MOLECULAR SYSTEMATICS OF THE GENUS *GONIOTHALAMUS* AND RELATED GENERA IN SOUTH-EAST ASIA

Miss Maliwan Nakkuntod

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biological Sciences
Faculty of Science
Chulalongkorn University
Academic Year 2005

ISBN 974-14-1762-4

228/50

โครงการพัฒนาองค์ความรู้และศึกษานโยบายการจัดการทรัพยากรชีวภาพในประเทศไทย
c/o ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ
อาคารสำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ
73/1 ถนนพระรามที่ 6 เขตราชเทวี
กรุงเทพฯ 10400

ซิสเต็มมาติกระดับโมเลกุลของพืชสกุลปาหนันช้างและสกุลใกล้เคียง ในเอเชียตะวันออกเฉียงใต้

นางสาวมลิวรรณ นาคขุนทด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาศาสตร์ชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2548

ISBN 974-14-1762-4

MOLECULAR SYSTEMATICS OF THE GENUS *GONIOTHALAMUS* AND RELATED GENERA IN SOUTH-EAST ASIA

Miss Maliwan Nakkuntod

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biological Sciences
Faculty of Science
Chulalongkorn University
Academic Year 2005
ISBN 974-14-1762-4

Thesis Title	Molecular Systematics of the Genus *Goniothalamus* and Related Genera in South-East Asia
By	Maliwan Nakkuntod
Filed of Study	Biological Sciences
Thesis Advisor	Tosak Seelanan, Ph.D.
Thesis Co-advisor	Associate Professor Richard M.K. Saunders, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctor's Degree

T. Vitidsant ..... Deputy Dean for Administrative Affairs,
Acting Dean, The Faculty of Science

(Associate Professor Tharapong Vitidsant, Ph.D.)

THESIS COMMITTEE

Pongtharin Lotrakul ..... Chairman

(Assistant Professor Pongtharin Lotrakul, Ph.D.)

..... Thesis Advisor

(Tosak Seelanan, Ph.D.)

..... Thesis Co-advisor

(Associate Professor Richard M.K. Saunders, Ph.D.)

Chumpol Khunwasi ..... Member

(Chumpol Khunwasi, Ph.D.)

..... Member

(Associate Professor Somsak Panha, Ph.D.)

Piya Chalermglin ..... Member

(Piya Chalermglin, Ph.D.)

S. Suddee ..... Member

(Somran Suddee, Ph.D.)

นางสาวมลิวรรณ นาคขุนทด : ซิสเต็มมาติกระดับโมเลกุลของพืชสกุลปาหนันช้างและสกุลใกล้เคียงในเอเชียตะวันออกเฉียงใต้ (MOLECULAR SYSTEMATICS OF THE GENUS *GONIOTHALAMUS* AND RELATED GENERA IN SOUTH-EAST ASIA) อาจารย์ที่ปรึกษา : อาจารย์ ดร. ต่อศักดิ์ สีลานันท์ อาจารย์ที่ปรึกษาร่วม : Associate Professor Dr. Richard M.K. Saunders จำนวนหน้า 75 หน้า. ISBN 974-14-1762-4.

พืชสกุลปาหนันช้าง (*Goniothalamus* Hook. f. & Thomson) เป็นสกุลใหญ่ที่สุดสกุลหนึ่งของพืชในวงศ์กระดังงา (Annonaceae) มีการกระจายพันธุ์อยู่ในเขตร้อนและกึ่งเขตร้อนในทวีปเอเชีย ที่ผ่านมาการศึกษาความสัมพันธ์ทางวิวัฒนาการของพืชสกุลนี้มีเพียงเล็กน้อย ดังนั้นเพื่อศึกษาประวัติการวิวัฒนาการและความสัมพันธ์ระหว่างสมาชิกในสกุลนี้ และเข้าใจถึงวิวัฒนาการของลักษณะสัณฐานบางประการ จึงใช้ลำดับเบสในคลอโรพลาสต์ คือ ลำดับเบสระหว่างยีน *trnL-F* และในนิวเคลียส คือ ส่วนของ ITS มาทำการวิเคราะห์ความสัมพันธ์ทางสายวิวัฒนาการ จากผลการวิเคราะห์สายวิวัฒนาการพบว่าพืชสกุลปาหนันช้างเป็นวงศ์วานเดี่ยว (monophyletic group) โดยมีสายวิวัฒนาการข้าวหลาม (*G. tamirensis*) และปาหนันจิ๋ว (*G. elegans*) เป็นสายวิวัฒนาการสายแรกที่แยกออกมา ส่วนชนิดอื่นที่เหลือในสกุลนี้ต่อมาจึงแยกย่อยและมีสายวิวัฒนาการใหญ่ๆ 4 สาย การศึกษาวิวัฒนาการของลักษณะทางสัณฐานวิทยาจำนวน 43 ลักษณะ พบว่าลักษณะส่วนใหญ่เป็นลักษณะที่เกิดขึ้นในหลายสายวิวัฒนาการที่ไม่เกี่ยวข้องกัน (homoplastic) แต่อย่างไรก็ตามใน 43 ลักษณะนี้มี 9 ลักษณะที่อาจเป็นประโยชน์ต่อการจัดหมวดหมู่ โดยอาจจะเป็นลักษณะก้าวหน้าร่วม (synapomorphic) ในสายวิวัฒนาการบางสาย ผลการวิเคราะห์ความสัมพันธ์ทางวิวัฒนาการมีความแตกต่างกับการจัดหมวดหมู่ในระดับต่ำกว่าสกุลของ Bân อย่างมาก ดังนั้นจึงควรมีการจัดหมวดหมู่พืชในสกุลนี้ใหม่โดยใช้ผลการวิเคราะห์ข้อมูลทางโมเลกุลและทางสัณฐานวิทยาร่วมกัน

สาขาวิชา.....วิทยาศาสตร์ชีวภาพ.....ลายมือชื่อนิสิต.....

ปีการศึกษา.....2548.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4473827223 : BIOLOGICAL SCIENCES

KEY WORDS: *Goniothalamus* / molecular systematics / phylogenetic analysis / *trnL-F* intergenic spacer /internal transcribed spacer/ Annonaceae

MALIWAN NAKKUNTOD : MOLECULAR SYSTEMATICS OF THE GENUS *GONIOTHALAMUS* AND RELATED GENERA IN SOUTH-EAST ASIA.

THESIS ADVISOR : TOSAK SEELANAN, Ph.D., THESIS CO-ADVISOR : RICHARD M.K. SAUNDERS, Ph.D. 75 pp. ISBN 974-14-1762-4.

The genus *Goniothalamus* Hook. f. & Thomson is one of the largest genera of the family Annonaceae. Its members are widespread in tropical and subtropical Asia. Little is known of the phylogenetic relationships within the genus. Thus, to evaluate evolutionary history and relationships among its members and to understand the evolution of selected morphological character, the *trnL-F* intergenic spacer and nuclear ITS region markers were employed. The results suggested that *Goniothalamus* is likely to be monophyletic, with the *G. tamirensis-G. elegans* clade sister to the rest of the genus. The larger clade was divided into four recognizable subclades. Almost 43 morphological characters when evaluated on the molecular tree were homoplastic. However, as many as 9 characters were partial informative as these are synapomorphic for some clades. The phylogeny was highly incongruent with Bân's classification; thus a new classification scheme should be proposed based on new evidence of molecular and morphological analyses.

Field of Study.....Biological Sciences...Student's signature.....Nakkuntod M.....

Academic year.....2005.....Advisor's signature.....

Co-advisor's signature.....

Acknowledgements

I would like to thank my advisor, Dr. Tosak Seelanan, and Associate Professor Dr. Richard M.K. Saunders, who have been admirable colleagues and mentors. Their expertise and accurate judgment has been invaluable for my work. Their kind support and encouragement has been appreciative. Acknowledgements are extended to my committee, Assistant Professor Dr. Pongtharin Lotrakul, Dr. Chumpol Khunwasi, Associate Professor Dr. Somsak Panha, Dr. Piya Chalermglin, and Dr. Somran Suddee, for their valuable comments and suggestions, which considerably improved this dissertation.

I would like to acknowledge these sources of plant materials, namely Dr. Piya Chalermglin, Dr. Shumpei Kitamura, Ms. Siriwan Nakkuntod, Hornbill Thailand Project, The University of Hong Kong, Nationaal Herbarium Nederland, Leiden branch and The Arnold Arboretum, Harvard University Herbaria. I appreciate for the research facilities at Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand, and Department of Ecology and Biodiversity, The University of Hong Kong, China. This study has been supported by the TRF/BIOTEC Special Program for Biodiversity Research and Training grant T_147016; Center of Excellence in Biodiversity, Faculty of Science, Chulalongkorn University (CEB_D_2_2005) and Graduate School; Commission on Higher Education, Ministry of Education, Thailand, and Biological Science Ph.D. Program, Faculty of Science, Chulalongkorn University. I am grateful for their support. The Department of Biology, Faculty of Science, Naresuan University, permitted the author to pursue Ph.D. study with no hesitation, which I am thankful.

I am deeply in debt to the assistance and kind supports of Yvonne Su, Zhou Lin Lin, Wang Jing and Heidi Kong, The University of Hong Kong. Acknowledgements are extended to Associate Professor Dr. Kumthorn Thirakhupt, Associate Professor Dr. Surin Piyachoknakul, Mrs. Nitaya Homchan, Mr. Nakarin Sukwatnangkul, all friends and staff in Department of Botany; also to colleagues in the Biological Science Ph.D. Program, Chulalongkorn University, and all mentors in Department of Biology, Faculty of Science, Naresuan University, Phitsanulok.

Last, but not least, family members of the Nakkuntods owed my gratefulness. They all, especially Mr. Chan and Mrs. Suwannee Nakkuntod, parents; Mrs. Bubpapan Wongphu-gna and Ms. Siriwan Nakkuntod, sisters; have been so helpful in completing this degree. Without whose endless patience and encouragement, I could not have been able to start nor finish this work. I would like to express my deep gratitude and dedicate this degree to them.

Table of Contents

List of Tables

List of Figures

Figures Page

CHAPTER I

GENERAL INTRODUCTION

The Annonaceae is a pantropical family, with approximately 200 genera and 2500 species. There are two related North America genera, but the remaining taxa are all tropical, displaying high continental generic endemism (Keßler, 1993; Doyle and Le Thomas, 1996). Annonaceae have been included in the order Magnoliales by various taxonomists (APG II, 2003). Morphological and more recent molecular cladistic analyses have continued to place Annonacece in the Magnoliales along with Magnoliaceae, Degeneriaceae, Himantandraceae, Eupomatiaceae and Myristicaceae (Donoghue and Doyle, 1989; Chase et al., 1993; Doyle and Endress, 2000; Soltis et al., 2000). Eupomatiaceae which were previously included as a subfamily within Annonaceae and have always been considered closely related based on morphological characters (Morawetz, 1988) was proposed by Qiu et al. (2000) as the sister to Annonaceae. From the analysis using *rbcL* and *trnL-F* DNA sequence data, Richardson et al. (2004) separated Annonaceae into 3 groups, namely the basal grade, the long branch clade and the short branch clade.

The genus *Goniothalamus* Hook. f. & Thomson, belonging to the long branch clade is one of the largest and most important genera of Annonaceae, with over 150 species distributed throughout tropical south-east Asia; the center of diversity lies in Indochina and western Malesia (Sumatra, Peninsular Maleysia and Borneo) (Saunders, 2003). Based on molecular clade, the estimated age of *Goniothalamus* is in the range of 3.6-4.8 ± 1.5 Myr ago (Richardson et al., 2004). Little is known of the evolution and biogeographical history of this genus. Many morphological characters are ambiguous and reveal little phylogenetically useful information. Thus molecular systematics has become an alternative approach for determining evolutionary relationships because morphological and other phenotypic characters are either absent or change too rapid to be useful for phylogenetic inference. So far, many studies in the genus *Goniothalamus* are based on morphological characters, thus it is impossible to conduct any tests on morphological homology and/or evolutionary changes in this genus. The objectives of

this dissertation were (1) to investigate the delimitations and phylogenetic relationships of the genus *Goniothalamus*, (2) to test the monophyly of *Goniothalamus*, (3) to infer the phylogenetic relationships within and among the genus *Goniothalamus* and related genera using nuclear and chloroplast genes, and (4) to use the phylogenetic reconstruction as a framework to infer character evolution in this group. These regions have been widely used to infer phylogenetic relationships within the Annonaceae (Doyle, Bygrave and Le Thomas, 2000; Meade, 2000; Erken, 2002; Mols, Keßler and Gravendeel, 2002; Chatrou et al., 2002; Richardson et al., 2004; Scharaschkin and Doyle, 2005)

The results from this study enable a clarification of the relationships and phylogeny within the genus *Goniothalamus* and related genera and understanding the evolutionary history and biogeography of this genus. Moreover, this study was to realize which morphological characters are phylogenetically informative in *Goniothalamus*.

CHAPTER II

THE OVERVIEW OF THE FAMILY ANNONACEAE AND GENUS *GONIOTHALAMUS*

Annonaceae (custard apple family) are a pantropical family of shrubs, trees and lianas. The family is a member of Magnoliales and basally positioned within the angiosperm (Soltis et al., 2000; APG II, 2003). The estimated age of stem Annonaceae according to Wikstrom, Savolainen and Chase (2001) is between 82 and 91 Myr ago. This proposed date is confirmed by Richardson et al. (2004) whose estimated age of the family obtained when calibrating the tree using *Archaeanthus* is 90.6 ± 1.3 Myr ago and placed the family at the stem of Magnoliaceae. The oldest remains of the family are seeds with a perichalazal ring and endosperm with lamelliform rumination that were found in the Maastrichtian of Nigeria (Chesters, 1955) and primitive pollen morphology from Columbia (Sole de Porta, 1971). This evidence suggests that the Annonaceae originated in west Gondwanaland (Richardson et al., 2004). Post-Eocene cooling presumably resulted in disjunctive distributions of taxa between tropical Africa, Asia and the New World. *Goniothalamus-Neostenanthera* African-Southeast Asian (34.9-41.1 ± 2.8 Myr ago) estimated splits could be the result of such a disruption of boreotropical vegetation (Pennington and Dick, 2004). From age estimates for the diversification of species-rich genera, the most rapid radiation seems to have taken place in the Southeast Asian genus *Goniothalamus*, whose crown is estimated at 3.6-4.8 ± 1.5 Myr ago (Richardson et al., 2004).

Although the position of Annonaceae among the flowering plants and their monophyly are not disputed (Doyle et al., 2004), the relationships of the genera within the family are not well understood. Morphological characters that are useful for the delimitation of genera and species are also overlaped at higher taxonomic levels (e.g. tribal level). More or less formal classification based on intuition or phenetic analyses of morphological characters (Hutchinson, 1923, 1964; Sinclair, 1955; Fries, 1959; Walker, 1971; Van Heusden, 1992; Van Setten and Koek-Noorman, 1992; Keßler, 1993; Koek-

Noorman, Van Setten and Van Zuilen, 1997) do not accurately predict relationships among genera in Annonaceae.

The genus *Goniothalamus* is one of the largest and most important genera of the Annonaceae in Asia. Widespread in tropical and subtropical Asia (Figure 2.1), they primarily occur in Malesia but a few species also occur in China, the Indian subcontinent and northern Australia. To date, more than 150 taxa have been described in *Goniothalamus*; about 120 species and 10 subspecies/varieties are currently recognized (Mat-Salleh, 1993; Saunders, 2002, 2003). Most species of *Goniothalamus* are small monocaulous treelets. Hence, they have not been used as major commercial timber forest products. Nevertheless, many of the species have promising medicinal value. They have been widely utilized in traditional medicinal practices. *Goniothalamus* are especially popular among Asian native women as post-natal medicines and abortifacients (Burkill, 1935; Perry, 1980; Mat-Salleh, 1989). The genus is characterized by axillary (or slightly supra-axillary) flowers that are generally pendent. As with most Annonaceae, the flowers have three sepals and two whorls of three petals. The outer petals are typically larger than the inner (although sometimes only slightly so), and the inner petals covers over the reproductive organs, forming a distinctive mitriform dome. The flowers are bisexual, with numerous free stamens and carpels. The stamens have broad apical connectives that are variable in shape, ranging from truncate to apiculate, and thecae that are septate; the pollen is released as tetrads. The apocarpous fruits are taxonomically very important, with variation in size, shape, indument, peduncle and stipe length (Saunders, 2003).

A widely used infrafamilial classification of the Annonaceae was introduced by Bentham and Hooker (1862), in which they placed the genera into five tribes based on the aestivation of calyx and corolla and the structure of the stamen connective. In their classification, the genus *Goniothalamus* was considered a member of the tribe *Mitrephoreae* (whose inner petals curve over the sexual organs forming a dome-shaped "mitriform" structure) together with *Mitrephora*, *Pseuduvaria*, *Friesodielsia*, *Orophea*, *Popowia* and *Neo-uvaria*. In Ridley's *Flora of the Malay Peninsula* (1922), the Annonaceae were grouped into six tribes. *Goniothalamus* was in the tribe *Mitrephoreae*,

along with *Orophea*, *Oxymitra*, *Mitrephora* and *Popowia*. Sinclair's (1955) revision of the Peninsular Malaysian Annonaceae, however, noted that while *Orophea* fits the circumscription of the tribe *Mitrephoreae* by virtue of the characters of its inner petals, some members of this genus also have unusual stamens that associate it with tribe *Miliuseae*. Sinclair further noted that the inner petals of *Mitrephora*, *Popowia* and *Neo-uvaria* are united only at the beginning of flower development and are separated at anthesis. Only flowers of *Goniothalamus*, *Friesodielsia* and *Pseuduvaria* have a true mitriform dome during anthesis. Based on floral characters, Sinclair (1955) suggested that *Friesodielsia* is perhaps the nearest relative of *Goniothalamus*. Members of *Friesodielsia*, however, are exclusively scandent lianas, a habit unknown in *Goniothalamus*, and the leaves are generally small. Furthermore, Sinclair inferred that the closest genus to *Goniothalamus* is probably *Mitrephora*, but this genus has different and unique chartaceous leafy outer petals and the mitriform inner petals are free before anthesis, spreading and generally much smaller than the inner petals of *Goniothalamus*.

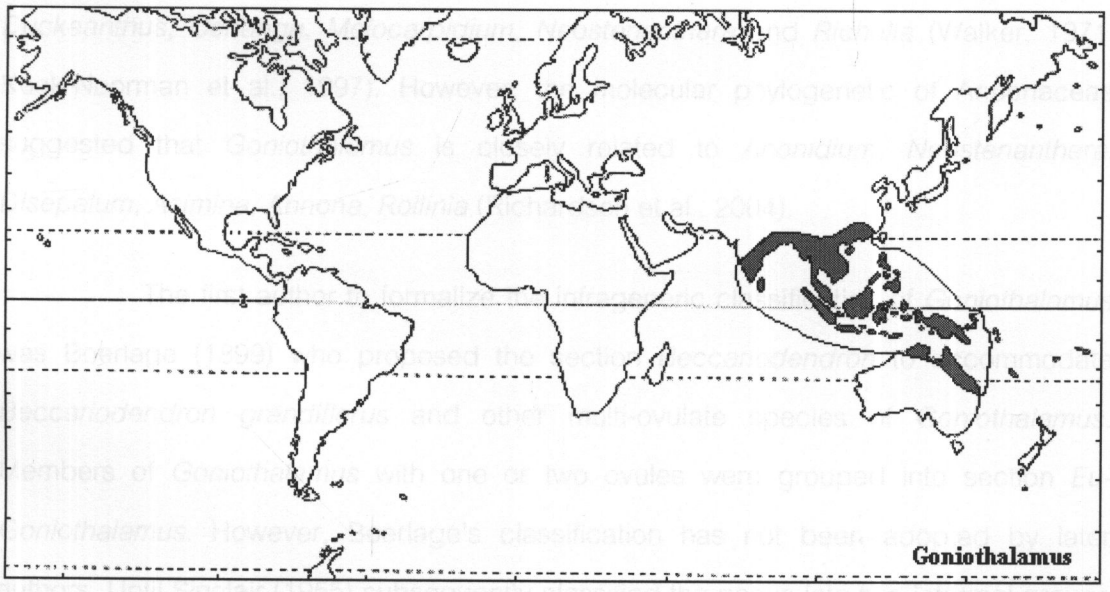


Figure 2.1 The distribution of *Goniothalamus*

Bentham and Hooker's classification (1862) was further modified by Fries (1959). Fries subdivided Bentham and Hooker's tribe into 14 new groups based on additional characters such as the position of the inflorescence, the number and position

of the ovules, fruit types, the presence of the floral bracts and stamen and thecae characters. Walker (1971) used Fries's classification as the basis of an infrafamilial classification of the family, enhanced yet using pollen characteristics. These characters are similar to the pollens of *Anaxagorea*, *Piptostigma*, *Xylopia*, *Fusaea*, *Duckeanthus*, *Cananga*, *Meiocarpidium*, *Neostenanthera* and *Richella*, thus grouped in "the *Fusaea* Subfamily" in Walker's classification. Recently, there have been several morphological and molecular cladistic studies of the angiosperm family Annonaceae, all of which have indicated that *Goniothalamus* is in the "long branch clade" together with *Dasymaschalon*, *Friesodielsia*, *Annona*, *Rollinia*, *Asimina*, *Disepalum*, *Anonidium* and *Neostenanthera* (Doyle and Le Thomas, 1994; Richardson et al., 2004).

There is conflicting opinion about the generic delimitation and relationships among *Goniothalamus* and related genera. On the one hand, it has been suggested that *Goniothalamus* is related to *Pseuduvaria*, *Orophea*, *Oxymitra*, *Mitrephora* and *Popowia* (Sinclair, 1955; Fries, 1959). On the other hand, it has been suggested that *Goniothalamus* is more closely related to *Anaxagorea*, *Piptostigma*, *Xylopia*, *Fusaea*, *Duckeanthus*, *Cananga*, *Meiocarpidium*, *Neostenanthera* and *Richella* (Walker, 1971; Koek-Noorman et al., 1997). However, the molecular phylogenetic of Annonaceae suggested that *Goniothalamus* is closely related to *Anonidium*, *Neostenanthera*, *Disepalum*, *Asimina*, *Annona*, *Rollinia* (Richardson et al., 2004).

The first author to formalize the infrageneric classification of *Goniothalamus* was Boerlage (1899) who proposed the section *Beccariodendron* to accommodate *Beccariodendron grandiflorus* and other multi-ovulate species of *Goniothalamus*. Members of *Goniothalamus* with one or two ovules were grouped into section *Eu-Goniothalamus*. However, Boerlage's classification has not been adopted by later authors. Until Sinclair (1955) subsequently classified the genus into two informal groups using stamen characters, apiculate and convex stamen connectives, because they are easier to be observed in herbarium specimen.

Walker (1971) studied pollen characteristics of nine species of *Goniothalamus*. His report indicated that the pollen of *Goniothalamus* consists of

tetrahedral or tetragonal tetrads, with heteropolar, bilateral, cataulcerate, disc-like concave-convex grains. The grains are comparatively large to very large, averaging 95 µm in diameter. They are microtectate, with no discernible columellae, and wide pitted or otherwise psilate exine. However, the morphology of pollen and tetrads observed in this study did not offer enough diversity to be useful for classification at the species level. The pollen of *Goniothalamus* is more or less homogenous throughout the genus. When Le Thomas (1981a, 1981b) disclosed more remarkable structural diversity of Annonaceae pollen, it was found that all Annonaceae pollen lacks endexine, a feature otherwise found in the monocotyledons. She also found that the reduction of pollen ultrastructure is paralleled by floral reduction and concluded that the family is perhaps finished an evolution.

Bân (1974a) reported that the single most taxonomically important part of the stamen is the connective, especially the tip. In fact, stamen connectives have been widely utilized in traditional classification to divide *Goniothalamus* into subgenera or sections. Thus Bân (1974b) proposed his infra-generic classification. Bân was doing little more than formalizing existing "dichotomous keys" as a supraspecific classification, and that none of his sections/subsections have been assessed to determine whether they are truly monophyletic. He divided the genus into two subgenera using staminal connective shape, *Goniothalamus* (apiculate stamens) and *Truncatella* (truncate stamens). He further proposed four sections, two sections in each subgenus, based on the shapes of the stigma and styles. In addition, he used number of ovules only at the subsection level. However, some species have intermediate stamen characters between two subgenera.

According to the surveys to establish the base chromosome number in the Annonaceae, the genus *Goniothalamus* is not well represented in accounts of chromosome data for the family. Only five papers provide original chromosome counts for the genus. Reports by Ehrendorfer et al. (1968), Sobha and Ramachandran (1979), Okada and Ueda (1984), Sauer and Ehrendorfer (1984) and Morawetz (1988) for *G. grandiflorus*, *G. microphyllus*, *G. opacus*, *G. wynadensis* and *G. australis* show a consistent count of $2n = 16$.

Annonaceae woods presented fine continuous tangential bands of parenchyma rays, occurring with remarkable uniformity and consistency among all genera. In contrast to the rather primitive floral characters, however, woods of Annonaceae seem to attain a high level of anatomical specialization (Wyk and Canright, 1956). Later Blunden and Jewers (1973, 1974a, b) mentioned the anatomical analysis for Bornean *Goniothalamus*, *G. andersonii*, *G. macrophyllus*, *G. malayanus* and *G. velutinus* that their leaf, stem and root anatomical features can be used to differentiate taxa.

Most *Goniothalamus* species are concentrated in West Malesia with high percentage of endemics in Indochina, Borneo, the Philippines and New Guinea. In his revision of Bornean representative, Mat-Salleh (1993) showed that Borneo has a remarkable representation of richness and diversity of the genus. A total of 30 species, including 11 new species, of the genus are currently recognized from Borneo, the largest number represented in any single biogeographic area. Based on inflorescence, inner petal dome, stamen, gynoecium and leaf characteristics, 11 informal species alliances were established for the Bornean species. Twenty-four species are local endemics and 10 Bornean species are known from very few localities. The distribution of these species in Borneo and other areas and the high percentage of endemics suggested that the most of these *Goniothalanus* species are locally evolved.

Mat-Salleh, Lim and Ratnam (2000) studied PCR-DAMD using M13 universal primer to determine generic relatedness between populations of *G. umbrosus*. The result showed that populations of kenerak in Kelantan are genetically close to each other. However, Penang populations were a distinct cluster of their own. In contrary, individuals from Perlis showed close relationship with other individuals from Kelantan. This might indicate that Penang population is not an introduced population but an isolated natural population. Genetic similarity of Perlis-Kelantan populations suggested that *G. umbrosus* is in fact a southern Thailand element, and they moved southward or introduced as a medicinal plant in the east and west coast of Peninsular.

CHAPTER III

ANALYSIS OF MOLECULAR DATA IN THE GENUS *GONIOTHALAMUS* USING NUCLEAR DNA AND CHLOROPLAST DNA MARKERS

3.1 INTRODUCTION

In the past, taxonomic data was generally collected from morphological variation. They are useful for the delimitation of genera and species but have overlap at higher taxonomic level (e.g. tribal level). More or less formal classification based on intuition or phenetic analyses of morphological characters do not accurately predict relationships between genera (Koek-Noorman et al., 1997). Then, the sources of taxonomic evidence such as anatomy, embryology, chromosomes, palynology, secondary plant compounds, proteins and DNA have been incorporated in classification and phylogenetic studies. Recently, DNA sequence data provide a source of characters suitable for building a phylogenetic classification particularly an independent scheme for classification.

3.1.1 The chloroplast encoded *trnL-F* region

Chloroplast (cp-) DNA genome is a circular molecule and subdivided into two single copy regions, the large single copy (LSC) region and the small single copy (SSC) region, which are separated by inverted repeats. The LSC region is slightly less conserved in sequence than the rest of the chloroplast genome (Clegg, Learn and Golenberg, 1991); hence it is potentially more useful for studies at lower taxonomic levels. Interspecific cpDNA polymorphisms are predominant in the form of length variation due to insertion/deletion mutations (McCauley, 1995) and variation in copy number of mononucleotide microsatellite repeats (Powell, Morgante and Andre, 1995). Sequences from noncoding regions of the cpDNA genome are often used in systematics because such regions tend to evolve relatively rapidly, for example the *trnL-F* intergenic spacer (Gielly and Taberlet, 1994), the *atpB-rbcL* intergenic spacer (Hodges and Arnold, 1994), the *rbcL-psaI* intergenic region (Morton and Clegg, 1993).

The cpDNA *trnT-L-F* region in land plants consists of the transfer RNA genes $trnT^{UGU}$, $trnL^{UAA}$ and $trnF^{GAA}$ arranged in tandem, separated by non-coding spacer regions. It is positioned in the large single copy region, approximately 8 Kbp downstream of *rbcL*. The *trnL* gene of cyanobacteria and a number of chloroplast genomes, including that of all land plants, contain a group-I intron positioned between the U and the A of the UAA anticodon loop. This intron is inferred by phylogenetic analysis to have been present in the cyanobacterial ancestor of the plastid lineages of Rhodophyta, Chlorophyta and Gluacocystophyta (Besendahl et al., 2000).

The succession of conserved *trn* genes and the apparent absence of gene rearrangements in the *trnL-F* region facilitated the design of plant universal primers by Taberlet et al., (1991); in particular the *trnL* intron and *trmL-F* spacer (collectively, the *trnL-F* region) has become one of the most widely used chloroplast markers for phylogenetic analysis in plants (Quandt et al., 2004).

3.1.2 Internal Transcribed Spacer (ITS) Region

Nuclear ribosomal DNA (nrDNA) is organized as individual chromosomal units that are repeated thousands of times in most higher plant genomes. Each of these units contains the three genes that encode the 18S, 5.8S and 26S ribosomal RNA subunits, as well as several different spacer DNA regions. The nucleotide sequence variation found in both of the internal transcribed spacer regions (ITS-1 and ITS-2) is used extensively for the systematic analysis of closely related taxa, at least in part due to the rapid rate of evolutionary change characterizing these DNA regions (Baldwin et al., 1995). The ITS sequences have proven to be a valuable source of characters to address phylogenetic relationships among closely related species in different plant families (Francisco-Ortega et al., 2001). The ITS sequences have also proven powerful in revealing hybridization and reticulate evolution (e.g. Sang, Crawford and Stuessy, 1997)

3.1.3 Systematic Studies of Annonaceae

Presently, there are a number of molecular phylogenetic investigations in Annonaceae. Meade (2000) used wide range of molecular data, including RAPDs,

RFLP, the *trnL-F* intergenic spacer and ITS sequences, to establish relationships within and between a small numbers of selected genera in Annonaceae. The early comprehensive molecular phylogeny of the family was carried out by Bygrave (2000; also partly published in Doyle et al. 2000) on 130 Annonacean taxa collected worldwide using *rbcL* gene.

Later, Erken (2002) studied the phylogenetic relationships among 47 taxa of *Guatteria* using DNA sequences from 6 regions in the plastid genome, including *rbcL* region, *matK* region, *trnL-F* spacer, *trnL-F* spacer and *trnL* intron and *psbA-trnH* spacer. The results suggested that *Guatteria* is a very well supported monophyletic group and the genus seems to have its origin in Central-America, rather than in South-America. However, these data are still insufficient to resolve the relationships between all closely related species.

Mols, Keßler and Gravendeel (2002) performed the phylogenetic investigation in *Miliusa* using cpDNA markers, including *rbcL*, *trnL* intron and *trnL-F* intergenic spacer, from more than 100 taxa occurring in Asia, Africa and America. The results showed that *Miliusa* proved to be monophyletic. *Polyalthia*, on the other hand, was highly polyphyletic.

Chatrou et al. (2002) presented the generic phylogenies of Neotropical Annonaceae based on cpDNA markers (*rbcL*, *trnT-L* intergenic spacer, *trnL* intron, *trnL-F* intergenic spacer and *psbA-trnH* spacer and partial *matK*) and the result indicated that *Guatteria* has the highest levels of divergence.

Annonaceae is included in the order Magnoliales (APG II, 2003). It has been suggested to be the sister taxon to Eupomatiaceae based on cladistic analysis of morphological and molecular data (Sauquet et al., 2003). Within the family, *Anaxagorea* resolved as the basal group to the rest of the family (Doyle, Bygrave and Le Thomas, 2000; Sauquet et al., 2003). The further use of morphology for phylogeny reconstruction in Annonaceae has been problematic due to difficulties in homology assessment and high levels of homoplasy (Doyle and Le Thomas, 1996). Results from phylogenetic

analyses based on *rbcL* and *trnL-F* sequence data (Mols et al., 2004; Richardson et al., 2004) supported the position of *Anaxagorea* and further divided the rest of Annonaceae between the small clades including *Cananga* and *Cleistopholis*, sister group to the larger clade including the majority of species of the family.

Later, sequences from the *trnL-F* region (not including the *trnT-L* region and *trnL* 5' exon) had been used in combination with those from further chloroplast markers (*rbcL* and *matK*) in phylogenetic reconstruction of the family Annonaceae (Sauquet et al., 2003; Mols et al., 2004; Richardson et al., 2004). The majority of species of Annonaceae fall within two large clades. The informally named 'long branch clade' (LBC) represents around 1,500 of the total 2,500 species, and is characterized by an inaperturate pollen condition. The LBC are further divided into 7 species-rich genera, i.e. *Annona*, *Artabotrys*, *Duguetia*, *Goniothalamus*, *Guatteria*, *Uvaria* and *Xylopia*. The sister group to the LBC is so-named the 'short branch clade' (SBC), representing yet another 700 species. The remaining species formed the basal grade.

Scharaschkin and Doyle (2005) investigated phylogeny and historical biogeography of the genus *Anaxagorea* using 75 morphological characters and molecular sequences from the *atpB-rbcL*, *psbA-trnH* and *trnL-trnF* spacers and the *trnL* intron. Molecular analyses alone did not support the monophyly of the Asian species, but the morphological and combined molecular and morphological analyses did.

3.2 MATERIALS AND METHODS

3.2.1 Taxon Sampling

Forty-one species of *Goniothalamus* were included and representing the geographical distribution of the genus (Table 3.1). Selected species from the LBC, *Annona squamosa* L., *Dasymaschalon lomentaceum* Finet & Gagnep., *Rollinia herzogii* R.E. Fries (AY841734), *Asimina triloba* (L.) Dunal (AY220359), *Disepalum* sp. (AY841690), *Anonidium* sp. (AY841675), *Neostenanthera myristicifolia* (Oliv.) Exell (AY743467) and *Friesodielsia desmoides* (Craib) Steenis, and the SBC, *Mitrephora keithii* Ridl. and *Polyalthia viridis* Craib, were selected as outgroup taxa. Seventeen

specimens from fresh leaves, nine specimens from silica-dried specimens and seventeen specimens from herbarium specimens were used for DNA extraction. All voucher specimens were deposited at Department of Botany Herbarium, Faculty of Science, Chulalongkorn University (BCU); The University of Hong Kong Herbarium (HKU); Nationaal Herbarium Nederland, Leiden branch (L) and The Arnold Arboretum (A).

3.2.2 DNA Extraction

Total genomic DNA was extracted from fresh leaf materials followed a modified cetyl trimethyl ammonium bromide (CTAB) method (Agrawal et al., 1992). For silica-gel dried materials or herbarium materials, the genomic DNA extraction followed the DNeasy Plant Mini Kit (QIAGEN, Leusden, Netherlands) according to the manufacturer's instructions or a modified CTAB method together with QIAquick™ PCR purification Kit (QIAGEN, Leusden, Netherlands) (Appendix A).

3.2.3 PCR amplification and sequencing

PCR reactions were set up in 50 µl reactions, usually containing 1X PCR buffer, 0.2mM dNTPs in equimolar ratio, 0.2pmol primer, 1U of *Taq* DNA polymerase (QIAGEN, Leusden, Netherlands), 50-100ng of genomic DNA and 2.5mM $MgCl_2$ for amplifying the *trnL-F* intergenic spacer or 1X Q-solution for amplifying the ITS fragment. Other modifications included increasing the amount of $MgCl_2$ or substituting 0.5% BSA for $MgCl_2$. Amplification was carried out using the PTC-100 MJ Research

The *trnL-F* intergenic spacer was amplified using the primer combinations e/f, 'e' (5'-GGTTCAAGTCCCTCTATCCC-3') and 'f' (5'-ATTTGAACTGGTGACACGAG-3') (Taberlet et al., 1991). The thermal cycling protocol comprised of 30 cycles, each with 1 minute 30 second of denaturation at 94 °C, 2 minutes of annealing at 49-50 °C, and 3 minutes of extension at 72 °C, concluding with an additional extension of 10 min at 72 °C after the final cycle.

Table 3.1 List of taxa used for phylogenetic analyses. Herbarium abbreviation was followed Index Herbariorum I (Holmgren, Keuken and Schofield, 1981)

No.	Species	Origin	Voucher specimen (Herbarium)
1	*G. aruensis* Scheff.	New Guinea	J. Regalado and W. Takcuchi 1409 (HKU)
2	*G. aurantiacus* sp. nov.	Kanchanaburi, Thailand	Saunders and Chalermglin 04/30 (HKU)
3	*G. australis* Jessup	Australia	A. Ford 4758 (HKU)
4	*G. borneensis* Mat-Salleh	Borneo	K. Sidiyasa et al. 2637 (HKU)
5	*G. cheliensis* Hu	Nan, Thailand	P. Chalermglin 470228 (BCU)
6	*G. clemensii* Bân	Borneo	A.C. Church 436 (A)
7	*G. costulatus* Miq.	Sumatra	Martati 169 (HKU)
8	*G. curtisii* King	Peninsular Malaysia	V. Balgooy 2122 (HKU)
9	*G. dewildei* R.M.K. Saunders	Sumatra	De Wilde and de Wilde Duyfies 201229 (L)
10	*G. dolichopetalus* Merr.	Borneo	A.C. Church 258 (A)
11	*G. elegans* Ast	Ubon Ratchathani, Thailand	M. Nakkuntod 40 (BCU)
12	*G. gardneri* Hook. f. & Thomson	Sri Lanka	H. Tillekeratne s.n. (HKU)
13	*G. giganteus* Hook. f. & Thomson	Bangkok, Thailand (cultivated)	M. Nakkuntod 60 (BCU)
14	*G. holttumii* J. Sinclair	Peninsular Malaysia	K.M. Kochummen FRI 16631 (HKU)
15	*G. hookeri* Thwaites	Sri Lanka	R.M.K. Saunders and A.D. Weerasooriya 00/09 (HKU)
16	*G. lanceolatus* (Bân) Mat-Salleh	Borneo	S.H. Rogstad 689 (A)
17	*G. laoticus*1 (Finet & Gagnep.) Bân	Nakhon Phanom, Thailand	P. Chalermglin 470425/1 (BCU)

Table 3.1 (Continued)

No.	Species	Origin	Voucher specimen (Herbarium)
18	*G. laoticus*2 (Finet & Gagnep.) Bân	Nakhon Ratchasima , Thailand	Saunders et al. 04/1 (HKU)
19	*G. macrophyllus* (Blume) Hook. f. & Thomson var. *macrophyllus*	Narathiwat, Thailand	S. Kitamura MN7 (BCU)
20	*G. macrophyllus* (Blume) Hook. f. & Thomson var. *siamensis* J. Sinclair	Ranong, Thailand	P. Chalermglin 461220 (BCU)
21	*G. majestatis* P. Kessler	Sulawesi	McDonald and Ismail 3896 (A)
22	*G. malayanus* Hook. f. & Thomson	Narathiwat, Thailand	M. Nakkuntod 16 (BCU)
23	*G. montanus* J. Sinclair	Peninsular Malaysia	E. Soepadmo and M. Suhaimi 43 (L)
24	*G. parallelivenius* Ridl.	Sumatra, Borneo	Z. Arifin AA3014 (HKU)
25	*G. repevensis* Pierre	Thailand	Saunders et al. 04/8 (HKU)
26	*G. rongklanus* sp. nov.	Kampangphet, Thailand	P. Chalermglin s.n. (BCU)
27	*G. rotundisepalus* Henderson	Peninsular Malaysia	T.C. Whitmore FRI 4208 (L)
28	*G. salicinus* Hook. f. & Thomson	Sri Lanka	Saunders et al. 01/3 (HKU)
29	*G. sawtehii* C.E.C. Fisch.	Petchburi, Thailand	Saunders et al. 04/14 (HKU)
30	*G. scortechinii* King	Narathiwat, Thailand	S. Kitamura MN21 (BCU)
31	*G. stenomitra* (a new sp. in prep)	Pathumthani, Thailand	M. Nakkuntod 1 (BCU)
32	*G. subevenius* King	Narathiwat, Thailand	S. Kitamura MN22 (BCU)
33	*G. tapis* Miq.	Pathumthani, Thailand (cultivated)	M. Nakkuntod 2 (BCU)
34	*G. tamirensis* Pierre ex Finet & Gagnap.	Mukdahan, Thailand	P. Chalermglin 470425/2 (BCU)
35	*G. thwaitesii* Hook. f. & Thomson	Sri Lanka	Saunders et al. 01/5 (HKU)

Table 3.1 (Continued)

No.	Species	Origin	Voucher specimen (Herbarium)
36	*G. tomentosus* R.M.K. Saunders	Peninsular Malaysia	K. Ogata KEP110361 (HKU)
37	*G. tortilipetalus* Henderson	Narathiwat, Thailand	S. Nakkuntod MN58 (BCU)
38	*G. umbrosus* J. Sinclair	Narathiwat, Thailand	S. Kitamura MN23 (BCU)
39	*G. undulatus* Ridl.	Narathiwat, Thailand	M. Nakkuntod 11 (BCU)
40	*G. uvaroides* King	Peninsular Malaysia	Z. Sohadi FRI14716 (L)
41	*G. wrayi* King	Peninsular Malaysia	T.C. Whitmore FRI810 (L)
42	*Goniothalamus* sp. (Panan Sroi)	Pathumthani, Thailand (cultivated)	M. Nakkuntod 4 (BCU)
43	*Goniothalamus* sp. (Phu Soi Dao2)	Utharadit, Thailand	Saunders et al. 04/40 (HKU)
44	*Annona squamosa* L.	Bangkok, Thailand	M. Nakkuntod 45 (BCU)
45	*Dasymaschalon lomentaceum* Finet & Gagnep.	Pathumthani, Thailand (cultivated)	M. Nakkuntod 5 (BCU)
46	*Anonidium* sp.	Africa	AY 841675*
47	*Neostenanthera myristicifolia* (Oliv.) Exell	Africa	AY 743467*
48	*Rollinia herzogii* R.E. Fries	America	AY 841734*
49	*Asimina triloba* (L.) Dunal	America	AY 220359*
50	*Disepalum* sp.	Asia	AY 841690*
51	*Frisodielsia desmoides* Craib	Pathumthani, Thailand (cultivated)	M. Nakkuntod 3 (BCU)
52	*Mitrephora keitii* Ridl.	Pathumthani, Thailand (cultivated)	M. Nakkuntod 8 (BCU)
53	*Polyalthia viridis* Craib	Bangkok, Thailand	M. Nakkuntod 62 (BCU)

* = DNA sequences from Genebank

The ITS fragment was amplified using the primer combinations ITS1/ITS4, 'ITS1' (5'-TCCGTAGGTGAACCTGCGG-3') and 'ITS4' (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). The thermal cycling protocol comprised of 30 cycles, each with 1 minute 30 second of denaturation at 94 °C, 2 minutes of annealing at 49-50 °C, and 3 minutes of extension at 72 °C, concluding with an additional extension of 10 min at 72 °C after the final cycle.

Primers used for amplification and sequencing of the *trnL-F* intergenic spacer and the ITS region were similar. Cloned products were sequenced using T7 and SP6 pGEM-T vector primers.

Double stranded PCR products were purified using the QIAquick™ PCR purification Kit (QIAGEN, Leusden, Netherlands) or PCR Purification Kit (MOBIO Laboratories, Inc., California, USA) according to manufacturer's instructions, and the purified products were eluted in 30 µl or 14 µl of autoclaved H_2O, respectively. Some samples that posted difficulties in DNA sequencing were cloned into pGEM-T vector (Promega, Madison, USA). Cloned products were purified using small-scale preparation of plasmid DNA lysis by alkali (Sambrook, Fritch and Maniatis, 1989). Automated DNA sequencing was done by Macrogen (Korea), Bio Service Unit (BSU, Thailand) and Genome Research Centre (Hong Kong).

3.2.4 Data Analysis

Upon completion of DNA sequencing, base calling was verified by examining fluorographs in Chromas version 1.45 (McCarthy, 1997), and corrections were made as necessary. Sequence alignment was done manually using the GeneDoc version 2.6.002 (Nicholas and Nicholas, 1997). Nucleotide frequencies, corrected distances and inferred numbers of transitions (ts) and transversions (tv) were determined by Mega 3.1 (Kumar, Tamura and Nei, 2004). Phylogenetic analyses were conducted using the parsimony method in PAUP* version 4.0b10 (Swofford, 1998). To search for the most-parsimonious (MP) tree, heuristic search with hundred random sequence additions, tree bisection reconnection (TBR) branch swapping and save all best trees were used. Characters were equally weighted. Bootstrap (Felsenstein, 1985) was used to obtain a

measure of support for each branch. Thousand bootstrap replications were carried out using full heuristic search. The strict consensus trees were presented for *trnL-F* and ITS data sets. Trees for *trnL-F* data set were shown in two consensus trees which as representative of each topology, whilst ITS tree was generated only one strict consensus tree.

Combined data analyses were performed in reduced *trnL-F* spacer data set and ITS data set. Partition homogeneity test was used to check incongruence between these two data sets. Phylogenetic analyses and bootstrap analyses were conducted in the same manner as described above.

3.3 RESULTS

3.3.1 *trnL-F* intergenic spacer analysis (full data: 44 ingroup + 10 outgroup)

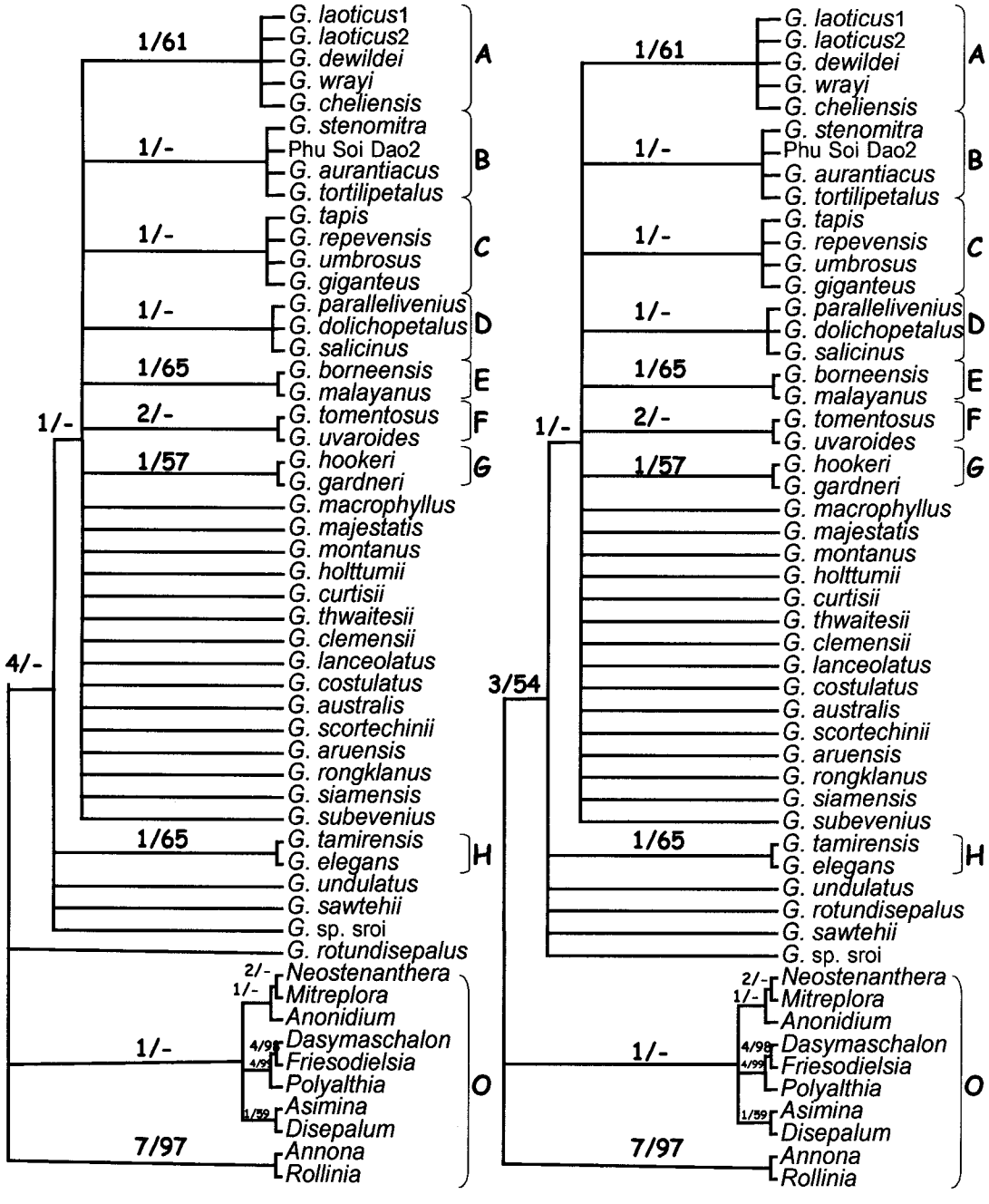
The length of the *trnL-F* spacer region ranged from 430 (*G. uvaroides*) to 476 bp (*Asimina triloba*). Within *Goniothalamus* the length ranged from 430 (*G. uvaroides*) to 465 bp (*G. majestatis*). The aligned *trnL-F* spacer sequence of 53 taxa was consisted of 617 bp. This non-coding region within *Goniothalamus* was quite variable and 18 indels ranging in size from 1 to 20 bp had to be inserted, three of which were phylogenetically informative. Of all characters used, 100 were variable, of which 34 (11.1%) were potentially phylogenetically informative while 75 (21.6%) were singleton. GC content was approximately 40%. Nucleotide pairs frequencies shows transition/transversion ratio to be about 1.2.

The parsimony analysis resulted in 38 equally most parsimonious trees, with a length of 137 steps, CI=0.8832 and RI=0.8431 (Table 3.2). Two topologies could be recognized (Figure 3.1). The difference between these two topologies was the position of *G. rotundisepalus*, either formed the sister clade to the larger clade or was a part of it. Regardless of the position of *G. rotundisepalus*, there was no difference between these two topologies. Although bootstrap values were low to moderate in the large clade, a few clades were recognized. In the large clade, 7 subclades formed a polytomy. However, there were only 3 recognizable subclades. The first clade composed of five

species, namely *G. wrayi*, *G. dewildei*, *G. laoticus* No.1, *G. laoticus* No.2 and *G. cheliensis*, with 61% bootstrap support. The second clade (65% bootstrap support) was consisted of two species, i.e. *G. borneensis* and *G. malayamus*. The last clade (57% bootstrap support) was of *G. hookeri* and *G. gardneri*. The rest subclades were shown with low bootstrap support, i.e. the *G. stenomitra*-Phu Soi Dao2-*G. aurantiacus*-*G. tortilipetalus* clade, the *G. tapis*-*G. repevensis*-*G. umbrosus*-*G. giganteus* clade, the *G. parallelivenius*-*G. dolichopetalus*-*G. salicinus* clade, the *G. tomentosus*-*G. uvaroides* clade. Overall branch supports were relatively low; few were more than 50% and none was more than 70%.

Table 3.2 Results from the maximum parsimony analyses of *trnL-F* intergenic spacer, ITS and combined data.

	*trn*L-F spacer (full data)	*trn*L-F spacer (reduced data)	ITS data	Combined data
No. of taxa	53	23	23	23
No. of characters	617	617	884	1374
Characters excluded	312	0	181	181
ts/tv ratio	1.2	1.2	1.1	-
No. of constant sites	205	559	500	932
No. of variable sites	100	58	203	261
No. of phylogenetically informative site	34	9	75	85
Tree length (steps)	137	65	304	373
No. of most parsimonious trees	38	1	6	1
Consistency index (CI)	0.8832	0.9538	0.7796	0.8016
Retention index (RI)	0.8431	0.8500	0.7100	0.7075



A B

Figure 3.1 The two topologies of the consensus trees of *Goniothalamus* from the full *trnL-F* data set. The clade indicated by "O" was an outgroup. A, B, C, D, E, F, G and H were groups for discussion. Number in front of slash was branch length and number after slash was bootstrap value from 100 replicates. Hyphen indicated bootstrap value below 50%.

3.3.2 ITS analysis

The length of ITS region ranged from 748 (*G. macrophyllus*, *G. tortilipetalus* and *G. repevensis*) to 792 bp (*G. giganteus*). The aligned sequence of 23 taxa was consisted of 884 bp. Twenty-three indels ranging in size from 1 to 26 bp had to be inserted. Of the 884 characters, 203 were variable, of which 75 (10.7%) were potentially phylogenetically informative while 128 (18.2%) were singleton. GC content was about 69%. Nucleotide pairs frequencies shows transition/transversion ratio to be about 1.1.

The parsimony analysis yielded 6 equally most parsimonious trees of 304 steps with CI=0.7796 and RI=0.7100 (Table 3.2). Three topologies could be recognized. The difference between these three topologies was the early branching clade. First topology showed *G. scortechinii* was the early branching clade, whilst clade H formed as a early branching clade in second topology. However, clade C that excluded *G. giganteus* was placed as a early branching clade in the last topology. Then the strict consensus tree was built and shown as Figure 3.2. The strict consensus tree formed polytomy with 4 recognizable clades, although generally low to moderate bootstrap support. The first clade (53% bootstrap support), *G. subevenius* was the first taxon to diverse, followed by two large subclades. The first subclades (89% bootstrap support) comprised of *G. malayanus* was sister to *G. cheliensis* with 99% bootstrap support and *G. laoticus* No.1 was sister to *G.laoticus* No.2 with 100% bootstrap support. The latter subclade, *Goniothalamus* sp. (sroi) was sister to the *G. undulatus*-*G. sawtehii* clade. The second clade (53% bootstrap support) consisted of four independent taxa and the *G. tapis*-*G. repevensis*-*G. umbrosus* subclade with 100% bootstrap support. Moreover, *G. tapis* was sister to *G. repevensis* with 66% bootstrap support. The third clade (88% bootstrap support) comprised four taxa, namely Phu Soi Dao2, *G. tortilipetalus*, *G. aurantiacus* and *G. stenomitra*. *G. tortilipetalus* was sister to *G. aurantiacus* with 82% bootstrap support. The last clade (79% bootstrap support) was of *G. tamirensis* and *G. elegens*. *G. scortechinii* didn't form the clade. The results from ITS locus yielded better resolved trees than that from the *trnL-F* data set, but still with generally low to moderate bootstrap supports on many branches.

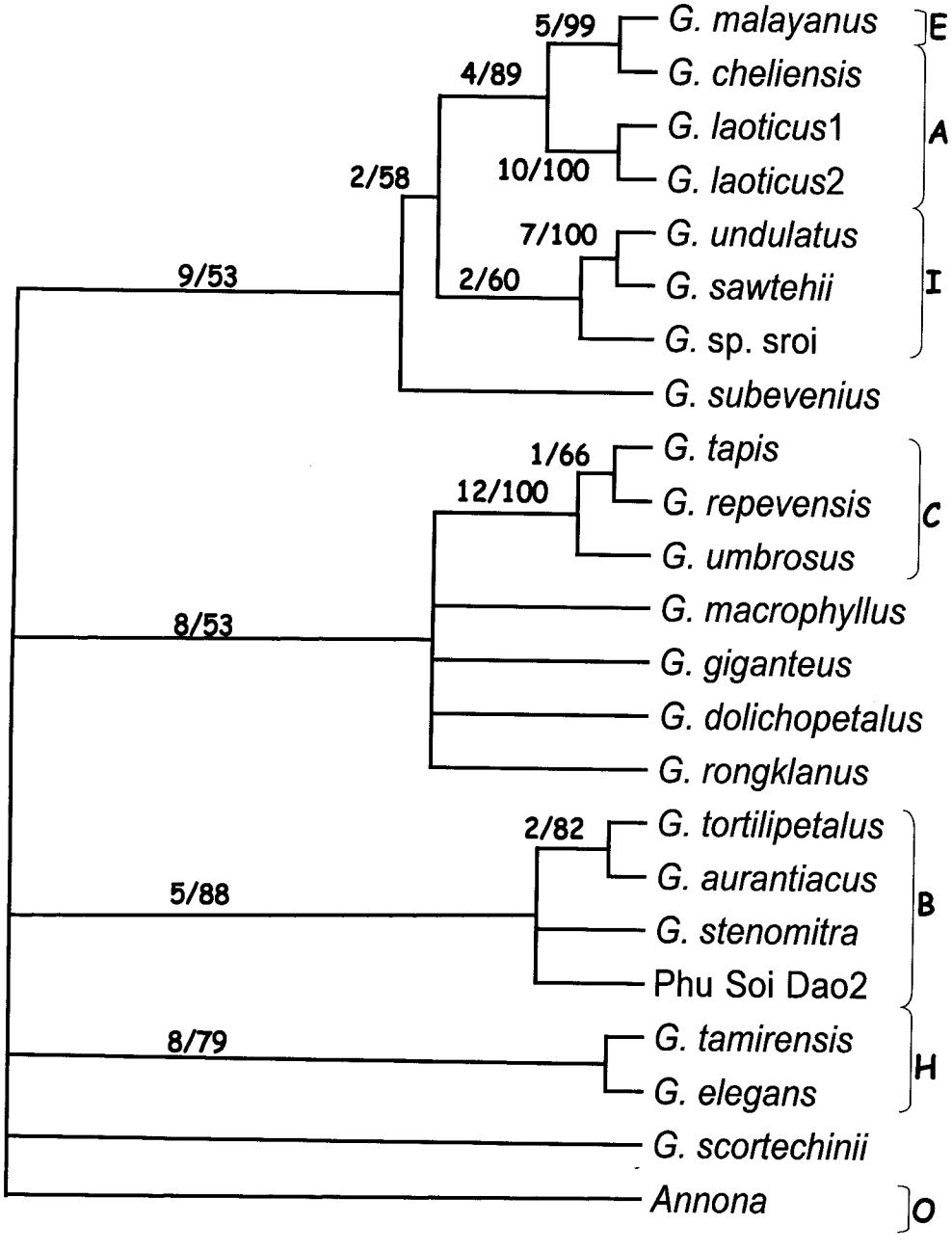


Figure 3.2 The strict consensus tree of *Goniothalamus* ITS sequence data with uninformative characters excluded. The clade indicated by "O" was an outgroup. A, B, C, E, H and I were groups for discussion. Number in front of slash was branch length and number after slash was bootstrap value from 100 replicates.

3.3.3 Combined data analysis

Due to the constraint on the number of taxa included in the ITS data set, few taxa had to be removed from the *trnL-F* spacer data set in order to be able to carry out the analyses on the combined data set. The aligned sequence of reduced *trnL-F* data set was consisted of 23 taxa and 617 bp. This non-coding region within *Goniothalamus* showed little variation, with only 8 indels ranging in size from 1 to 25 bp being inserted. Of 617 bp, 58 were variable, of these 9 (1.5%) were potentially phylogenetically informative while 49 were singleton. The parsimony analysis resulted in the most tree, with a length of 65 steps, CI=0.9538 and RI=0.8500 (Table 3.2). The tree topology was slightly different from that of full data set (Figure 3.1). The *G. tamirensis-G. elegans* clade still occupied the early branching lineage to the rest of the genus, although without bootstrap support. Therefore, the reduced *trnL-F* spacer data set can be used as representative of *trnL-F* spacer in combined analyses.

Prior to performing analyses on the combined *trnL-F* - ITS data set, partition homogeneity test (i.e. ILD, Mickevich and Ferris, 1981) was conducted to assess incongruence between these of two data sets. The test indicated these two data sets were not statistically incongruent (P value = 0.97). Thus, it was suggested that these data set could be combined.

The parsimony analysis of combined data set yielded only one most parsimonious tree of 373 steps with CI=0.8016 and RI=0.7075 (Table 3.2). This tree was more fully resolved. The monophyly of *Goniothalamus* may not be ascertained as only one outgroup taxon was included (Figure 3.3). Nonetheless, there were resolved clades with low to moderate branch supports. The first and likely to be early branching clade was the *G. tamirensis-G. elegans* clade (H). In the rest of the genus, *G.* cf. *scortechinii* formed the early branching lineage to the rest. The *G. tortilipetalus-G. aurantiacus-G. stenomitra*-Phu Soi Dao2 clade was then separated and two sister subclades, although lacking bootstrap support. However, the bootstrap supports in subterminal branches within these two clades were accasionally high, but often absent.

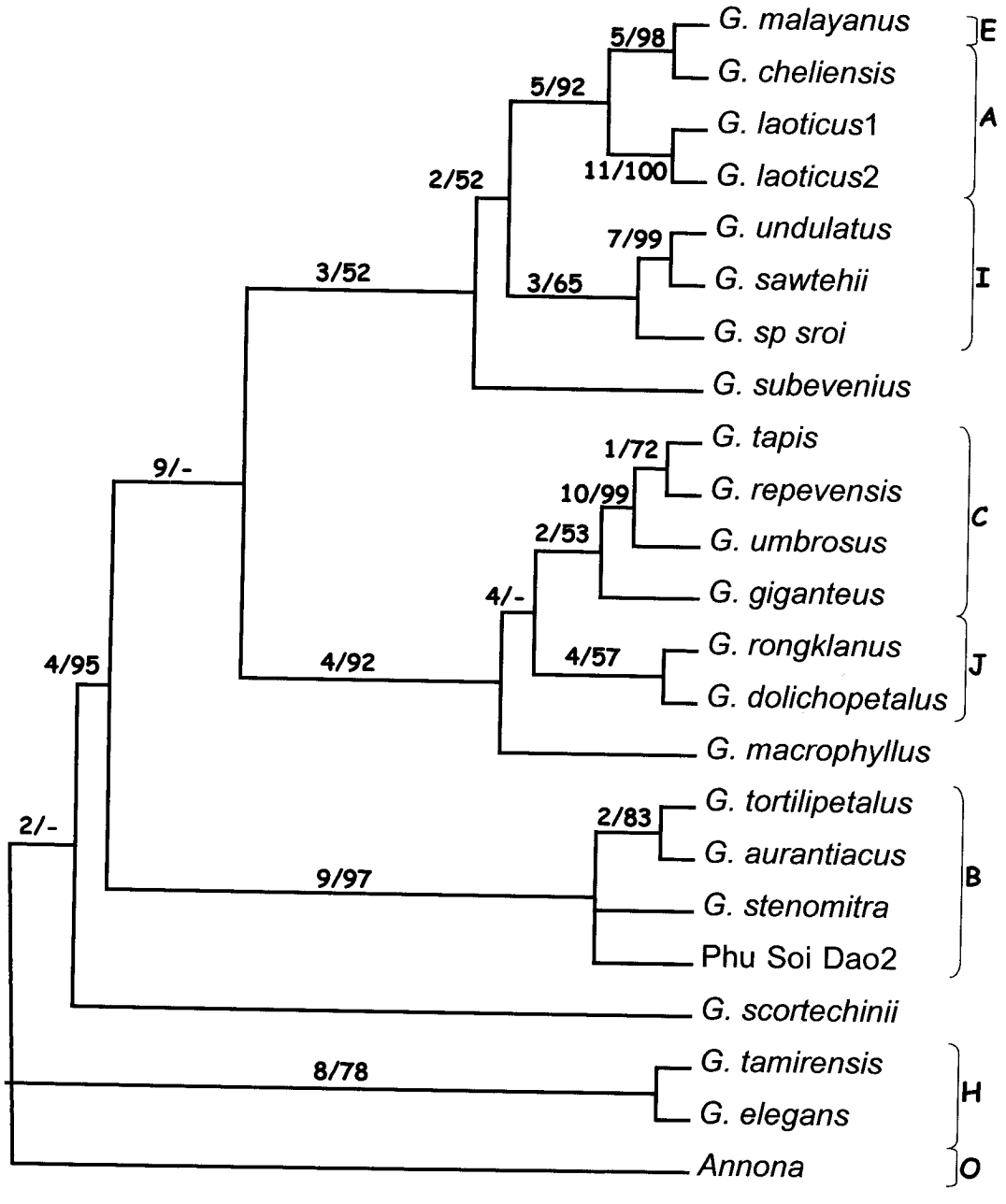


Figure 3.3 The most parsimonious tree from combined *trnL-F* and ITS data sets. The clade indicated by "O" was an outgroup. A, B, C, E, H, I and J were groups for discussion. Number in front of slash was branch length and number after slash was bootstrap value from 100 replicates. Hyphen indicated bootstrap value below 50%.

3.4 DISCUSSION

3.4.1 Comparisons among *trnL-F*/ITS/conbined data trees

The phylogenetic trees from *trnL-F* and ITS data sets showed little resolution in the consensus trees due to ambiguous characters supports, and thus low bootstrap values in many clades. The early branching position on the *trnL-F* tree was *G. rotundisepalus*,

whilst that on the ITS tree was the *G. tapis-G. repevensis-G. umbrosus* clade or *G.* cf. *scortechinii* or the *G. tamirensis-G. elegans* clade. For combined tree, the *G. tamirensis-G. elegans* clade was the early branching position, similar to the ITS tree.

Although a few clades were shown (i.e. clades A-I in Figure 3.1 and 3.2), the relationships among taxa and clades may not fully be inferred. In the *trnL-F* tree, clade A was not resolved but it were formed together as sister taxa to clade E in the ITS tree. Similarly, clade B was found on both trees, but the ITS tree indicated that within this clade *G. tortilipetalus* was the sister taxon to *G. aurantiacus*. In contrast, in clade C in the *trnL-F* tree is not present similar to in the ITS tree due to the exclusion of *G. giganteus*. Moreover, clades D, F and G were only present in the *trnL-F* tree but not in the ITS tree. This absence might be due to the limiting taxa in the ITS analysis or lack of bootstrap support in the *trnL-F* tree. *G. tamirensis* was sister to *G. elegans*, as clade H in both trees with moderate bootstrap support. In addition, clade I was only found in the ITS tree. It suggested that *G. undulatus* was closely related to *G. sawtehii*; this relationship was not recovered in the *trnL-F* tree.

From the combined data analyses (Figure 3.3), many subclades in Figure 3.1 and 3.2 existed in the combined tree, including clades A+E (92% bootstrap support), B (97% bootstrap support), C (53% bootstrap support), H (78% bootstrap support) and I (65% bootstrap support), whilst clade J (57% bootstrap support) was shown only in the combined tree. However, a few clades might be reliable. Clade H was early branching positioned as in the ITS tree, although lacking bootstrap support, with *G. scortechinii* early branching to the rest. Clade B was then still appeared and separated as the early

branching lineage to the larger clade. Two sister subclades situated at more latter position on the tree with low bootstrap support. In the first subclade, clades C and J appear as the sister groups (without bootstrap support) and *G. macrophyllus* was the early branching lineage of clade C+J. Clade C, excluding *G. giganteus*, was reliable due to high bootstrap support. This subclade was supported with 53% bootstrap value. In the other subclade, *G. subevenius* was sister to the large clade. Group A was paraphyletic to clade E, with *G. cheliensis* (in group A) sister taxon to *G. malayanus* (in clade E). *Goniothalamus* sp. (sroi) was sister taxon to the *G. undulatus-G. sawtehii* clade, as clade I appeared only in the combined tree. The *G. undulatus-G. sawtehii* clade was reliable due to high bootstrap support, alike the *G. malayanus-G. cheliensis* clade and the *G. laoticus*1-*G. laoticus*2.

In the light of combined analysis, the unclear taxa could be placed into the clades on all trees. *G. laoticus*2 was placed to *G. laoticus*1, suggesting that they are not significant different in molecular data although different location. Therefore, they are counted as one species in next chapter. *Goniothalamus* sp. (sroi) was sister to the *G. undulatus-G. sawtehii* clade. Its relationships might be reliable due to having shared some morphological characters, i.e. truncate staminal connective, glabrous style and stigma, funnel-shaped stigma. In other taxa, *G. rongklanus* was placed with *G. dolichopetalus*. *G. rongklanus* has hairy ovary and glabrous style similar to *G. dolichopetalus*, so their relationships seem likely. *Goniothalamus* from Phu Soi Dao No.2 was placed in the clade B together with *G. stenomitra*, *G. tortilipetalus* and *G. aurantiacus*. This species has large and erect sepals and connate at base similar to these three species, thus its placement might be reliable.

3.4.2 Infra-generic relationships within *Goniothalamus*

So far, the only infra-generic classification of *Goniothalamus* was that of Bân (1974b). However, this classification appeared to be somewhat artificial because it was based on 2-3 morphological characters to classify at subgeneric or section level. In addition, my data based on molecular evident was highly incongruent with Bân's classification. Several clades were reliable in our analyses of the *trnL-F*, ITS and

combined data sets (Figure 3.1, 3.2 and 3.3). Clade A comprised five species receiving 61% bootstrap support in *trnL-F* tree. In this clade, *G. wrayi* was likely to be closely related to *G. dewildei*, because of having apiculate staminal connective. This finding agreed with Saunders (2002) suggestion that *G. wrayi* was closely related to *G. dewildei* but differences are in its smaller leaves, fewer secondary vein in the leaves, axillary flowers, distinctively tapered staminal connectives, sparely hairy styles, and ellipsoid monocarps, although these two species was not in ITS and combined trees. A possible morphological synapomorphy for this clade A is erect sepals. Nonetheless, clade A seems as paraphyletic to *G. malayanus* in clade E with 89% and 92% bootstrap supports in ITS and combined trees, respectively.

Clade B was always appeared in all analysis, with high bootstrap supports in ITS and combined trees. Therefore, this clade might be reliable although this clade lacks bootstrap support in *trnL-F* tree. The possible morphological synapomorphies of this clade are connate sepals, sepal persistence in fruit and glabrous seed testa. Moreover, *G. tortilipetalus* was closely related to *G. aurantiacus* with shared some morphological character, i.e. distinctly prominence of secondary vein on adaxial leaf surface, percurrent tertiary vein arrangement, copious mucilage around seed.

Clade C consisted of four species with low bootstrap support in both *trnL-F* and combined trees. Clade C, excluded *G. giganteus*, might be more reliable due to high bootstrap support in ITS and combined trees. The possible morphological synapomorphies of this clade are hairy style and ovary.

Clades D and F are not reliable due to none bootstrap support in *trnL-F* tree and a number of these two clades were not appeared in ITS and combined trees. It is resulted of limiting taxa or none bootstrap support.

Clade E (65% bootstrap support in *trnL-F* tree) showed that *G. malayanus* was sister to *G. borneensis*. Also, somewhat in the same line of Mat-Salleh (2001) and Saunders (2002) suggestion that *G. malayanus* was closely related to *G. borneensis* and *G. giganteus*. My data, however, *G. giganteus* was not included in the clade E due

to having the warty fruits, while the fruits of *G. malayanus* and *G. borneensis* are smooth.

Clade G presented that *G. hookeri* was closely related to *G. gardneri* with 57% bootstrap support in *trnL-F* tree and with the possible synapomorphic character of hairy ovary and shared the same geographic distribution.

Clade H, with 65%, 79% and 78% bootstrap support in *trnL-F*, ITS and combined trees, respectively. This clade explained that *G. tamirensis* was sister to *G. elegans*. The possible synapomorphies are truncate staminal connective, glabrous pistil with large convoluted stigma.

Clade I consisted three taxa with moderate bootstrap support in ITS and combined trees, although this clade was not found in *trnL-F* tree. *G. undulatus* was sister to *G. sawtehii* with 100% bootstrap support. Thus this relationship is reliable and shared morphological characters, namely truncate staminal connective, glabrous style and stigma. Besides clade I might be related clades A and E but low bootstrap support (58% in ITS tree and 52% in combined tree)

Clade J showed the relationship of *G. rongklanus* and *G. dolichopetalus* with 57% bootstrap support in combined tree and shared two morphological synapomorphies, namely reflexed sepal and hairy of seed testa. Nevertheless, this relationship might be not certain, alike the relationship between clade J and clade C with none bootstrap support.

As discussion above, a few clade from these molecular phylogenies was reliable. These clades seem not to agree with Bân (1974b) infra-generic classification of *Goniothalamus*. In many aspects, however, these results seem to agree with Saunders (2002, 2003) view of *Goniothalamus* species relationships. Thus, new classification scheme should be proposed based on new evidence of molecular and morphological analyses.

CHAPTER IV

CHARACTER EVOLUTION IN THE GENUS *GONIOTHALAMUS*

4.1 INTRODUCTION

Evolution is a difficult phenomenon to study. It is rarely fast enough to be observed directly and only in exceptional cases is it possible to find physical evidence, such as fossils or ancient DNA, of past states and events. Fortunately, evolution leaves its footprint in the distribution of traits among living organisms. By studying this footprint, we can infer how organisms originated through the successive splitting of ancestral lineages, a process depicted in phylogenetic trees. Given a phylogenetic tree, the evolutionary history of individual traits of interest can be reconstructed. The evolution of characters among taxa can be studied both to infer history and interpret processes of change.

Saunders (2002) proposed that there are many characters of the flowers that are taxonomically important in *Goniothalamus*, i.e. size, shape, color and indument. The relative size of the outer and inner petals is often used as a taxonomic character at the generic level. In addition, the staminal connective shape is diagnostically important at the species level. In interspecific classification, the structure and indument of the carpel are also taxonomically important. Besides the structure of the seeds, i.e. size, shape, indument and color as well as the presence of exotestal hairs, with differences in length, color and density are also particularly important in infrageneric classification.

Scharaschkin and Doyle (2006) studied character evolution in *Anaxagorea* using a combined morphological and molecular phylogenetic analysis. A high level of homoplasy in stamen and leaf venation characters was observed. The distributions of characters on the tree confirm assumptions that several distinctive similarities between *Anaxagorea* and other Magnoliales are primitive retentions. A number of morphological synapomorphies were identified for a clade containing most Central American species and another comprising all Asian species.

The objective of this study is to assess the evolution of selected morphological characters using the phylogenetic reconstruction generated from combination of *trnL-F* and ITS data set as a framework. The combined molecular tree from Figure 3.3 in Chapter III was the most parsimonious tree with moderate to high bootstrap support. Therefore this study will use original tree without collapsing branches to avoid polytomy condition.

4.2 MATERIALS AND METHODS

4.2.1 Phylogenetic framework

The phylogenetic tree derived from combined ITS-*trnL-F* data (this study; Chapter III) is employed. Due to the limited number of taxa in this tree, the taxa sampled for evaluating morphological evolution will also be restricted. Only 23 species of *Goniothalamus* were included in this study (Table 3.1). *Annona squamosa* was selected as the outgroup based on molecular phylogenetic analyses in this study (Chapter III).

4.2.2 Morphological characters

A total of 43 characters (1 habit, 1 shoot, 3 leaf, 26 flower and 12 fruit and seed traits) were investigated. The list of morphological characters and the assigned character states is given in Appendix B. The character states for each taxon were scored from herbarium and descriptions from various journal articles and flora accounts (Sinclair, 1955; Keßler, 1996; Saunders, 2002, 2003, unpublished data).

4.2.3 Character evolution

Evaluation of morphological character evolution was performed in MacClade version 3.08 (Maddison and Maddison, 2001). Tracing of discrete-valued characters indicated nodes fixed in traced character, with unordered type and equal weight. The root was placed at the *Anona squamosa* clade.

4.3 RESULTS AND DISCUSSION

Although definitions of characters and their states are presented in the Appendix C, most of the practical and conceptual problems encountered and the arguments used in defining analysis of morphological characters were treated in this section because we viewed our analysis of morphological characters as one of the main results of this study.

4.3.1 Habit and vegetative characters

In general, the plesiomorphic character state (small tree habit) dominates in the genus, with only one taxon evolving to the derived state (larger trees) namely *G. giganteus* (character 1, Figure 4.1A). Likewise, most of the members of the genus are not too hairy (plesiomorphic) except in three unrelated lineages. Densely hairy or velutinous primary shoots is partially synapomorphic in the *G. undulatus-G. sawtehii-G.* sp. (sroi) clade (character 2, Figure 4.1B). In contrast, although that nitid adaxial leaf surface is the apomorphic, this state is very homoplastic (character 3, Figure 4.1C). This character serves as partial synapomorphic in the *G. malayanus-G. cheliensis* clade.

Prominence of secondary veins on the adaxial leaf surface is also homoplastic (character 4, Figure 4.2A). The ancestral node is equivocal, and consequently the plesiomorphic and apomorphic character states within the genus could not be ascertained. Nonetheless, it seems that there are three (possibly four) clades in which distinctly prominent secondary veins have been derived from impressed or slightly prominent veins. Also, the distinctly prominent secondary vein on the adaxial leaf surface may be synapomorphic for clade I and II, in which the subclade of one taxon has reversal condition, i.e. *G. tapis*.

The last character in this category, tertiary vein arrangement (character 5, Figure 4.2B), is inconclusive due to the fact that the various taxa with percurrent venation are linked by nodes that equivocal. Reticulate venation appears certain for two clades, namely the *G. tamirensis-G. elegans* clade and clade I, of which one contains two taxa, namely *G. malayanus* and *G. cheliensis*, with reversed condition. However, as outgroup node is equivocal, character state polarity could not be determined.

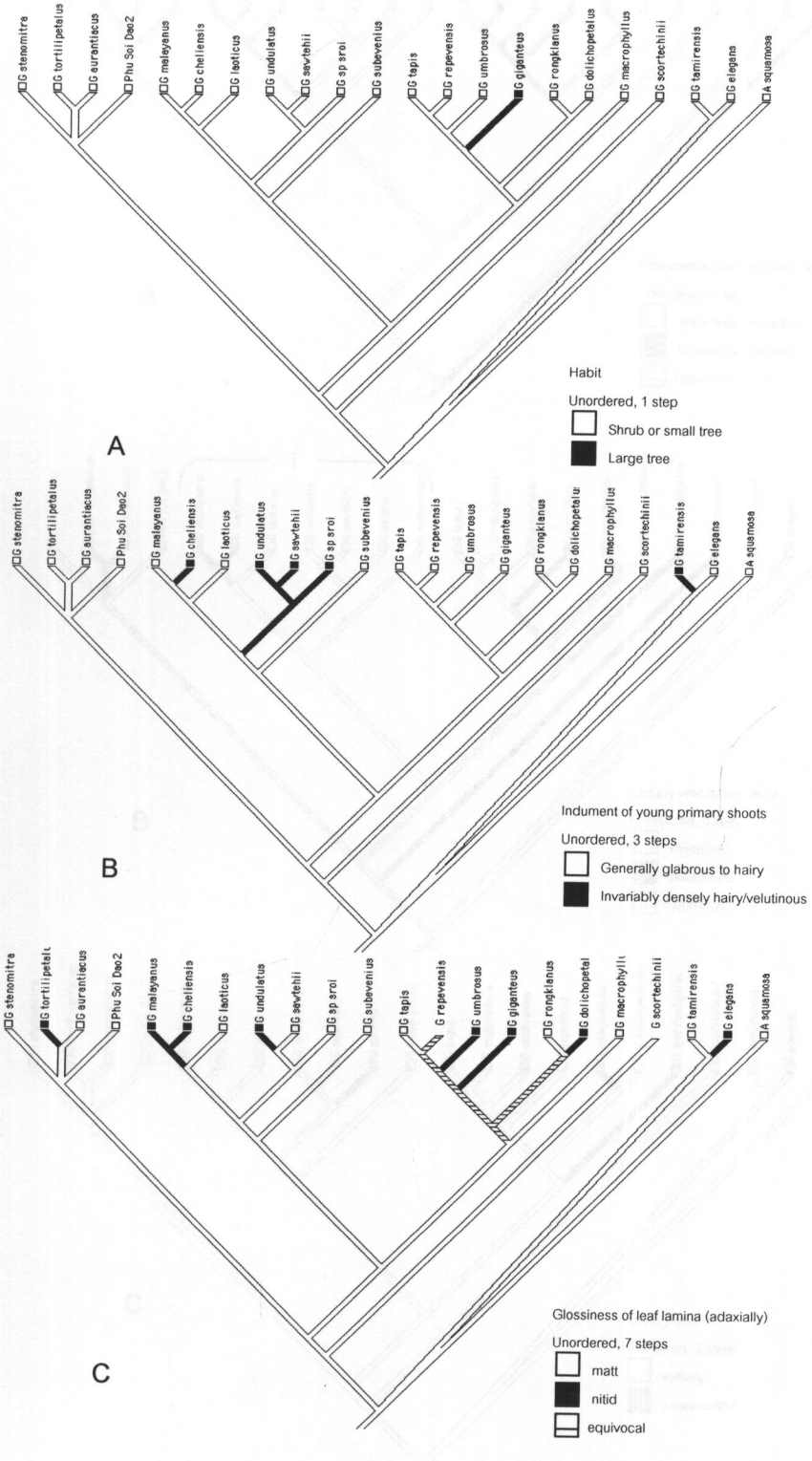


Figure 4.1 Trees showing inferred evolution of habit (A), indument of young primary shoots (B) and glossiness of leaf lamina (adaxially) (C) in *Goniothalamus* .

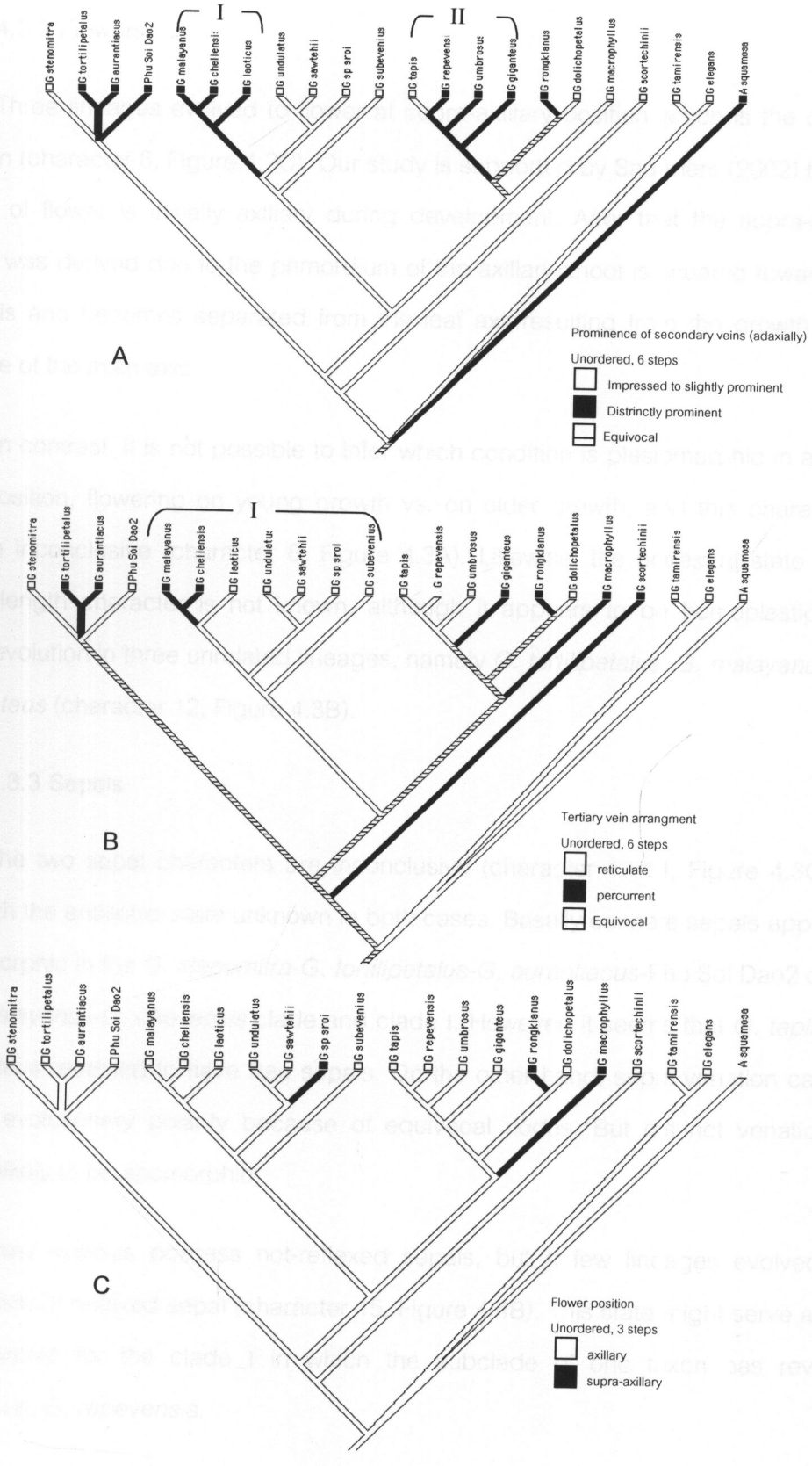


Figure 4.2 Trees showing inferred evolution of prominence of secondary veins (adaxially) (A), tertiary vein arrangement (B) and flower position (C) in *Goniothalamus*.

4.3.2 Flowers

Three lineages evolved to flower at supra-axillary position which is the derived condition (character 6, Figure 4.2C). Our study is supported by Saunders (2002) that the position of flower is initially axillary during development. After that the supra-axillary position was derived due to the primordium of the axillary shoot is situated towards the main axis and becomes separated from the leaf axil resulting from the growth of the internode of the main axis.

In contrast, it is not possible to infer which condition is plesiomorphic in another flower position, flowering on young growth vs. on older growth, and this character is therefore inconclusive (character 8, Figure 4.3A). Likewise, the ancestral state of the pedicel length character is not known, although it appears to be homoplastic, with parallel evolution in three unrelated lineages, namely *G. tortilipetalus*, *G. malayanus* and *G. giganteus* (character 12, Figure 4.3B).

4.3.3 Sepals

The two sepal characters are inconclusive (character 13-14, Figure 4.3C and 4.4A), with the ancestral state unknown in both cases. Basally connate sepals appear to synapomorphic in the *G. stenomitra-G. tortilipetalus-G. aurantiacus*-Phu Soi Dao2 clade, the *G. malayanus-G. cheliensis* clade and clade I. However, it seems that *G. tapis* and *G. giganteus* reversed to have free sepals. On the other hand, sepal venation can not infer the evolutionary polarity because of equivocal nodes. But distinct venation on sepals is likely to be apomorphic.

Many species possess not-reflexed sepals, but a few lineages evolved the derived state of reflexed sepal (character 15, Figure 4.4B). This state might serve as the synapomorphic for the clade I in which the subclade of one taxon has reversal condition, i.e. *G. repevensis*.

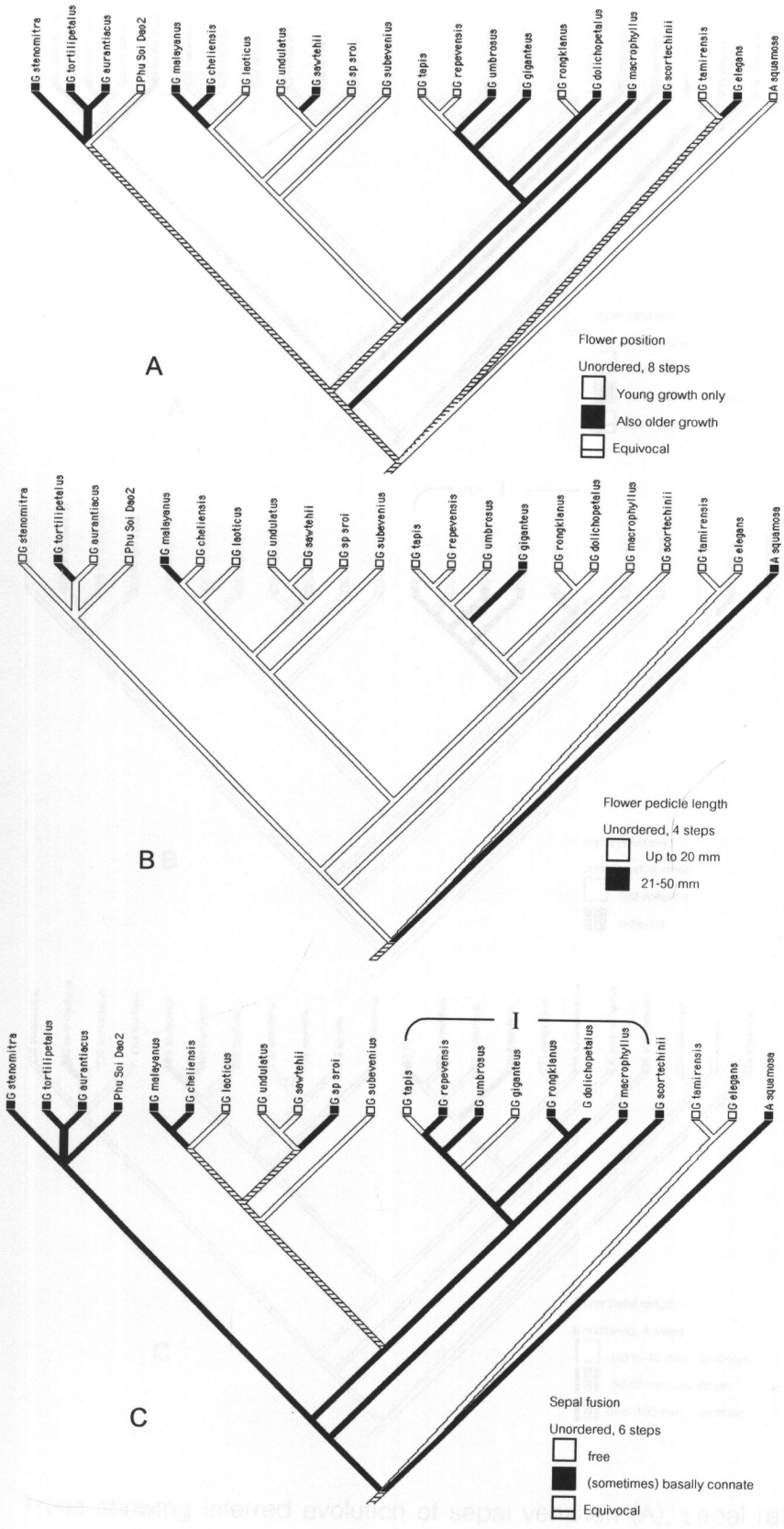


Figure 4.3 Trees showing inferred evolution of flower position (A), flower pedicle length (B) and sepal fusion (C) in *Goniothalamus*.

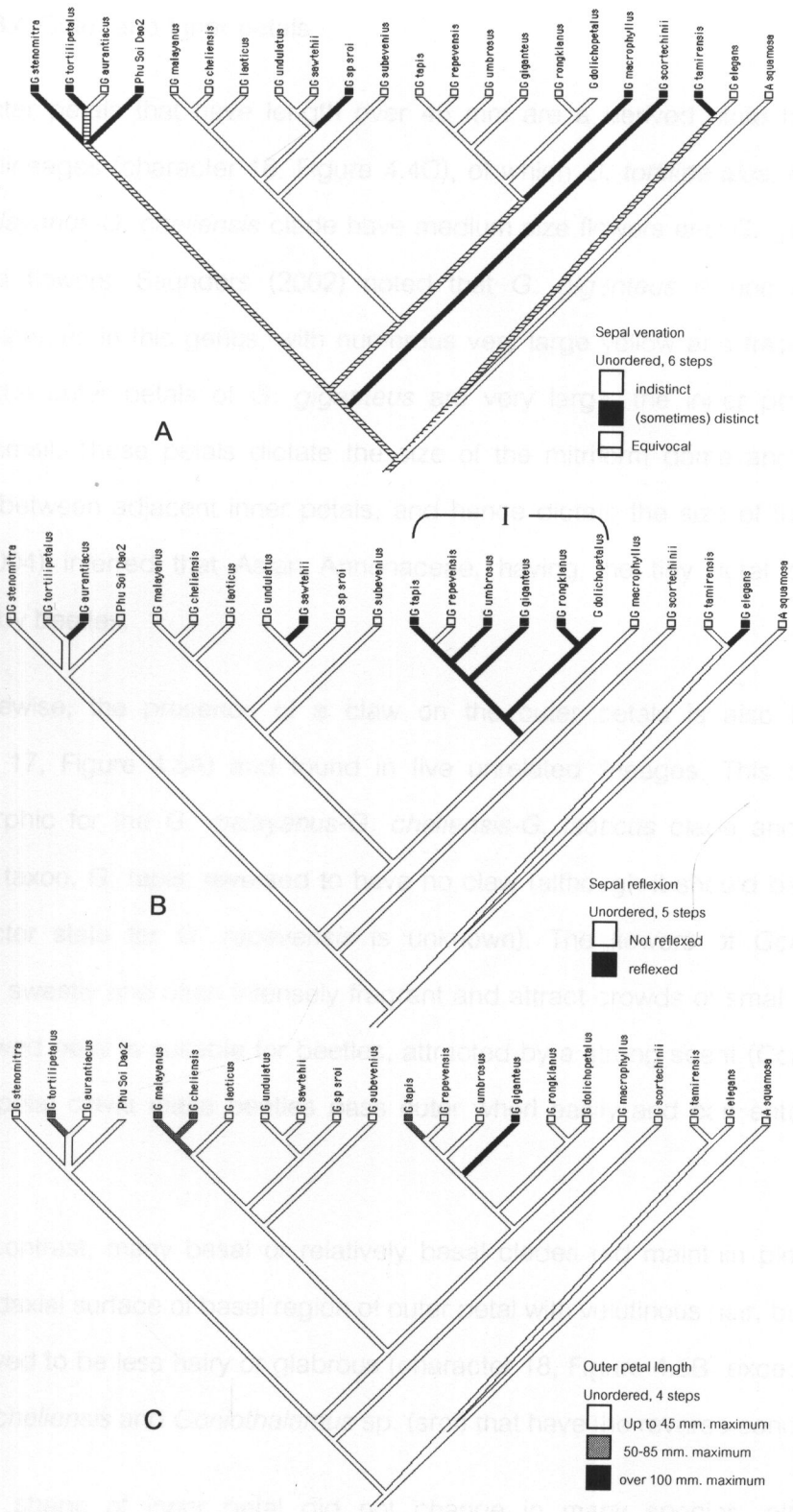


Figure 4.4 Trees showing inferred evolution of sepal venation (A), sepal reflexion (B) and outer petal length (C) in *Goniothalamus*.

4.3.4 Outer and inner petals

Outer petals that have length over 45 mm are a derived state found in four unrelated lineages (character 16, Figure 4.4C), of which *G. tortilipetalus*, *G. tapis* and the *G. malayanus-G. cheliensis* clade have medium size flowers and *G. giganteus* has the largest flowers. Saunders (2002) noted that *G. giganteus* is one of the most attractive species in this genus, with numerous very large yellow and fragrant flowers. Although the outer petals of *G. giganteus* are very large, the inner petals are still relatively small. These petals dictate the size of the mitriform dome and the size of apertures between adjacent inner petals, and hence dictate the size of the pollinator. Corlett (2004) inferred that Asian Annonaceae, having the tiny floral chamber, is pollinated by beetles.

Likewise, the presence of a claw on the outer petals is also homoplastic (character 17, Figure 4.5A) and found in five unrelated lineages. This character is synapomorphic for the *G. malayanus-G. cheliensis-G. laoticus* clade and clade I in which one taxon, *G. tapis*, reversed to have no claw (although it should be noted that the character state for *G. repevensis* is unknown). The flowers of *Goniothalamus* species as sweetly and often intensely fragrant and attract crowds of small beetles, so having clawed petal is suitable for beetles, attracted by a strong scent (Corner, 1988). The outer petal claws make beetles pass outer whorl easily and can enter the floral chamber.

In contrast, many basal or relatively basal clades did maintain plesiomorphic state, i.e. adaxial surface of basal region of outer petal with velutinous hair, but in clade I and II evolved to be less hairy or glabrous (character 18, Figure 4.5B) except few taxa, namely *G. cheliensis* and *Goniothalamus* sp. (sroi) that have the reverse condition.

The shape of inner petal did not change in many species, although the apomorphic condition is observed in six species, *G. stenomitra*, *G. aurantiacus*, *G. cheliensis*, *G. repevensis*, *G. umbrosus* and *G. rongklanus*, whose extensive contiguous area of inner petal are developed (character 19, Figure 4.5C). The *G. undulatus-G. sawtehii-G.* sp. (sroi) clade maintains the plesiomorphic condition.

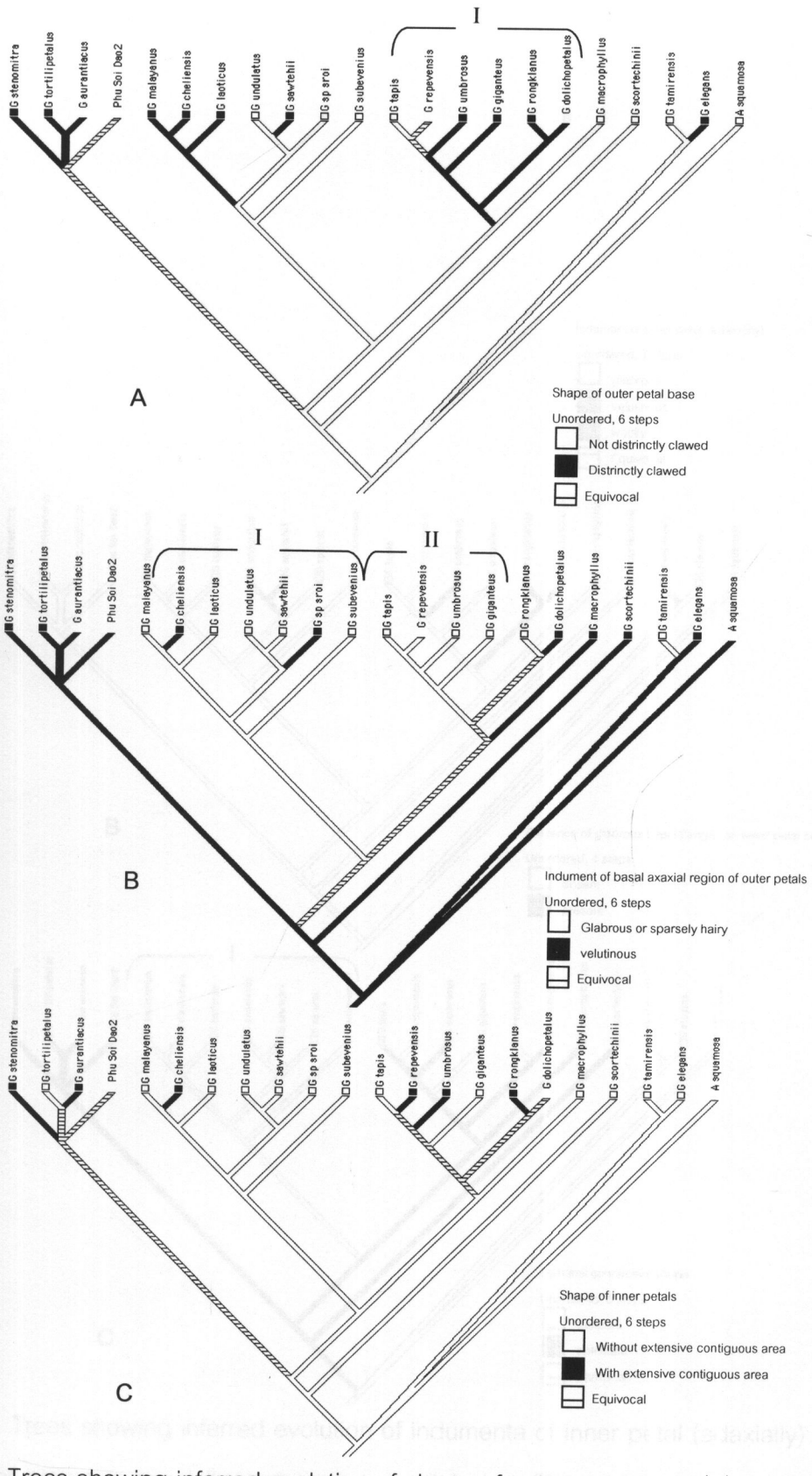


Figure 4.5 Trees showing inferred evolution of shape of outer petal base (A), indument of basal adaxial region of outer petals (B) and shape of inner petals (C) in *Goniothalamus*.

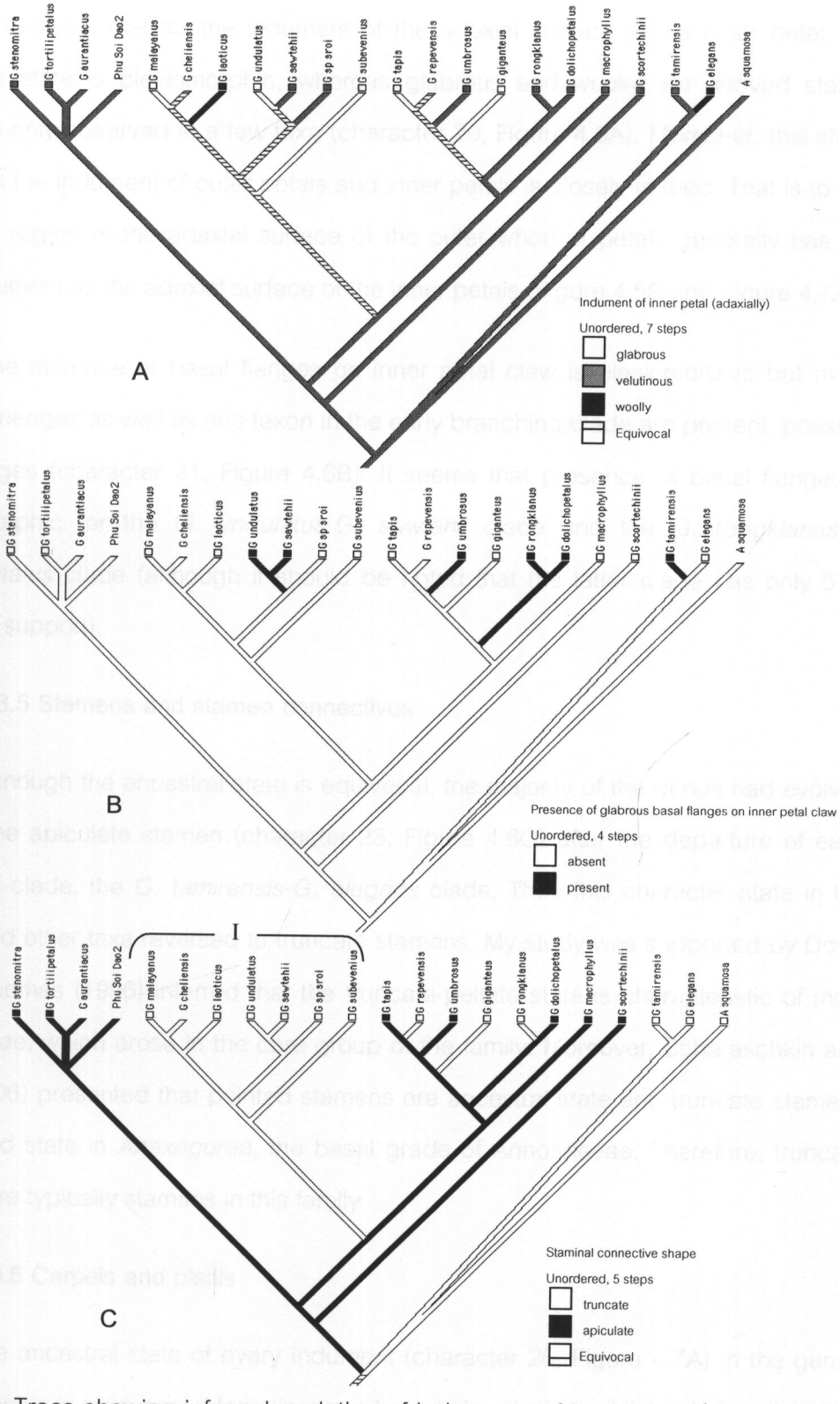


Figure 4.6 Trees showing inferred evolution of indumenta of inner petal (adaxially) (A), presence of glabrous basal flanges on inner petal claw (B) and staminal connective shape (C) in *Goniothalamus*.

It appears that for the indument of the adaxial surface of the inner petal, the velutinous state is plesiomorphic, whereas glabrous and woolly are derived states, which are only observed in a few taxa (character 20, Figure 4.6A). Moreover, this study found that the indument of outer petals and inner petals is closely related. That is to say the basal region of the adaxial surface of the outer whorl of petals generally has the same indument as the adaxial surface of the inner petals (Figure 4.5B and Figure 4.7A).

The absence of basal flanges on inner petal claw is plesiomorphic but many terminal lineages as well as one taxon in the early branching clade are present, possess such flanges (character 21, Figure 4.6B). It seems that presence of basal flanges is synapomorphic for the *G. undulatus-G. sawtehii* clade and the *G. rongklanus-G. dolichopetalus* clade (although it should be noted that the latter clade has only 57% bootstrap support).

4.3.5 Stamens and stamen connectives

Although the ancestral state is equivocal, the majority of the genus had evolved to have the apiculate stamen (character 23, Figure 4.6C) after the departure of early branching clade, the *G. tamirensis-G. elegans* clade. Then this character state in the clade I and other taxa reversed to truncate stamens. My study was supported by Doyle and Le Thomas (1996) inferred that the truncate-peltate state is characteristic of most Annonaceae, which arose in the core group of the family. Moreover, Scharaschkin and Doyle (2006) presented that pointed stamens are ancestral state and truncate stamens are derived state in *Anaxagorea*, the basal grade of Annonaceae. Therefore, truncate stamens are typically stamens in this family.

4.3.6 Carpels and pistils

The ancestral state of ovary indument (character 26, Figure 4.7A) in the genus *Goniothalamus* is equivocal. Many lineages have hairy ovaries, whilst only four lineages have glabrous ovaries, i.e. the *G. tamirensis-G. elegans* clade in early branching clade, the *G. undulatus-G. sawtehii* clade, *G. laoticus*, *G. subevenius*. This character may be

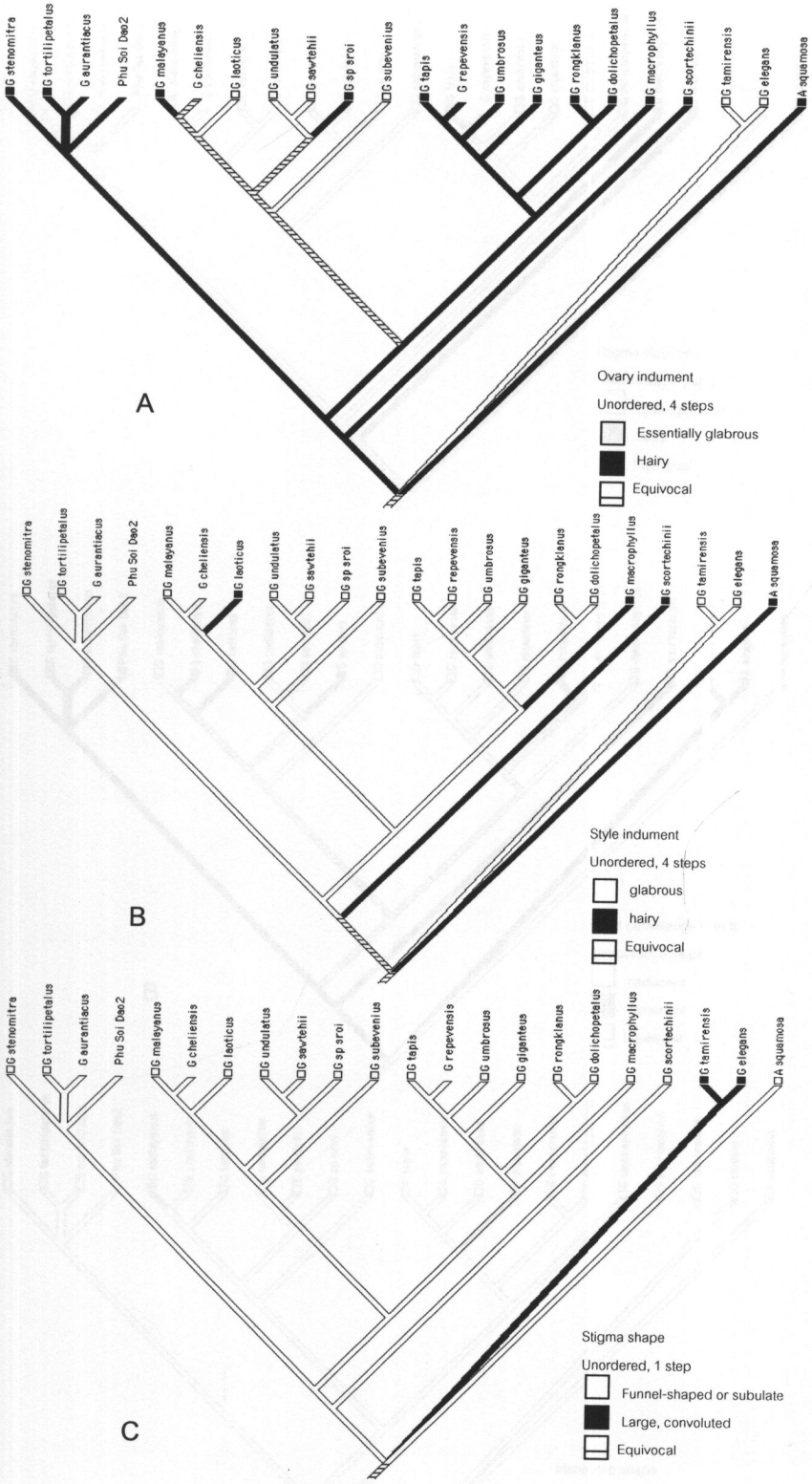


Figure 4.7 Trees showing inferred evolution of ovary indument (A), style indument (B) and stigma shape (C) in *Goniothalamus*.

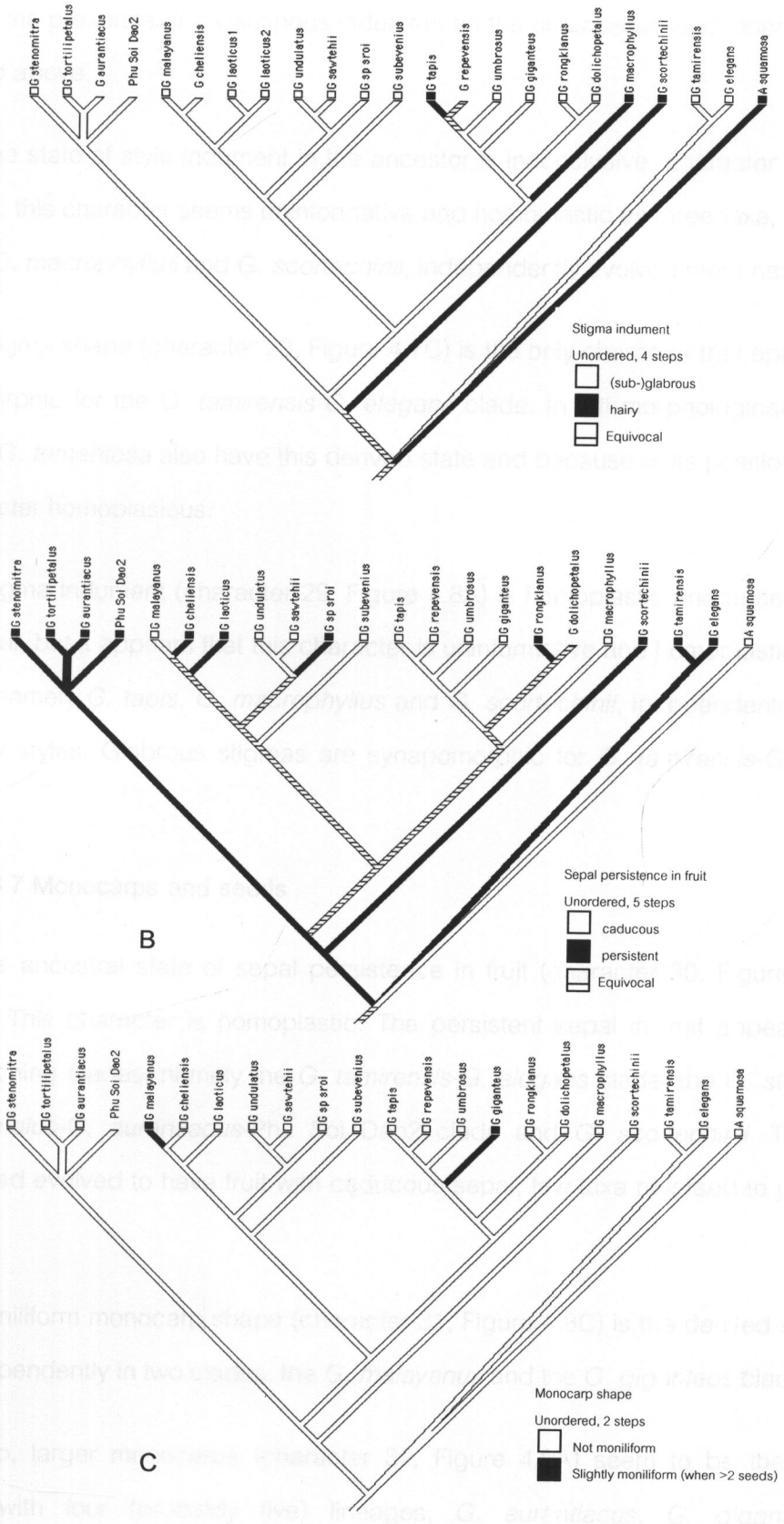


Figure 4.8 Trees showing inferred evolution of stigma indument (A), sepal persistence in fruit (B) and monocarp shape (C) in *Goniothalamus*.

related to the presence of a velutinous indument on the outer petals and inner petals, as discussed above.

The state of style indument in the ancestor is inconclusive (character 27, Figure 4.7B). Yet, this character seems uninformative and homoplastic as three taxa, namely *G. laoticus*, *G. macrophyllus* and *G. scortechinii*, independently evolved more hairy styles.

Stigma shape (character 28, Figure 4.7C) is the only character that appear to be synapomorphic for the *G. tamirensis-G. elegans* clade. In full morphological data set, however, *G. tomentosa* also have this derived state and because of its position, renders this character homoplasious.

Stigma indument (character 29, Figure 4.8A) is homoplastic and ancestral state is equivocal, but it appears that this character is uninformative and homoplastic as three lineages, namely *G. tapis*, *G. macrophyllus* and *G. scortechinii*, independently evolved more hairy styles. Glabrous stigmas are synapomorphic for *G. tamirensis-G. elegans* clade.

4.3.7 Monocarps and seeds

The ancestral state of sepal persistence in fruit (character 30, Figure 4.8B) is equivocal. This character is homoplastic. The persistent sepal in fruit appears in the early branching clades, namely the *G. tamirensis-G. elegans* clade, the *G. stenomitra-G. tortilipetalus-G. aurantiacus*-Phu Soi Dao2 clade and *G. scortechinii*. Then, few lineages had evolved to have fruit with caducous sepal, few taxa reversed to persistent sepal.

Moniliform monocarp shape (character 31, Figure 4.8C) is the derived state and found independently in two clades, the *G. malayanus* and the *G. giganteus* clades.

Also, larger monocarps (character 32, Figure 4.9A) seem to be the derived condition with four (probably five) lineages, *G. aurantiacus*, *G. giganteus*, *G. dolichopetalus*, *G. malayanus* and *G. laoticus*, evolving this condition independently,

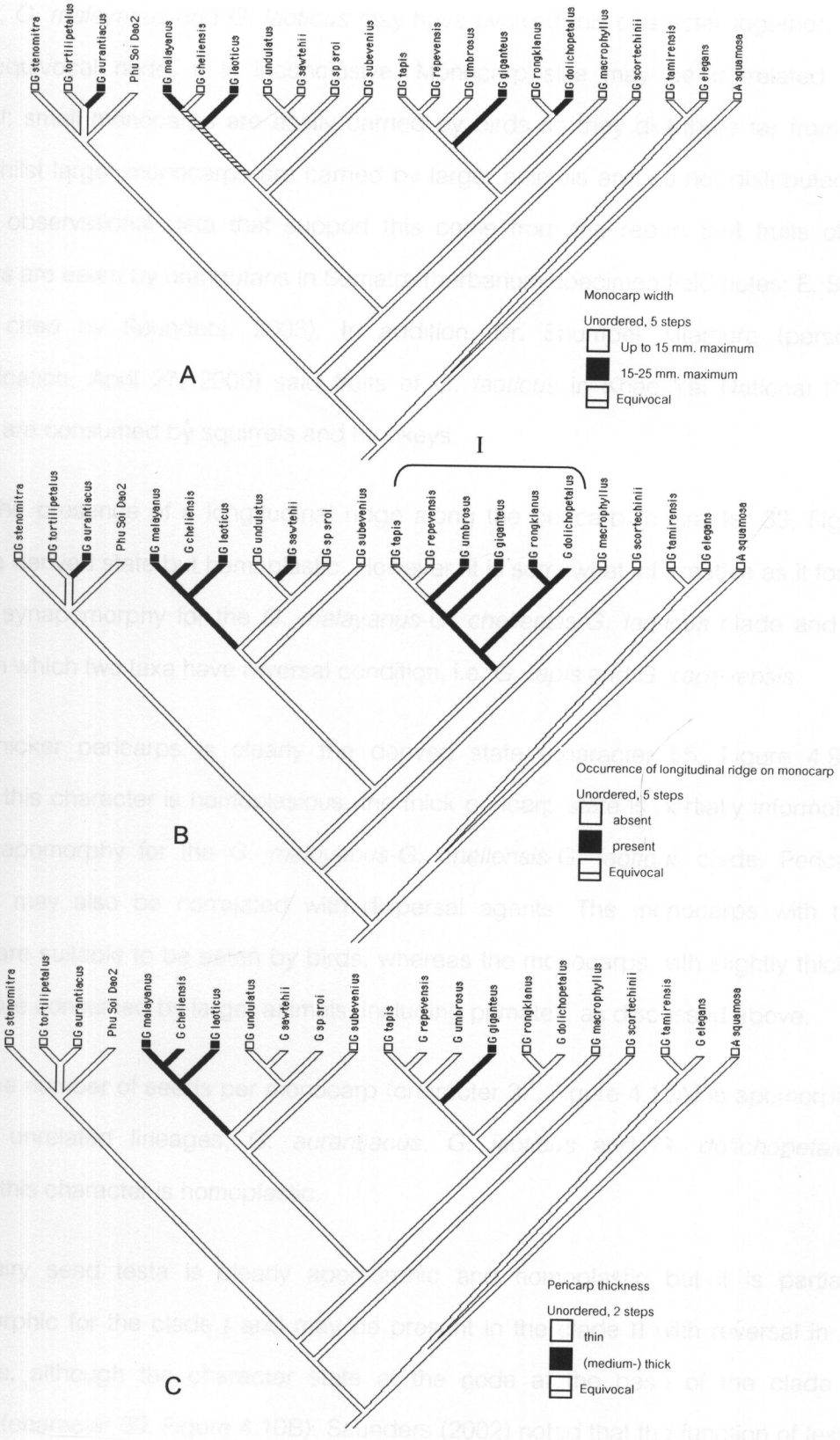


Figure 4.9 Trees showing inferred evolution of monocarp width (A), occurrence of longitudinal ridge on monocarp (B) and pericarp thickness (C) in *Goniothalamus*.

however, *G. malayanus* and *G. laoticus* may have evolved this character together. Due to the equivocal node, it is inconclusive. Monocarp size may be correlated with dispersal: small monocarps are easily carried by birds so they distribute far from the origin, whilst larger monocarps are carried by larger animals and so not distributed so far. The observational data that support this come from the report that fruits of *G. giganteus* are eaten by orangutans in Sumatra (herbarium specimen field notes: E. Sterk 185, L; cited by Saunders, 2003). In addition, Dr. Shumpei Kitamura (personal communication, April 27, 2006) said fruits of *G. laoticus* in Khao Yai National Park, Thailand are consumed by squirrels and monkeys.

The presence of a longitudinal ridge along the pericarp (character 33, Figure 4.9B) is a derived state but homoplastic. However, it is somewhat informative as it forms a partial synapomorphy for the *G. malayanus-G. cheliensis-G. laoticus* clade and for clade I, in which two taxa have reversal condition, i.e. *G. tapis* and G. *repevensis*.

Thicker pericarps is clearly the derived state (character 35, Figure 4.9C). Although this character is homoplasious, the thick pericarp state is partially informative as a synapomorphy for the *G. malayanus-G. cheliensis-G. laoticus* clade. Pericarp thickness may also be correlated with dispersal agents. The monocarps with thin pericarp are suitable to be eaten by birds, whereas the monocarps with slightly thicker pericarp are consumed by larger animals, including primates, as discussed above.

The number of seeds per monocarp (character 37, Figure 4.10A) is apomorphic for three unrelated lineages, *G. aurantiacus*, *G. laoticus* and *G. dolichopetalus*, although, this character is homoplastic.

Hairy seed testa is clearly apomorphic and homoplastic but it is partially synapomorphic for the clade I and may be present in the clade II with reversal in *G. rongklanus*, although the character state at the node at the base of the clade is equivocal (character 39, Figure 4.10B). Saunders (2002) noted that the function of testal hair is unclear, but may possibly be involved in mucilage production. This study supported a positive correlation between testal hairs and mucilage production around

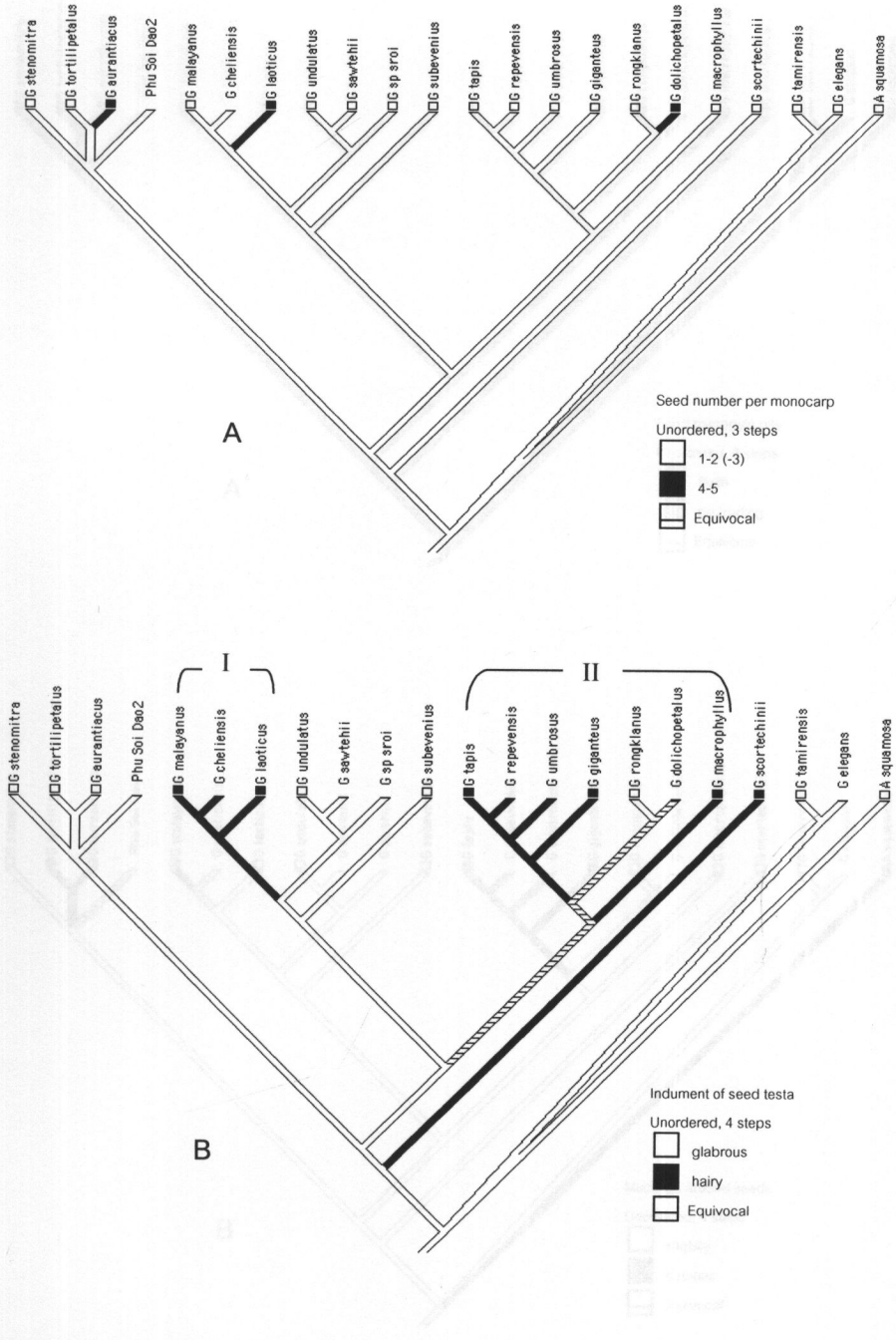


Figure 4.10 Trees showing inferred evolution of seed number per monocarp (A) and indument of seed testa (B) in *Goniothalamus*.

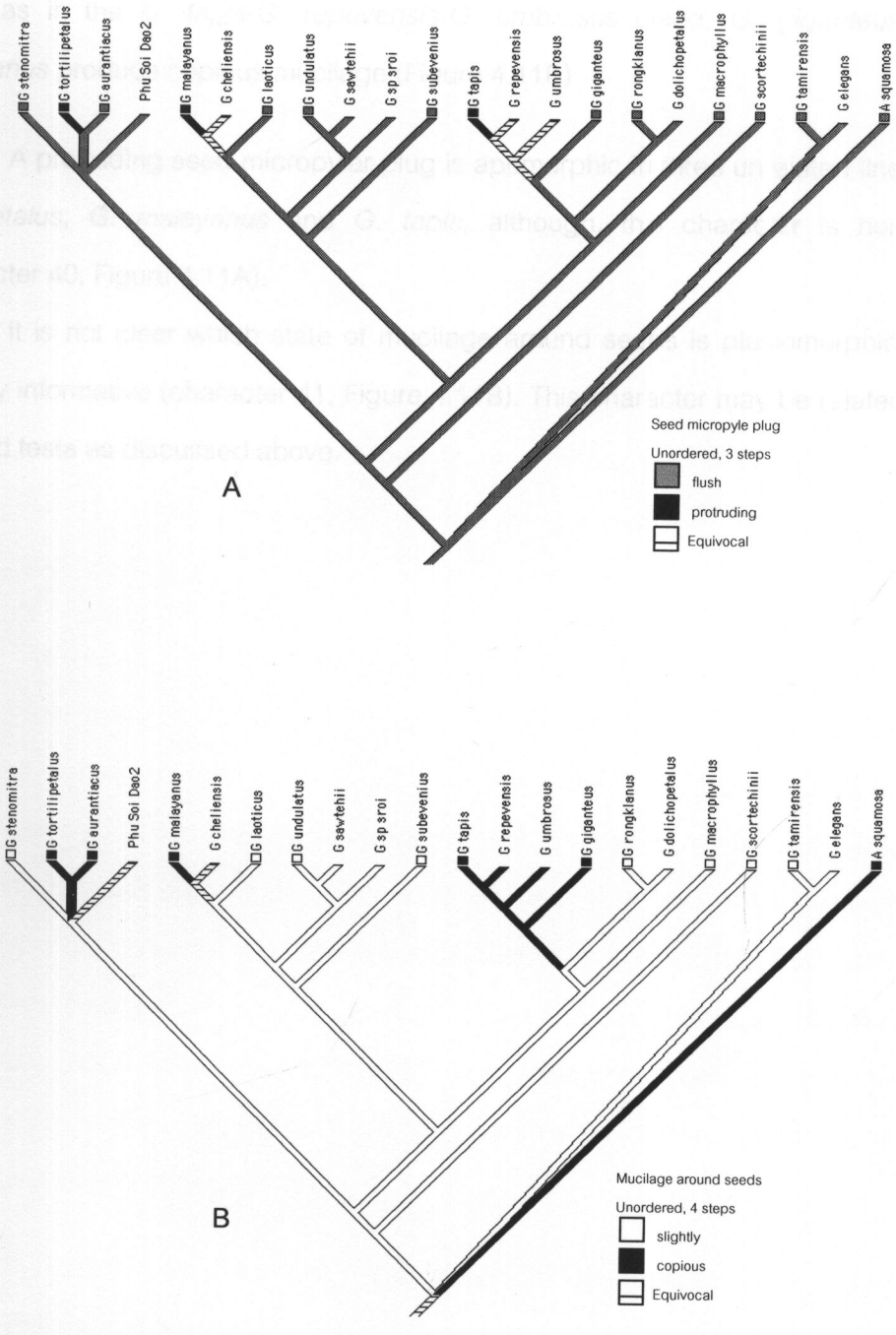


Figure 4.11 Trees showing inferred evolution of seed micropylar plug (A) and mucilage around seeds (B) in *Goniothalamus*.

seed, as in the *G. tapis-G. repevensis-G. umbrosus* clade, *G. giganteus* and *G. malayanus* produce copious mucilage (Figure 4.11B)

A protruding seed micropylar plug is apomorphic in three unrelated lineages, *G. tortilipetalus*, *G. malayanus* and *G. tapis*, although, this character is homoplastic (character 40, Figure 4.11A).

It is not clear which state of mucilage around seeds is plesiomorphic but it is partially informative (character 41, Figure 4.11B). This character may be related to hairs on seed testa as discussed above.

CHAPTER V

CONCLUSION

Results from the combined *trnL-F* spacer and ITS data sets showed better resolution of relationships within *Goniothalamus* than either *trnL-F* or ITS alone. The tree topology appeared fully resolved and the bootstrap supports were moderate to high. The phylogenetic tree indicated that *Goniothalamus* is likely to be monophyletic, with the *G. tamirensis-G. elegans* clade basal to the rest of *Goniothalamus*. The larger clade was divided into four recognizable subclades. None of the clades were congruent with Bân's infra-generic classification, suggesting as a new infra-generic classification scheme should be proposed.

The results from the assessment of the morphological character evolution showed that most of 43 characters were homoplastic. Some of 43 characters were apparently autapomorphic (e.g. habit, stamen number per flower and monocarp ornamentation), but these certainly were due to limited number of taxon in the assessment. There are only 9 out of 43 characters that were partial informative, i.e. synapomorphic characters for some clades, including velutinous primary shoots, nitid leaf surface adaxially, large outer petals, glabrous indument of the basal adaxial region of the outer petals, basally clawed outer petals, presence of glabrous basal flanges on the inner petal claws, large convoluted stigmas, the occurrence of a longitudinal ridge on monocarps and thick pericarp.

The results from this study indicated that the infra-generic classification scheme of *Goniothalamus* should emphasize to the synapomorphic characters discussed above in order to reflex the "true" phylogeny of the genus *Goniothalamus*.

REFERENCES

Agrawal, G.K., Pandey, R.N. and Agrawal, V.P. 1992. Isolation of DNA from *Cheorospondias asillaris* leaves. Biotech. Biodiv. Lett. 2 : 19-24.

APG. 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 : 339-436.

Baldwin, B.G., Sanderson, M.J., Porter, J.M., Wojciechowski, M.F., Campbell, C.S. and Donaghue, 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.

Bân, N.T. 1974a. On the taxonomy of the genus *Goniothalamus* (Blume) Hook.f. et Thoms.(Annonaceae). II. Bot. Zhurn. (Moscow & Leningrad) 59(4) : 547-555.

Bân, N.T. 1974b. On the taxonomy of the genus *Goniothalamus* (Blume) Hook.f. et Thoms. (Annonaceae). II. Bot. Zhurn. (Moscow & Leningrad) 59(5) : 660-672.

Bentham, G. and Hooker, J.D. 1862. Annonaceae. Genera Plantarum 2 : 20-29.

Besendahl, A., Qiu, Y.-L., Lee, J., Palmer, J.D. and Bhattacharya, D. 2000. The cyanobacterial origin and vertical transmission of the plastid $tRNA_{LUE}$ group-I intron. Current Genetics 37 : 12-23.

Blunden, G.A.K. and Jewers, K. 1973. The comperative leaf anatomy of *Goniothalamus andersonii*, *G. macrophyllus*, *G. malayanus* and *G. velutinus*. Botanical Journal of the Linnean Society 67 : 361-376.

Blunden, G.A.K. and Jewers, K. 1974a. The comperative stem and root anatomy of *Goniothalamus andersonii*, *G. macrophyllus*, *G. malayanus* and *G. velutinus* (Annonaceae) from peat swamps of Sarawak. Botanical Journal of the Linnean Society 68 : 209-225.

Blunden, G.A.K. and Jewers, K. 1974b. The morphology and anatomy of the fruit of *Goniothalamus andersonii*. Lloydia 37 : 17-22.

Boerlage, J.G. 1899. Notes sur les Annonacees du Jardin Botanique de Buitenzorg. Icon. Bogor. 1 : 79-208.

Burkill, I.H. 1935. A dictionary of the economic products of the Malay Peninsula. 2 vols. Kuala Lumpur: Ministry of Agriculture and Co-operative.

Bygrave, P.C. 2000. Molecular systematics of Annonaceae Juss. Ph.D. dissertation, University of Reading, Reading, UK.

Chase M.W. et al. 1993. Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene *rbc*L. Annuals of the Missouri Botanical Garden 80 : 528-580.

Chatrou, L.W., Pirie, M.D., Erkens, R.H.J., Niet van der T., Mols, J.B. and Maas, J.W. 2002. Species-level phylogenetics in Neotropical Annonaceae. Annonaceae Newsletter No.14 page15-36.

Chesters, K.I.M. 1955. Some plant remains from the Upper Cretaceous and Tertiary of West Africa. Annals and Magazine of Natural History 12 : 489-504.

Clegg, M.T., Learn, G.H. and Golenberg, E.M. 1991. Molecular evolution of chloroplast DNA. In Evolution at the Molecular Level (eds Selander, R.K., Clark, A.G. and Whittam, T.S.), pp. 135-149. Sinauer associates, Sunderland, MA.

Corlett, R.T. 2004. Flower visitors and pollination in the Oriental (Indomalayan) Region. Biol. Rev. 79 : 497-532.

Corner, E.J.H. 1988. Wayside Trees of Malaya, 3rd Edn. Malayan Nature Society, Kuala Lumpur, Malaysia.

Donoghue, M.J. and Doyle, J.A. 1989. Phylogenetic analysis of angiosperms and the relationships of Hamamelidae. Pp. 17-45 in Evolution, systematics and fossil history of the Hamameilidae, vol. 1, ed. P.R. Crane and S. Blackmore, Oxford: Clarendon Press.

Doyle, J.A. and Le Thomas, A. 1994. Cladistic analysis and pollen evolution in Annonaceae. Acta Botanica Gallica 141 : 149-170.

Doyle, J.A. and Le Thomas, A. 1996. Phylogenetic analysis and character evolution in Annonaceae. Bulletin du Museum National d'Histoire Naturelle, 4e serie, Sect. B, Adansonia 18 : 279-334.

Doyle, J.A. and Endress, P.K. 2000. Morphological phylogenetic analysis of basal angiosperm: comparison and combination with molecular data. International Journal of Plant Sciences 16(Supplement) : S121-S153.

Doyle, J.A., Bygrave, P. and Le Thomas, A. 2000. Imbrications of molecular data for pollen evolution in Annonaceae. Pollen and spores: morphology and biology (ed. By M.M. Harley, C.M. Morton and S. Blackmore), pp. 259-284. Royal Botanical Garden, Kew, UK.

Doyle, J.A., Sauquet, H., Scharaschkin, T. And Le Thomas A. 2004. Phylogeny, molecular and fossil dating, and biogeographic history of Annonaceae and Myristicaceae (Magnoliales). International Journal of Plant Sciences 165 : S55-S67.

Ehrendorfer, F., Kendall, F., Habeler, E.E. and Suer, W. 1968. Chromosome numbers and evolution in primitive angiosperm. Taxon 17 : 337-353.

Erkens, R.H.J. 2002. A central America origin for *Guatteria* (Annonaceae) based on 5 chloroplast markers. Annonaceae Newsletter No.14 page16.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstap. Evolution 39 : 783-791.

Francisco-Ortega, J., Park, S.-J., Santos-Guerra, A., Benabid, A. and Jansen R.K. 2001. Origin and evolution of the endemic Macaronesian Inuleae (Asteraceae): evidence from the internal transcribed spacers of nuclear ribosomal DNA. Biological Journal of the Linnean Society 72 : 77-97.

Fries, R.E. 1959. Annonaceae. In Die Naturlichen Pflanzenfamilien Band. 17a II : 1-171. Duncker & Humblot, Berlin.

Gielly, L. and Taberlet, P. 1994. The use of chloroplast DNA to resolve plant phylogenies: noncoding versus *rbcL* sequences. Molecular Biology and Evolution 11 : 768-777.

Hodges, S.A. and Arnold, M.L. 1994. Columbines: A geographically widespread species flock. Proceeding of the National Academy of Sciece (USA) 91 : 5129-5132.

Holmgren, P.K., Keuken, W. and Schofield, E.K. 1981. Index Herbariorum I. Edition 7, Frans A. Stafleu, Utrecht, The Netherlands.

Hutchinson, J. 1923. Contributions towards a phylogenetic classification of flowering plants 2. The genera of Annonaceae. Kew Bulletin 7 : 241-261.

Hutchinson, J. 1964. The genera of flowering plants. Dicotyledons 1. Clarendon Press, Oxford, UK.

Keßler, P.J. A. 1993. Annonaceae, In K. Kubitzki, J.G. Rohwer, and V. Bittrich [eds.], The families and genera of vascular plants, vol. 2, 93-129. Springer-Verlag, Berlin, Germany.

Keßler, P.J. A. 1996. *Goniothalamus majestatis*, a new species of Annonaceae from Sulawesi, Indonesia. Blumea 41 : 27-28.

Kitamura, S. Post-doctoral Student, Hornbill Thailand Project, Mahidol University. Personal communication, 27 April 2006.

Koek_Noorman, J., Van Setten, A.K. and Van Zuilen, C.M. 1997. Studies in Annonaceae XXVI. Flower and fruit morphology in Annonaceae. Their contribution to patterns in cluster analysis. Botanische Jahrbücher für Systematik 119(2) : 213-230.

Kumar, S., Tamura, K. and Nei, M. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and Sequence Alignment. Briefings in Bioinformatics 5:150-163.

Le Thomas, A. 1981a. Ultrastructure characters of the pollen grains of African Annonaceae and their significance for the phylogeny of primitive angiosperm (first part). Pollen Spores 22 : 267-342.

Le Thomas, A. 1981b. Ultrastructure characters of the pollen grains of African Annonaceae and their significance for the phylogeny of primitive angiosperm (second part). Pollen Spores 23 : 5-36.

Maddison, D.R. and Maddison, W.P. 2001. MacClade4: analysis of phylogeny and character evolution. Version 4.03. Sunderland: Sinauer Associates.

Mat-Salleh, K.B. 1989. Ethnobotanical significance of Asiatic Annonaceae. Pp. 80-87 in Malaysian Traditional Medicines, eds. Soepadmo, E., Goh, S.H., Wong, W.H., Din, L.B. and Chuah, C.H. Kuala Lumpur: Institute of Advanced Studies, Universiti Malaya.

Mat-Salleh. K.B. 1993. Revision of the genus *Goniothalamus* (Annonaceae) of Borneo. Michigan State University, 261 pp.

Mat-Salleh, K.B. 2001. New and noteworthy species of Bornean *Goniothalamus* (Annonaceae). Folia Malaysiana 2 : 75-116.

Mat-Salleh, K.B., Lim, J.S.H. and Ratnam, W. 2000. Genetic relatedness between populations of *Goniothalamus umbrosus* J. Sinclair (Annonaceae). Proceeding International Conference on in-situ and ex-situ Biodiversity Conservation in the New Millennium, 19-22 June 2000, Shangri La Tanjung Aru Resort, Kota Kinabalu.

McCarthy, C. 1997. Chromas version 1.45. School of Biomolecular and Biomedical Science, Brisbane, Queensland, Australia.

McCauley, D.E. 1995. The use of chloroplast DNA polymorphism in studies of gene flow in plants. Tree 10 : 198-202.

Meade, C.V. 2000. A systematic revision of the *Uvaria* L. group (Annonaceae) in continental Asia. Ph.D Thesis of the University of Dublin. 311 pp.

Mickevich, M.F. and Ferris, J.S. 1981. The implications of congruence in Menidia. Systematic Zoology 30 : 351-370.

Mols, J.B., Gravendeel, B., Chatrou, L.W., Pirie, M.D., Bygrave, P.C., Chase, M.W. and Keßler, P.J.A. 2004. Identifying clades in Asian Annonaceae: monophyletic genera in the polyphyletic Miliuseae. American Journal of Botany 91 :590-600.

Mols, J.B., Keßler, P.J.A. and Gravendeel, B. 2002. Identifying clades in Asian soursops-molecular phylogeny of *Miliusa* and *Polyalthia* (Annonaceae). Annonaceae Newsletter No.14 page19-20.

Morawetz, W. 1988. Karyosystematics and evolution of Australian Annonaceae as compared with Eupomatiaceae, Himantandraceae and Austrobaileyaceae. Plant Systematic Evolution. 159 : 19-79.

Morton, B.R. and Clegg, M.T. 1993. A chloroplast DNA mutational hotspot and gene conversion in a non-coding region near *rbc*L in the grass family (Poaceae). Current Genetics 24 : 357-365.

Nicholas, K.B. and Nicholas H.J.B. 1997. Genedoc: a tool for editing and annotating multiple sequence alignment. www.psc.edu/biomed/genedoc.

Okada, H. and Ueda, K. 1984. Cytotaxonomical studies on Asian Annonaceae. Plant Systematic Evolution 144 : 165-177.

Pennington, R.T. and Dick, C.W. 2004. The role of immigrants in the assembly of the South American rainforest tree flora. Philosophical Transactions of the Royal Society of London, Series B 359 : 1611-1622.

Perry, L.M. 1980. Medicinal Plants of East and Southeast Asia. Cambridge: MIT Press.

Powell, W.M., Morgante, M. and Andre, C. 1995. Hypervariable microsatellites provide a general source of polymorphic DNA markers for the chloroplast genome. Current Biology 5 : 1023-1029.

Qiu, Y.L., Lee, J., Bernasconi-Quadroni, F., Soltis, D.E., Soltis, P.S., Zanis, M., Zimmer, E.A., Chen, Z., Savolainen, V. and Chase, M.W. 2000. Phylogeny of basal angiosperm: analysis of five genes from three genomes. International Journal of Plant Sciences 161, Supplement 6 : 3-27.

Quandt, D., Muller, K., Stech, M., Fraham, J.-P., Frey, W., Hilu, K. And Borsch, T. 2004. Molecular evolution of the chloroplast *trn*L-F region in land plants. Annals of the Missouri Botanical Garden 13-37.

Richardson, J.E., Chatrou, L.W., Mols, J.B., Erkens, R.H.J. and Pirie, M.D. 2004. Historical biogeography of two cosmopolitan families of flowering plants: Annonaceae and Rhamnaceae. Philosophical Transactions of the Royal Society of London, Series B 359 : 1495-1508.

Ridley, H.N. 1922. The Flora of the Malay Peninsula. L. Reeve&Co., London.

Sambrook, J., Fritch, E.F. and Maniatis, T. 1989. Molecular Cloning a Laboratory Manual 2nd eds. Cold Springs Harbor, New York: Cold Spring Harbor Laboratory Press.

Sang, T., Crawford, D.J. and Stuessy, T.F. 1997. Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer of nuclear ribosomal DNA: implications for biogeography and concerted evolution. Proceeding of the National Academy of Sciences (USA) 92 : 6813-6817.

Saunders, R.M.K. 2002. The genus *Goniothalamus* (Annonaceae) in Sumatra. Botanical Journal of the Linnean Society 139 : 225-254.

Saunders, R.M.K. 2003. A synopsis of *Goniothalamus* species (Annonaceae) in Peninsular Malaysia, with a description of a new species. Botanical Journal of the Linnean Society 142 : 321-339.

Sauer, W. and Ehrendirfer, F. 1984. Notes on Karyosystematics of Annonaceae. Plant Systematic Evolution 146 : 47- 55

Sauquet, H., Doyle, J.A., Scharaschkin, T., Borsch, T., Hilu, K.W., Chatrou, L.W. and Le Thomas, A. 2003. Phylogenetic analysis of Magnoliales and Myristicaceae based on multiple datasets: implication for character evolution. Botanical Journal of the Linnean Society 142 : 125-186.

Scharaschkin, T. and Doyle, J.A. 2005. Phylogeny and Historical Biogeography of Anaxagorea (Annonaceae) Using Morphology and Non-Coding Chloroplast Sequence Data. Systematic Botany 30 : 712-735.

Scharaschkin, T. and Doyle, J.A. 2006. Character evolution in Anaxagorea (Annonaceae). American Journal of Botany 93 : 36-54.

Sinclair, J. 1955. A revision of the Malayan Annonaceae. Grad. Bull. Singapore 14 : 149-516.

Sobha, V. and Ramachandran, K. 1979. IOPB chromosome number reports LXVI. Taxon 28 : 269.

Sole de Porta, N. 1971. Algunos generos nuevos de polen procedentes de la Formacion Guaduas (Maastrichtiense-Paleoceno) de Colombia. Studia Geologica Salmanticensia 2 : 133-143.

Soltis, P.S., Soltis, D.E., Zanis, M.J. and Kim, S. 2000. Basal lineages of angiosperms: relationships and imbrications for floral evolution. International Journal of Plant Sciences 161(Supplement) : S97-S107.

Swofford, D.L. 1998. PAUP*: phylogenetic analysis using parsimony. Sinauer Associates, Sunderland, Massachusetts, USA.

Taberlet, P., Gielly, L., Pautou, G. and Bouvet, J. 1991. Unversal primers for amplification of three non-coding regions of Chloroplast DNA. Plant Molecular Biology 17 : 1105-1109.

Van Heusden, E.C.H. 1992. Flowers of Annonaceae: morphology, classification and evolution. Blumea Supplement 7 : 1-218.

Van Setten, A.K. and Koek-Noorman, J. 1992. Fruits and seeds of Annonaceae: morphology and its significance for identification. Bibliotheca Botanica 142 : 1-101.

Walker, G.H.K. 1971. Pollen morphology, phytogeography and phylogeny of the Annonaceae. Contributions from the Gray Herbarium of Harvard University 202 : 1-132.

Wikstrom, N., Savolainen, V. and Chase, M.W. 2001. Evolution of the angiosperms: calibrating the family tree. Proceedings of the Royal Society of London, Biological Sciences 1482 : 2211-2220.

White, T.J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315-322. In PCR protocols: a guide to methods and amplifications eds. M. Innis, D. Gelfand, J. Sninsky and T. White. Academic Press, San Diego, California, USA.

Wyk, R.W. van der and Canright, J.E. 1956. The anatomy and the relationship of the Annonaceae. Tropical Woods 104 : 1-24.

APPENDICES

APPENDIX A

PROTOCOL FOR DNA EXTRACTION OF HERBARIUM SPECIMENS

This is a protocol for DNA extraction from herbarium specimens using a combination of CTAB method (Agrawal et al., 1992) and QIAquick PCR Purification Kit (QIAGEN).

Materials:

1. Pestles and mortars
2. 1.5 µl microcentrifuge tubes
3. CTAB buffer
4. ß-mercaptoethanol
5. Chloroform : isoamylalcohol (24 :1) mixture
6. QIAquick PCR Purification Kit (QIAGEN)
7. Water-bath
8. Liquid nitrogen
9. RNase A (100mg/ml)

Preparation:

1. Preheat CTAB buffer in water-bath to 65°C
2. Set the microcentrifuge tube filled with extraction buffer containing 650 µl CTAB buffer, 20 µl ß-mercaptoethanol and 10 µl RNase A at 65 °C
3. Clean leaf with 70% alcohol, then remove midrib and weigh 50 mg leaf material of each sample

Extraction:

1. Add liquid nitrogen to a pestle containing leaf material and grind to powder with a mortar.

2. Transfer powder to extraction buffer in a microcentrifuge tube, vertex 10 second, and incubate 65 °C for 3 hours, mixing every 10 minutes.

3. Add 650 µl of Chloroform : Isoamylalcohol mixture, invert gently 5 times and incubate by gently shaking at room temperature. Leave for at least 1½ hours

4. Centrifuge at 10,000 rpm at RT for 10 minutes

5. Transfer supernatant into a new microcentrifuge tube

6. Add 1ml Buffer PB (provided in QIAquick PCR Purification Kit) and mix by pipetting

7. Place a QIAquick spin column in a collecting tube

8. To bind DNA, apply the sample from step 6. to the QIAquick column and centrifuge for 30-60 second

9. Discard flow-through and place the QIAquick column back into the same tube

10. To wash, add 500 µl Buffer PE to the QIAquick column and centrifuge for 30-60 second

11. Discard flow-through and place the QIAquick column back into the same tube. Centrifuge the column for an additional 1 minute

12. Place QIAquick column in a clean microcentrifuge tube

13. To elute DNA, add 30 µl Buffer EB or H_2O to the center of the QIAquick membrane, let the column stand for 1 minute and centrifuge the column for 1 minute

14. DNA is ready to use

APPENDIX B

CHARACTER SCORING FOR MORPHOLOGICAL DATA MATRIX

1. Habit: (0) shrubs or small trees; (1) large trees
2. Indument of young primary shoots: (0) generally glabrous to hairy; (1) invariably densely hairy/velutinous
3. Glossiness of leaf lamina (adaxially): (0) matt; (1) nitid
4. Prominence of secondary veins (adaxially): (0) impressed to slightly prominent; (1) distinctly prominent
5. Tertiary vein arrangement: (0) reticulate; (1) percurrent
6. Flower position: (0) axillary; (1) (slightly) supra-axillary
7. Occurrence of flower fascicles: (0) absent; (1) present
8. Flower position on branches: (0) young growth only; (1) also older growth
9. Flower position on trunk: (0) not exclusively at base; (1) exclusively at base
10. Number of flowers: (0) solitary (occasionally paired); (1) invariably paired
11. Flower orientation: (0) pendent; (1) erect
12. Flower pedicel length: (0) up to 20 mm maximum; (1) 21-50 mm maximum; (2) 51-110 mm maximum
13. Sepal fusion: (0) free; (1) (sometimes) basally connate
14. Sepal venation: (0) indistinct; (1) (sometimes) distinct
15. Sepal refexion: (0) not reflexed; (1) reflexed
16. Outer petal length: (0) up to 45 mm maximum; (1) 50-85 mm maximum; (2) over 100 mm maximum
17. Shape of outer petal base: (0) not distinctly clawed; (1) distinctly clawed
18. Indument of basal adaxial region of outer petals: (0) glabrous or sparsely hairy; (1) velutinous
19. Shape of inner petals: (0) without extensive contiguous area: (1) extensive contiguous area
20. Indument of inner petal (adaxially): (0) glabrous; (1) velutinous; (2) woolly
21. Presence of glabrous basal flanges on inner petal claw: (0) absent; (1) present

22. Stamen number per flower: (0) up to 320 maximum; (1) 450-570 maximum
23. Staminal connective shape: (0) truncate; (1) apiculate;
(2) very long apiculate
24. Apiculate staminal connective shape:
(0) not distinctly tapered; (1) distinctly tapered
25. Carpel number per flower: (0) up to 60 maximum; (1) 90-105 maximum
26. Ovary indument: (0) essentially glabrous; (1) hairy
27. Style indument: (0) glabrous; (1) hairy
28. Stigma shape: (0) funnel-shaped or subulate; (1) large, convoluted
29. Stigma indument: (0) (sub-) glabrous; (1) hairy
30. Sepal persistence in fruit: (0) caducous; (1) persistent
31. Monocarp shape: (0) not moniliform; (1) slightly moniliform (when > 2 seeds)
32. Monocarp width: (0) up to 15 mm maximum; (1) 15-25 mm maximum;
(2) 26-30 mm maximum
33. Occurrence of longitudinal ridge on monocarp: (0) absent; (1) present
34. Monocarp ornamentation: (0) smooth; (1) verrucose
35. Pericarp thickness: (0) thin; (1) (medium-) thick
36. Seed width: (0) up to 16 mm maximum; (1) 17-20 mm maximum
37. Seed number per monocarp: (0) 1-2 (-3); (1) 4-5
38. Seed shape around micropyle: (0) not elongated; (1) elongated
39. Indument of seed testa: (0) glabrous; (1) hairy
40. Seed micropylar plug: (0) sunken; (1) flush; (2) protruding
41. Mucilage around seeds: (0) slight; (1) copious
42. Inner petal arrangement: (0) not mitriform; (1) mitriform
43. Stamen septation: (0) aseptate; (1) septate

BIOGRAPHY

Maliwan Nakkuntod was born in Nakhon Ratchasima Province, Thailand, on 7 January 1975. She earned her Bachelor Degree in Science in Biology from the Department of Biology, Faculty of Science, Khon Kaen University, Khon Kaen, in 1995. In 1998, she received her Master of Science Degree in Botany from Department of Botany, Kasetsart University, Bangkok. After graduation, she worked as lecturer at Department of Biology, Faculty of Science, Naresuan University, Phitsanulok. Since June 2001, she pursued her Ph.D. study in Biological Science Ph.D. program, Faculty of Science, Chulalongkorn University, Bangkok.