GENETIC RELATIONSHIP BETWEEN SPECIES OF *Gonystylus spp.*

AYPBC Widyatmoko

ITTO CITES PROJECT
IN COOPERATION WITH
CENTER FOR FOREST AND NATURE CONSERVATION RESEARCH AND DEVELOPMENT
MINISTRY OF FORESTRY

Bogor, 2010

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Cover: *Gonystylus macrophyllus, G. bananus, G. affinis, and G. consanguineus*

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SUMMARY

We reported genetic relationship of ramin (Gonystylus spp.), a CITES-listed genus subject to illegal international trade. Samples representing 9 different ramin species. DNA extraction from leaf material was achieved using modified CTAB method. ITS rDNA (ITS3) and 3 chloroplast non coding regions sequences were examined identifying genetic relationship among the species. No variation within species and sequence differentiation between species were recognized among the 9 species, included 3 unidentified Gonystylus sp. Based on ITS3 sequence, 9 species were divided into 3 clades. The first clade was G. bancanus. The second clade was consisted of 5 species, G. brunescens, G. velutinus and 3 Gonystylus sp. The third clade was consisted of 3 species, G. consangineus, G. keithii and G. macrophyllus. However, using combination of ITS3 and 3 chloroplast non coding regions, the 9 species were divided into 4 groups. The first group was G. bancanus and G. macrophyllus; the second group was G brunescens, G. consangineus, G. velutinus (2 samples) and 1 Gonystylus sp. The third group was consisted of G. velutinus (2 samples) and 2 Gonystylus sp. The other group was only consisted of G. keithii.

INTRODUCTION

Ramin (*Gonystylus* spp.) is a tropical hardwood tree found primarily in the peat-swamp forests of Indonesia and Malaysia. The genus *Gonystylus,* consisting of about 30 species, is distributed throughout the Malesian area (Indonesia, Malaysia, the Philippines, Papua New Guinea, Singapore and Brunei Darussalam) with the majority of species found in Borneo. Ramin has been heavily depleted throughout its range to supply international markets for ramin timber and processed wood products. Six species (*G. affinis, G. bancanus, G. forbesii, G. macrophyllus, G. maingayi* and *G. velutinus*) are known to be commercially valuable, of which *G. bancanus* is the most heavily traded. Fifteen species in the genus, including *G. bancanus*, are classified as vulnerable in the IUCN's Red List of Threatened Species due to observed, inferred or projected habitat loss and over-exploitation. Logging—both selective logging and clear-cutting—is recognized as the major threat to these species (IUCN 2004). The decline is mainly due to the naturally slow generation process of ramin, thus its natural populations have yet to recover. Much of Indonesia`s remaining ramin is found in national park and protected areas that provide vital habitat for many other threatened species. Although the species' current listing on Appendix III has provided important enforcement benefits in a number of areas, these benefits are being undermined by illegal logging operations and laundering activities and thus do not afford adequate protection for the species. Therefore, conservation effort is urgently required and several are still going to support sustainable use of ramin.

Molecular data are often considered to be more reliable than morphological data when inferring phylogenetic relationships at lower taxonomic levels. For recently diverged taxa in which morphological characters are prone to phenotypic plasticity, molecular characters, such as DNA sequences, may provide greater resolution of phylogenetic relationships. The internal transcribed spacers (ITS) of nuclear ribosomal DNA are frequently used for phylogenetic analysis at the species level.

Although nuclear ribosomal DNA is multicopy in large arrays of repeats, it is useful for phylogenetics as the copies are usually highly homogenous due to concerted evolution.

Non-coding region has been used for elucidating phylogenetic relationship of different taxa and sequence variation. Compared with coding regions, non-coding regions may provide more informative characters in phylogenetic studies at species level because of their high variability due to the lack of functional constrains.

The present study uses DNA sequences from the internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) and the 3 non coding regions of chloroplast DNA (cpDNA) to elucidate genetic relationship of 9 species of *Gonystylus* sp.

MATERIALS AND METHODS

Plant materials and DNA extraction

Leaf of individual trees was selected from 9 different species. In order to identify sequence variation within species, 2 to 5 different individual trees from different species were used. Detail of species has been collected and list of samples used in this activity was shown in Table 1 and Table 2. Three species, namely *G. maingayi*, *G. fornesii* and *G. affinis*, could not be analyzed because their leaves were decomposed when arrived in DNA Molecular Laboratory.

Table 1. List of *Gonystylus* non-*bancanus* have been collected

No.	Species
1.	*Gonystylus maingayi* Hk.f.
2.	*G. fornesii* Gilg
3.	*G. affinis* A. Shaw
4.	*G. velutinus* A. Shaw
5.	*G. keithii* A. Shaw
6.	*G. macrophyllus* A. shaw
7.	*G. brunescens* A. Shaw
8.	*G. malacensis*
9.	*G. consanguineus*
10.	*G. bancanus*
11.	*Gonystylus sp*
12.	*Gonystylus sp*
13.	*Gonystylus sp*

Note: Shade in grey color were decomposed leaves species

Total genomic DNA was extracted using a modified Cetyl Trimethyl Ammonium Bromide (CTAB) protocol reported by Shiraishi and Watanabe (1995). The DNA

was purified using GENECLEAN III (BIO101) as a template for the further analyses.

PCR Amplification

PCR of ITS3 and three chloroplast non coding regions (the *trn*L intron, the intergenic spacer between *trn*L - *trn*P, and *trn*D - *trn*Y) was performed in a total volume of 20 µl containing 4 ng of genomic DNA, 0.25 µM of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.0 mM $MgCl_2$, 200 mM of each dNTP, and 0.25 unit/10µl *Ex Taq* DNA polymerase. DNA amplification was performed with a Gene Amp PCR System Model 9600 (Perkin-Elmer) programmed as follows: 95^{o}C for 90 s, 30 cycles of 30 s at 94^{o}C, 30 s at 55^{o}C, and 90 s at 72^{o}C, followed by 60 s at 72^{o}C. The PCR product was separated by electrophoresis in 1.5% agarose gel and the target fractions were excised from the gel. DNA was recovered from the gel particles and was purified using QIAEX II Gel Extraction (QIAGEN). The sequence reaction was carried out using a Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech), the template DNA, and -21M13 (TGTAAAACGACGGCCAGT) / M13Rev (CAGGAAACAGCTATGA-CC) sequence primer 5'-labeled with Texas Red fluorescent dye (Amersham Pharmacia Biotech). The sequence was analyzed with an ABI 3100 DNA Sequencer.

Table 2. Detail of samples

No	Species	Population	Code
1	*G. macrophyllus* A. shaw	Jawa/Bodogol	
2	*G. macrophyllus* A. shaw	Jawa/Bodogol	
3	*G. macrophyllus* A. shaw	Jawa	
4	*G. macrophyllus* A. shaw	Jawa	
5	*G. velutinus* A. Shaw	KRB	
6	*G. velutinus* A. Shaw	KRB	
7	*G. velutinus*		WR
8	*G. velutinus*		WR
9	*G. keithii* A. Shaw	CNV 7	No. 18
10	*G. malacensis*		2
11	*Gonystylus sp*	Plot CNV 9	No. 82
12	*Gonystylus sp*		
13	*Gonystylus sp*		
14	*G. brunescens*		BK 1195
15	*G. brunescens*		BK 153
16	*G. brunescens*		A14-A15.48
17	*G. brunescens*		A17-A18.65
18	*G. brunescens*		bb. 31633
19	*G. consanguineus*		WR
20	*G. consanguineus*		WR
21	*G. bancanus*		
22	*G. bancanus*		
23	*G. bancanus*		
24	*G. bancanus*		

RESULT AND DISCUSSION

Sequence variation within and between species

Total of 85 based form 4 regions (ITS3 and 3 chloroplast non-coding regions) was recognized as insertion/deletion and substitution among the nine species of *Gonystylus*. No sequence variation was within species for internal transcribe spacer (ITS3) region. Thirty two bases of ITS3 can be used to differentiate the nine species. Internal transcribe spacer is very useful to identify species because almost no variation within species and different species possess different ITS sequence.

Chloroplast non-coding regions have high variability within and between species. Therefore, these regions are used to elucidate genetic variation within species. In this research, 3 non-coding regions of chloroplast DNA were used, namely the *trn*L intron, the intergenic spacer between *trn*L - *trn*P, and *trn*D – *trn*Y. Total of 53 different bases were variable among the species. Within and between species sequence variation were found in this research. Among the nine species, *G. macrophyllus*, *G. brunescens* and *G. velutinus* have high sequence variation within species. Only *G. bancanus* did not reveal sequence variation within species.

Genetic relationship among species

Genetic relationship of 24 samples of *Gonystylus* spp was revaled by dendrogram (Fig. 1 and Fig. 2). Fig. 1 revealed genetic relationship between 9 species of *Gonystylus* based on internal transcribed spacer sequence only. The dendrogram divided the 9 species into 3 different groups. *G. bancanus* was separated with the others 8 species. G. brunescens, G. velutinus and 3 Gonystylus spp were grouped together, and the third group was consisted of. *G. consangineus*, *G. keithii* and *G. macrophyllus*. Among the 9 species, 3 unidentified *Gonystylus* spp have closed relationship, followed by *G. consangineus* and *G. keithii*.

Based on sequence of the four regions, different dendrogram was produced (Fig. 2). All 4 samples of *G. bancanus* were grouped together because no variation was found within this species. Different with Fig.1, in Fig. 2 *G. keithii* was separated with another 8 species. *G. bancanus* was closed with *G. macrophyllus*, and *G. consangineus* was closed *G. velutinus*. Similar relationship was revealed by 3 *Gonystylus* spp. Two of the species was closed related with *G. velutinus*, and the other *Gonystylus* spp was closed with *G. brunescens*. The dendrogram (Fig. 2) was divided the 9 species into 3 groups. The first group was consisted of *G. bancanus* and *G. macrophylla*. The second group was consisted of *G. keithii*, and the other 6 species placed into the third group. The third group can be separated into 3 small clades, those were *G. brunescens* and *Gonystylus* spp (Clade I); *G. consangineus* and *G. velutinus* (Clade II); and *G. velutinus* and 2 *Gonystylus* spp.

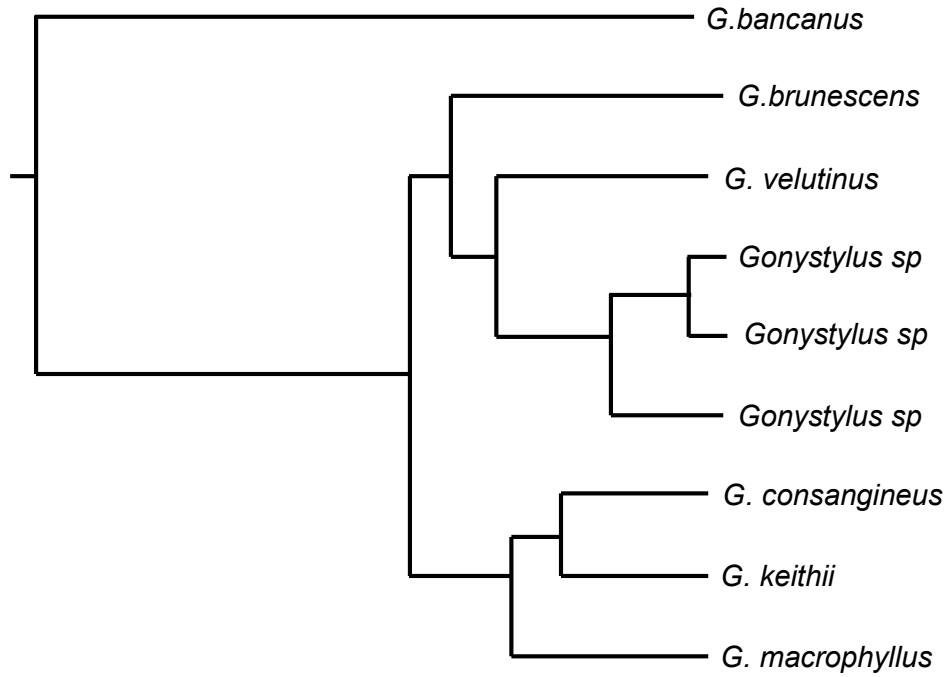

Figure 1. Dendrogram of *Gonystylus* sp based on sequence of ITS-3

CONCLUSION

1. Leaves of nine *Gonystylus* spp, include 3 unidentified species, can be used to elucidate genetic relationship between species.
2. No sequence variation was found within species for internal transcribe spacer (ITS3), however, each species could be differentiated using this region.
3. Sequence variation within species was found for three chloroplast non coding regions, except for *G. bancanus*.
4. Based on ITS3 sequence, 9 species were divided into 3 clades. The first clade was *G. bancanus*; the second clade was consisted of 5 species, *G. brunescens*, *G. velutinus* and 3 *Gonystylus* sp; and the third clade was consisted of 3 species, *G. consangineus*, *G. keithii* and *G. macrophyllus*
5. Nine species were separated into 4 groups based on sequence combination of ITS3 and 3 chloroplast non coding regions. The first group was *G. bancanus* and *G. macrophyllus*; the second group was *G brunescens*, *G. consangineus*, *G. velutinus* (2 samples) and 1 *Gonystylus* sp; and the third group was consisted of *G. velutinus* (2 samples) and 2 *Gonystylus* sp. *G. keithii* was placed into different group.

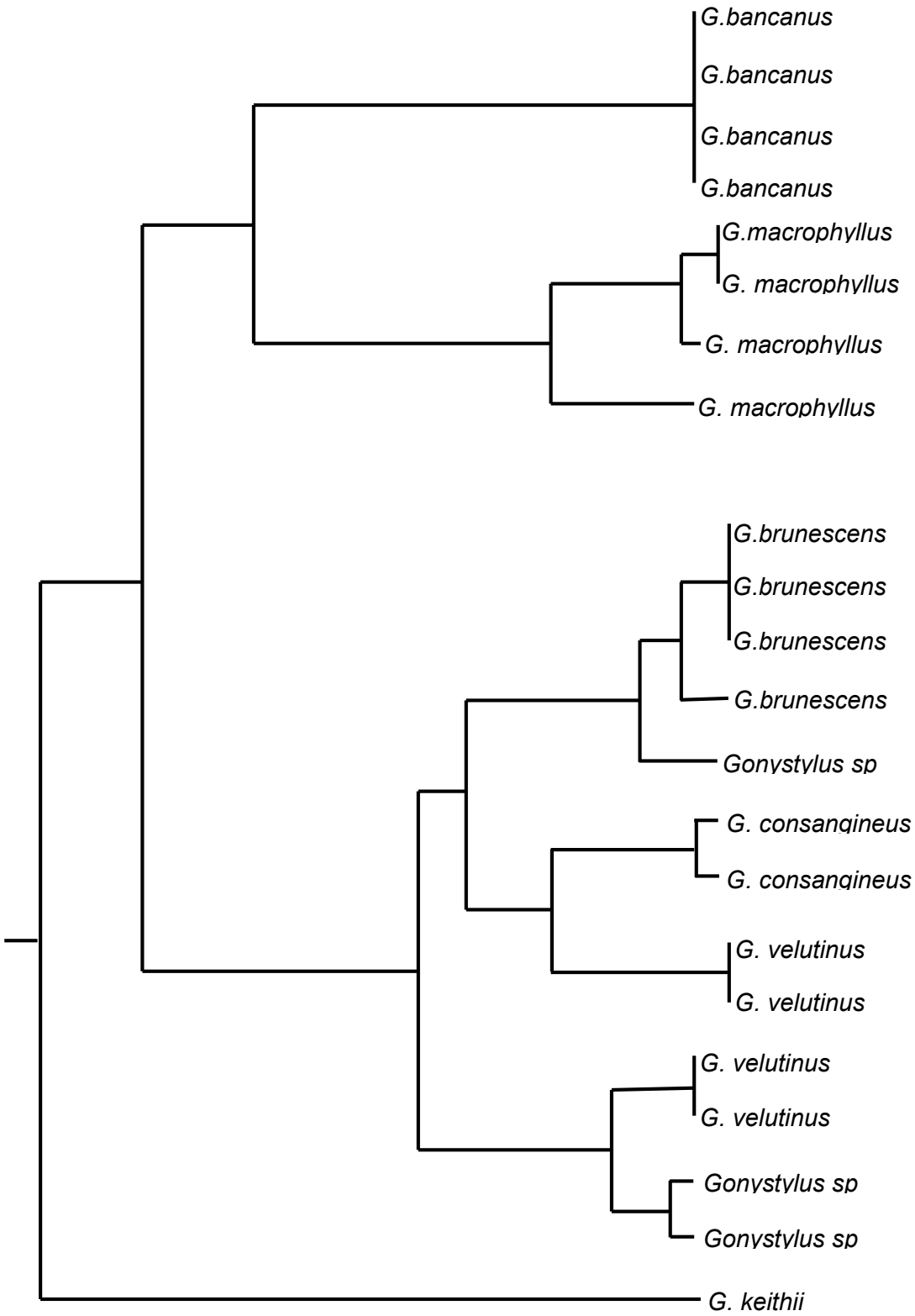

Figure 2. Dendrogram of *Gonystylus* sp based on sequence of ITS-3 and cpDNA non-coding region

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