A STUDY OF *GONIOTHALAMUS* (ANNONACEAE) IN THAILAND BASED ON CHLOROPLAST *trn*L AND *trn*G INTRON SEQUENCES

Yuttaya Yuyen[1], Jessada Denduangboripant[2], Piya Chalermglin[3], Quentin C. B. Cronk[4] and Vilaiwan Anusarnsunthorn[1]

ABSTRACT

Goniothalamus (Annonaceae) is a large (ca. 120 species) and highly diverse genus in Southeast Asia. Numerous infrageneric (subgeneric and sectional) classification systems have been proposed in the past, mainly on the basis of stamen characteristics and the number of ovules. To investigate the relationships of the *Goniothalamus* species occurring in Thailand, we performed a molecular phylogenetic analysis of 20 Thai accessions using comparative nucleotide sequences of chloroplast *trn*L and *trn*G introns. Our sequence alignment resulted in a combined data-matrix of 1,127 basepairs, though with only 5.9% parsimonious informative characters. Maximum-parsimony, neighbor-joining and maximum-likelihood analyses produced similar trees. Our results suggest that there are probably six groups of *Goniothalamus* in Thailand, but only one of these has high bootstrap support. The six groups reflect similarities in floral morphology but not biogeography. Elements of both Boerlage's and Bân's infrageneric classifications are reflected in our analysis, although our results are not strictly congruent with either of theirs.

Keywords: Annonaceae, *Goniothalamus*, molecular phylogeny, Thailand, *trn*L intron, *trn*G intron

INTRODUCTION

The Genus *Goniothalamus* (Blume) Hook. f. & Thoms.

Annonaceae are a pantropical family of shrubs, trees and lianas. The family consists of about 130 genera and 2,300 species. The largest number of genera and species are known from Asia (including Australia and the Pacific), with ca. 60 and 1000, respectively (MOLS & KESSLER, 2003). Among the Asian Annonaceae, *Goniothalamus* (Blume) Hook. f. & Thoms. is a large important genus of some 120 species (MAT-SALLEH, 2001). It is widely distributed

[1] Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand.
[2] Department of Biology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand.
[3] Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand.
[4] UBC Botanical Garden and Centre for Plant Research, University of British Columbia, 6804 SW Marine Drive, Vancouver, Canada V6T 1Z4.

in lowland and submontane tropical forests in Southeast Asia, with a center of species diversity in Indochina and Western Malesia (SAUNDERS, 2002; 2003). They are shrubs or small trees, characterised by having outer petals which are slightly or not spreading, while the inner petals are smaller, clawed and curving over the sexual organs to form a dome-shaped structure (mitreform dome) during anthesis. The stamens have broad apical connectives and their shapes range from truncate to apiculate.

Based on the morphological characters, SINCLAIR (1955) classified the family into 6 tribes (Uvarieae, Unonieae, Miliuseae, Mitrephoreae, Annonieae and Xylopieae). *Goniothalamus* was placed in the tribe Mitrephoreae by the character of inner petals curving over the sexual organs forming a dome-shaped (mitreform) structure, along with *Pseuduvaria, Neo-uvaria, Oxymitra, Mitrephora* and *Popowia*. In contrast, results from RICHARDSON *ET AL.* (2004), who studied the historical biogeography of Annonaceae using *rbc*L and *trn*L-F plastid DNA sequences, suggested that *Goniothalamus* was totally unrelated to other mitreform genera of Annonaceae. They found that *Goniothalamus* was a strongly supported group, but the sister group to the genus was unclear. Their molecular data indicated that *Goniothalamus* is closely related to *Anonidium, Neostenanthera, Disepalum, Asimina, Annona*, and *Rollinia*.

History of Classification

Goniothalamus is an interesting genus that is in need of study. The genus is poorly understood, both in terms of basic morphological taxonomy and phylogenetic relationships. It has been subject to several different subgeneric, sectional and subsectional classifications (Table 1). The name *Goniothalamus* was first proposed by BLUME (1830) in *Flora Javae* as a section of the genus *Polyalthia* to accommodate *Polyalthia macrophylla*, a species with an "angled receptacle". HOOKER & THOMSON (1855) later elevated this section to a genus in their *Flora Indica*. BOERLAGE (1899) incorporated *Beccariodendron*, a monospecific New Guinean genus, into *Goniothalamus*. The section *Beccariodendron* of BOERLAGE was founded to accommodate *Beccariodendron grandiflorus* and the multi-ovulate *Goniothalamus* species. Other *Goniothalamus* with only one or two ovules were then grouped into the section *Eu-Goniothalamus*.

BÂN (1974) proposed a more hierarchical infrageneric classification based mainly on stamen characters. He divided the genus into two subgenera: subgenus *Goniothalamus* (apiculate stamens) and subgenus *Truncatella* (truncate stamens). He also suggested that subgenus *Goniothalamus* could be divided into two sections: section *Goniothalamus* (with subsections *Goniothalamotypus* and *Pleiospermi*) and section *Longistigma*. The other subgenus *Truncatella* was also divided into two sections: section *Infundibulistigma* (with subsections *Polyspermi* and *Infundibuliformes*) and section *Truncatella* (with subsections *Multiseminales* and *Pauciseminales*). However, MAT-SALLEH (1993, 2001) in studying *Goniothalamus* species from Borneo, suggested that an infrageneric classification would be more natural if based on floral/leaf characters and habits. Recently, further Southeast Asian *Goniothalamus* collections (especially from Peninsular Malaysia and Sumatra) have been critically studied (SAUNDERS, 2002; 2003) and the usefulness of MAT-SALLEH's taxonomic characters further emphasised.

Table 1. Infrageneric classifications of the genus *Goniothalamus* proposed by Boerlage (1899) and Bân (1974).

Authors	Infrageneric classification	Morphological characters
Boerlage (1899)	Section 1. *Beccariodendron*	More than one ovule
	Section 2. *Eu–Goniothalamus*	One or two ovules
Bân (1974)	Subgenus 1. *Truncatella*	Connective truncate stamen
	Section 1. *Infundibulistigma*	Style short; stigma funnel-shaped or fusiform, broad
	Subsection 1. *Polyspermi*	Ovary with 3-10 ovules
	Subsection 2. *Infundibuliformes*	Ovary with 1-2 ovules
	Section 2. *Truncatella*	Style long, cylindrical or subuliform; stigma minute, integral or slightly bilobed
	Subsection 3. *Multiseminales*	Ovary with 3-8 ovules
	Subsection 4. *Pauciseminales*	Ovary with 1-2 ovules
	Subgenus 2. *Goniothalamus*	Connective apiculate stamen
	Section 3. *Goniothalamus*	Style distinctive, cylindrical or subuliform; stigma minute, integral or bilobed
	Subsection 5. *Goniothalamotypus*	Ovary with 1-2 ovules
	Subsection 6. *Pleiospermi*	Ovary with 3-10 ovules
	Section 4. *Longistigma*	Style cone-shaped; stigma filiform and extremely long

Economic Importance of *Goniothalamus*

The genus is not normally recognised as an important forest product. However, some species are used as ornamental plants because of their small size and strongly fragrant flowers. The species most commonly cultivated are *G. griffithii* Hook. f. & Thomson, *G. laoticus* (Finet & Gagnep.) Bân, *G. macrophyllus* (Blume) Hook. f. & Thomson, *G. malayanus* Hook. f. & Thomson, *G. repevensis* Pierre ex Finet & Gagnep., *G. sawtehii* Fischer, *G. tapis* Miq., *G. tortilipetalus* Henderson, *G. umbrosus* J. Sinclair and *G. undulates* Ridl.

BURKILL (1935) reported that *G. macrophyllus, G. umbrosus*, and *G. tapis* were widely utilised by village midwives in the Malay Peninsula in their traditional practices. *Goniothalamus malayanus* and *G. macrophyllus* have been used too in Borneo for the same purpose (MAT-SALLEH, 1993). Javanese mountain dwellers also treated patients suffering with fevers with aromatic roots of *G. macrophyllus* (BURKILL, 1935).

There have been several recent phytochemical studies on *Goniothalamus* focusing on their potential for anti-cancer therapies. Styryl-pyrrone and styryl lactone derivatives from *Goniothalamus* have been suggested to have anti-cancer activities (LI *ET AL.*, 1998; INAYAT-HUSSAIN *ET AL.*, 2002; UMAR-TSAFE *ET AL.*, 2004; TIAN *ET AL.*, 2006).

Goniothalamus in Thailand

Thailand is an important distributional area for *Goniothalamus*. Although only nine species were reported in Thailand by CRAIB (1925), a more recent checklist (BYGRAVE, 1997) listed 21 species. A recent survey by CHALERMGLIN (2001) found 23 species of Thai *Goniothalamus*. We suspect that the real number of *Goniothalamus* species in Thailand is likely to be considerably higher.

So far, no comprehensive treatment of *Goniothalamus* in Thailand has been undertaken and existing studies of Thai species are based on morphological characters alone. The use of molecular data is of potential value in helping to assess the utility of morphological taxonomic characters, and to assist in the natural classification of the genus.

For this reason we conducted a molecular systematic investigation on the Thai species using chloroplast *trn*L and *trn*G intron sequences. The *trn*L intron sequences have been used successfully in other genera of the Annonaceae, for instance in a molecular phylogeny of *Miliusa* and its allies in the tribe Saccopetaleae (MOLS *ET AL.*, 2000), and phylogenies of some Neotropical genera (CHATROU *ET AL.*, 2002). The *trn*G intron has never previously been used for phylogenetic studies in the Annonaceae, but it has occasionally been used in other groups of plants (PEDERSEN & HEDENÄS, 2003; SHAW & SMALL, 2004).

In this study, we reconstruct a molecular phylogeny of 20 Thai *Goniothalamus* taxa using the combined *trn*L and *trn*G intron sequences. The results are discussed in the context of the infrageneric classifications of BOERLAGE (1899) and BÂN (1974).

MATERIALS AND METHODS

Ingroup and Outgroup Taxa

Fresh leaves of 20 Thai *Goniothalamus* taxa were collected in the wild and kept dry in silica gel until used (Fig. 1). Plant specimens were identified to species following CRAIB (1925), SINCLAIR (1955), MAT-SALLEH (1993), and SAUNDERS (2002, 2003). Some plants were unidentifiable and had to be informally named (using the collecting locality) pending either identification with species not currently recorded from Thailand or description as new species. Work on describing these informally named specimens is in progress. Herbarium specimens and spirit materials were deposited at CMU (Chiang Mai University) Herbarium, Chiang Mai, Thailand.

We used all 20 *Goniothalamus* as ingroup taxa (Table 2) while two other Thai Annonaceae, *Orophea enterocarpa* Maingay ex Hook. f.&Thoms. and *Artabotrys spinosus* Craib were used as outgroups to root the phylogeny. RICHARDSON *ET AL.* (2004) reported other genera more phylogenetically closely related to *Goniothalamus*, but *Artabotrys* is the only genus of the tribe Mitrephoreae that we could collect in Thailand and successfully sequence its DNA. In fact, two more outgroups from the tribe Mitrephoreae (*Friesodielsia desmoides* (Craib) Steenis and *Mitrephora kethii* Ridley) were also tested but none of the PCR products gave a readable sequence. On the other hand, *Orophea enterocarpa* was also selected as the outgroup because it is in tribe Miliuseae (SINCLAIR, 1955) which is closely related to the tribe Mitrephoreae.

Genomic DNA Extraction, PCR Amplification, and DNA Sequencing

Total genomic DNA was extracted from a leaf sample of each plant using DNeasy® Plant Mini kit (QIAGEN GmbH, Germany) or the CTAB method (DOYLE & DOYLE, 1987). DNA quantity and quality was examined by agarose gel electrophoresis. Chloroplast *trn*L intron sequences were amplified with the Polymerase Chain Reaction (PCR) technique using a forward primer *trn*L-C (5'-CGA AAT CGG TAG ACG CTA CG-3') and a reverse primer *trn*L-D (5'-GGG GAT AGA GGG ACT TGA AC-3'), following TABERLET *ET AL.* (1991). Amplifications were carried out in 50-μl reaction volume with 0.8 μl of 2.5 unit of *Taq* DNA Polymerase (Finnzyme, Finland), 5 μl of 10× optimised *Taq* Polymerase buffer with 1.5 mM MgCl$_2$ included (Finnzyme, Finland), 1μl of 10-mM dNTP, 5 μl of 10-μM each primer, and 10–20 ng of genomic DNA. The PCR cycling profile to amplify *trn*L intron sequences was modified from BRUNEAU *ET AL.* (2001). The PCR amplification was performed using a GeneAmp® PCR system 9700 (Applied Biosystems, Singapore), programmed for pre-denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C 1 min, annealing 55°C 1 min and extension 72°C 2 min, with final extension step at 72°C for 7 min.

Chloroplast *trn*G intron sequences were amplified using a forward primer 3'*trn*G (5'-GTA GCG GGA ATC GAA CCC GCA TC-3') and a reverse primer 5'*trn*G2G (5'-GCG GGT ATA GTT TAG TGG TAA AA-3') following SHAW *ET AL.* (2005). Amplifications were carried out in 25-μl reaction volume with 0.25 μl of 5 unit of *Taq* DNA Polymerase (New England BioLabs, USA), 2.5 μl of 10× *Taq* DNA Polymerase buffer with 1.5 mM MgCl$_2$ included (New England BioLabs, USA), 2.5 μl of 2 mM dNTP and 1 μl of 10 μM each primer (forward and reverse), and 1 μl (10–20 ng) of total DNA. The PCR cycling profile to amplify
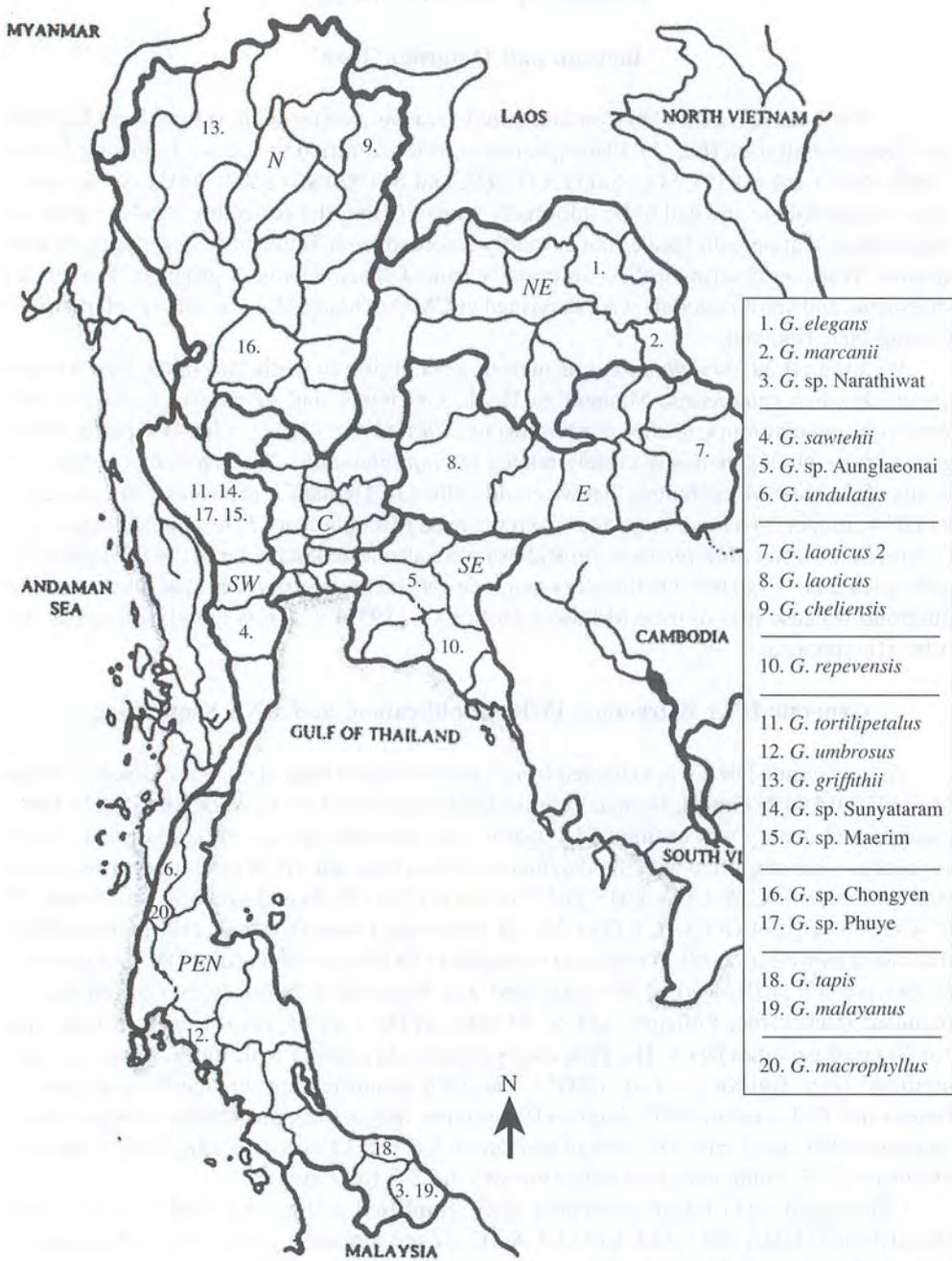
Figure 1. Collecting localities of 20 Thai *Goniothalamus* specimens used in this study. The floristic-region map
of Thailand is after Smitinand (2001). Abbreviations of the floristic-regions are as follows: *N* = North,
NE = Northeast, *E* = East, *SW* = Southwest, *C* = Central, *SE* = Southeast, *PEN* = Peninsular.

Table 2. Collector number and GenBank accession numbers of 20 *Goniothalamus* samples and two outgroups (*Artabotrys spinosus* and *Orophea enterocarpa*) collected and examined in this study.

No.	Taxon	Locality (province)	Collector no.	GenBank accession no.
1.	*G. cheliensis* Hu	Doi Phu Kha (Nan)	Yuyen 220	EU249788, EU249766
2.	*G. elegans* Ast	Phu Phan (Sakon Nakhon)	Yuyen 229	EU249783, EU249761
3.	*G. griffithii* Hook. f. & Thomson	Doi Saket (Chiang Mai)	Yuyen 208	EU249798, EU249776
4.	*G. laoticus* (Finet & Gagnep.) Bân (sample 1)	Khao Yai (Nakhon Ratchasima)	Yuyen 250	EU249800, EU249778
5.	*G. macrophyllus* (Blume) Hook. f. & Thomson	Khao Pho Ta Luang Kaew (Ranong)	Yuyen 211	EU249789, EU249767
6.	*G. marcanii* Craib	Phu Moo (Mukdahan)	Chalermglin 25-4-47(1)	EU249787, EU249765
7.	*G. malayanus* Hook. f. & Thomson	Ba Cho (Narathiwat)	Yuyen 209	EU249796, EU249774
8.	*G. repevensis* Pierre ex Finet & Gagnep.	Khao Soi Doaw (Chanthaburi)	Yuyen 232	EU249794, EU249772
9.	*G. sawtehii* Fischer	Phanoenthong (Phetchaburi)	Yuyen 235	EU249784, EU249762
10.	*G. tapis* Miq.	Sai Buri (Pattani)	Yuyen 205	EU249793, EU249771
11.	*G. tortilipetalus* Henderson	Thong Pha Phum, (Kanchanaburi)	Yuyen 234	EU249790, EU249768
12.	*G. umbrosus* J. Sinclair	Khlong Thom (Krabi)	Yuyen 236	EU249797, EU249775
13.	*G. undulatus* Ridl.	Khlong Na Kha (Ranong)	Yuyen 217	EU249786, EU249764
14.	*G.* sp. Aunglaeonai (อ่างฤๅใน)	Aunglaeonai (Chachoengsao)	Yuyen 213	EU249785, EU249763
15.	*G.* sp. Chongyen (ช่องเย็น)	Chong Yen (Kamphaeng Phet)	Chalermglin 6-3-47(1)	EU249791, EU249769
16.	*G.* sp. Maerim (แม่ริม)	Kanchanaburi	Yuyen 239	EU249802, EU249780
17.	*G.* sp. Narathiwat (นราธิวาส)	Mueng (Narathiwat)	Yuyen 206	EU249795, EU249773
18.	*G.* sp. Phuye (พุเย)	Thong Pha Phum (Kanchanaburi)	Yuyen 219	EU249792, EU249770
19.	*G.* sp. Sunyataram (สุญตาราม)	Thong Pha Phum (Kanchanaburi)	Yuyen 218	EU249801, EU249779
20.	*G. laoticus* 2 (Finet & Gagnep.) Bân (sample 2)	Dongphahuan (Ubon Ratchathani)	Yuyen 228	EU249799, EU249777
	Out group *Artabotrys spinosus* Craib	Dongphahuan (Ubon Ratchatani)	Yuyen 247	EU249782, EU249760
	Out group *Orophea enterocarpa* Maingay ex Hook. f. & Thomson	Dongphahuan (Ubon Ratchatani)	Yuyen 246	EU249781, EU249759

*trn*G intron sequences was modified from SHAW *ET AL.* (2005). The PCR amplification was performed on Mastercycler® PCR gradient system (Eppendorf AG, Hamburg) programmed for pre-denaturation at 80°C for 5 min, 30 cycles of denaturation at 95°C 45 sec, annealing 48–52°C 30 sec and extension 72°C 1 min; with final extension step at 72°C for 5 min.

PCR products were purified using QIAquick PCR purification kit (QIAGEN GmbH, Germany) prior to cycle sequencing reaction. Sequencing products were analysed on an automated DNA sequencer ABI Prism® 377 (Applied Biosystems, USA) or an ABI 3730XL sequencer of Macrogen Inc. (in Seoul, South Korea). Both forward and reverse sequencing reactions were performed for sequence confirmation of each sample.

Phylogenetic Analyses

The *trn*L and *trn*G intron sequences were aligned using the alignment program ClustalX with minor manual adjustments. Sequence boundaries of *trn*L and *trn*G intron were determined using published sequences of other genera in the GenBank nucleotide database. Single and combined *trn*L and *trn*G intron sequence matrices were analysed with a phylogenetic reconstruction program PAUP* version 4.0b10 (SWOFFORD, 1998) running on Macintosh Powerbook (CPU G3-300MHz, 192Mb RAM) with character states unordered and equally weighted. Phylogenetic trees were reconstructed using a maximum-parsimony (MP) method as implemented in the program. Gaps (indels) were treated as missing data. All sequence characteristics of the single *trn*L intron, *trn*G intron, and combined sequence data matrices were obtained from PAUP*.

We performed branch-and-bound search in order to find the most parsimonious trees (MPTs). The analyses were carried out without using a gap matrix since no different tree-topology was found from the initial analysis. A semistrict consensus tree was calculated from all found MPTs. Bootstrap (FELSENSTEIN, 1985) and jackknife (LANYON, 1985) analyses were performed 1,000 replicates using PAUP* set to a heuristic-search option and a simple sequence addition. Descriptive statistic indices reflecting amounts of phylogenetic signals in the parsimony analyses were given by consistency index (CI), retention index (RI), and rescaled consistency (RC) index.

A neighbour-joining (NJ) tree and a maximum likelihood (ML) tree from the 1227-bp combined sequence data matrix were also reconstructed using PAUP* program to compare with the MP results. Evolutionary model and parameter settings for the ML analysis were suggested by a program ModelTest version 3.06 (POSADA & CRANDALL, 1998). TVM+G model was selected with a substitution-rate matrix of 0.5093, 1.7681, 0.3491, 0.7361, and 1.7681; nucleotide frequencies A = 0.34250, C = 0.15890, G = 0.19350, and T = 0.30510; and a gamma distribution parameter (G) = 0.9324. The ML analysis was executed with a heuristic search strategy using a random sequence addition and TBR branch-swapping.

RESULTS

Collection of *Goniothalamus* Species in Thailand

From our field expeditions (focusing mainly on the north of the country), we were able to locate 20 taxa of *Goniothalamus* in Thailand. Fourteen of these were identifiable with known

species. The other six were unknown and had to be named following the collecting localities: *G.* sp. Narathiwat, *G.* sp. Aunglaeonai, *G.* sp. Sunyataram, *G.* sp. Maerim, *G.* sp. Chongyen, and *G.* sp. Phuye. Of the 14 identifiable taxa, five were new records for Thailand. We found five of the nine species reported from Thailand by CRAIB (1925), eight of the 21 species listed by BYGRAVE (1997) and 16 of the 23 taxa found by CHALERMGLIN (2001).

Sequence Alignment and Matrix Characteristics

Sequence characteristics of the *trn*L and *trn*G intron data for 20 Thai *Goniothalamus* and two outgroups are summarised in Table 3. The aligned *trn*L and *trn*G matrices can be obtained from the first author. From the 1,227-basepair combined data matrix, only 73 nucleotides (5.9%) were found to be parsimony-informative, while 127 nucleotides (10.4%) were autapomorphic characters. An initial MP analysis of the whole 1,227-bp alignment yielded more MPTs than could be held in the computer memory; therefore, only the 73 parsimony-informative characters were used in further analyses. The consistency index (CI) and retention index (RI) values were 0.8667 and 0.8824, respectively, while the rescaled consistency index (RC) was 0.7674. When all 1,227 nucleotides of the matrix were analysed, the indices were slightly higher (CI = 0.9492, RI = 0.8824, and RC = 0.8375).

Table 3.   Sequence characteristics of the single *trn*L intron, *trn*G intron, and combined sequence data matrices used in this study.

Parameters	*trn*L intron	*trn*G intron	Combined sequence
Length range (total)	519–575 bp	602–723 bp	1143–1284 bp
Length mean (total)	555 bp	635 bp	1190 bp
Length range (ingroups only)	523–575 bp	602–723 bp	1152–1284 bp
Length mean (ingroups only)	557 bp	636 bp	1193 bp
Length range (outgroups only)	519–561 bp	624–631 bp	1143–1192 bp
Length mean (outgroups only)	540 bp	627 bp	1168 bp
G + C content range	35–39%	33–37%	35–38%
G + C content mean	38%	36%	37%
Number of excluded sites	52 bp	80 bp	132 bp
Number of sites after exclusion	540 bp	687 bp	1227 bp
Aligned length (total)	592 bp	767 bp	1359 bp
Sequence divergence (ingroups)	0.00–1.91%	0.00–3.52%	0.09–2.43%
Sequence divergence (in/outgroups)	10.56–12.12%	6.10–9.09%	8.55–10.11%
Number of indels (ingroups)	6	72	78
Number of indels (total)	8	73	81
Size of indels (ingroups)	1–8 bp	1–21 bp	1–21 bp
Size of indels (total)	1–9 bp	1–21 bp	1–21 bp
Number of constant sites	459 bp (85%)	568 bp (82.7%)	1027 bp (83.7%)
Number of variable sites	81 bp (15%)	119 bp (17.3%)	200 bp (16.3%)
Number of MP-informative sites	51 bp (9.4%)	22 bp (3.2%)	73 bp (5.9%)
Number of autapomorphic sites	30 bp (5.6%)	97 bp (14.1%)	127 bp (10.4%)
Transition: transversion ratio	1 : 0	3 : 2	4 : 2

Maximum parsimonious Semistrict consensus

Semistrict consensus tree with clades labeled I–VI and branch support values (100/74/<50, 100/66,59, 100/91/<50, 100/80/<50, 46/<50, 100/94,87, 100/57,<50, 100/55,51, 100/<50, 100/100,100).

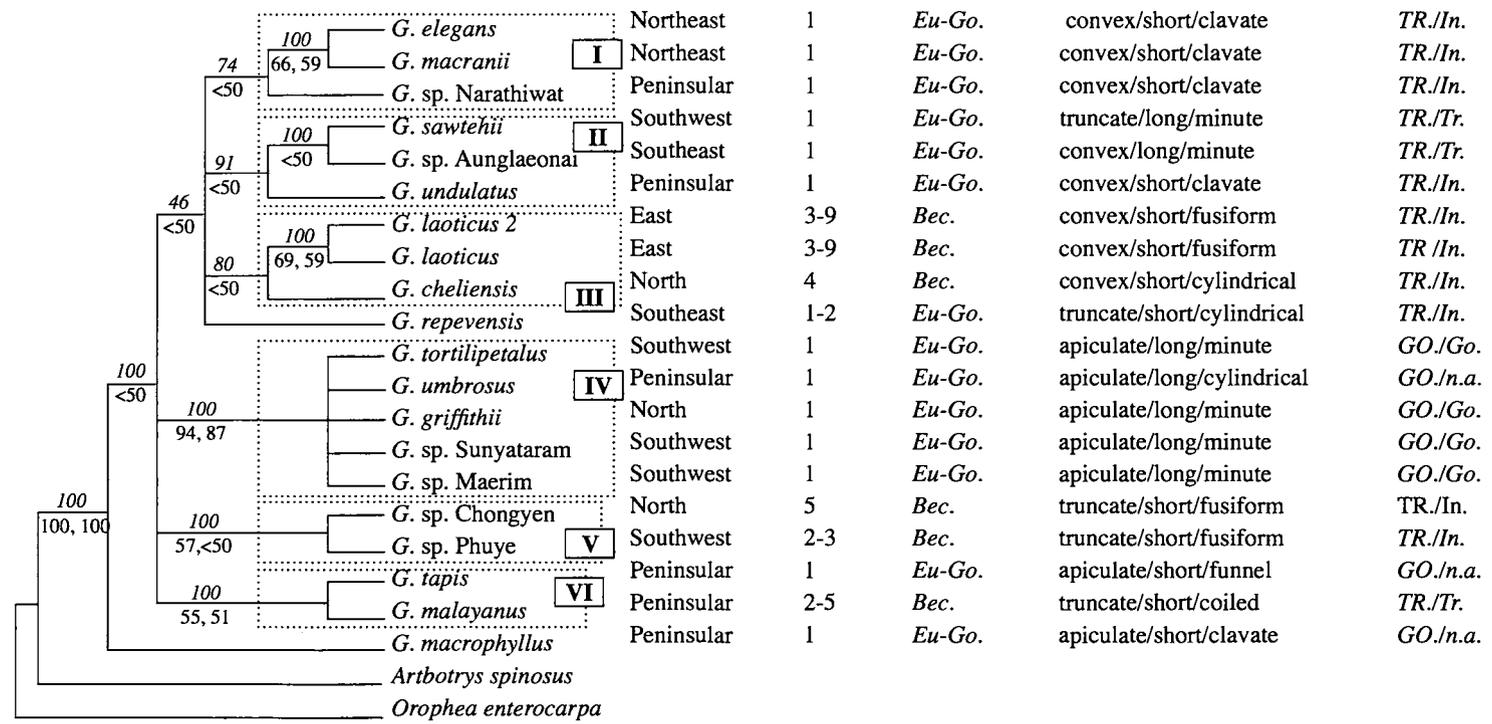| Taxon	Region	Ovule	Boerlage's Sect.	Stamen/Style/Stigma	Ban's Subgen./Sect.
G. elegans (I)	Northeast	1	*Eu-Go.*	convex/short/clavate	*TR./In.*
G. macranii (I)	Northeast	1	*Eu-Go.*	convex/short/clavate	*TR./In.*
G. sp. Narathiwat (I)	Peninsular	1	*Eu-Go.*	convex/short/clavate	*TR./In.*
G. sawtehii (II)	Southwest	1	*Eu-Go.*	truncate/long/minute	*TR./Tr.*
G. sp. Aunglaeonai (II)	Southeast	1	*Eu-Go.*	convex/long/minute	*TR./Tr.*
G. undulatus	Peninsular	1	*Eu-Go.*	convex/short/clavate	*TR./In.*
G. laoticus 2	East	3-9	*Bec.*	convex/short/fusiform	*TR./In.*
G. laoticus	East	3-9	*Bec.*	convex/short/fusiform	*TR /In.*
G. cheliensis (III)	North	4	*Bec.*	convex/short/cylindrical	*TR./In.*
G. repevensis	Southeast	1-2	*Eu-Go.*	truncate/short/cylindrical	*TR./In.*
G. tortilipetalus	Southwest	1	*Eu-Go.*	apiculate/long/minute	*GO./Go.*
G. umbrosus (IV)	Peninsular	1	*Eu-Go.*	apiculate/long/cylindrical	*GO./n.a.*
G. griffithii	North	1	*Eu-Go.*	apiculate/long/minute	*GO./Go.*
G. sp. Sunyataram	Southwest	1	*Eu-Go.*	apiculate/long/minute	*GO./Go.*
G. sp. Maerim	Southwest	1	*Eu-Go.*	apiculate/long/minute	*GO./Go.*
G. sp. Chongyen	North	5	*Bec.*	truncate/short/fusiform	*TR./In.*
G. sp. Phuye (V)	Southwest	2-3	*Bec.*	truncate/short/fusiform	*TR./In.*
G. tapis (VI)	Peninsular	1	*Eu-Go.*	apiculate/short/funnel	*GO./n.a.*
G. malayanus	Peninsular	2-5	*Bec.*	truncate/short/coiled	*TR./Tr.*
G. macrophyllus	Peninsular	1	*Eu-Go.*	apiculate/short/clavate	*GO./n.a.*
Artbotrys spinosus					
Orophea enterocarpa					

Figure 2. Semistrict consensus tree of 3,273 equally most-parsimonious trees based on the 73-basepair combined *trnL/trnG* intron sequence matrix of 20 Thai *Goniothalamus* with two Annonaceae outgroups. Numbers above branches (in italics) are identical percentages of the branch among all congruent parsimonious trees. Numbers below branches designate bootstrap and jackknife support of 1,000 replicates, respectively. Collecting regions, important floral characteristics, and Boerlage's and Bân's taxonomic assignments were also indicated. Abbreviations of subgenera and sections as follow: *Eu-Go.* = section *Eu-Goniothalamus*, *Bec.* = section *Beccariodendron*, *TR.* = subgenus *Truncatella*, *GO.* = subgenus *Goniothalamus*; *In.* = section *Infundibulistigma*, *Tr.* = section *Truncatella*, *Go.* = section *Goniothalamus*, and n.a. = unable to classify to section.

Phylogenetic Analyses

A branch-and-bound search on the 73-basepair *trnL/trnG* intron sequence matrix gave 3,273 equally most-parsimonious trees, of 227 steps in length (phylograms not shown). A semistrict consensus tree (Fig. 2) from the entire 3,273 MPTs showed that all 20 *Goniothalamus* were separated from the outgroup species as a single clade with 100% bootstrap and jackknife supporting values. From the semistrict consensus analysis, six plausible groups were recognised within the Thai *Goniothalamus* group. Each group was present in the majority of MP trees. Groups I–VI were recovered in 74%, 91%, 80%, 100%, 100%, and 100% of trees, respectively. Likewise, the neighbour-joining tree and the maximum-likelihood tree of the 1,227-bp combined sequence data matrix of the 20 Thai *Goniothalanus* taxa (Fig. 3) also revealed the same six subgroups. Two species, *G. repevensis* and *G. macrophyllus*, were the only taxa that did not cluster with any of the six groups.

Group I contains *G. elegans*, *G. marcanii*, and *G.* sp. Narathiwat; group II has *G. sawtehii, G.* sp. Aunglaeonai, and *G. undulatus*; group III, *G. laoticus* 2, *G. laoticus*, and *G. Cheliensis*; group IV, *G. tortilipetalus, G. umbrosus, G. griffithii, G.* sp. Sunyataram, and *G.* sp. Maerim; group V, *G.* sp. Chongyen and *G.* sp. Phuye; and group VI contains *G. tapis* and *G. malayanus*. Only three groups (IV, V, and VI) have bootstrap support, but generally low. The exception is group IV, which has high support in all three analyses (bootstrap values of 94%, 92%, and 95% on the MP, NJ, and ML trees, respectively).

DISCUSSION

Goniothalamus Chloroplast trnL/trnG Introns vs. Nuclear ITS Regions

We found that the average lengths of *Goniothalamus trnL* and *trnG* introns (557 bp and 636 bp, respectively) were similar to those of other plants. SHAW *ET AL.* (2005) investigated phylogenetic utilities of many noncoding chloroplast DNA sequences of phanerogams and reported the average length of their *trnL* introns as 499 basepairs (ranging from 697 to 1008 bp) while the average *trnG*-intron length was 636 basepairs (395 to 602 bp). Moreover, we also found that the *trnL* and *trnG* introns of *Goniothalamus* had fairly low GC-content values, only 38% and 36% in average, respectively.

Problematically, the numbers of parsimony-informative characters of *Goniothalamus trnL/trnG* introns are remarkably low, only 9.4% and 3.2%, respectively. The average value was even lower (5.9%) in the combined sequence matrix. Data of RICHARDSON *ET AL.* (2004), suggested that *Goniothalamus* is a very young genus, with an estimated age of only 3.6–4.8 Ma. Therefore, the very low informative sites of *Goniothalamus* chloroplast *trnL/trnG* introns found in this study could support their hypothesis that this genus has a short evolutionary history.

Our finding that *Goniothalamus trnL* intron sequences had comparatively higher usefulness than the *trnG* introns is contrary to the study of SHAW *ET AL.* (2005). They suggested that the *trnG* introns of phanerogams were generally more informative than *trnL* introns. Nevertheless, PACAK & SZWEYKOWSKA-KULINSKA (2000) found that liverwort *trnG* intron sequences provided more nucleotide substitutions than the *trnL* introns. Moreover, PEDERSEN & HEDENÄS (2003) reported that the *trnG* intron of an apocarpous moss provided nearly twice as many as the *trnL-trnF* sequence.
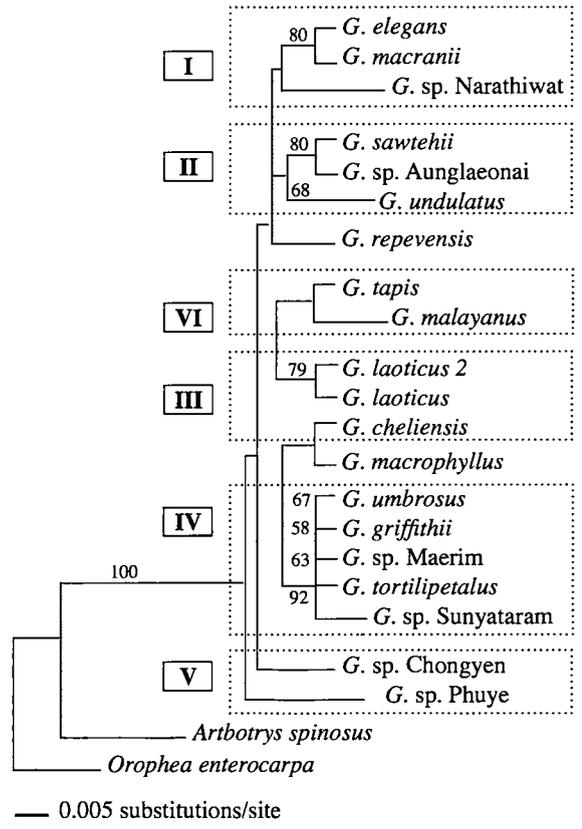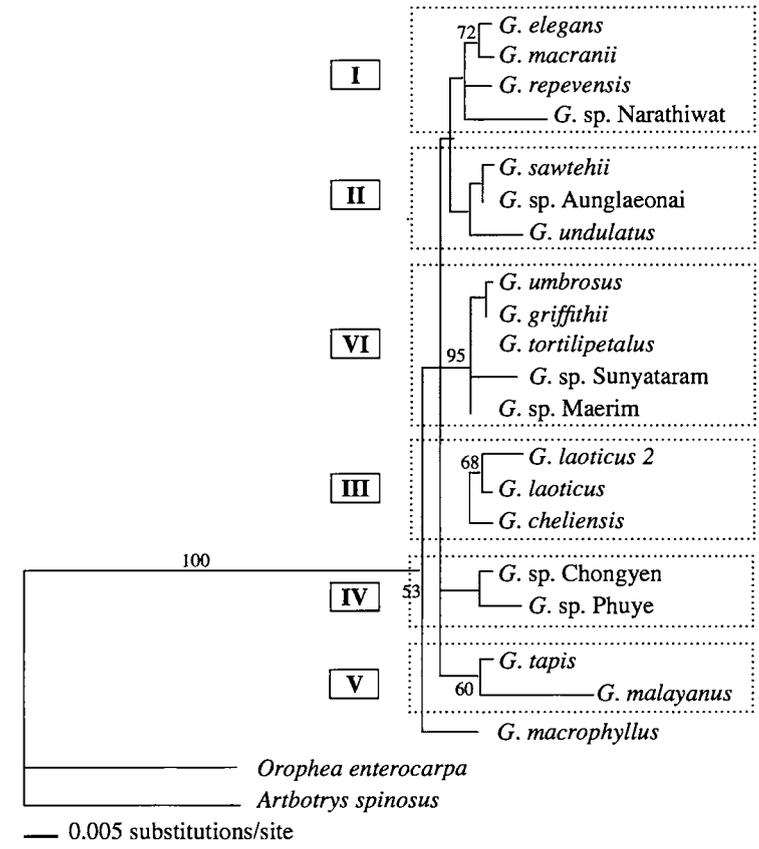
Figure 3. Neighbour-joining (left) and maximum likelihood (right) trees based on the 1,223-basepair combined sequence matrix of 20 Thai *Goniothalamus* taxa with two outgroups based on the combined *trn*L/*trn*G intron sequence analyses. The ML tree was reconstructed using TVM+G model (−Ln = 2983.49826). Six recurring subgroupings congruently found on all MP, NJ, and ML trees were proposed. Numbers along branches indicate 1000-replicate bootstrap supporting-values. No number was assigned to any branch having less than 50% support.

To solve our problem on the low parsimony-informative level of *Goniothalamus* chloroplast *trn*L and *trn*G introns, we had also tried to implement other chloroplast DNA regions and ITS (Internal Transcribed Spacer) regions of nuclear ribosomal genes. Unfortunately, all sequencing experiments on chloroplast DNA *trn*S-*trn*G, *rpo*B-*trn*C, *trn*T-*trn*DF, and *trn*fM-*trn*S regions followed SHAW *ET AL.* (2005) could not give amplifiable PCR-products from all 22 plant specimens. We also performed additional experiments on ITS amplifications followed the works of MÖLLER & CRONK (1997) on *Saintpaulia* (Gesneriaceae) and MEADE (2000) on *Uvaria* (Annonaceae). Only MEADE'S ITS primers could amplify all our 22 plant specimens, resulting in approximately 900-bp PCR products. However, none of the PCR products gave readable sequence. Further studies (beyond the scope of this project) involving cloning experiments of the PCR products are needed to solve this problem.

Goniothalamus Molecular Phylogeny vs. Biogeography

We suggest that the genus *Goniothalamus* in Thailand may be divided into six putative groups on the basis of the phylogenetic analysis. However, the supporting values for these groups (with the exception of group IV) were not high, due to the generally low number of parsimony informative characters. In addition, the positions of *G. repevensis* and *G. macrophyllus* were unstable in the trees and probably contribute to the low support values of the other groups. On morphological grounds it is surprising that *G. repevensis* does not group with *G. undulatus* with which it shares similar ovule number.

There is very little biogeographical patterning evident in the clade structure. For instance, although the two northeastern species, *G. elegans* and *G. marcanii*, occur as sister taxa in group I, they were allied with *G.* sp. Narathiwat from the southern border. Likewise, the eastern taxon-pair of *G. laoticus* 2 and *G. laoticus* are allied to the northern species, *G. cheliensis*, to form group III. These two cases may be explained by the low support for the group; however, the same pattern is evident in the very strongly-supported group IV of five *Goniothalamus* taxa. Members of this clade come from distant regions of the country: *G. tortilipetalus*, *G.* sp. Maerim and *G.* sp. Suyataram are found in the southwest; *G. griffithii* from the north; and *G. umbrosus* from the peninsular.

Widely distributed areas of members of each group may be due to the effective fruit dispersal of the genus. Birds and mammals are the most likely seed vectors, even though studies of seed dispersal in the genus are still lacking. SAUNDERS (2002, 2003) suggested that *Goniothalamus* has its center of diversity in Indochina and western Malesia. Notably, YUYEN & BOONKERD (2002) had suggested before that Thailand (and particularly Prachuap Khiri Khan Province) has long been a meeting point of Malesian, Indo-Burmese, and Indo-Chinese elements.

Taxonomic Implications of the Thai *Goniothalamus* Phylogeny

Though with low supporting levels in some, the six groups found in this study to some extent could reflect floral morphology including the number of ovules and the shape of the stamen connective, characteristics used in the infrageneric classification systems of BOERLAGE (1899) and BÂN (1974) respectively.

All taxa in the groups I, II, IV, and VI had only one or two ovules (i.e. section *Eu-oniothalamus* (one or two ovules). On the other hand, the groups III and V were the only two
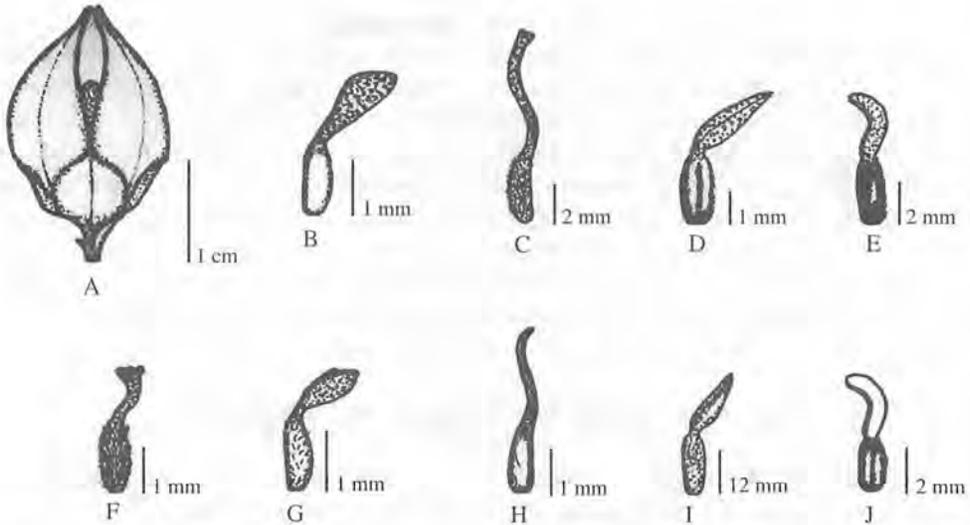
Figure 4. A, Flower of *Goniothalamus marcanii*; B–J main types of pistils showing the range of style and
stigma variation in the genus; F, Funnel-shaped stigma: B, G, clavate stigma; C, H, stigma minute;
D, I, fusiform stigma; E, J, cylindrical stigma. Plants with minute stigmas have long styles, all other
styles are short. Species: B, *G. marcanii*, C, *G. griffithii*, D, *G.* sp Phuye, E, *G. cheliensis*, F, *G.
tapis*, G, *G. undulatus*, H, *G. sawtehii*, I, *G. laoticus*, J, *G. repevensis*. Drawn by Y. Yuyen.

subgroups that had many ovules (i.e. section *Beccariodendron*). However, in our tree section
Beccariodendron appears to be a paraphyletic group, as *G. repevensis* and the clades I, II,
and III are clustered together. However, support for this grouping (that suggests paraphyly)
is very weak, so a monophyletic section *Beccariodendron* cannot be ruled out.

Similarly our six proposed groups can be compared to BÂN's subgeneric classification
using the character of truncate or convex vs. apiculate stamen connectives. The clustering
of the truncate or convex taxa, *G. repevensis* and clades I, II, and III, is possibly suggestive
of a natural group of truncate species (subgenus *Truncatella*), separated from the subgenus
Goniothalamus (exemplified by group IV). However, group VI of *G. tapis* (apiculate) and
G. malayanus (truncate), have mixed stamen connective characteristics. A relationship study
of these two species is needed in the future.

In addition to the importance of the stamen connective, BÂN (1974) recommended us-
ing style and stigma features to further divide the two subgenera of *Goniothalamus* into four
sections. It seems that stigma and style characters may be more complex than detailed by
BÂN. For instance in the Thai species we note five types of stigmas (Fig. 4). In addition to
minute, fusiform and funnel-shaped stigmas, there are the clavate stigmas of *G. elegans*, *G.
marcanii*, and *G.* sp. Narathiwat, and the cylindrical stigmas of *G. repevensis*, *G. umbrosus*,
and *G. undulatus* (see Fig. 4).

Although, our findings and attempts to reconstruct the phylogeny of *Goniothalamus*
should be useful for Annonaceae systematists, it is important to note that *Goniothalamus* is
a large and complex genus (ca. 120 species) and consequently this study is very preliminary.
Less than 17% of the genus was sequenced in our studies and the addition of other genes and
more taxa are clearly needed to further test our results and hypotheses.

ACKNOWLEDGMENTS

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