Calamus* and related rattan genera palms, adequate phylogenetically informative variation was detected using 5S spacer sequences, but the study also identified moderate levels of intragenome polymorphism, implicating incomplete concerted evolution to homogenize the multiple copies of the 5S nrDNA in the nuclear genome (Baker *et al*., 2000b). The phylogenetic study of the Calamoideae palms using the ITS region of the 18S–26S nrDNA also showed high levels of within-individual polymorphism (Baker *et al*., 2000a). Thus, sufficient genome sampling is required to isolate different copy

types to produce robust species trees. Despite the fact that the two nrDNA regions yielded well-resolved topologies, they possessed limited value in palm phylogenetic studies owing to the lack of homogeneity among repeats within individual genomes (Baker *et al*., 2000a; 2000b).

The problems encountered with paralogous sequences found in the two nrDNA urged palm systematists to explore other nuclear genes that do not belong to large multigene families. There were several reviews on the utility of low-copy nuclear genes in plant molecular phylogenetics (e.g., Sang, 2002; Mort and Crawford, 2004; Small *et al*., 2004). The advantages of low-copy nuclear genes included an elevated rate of sequence evolution compared to organeller genome, presence of multiple unlinked loci that may be useful for independent phylogenetic inference, and explicitly biparental inheritance, thus making them ideal for identifying parental donors of suspected hybrids or polyploids. In contrast, the phylogenetic utility of low-copy nuclear genes has been confounded by the following challenges: 1) Difficulties in identifying and isolating orthologous loci owing to the more complex evolutionary dynamics of the nuclear genome; 2) The possibilities of concerted evolution and recombination among paralogous sequences; and 3) The presence of intraspecific, intrapopulational and intraindividual polymorphism (heterozygosity). Despite these limitations, low-copy nuclear genes have been targeted as potential molecular markers to study plant phylogeny (Sang, 2002; Small *et al*., 2004).

In the effort to search for suitable low-copy nuclear gene candidates, three low-copy number nuclear regions have been employed to study palm phylogeny, viz., genes encoding for malate synthase (*MS*, a glyoxylate cycle enzyme) exons 2–3 (Lewis and Doyle, 2001), phosphoribulokinase (*PRK*, a Calvin cycle enzyme) intron 4 (Lewis and Doyle, 2002) and the second largest subunit of RNA polymerase II

(*RPB*2) intron 23 (Roncal *et al*., 2005). None of these genes has been reported to exist in multiple copies within plant genomes. Additionally, the angiosperm sequence data are available for both *MS* and *PRK* and hence eased primer design (Lewis and Doyle, 2001; 2002). Among the three low-copy nuclear genes, *MS* was found to be the least variable and only providing about half of the parsimony informative sites compared to *PRK* when the same taxon sample was studied (Baker *et al*., unpublished). However, in a family-level study, *MS* was shown to be useful to reconstruct relationships among some of the major lineages of palms (Lewis and Doyle, 2001).

4.1.4 *PRK*

Phosphoribulokinase is a Calvin cycle enzyme, unique to the photosynthetic carbon reduction cycle and catalyzes the irreversible ATP-dependent synthesis of ribulose-1, 5-bisphosphate (Lloyd *et al*. 1991). Lewis and Martinez (2000) first published the use of *PRK* to study palm phylogeny. This study was conducted on five species of the genus *Hyophorbe*, revealed that one of the two paralogues of the *PRK* region was variable and informative at the species-level. The same *PRK* paralogue was then employed to a more comprehensive study — the phylogeny of the largest tribe of palms, the Areceae, of which *PRK* provided sufficient resolution to infer tribal- and generic-level relationships (Lewis and Doyle, 2002). Subsequently, this region has been targeted to study the phylogeny of palms (e.g., Gunn, 2004; Roncal *et al*., 2005; Thomas *et al*., 2006; Loo *et al*., 2006).

Lewis and Doyle (2002) designed the degenerate *PRK* primers (*PRK*488f and *PRK*1167r; Figure 4.1) from the cDNA sequences of flowering plants (e.g., *Arabidopsis thaliana*, *Pisum sativum* and *Triticum aestivum*) available in GenBank. The specific primers (*PRK*717f and *PRK*969r; Figure 4.1) were designed against a set

of palm sequences obtained from the degenerate primers. Two paralogues of *PRK* were identified, the larger size copy corresponding to about 1,300 bp (paralogue 1) and the smaller size copy corresponding to about 700 bp (paralogue 2), from exons 4 and 5 flanking the fourth intron (Figure 4.1).

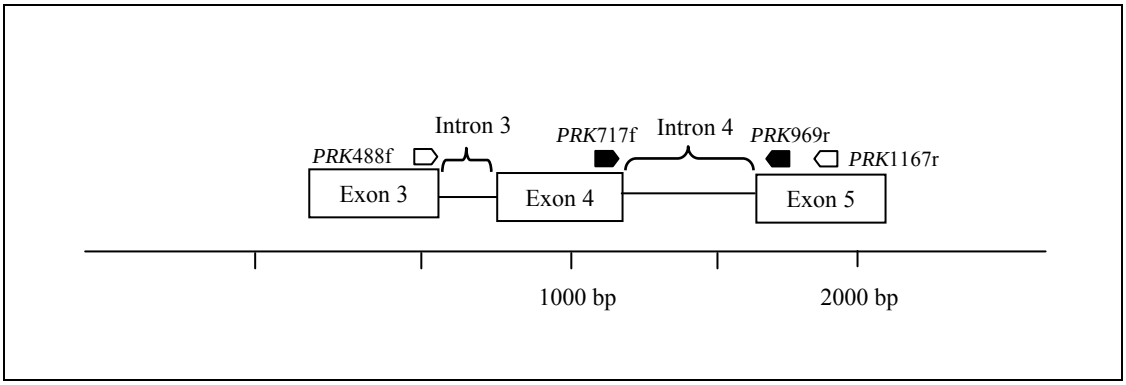

Figure 4.1. A schematic diagram of *PRK* paralogues 1 and 2, based on sequences from *Hyophorbe lagenicaulis*. The degenerate primers are *PRK*488f and *PRK*1167r (paralogue 1) and primers specific to palms are *PRK* 717f and *PRK*969r (paralogue 2) (adapted from Lewis and Doyle, 2002).

Gun (2004) conducted a study of the tribe Cocoeae using *PRK* primers for paralogue 2 designed by Lewis and Doyle (2002). The study found between three to five similar copies of paralog 2 from nearly every accession but all clones from the same species resolved as being monophyletic in all phylogenetic analyses. Roncal *et al*. (2005) and Loo *et al*. (2006) used the same primers to study the relationships within the tribe Geonomeae and subtribe Arecinae, respectively. In both studies, no additional paralogs were detected and paralog 2 was informative enough to resolve the relationships for the above two studies. However, the study conducted by Thomas *et al*. (2006) on the palm genus *Chamaedorea* found a new paralogue and named it paralogue 3 with respect to paralogues 1 and 2 previously identified by Lewis and Doyle (2002). Paralogue 3 was observed in five *Chamaedorea* species, and despite

having a similar length with the target copy of *PRK*, it was easily distinguished from the latter in sequence alignment and possessed a larger uncorrected pairwise distance with any other taxon compared to the target copy.

Despite the possibility of additional paralogues, *PRK* was shown to be useful for examining the relationships at lower taxonomic levels (e.g., Gunn, 2004; Roncal *et al*., 2005; Thomas *et al*., 2006; Loo *et al*., 2006). Hence, the use of *PRK* in phylogenetic studies must be done with care so that orthologues can be accurately identified. To date, the use of *PRK* for molecular phylogenetic studies in other plant families is unknown.

4.1.5 *RPB2*

*RPB*2 encodes the second largest subunit of RNA polymerase II, which is responsible for catalysing messenger RNA synthesis in eukaryotic cell nuclei. This gene is ubiquitous and consists of motifs that are conserved across different kingdoms of life. Hence, it has been shown to be useful in evolutionary studies at many taxonomic levels (Allison *et al*., 1985; Iwabe *et al*., 1991). The utility of this gene in phylogenetic studies has been explored in angiosperms and other seed plants (e.g., Denton *et al*., 1998; Oxelman and Bremer, 2000; Nickerson and Drouin, 2004; Oxelman *et al*., 2004; Pfeil *et al*., 2004). These studies found that *RPB*2 were phylogenetically informative notwithstanding the paralogy that was discovered in some of these studies. For instance, two different copies were found in the angiosperm order Gentianales, of which one of the copies lacks an intron and another possessing an intron at locations corresponding to those in the green plants that were previously investigated (Oxelman and Bremer, 2000). Thereafter, more than one *RPB*2 sequence was reported in two asterid groups (Oxelman *et al*., 2004). The study

conducted by Pfeil *et al*. (2004) also identified two copies of *RPB*2 in the family Malvaceae and its relatives. The two paralogues demonstrated a congruent phylogenetic pattern and were largely in agreement with cpDNA topologies.

Roncal *et al*. (2005) developed palm-specific primers for *RPB*2 intron 23 to study the phylogeny of the Neotropical palm tribe Geonomeae. No *RPB*2 paralogues were identified for the tribe Geonomeae and the data added support to the previously reported monophyly of the tribe. Thereafter, the same primers were employed to investigate the relationships in *Chamaedorea* (Thomas *et al*., 2006) and subtribe Arecinae (Loo *et al*., 2006). No paralogues were identified in both studies and *RPB*2 was informative enough to study the relationship at lower taxonomic levels. Despite the fact that no paralogues were identified in the above studies the possibility of the occurrence of multiple copies may yet to be detected in the Arecaceae. Hence, the use of low-copy nuclear genes must be done with great caution to avoid incorrect phylogenetic estimation.

4.1.6 *Objectives*

In Chapter 3, AFLP fingerprinting was shown to be useful to study the systematics of *Johannesteijsmannia*. However, AFLP fingerprinting was interpreted phenetically, where taxa are grouped by overall similarity as opposed to phylogenetically, where taxa are grouped by shared derived features (synapomorphies) (Radford, 1986). In respect to the AFLP-derived profiles, banding similarities were based on distance measures, for which bands sharing identical sizes were assumed to be homologous. On the other hand, DNA sequences of a homologue among individuals were used to infer phylogenetic relationships of a group of taxa when cladistic methods are applied. Despite the fact that AFLP markers are highly

homologous (Rouppe Van Der Voort *et al*., 1997), the use of DNA sequencing to infer the phylogenetic relationships of *Johannesteijsmannia* would complement the AFLP results as DNA sequencing is based on homologous characters. Also, another advantage of DNA sequencing compared to AFLP fingerprinting is the ease of data visualization. In AFLP fingerprinting, a reference ladder must be run simultaneously with the OTUs in the polyacrylamide gel for accurate comparison. This requirement is not needed in DNA sequencing. The data can be stored as base sequences and extracted anytime for alignment and comparison with the sequences of other OTUs.

Hence, the main aim of this chapter was to conduct a phylogeny of *Johannesteijsmannia* using two low-copy nuclear genes (*PRK* and *RPB*2). This study also attempted to examine the relationship of *Johannesteijsmannia* with its sister group, *Licuala* and some selected taxa from its subfamily, the Coryphoideae, with a view to answer the following questions: 1) Is *Johannesteijsmannia* monophyletic?; 2) Is *Licuala* a sister group to *Johannesteijsmannia*?; 3) Are the low-copy nuclear genes *PRK* and *RPB*2 useful to study the species-level relationships in the genus *Johannesteijsmannia*?

4.2 Materials and Methods

4.2.1 *Taxon Sampling*

Thirty-two taxa were sampled, representing 16 species from nine genera within two subtribes and three tribes of the subfamily Coryphoideae. The ingroup included the four *Johannesteijsmannia* species collected from different populations, one cultivated *Jt. altifrons* and five *Licuala* species. The outgroup members were *Chaemaerops humilis*, *Kerriodoxa elegans*, *Phoenix reclinata Rhapidophyllum hystrix*, *Sabal bermudana*, *Serenoa repens* and *Trachycarpus fortunei*. The outgroup

members were selected based on their relationships resolved by Asmussen and Chase (2001) and the availability of materials from the DNA Bank at the Royal Botanic Gardens, Kew, UK. The first four taxa belong to the main clade where *Licuala* fits and the last three fall outside the clade and hence were chosen to root the tree.

4.2.2 *DNA Sources*

Genomic DNA of all *Johannesteijsmannia* species was obtained from the field collection in the present study except for the cultivated *Jt. altifrons* individual. Genomic DNA of the cultivated *Jt. altifrons*, *Chaemaerops humilis*, *Kerriodoxa elegans*, *Licuala grandiflora*, *Licuala kunstleri*, *Licuala lauterbachii*, *Licuala tanycola*, *Licuala telifera*, *Phoenix reclinata*, *Rhapidophyllum hystrix*, *Sabal bermudana*, *Serenoa repens* and *Trachycarpus fortunei* was obtained from the DNA Bank at the RBG, Kew. The purposes of obtaining genomic DNA from the DNA Bank at the RBG, Kew were that the taxa used were correctly identified and the same DNAs used could be compared with other published data using the same DNA samples.

4.2.3 *DNA Extraction Protocol*

The DNA extraction protocol was carried out as described in Chapter 3, section 3.2.2.

Table 4.1. Taxonomic sampling based on Asmussen and Chase (2001) and voucher information. K: Herbarium, Royal Botanical Gardens, Kew; SINU: Herbarium, Raffles Museum of Biodiversity Research, Department of Biological Sciences, National University of Singapore.

Tribe: Subtribe (Asmussesn and Chase, 2001)	**Species**	**Taxa Code (Population Code)**	**Voucher/ Herbarium**	**DNA Source**
Ingroup				
Corypheae: Livistoninae	*Johannesteijsmannia altifrons* (Reichb.*f. et* Zoll.) H.E. Moore	*J. altifrons* 1 (GJA)	Look 084/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia altifrons* (Reichb.*f. et* Zoll.) H.E. Moore	*J. altifrons* 2 (GEE)	Look 104/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia altifrons* (Reichb.*f. et* Zoll.) H.E. Moore	*J. altifrons* 3 (ASLA)	Look 135/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia altifrons* (Reichb.*f. et* Zoll.) H.E. Moore	*J. altifrons* 4 (BAK)	Look 161/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia altifrons* (Reichb.*f. et* Zoll.) H.E. Moore	*J. altifrons* 5 (BPA)	Look 170/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia altifrons* (Reichb.*f. et* Zoll.) H.E. Moore	*J. altifrons* 6 (SDU)	Look 179/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia altifrons* (Reichb.*f. et* Zoll.) H.E. Moore	*J. altifrons* 7 (SNI)	Look 414/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia altifrons* (Reichb.*f. et* Zoll.) H.E. Moore	*J. altifrons* 8 (LUM)	Look 429/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia altifrons* (Reichb.*f. et* Zoll.) H.E. Moore	*J. altifrons* 9 (LAH)	Look 443/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia altifrons* (Reichb.*f. et* Zoll.) H.E. Moore	*J. altifrons* Cult.	Kew 1985–515/ K	DNA Bank, Kew
Corypheae: Livistoninae	*Johannesteijsmannia lanceolata* J.Dransfield	*J. lanceolata* 1 (LSLA)	Look 020/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia lanceolata* J.Dransfield	*J. lanceolata* 2 (ANG)	Look 062/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia lanceolata* J.Dransfield	*J. lanceolata* 3 (JBR)	Look 405/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia lanceolata* J.Dransfield	*J. lanceolata* 4 (RAU)	Look 436/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia magnifica* J.Dransfield	*J. magnifica* 1 (BUN)	Look 070/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia magnifica* J.Dransfield	*J. magnifica* 2 (GAT)	Look 130/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia magnifica* J.Dransfield	*J. magnifica* 3 (KIN)	Look 421/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia perakensis* J.Dransfield	*J. perakensis* 1 (KSA)	Look 035/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia perakensis* J.Dransfield	*J. perakensis* 2 (BUB)	Look 040/ SINU	Own collection
Corypheae: Livistoninae	*Johannesteijsmannia perakensis* J.Dransfield	*J. perakensis* 3 (BSU)	Look 147/ SINU	Own collection
Corypheae: Livistoninae	*Licuala grandiflora* Ridley	*Licuala grandiflora*	Dransfield 7719/ K	DNA Bank, Kew
Corypheae: Livistoninae	*Licuala kunstleri* Becc.	*Licuala kunstleri*	Chase 2282/ K	DNA Bank, Kew
Corypheae: Livistoninae	*Licuala lauterbachii* Damm. & K.Schum	*Licuala lauterbachii*	Heatuban 187/ K	DNA Bank, Kew
Corypheae: Livistoninae	*Licuala tanycola* H.E. Moore	*Licuala tanycola*	Baker 1139/ K	DNA Bank, Kew
Corypheae: Livistoninae	*Licuala telifera* Becc.	*Licuala telifera*	Baker 1054/ K	DNA Bank, Kew

Table 4.1. Taxonomic sampling based on Asmussen and Chase (2001) and voucher information. K: Herbarium, Royal Botanical Gardens, Kew (continued).

Tribe: Subtribe (Asmussesn and Chase, 2001)	**Species**	**Taxa Code (Population Code)**	**Voucher/Herbarium**	**DNA Source**
Outgroup				
Corypheae: Thrinacinae	*Chamaerops humilis* Linn.	*Chamaerops humilis*	Barrow 76/ K	DNA Bank, Kew
Corypheae: Coryphinae	*Kerriodoxa elegans* J.Dransfield	*Kerriodoxa elegans*	Kew 1987–2685/ K	DNA Bank, Kew
Phoeniceae: Nil	*Phoenix reclinata* Jacq.	*Phoenix reclinata*	Goyder *et al*. 3928/ K	DNA Bank, Kew
Corypheae: Thrinacinae	*Rhapidophyllum hystrix* H.Wendl. & Drude	*Rhapidophyllum hystrix*	Kew 1967–1301/ K	DNA Bank, Kew
Corypheae: Sabalinae	*Sabal bermudana* L.H. Bailey	*Sabal bermudana*	Kew 1982–5602/ K	DNA Bank, Kew
Corypheae: Livistoninae	*Serenoa repens* (Bartram) J.K.Small	*Serenoa repens*	Kew 1958–66102/ K	DNA Bank, Kew
Corypheae: Thrinacinae	*Trachycarpus fortunei* H. Wendl.	*Trachycarpus fortunei*	Dransfield s.n./ K	DNA Bank, Kew

4.2.4 *Polymerase Chain Reaction (PCR) Protocol*

Both primer sequences of *PRK* and *RPB*2 were obtained from published sources. Primers for *PRK* and *RPB*2 were designed by Lewis (Lewis and Doyle, 2002; Roncal *et al*., 2005). The sequences of both primers are as listed in Table 4.2. PCR reactions were performed with a final amplification reaction mixture of 25 µl containing 1× PCR buffer with $(NH_4)_2SO_4$ (Fermentas, www.fermentas.com), 0.4 mM dNTPs, 4 mM $MgCl_2$ (Fermentas), 0.3 µM of each primer, 1.0 U *Taq* DNA polymerase (Fermentas), 10 ng template DNA and autoclaved deionized water. The same PCR reaction mixture was used for both genes. The amplification reactions for *PRK* were performed for one cycle at 94°C for 4 min, followed by 33 cycles of 1 min at 94°C, 1 min for 56°C, 2 min at 72°C and followed by 7 min at 72°C. The amplification reactions for *RPB*2 were performed for one cycle at 94°C for 4 min, followed by 36 cycles of 1 min at 94°C, 1 min for 57°C, 2 min at 72°C and followed by 7 min at 72°C. All the PCR reactions were carried out using the GeneAmp PCR System 9700 (PE Applied Biosystems). The amplified products were fractionated by 1.5% (w/v) agarose gel electrophoresis and visualized under ultraviolet light after staining with ethidium bromide.

Table 4.2. Primers employed in this study.

Primer	**Sequence 5′ → 3′**	**Tm**
PRK717F (Forward)	GTGATATGGAAGAACGTGG	58 °C
PRK969R (Reverse)	ATTCCAGGGTATGAGCAGC	60.2°C
RPB2-INT23F (Forward)	CAACTTATTGAGTGCATCATGG	58.9°C
RPB2-INT23R (Reverse)	CCACGCATCTGATATCCAC	60.2°C

4.2.5 *Gel Extraction and Cloning Protocol*

Bands of the expected size were excised from the gel using a razor blade, and the DNA was recovered with QIAEX®II Gel Extraction System (QIAGEN, Germany). Purified products were then quantified and cloned into pGEM-T vector (Promega, USA) by TA cloning. Ligation was set up following the instructions of the manufacturer's manual using T4 ligase (Promega, USA). After 3 h at room temperature and then overnight ligation at 4ºC, the total ligation mix of 10 µl was used to transform chemically competent *E. coli* DH5α cells by heat-shock transformation.

4.2.6 *Preparation of* Escherichia coli *Competent Cells*

Escherichia coli competent cells were prepared as described by Inoue *et al*. (1990) with some modifications. Frozen stock *E. coli* cells were thawed, streaked on an LB agar plate, and cultured overnight at 37º C. A single colony was inoculated into 1.5 ml SOB medium (Tryptone 20 g/l, yeast extract 5 g/l, NaCl 0.58 g/l, KCl 0.19 g/l, $MgCl_2 \cdot 6H_2O$ 2.03 g/l, $MgSO_4 \cdot 7H_2O$ 2.46 g/l) in a 15 ml culture tube, and grown for 12 h with vigorous shaking (200 rpm) at 37 ºC. 500 µl of the above culture was then inoculated to 100 ml SOB medium in a 1-liter flask, and grown to an A_{600} of 0.6 at about 20ºC with vigorous shaking at 225 rpm. The culture was then transferred to 2 ice-cold Falcon tubes, and placed on ice for 10 min before centrifugation at 3,000 rpm for 5 min at 4 ºC. The pellet was gently resuspended in 20 ml of freshly prepared ice-cold TB (10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7), incubated on ice for 10 min, and centrifuged as above. The cell pellet was gently resuspended in 4 ml of TB, and DMSO was added with gentle swirling to a final concentration of 7%. After incubating

on ice for 10 min, the cell suspension was aliquoted (100µl) into pre-chilled 1.5 ml microfuge tubes, immediately frozen by immersion in liquid nitrogen, and stored at −80ºC.

4.2.7 *Heat-shock Transformation of* Escherichia coli *Competent Cells*

A tube with 100 µl frozen competent cells was thawed by holding in the palm. Just as the cells thaw, the tube was put on ice immediately. The ligation mixture was added to the tube and mixed well with the cells by gently tapping, and the cells were incubated on ice for 30 min. The cells were heat shocked for 90 s at 42ºC in a heat block and transferred to ice, incubated for 3 min. After 1 ml SOB was added, the cells were allowed to recover at 37º C for 1 h with shaking at 200 rpm. The cultured cells were centrifuged in an Eppendorf Centrifuge 5417C at 9,000 rpm for 1 min at room temperature, and 800 µl of SOB medium from the top was discarded. The cells in the rest of the medium were resuspended and spread onto LB ampicilin (100 mg ml^{-1}) plates containing X-gal (30 µg ml^{-1}) and IPTG (30 µg ml^{-1}) using a sterile glass rod. The plates were incubated overnight at 37º C for growth of colonies.

4.2.8 *Screening of Clones*

A sterilized white tip was lightly touched on the surface of the test white colony on the agar medium to collect the bacteria. Five to 10 clones per plate were selected for each taxon. The collected sample was then resuspended in 6 µl of sterile water in a sterile 0.6 ml microfuge tube. Two µl of this suspension was used as a template for the PCR

reaction with SP6 and T7 primers, which are within the pGEM-T vector backbone. The colonies that gave the expected sized band were cultured overnight at 37ºC.

4.2.9 *Isolation and Purification of Recombinant Plasmids*

A 1.5 ml overnight culture was aliquoted into a sterile 2 ml microcentrifuge tube and an equal volume of 50% glycerol was added and the solution was gently mixed before storage at −80ºC. The remaining culture was used for plasmid isolation. Plasmid DNA was isolated using the Miniprep kit (Promega, USA) according to the manufacturer's instructions.

4.2.10 *DNA Sequencing Protocol*

The cloned fragment for both strands was sequenced by the dideoxy method (Sanger *et al.*, 1977) using the ABI PRISM BigDye^{TM} Terminator Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems, USA). The cycle sequencing reaction was prepared with a mixture of 200 ng plasmid DNA, 1.6 pmol appropriate primers, 4 µl Terminator Ready Reaction Mix mixed with deionized water to a reach a final volume of 10 µl. Then the PCR was performed using 25 cycles of denaturation at 96º C for 10 s, annealing at 50ºC for 5 s and extension at 60ºC for 4 min. The amplified products were precipitated for 15 min in sodium acetate-ethanol mixture (1.0 µl 3 M sodium acetate, pH 4.6 and 25 µl 95% ethanol) at room temperature and centrifuged for 15 min at 14,000 rpm. The pellet was rinsed with 250 µl 70% ethanol, vortexed briefly and centrifuged for 5 min at 14,000 rpm. The supernatant was discarded and the pellet was air dried. Prior to sequencing, the pellet was dissolved in 12 µl Hi-diformamide (Applied Biosystems),

heat-denatured for 2 min at 95ºC and cooled on ice. Sequencing was performed using an ABI PRISM^{TM} 3100 DNA sequencer (Perkin-Elmer Applied Biosystems, USA). DNA sequences obtained were checked for homology to other palm sequences at the nucleotide level using the web-based programme BLAST (Basic Local Alignment Search Tool) at the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/BLAST/).

4.2.11 *Sequence Alignment*

Editing and assembling of individual sequences was conducted using ContigExpress in Vector NTI Suite 8 (http://trial.informaxinc.com/vntitrial/vntitrial.cgi). All clones of each taxon for both genes, forward and reverse sequences, had overlaps of at least 70%. The sequences were then entered into MegAlign (Lasergene, DNA Star) and the final alignments were refined by eye. Within *Johannesteijsmannia*, both intron regions are highly conserved in length, showing only a few short indels. Despite the presence of poly-AG homopolymer regions in *PRK* and poly-AT homopolymer regions in *RPB*2, cloning yielded clean sequences and hence the alignment was relatively easy. The conserved regions permitted confident alignment of all species for both genes.

4.2.12 *Cladistic Analysis*

Cladistic analyses were conducted using the maximum-parsimony (MP) optimality criterion as implemented in PAUP* version 4b10 (Swofford, 2002). The two DNA regions were first analyzed separately and then combined for a simultaneous analysis of all available sequence data. Characters were treated as unordered, unweighted

(Fitch parsimony; Fitch, 1971) and only parsimony informative characters were included in all searches and analyses. Initially, the analysis employed 100 heuristic searches, each with starting trees obtained by random taxon addition, tree-bisection-reconnection (TBR) swapping and retaining multiple trees per step (MULTREES on). These heuristic searches resulted in a pool of trees owing to the presence of numerous zero-length branches. Hence, the heuristic search could not be completed. An alternative method was carried out to minimise the time spent on sub-optimal islands but gather trees from different parts of the tree space (Maddison, 1991). One thousand consecutive heuristic searches were employed using the settings as described earlier, but no more than 10 trees were saved in each search. The trees saved in these searches were then used as starting trees in a subsequent search. A maximum of 20,000 trees were saved and swapped to completion using TBR. One thousand bootstrap replicates were employed to determine the internal support for the clades. Each replicate was subject to one heuristic search with TBR swapping and saving 10 trees per replicate to save time in swapping on islands with a large number of trees. Salamin *et al*. (2003) evaluated this strategy and they found this strategy to be reliable. Groups that were found in strict consensus tree and 50% or more of the replicates were recorded.

The combined analysis was conducted by randomly choosing one representative clone for each species. Branch and bound search (a more exhaustive technique) was conducted instead of doing a heuristic search owing to the smaller number of species and a more exhaustive search could be employed. The bootstrap support for the combined analysis was as described above.

4.3 Results and Discussion

4.3.1 *Description of the Genes PRK and RPB2*

Amplified products for *PRK* and *RPB2* were consistent, viz., approximately 700 bp and 900 bp, respectively across the subfamily. The amplification of *Kerriodoxa elegans* for both genes was unsuccessful and this taxon was omitted in the present study. Occasionally, more than one band was seen on the agarose gel and the band size was slightly smaller than the targeted fragment. The extra bands were excised, purified, sequenced and checked by a BLAST search for the presence of paralogues. None of the extra bands checked corresponded to the targeted gene when BLAST search was performed.

Even though direct sequencing of PCR products was straightforward, the electropherograms showed much noise and unreadable results. Hence cloning was conducted for all species in the present study to obtain clean and reliable sequences. Cloning yielded clean electropherograms for both genes despite the presence of the AG homopolymer in *PRK* and AT homopolymer in *RPB*2. The numbers of clones ranged from two to 10 for each taxon and were included in the analysis to verify if they are paralogues of the targeted copy. The name of the clones for each *Johannesteijsmannia* accession in present study was given in the number after the taxon code. For instance, *Jt. altifrons* 1.3 represents *Jt. altifrons* individual one (collected from a given population) and clone number three. For *Licuala* species and other outgroup members, the name of the clone is straightforward, e.g. *Licuala kunstleri* 1 representing *Licuala kunstleri* clone number one. In total, 280 sequences in the *PRK* dataset and 246 sequences in the *RPB*2

dataset representing 15 taxa and eight genera. The combined data set was composed of 15 taxa in which only one clone was randomly chosen to represent a given taxon.

4.3.2 *PRK Analysis*

The final matrix of *PRK* sequences consisted of 712 characters, of which 193 were potentially parsimony informative. The pairwise Juke-Cantor distance (excluding uninformative characters) for the whole data set ranged from 0 to 0.0283 (mean = 0.0063, SD = 0.0056). The parsimony analysis yielded 20,000 equally parsimonious trees with a length of 354 steps, CI = 0.61 and RI = 0.95.

The *PRK* strict consensus tree (Figure 4.2A) resolved *Johannesteijsmannia* as a monophyletic clade with high bootstrap support, 99%. Nevertheless, clones of *Johannesteijsmannia* species did not resolve, but nested with clones of other species. It should be highlighted that none of the clones from the same accession resolved as being monophyletic. Sequences of *Johannesteijsmannia* exhibited high allelic polymorphism and provided no resolution of the species clade in the tree. *PRK* data showed that *Licuala* is a sister group to *Johannesteijsmannia* (94% bootstrap support) adding support to the studies of Uhl *et al*. (1995), Baker *et al*. (1999) and Asmussen *et al*. (2000) (Figure 4.2B). This genus also formed a monophyletic clade with high bootstrap support (97%). Also, all clones of *Licuala* species were resolved within their own species (except for those of *Licuala lauterbachii*) with moderate to high bootstrap support (ranged from 64% to 98%). Clones of the remaining taxa (*Chaemaerops humilis*, *Phoenix reclinata*, *Rhapidophyllum hystrix*, *Sabal bermudana*, *Serenoa repens* and *Trachycarpus fortunei*) also resolved as being monophyletic for each species (Figure 4.2B). *Serenoa repens* is a

sister group to the *Johannesteijsmmania* and *Licuala* clade, and these three species formed a subtribe Livistoninae clade with high bootstrap support (98%). *Rhapidophyllum hystrix* is a sister group to *Chaemaerops humilis* but with low bootstrap support (55%). Nevertheless, together with *Trachycarpus fortunei*, these members formed a subtribe Thrinacinae clade with high bootstrap support (86%). *Phoenix reclinata* and *Sabal bermudana* were used to root the tree because they are in a different clade compared to the other taxa according to Asmussen and Uhl (2001) and hence were chosen as outgroup members. Results from the *PRK* data set yielded a congruent topology with the study of Asmussen and Chase (2001).

4.3.3 *RPB2 Analysis*

The final matrix of the *RPB*2 sequences consisted of 991 characters, of which 222 were potentially parsimony informative. The pairwise Juke-Cantor distance excluding uninformative characters for the whole data set ranged from 0.000 to 0.0185 (mean = 0.0045, SD = 0.0037). The parsimony analysis yielded 20,000 shortest trees with a length of 542 steps, CI = 0.83 and RI = 0.97.

The *RPB*2 strict consensus tree (Figures 4.3, 4.3A and 4.3B) was in great agreement with that of *PRK*. *Johannesteijsmannia* was resolved as being monophyletic with 100% bootstrap support (Figure 4.3A). All clones from each *Johannesteijsmannia* accession did not resolve except for the cultivated *Jt. altifrons* accession and *Jt. magnifica* accession 2 (bootstrap support = 60% and 62%, respectively). High allelic polymorphism was also observed in the genus when *RPB*2 was employed, suggesting a recent duplication event had occurred in this genus. *Licuala* did not resolve as being

monophyletic when *RPB2* sequences were employed (Figure 4.3B). However, all clones of *Licuala* were resolved within their own species except for *Licuala telifera*. It should be highlighted that *Licuala lauterbachii* was resolved in the *RPB*2 data set (83% bootstrap support) compared to that of *PRK*. Clones of other outgroup species were resolved within the species with 100% bootstrap support. *Serenoa repens* was closer to the unresolved *Licuala* clade in this data set. *Rhapidophyllum hystrix* is a sister group to *Chaemaerops humilis*, in congruence with the results from the *PRK* data analysis but with higher bootstrap support (73%). Again, together with *Trachycarpus fortunei*, these members formed a subtribe Thrinacinae clade with 91% bootstrap support. In general, the *RPB*2 data set provided similar resolution of the taxa but was less informative than the *PRK* data set.

4.3.4 *Combined Analysis*

Combined analysis of both genes was based on one clone from each taxon. Hence, a limited number of taxa were included in this analysis. When *PRK* and *RPB*2 data sets were combined, the final matrix consisted of 1703 characters, of which 99 were potentially parsimony informative. The parsimony analysis yielded only one tree with a length of 141 steps, CI = 0.76 and RI = 0.87. The strict consensus tree (Figures 4.4) was well resolved with high bootstrap support. *Johannesteijsmannia* was resolved as being monophyletic with a high bootstrap support (100%). The four *Johannesteijsmannia* species were resolved with *Jt. altifrons* as a sister to *Jt. perakensis* (bootstrap support = 94%) and *Jt. lanceolata* as a sister to *Jt. magnifica* (bootstrap support = 71%). This result

is congruent with the AFLP fingerprinting results, suggesting that *Jt. perakensis* may be a subspecies of *Jt. altifrons*.

The strict consensus tree generated with combined data set analysis is in congruence with the *PRK* data set. Again, *Licuala* resolved as monophyletic group. *Licuala* is a sister group to *Johannesteijsmannia* (62% bootstrap support), *Serenoa repens* is sister to the *Johannesteijsmannia* and *Licuala* clade (100% bootstrap support), supported the placement of these taxa in subtribe Livistoninae. *Chamaerops humilis* is sister to *Rhapilophyllum hystrix* with 80% bootstrap support and formed a subtribe Thrinacinae clade (99% bootstrap support) with *Trachycarpus fortunei*.

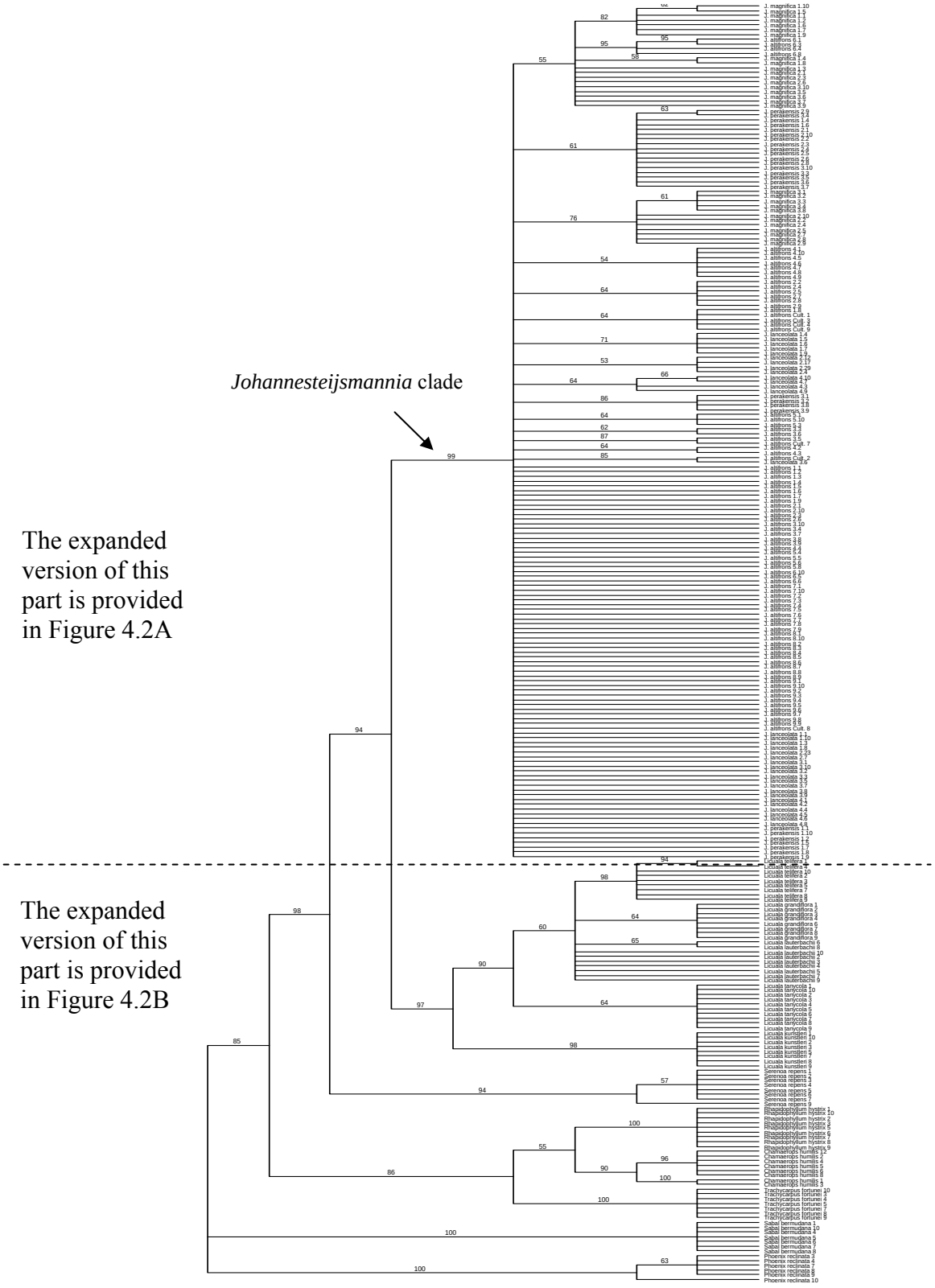

Figure 4.2. Strict consensus tree of 20,000 equally most parsimonious trees from analysis of the *PRK* data set. Numbers above the branches indicate the bootstrap percentages. Dash lines showing the expanded versions of this tree are provided in Figures 4.2A to B.

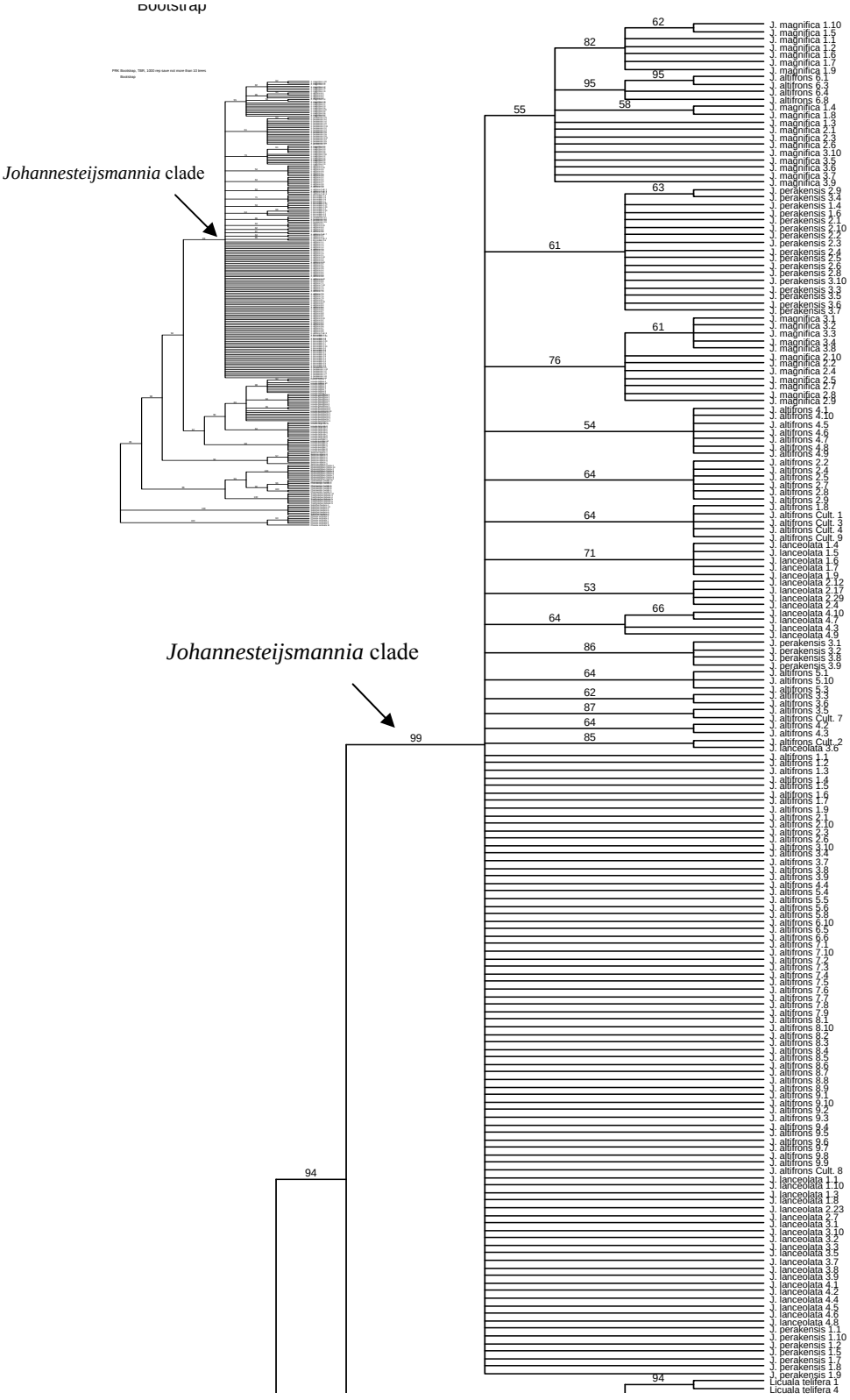

Figure 4.2A. Strict consensus tree of 20,000 equally most parsimonious trees from analysis of the *PRK* data set. Numbers above the branches indicate the bootstrap percentages.

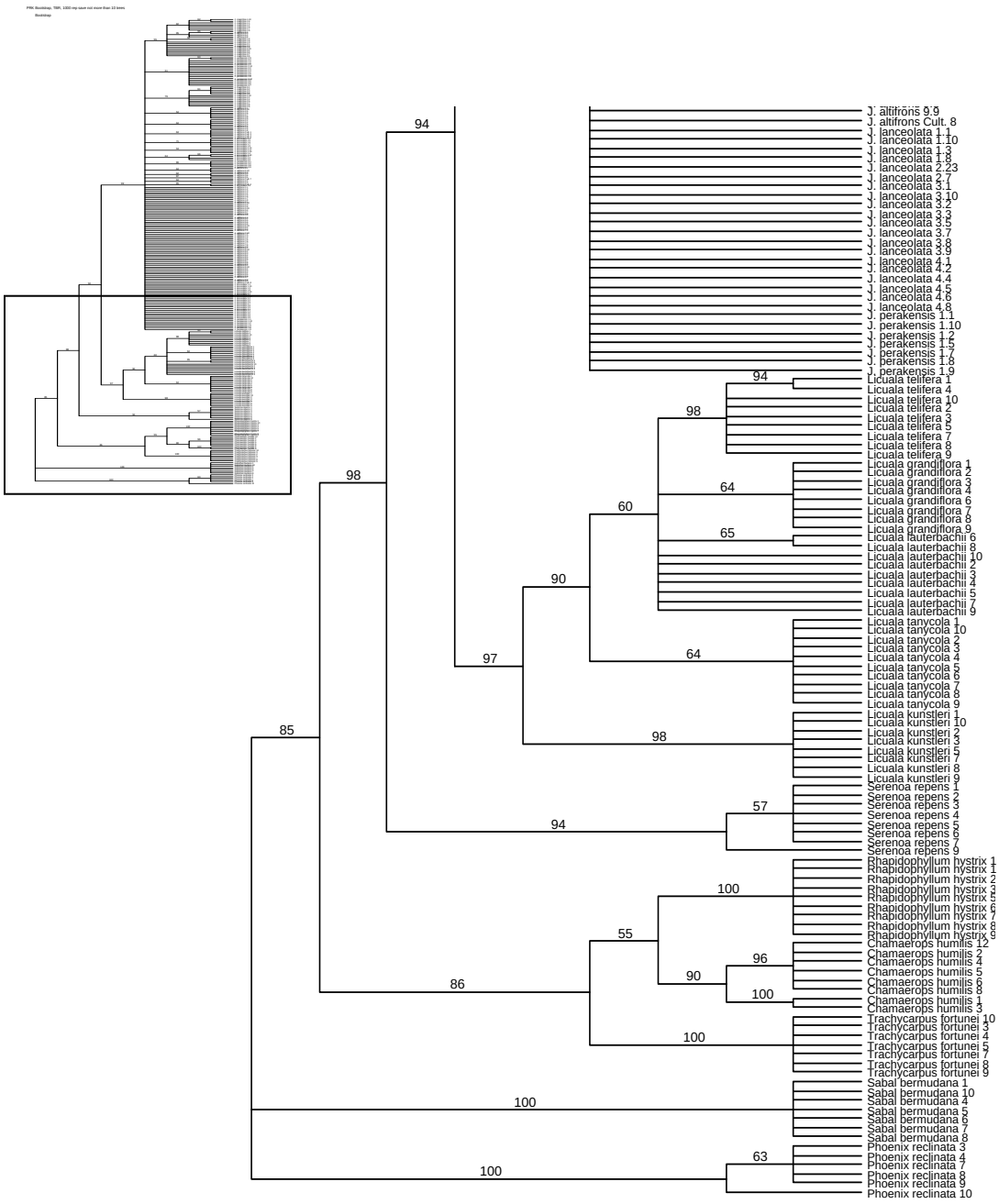

Figure 4.2B. Strict consensus tree of 20,000 equally most parsimonious trees from analysis of the *PRK* data set. Numbers above the branches indicate the bootstrap percentages.

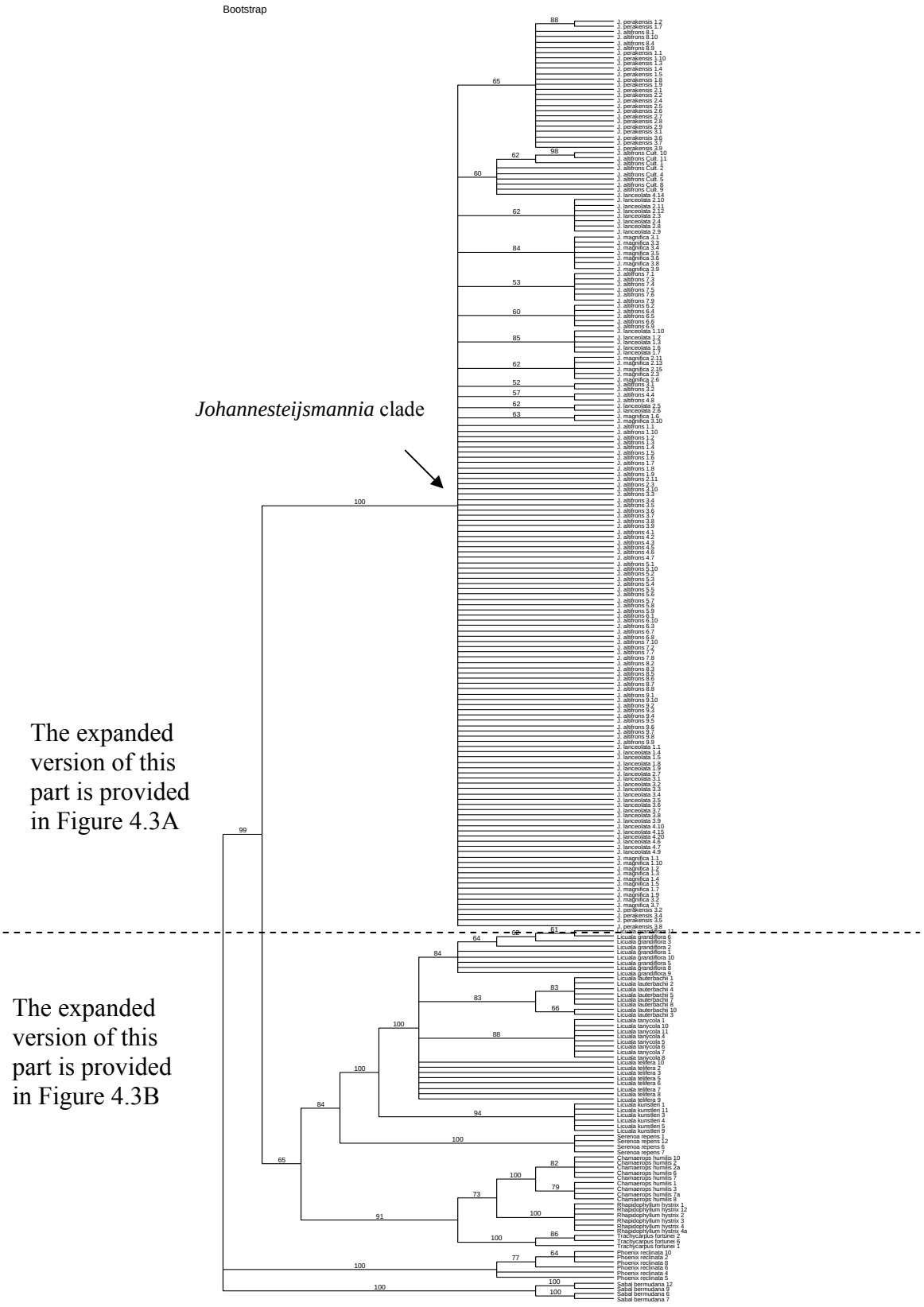

Figure 4.3. Strict consensus tree of 20,000 equally most parsimonious trees from analysis of the *RPB*2 data set. Numbers above the branches indicate the bootstrap percentages. Dash lines showing the expanded versions of this tree are given in Figures.4.3A to B.

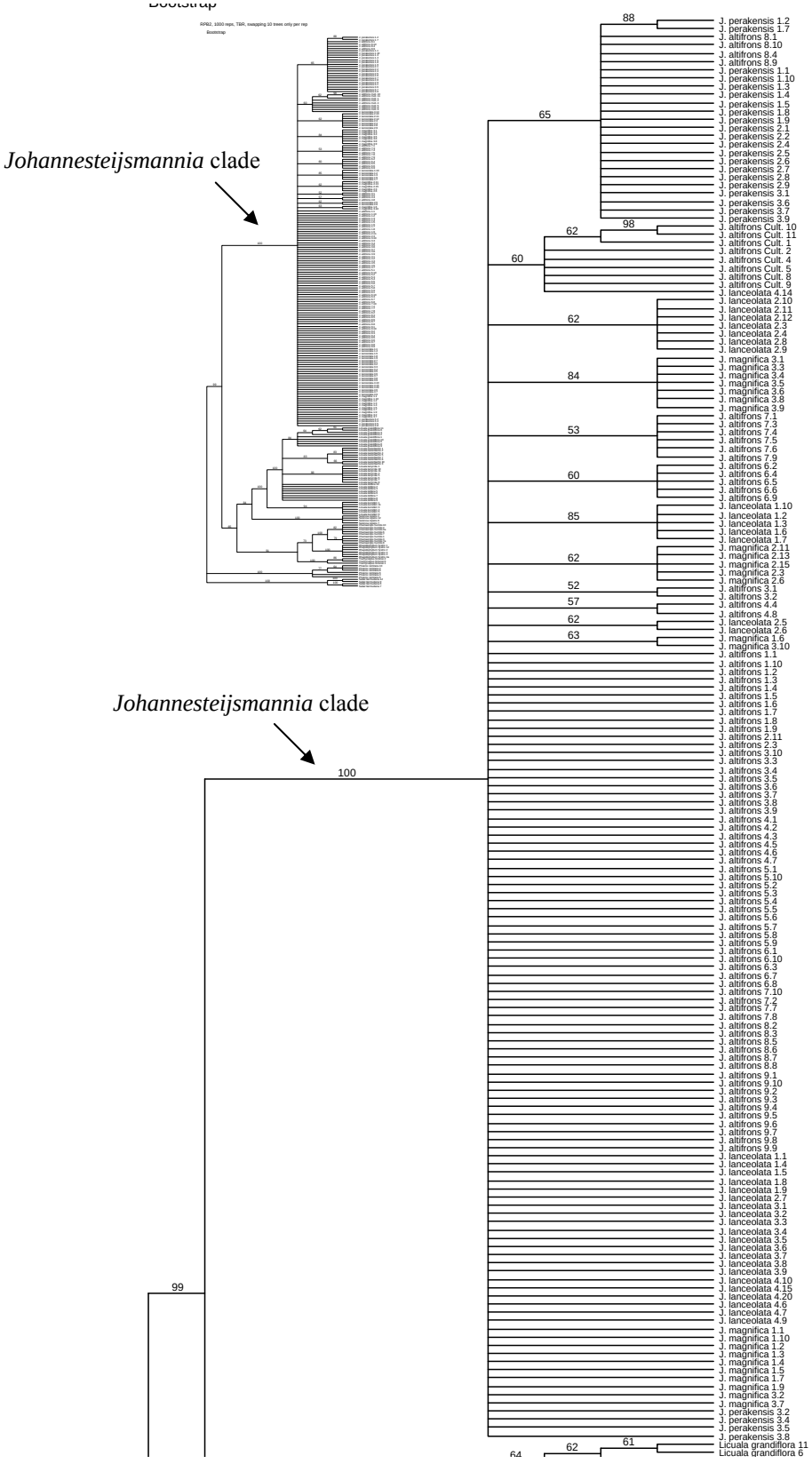

Figure 4.3A. Strict consensus tree of 20,000 equally most parsimonious trees from analysis of the *RPB*2 data set. Numbers above the branches indicate the bootstrap percentages.

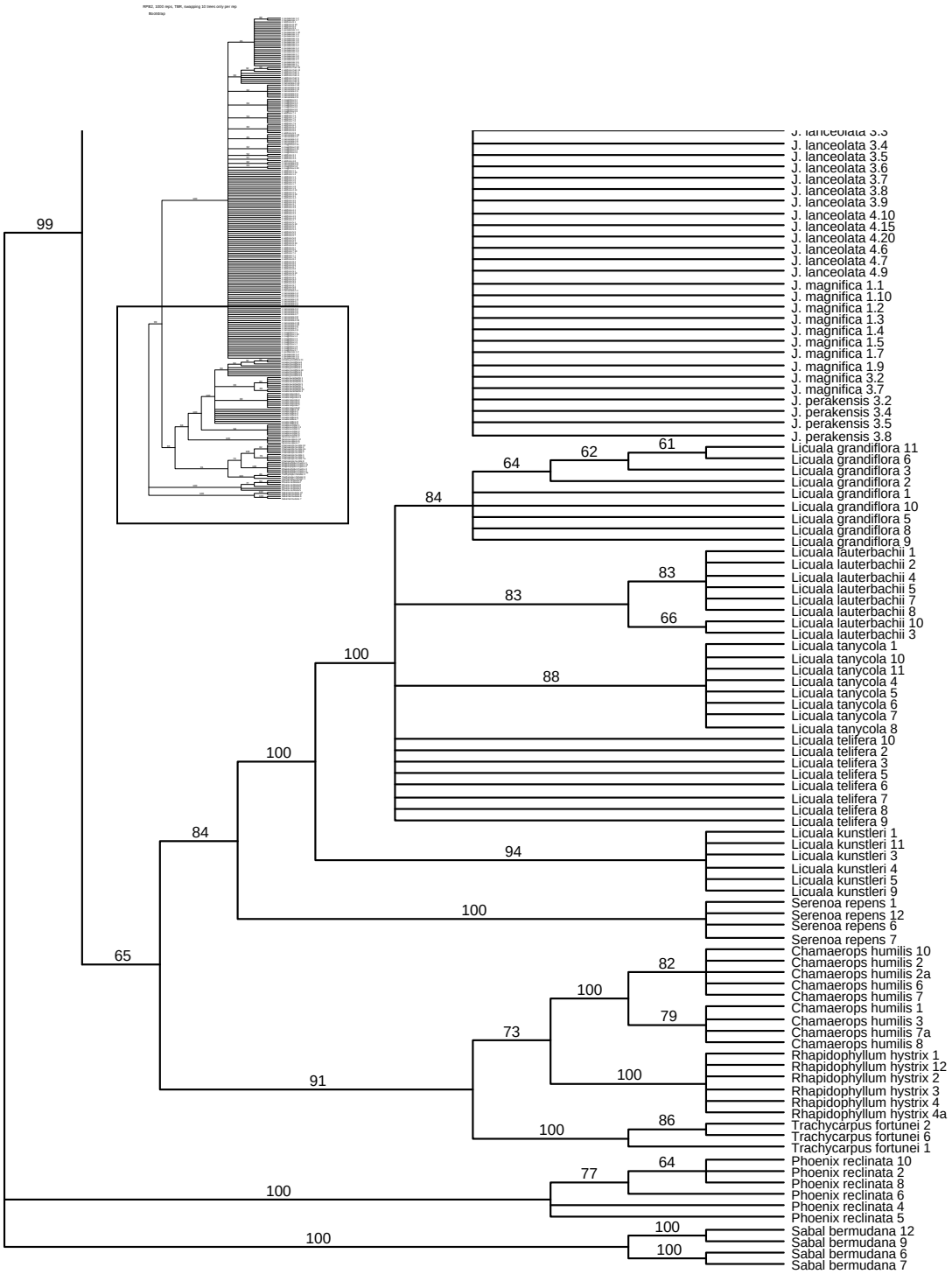

Figure 4.3B. Strict consensus tree of 20000 equally most parsimonious trees from analysis of the *RPB*2 data set. Numbers above the branches indicate the bootstrap percentages.

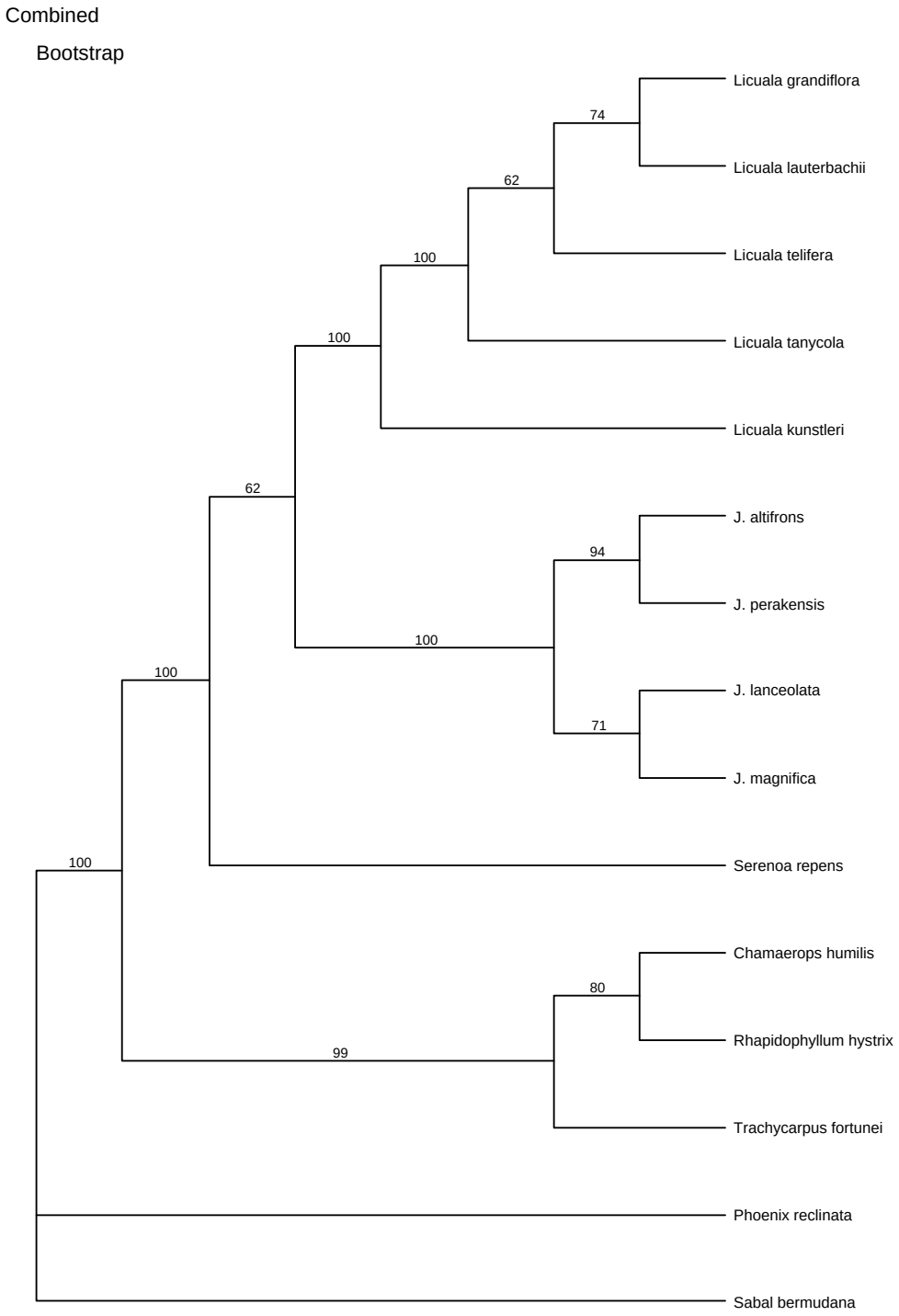

Figure 4.4. Strict consensus of most parsimonious tree from the combined analysis of the *PRK* and *RPB*2 data set. Numbers above the branches indicate the bootstrap percentages.

4.3.5 *Paralogues and Pseudogenes* PRK *and* RPB*2*

While analyses of the datasets based on both *PRK* and *RPB*2 regions did not resolve the relationship among *Johannesteijsmannia* species, they did show that the relationships of the other taxa examined were consistent with current classification schemes.

When translated into amino acid sequences, seven *PRK* sequences were found to have a stop codon at the coding region. The stop codon either resulted from a point mutation (identified in clones *Jt. altifrons* cultivated 9, *Jt. lanceolata* 2.23, *Phoenix reclina* 9 and *Serenoa repens* 5) or an indel that causes frameshift (identified in clones *Jt. magnifica* 1.4, *Jt. magnifica* 2.8 *and Licuala lauterbachii* 10). These stop codons may be pseudogenes or functional copies of the gene. Recovery of pseudogenes is a concern when assessing orthology. On the other hand, the sequences may appear to be functional and coding for a truncated protein that might have acquired new functions (Paralogues; http://homepage.usask.ca/~ctl271/857/def_homolog.shtml).

More detailed analysis, especially functional studies are needed, before one can conclusively determine if the stop codons are indeed pseudogenes or functional copies of the gene *PRK*. When translated into amino acid sequences, no stop codon was found in the *RPB*2 sequences. Also, no identifiable paralogous sequences of *RPB*2 were observed in this study.

4.3.6 *The Phylogenetic Relationships of* Johannesteijsmannia

Although the two low-copy nuclear genes *PRK* and *RPB2* have been shown to be informative in resolving species relationship in other studies (Thomas *et al*, 2006), the phylogeny of *Johannesteijsmannia* was not resolved using them in this study because of the high allelic variation observed in *Johannesteijsmannia* species.

High allelic variation may result from lineage sorting within the genus. According to Tsang (2002), lineage sorting (or deep coalescence) occurs with random fixation of ancestral polymorphic alleles in descendant taxa. If the allelic variation spans species boundaries, some alleles of a species are more closely related to alleles of other species than they are to those of the same species (Small *et al*., 2004). The primary cause of lineage sorting depends very much on the population genetics of the nuclear genes. Owing to the greater effective population size and faster mutation rates of nuclear genes compared to those of organelle genes, together with process of recombination, interspecific allelic variation is expected and observed in a species with sufficiently large population size. When speciation occurs, it is very likely that both descendant and ancestral species will contain some, if not all of the allelic variation present in the ancestral species. If the time for divergence between the studied taxa decreases, the chance of lineage sorting increases. Recent speciation and a lack of time for the phylogenetic sorting of lineages can result in the individuals from different species sharing ancestral DNA haplotypes, as is likely in the case of *Johannesteijsmannia*.

4.3.7 *Comparison of AFLP Fingerprinting and DNA Sequence Evidence*

AFLP fingerprinting was shown to be useful for resolving the phylogenetic relationship of the *Johannesteijsmannia* species in this study. Results from AFLP fingerprinting suggested it is very likely that there may be only three *Johannesteijsmannia* species, i.e., *Jt. altifrons, Jt. lanceolata* and *Jt. magnifica*, with *Jt. perakensis* as a subspecies of *Jt. altifrons*, rather than four as originally erected by Dransfield (1972).

However, DNA sequence data of both *PRK* and *RPB*2 yielded limited nucleotide variation to infer the species delimitations. The clones of each accession did not resolve as accession clades, and neither did they for their corresponding species, so AFLP data provided better resolution compared to the nuclear genes employed. AFLP fingerprinting can generate many polymorphic markers widely distributed across the genome of the plants studied. A study conducted by Despres *et al*. (2003) successful using AFLP to resolve phylogenetic relationships in a morphologically diversified plant species complex when nuclear and chloroplast sequences fail to reveal variability. Nevertheless, AFLP data set was analyzed phenetically. It has not been widely used in phylogenetic inference, presumably because they are believed to be homoplasious markers (Despres *et al*., 2003).

4.4 Conclusions

Results from this chapter revealed that *Johannesteijsmannia* is monophyletic. However, the presence of allelic polymorphism in both *PRK* and *RPB2* genes did not resolve the phylogenetic relationship of the species. The high percentage of duplications of *PRK* and *RPB2* indicated that the four species are rapidly evolving. *Licuala* is a sister group to *Johannesteijsmannia* based on the *PRK* and combined *PRK* and *RPB2* sequence data set analyses but this was not supported by analysis of the *RPB*2 data set. On the other hand, the relationships of the remaining taxa from the Coryphoideae based on *PRK* and/or *RPB2* sequence data set analyses were consistent with current classification schemes.

CHAPTER 5

GENETIC VARIABILITY AND STRUCTURE OF NATURAL POPULATIONS OF *JOHANNESTEIJSMANNIA* SPECIES REVEALED BY AFLP FINGERPRINTING

5.1 Introduction

The work described in this chapter deals with the study of genetic diversity present in each natural population of four *Johannesteijsmannia* species as well as genetic variation within and between populations. From the field observations, there is no significant morphological variation observed within a population for the four species except for the following three minor variations in *Jt. lanceolata*. As discussed in Chapter 2, there were differences in lamina dimensions, the extent of branching and number of rachillae in different populations of *Jt. lanceolata* (Figure 2.8 and 2.9). Such differences were absent within a given population, suggesting the possibility of genetic differences between populations of this species. It is also probable that such differences in morphology may be entirely due to environmental factors rather than genetic differences.

Population genetics is the quantitative study of the amount of distribution of genetic variation in populations, and the dynamics of the underlying genetic processes (Yeh, 2000). One of the main objectives of population genetics is to describe the amount of genetic variability in populations and to study the mechanism of maintenance of the variation. A better knowledge of genetic variation of the various populations of a species is essential to develop effective strategies for conservation of the gene pools of highly endangered and endemic species such as *Johannesteijsmannia*.

As discussed in Chapter 1, the different *Johannesteijsmannia* species are of limited distribution. Of the four species, three are endemic to Peninsular Malaysia and

one is more widespread, being distributed in southern Thailand, Peninsular Malaysia, Sumatra and western Borneo. All *Johannesteijsmannia* species are understorey palms and are obligate shade plants. They cannot survive if the forest canopy is removed, e.g., owing to deforestation (Figure 5.1) leading to the loss of entire populations. If they are of unique genetic make up loss of such populations would be a tremendous blow to the conservation of the species. It should be highlighted that the four *Johannesteijsmannia* species have been listed in the 1997 IUCN red list.

Figure 5.1. Deforestation at Gunung Lee Pa, Narathiwat, southern Thailand, leading to leaf searing and eventually death of the *Jt. altifrons* plants.

In view of the limited distribution of the four *Johannesteijsmannia* species and their sensitivity towards forest disturbance, the conservation of this genus is highly desired before it is too late. Knowledge of the levels and patterns of genetic diversity within a population as well as genetic differentiation between populations is crucial for developing conservation strategies.

In this chapter, results from AFLP fingerprinting of the populations of four *Johannesteijsmannia* species are presented. The major advantage of using DNA markers (e.g., AFLPs) in this study is that they are not affected by environmental factors and epistatic interactions. The technique of AFLPs has been discussed in Chapter 3. The diversity of the four species was computed using Nei's genetic diversity using the software package AFLP-SURV 1.0 (Vekemans, 2002) and the Shannon Information Index, I, from the software package POPGENE 1.31 (Yeh *et al*., 1999). In addition, analysis of molecular variance (AMOVA; Excoffier *et al*., 1992) was conducted as described in the software package ARLEQUIN 2.0 (Schneider *et al*., 2000) to partition the genetic variability within and among populations of each species. The Bayesian Method using the software package HICKORY 1.0 (Holsinger *et al*., 2002) was used to infer the population structure of each species. The correlation between geographical and genetic distances was analyzed using the Mantel Test (Mantel, 1967). Based on these data and field observations, recommendations for developing efficient conservation strategies for the species are presented.

5.2 Materials and Methods

5.2.1 *Plant Materials*

The sampling and collection localities were as described in Chapter 2 (section 2.2.1; Tables 2.2, 2.3, 2.4 and 2.5).

5.2.2 *DNA Extraction*

The DNA extraction was carried out as described in Chapter 3, section 3.2.2.

5.2.3 *Protocol for AFLP Fingerprinting Reactions and Scoring AFLP Bands*

The AFLP fingerprinting reactions, the primer combinations used and the scoring of AFLP bands were as described in Chapter 3, sections 3.2.3 and 3.2.4.

5.2.4 *Statistical Analysis*

The statistical analyses of AFLP markers were based on the following assumptions: 1) An AFLP is a dominant marker, with dominant alleles coding for the presence of a band at a given locus and a recessive null-allele coding for the absence of a band; 2) Comigrating fragments represent homologous loci; 3) Populations are at the Hardy-Weinberg Equilibrium (HWE).

The levels of genetic diversity within populations or species are described in terms of the number of polymorphic loci, mean number of fragments per individual, the total genetic diversity [analogous to Nei's gene diversity (Nei, 1987)], the expected heterozygosity under Hardy-Weinberg genotypic proportion [also called Nei's gene diversity (Nei, 1987), analogous to H or H_e in most publications].

The above parameters are computed using the AFLP-SURV 1.0 programme (Vekemans, 2002). The programme starts by estimating allele frequencies at each marker locus in each population. The primary problem in population studies using multilocus dominant DNA markers is the estimation of frequency of the null-allele. It should be highlighted that the null-alleles are relatively rare and non-uniformly distributed over populations for each single locus. The Bayesian Method with non-uniform prior distribution of allele frequencies (the default method in the programme) (Zhivotovsky, 1999) was adopted in the analysis. Data simulations showed that this approach did not overestimate the frequency of null-alleles but produced more satisfactory results. The programme then computed the statistics of genetic diversity

and population genetic structure according to the treatment of Lynch and Milligan (1994). The programme also produced matrices of pairwise genetic distances between populations with bootstrap support. The genetic distances computed included Nei's genetic distance (after Lynch and Millgan, 1994) and the fixation index of Wright (1965), F_{st}. Both the number of bootstraps for genetic distances and the permutations test on F_{st} was 1000. To infer bootstrap confidence on the branches of dendrograms, the files written with many distance matrices computed by bootstrapping over AFLP loci were then used as input file for the procedures NEIGHBOR and CONSENSE from the PHYLIP 3.5c software package (Felsenstein, 1993). The dendrograms constructed with the unweighted pair group method using arithmetic mean (UPGMA; Sneath and Sokal, 1973) algorithm in the programme PHYLIP were viewed using a tree drawing software package, TREEVIEW 1.6.6 (Page, 1996).

The Shannon Information Index, I, is an alternative measure of genetic diversity. This measure was applied because it is relatively insensitive to the inability of AFLP analysis to detect heterozygous loci (Dawson *et al*., 1995). It was computed using the software POPGENE 1.31 (Yeh *et al*., 1999).

Hierarchical genetic structure was assessed using AMOVA (Excoffier *et al*., 1992) from the ARLEQUIN 2.0 programme (Schneider *et al*., 2000). In order to evaluate differentiation between species, between populations and within populations, the total genetic diversity was partitioned between species, between populations and between individuals within populations. For each species, two hierarchical analyses were conducted, i.e., to partition the total genetic variation among populations and among individuals within populations. The variance components were used to compute fixation indices following Wright (1965). The significance of the fixation indices was then tested using a hierarchical, nonparametric permutation approach

(Excoffier *et al*., 1992). The number of permutations used to test the significance of all fixation indices is 10,100.

As an alternative to AMOVA, the Bayesian approach (Holsinger *et al*., 2002) from the Hickory 1.0 (Holsinger and Lewis, 2003) programme was used to infer the population structure independently. This statistical approach demonstrated the aptitude to alleviate the bias related to the dominant nature in estimating population genetic parameters when dominant markers are applied (Zhivotovsky, 1999; Holsinger *et al*., 2002). The AFLP data were fitted into four models: 1) A full model; 2) A 'f = 0' model; 3) A 'theta = 0' model; 4) A f-free model. The f mentioned above is the inbreeding coefficient and theta (Holsinger *et al*., 2002) is the analogue of the coancestry parameter of Weir and Cockerham (1984) that is equivalent to $F_{st.}$ The Markov Chain Monte Carlo (MCMC) simulations were used to numerically approximate the posterior distributions. The computation was carried out with a burn-in period of 5,000 iterations, sampling run of 25,000 iterations and thin factor 10. The sampling parameters used were according to the default values in the user's manual except a modification on the thinning factor, 10 instead of 5 (default value). As stated in the user's manual, it is recommended to run the sampler for a larger number of generations and thinning factor to obtain consistent results. Subsequently, the deviance information criterion (DIC; Spiegelhalter *et al*., 2002) was used to estimate how well each model fit the data and choosing among the models.

The correlation, r, between geographical distance and genetic distance was verified with Mantel Test (Mantel, 1967). The geographical coordinates collected at each study locality were used to generate pairwise geographical distance matrices of each species. The pairwise F_{st} matrix computed with AMOVA and the geographical distance matrix of each species were then combined together to form a single file with

the data in columnar form to plot the graph in Microsoft Excel. The number of permutation tests carried out was 10100. The significance of the correlation was determined by the percentage point for absolute regression coefficients (P-value). The P-value is given as percentage in the result files.

5.3 Results and Discussion

The number of primer combinations screened and chosen for further anlaysis were the same as listed in Chapter 3 (section 3.3; Table 3.3). The use of AFLP fingerprinting to assess genetic variability among individuals and populations is promising because it can produce many polymorphic loci fairly easily in a relatively short time. In this study, AFLP analysis proved to be a powerful method to detect genetic diversity and population differentiation.

5.3.1 *Genetic Diversity*

There were 222 samples from four species from 27 populations (each species with a different number of populations, Tables 2.2, 2.3, 2.4 and 2.5) that were analyzed using the statistical analyses as described in section 5.2.4. All 222 samples revealed different AFLP haplotypes. The mean number of fragments per individual recorded was 83 and the total number of segregating fragments were 282 (97.9%). The numbers and percentages of polymorphic loci, mean number of fragments per individual, Shannon information index and the total genetic diversity of each species are recorded in Table 5.1.

The percentage of polymorphic loci recorded for each species ranged from the lowest 26.7% (*Jt. magnifica*), followed by 30.6% (*Jt. perakensis*), 59.7% (*Jt. lanceolata*) to the highest at 75.0% (*Jt. altifrons*). The mean number of fragments per

individual recorded ranged from 78.5 (*Jt. lanceolata*) to 84.5 (*Jt. altifrons*). *Jt. magnifica* exhibited the lowest genetic diversity followed by *Jt. perakensis* when estimated using the Shannon Information Index, I and Nei's genetic diversity, H_t (I = 0.1068, H_t = 0.0964 for *Jt. magnifica*; I = 0.1138, H_t = 0.1024 for *Jt. perakensis*).

Table 5.1 Number of polymorphic loci, Shannon Information Index and total genetic diversity of each *Johannesteijsmannia* species.

Species	**P [(a)] (%)**	**Mean[(b)]**	**Shannon Index (I)**	**H_t**
Jt. altifrons	216 (75.0)	84.5	0.1989	0.1513
Jt. lanceolata	172 (59.7)	78.5	0.1395	0.1729
Jt. magnifica	77 (26.7)	84.4	0.1068	0.0964
Jt. perakensis	88 (30.6)	80.1	0.1138	0.1024

Notes:
$P^{(a)}$ — Number of polymorphic loci at the 5% level.
$Mean^{(b)}$ — Mean number of fragments per individual.
H_t — The total genetic diversity (analogous to Nei's analysis of gene diversity).

Among the four species, *Jt. altifrons* demonstrated the highest genetic diversity when estimated with the Shannon Information Index (I = 0.1989). This result is contradicted by the one computed with Nei's genetic diversity, where *Jt. lanceolata* exhibited the highest genetic diversity (H_t = 0.1729) compared to *Jt. altifrons* (H_t = 0.1513). Dawson *et al*. (1995) pointed out that the Shannon Information Index has general applications in ecology and is relatively insensitive to the skewing effects caused by the inability to detect heterozygous loci. Hence, it is more reliable in this case compared to the estimation provided by Nei's genetic diversity. On the other hand, *Jt. altifrons* displayed much wider distribution ranges compared to *Jt. lanceolata*. Geographic range is one of the factors to determine the genetic diversity of a plant species. It is regarded as an approximate measure of the total number of individuals of the species and thus species with a wider distribution tend to have a larger genetic diversity than a more narrowly distributed species, given the other

conditions are equivalent (Karron, 1987). Many studies reported that overall, geographically restricted species tend to have a lower genetic diversity compared to that of widespread species (e.g., Karron, 1987; Baskauf *et al*. 1994; Maki and Horie, 1999; Maki *et al*., 2002) though the opposite trend was also reported in some of the studies (Vogelmann and Gastony, 1987; Ranker, 1994; Young and Brown, 1996).

When compared with some of the palms studied, *Johannesteijsmannia* species displayed similar genetic diversity with *Euterpe edulis* (heart-of-palm), a long generation time rainforest palm (Cardoso *et al*., 2000). The Nei's unbiased genetic diversity value recorded was 0.119, slightly higher than those for *Jt. magnifica* and *Jt. perakensis* but lower than the values for *Jt. altifrons* and *Jt. lanceolata*.

The population data analyzed included the number and percentage of polymorphic loci, sample size, Nei's genetic diversity under Hardy-Weinberg genotypic proportion and the Shannon Information Index of each population of each species (Table 5.2).

Fourteen *Jt. altifrons* populations were analyzed showing that the percentage of polymorphic loci ranged from 35.8% (populations BAK and MUT) to 47.6% (population LUM). Nei's genetic diversity, H_j (assuming Hardy-Weinberg equilibrium) ranged from 0.0861 (populations BAK and MUT) to 0.1557 (population LUM). The Shannon Information Index (I) ranged from 0.0677 (population and MUT) to 0.1595 (population LUM).

There were only four populations of *Jt. lanceolata* that were sampled throughout its distribution range because this species is very rare. The percentage of polymorphic loci calculated ranged from 37.2% (population JBR) to 49.0% (population LSLA). The Nei's genetic diversity value ranged from 0.0984 (populations JBR) to 0.1958 (population LSLA). The Shannon Information Index (I)

ranged from 0.0871 (population JBR) to 0.2355 (population LSLA). Despite the restricted distribution of this species, it exhibited a wider range in genetic diversity.

Jt. magnifica exhibited the lowest genetic diversity among the four species as discussed earlier. Five populations were examined and the percentage of polymorphic loci ranged from 35.4% (population GAT) to 38.5.0% (population KIN). Nei's genetic diversity value ranged from 0.08614 (populations SER) to 0.0950 (population KIN). The Shannon Information Index (I) ranged from 0.0751 (population SER) to 0.0831 (population KIN).

Only four *Jt. perakensis* populations were sampled throughout its distribution range and was thus of a similar sample size to that of *Jt. lanceolata*. The percentage of polymorphic loci ranged from 35.4% (population BUB) to 38.5% (population BSU). Nei's genetic diversity value ranged from 0.0935 (populations KSA) to 0.1026 (population KSB). The Shannon Information Index (I) recorded ranged from 0.0841 (population BSU) to 0.0939 (population KSB). Notwithstanding the same number of populations sampled with *Jt. lanceolata*, this species demonstrated much lower within population genetic diversity compared to the latter.

Population SER of *Jt. magnifica* displayed the lowest genetic diversity (H_j = 0.0861; I = 0.0751) compared to all other populations from all *Johannesteijsmannia* species. The overall low genetic diversity in populations of *Jt. magnifica* contributed to the lowest genetic diversity in this species. In contrast, populations LSLA and ANG of *Jt. lanceolata* demonstrated the highest genetic diversity (H_j = 0.1958; I = 0.2355 and H_j = 0.1860; I = 0.2075 for LSLA and ANG, respectively) among all *Johannesteijsmannia* species. Hence, this contributed to the relatively high total genetic diversity in this species.

Table 5.2. Number and percentage of polymorphic loci, sample size, Nei's genetic diversity value and Shannon Information Index of each population of the four *Johannesteijsmannia* species.

Species	Population	Sample Size	P [(a)] (%)	H_j [(b)] (S.E.)	Shannon Information Index, I
Jt. altifrons	KMR	13	110 (38.2%)	0.1065 (0.0089)	0.1129
	MUT	6	103 (35.8%)	0.0861 (0.0085)	0.0677
	GJA	8	128 (44.4%)	0.1250 (0.0095)	0.1181
	UGU	8	116 (40.3%)	0.1005 (0.0087)	0.0899
	GEE	8	119 (41.3%)	0.1078 (0.0091)	0.0987
	LEP	9	117 (40.6%)	0.1036 (0.0089)	0.0971
	ASLA	9	131 (45.5%)	0.1506 (0.0104)	0.1492
	MAT	8	113 (39.2%)	0.0999 (0.0088)	0.0910
	BAK	8	103 (35.8%)	0.0861 (0.0083)	0.0764
	BPA	8	115 (39.9%)	0.1032 (0.0089)	0.0941
	SDU	8	125 (43.4%)	0.1233 (0.0095)	0.1183
	SNI	8	128 (44.4%)	0.1307 (0.0095)	0.1233
	LUM	8	137 (47.6%)	0.1557 (0.0105)	0.1595
	LAH	8	122 (42.4%)	0.1208 (0.0095)	0.1125
Jt. lanceolata	LSLA	9	141 (49.0%)	0.1958 (0.0123)	0.2355
	ANG	8	138 (47.9%)	0.1860 (0.0120)	0.2075
	JBR	8	107 (37.2%)	0.0984 (0.0089)	0.0871
	RAU	8	121 (42.0%)	0.1207 (0.0095)	0.1101
Jt. magnifica	BUN	8	110 (38.2%)	0.0908 (0.0084)	0.0789
	SER	8	104 (36.1%)	0.0861 (0.0084)	0.0751
	MSLS	8	107 (37.2%)	0.0879 (0.0084)	0.0777
	GAT	8	102 (35.4%)	0.0884 (0.0088)	0.0794
	KIN	8	111 (38.5%)	0.0950 (0.0086)	0.0831
Jt. perakensis	KSA	8	107 (37.2%)	0.0935 (0.0086)	0.0847
	BUB	8	102 (35.4%)	0.0955 (0.0090)	0.0876
	BSU	8	109 (37.8%)	0.0943 (0.0084)	0.0841
	KSB	8	111 (38.5%)	0.1026 (0.0090)	0.0939

Notes:
$P^{(a)}$ — Number of polymorphic loci at the 5% level.
$H_j^{(b)}$ — Expected heterozygosity under Hardy-Weinberg genotypic proportion, also called Nei's gene diversity (analogous to H or H_e in most publications).
S.E. — Standard error.

Genetic diversity within the population can be affected by many factors, mainly the life history traits of a species, e.g., geographical range, mating system, life span, pollen and seed dispersal mechanisms and fecundity (Hamrick and Godt, 1989; Hamrick *et al*., 1992; Hamrick, 1993; Gitzendanner and Soltis, 2000). All *Johannesteijsmannia* species are long-lived perennial plants and the only known difference among the four species is the geographical range (*Jt. altifrons* is the widespread congener of the remaining endemic species). The mating system, pollen and seed dispersal mechanisms and fecundity of each *Johannesteijsmannia* species are yet to be investigated. Hence, it is not possible to firmly conclude that the highest genetic diversity in *Jt. altifrons* is owed to its wider geographical range.

5.3.2 *Genetic Structure at Different Hierarchical Levels*

The total genetic diversity was partitioned into three levels, viz., between species, between populations within species and between individuals within populations. For each species, the total genetic diversity was also partitioned into two hierarchical levels, i.e., between populations and between individuals within populations (Table 5.3).

The AMOVA results (Table 5.3) revealed that 32.8% of the molecular variance was between species, 19.5% of the molecular variance was between populations within a species and 47.8% of the molecular variance was between individuals within populations. The between species molecular variance detected was moderate (32.8%) but highly significant ($\Phi_{ST} = 0.3277$; $P < 0.001$), which suggests that the four species are relatively similar in their genetic makeup.

Table 5.3. Results of AMOVA, including the percentage of variation explained by the different hierarchical levels and fixation index.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation index, Φ_{ST}
Among species	3	1346.299	8.2823	32.77	0.3277 (P < 0.001)
Among populations within species	23	1208.135	4.9196	19.46	0.2895 (P < 0.001)
Within populations	195	2354.719	12.0755	47.77	0.52228 (P < 0.001)
Total	221	4909.153	25.2774		
Jt. altifrons					
Among populations	13	939.822	7.1956	36.91	0.3691 (P < 0.001)
Within population	103	1266.691	12.2980	63.09	
Total	116	2206.513	19.4935		
Jt. lanceolata					
Among populations	3	126.389	3.0100	14.81	0.1481 (P < 0.001)
Within population	29	502.278	17.3199	85.19	
Total	32	628.667	20.3299		
Jt. magnifica					
Among populations	4	75.925	1.2575	12.35	0.1235 (P < 0.001)
Within population	35	312.250	8.9214	87.65	
Total	39	388.175	10.1789		
Jt. perakensis					
Among populations	3	66.000	1.5290	13.53	0.1354 (P < 0.001)
Within population	28	273.500	9.7679	86.47	
Total	31	339.500	11.2969		

Notes:
d.f.– Degree of freedom
Φ_{ST} – Wright's F_{st}, also known as population differentiation

When the AMOVA was conducted on each species at two hierarchical levels, the proportions of genetic variation attributable to within population differences was again higher than between populations for all four species. For *Jt. altifrons*, between populations molecular variation was 36.91% but within population molecular variation was 63.09%. For *Jt. lanceolata*, between populations molecular variation was 14.81% but within population molecular variation was 85.19%. A similar pattern

found in *Jt. magnifica* and *Jt. perakensis* i.e., between populations molecular variation was 12.35% but within populations molecular variation was 87.65%, between populations molecular variation was 13.53%; within population molecular variation was 86.47%, respectively. Such a finding of higher genetic variation within populations than between populations was reported in many plant studies (e.g., Cardoso, 2000; Zawko *et al*., 2001; Sheng *et al*., 2005).

Long-lived and woody plants generally harbour more genetic variation within populations (Hamrick *et al*., 1992). On the other hand, Hamrick and Godt (1989) also pointed out that reproductive biology is the key factor to determine the genetic structure of plant populations. They pointed out that selfing species have an average of 50% genetic variation between populations, whereas outcrossing species harbour only 10–20% genetic variation among populations. Results of AMOVA implied that *Jt. lanceolata*, *Jt. magnifica* and *Jt. perakensis* may possess an outcrossing mating system, because the genetic variation between populations ranged from 12–15%. *Jt. altifrons* showed a higher genetic variation among populations (37%), and this may imply that this species is predominantly outcrossing. According to Hamrick and Godt (1996), life history traits alone explain a relatively low amount of genetic variation among species. The high genetic variation maintained within populations and genetic homogeneity among populations may be the effect of high gene flow, or the populations have not been separated sufficiently long to accumulate detectable genetic differences. The genetic diversity maintained within and between populations is a function of historical events and recent evolutionary processes. Owing to the limited knowledge in the evolutionary history and ecology of *Johannesteijsmannia* species, explanations on the levels and patterns of genetic diversity within and among populations rely primarily on the inference from the molecular data.

The F_{st} (known as population differentiation) ranged from 0.1235 (*Jt. magnifica*) to 0.3691 (*Jt. altifrons*) and is highly significant ($P < 0.001$). This finding suggests that there is gene flow among populations of each species. The highest F_{st} found in *Jt. altifrons* may suggest that this species has limited gene flow among populations compared to the other species. The limited gene flow found in this species may be owed to the greater geographical distances between the populations sampled. More studies on the ecology and phenology should be carried to further understand the genetic diversity and structure within this genus as well as to complement the data gathered in the present study. On the other hand, the three other *Johannesteijsmannia* species with narrower distributions demonstrated relatively low F_{st}— the high rate of historical gene flow among populations might suggest that the populations studied were probably part of one continuous population in the past (Lee *et al*., 2002).

The AMOVA results were corroborated by the Bayesian analysis of population structure, with significant F_{st} values between populations of each species. The best DIC and Dbar values were obtained with 'f = 0' model for all four species (Table 5.4). The DIC and Dbar values are the measures of how well the model fits the data. Models with smaller DIC and Dbar values are preferred because the model fits the data better. The choice of the 'f = 0' model also implies no inbreeding in the populations of all the four species and the F_{st} computed for each species is significant.

Table 5.4. Results of Bayesian analysis, including the fixation index estimated for each species.

Species	'f = 0' model	'Theta = 0' model
Jt. altifrons	DIC = 4699.5160 Dbar = 3763.4183 F_{st} = 0.2771 95% credible interval did not include zero Lower bound = 0.2541 Upper bound = 0.3018	DIC = 9039.2641 Dbar = 8623.7445
Jt. lanceolata	DIC = 1701.6024 Dbar = 1397.4857 F_{st} = 0.1287 95% credible interval did not include zero Lower bound = 0.0992 Upper bound = 0.1619	DIC = 2144.6104 Dbar = 1979.4458
Jt. magnifica	DIC = 1019.0135 Dbar = 849.3396 F_{st} = 0.0984 95% credible interval did not include zero Lower bound = 0.0681 Upper bound = 0.1328	DIC = 1223.2526 Dbar = 1149.0925
Jt. perakensis	DIC = 927.5593 Dbar = 769.9384 F_{st} = 0.1064 95% credible interval did not include zero Lower bound = 0.0697 Upper bound = 0.1474	DIC = 1136.9086 Dbar = 1052.7503

Notes:
f — inbreeding coefficient.
Theta — F_{st} (known as population differentiation).

5.3.3 *Pairwise Genetic Distance and the Mantel Test*

The pairwise genetic distance (Wright's F_{st} and Nei's genetic distance) between populations of each species are recorded in Tables 5.5 to 5.12. The UPGMA dendrograms generated with both Wright's F_{st} and Nei's genetic distance values are shown in Figure 5.2 to Figure 5.11.

The UPGMA dendrograms constructed with both Wright's F_{st} and Nei's genetic distance values revealed identical topology in the dendrograms for *Jt. lanceolata*, *Jt. magnifica* and *Jt. perakensis*, except two minor differences were

detected on the branches of the dendrograms of *Jt. altifrons*. The coherent results observed in the dendrograms produced with both genetic distance methods may be taken as an indication that the data collected in the present study are robust and the populations followed the conceptual models well.

Briefly, there were five clusters found on both dendrograms based on Wright's F_{st} and Nei's genetic distance values for *Jt. altifrons*, namely: 1) Cluster of southern Peninsular Malaysia (populations GJA, UGU, KMR and MUT) with population ASLA; 2) Cluster of southern Thailand (populations LEP and GEE); 3) Cluster of east Malaysia (populations MAT and BAK); 4) Cluster of populations LAH and SNI; 5) Cluster of Kelantan (populations SDU and BPA) (Figures 5.2 and 5.3). Population LUM was genetically closer to populations LAH and SNI compared to the remaining populations.

Of the five clusters mentioned above, dendrograms based on Wright's F_{st} and Nei's genetic distance values of *Jt. altifrons* reflected three highly supported clusters: 1) Cluster of southern Thailand (populations LEP and GEE; 100% bootstrap support); 2) Cluster of east Malaysia (populations MAT and BAK; 100% bootstrap support); 3) Cluster of populations LAH and SNI (89% bootstrap support) (Figures 5.2 and 5.3). These results were congruent with the geographical distances of the populations, of which, 1) LEP and GEE populations (46.9 km apart) are located within Narathiwat Province, southern Thailand; 2) populations MAT and BAK (36 km apart) are located within Kuching, the state of Sarawak, east Malaysia and 3) LAH and SNI populations (34 km apart) are located in neighboring states in the Peninsular Malaysia (population LAH located within Jerantut, the state of Pahang and population SNI located within Kemaman, the state of Terengganu).

Populations SDU and BPA (110 km apart) formed another cluster on the dendrogam based on Nei's genetic distance values but with low bootstrap support (37%) (Figure 5.3). This distinct cluster was not seen on the dendrogram based on Wright's F_{st} value, and instead the two populations were just clustered closer to each other (Figure 5.2). Despite the fact that populations SDU and BPA are located within the state of Kelantan, Peninsular Malaysia, they were about 1000 km apart. Hence, this led to the low bootstrap support on the branch (37%) even the populations clustered together on the dendrogram based on Nei's genetic distance. Another minor swapping was found for populations MUT and ASLA in both dendrograms. Population MUT was genetically closer to population KMR (55% bootstrap support) in the dendrogram based on Nei's genetic distance values (Figure 5.3). In fact, they are located within the state of Johor, Peninsular Malaysia and only about 32 km apart. Nevertheless, population ASLA was found to be more genetically similar to population KMR (63% bootstrap support) based on Wright's F_{st} value (Figure 5.2) despite the fact that they are geographically separated (215 km apart). Regardless of the minor dissimilarities based on the two dendrograms, the remaining populations are topologically identical.

The pairwise F_{st} and pairwise Nei's genetic distance values between *Jt. altifrons* populations ranged from 0 (SNI and LAH) to 0.4408 (BAK and LEP) and from 0 (SNI and LAH) to 0.0931 (MAT and GEE), respectively (Table 5.5 and 5.6). The geographical distance between *Jt. altifrons* populations ranged from about 6 km (UGU and GJA) to about 4500 km (KMR and GEE). The highest pairwise genetic distance was demonstrated by the east Malaysia populations, most likely because these populations were geographically isolated for a long time.

The correspondence between genetic and geographical distance inferred from the dendrograms was confirmed by Mantel test. Results of Mantel test indicated that there was a significant correlation between geographical and genetic distances for *Jt. altifrons* (r = 0.4774; P-value = 0.002) (Figure 5.10). This was in agreement with the theory of 'isolation by distance' (Wright, 1943), which described the accumulation of local genetic differences through geographically restricted dispersal. Field observations suggested that rodents and bees are the likely agents for fruit and pollen dispersal, respectively of *Jt. altifrons*. The gene flow would be restricted because there is a limitation on the dispersal range by rodents and bees. In addition, results of AMOVA suggested that this species has a higher F_{st} value, hence restricted gene flow between populations responsible for the observed differentiation. To date, there is no study done on the dispersal of *Jt. altifrons*. Hence, more work needs to be completed before any conclusions can be drawn.

Dendrograms based on both Wright's F_{st} and Nei's genetic distance values for *Jt. lanceolata* (Figures 5.4 and 5.5) yielded congruent results. Populations JBR and RAU formed a highly supported cluster (100% bootstrap support). This was expected as these populations are located within the state of Pahang, Peninsular Malaysia and only about 43 km apart. Population ANG was more genetically similar to the above cluster (100% bootstrap support) than population LSLA despite the fact that the latter was geographically closer to the populations JBR and RAU (105 km and 68 km apart, respectively). The pairwise F_{st} and pairwise Nei's genetic distance values between *Jt. lanceolata* populations ranged from 0.0620 (ANG and LSLA) to 0.1994 (JBR and LSLA) and from 0.0015 (ANG and LSLA) to 0.0458 (RAU and LSLA), repectively (Table 5.7 and 5.8). The geographical distances between *Jt. lanceolata* populations ranged from about 43 km (JBR and RAU) to about 385 km (ANG and JBR). The

Mantel test indicated that there was no significant correlation between geographical and genetic distances (r = −0.6336; P-value = 0.2149) (Figure 5.11) for *Jt. lanceolata*.

Jt. magnifica produced similar results as *Jt. lanceolata*, and both dendrograms (Figures 5.6 and 5.7) based on the two genetic distance methods gave rise to identical topologies. Populations GAT and MSLA formed a distinct cluster with moderate bootstrap support (53% and 55% for Wright's F_{st} and Nei's genetic distance values, respectively). This was not surprising because populations GAT and MSLA are located adjacent to each other (only 3 km apart). The most unexpected finding was with population KIN, a small population located further north from the other populations and geographically isolated from others (geographic distances with the remaining populations ranged from 149 km to 213 km). Results showed that population KIN was genetically closer to the GAT and MSLA cluster (70% and 71% bootstrap support) despite the geographical distance. Population SER was more genetically similar to the above three populations (100% bootstrap support) whilst population BUN exhibited the furthest genetic distance with all other *Jt. magnifica* populations. The pairwise F_{st} and pairwise Nei's genetic distance values between *Jt. manifica* populations ranged from 0.0354 (GAT and MSLA) to 0.1374 (BUN and GAT) and from 0.0036 (GAT and MSLA) to 0.0158 (BUN and GAT), respectively (Table 5.9 and 5.10). The geographical distance between *Jt. magnifica* populations ranged from about 2.87 km (GAT and MSLA) to about 213 km (BUN and KIN). Results of the Mantel test indicated that there was no significant correlation between geographical and genetic distances (r = −0.0346; P-value = 0.9255) (Figure 5.12) for *Jt. magnifica*.

The four *Jt. perakensis* populations demonstrated unexpected results in the dendrograms (Figures 5.8 and 5.9). Populations KSB and BSU formed a distinct

cluster (65% bootstrap support) despite the fact that the population BSU was the furthest from the other populations (ranging from 84.1 km to 95.67 km). This is a newly discovered population, found in the state of Kedah, further north from the other three populations located within the state of Perak. Population BUB was more genetically similar to the above mentioned cluster (100% bootstrap support) compared to population KSA. Of all the four *Jt. perakensis* populations sampled, populations KSA and KSB are located adjacent to each other at only 0.64 km apart. Nevertheless, these populations did not exhibit small genetic distances. The pairwise F_{st} and pairwise Nei's genetic distance values between *Jt. perakensis* populations ranged from 0 (KSA and BUB) to 0.1334 (populations BUB and BSU) and from 0 (KSA and BUB) to 0.0163 (BUB and BSU), respectively (Table 5.11 and 5.12). The geographical distance between *Jt. magnifica* populations ranged from about 2.87 km (GAT and MSLA) to about 213 km (BUN and KIN). Results of the Mantel test indicated that there was no significant correlation between geographical and genetic distances for *Jt. perakensis* (r = 0.9227; P-value = 0.06262) (Figure 5.13).

For P-values greater than 0.05, the correlation would not be significant. *Jt. lanceolata*, *Jt. magnifica* and *Jt. perakensis* showed no significant correlation between geographical and genetic distances. AMOVA results showed that these species demonstrated low F_{st} value, indicating the possibility of the presence of gene flow between the populations. In contrast, the presence of mountain ranges may serve as barriers to gene flow and hence the populations were isolated despite the fact that they are located fairly close to each other. Alternatively, it may because of the possibility that the populations recently separated, which may not be long enough to gather detectable genetic differences.

Table 5.5. Pairwise F_{st} (below the diagonal) values and geographic distance (in km; above the diagonal) between populations of *Jt. altifrons.*

	KMR	MUT	GJA	UGU	GEE	LEP	ASLA	MAT	BAK	BPA	SDU	SNI	LUM	LAH
KMR	0.0000	32.32	38.87	43.09	4499.72	496.41	215.32	720.02	754.05	340.31	400.70	238.95	376.06	230.20
MUT	0.1249	0.0000	53.17	54.33	465.42	511.76	212.68	735.32	769.81	353.11	420.11	260.99	382.53	248.90
GJA	0.0349	0.1046	0.0000	5.61	413.69	460.21	178.10	752.79	786.46	302.86	367.14	207.87	337.25	195.98
UGU	0.0577	0.1700	0.0296	0.0000	411.51	457.96	173.05	758.28	791.96	300.13	365.79	207.34	333.46	194.58
GEE	0.1817	0.2167	0.1364	0.1861	0.0000	46.91	304.06	1047.63	1075.18	117.83	75.82	223.35	149.00	221.21
LEP	0.1846	0.2364	0.1278	0.2122	0.0097	0.0000	345.26	1089.24	1116.30	161.65	115.75	269.89	178.59	268.12
ASLA	0.1179	0.1886	0.0763	0.1240	0.1783	0.1713	0.0000	927.05	960.07	187.27	288.02	190.33	184.09	157.07
MAT	0.3902	0.4263	0.3622	0.4220	0.4344	0.4375	0.2583	0.0000	36.30	980.87	973.58	841.43	1047.05	860.70
BAK	0.3985	0.4385	0.3557	0.4196	0.4396	0.4408	0.2547	0.0483	0.0000	1010.53	1000.76	871.04	1077.64	890.98
BPA	0.3349	0.3334	0.2831	0.3468	0.3407	0.3347	0.1524	0.2494	0.2367	0.0000	109.57	139.50	81.29	123.05
SDU	0.3151	0.3240	0.2596	0.3313	0.3169	0.3144	0.1293	0.2072	0.1925	0.0556	0.0000	164.10	175.94	171.22
SNI	0.2939	0.3333	0.2568	0.3175	0.3232	0.3122	0.1214	0.2311	0.2083	0.0585	0.0437	0.0000	211.12	34.21
LUM	0.3003	0.3213	0.2595	0.3169	0.2744	0.2678	0.1288	0.2200	0.2247	0.1102	0.0698	0.0534	0.0000	187.11
LAH	0.3197	0.3487	0.2682	0.3407	0.3370	0.3280	0.1323	0.2293	0.2475	0.0676	0.0500	0.0000	0.0266	0.0000

Table 5.6. Nei's genetic distance values after Lynch and Milligan (1994) (below the diagonal) and geographic distance (in km; above the diagonal) between populations of *Jt. altifrons.*

	KMR	**MUT**	**GJA**	**UGU**	**GEE**	**LEP**	**ASLA**	**MAT**	**BAK**	**BPA**	**SDU**	**SNI**	**LUM**	**LAH**
KMR	0.0000	32.32	38.87	43.09	4499.72	496.41	215.32	720.02	754.05	340.31	400.70	238.95	376.06	230.20
MUT	0.0152	0.0000	53.17	54.33	465.42	511.76	212.68	735.32	769.81	353.11	420.11	260.99	382.53	248.90
GJA	0.0047	0.0136	0.0000	5.61	413.69	460.21	178.10	752.79	786.46	302.86	367.14	207.87	337.25	195.98
UGU	0.0071	0.0212	0.0038	0.0000	411.51	457.96	173.05	758.28	791.96	300.13	365.79	207.34	333.46	194.58
GEE	0.0270	0.0300	0.0209	0.0269	0.0000	46.91	304.06	1047.63	1075.18	117.83	75.82	223.35	149.00	221.21
LEP	0.0269	0.0329	0.0190	0.0311	0.0012	0.0000	345.26	1089.24	1116.30	161.65	115.75	269.89	178.59	268.12
ASLA	0.0196	0.0310	0.0132	0.0201	0.0324	0.0302	0.0000	927.05	960.07	187.27	288.02	190.33	184.09	157.07
MAT	0.0764	0.0791	0.0745	0.0847	0.0931	0.0921	0.0507	0.0000	36.30	980.87	973.58	841.43	1047.05	860.70
BAK	0.0730	0.0763	0.0670	0.0771	0.0877	0.0860	0.0462	0.0052	0.0000	1010.53	1000.76	871.04	1077.64	890.98
BPA	0.0607	0.0536	0.0520	0.0620	0.0628	0.0597	0.0261	0.0382	0.0329	0.0000	109.57	139.50	81.29	123.05
SDU	0.0614	0.0574	0.0509	0.0643	0.0624	0.0603	0.0237	0.0333	0.0280	0.0075	0.0000	164.10	175.94	171.22
SNI	0.0575	0.0623	0.0519	0.0625	0.0667	0.0619	0.0228	0.0398	0.0322	0.0081	0.0067	0.0000	211.12	34.21
LUM	0.0665	0.0664	0.0587	0.0700	0.0586	0.0555	0.0271	0.0416	0.0398	0.0181	0.0120	0.0094	0.0000	187.11
LAH	0.0620	0.0635	0.0527	0.0663	0.0677	0.0635	0.0241	0.0375	0.0384	0.0091	0.0073	0.0000	0.0042	0.0000

Table 5.7. Pairwise F_{st} values (below the diagonal) and geographic distance (in km; above the diagonal) between populations of *Jt. lanceolata*.

	LSLA	**ANG**	**JBR**	**RAU**
LSLA	0.0000	310.43	104.90	67.68
ANG	0.0062	0.0000	384.71	343.68
JBR	0.1994	0.1521	0.0000	42.85
RAU	0.1957	0.1347	0.0854	0.0000

Table 5.8. Nei's genetic distance values after Lynch and Milligan (1994) (below the diagonal) and geographic distance (in km; above the diagonal) between populations of *Jt. lanceolata*.

	LSLA	**ANG**	**JBR**	**RAU**
LSLA	0.0000	310.43	104.90	67.68
ANG	0.0015	0.0000	384.71	343.68
JBR	0.0422	0.0289	0.0000	42.85
RAU	0.0458	0.0278	0.0115	0.0000

Table 5.9. Pairwise F_{st} values (below the diagonal) and geographic distance (in km; above the diagonal) between populations of *Jt. magnifica*.

	BUN	**SER**	**MSLA**	**GAT**	**KIN**
BUN	0.0000	65.02	29.95	28.35	213.03
SER	0.0403	0.0000	35.09	36.76	149.45
MSLA	0.0626	0.0945	0.0000	2.87	183.50
GAT	0.1374	0.0977	0.0354	0.0000	185.58
KIN	0.0725	0.0554	0.0444	0.0549	0.0000

Table 5.10. Nei's genetic distance values after Lynch and Milligan (1994) (below the diagonal) and geographic distance (in km; above the diagonal) between populations of *Jt. magnifica*.

	BUN	**SER**	**MSLA**	**GAT**	**KIN**
BUN	0.0000	65.02	29.95	28.35	213.03
SER	0.0041	0.0000	35.09	36.76	149.45
MSLA	0.0066	0.0100	0.0000	2.87	183.50
GAT	0.0158	0.0104	0.0036	0.0000	185.58
KIN	0.0080	0.0058	0.0047	0.0059	0.0000

Table 5.11. Pairwise F_{st} values (below the diagonal) and geographic distance (in km; above the diagonal) between populations of *Jt. perakensis*.

	KSA	**BUB**	**KSB**	**BSU**
KSA	0.0000	21.43	0.64	84.10
BUB	0.0000	0.0000	21.59	95.67
KSB	0.0040	0.0217	0.0000	84.59
BSU	0.0895	0.1334	0.0900	0.0000

Table 5.12. Nei's genetic distance values after Lynch and Milligan (1994) (below the diagonal) and geographic distance (in km; above the diagonal) between populations of *Jt. perakensis*.

	KSA	**BUB**	**KSB**	**BSU**
KSA	0.0000	21.43	0.64	84.10
BUB	0.0000	0.0000	21.59	95.67
KSB	0.0004	0.0024	0.0000	84.59
BSU	0.0102	0.0163	0.0108	0.0000

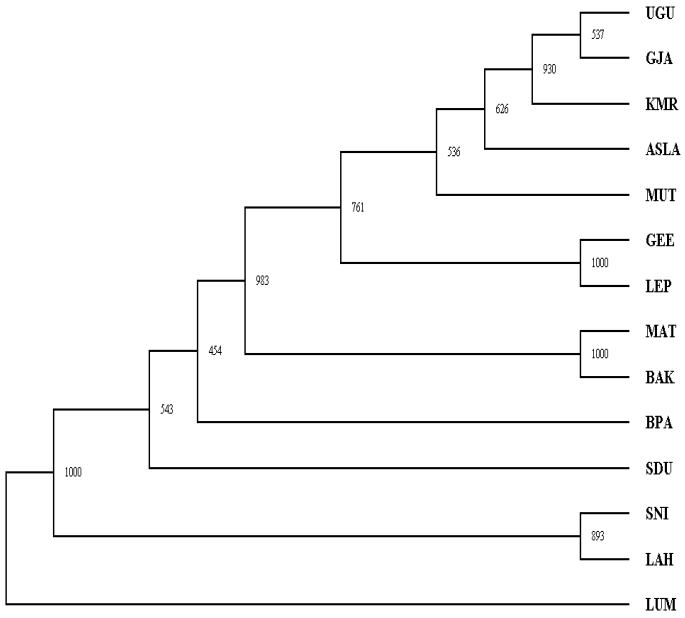

Figure 5.2 UPGMA dendrogram based on Wright's F_{st} values of 14 populations of *Jt. altifrons*. Bootstrap values are given at each node (1000 replications).

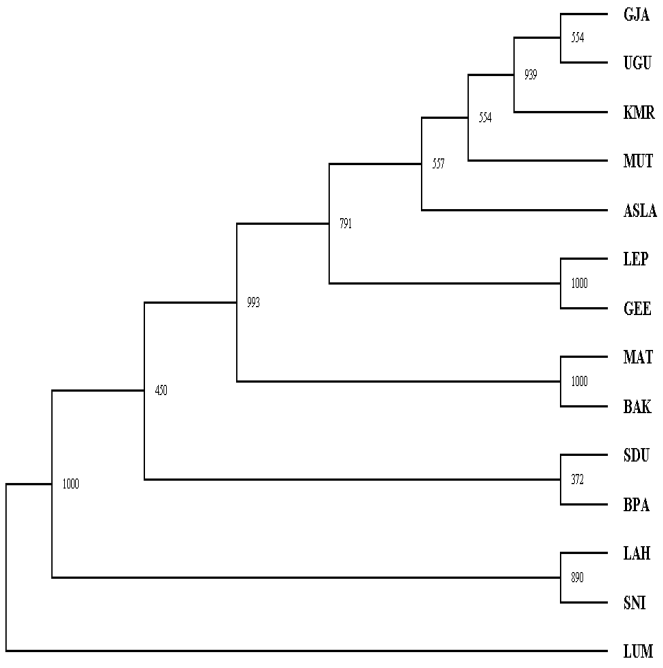

Figure 5.3 UPGMA dendrogram based on Nei's genetic distance values of 14 populations of *Jt. altifrons*. Bootstrap values are given at each node (1000 replications).

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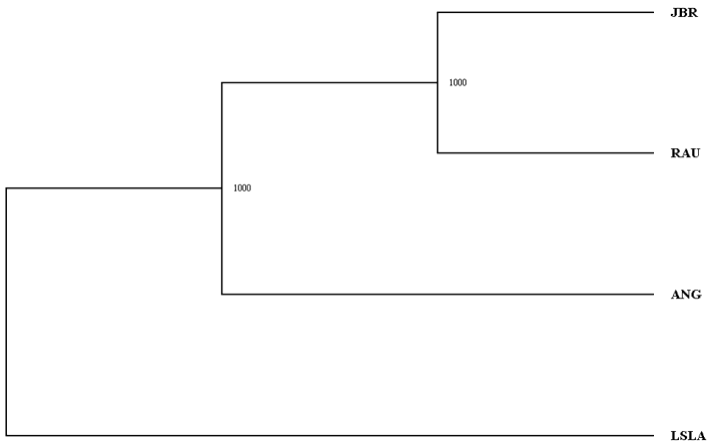

Figure 5.4 UPGMA dendrogram based on Wright's F_{st} values of four populations of *Jt. lanceolata*. Bootstrap values are given at each node (1000 replications).

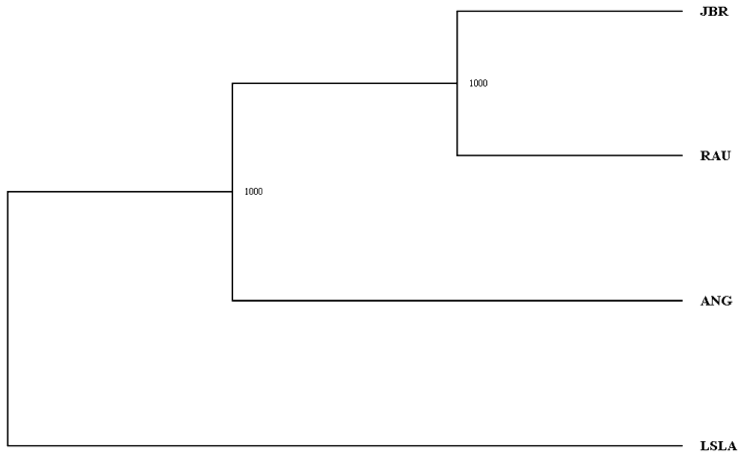

Figure 5.5 UPGMA dendrogram based on Nei's genetic distance values of four populations of *Jt. lanceolata*. Bootstrap values are given at each node (1000 replications).

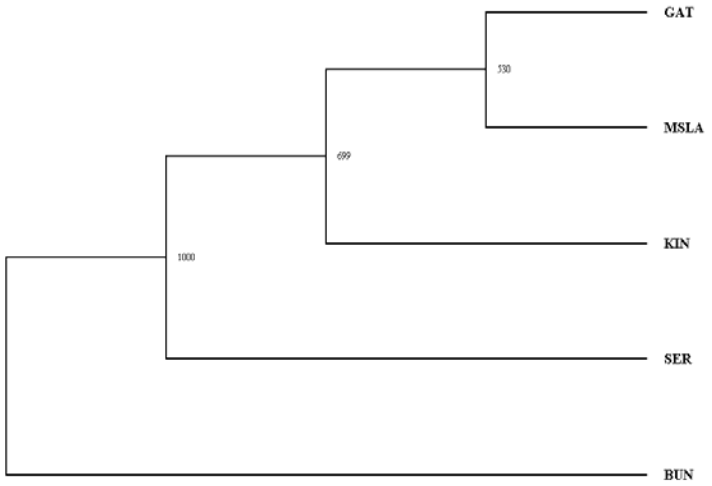

Figure 5.6 UPGMA dendrogram based on Wright's F_{st} values of five populations of *Jt. magnifica*. Bootstrap values are given at each node (1000 replications).

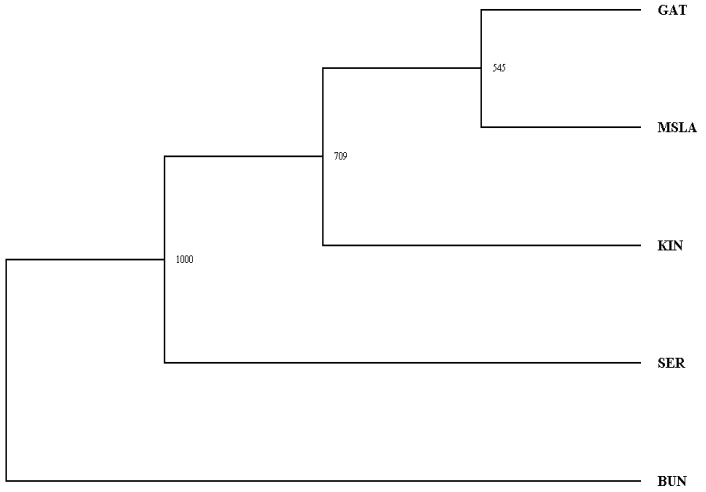

Figure 5.7 UPGMA dendrogram based on Nei's genetic distance values of five populations of *Jt. magnifica*. Bootstrap values are given at each node (1000 replications).

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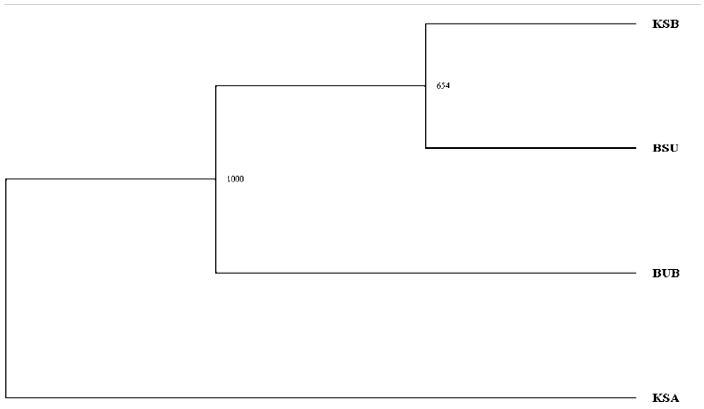

Figure 5.8 UPGMA dendrogram based on Wright's F_{st} values of four populations of *Jt. perakensis*. Bootstrap values are given at each node (1000 replications).

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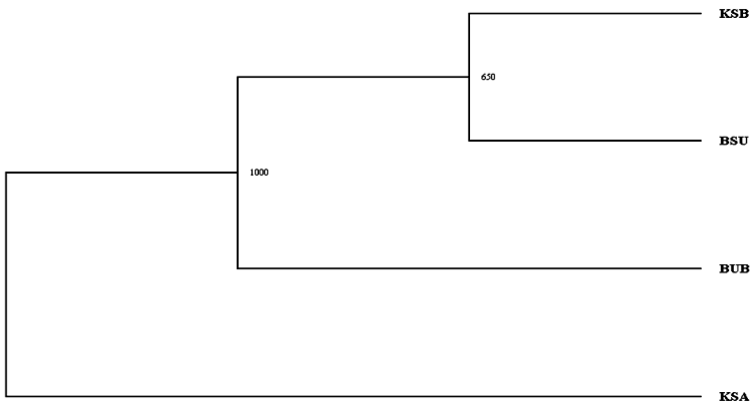

Figure 5.9 UPGMA dendrogram based on Nei's genetic distance values of four populations of *Jt. perakensis*. Bootstrap values are given at each node (1000 replications).

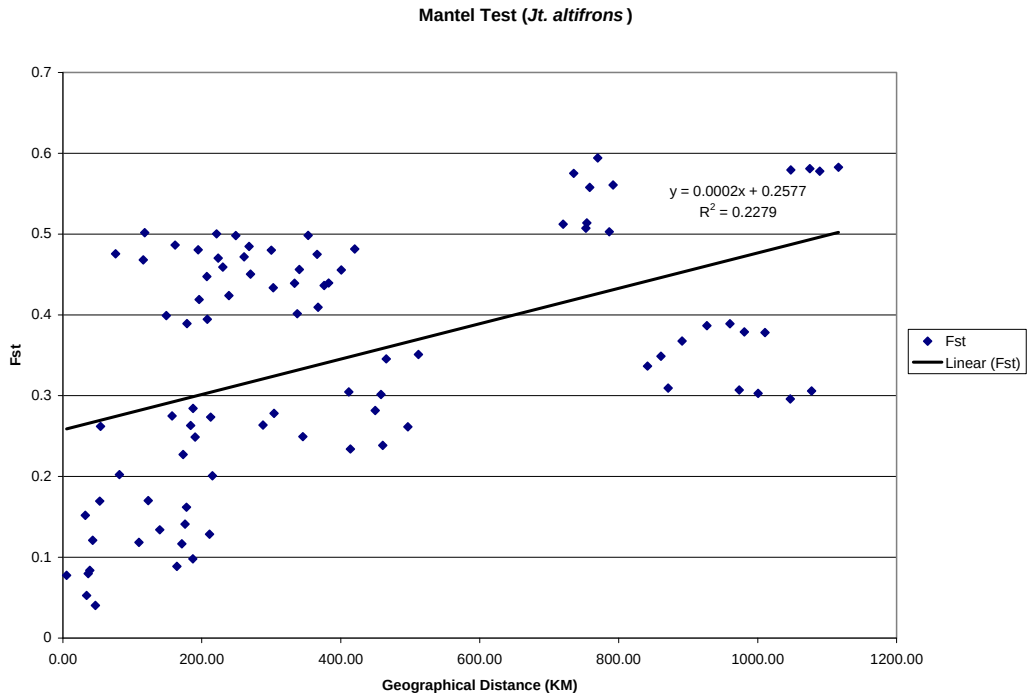

Figure 5.10. Mantel test for correlation between genetic and geographical distances in *Jt. altifrons*. r = 0.4774; P-value = 0.002.

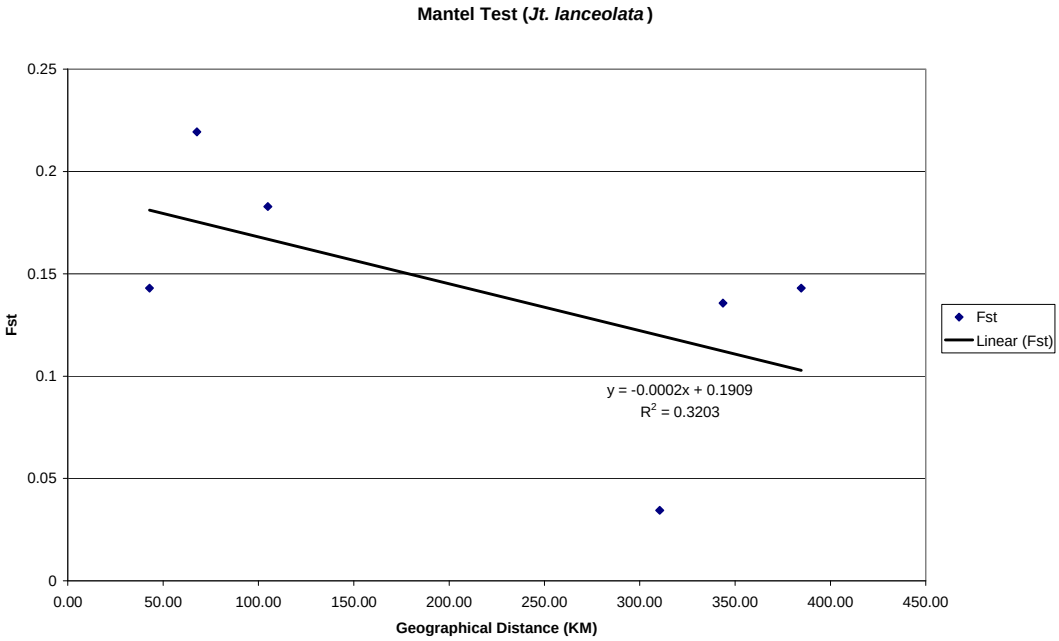

Figure 5.11. Mantel test for correlation between genetic and geographical distances in *lanceolata*. r = −0.6336; P-value = 0.2149

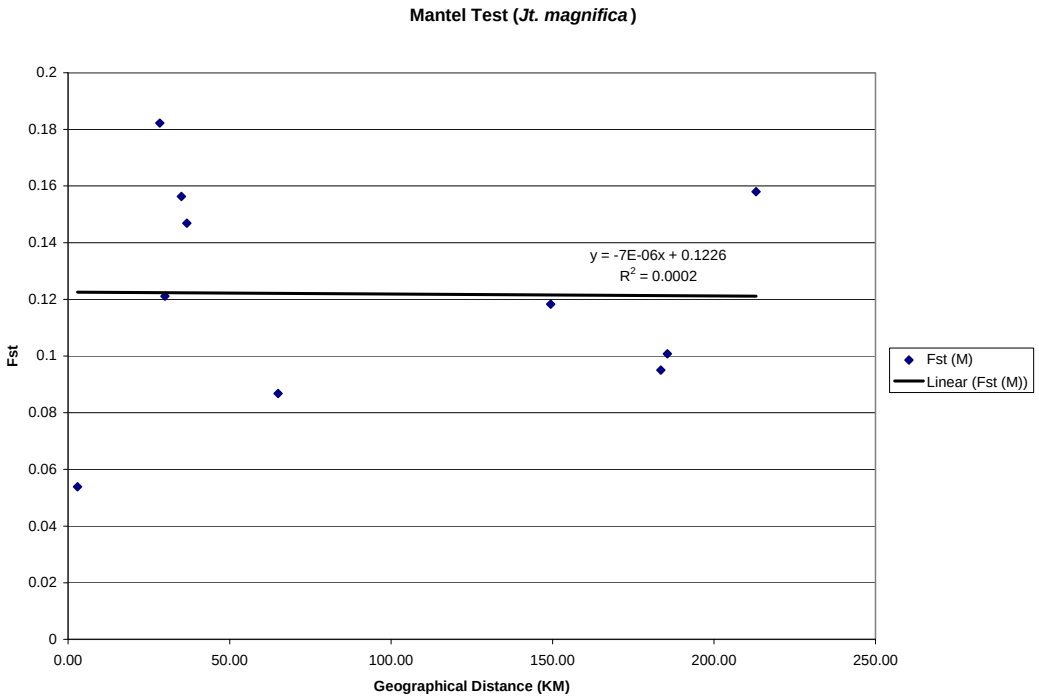

Figure 5.12. Mantel test for correlation between genetic and geographical distances in *Jt. magnifica*. r = −0.0346; P-value = 0.9255.

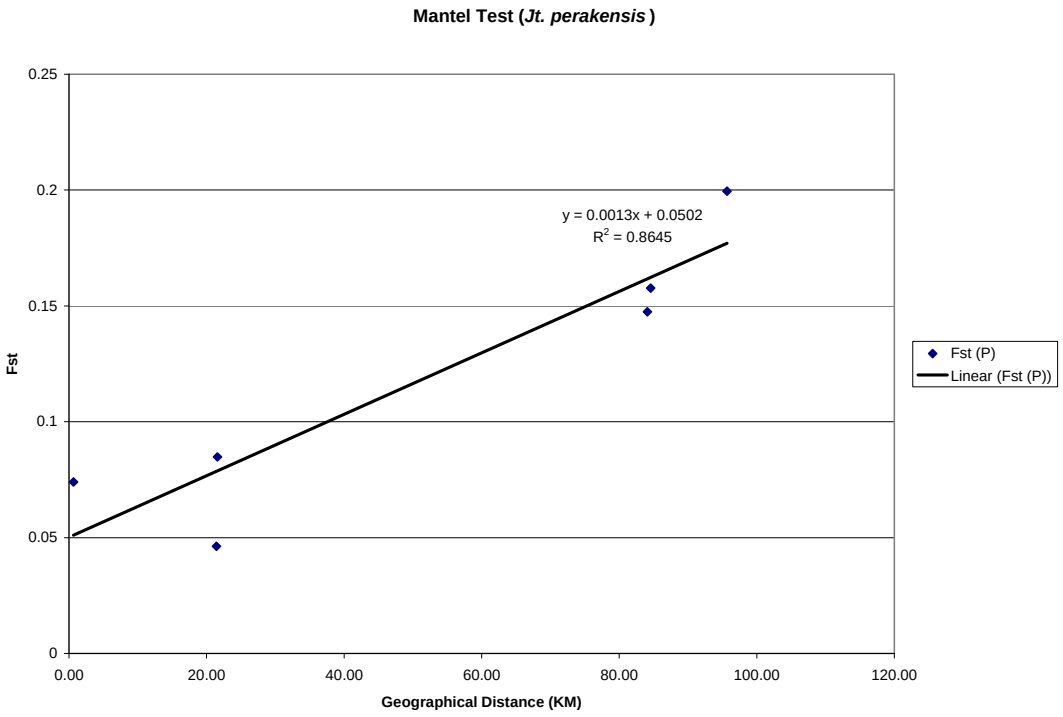

Figure 5.13. Mantel test for correlation between genetic and geographical distances in *Jt. perakensis*. r = 0.9227; P-value = 0.06262.

5.3.4 *Implications for Conservation*

The genetic variability and structure of *Johannesteijsmannia* species revealed in this study can be used as guidelines to design strategies for conservation. The population with the highest genetic diversity should be given priority because the probability to conserve the unique alleles in the population is higher.

Three *Johannesteijsmannia* species endemic to peninsular Malaysia demonstrated a high proportion of genetic variation within the populations. This suggested a smaller number of populations are required to effectively conserve the populations compared with island endemics with many and strongly isolated populations (Sheng *et al*., 2005).

In situ conservation may not be feasible to cover all populations but it provides the best environment for plants to survive. *In situ* conservation concentrates on the management and maintenance of genetic diversity within the wild populations in forest and has the advantage of allowing the operation of genetic processes to be continued, e.g., selection and gene flow. Hence, there is the possibility of conserving the dynamic gene pool capable of evolutionary response to the changing environment (Young *et al*., 2000). Another consideration about sampling wide-ranging species such as *Jt. altifrons* is the geographical placement of units. Millar and Libby (1991) pointed out that if a few of the populations were strategically placed on the basis of genetic variation, much of the genetic variability can be captured. For tropical trees, if 80% of the genetic diversity resides within a population, five strategically placed populations should capture 99% of their total genetic diversity (Hamrick, 1993). However, as the mating system and variation in adaptive traits of *Johannesteijsmannia* species are not known, more than five populations should be conserved

5.4 Conclusions

Results from this chapter revealed that *Jt. altifrons* displayed the highest genetic diversity among the four species, followed by *Jt. lanceolata*, *Jt. perakensis* and *Jt. magnifica* in descending order. AMOVA results indicated the proportions of genetic variation attributable to within population differences were higher than between populations for all four species. Hamrick and Godt (1989) pointed out that the selfing species have an average of 50% genetic variation between populations, whereas outcrossing species harbour only 10–20% of the genetic variation between populations. Results of AMOVA implied that *Jt. lanceolata*, *Jt. magnifica* and *Jt. perakensis* may exhibit an outcrossing mating system owing to the fact that genetic variation between populations ranged from 12–15%. *Jt. altifrons* showed higher a genetic variation between populations (37%), so this may imply that this species exhibits a predominantly outcrossing mating system. Field observations, reproductive biology and ecological studies should be carried out to determine the mating systems of *Johannesteijsmannia* species as well as to complement the results generated from this chapter.

The F_{st} values (known as population differentiation) are highly significant ($P < 0.001$), ranged from 0.1235 (*Jt. magnifica*) to 0.3691 (*Jt. altifrons*). This suggested that there is gene flow among populations of each species. The highest F_{st} value found in *Jt. altifrons* may indicate that this species has limited gene flow between populations compared to the other species. The AMOVA results were corroborated by the Bayesian analysis of population structure, with significant F_{st} values among populations of each species. The choice of 'f = 0' model also implied no inbreeding in the populations of all four species.

Mantel test indicated that there was a significant correlation between geographical and genetic distances for *Jt. altifrons* (r = 0.4774; P-value = 0.002) but was insignificant for the remaining species. In addition, results of the AMOVA suggested that *Jt. altifrons* has higher F_{st}, hence restricted gene flow between populations that are responsible for the observed differentiation.

Jt. lanceolata, *Jt. magnifica* and *Jt. perakensis* showed no significant correlation between geographical and genetic distances. The AMOVA results showed that these species demonstrated relatively low F_{st} values, indicating that there is gene flow between the populations. In contrast, the presence of mountain ranges may serve as barriers to restrict gene flow and hence the populations are isolated despite being located near each other. Alternatively, it may be due to possibility that the populations recently separated, which is not long enough for them to gather detectable genetic differences.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Since the revision of the Malesian palm genus *Johannesteijsmannia* by Dransfield (1972), no further studies on its systematics was published. The attractive congeners, particularly *Jt. altifrons* and *Jt. magnifica*, have received increasing attention from plant lovers owing to their use as beautiful ornamental plants. The increasing demands for these plants are leading to their depletion in the wild. Also, three of the four congeners, *Jt. lanceolata*, *Jt. magnifica* and *Jt. perakensis*, have a very limited distribution, being endemic, and only found at a few localities in Peninsular Malaysia. To effectively conserve, manage and better preserve the species, there is need to understand their population genetics, systematics as well as evolutionary history.

6.1 General Conclusions

From the work done in this study, several conclusions can be made regarding the population genetics, systematic and phylogeny of the genus *Johannesteijsmannia.* The conclusions are:

1) Morphometric analysis using only reproductive or vegetative characters supported Dransfield's hypothesis that there are four putative species in *Johannesteijsmannia*. However, when stem characters were excluded in the analysis, morphological evidence did not support Dransfield's hypothesis but suggested that *Jt. perakensis* may be a subspecies of *Jt. altifrons*. Molecular evidence also supported this finding, so both morphological and molecular data

were consistent. Hence, a combination of both sets of data resulted in more reliable and robust findings.

2) AFLP data showed that there is no hybridization among *Jt. altifrons*, *Jt. lanceolata* and *Jt. magnifica* despite their sympatric occurrence at Sungai Lalang Forest Reserve, Selangor, Peninsular Malaysia. Therefore, they are separate species and apparently reproductively isolated.

3) When both nuclear genes *PRK* and *RPB*2 were employed to infer the phylogeny, *Johannesteijsmannia* resolved as a monophyletic group with high bootstrap support (99% and 100%). Nevertheless, the relationship among the congeners was not resolved. High allelic polymorphism was observed in the clone sequences of the four congeners in both data sets, suggesting recent speciation has taken place. Only the *PRK* and combined data supported *Licuala* being a sister group to *Johannesteijsmannia*.

4) *Jt. altifrons* exhibited the highest genetic diversity, followed by *Jt. lanceolata*, *Jt. perakensis* and *Jt. magnifica*, in descending order. Populations of each species exhibited moderate genetic diversity (Nei's genetic diversity values ranging from 0.086–0.197 and the Shannon information index, from 0.068–0.236). Results of the AMOVA showed that all congeners partition higher genetic diversity within population (63%–88%) than between populations (12%–37%). The population differentiation measure, the F_{st} value, was highly significant ($P < 0.001$) and ranged from 0.1235 (*Jt. magnifica*) to 0.3277 (*Jt. altifrons*) indicating that there is gene flow between populations of each species. AMOVA results were corroborated by Bayesian analysis with significant F_{st} values for all species. The

5) Results from Mantel test demonstrated there is a significant correlation between geographical and genetic distance in *Jt. altifrons* but insignificant for the others.

6.2 Future Perspectives

The need to explore new genes to construct the phylogeny of the genus is much desired. Also, functional studies are required to confirm if the stop codon found on some of the clones are indeed functional copies of a gene that codes for truncated proteins or are merely pseudogenes. Network and intraspecific genealogies analysis within the genus may be suitable to detect low-level variation using the statistical parsimony programme TCS (Clement *et al.*, 2000). The mating systems of each *Johannesteijsmannia* species should also be determined to provide more information for use to design management strategies for conservation.

6.3 Recommendation for Conservation of Genetic Resources of *Johannesteijsmannia*

Because three of the four *Johannesteijsmannia* species are highly endemic and all are obligate tropical rain forest understorey palms and sensitive to forest disturbance, there is a great need for conservation for this genus. Hence, here are the recommendations based on findings from this study:

1) Populations exhibiting highest genetic diversity (populations LUM, LSLA, KIN and KSB) for each species should be given priority for conservation. In general, the more the polymorphic loci the higher the genetic diversity for any given

population. Hence, conserving such populations will ensure a higher chance to safeguard more unique alleles, which may not be present in other populations.

2) The F_{st} value for each species is highly significant. This implies that there is genetic differentiation between the populations of each species, especially in *Jt. altifrons* because it exhibits the highest F_{st} value. Hence, it is advisable to conserve all available populations whenever possible, because we do not know how distinct the genetic compositions contributing to such differentiation are. In addition, the Sumatran populations were omitted in this study; *Jt. altifrons* in this region should also be conserved because one cannot rule out that Sumatran populations may have evolved to distinct genotypes due to geographical isolation, as shown in the East Malaysian populations of the species in our AFLP analysis.

3) The genetic diversity estimated for each population of *Johannesteijsmannia* is moderate, so any breeding programme under cultivation will be of limited genetic diversity. Hence, to obtain genetic stock for breeding, it is imperative that all available natural populations should be conserved, preferably *in situ*. Also, *ex situ* conservation efforts in designated locations, e.g., arboreta or botanical gardens, should have replicate collections from all known natural populations to ensure the maximum genetic diversity in these 'gene banks'.

4) The present study showed that all *Johannesteijsmannia* species are characterized by a high level of genetic diversity partitioned within their populations (ranging from 63% to 88%). For tropical trees, if 80% of the genetic diversity resides within a population, five strategically placed populations should capture 99% of their total genetic diversity (Hamrick, 1993). Therefore, sampling five

strategically placed populations of *Jt. lanceolata*, *Jt. magnifica* and *Jt. perakensis* should safely capture most of the genetic diversity, assuming that Hamrick's findings apply here. However, for a given *Jt. altifrons* population, only about 63% genetic variation resides within it. Hence, sampling more than five populations is recommended for *Jt. altifrons*.

5) Population KSB of *Jt. perakensis* was observed to be disturbed and relatively small in size. Logging was observed near this locality and can threaten the population. Additionally, this population exhibits the highest genetic diversity among the populations examined for this species. Therefore, conservation is needed urgently to protect this population.

6) All four *Johannesteijsmannia* species were listed in the 1997 IUCN red list of threatened plants as mentioned in Chapter 1 but these four species have been omitted from the latest version. Our findings highlight that all *Johannesteijsmannia* species should be reinstated and included in the upcoming edition of the IUCN red list.

7) *Johannesteijsmannia* species are attractive plants and can be easily identified in forests. Hence, they have the potential to be flagship or keystone species (Caro and O' Doherty, 1999) to convince the public and the relevant authorities of the need to conserve a given habitat.

REFERENCES

Allison, L., Moyle, M., Shales, M. and Ingles, C. 1985. Extensive homology among the largest subunit of eukaryotic and prokaryotic RNA polymerases. *Cell* 42: 599–610.

APG II. 2003. An update of the angiosperm phylogeny group classification for the orders and families of flowering plants: APG II. *Botanical Journal of the Linnean Society* 141: 399–436.

Asmussen, C.B. 1999. Toward a chloroplast DNA phylogeny of the tribe Geonomeae (Palmae). *Memoirs of the New York Botanical Garden* 83: 121–129.

Asmussen, C.B., Baker, W.J. and Dransfield, J. 2000. Phylogeny of the palm family (Arecaceae) based on *rps*16 intron and *trn*L-*trn*F plastid DNA sequences. In Wilson, K.L. and Morrison, D.A. (eds.). *Monocots: Systematics and Evolution*. Pg. 525–537. CSIRO: Melbourne, Australia.

Asmussen, C.B. and Chase, M.W. 2001. Coding and noncoding plastid DNA in palm family systematics. *American Journal of Botany* 88: 1103–1117 .

Bacon, C.D. and Bailey, C.D. 2006. Taxonomy and conservation: a case study from *Chamaedorea alternans*. *Annals of Botany* 98: 755–763.

Baker, W.J., Asmussen, C.B., Barrow, S.C., Dransfield, J. and Hedderson, T.A. 1999. A phylogenetic study of the palm family (Palmae) based on chloroplast DNA sequences frm the *trn*L-*trn*F region. *Plant Systematics and Evolution* 219: 111–126.

Baker, W.J., Hedderson, T.A. and Dransfield, J. 2000a. Molecular phylogenetics of subfamily Calamoideae (Palmae) based on nrDNA ITS and cpDNA *rps*16 intron sequence data. *Molecular Phylogenetics and Evolution* 14: 195–217.

Baker, W.J., Hedderson, T.A. and Dransfield, J. 2000b. Molecular phylogenetics of *Calamus* (Palmae) and related rattan genera based on 5S nrDNA spacer sequence data. *Molecular Phylogenetics and Evolution* 14: 218–231.

Baldwin, B.G., Sanderson, M.J., Porter, M.J., Wojeiechowski, M.F., Campbell, C.S., Donghue, M.J. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Annals of the Missouri Botanical Garden* 82: 247–277.

Barrow, S.C. 1999. Systematic studies in *Phoenix* L. (Palame: Coryphoideae). *Memoirs of the New York Botanical Garden* 83: 215–223.

Baskauf, C.J., MacCauley, D.E. and Eickmeier, W.G. 1994. Genetic analysis of rare and widespread species of *Echinacea* (Asteraceae). *Evolution* 48: 180–188.

Blears, M.J., Grandis, SA. De., Lee, H. and Trevors, J.T. 1998. Amplified fragment length polymorphism (AFLP): a review of the procedure and its applications. *Journal of Industrial Microbiology and Biotechnology* 21: 99–114.

Briggs, D. and Walters, S.M. 1993. *Plant variation and evolution*. 3rd Edition, Cambridge University Press, UK.

Cardoso, S.R.S., Eloy, N.B., Provan, J., Cardoso, M.A. and Ferreira, P.C.G. 2000. Genetic differentiation of *Euterpe edulis* Mart. populations estimated by AFLP analysis. *Molecular Ecology* 9: 1753–1760.

Cardoso, S.R.S., Provan, J., Lira, C.F., Pereira, L.O.R., Ferreira, P.C.G. and Cardoso, M.A. 2005. High levels of genetic structuring as a result of population fragmentation in the tropical tree species *Caesalpinia echinata* Lam. *Biodiversity and Conservation* 14: 1047–1057.

Caro, T.M. and O'Doherty, G. 1999. On the use of surrogate species in conservation biology. Conservation Biology 13: 805–814.

Chase, M.W., Soltis, D.E., Olmstead, R.G., Morgan , D. Les, D.H. and Mischler, B.H. 1993. Phylogenetic of seed plants: an analysis of nucleotide sequence from the plastid gene *rbc*L. *Annals of the Missouri Botanical Garden* 80: 528–580.

Chase, M.W., Soltis, D.E., Soltis, P.S., Rudall, P.J., Fay, M.F., Hahn, W.J., Sullivan, S., Joseph, J, Molvray, M., Kores, P.J., Givnish, T.J., Sytsma, K.J. and Pires, J.C. 2000. Higher-level systematics of the monocotyledons: an assessment of current knowledge and a new classification. In Wilson, K.L. and Morrison, D.A. (eds.). *Monocots: Systematics and Evolution.* Pg. 3–16. CSIRO: Melbourne, Australia.

Clement, M., Posada, D and Crandall, K. 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* 9: 1657–-1660.

Davis, J. and Nixon, K. 1992. Populations, genetic variation, and the delimitation of phylogenetic species. *Systematic Biology* 41: 421–435.

Dawson, I.K., Simon, A.J., Waugh, R. and Powell, W. 1995. Diversity and genetic differentiation among subpopulations of *Gliricidia sepium* revealed by PCR-based assays. *Heredity* 75:10–18.

Denton, A.L., McConaughy, B.L. and Hall, B.D. 1998. Usefulness of RNA polymerase II coding sequences for estimation of green plant phylogeny. *Molecular Biology and Evolution* 15:1082–1085.

Despres, L., Gielly, L., Redoutet, B. and Taberlet, P. 2003. Using AFLP to resolve phylogenetic relationships in a morphologically diversified plant species complex when nuclear and chloroplast sequences fail to reveal variability. *Molecular Phylogenetics and Evolution* 27: 185–196.

Dijkshoorn, L., Aucken, H., Gerner-Smidt, P., Janssen, P., Kaufmann, M.E., Garaizar, J., Ursing, J. and Pitt, T.L. 1996. Comparison of outbreak and nonoutbreak *Acinetobacter baumannii* strains by genotypic and phenotypic methods. *Journal of Clinical Microbiology* 34: 1519–1525.

Dransfield, J. 1972. The genus *Johannesteijsmannia* H.E. Moore Jr. *Gardens' Bulletin, Singapore* 26: 63–83.

Dransfield, J., Ferguson, I.K. and Uhl, N.W. 1990. The Coryphoid palms: patterns of variation and evolution. *Annals of the Missouri Botanical Garden* 77: 802–815.

Dransfield, J. 1999. Species concepts in the Old World Palms. In Henderson, A. and Borchsenius, F (eds.). *Evolution, Variation and Classification of Palms*. Memoirs of the Missouri Botanical Garden v. 83.

Dransfield, J., Uhl, N.W., Asmussen, C.B., Baker, W.J., Harley, M.M. and Lewis, C.E. 2005. A new phylogenetic classification of the palm family, Arecaceae. *Kew Bulletin* 60: 559–569.

Dransfield, J., Uhl, N.W., Baker, W.J., Harley, M.M., Asmussen, C.B. and Lewis, C.E. (In prep.). *Genera Palmarum Edition 2*. Royal Botanic Gardens, Kew, Richmond, Surrey, UK.

Excoffier, L., Smouse, P.E. and Quattro, J.M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* 131: 479–491.

Felsenstein, J. 1993. *PHYLIP (Phylogeny Inference Package) version 3.5c.* Distributed by the author. Department of Genetics, University of Washington, Seattle.

Fitch, W.M. 1971. Toward defining the course of evolution: minimal change for a specific tree topology. *Systematic Zoology* 20: 406–416.

Gitzendanner, M.A. and Soltis, P.S. 2000. Patterns of genetic variation in rare and widespread plant congeners. *American Journal of Botany* 87: 783–792.

González-Pérez, M.A.; Caujapé-Castells, J. and Sosa P.A. 2004. Molecular evidence of hybridisation between endemic *Phoenix canariensis* and the widespread *P.*

dactylifera with Random Amplified Polymorphic DNA (RAPD) markers. *Plant Systematics and Evolution*. 247: 165–175.

Govaerts, R. and Dransfield, J. 2005. *World checklist of palms*. Royal Botanic Gardens, Kew, Richmond, Surrey, UK.

Gower, J. 1971. A general coefficient of similarity and some of its properties. *Biometrics* 27: 857–871.

Gunn, B.F. 2004. The phylogeny of the Cocoeae (Arecaceae) with emphasis on *Cocos nucifera*. *Annals of the Missouri Botanical Garden* 91: 502–522.

Hahn, W.J. 2002a. A molecular phylogenetic study of the Palmae (Arecaceae) based on *atp*B, *rbc*L, and 18S nrDNA sequences. *Systematic Biology* 51: 92–112.

Hahn, W.J. 2002b. A phylogenetic analysis of the Arecoid line of palms based on plastid DNA sequence data. *Molecular Phylogenetics and Evolution* 23: 189–204.

Hamrick, J.L. 1993. Genetic diversity and conservation in tropical forest. In Drysdale, R.M., John, S.E.T. and Yapa, A.C. (eds.). *Proceedings of the ASEAN-Canada Symposium on Genetic Conservation and Production of Tropical Tree Seed*. Pg. 1–9. ASEAN-Canada Forest Tree Seed Center, Muaklek, Saraburi, Thailand.

Hamrick, J.L., and Godt, M.J.W. 1989. Allozyme diversity in plant species. In Brown, A.H.D., Clegg, M.J., Kahler, A.L. and Weir, B.S. (eds.). *Plant Population Genetics, Breeding and Genetic Resources.* Pg. 43–63. Sunderland: Sinauer Associate Press.

Hamrick, J.L. and Godt, M.J.W. 1996. Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions of the Royal Society of London: Series B* 351: 1291–1298.

Hamrick, J.L., Godt, M.J.W. and Sherman-Broyles, S.L. 1992. Factor influencing levels of genetic diversity in woody plant species. *New Forest* 6: 95–124.

Hartl, L. and Seefelder, S. 1998. Diversity of selected hop cultivars detected by fluorescent AFLPs. *Theoretical and Applied Genetics* 96: 112–116.

Henderson, A. 2002. Phenetic and phylogenetic analysis of *Reinhardtia* (Palmae). *American Journal of Botany* 89:1491–1502.

Henderson, A. 2004. A multivariate analysis of *Hyospathe* (Palmae). *American Journal of Botany* 91: 953–965.

Henderson, A. and Ferreira, E. 2002. A morphometric study of *Synechanthus* (Palmae). *Systematic Botany* 27: 693–702.

Hillis, D.M. 1987. Molecular versus morphological approaches to systematics. *Annual Review of Ecology and Systematics*. 18: 23–42.

Holsinger, K.E., Lewis, P.O. and Dey, D.K. 2002. A Bayesian approach to inferring population structure from dominant markers. *Molecular Ecology* 11: 1157–1164.

Holsinger, K.E. and Lewis, P.O. 2003. *Hickory: A package for analysis of population genetic data v1.0.* Department of Ecology and Evolutionay Biology, University of Connecticut, Storrs, Connecticut, U.S.A. Available at website http://www.eeb.uconn.edu

Huys, G., Coopman, R., Janssen, P. and Kersters, K. 1996. High-resolution genotypic analysis of the genus *Aeromonas* by AFLP fingerprinting. International *Journal of Systematic Bacteriology* 46: 572–580.

Inoue, H., Nohima, H. and Okayama, H. 1990. High efficiency transformation of *Escherichia coli* with plasmids. *Gene* 96 23–28.

Iwabe, N., Kuma, K., Kashino, H., Hasegawa, M. and Miyata, T. 1991. Evolution of RNA polymerases and branching patterns of the three major groups of archaebacteria. *Journal of Molecular Evolution* 32: 70–78.

Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., Zabeau, M. and Kersters, K. 1996. Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiology* 142: 1881–1893.

Jones, C.J., Edwards, K.J., Castaglione, S., Winfield, M.O., Sala, F., Van der Wiel, C., Vosman, B., Matthes, Daly, A., Brettschneider, R., Maestri, E., Marmiroli, N., Aert, R., Volckaert, G. and Karp, A. 1997. Reproducibility testing of AFLPs by a network of European laboratories. *Molecular Breeding* 3: 381–390.

Juan, A., Crespo, M.B., Cowan, R.S., Lexer, C. and Fay, M.F. 2004. Patterns of variability and gene flow in *Medicago citrina*, an endangered endemic of island in the western Mediterranean, as revealed by amplified fragment length polymorphism (AFLP). *Molecular Ecology* 13: 2679–2690.

Kalinowski, S.T. 2005. Do polymorphic loci require large sample sizes to estimate genetic distances? *Heredity* 94: 33–36.

Karron, J.D. 1987. A comparison of levels of genetic polymorphism and self-compatibility in geographically restricted and widespread plant congeners. *Evolution* 1: 47–58.

Kjaer, A. Barford, A.S. Asmussen, C.B. and Seberg, O. 2004. Investigation of genetic and morphological variation in sago palm (*Metroxylon sagu*; Arecaceae) in Papua New Guinea. *Annals of Botany* 94: 109–117.

Koopman, W.J.M., Zevenbergen, M.J. and Van Den Berg, R.G. 2001. Species relationships in *Lactuca* s.l. (Lactuceae, Asteraceae) inferred from AFLP fingerprintings. *American Journal of Botany* 88: 1881–1887.

Kovach Computing Services. 1999. *Multi-Variate Statistical Package (MVSP) version 3.10b.* Wales, UK.

Lee, S.L., Ng, K.K.S., Saw, L.G., Norwati, A., Siti Salwana, M.H., Lee, C.T., Norwati, M. 2002. Population genetics of *Intsia palembanica* (Leguminosae) and genetic conservation of Virgin Jungle Reserves in Peninsula Malaysia. *American Journal of Botany* 89: 447–459.

Legendre, L. and Legendre, P. 1983. *Numerical Ecology*. Elsevier Scientific Publishing Company, New York, USA.

Lewis, C.E. and Doyle, J.J. 2001. Phylogenetic utility of the nuclear gene malate synthase in the palm family (Arecaceae). *Molecular Phylogenetics and Evolution* 19: 409–420.

Lewis, C.E. and Doyle, J.J. 2002. A phylogenetic analysis of tribe Areceae (Arecaceae) using two low-copy nuclear genes. *Plant Systematics and Evolution* 236: 1–17.

Lewis, C.E. and Martinez, N. 2000. Identify of the *Hyophorbe* palms at the Botanical Gardens of Cienfuegos, Cuba. *Palms* 44: 93–97.

Lihova, J., Marhold, K., Tribsch, A. and Stuessy, T. 2004. Morphometric and AFLP re-evaluation of tetraploid *Cardamine amara* (Brassicaceae) in the Mediterranean. *Systematic Botany* 29: 134–146.

Lim, C.K. and Whitmoore, T.C. 2000. The magnificent *Johannesteijsmannia* palms of Malaysia. *Folia Malaysiana* 2: 13–26.

Lloyd, J.C., Horsnell, P.R., Dyer, T.A. and Raines, C.A. 1991. Structure and sequence of a wheat phosphoribulokinase gene. *Plant Molecular Biology* 17: 167–168.

Loo A.H.B., Dransfield, J., Chase, M.W. and Baker, W.J. 2006. Low-copy nuclear DNA, phylogeny and the evolution of dichogamy in the betel nut palms and

their relatives (Arecinae; Arecaceae). *Molecular Phylogenetics and Evolution* 39: 598–618.

Loo A.H.B., Tan, H.H.W., Kumar, P.P. and Saw, L.G. 1999. Population analysis of *Licuala glabra* Griff. Var. *glabra* (Palmae) using RAPD profiling. *Annals of Botany* 84: 421–427.

Loo, A.H.B., Tan, H.T.W., Kumar, P.P. and Saw, L.G. 2001. Intraspecific variation in *Licuala glabra* Griff. (Palmae) in Peninsular Malaysia — a morphometric analysis. *Biological Journal of the Linnean Society* 72: 115–128.

Lynch, M. and Milligan, B.G. 1994. Analysis of population genetic structure with RAPD markers. *Molecular Ecology* 3: 91–99.

Maddison, D.R. 1991. The discovery and importance of multiple islands of most-parsimonious trees. *Systematic Zoology* 40: 315–328.

Maki, M. and Horie, S. 1999. Random amplified polymorphic DNA (RAPD) markers reveal less genetic variation in the endangered plant *Cerastium fisherianum* var. *molle* than in the widespread conspecific *C. fisherianum* var. *fisherianum* (Caryophyllaceae). *Molecular Ecology* 8: 145–150.

Maki, M., Horie, S. and Yokoyama, J. 2002. Comparison of genetic diversity between narrowly endemic shrub *Menziesia goyozanensis* and its widwspread congener *M. pentandra* (Ericaceae). *Conservation Genetics* 3: 421–425.

Mantel, N. 1967. The detection of disease clustering and generalized regression approach. *Cancer Research* 27: 209–220.

Millar, C.I. and Libby, W.J. 1991. Strategies for conserving clinal ecotypic, and disjunct population diversity in widespread species. In Falk, D.A. and Holsinger, K.E. (eds.) 1991. *Genetic and Conservation of Rare Plants*. Pg. 149–170. Oxford University Press, New York, New Yok, USA.

Miller, M.P., Stevens, L.E., Busch, J.D. Sorensen, J.A. and Keim. P. 2000. Amplified fragment length polymorphism and mitochondrial sequence data detect genetic differentiation and relationships in endangered southwestern U.S.A. ambersnails (*Oxyloma* spp.). *Canadian Journal of Zoology* 78: 1845–1854.

Mitchell, A.D. and Heenan, P.B. 2002. Genetic variation within the *Pachycladon* (Brassicaceae) complex based on fluorescent AFLP data. *Journal of the Royal Society of New Zealand* 32: 427–443.

Moore, H.E. 1961. *Johannesteijsmannia* – a new name for the palm genus *Teysmannia*. *Principes* 5: 116.

Moore, H.E. 1973. The major groups of palms and their distribution. *Gentes Herbarium* 11: 27–141.

Moore, W.S. 1995. Inference of phylogenies from mtDNA variation: mitochondrial gene tree versus nuclear-gene trees. Evolution 49: 718–726.

Mort, M.E. and Crawford, D.J. 2004. The continuing search: low-copy nuclear sequences for lower-level plant molecular phylogenetic studies. *Taxon* 53: 257–261.

Mueller, U.G., Lipari, S.E. and Milgroom, M.G. 1996. Amplified fragment length polymorphism (AFLP) fingerprinting of symbiotic fungi cultured by the fungus-growing ant *Cyphomyrmex minutus*. *Molecular Ecology* 5: 119–122.

Muller, U.G. and Wolfenbarger, L. LaReesa 1999. AFLP genotyping and fingerprinting. *Trends of Ecology and Evolution* 14: 389–394.

Murray, M.G. and Thompson, W.F. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research* 8: 4321–4325.

Nei, M. 1987. *Molecular evolutionary genetics*. Columbia University Press, New Yok, USA.

Nickerson, J. and Drouin, G. 2004. The sequence of the largest subunit of RNA polymerase II is a useful marker for inferring seed plant phylogeny. *Molecular Phylogenetics and Evolution* 31: 403–415.

Nielsen, L.R. 2004. Molecular differentiation within and among island populations of the endemic plant *Scalesia affinis* (Asteraceae) from the Galapagos Islands. *Heredity* 93: 434–442.

Odat, N., Jetschke, G. and Hellwig, F.H. 2004. Genetic diversity of *Ranunculus acris* L. (Ranunculaceae) populations in relation to species diversity and habitat type in grass communities. *Molecular Ecology* 13: 1251–1257.

O'Hanlon, P.C., Peakall, R. and Briese, D.T. 2000. A review of new PCR-based markers and utility to weed ecology. *Weed Research* 40: 239–254.

Oxelman, B. and Bremer, B. 2000. Discovery of paralogous nuclear gene sequences coding for the second-largest subunit of RNA polymerase II (*RPB*2) and their phylogenetic utility in Gentianales of the Asterids. *Molecular Biology and Evolution* 17: 1131–1145.

Oxelman, B. Yoshikawa, N., McConaughy, B.L., Luo, J., Denton, A.L. and Hall, B.D. 2004. *RPB*2 gene phylogeny in flowering plants, with particular emphasis on asterids. *Molecular Phylogenetics and Evolution* 32: 462–479.

Page, R. D. M. 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences* 12: 357–358.

Palm, B.T. and Jochems, S.C.J. 1924. De sang-palm van Sumatra. *Trop. Natuur* 13:9–12.

Peres-Neto, P.R., Jackson, D.A. and Somers, K.M. 2003. Giving meaningful interpretation to ordination axes: assessing loading significant in principal component analysis. *Ecology* 84: 2347–2363.

Pfeil, B.E., Brubaker, C.L., Craven, L.A. and Crisp, M.D. 2004. Paralogy and orthology in the Malvaceae *rpb*2 gene family: investigation of gene duplication in *Hibiscus*. *Molecular Biology and Evolution* 21: 1428–1437.

Radford, A.E., 1986. *Fundamentals of Plant Systematics*. Harper and Row, Publishers, Inc., New Yok, USA.

Rafalski, J.A. and Tingey, S.V. 1993. Genetic diagnostics in plant breeding: RAPDs, microsatellites and machines. *Trends in Genetics* 9: 275–280.

Ranker, T.A. 1994. Evolution of high genetic variability in the rare Hawaiian fern *Adenophorus periens* and implications for conservation management. *Biological Conservation* 70: 19–24.

Roncal, J., Fransisco-Ortega, J., Asmussen, C.B. and Lewis, C.E. 2005. Molecular phylogenetics of tribe Geonomeae (Arecaceae) using nuclear DNA sequences of phosphoribulokinase and RNA polymerase II. *Systematic Botany* 30: 275–283.

Rottenberg, A. and Parker, J.S. 2003. Conservation of the critically endangered *Rumex rothschildianus* as implied from AFLP diversity. *Biological Conservation* 114: 299–303.

Rouppe Van Der Voort, J.N.A.M., P. van Zandvoort, van Eck, H.J., Folkertsma, R.T., Hutten, R.C.B., Draaistra, J., Gommers, F.J., Jacobsen, E., Helder, J. and Bakker, J. 1997. Use of allele specificity of comigrating AFLP markers to align genetic maps from different potato genotypes. *Molecular and General Genetics* 255: 438–447.

Salamin, N., Chase, M.W., Hodkinson, T.R. and Sovolainen, V. 2003. Assesment internal support with large phylogenetic DNA matrices. *Molecular Phylogenetics and Evolution* 27: 528–539.

Sang, T. 2002. Utility of low-copy nuclear gene sequences in plant phylogenetics. *Critical reviews in Biochemistry and Molecular Biology* 37: 121–147.

Sanger F., Nicklen, S. and Coulson, A.R. 1977. DNA sequencing with chain termination inhibitors. *Proceeding of the National Academy of Science USA* 74: 5463–5467.

Sastri, D.C., Hilu, K., Appels, R., Lagudah, E.S., Palyford, J. and Baum, B.R. 1992. An overview of evolution in plant 5S DNA. *Plant Systematics and Evolution* 183: 169–181.

Schneider, S., Roessli, D. and Excoffier, L. 2000. *Arlequin ver. 2.000: A software for population genetics data analysis*. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Sheng, Y., Zheng, W., Pei, K. and Ma, K. 2005. Genetic variation within and among populations of a dominant desert tree *Haloxylon ammodendron* (Amaranthaceae) in China. *Annals of Botany* 96: 245–252.

Small, R.L., Cronn, R.C. and Wendel, J.F. 2004. L.A.S. Johnson Review No.2 Use of nuclear genes for phylogeny reconstruction in plants. *Australian Systematic Botany* 17: 145–170.

Sneath, P.H.A. and Sokal, R.R. 1973. *Numerical Taxonomy*. W.H. Freeman and Company, San Francisco, USA.

Sokal, R.R. and Michener, C.D. 1958. A statistical method for evaluating systematic relationships. *University of Kansas Scientific Bulletin* 38: 1409–1438.

Soltis, P.S. and Soltis, D.E. 2004. The origin and diversification of angiosperms. *American Journal of Botany* 91: 1614–1626.

Soltis, P.S. and Soltis, D.E. 1998. Choosing an approach and appropriate gene for phylogenetic analysis. In Soltis, D.E., Soltis, P.S. and Doyle, J.J. (eds.). *Molecular Systematics of Plants II: DNA Sequencing*. Pg. 1–42. Kluwer Academic Publishers, Boston, US.

Soltis, P.S., Soltis, D.E. and Chase, M.W. 1999. Angiosperm phylogeny inferred from multiple genes as a tool for comparative biology. *Nature* 402: 402–404.

Spiegelhalter, D.J., Best, N.G., Carlin, B.P. and van der Linde, A. 2002. Bayesian measures of model complexity and fit. *Journal of the Royal Statistical Society Series B* 64: 483–689.

Stevens, P. F. 2001 onwards. *Angiosperm Phylogeny Website*. Version 7, May 2006 [and more or less continuously updated since]. http://www.mobot.org/MOBOT/research/APweb/.

Thomas, M.M., Garwood, N.C., Baker, W.J., Henderson, S.A., Russell, S.J., Hodel, D.R. and Bateman, R.M. 2006. Molecular phylogeny of the palm genus *Chamaedorea*, based on the low-copy nuclear genes *PRK* and *RPB*2. *Molecular Phylogenetics and Evolution* 38: 398–415.

Thorpe, R.S. 1976. Biometric analysis of geographic variation and racial affinities. *Biological Reviews* 51: 407–452.

Thorpe, R.S. 1983. A review of numerical methods for recognizing and analyzing racial differentiation. In Felsenstein, J. (ed.). *Numerical Taxonomy*. Pg. 404–423. Springer, New Yok, USA.

Ude, G., Pillay, M., Nwakanma, D. and Tenkouano, A. 2002. Analysis of genetic diversity and sectional relationships in *Musa* using AFLP markers. *Theoretical and Applied Genetics* 104:1239–1245.

Uhl, N.W. and Dransfield, J. 1987. *Genera Palmarum. A classification of Palms based on the work of Harold E. Moore, Jr.* The L. H. Bailey Hortorium and the International Palm Society, Kansas, Lawrence, Allen Press. USA.

Uhl, N.W. and Dransfield, J. 1999. Genera Palmarum after ten years. *Memoirs of the New Yok Botanical Garden* 83: 245–253.

Uhl, N.W., Dransfield, J., Davis, J.I., Luckow, M.A., Hansen, K.S. and Doyle, J.J. 1995. Phylogenetic relationships among palms: cladistic analysis of morphological and chloroplast DNA restriction site variation. In Rudall, P.J., Cribb, P.J., Cutler, D.F. and Humphries, C.J. (eds.). *Monocotyledons: Systematics and Evolution*. Pg. 623–661. Royal Botanical Gardens, Kew, Richmond, Surrey, UK.

van de Wouw, M., Maxted, N., Chabane, K. and Ford-Lloyd, B.V. 2001. Molecular taxonomy of *Vicia* ser. *Vicia* based on amplified fragment length polymorphisms. *Plant Systematics and Evolution* 229: 91–105.

Vekemans, X. 2002. *AFLP-SURV version 1.0*. Distribution by the author. Laboratoire de Génétique et Ecologie Végétale, Université Libre de Bruxelles, Belgium.

Vogelmann, J.E. and Gastony, G.J. 1987. Electrophoresis enzyme analysis of North American and eastern Asian populations of *Agastache* sect. *Agastache* (Labiatae). *American Journal of Botany* 74: 385–393.

Vos, P., Hogers, R., Bleeker, M., Rijans, M., van de Lee, T., Hornes, M., Frijters, A., Plot, J. Peleman, J., Kuiper, M., Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* 23: 4407–4414.

Waugh, R., Bonar, N., Baird, E., Thomas, B., Graner, A. Hayes, P. and Powell, W. 1997. Homology of AFLP products in the three mapping populations of barley. *Molecular Genetics and Genomics* 255: 311–321.

Weir, B.S. and Cockerham, C.C. 1984. Estimating F-statistics for the analysis of population structure. *Evolution* 38: 1358–1370.

Wiens, J.J. and Servedio, M.R. 2000. Species delimitation in systematics: inferring diagnostic differences between species. *Proceeding the Royal Society of London B* 267: 631–636.

Wilson, M.A., Gaut, B. and Clegg, M.T. 1990. Chloroplast DNA evolves slowly in the palm family (Arecaceae). *Molecular Biology and Evolution* 7: 303–314.

Wright, S. 1965. The interpretation of genetic structure by F-statistics with special regard to systems of mating. *Evolution* 19: 355–420.

Yeh, F.C. Population genetics. In Young, A., Boshier, D. and Boyle, T. (eds.) 2000. *Forest Conservation Genetics: Principle and Practice*. Pg. 21–37. CSIRO Publishing, Australia.

Yeh, F.C., Yang, R.C. and Boyle, T. 1999. *POPGENE version 1.31. Microsoft Window-based Freeware for Population Genetic Analysis* (Available at http://www.ualberta.ca/~fyeh/). University of Alberta and Centre for International Forestry Research.

Young, A.B. and Brown, A.H.D. 1996. Comparative population genetic structure of the rare woodland shrub *Daviesia suaveolens* and its common congener *D. mimosoides*. *Conservation Biology* 74: 385–393.

Young, A., Boshier, D. and Boyle, T. 2000. *Forest Conservation Genetics: Principle and Practice*. CSIRO Publishing, Australia.

Zabeau, M. and Vos, P. 1993. Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Application Number: 9240269.7, Publication Number: 0 534 858 A1.

Zawko, G., Krauss, S.L., Dixon, K.W. and Sivasithamparam, K. 2001. Conservation genetics of the rare and endangered *Leucopogon obtectus* (Ericaceae). *Molecular Ecology* 10: 2389–2396.

Zhivotovsky, L.A. 1999. Estimating population structure in diploids with multilocus dominant DNA markers. *Molecular Ecology* 8: 907–913.

Appendix 1. Matrix of reproductive and vegetative data of samples for multivariate analysis using MVSP 3.1.

Label	Lamlen	Lamwid	Lamwb	Petlen	Petwid	Petdep	Pleats	B_Stem	Stemhei	Stemdia	B_Lamund
A1	203	70.5	108	75.5	2.1	1.7	58	0	0	0	0
A2	214.5	78.5	116.5	108	2.4	1.7	57	0	0	0	0
A3	244.4	66.3	132	150.5	2.1	1.6	53	0	0	0	0
A4	218.5	92	79.5	100.5	2.1	1.5	59	0	0	0	0
A5	231.2	72.4	113.8	117	2	1.6	57	0	0	0	0
A6	241.2	100.4	133	80	2.4	2	64	0	0	0	0
A7	214.8	113.4	120.6	68	2.5	1.6	64	0	0	0	0
A8	231.8	96.7	120.3	127.5	2.2	1.4	63	0	0	0	0
A9	234.5	96	105.5	148.5	2.1	1.5	57	0	0	0	0
A10	250.5	63.2	110	164.5	1.8	1.3	58	0	0	0	0
A11	259.5	87.2	121.8	144.3	1.9	1.8	53	0	0	0	0
A12	215.9	79	125	127	1.8	1.5	59	0	0	0	0
A13	208	56.8	110	131.8	1.5	1.3	50	0	0	0	0
A14	245.6	72.2	140.8	143.5	1.8	1.3	53	0	0	0	0
A15	270.5	74	136.5	220.1	2	1.9	59	0	0	0	0
A16	240.9	68.8	99.4	247.5	2.7	1.8	58	0	0	0	0
A17	239.6	76.6	107.4	188.5	2.2	1.7	59	0	0	0	0
A18	204	93	122	232	2	1.5	65	0	0	0	0
A19	193	129	101	176	2.5	2	65	0	0	0	0
A20	207	75	95	168	2.1	1.3	56	0	0	0	0
A21	246	104	124	165	2.6	2	64	0	0	0	0
A22	232	84	106	118	2.3	1.7	59	0	0	0	0
A23	224	105	94	205.5	2.6	1.8	62	0	0	0	0
A24	217	85	90	210	2.2	1.7	56	0	0	0	0
A25	216	101	109	221	2.5	2.1	62	0	0	0	0
A26	248	116	121	210	2.7	3	65	0	0	0	0
A27	210	100	97	199	2.2	1.7	63	0	0	0	0
A28	251	94	113	192	2.3	1.8	64	0	0	0	0
A29	244	100	144	207	2.1	1.9	60	0	0	0	0
A30	251	124	129	123	2.7	1.7	74	0	0	0	0
A31	186	97	103	126	2.4	1.5	74	0	0	0	0
A32	249	115	135	221	2.5	2.4	65	0	0	0	0
A33	221	115	129	159	2.6	2.2	73	0	0	0	0
A34	236	107	104	106	2.4	1.8	67	0	0	0	0
A35	256	96	104.5	121	3	2.2	63	0	0	0	0
A36	237.5	100	110	172	2.5	2.3	58	0	0	0	0
A37	229	110	115	117	2.5	1.6	65	0	0	0	0
A38	269	113	146	213	2.6	2.4	61	0	0	0	0
A39	277	98	111	151	2.7	1.6	59	0	0	0	0
A40	268	94	140	148	2.5	2.3	66	0	0	0	0
A41	261	112	105	205	3.2	3	60	0	0	0	0
A42	280	113	112	164	3	2.9	60	0	0	0	0
A43	288	93	162	126	2.6	2.5	66	0	0	0	0
A44	290	75	151	163	2.5	2	54	0	0	0	0
A45	278	72	135	155	2.3	2.2	52	0	0	0	0
A46	266	59	132	68	2	1.8	44	0	0	0	0
A47	269	64	136	98	2	2	47	0	0	0	0
A48	231.5	51.5	91	61.5	2.5	2.2	52	0	0	0	0
A49	231.5	69	129	66	2.5	2.3	50	0	0	0	0
A50	280	64	143	141	2	2	49	0	0	0	0
A51	229	97	96	102	1.8	1.6	43	0	0	0	0
A52	320	68	158	147	2.5	2.7	49	0	0	0	0
A53	283.5	56	127	107	2.5	1.4	39	0	0	0	0
A54	224	58	123	104	2.5	2	42	0	0	0	0
A55	237	58	142	129.5	2.5	1.5	44	0	0	0	0
A56	221	70.5	122	146	2.5	2	49	0	0	0	0
A57	234.5	77	132	165.5	2.5	2	52	0	0	0	0
A58	282	78	139	157	2.7	2.6	53	0	0	0	0
A59	241	71	140	140.5	2.4	2.5	54	0	0	0	0
A60	281	73	159	120	2.4	2.5	46	0	0	0	0
A61	263	71	137	92.5	2.3	2	49	0	0	0	0
A62	275	77.5	136	98	2.6	2.5	48	0	0	0	0
A63	325	85	143	152	2.5	2.6	49	0	0	0	0
A64	233.5	120.5	110	199	3	2.5	52	0	0	0	0
A65	272	102	116	221	2.7	2.3	63	0	0	0	0
A66	274	85	151	203	2.4	2.2	56	0	0	0	0
A67	291	108	164	242.5	2.9	2.5	66	0	0	0	0
A68	221	96	125	132	2.1	1.7	62	0	0	0	0
A69	253	99	142	152	2.4	1.8	63	0	0	0	0
A70	251	90	116	204	2	1.7	58	0	0	0	0
A71	259	88	132	174	2.4	1.9	61	0	0	0	0
A72	210	85.5	122	232	2.4	2.1	59	0	0	0	0
A73	228.5	100	109.5	194	2.5	2.3	52	0	0	0	0
A74	238.5	93	113	92.5	2.5	2	50	0	0	0	0
A75	254.1	79	144.5	186	2.6	2	59	0	0	0	0
A76	177.5	96	91	117	2.1	1.8	56	0	0	0	0
A77	247	92	113	232.5	2.5	2	54	0	0	0	0
A78	250.5	80	120.5	246.5	2.5	2.2	55	0	0	0	0
A79	251	89	120	173.5	2.6	2.2	56	0	0	0	0
A80	238.5	100	120	129	2.5	2	53	0	0	0	0
A81	187.5	95	91	207	2.5	2.2	59	0	0	0	0
A82	222	78	120	167.5	2.2	2.1	55	0	0	0	0
A83	210	80	103.5	130	2.1	1.8	53	0	0	0	0
A84	197.5	90	98	181	2.4	2.2	54	0	0	0	0

Label	Lamlen	Lamwid	Lamwb	Petlen	Petwid	Petdep	Pleats	B_Stem	Stemhei	Stemdia	B_Lamund
A85	199	103	96	145.7	2.4	2	61	0	0	0	0
A86	244.5	106	122	181	2.9	2.3	63	0	0	0	0
A87	240	110	125	230	2.5	2.3	70	0	0	0	0
A88	241.5	83.5	140	183.5	2	1.8	59	0	0	0	0
A89	165	84.5	82	124	1.8	1.5	63	0	0	0	0
A90	160	89	70	127	2	1.6	62	0	0	0	0
A91	180	79	83	136	2.1	1.9	57	0	0	0	0
A92	175.5	73.5	93	122	1.7	1.5	60	0	0	0	0
A93	217.2	93	89	167	2.1	1.9	63	0	0	0	0
A94	189	93	76	194	2.4	1.9	54	0	0	0	0
A95	221.8	84	108	117.5	2.5	2	52	0	0	0	0
A96	170	87	84	160	2	1.5	56	0	0	0	0
A97	180	87	88	137	1.9	1.4	59	0	0	0	0
A98	267	105.5	133	215	3.2	2.8	59	0	0	0	0
A99	214.6	79	95	149	2.1	1.8	49	0	0	0	0
A100	186	84	100.5	181.5	2.5	1.8	54	0	0	0	0
A101	224.5	105	123	177	2.5	2.2	61	0	0	0	0
A102	197	94	100	146.5	2	1.9	58	0	0	0	0
A103	243	95	128	243	2.4	1.9	65	0	0	0	0
A104	238	105	112	178	2.5	2.3	55	0	0	0	0
A105	186	70	95	133	1.8	1.6	50	0	0	0	0
L1	223	45	124	157	2	1.7	41	0	0	0	0
L2	215	43	126	144.5	1.9	1.8	43	0	0	0	0
L3	248	48	140	120	2.1	1.5	40	0	0	0	0
L4	228	61	119	126	2.2	2	52	0	0	0	0
L5	195	53	89	104	1.7	1.8	45	0	0	0	0
L6	241.5	40	94	139	1.9	1.6	44	0	0	0	0
L7	190	51	108	133	1.7	1.3	44	0	0	0	0
L8	173	39	96	136	1.6	1.3	42	0	0	0	0
L9	242.5	34.5	102	105	1.7	1.4	44	0	0	0	0
L10	211.5	41.5	79.5	64	2	1.5	45	0	0	0	0
L11	217	31.5	95	27.5	1.7	1.2	43	0	0	0	0
L12	223.5	45.5	81	73.5	1.8	1.5	41	0	0	0	0
L13	240	46	111.5	121	1.8	2	45	0	0	0	0
L14	222	36.5	112	159	2	1.5	46	0	0	0	0
L15	215	40	80.5	71	1.7	1	38	0	0	0	0
L16	253	45	116	192	1.4	2.2	44	0	0	0	0
L17	294	34.5	142	138.5	2.5	2	47	0	0	0	0
L18	218	26	68	135.9	1.5	1.3	35	0	0	0	0
L19	225.5	35	106	161	1.8	1.7	44	0	0	0	0
L20	210	27	104	118	1.3	1.4	40	0	0	0	0
L21	280	32.5	115	115	2.2	2.3	40	0	0	0	0
L22	253	25	122.5	142	1.3	1.3	35	0	0	0	0
L23	212	28	83.5	89	1.3	1.2	36	0	0	0	0
L24	299	26.5	126	137	1.8	1.6	39	0	0	0	0
L25	257.5	35	122	135	1.8	1.7	42	0	0	0	0
M1	220	123	115	180	2.6	1.9	61	0	0	0	1
M2	219	99	107	233	2.3	2	61	0	0	0	1
M3	198	102	100	167	2.1	1.9	64	0	0	0	1
M4	236	120	123	223	2.6	2.3	61	0	0	0	1
M5	195	109	104	171	2.3	2	61	0	0	0	1
M6	180	85	95	143	1.9	1.9	52	0	0	0	1
M7	173	113	84	171	2.2	2	63	0	0	0	1
M8	248	98	110	239	2.5	2	61	0	0	0	1
M9	289	134	139	249	3	2.1	70	0	0	0	1
M10	237	129	119	208	2.8	2.3	73	0	0	0	1
M11	257	135	121	220	3.5	2.6	73	0	0	0	1
M12	186	115	97	205	2.7	2	58	0	0	0	1
M13	252	112	113	222.5	2.9	2.4	71	0	0	0	1
M14	246	118	107	224	3	2.5	71	0	0	0	1
M15	222	138	102	139	2.7	2.2	68	0	0	0	1
M16	243	125	119	180	3	2.8	69	0	0	0	1
M17	309	117	153	183	2.5	2.3	66	0	0	0	1
M18	295	125.5	167	182	2.7	2.5	67	0	0	0	1
M19	276	123	113	147	3.1	2.4	64	0	0	0	1
M20	277	121	127	174	3	2.4	62	0	0	0	1
M21	253	109	129	186	2.4	2	65	0	0	0	1
M22	250	148	128	216	3	2.1	65	0	0	0	1
M23	315	113	136	247	3.5	2.5	67	0	0	0	1
M24	221	130	123	237	3	2.4	67	0	0	0	1
M25	221	119	120	142	2.1	1.8	62	0	0	0	1
M26	231	97	109	183	2.1	2	58	0	0	0	1
M27	227	89	107	134	2.1	1.9	59	0	0	0	1
M28	255	129	146	131	2.7	2	74	0	0	0	1
M29	195	117	97	82	2.1	1.9	61	0	0	0	1
M30	201	99	118	107	2.9	2.1	62	0	0	0	1
M31	224	103	94	89	2.5	2	58	0	0	0	1
M32	226	121	117	120	2.7	2	62	0	0	0	1
M33	237.5	102.5	134	183.5	2.7	2.5	58	0	0	0	1
M34	194.5	71.5	118	160	1.9	1.8	57	0	0	0	1
M35	198	73	103	135	2.4	2.2	54	0	0	0	1
M36	231	101	115	196	2.8	2.5	60	0	0	0	1
M37	194	74	96	124	1.8	1.5	53	0	0	0	1
M38	243	112	144	197	2.4	2.1	68	0	0	0	1
M39	191.5	100	103	158	2.2	1.9	59	0	0	0	1
M40	221	100	132	232	2.5	2	64	0	0	0	1

Label	Lamlen	Lamwid	Lamwb	Petlen	Petwid	Petdep	Pleats	B_Stem	Stemhei	Stemdia	B_Lamund
P1	293	112	156	157	3	2	66	1	43	49	0
P2	208	105	126	92	5	3	60	1	176	48	0
P3	254	108	123	239	2.4	1.8	68	1	88	50	0
P4	225	81	127	128	2	1.2	58	1	131	41	0
P5	261	84	148	195	2.3	1.7	54	1	227	38	0
P6	265	64	121	224	2	1.8	31	1	25	51	0
P7	349	89	124	303	3.1	2.6	76	1	35	57	0
P8	268	94	133	217	2.5	1.8	63	1	70	49	0
P9	295	77	112	211	1.9	1.7	62	1	90	52	0
P10	199	77	87	169	1.7	1.8	58	1	62	62	0
P11	242	92	120	224	2.5	2.5	62	1	87	105	0
P12	208	81	97	138	2	1.5	62	1	74	33	0
P13	162	80	76	163	1.4	1.1	62	1	50	58	0
P14	211	79	94	188	1.7	1.5	60	1	75	46	0
P15	243	96	131	151	2.1	1.9	60	1	164	38	0
P16	216	92	103	124	1.9	1.6	58	1	181	46	0
P17	235	91	115	204	2.1	1.9	64	1	255	42	0
P18	260	87	103	246	2	1.7	63	0	0	0	0
P19	261	98	122	216	2.2	1.6	62	1	58	64	0
P20	237	80	125	144	1.6	1.8	60	0	0	0	0
P21	275	96	168	287	2.1	1.7	55	0	0	0	0
P22	213	78	121	163	1.5	1.9	56	1	145	43	0
P23	230	78	102	93	2	1.7	62	1	118	55	0
P24	196	69	112	107	1.5	1.7	48	1	96	39	0
P25	258	108	129	121	2.6	2.1	69	1	126	16	0
P26	266	109	111	163	3	3	65	1	145	49	0
P27	243	107	120	169	2.8	2.2	76	1	72	45	0
P28	244	113	104	119	2.5	2	62	1	106	34	0
P29	214	97	89	120	2.4	2	67	1	285	39	0
P30	250	111	104	120	2.1	2	67	1	193	39	0
P31	266	95	115	130	2.5	2.3	59	1	122	45	0
P32	246	98	119	127	2.5	2	67	1	520	48	0