SEARCHING FOR THE RELATIVES OF THE PHILIPPINE ENDEMIC *Gloeocarpus* Radlk. (SAPINDACEAE): EVIDENCE FROM MOLECULAR SEQUENCE DATA

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ABSTRACT

Gloeocarpus is a monotypic genus and is endemic to the Philippines. There are no available molecular data for *Gloeocarpus,* and its generic status as well as its position within the Sapindaceae has never been challenged, hence, it was not included in any molecular phylogenetic study within the family. Therefore, to determine its phylogenetic position and to evaluate its monophyly, the ITS (nrDNA) regions were sequenced and analyzed together with the previously published sequences of closely related genera. The aligned ITS dataset contained a total of 694 positions, 239 of which are parsimony informative. The strict consensus tree confirmed the phylogenetic position of Gloeocarpus within the tribe Cupanieae with strong support (BS=100) and the monophyly of the genus is highly supported (BS=100). Molecular results support morphological evidences characterizing the genus by having the sinuous branchlets and hairy petals with folded margin.

INTRODUCTION

Sapindaceae *s.l.* (soapberry family) is a cosmopolitan family with c.142 genera and 1900 species of trees, shrubs and lianas that are widely distributed within the tropical region but with some temperate representatives (*Acer* L. and *Aesculus* L.) (Buerki et al., 2010). The family is known as a source of edible fruits and seeds, hence widely cultivated (e.g., *Litchi chinensis* Sonn., *Dimocarpus longan* Lour. and *Nephelium lappaceum* L.). The wood of several

species such as *Harpulia arborea* (Blanco) Radlk., *Ganophyllum falcatum* Blume and *Dodonaea viscosa* Jacq. are good sources of timber for furniture and construction purposes. The family is also represented by ornamental genera such as *Cardiospermum* L., *Koelreuteria* Laxm. and *Lepisanthes* Blume. Representatives within the family are capable of producing large quantities of saponin, a secondary metabolite used as fish poison and detergent (Adema et al., 1994).

The very first comprehensive treatment of the family sensu stricto (s.s.) was done by Radlkofer (1931-1934) based on the number of ovules per locule, fruit morphology and cotyledon shape. Sapindaceae *s.s.* is delimited with two subfamilies namely; Sapindoideae divided into nine tribes (Paullinieae, Thouinieae, Sapindeae, Aphanieae, Lepisantheae, Melicocceae, Schleicherae, Nephelieae and Cupanieae) and Dodonaeoideae with five tribes (Koelreuterieae, Cossignieae, Dodonaeeae, Doratoxyleae, and Harpullieae). However, as a result of various taxonomic treatments, several studies considered the close relationship of some genera of the Aceraceae and the Hippocastanaceae to the Sapindaceae and therefore combined the three into a broader family of Sapindaceae sensu lato (*s.l.*) (Adema, 1994). For instance, Muller and Leenhouts (1976) did some modifications of the circumscription developed by Radlkofer by providing changes to the tribal position of some species based mainly on the morphological characters of pollen grains. In addition, Umadevi and Daniel (1991) supported the inclusion of the closely related species of Aceraceae and Hippocastanaceae based on the presence of various secondary metabolites in the leaves. The merging of the three families into a broader Sapindaceae was also affirmed by the earlier molecular work of Gadek et al. (1996) and APG II (2003). Using nuclear and plastid DNA genomes, Harrington et al. (2005) and Buerki et al. (2009) provided a more reliable phylogenetic inference in the establishment of a monophyletic Sapindaceae s.l. From Radlkofer's traditional two subfamilies, Buerki et al. (2009) proposed four subfamilies, namely Xanthoceroideae, Hippocastanoideae, Dodonaeoideae and Sapindoideae. However, within the more natural relationship in Sapindaceae *s.l.*, polyphyly within subfamilial and tribal relationships was still observed (e.g., Harpullieae with *Xathoceras* Bunge). Therefore, in an effort to ensure monophyly the subfamily Xanthoceroideae was elevated into the familial rank of Xanthoceraceae (Buerki et al., 2010). (Table 1)

The use of morphological data provided significant advantages, but it is only capable of offering very minimal information, which may become intrinsically problematic. Hence, it should not be used solely in reconstructing

phylogeny (Scotland, 2003). Recent advancement in molecular biology and the age of comparative genomics have bolstered the goal for constructing more natural groupings (Weins, 2004) and therefore developed strong confidence in phylogenetic reconstruction efforts using molecular-based trees (Soltis et al., 2004). Molecular approach has contributed much to the understanding of evolutionary relationships (Downie et al., 2000) within the subfamilial and tribal classification of the Sapindaceae (e.g., Li et al., 2006; Buerki et al., 2009; Zhang et al., 2010). However, despite all these molecular phylogenetic studies carried out, no modern classification has included all genera of the Sapindaceae, like the Philippine endemic genus *Gloeocarpus* Radlk.

Table1. Summary of the delimitation and circumscription of Sapindaceae

Authors	Circumscription and Delimitation
Radlkofer (1890)	Sapindaceae s.s. - Subfamily Dodonaeoideae - Subfamily Sapindoideae
Muller and Leenhouts (1976) Umadevi and Daniel (1991) APG II (2003)	Sapindaceae s.l. - Family Sapindaceae sensu stricto Subfamily Dodonaeoideae Subfamily Sapindoideae - Family Aceraceae (subfamily Aceroideae) - Family Hippocastanceae
Buerki et al. (2009)	Sapindaceae s.l. - Subfamily Xanthoceroideae - Subfamily Hippocastanoideae - Subfamily Dodonaeoideae - Subfamily Sapindoideae

Authors	Circumscription and Delimitation
Buerki et al. (2010)	Sapindaceae s.l. - Subfamily Hippocastanoideae - Subfamily Dodonaeoideae - Subfamily Sapindoideae Family Xanthoceraceae (newly delimited)

The monotypic *Gloeocarpus* is currently represented by *G. patentivalvis* (Radlk.) Raldk. The genus was established by Radlkofer (1913) but has never been included in any molecular study, therefore, its circumscription is still based on Radlkofer's (1890) treatment wherein it is delimited under subfamily Sapindoideae and grouped to the tribe Cupanieae. The subfamilial and tribal position of the genus has never been challenged in any molecular phylogenetic study because leaf samples suitable for DNA extraction are wanting. Furthermore the delimitation of the genus which is currently based on morphology is ambiguous. Radlkofer (1913) first described the species as Cupaniopsis patentivalvis, however, he later on recognized it as Gloeocarpus patentivalvis (1920) based on morphology and biogeography. Moreover, two other names were referred for *Gloeocarpus patentivalvis*, the *G. crenatus* Radlk. which is considered as synonym and *G. philippinensis* Elmer ex Merr. that is invalidly published (Adema et al., 1994).

In this first molecular study of *Gloeocarpus*, the ITS region (ITS1, 5.8s gene and ITS2) of the nuclear ribosomal DNA (nrDNA) was sequenced and analyzed together with those of closely related taxa of Sapindaceae. The ITS region has resolved phylogenetic inferences specifically at the generic level because of features such as 1) being highly repetitive, 2) the gene undergoes rapid concerted evolution and 3) the total size is small with higher conserved sequences, which makes it easy to amplify (Baldwin et al., 1995). Therefore, the present study aims to determine the phylogenetic position of *Gloeocarpus* and to test its monophyly.

MATERIALS AND METHODS

Taxon Sampling

Herbarium specimens of *Gloeocarpus patentivalvis* from the PNH as well as type specimens from the L, NY and US databases were observed and studied.

Fieldwork was conducted in Tayabas, Quezon wherein samples were collected for preparation of herbarium specimens, leaf material for molecular study as well as preservation of reproductive structures in 70% ethanol. Two samples of *G. patentivalvis* coded as GP01 and GP02 were deposited at the University of Santo Tomas Herbarium (USTH). Leaf samples for DNA extraction were preserved in silica-gel following the work of Chase and Hills (1991).

Molecular Methods

DNA was extracted from silica-gel-dried leaf samples using the DNeasy Plant Minikit (Quiagen, Germany). The ITS region of the nrDNA were amplified using primers P17F (5'-CTACCGATTGAATGGTC-CGGTGAA-3') and 26S-82R (5'- TCCCGGTTCGCTCGCCGTTACTA - 3') (Popp and Oxelman 2001). The PCR cocktail is composed of 15.3 µL nuclease free water, 2.5 µL 10x PCR buffer, 2.0 µL MgCl and 1.5 µL dNTP (2mM). PCR reactions were run on MJ Research Tetrad PTC 100 Thermal Cycler with the following settings: initial denaturation of 97^{o}C for 1 min and 30 sec, followed by 35 cycles of 20 sec at 97^{o}C, 90 sec at 72^{o}C, 30 sec at 72^{o}C, and a final extension of 7 min at 72^{o}C. Gel electrophoresis was carried out for the confirmation of the presence of bands of the amplified PCR products. The gel was run for 30 min under a power supply (BIORAD Power Pac 300) and was maintained at 70 volts. Gel was viewed using AUV DIGI Doc 1T Digital Gel Documentation System. Amplified DNA were purified (Figure 1) using the QIA-quick Purification Kit (Qiagen, Germany). Purified DNA were sent to MACROGEN Inc. Seoul, South Korea for sequencing with the following forward and reverse sequences: P16F (5'-TCA CTG AAC CTT ATC ATT TAG AGG-3') and P25R (5'-GGG TAG TCC CGC CTG ACC TG-3') (Popp and Oxelman, 2001).

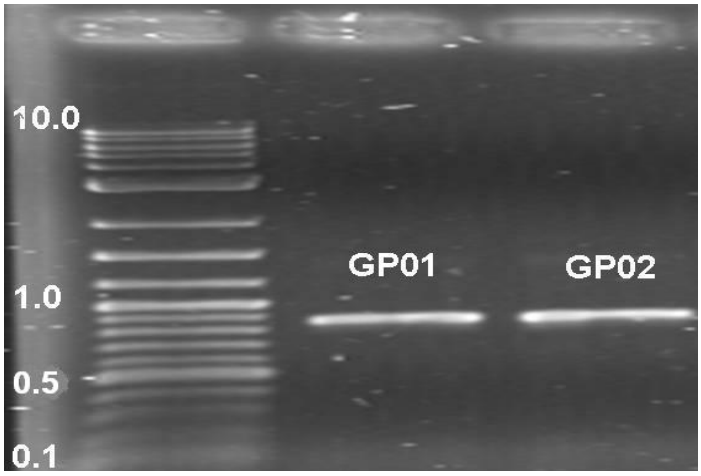

Figure 1. Purified DNA bands of the two isolates of *G. patentivalvis*

Sequences Alignment

DNA sequences of the ITS region were manually assembled and edited using the CodonCode Aligner v3.0.1. Edited ITS sequence was uploaded in ClustalX wherein boundaries of ITS1 and ITS2 regions were compared with the previously published sequences of Sapindaceae. A total of 26 sequences (Table 2) under the tribe Cupanieae was downloaded from the Genbank (NCBI) and were also assembled and aligned using ClustalX. In addition, novel sequences of *G. patentivalvis* were submitted and uploaded into GenBank with accession codes of JF912445 and JF912446.

Table 2. Species information and GenBank accession numbers used in the study

SPECIES	VOUCHER	ITS GenBank*
Cupania rubiginosa (Poir.) Radlk.	Mori 886	EU720841
Cupania scrobiculata Rich.	Acevedo 111000	EU72052
Cupaniopsis anacardioides (A. Rich.) Radlk.	Chase 217	EU720438
Cupaniopsis flagelliformis (Bailey) Raldk.	Edwards KE72	EU720432
Diploglottis campbelli Cheel	Chase 2048	EU720457

SPECIES	VOUCHER	ITS GenBank*
Elattostachys apetala (Labill.) Radlk.	McPherson 18184	EU720538
Elattostachys nervosa (F. Muel.) Radlk.	Chase 2022	EU720455
Gloeocarpus patentivalvis (Radlk.) Radlk.	GP01	JF912445
Gloeocarpus patentivalvis (Radlk.) Radlk.	GP02	JF912446
Guioa diplopetala Radlk.	Chase 1352	EU720447
Guioa microsepala Radlk.	Munzinger 744	EU720546
Guioa semiglauca (F. Muel.) Radlk.	Chase 2058	EU720458
Guioa villosa Radlk.	McPherson 18040	EU720544
Harrisonia abyssinica Oliv. (OUTGROUP)	Edwards KE510	EU720440
Jagera javanica (Blume) Blume	Chase 2130	EU720468
Jagera javanica subsp. australiana Leenh.	Edwards KE178	EU720442
Matayba guianensis Aubl.	Acevedo 12342	EU720527
Matayba laevigata Radlk.	Acevedo 12357	EU720528
Matayba elaeagnoides Radlk.	Zardina 43278	EU720553
Mischocarpus sp.	Edwards KE37	EU720437
Mischocarpus exangulatus (F. Muel.) Radlk.	Edwards KE30	EU720434
Sarcopteryx martyana (F. Muel.) Radlk.	IRV1810	EU720426
Sarcopteryx reticulata S.T. Reynolds	BG 1137	EU720439
Tina isaloensis Drake	Ranirison PR827	EU720520
Tina striata Radlk.	Vary 45	EU720509

SPECIES	VOUCHER	ITS GenBank*
Toechima erythrocarpum (F. Muel.) Radlk	Edwards KE20	EU720597
Toechima plurinerve Radlk	Chase 1357	EU720452
Toechima tenax (Cunn. ex Benth) Radlk	Chase 2046	EU720456

* ACCESSION NUMBER

Phylogenetic Analysis

Parsimony analysis was carried for the 28 taxa using the Molecular Evolutionary Genetics Analysis ver. 4 (MEGA 4) (Tamura et al., 2007). The MP tree was obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with search level 2 (Eck and Dayhoff, 1996 and Nei and Kumar, 2000) wherein initial trees were obtained with the random addition of sequences (5000 replicates). All positions containing gaps and missing data were eliminated from the dataset. The following were calculated: base frequency, tree length, consistency index (CI) and retention index (RI). Bootstrap values were obtained with 5000 replicates to determine the relative support wherein values between 86% - 100% are considered strongly supported, 70% - 85% moderately supported and values between (50% - 69%) are weakly supported. Harrisonia abyssinica Oliv. (Rutaceae) was used as the outgroup in this study, in reference to the works of Harrington et al., (2005), Buerki et al., (2009) and Buerki et al., (2010).

RESULTS AND DISCUSSION

Sequence Characteristics

The ITS analysis of 28 sequences contained an average of 624 base pairs (bp). The GC content ranged from 30% to 34% while AT content ranged from 13% to 22% which corroborated with the report of Hershkovitz and Zimmer (1996) that the ITS region is GC rich.

The aligned matrix of the 28 taxa included a total of 694 characters, 239 of which were phylogenetically informative. Parsimony analysis of the ITS data resulted in 93 equally parsimonious trees with consistency index (CI) and retention index (RI) of 0.61 and 0.74, respectively. The values indicated that the data were far from being homoplasious, therefore very reliable in

reconstructing phylogeny (Farris, 1989; Simpson, 2006). The strict consensus tree of the parsimony analysis (SCT) is shown in Figure 2.

Tribal Position and Monophyly of Gloeocarpus patentivalvis

The phylogentic analysis of the two ITS sequences of *Gloeocarpus* with the inclusion of those from closely related taxa showed a robust clade that was highly supported (BS=100). The SCT generated in this study was almost congruent with the *Cupania* clade of Harrington et al., (2005), Buerki et al., (2009) and Buerki et al., (2010) in their multiple sequence analysis. Buerki et al. (2009) divided the *Cupania* group into two major clades based on phytogeography: Australasian and Malagasy. As shown in Figure 2, the two samples of *Gloeocarpus patentivalvis* were nested within the tribe Cupanieae specifically the Malagasy clade with high support (BS=100). This is the very first study that confirms the position of *Gloeocarpus* within the tribe using molecular data. The molecular results were supported also with morphological characters such as ramiflorous infloresence, the presence of aril, and a capsular fruit. Although Van Welzen (1990), Adema (1994) and Muellner et al. (2003) stated that there is a strong tendency for the fruit type of this group to exhibit plasticity, Harrington et al. (2005) considered that the majority of the species under the tribe is characterized by the aforementioned synapomorphies.

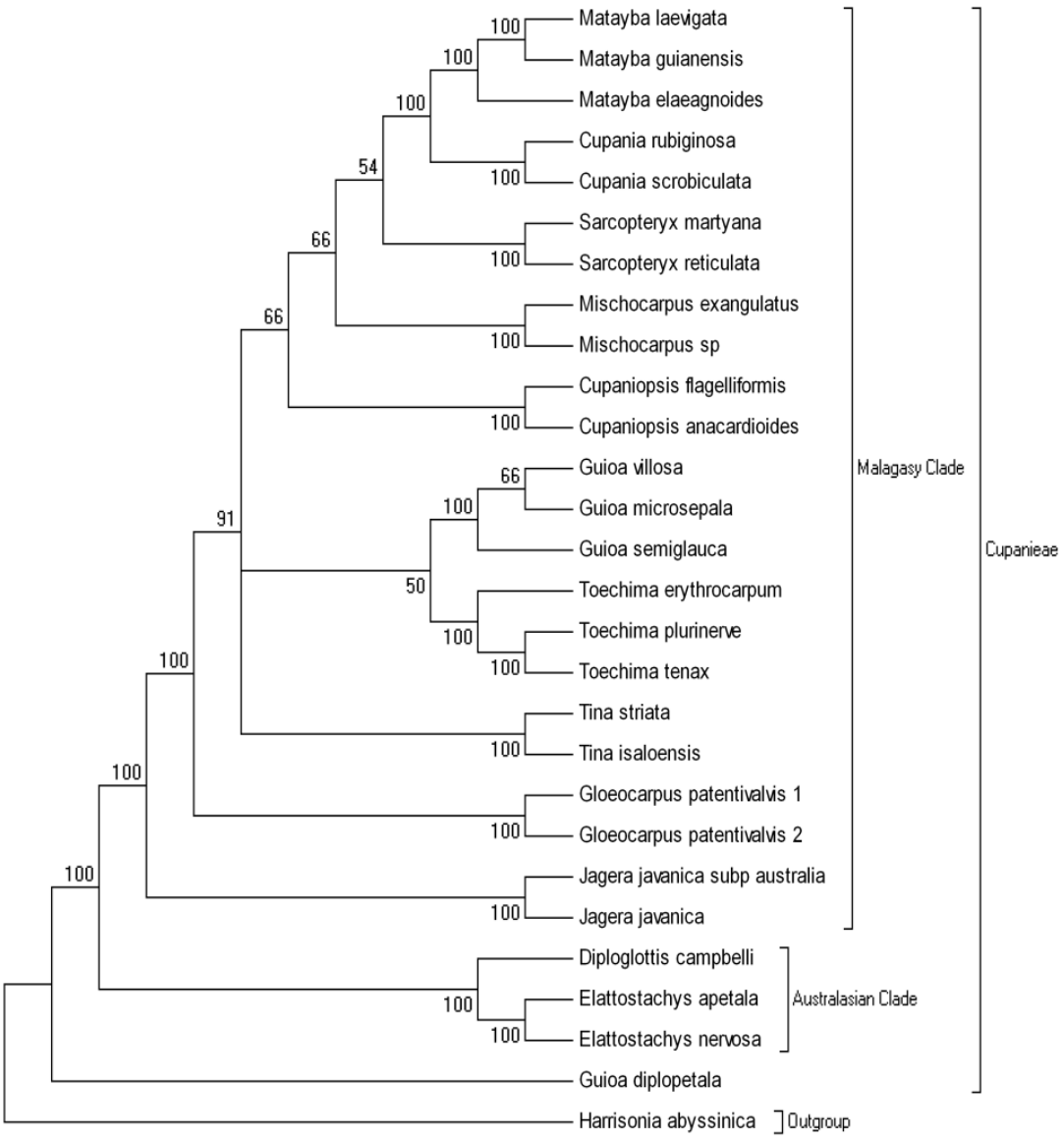

Figure 2. Strict consensus tree derived from 93 equally parsimonious trees based on the phylogenetic analysis of the ITS sequence data. Numbers at each nodes indicate the bootstrap values >50%.

Furthermore, *Gloeocarpus* formed a natural assemblage on its own, which was highly supported (BS=100). In addition, our results supported the transfer made by Radlkofer from *Cupaniopsis* to *Gloeocarpus.* Morphological

distinctions of *Gloeocarpus* include the presence of hairy petals with slightly folded margins as well as branchlets having sinuses that are shallow, smooth and wavy in horizontal plane (sinuate). In contrast, *Cupaniopsis* is characterized by branchlets that are straight and the presence of scales in petals.

CONCLUSION AND RECOMMENDATION

This first molecular study involving the ITS region of the endemic genus *Gloeocarpus* confirmed its placement within the tribe Cupanieae specifically nested in the Malagasy clade. In addition, the study also affirmed the hypothesis of Radlkofer regarding the monophyly of the genus and herein reinforced further with the following apomorphic characters like hairy petals with slight folded margins and -sinuate branchlets.

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