MOLECULAR CONFIRMATION ON THE PHYLOGENETIC POSITION OF THE GENUS *CLEMENSIELLA* SCHLTR. IN MARSDENIEAE (APOCYNACEAE - ASCLEPIADOIDEAE)

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ABSTRACT

The Philippine Marsdenieae (Apocynaceae-Asclepiadoideae) comprises a total of seven genera including the imperfectly known *Clemensiella* Schltr. Due to its unique morphology, the position of the small genus in the tribe Marsdenieae of Asclepiadoideae was previously in question. In this first molecular study of *Clemensiella*, the internal transcribed spacer (ITS, nrDNA) region of three isolates were newly generated and analyzed together with previous published sequences. The present aims to assess the phylogenetic positon, monophyly and closely-related genera of *Clememsiella*. The three *Clemensiella* isolates yielded a total of 630 to 642 base pairs (bp) and the average GC content ranged from 63.21% to 63.86%. Based on the strict consensus tree, the monophyly of *Clemensiella* is highly supported with BS = 100% and nested within the tribe Marsdenieae with BS = 61%. This study confirms the phylogenetic position of *Clemensiella* in Marsdenieae and its close relation to *Dischidia* and *Hoya*. Although the three Marsdenieae are distinct in their seed structures, they share common features in habit, inflorescence and corolla aestivation.

KEYWORDS: *Clemensiella*, ITS, Marsdenieae, Apocynaceae, Asclepiadoideae

INTRODUCTION

The Apocynaceae sensu lato (s.l.), commonly known as the dogbane family, consist of ca. 424 genera and 1,500 species of trees, shrubs, herbs and lianas widely distributed in the cosmopolitan regions (Endress and Bruyns, 2000). The family is characterized by a simple, usually opposite and decussate, or whorled leaves; usually showy, 5-lobed calyx, actinomorphic flowers; cymose or racemose, terminal or axillary inflorescences; inserted stamens; usually superior ovary; and a drupe, berry, capsule, or follicle fruits (Endress and Bruyns, 2000). Notable phytochemical compounds and economic uses had been observed within the members of Apocynaceae s.l. Some medicinally

known species are *Rauvolfia* L. with reserpine (a hypertensive drug), *Strophanthus* DC. with strophantine (a cardiac glycosides), and *Catharanthus* L. with vincristine and vinblastine (potential drug treatment for cancer) (Zomlefer, 1994). Ornamental species of the family includes *Allamanda* L. (yellow bell), *Amsonia* Walter (blue star), *Asclepias* L. (milkweed), *Carissa* L. (natal plum), *Hoya* R. Br. (wax plant), *Nerium* L. (oleander), *Plumeria* L. (frangipani), *Stapelia* L. (carrion flower), *Thevetia* L. (lucky nut), and *Vinca* L. (periwinkle) (Zomlefer, 1994).

Prior to the worldwide revision of the family, Apocynaceae sensu stricto (s.s.) are identified separately from Asclepiadaceae. Several synapomorphies of the members of both families despite the segregation were nevertheless acknowledged (Rosati, 1989). Based on molecular analyses (e.g., Sennblad, 1997; Civeyrel et al., 1998; Sennblad and Bremer, 2000), the family Apocynaceae is considered paraphyletic while the family Asclepiadaceae is monophyletic. With the recent phylogenetic studies, however, the segregation of Asclepiadaceae from Apocynaceae was questioned in favor of a broad circumscription of Apocynaceae with Asclepiadaceae (e.g., Judd et al., 1994; Civeyrel et al., 1998; Endress and Bruyns 2000; Sennblad and Bremmer, 2000; Potgeiter and Albert 2001). For this reason, the two families were merged into a single family called the Apocynaceae including Asclepiadaceae.

Apocynaceae s.l. consists of five subfamilies: Rauvolfioideae, Apocynoideae, Periplocoideae, Secamonoideae and Asclepiadoideae sensu Endress and Bruyns (2000). Prior to the circumscription of Asclepiadoideae sensu Endress and Bruyns (2000), there are five tribes of the subfamily: Fockeeae, Marsdenieae, Stapelieae (=Ceropegieae), Gonolobeae, and Asclepiadeae (Kunze, 1994). However, due to several taxonomic revisions based on morphological and molecular data, Endress and Bruyns (2000) reduced the number of tribes into three, abandoning Gonolobeae and Fockeeae. Gonolobeae was merged to Asclepiadeae as suggested by several researchers (e.g., Swarupanandan et al., 1996; Sennblad and Bremer, 1996; Liede, 1997; Potgieter, 1999) while Fockeae in Marsdenieae as proposed by Endress and Bruyns (2000). This leaves the current accepted tribal circumscription of Asclepiadoideae to three which are the Asclepiadeae, Ceropegieae, and Marsdenieae.

The genus *Clemensiella* of the tribe Marsdenieae comprises only two species, the *Clemensiella mariae* (Schltr.) Schltr. (a Philippine endemic) and *Clemensiella omlori* Livsh. & Meve (Borneo and Sumatra). *Clemensiella mariae* was initially placed under family Meliaceae (Merrill, 1908). However, Schlecter (1915) transferred *Clemensilella* to Apocynaceae due to several incongruent morphological features against Meliaceae (e.g., alternate leaves; regular, perfect flowers; imbricate or valvate corolla). The leaf arrangement of *Clemensiella* is opposite, occasionally decussate which corresponds with the morphology of Apocynaceae (Schlecter, 1915). Recently, Omlor (1998) suggested that *Clemensiella mariae* does not agree with the morphology of the

rest of Marsdenieae. Omlor (1998) noted, however, the similarities of *Clemensiella* to *Marsdenia* (pollinaria morphology) and *Hoya* (habit). This raises question on the placement of *Clemensiella* in Marsdenieae.

In this first molecular study of the Philippine endemic *Clemensiella mariae*, the internal transcribe spacers (ITS) region of the nuclear ribosomal DNA was sequenced and analyzed together with previous published sequences. The employment of molecular data has been extensively used to answer phylogenetic questions in flowering plants (e.g., Baldwin, 1992; Baldwin et al., 1995; Jobst et al., 1998; Andrerson, and Rova, 1999; Meve and Liede, 2002; Meve and Liede, 2004; Alejandro et al., 2005). Based on ITS sequence data, the present study investigates the phylogenetic position, monophyly and closely related genera of *Clemensiella*.

MATERIALS AND METHODS

Taxon Sampling

A total of three *Clemensiella mariae* isolates was collected in Irosin, Sorsogon. Plant samples (Fig. 1) were prepared for herbarium and two to three leaves were preserved in silica gel for DNA extraction (Chase and Hills, 1991). Reproductive parts were preserved in 70% ethanol, FAA (Radford et al., 1974) to allow the study of its detailed parts.

Fig. 1. *Clemensiella mariae* showing its flower morphology.

DNA Extraction, Amplification and Sequencing

Genomic DNA was extracted from silica-gel dried leaf tissue following the protocol of DNeasy Plant Mini Kit (Qiagen®, Germany). The ITS region (ITS1, 5.8s gene and ITS2) was amplified using primers P17F (5'-CTA CCG ATT GAA TGG TCC GGT GAA-3') and 26S-82R (5'-TCC CGG TTC GCT CGC CGT TAC TA-3') (Popp and Oxelman, 2001). PCR cocktail consisted of 15.3 µl of H_20, 2.5 µl of MgCl, and 2.0 µl of DNTPs (2mM). The PCR

reactions amounting to 25µl were run on MJ Research Tetrad PTC 100 Thermal Cycler®. The program was set to have an initial denaturation of 97ºC for 1 min and 30 sec, followed by 35 cycles of 20 sec at 97ºC, 90 sec at 72ºC, 30 sec at 72ºC, and a final extension of 7 min at 72ºC.

The amplified PCR products were subjected to agarose gel electrophoresis for confirmation of the presence of single DNA bands. The gel was ran for 35-45 min in a power supply (BIORAD Power Pac 300®), which was calibrated at 80 volts. For visualization of bands, the gel was viewed using the AUV DIGI Doc 1T Digital Gel Documentation System. After the amplification of DNA, purification of PCR products was done using the QIA-quick Purification Kit (Qiagen®, Germany). The purified bands of the three *Clemesiella* isolates (labeled as CM1a, CM1b and CM2) are shown in Fig. 2.

The purified DNA samples were were sent to Macrogen Inc. in Seoul, South Korea for sequencing. The primers used for the forward and reverse sequences are P16F (5'-TCA CTG AAC CTT ATC ATT TAG AGG-3') and P25R (5'-GGG TAG TCC CGC CTG ACC TG-3') (Popp and Oxelaman, 2001).

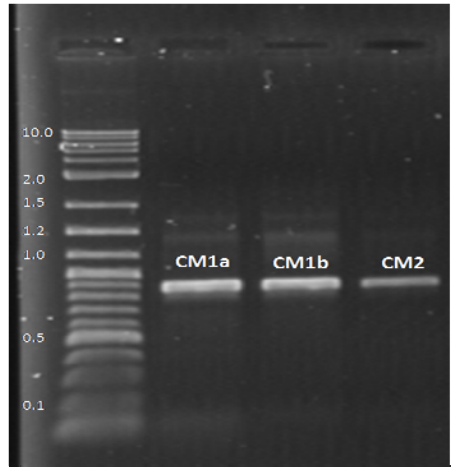

Fig. 2. Purified DNA bands of the three isolates of *Clemensiella mariae*.

Alignment and Phylogenetic Analysis

The ITS sequences were manually edited using the Bioedit and Perkin Elmer Sequence Navigator®, version 1.0.1. Subsequently, the sequences were assembled using MacClade version 4. The boundaries for the three coding regions were determined by comparing to the previous published Marsdenieae sequences in the Genbank.

Aside from the three *Clemensiella* isolates, a total of 30 sequences (Table 1) representing the three tribes of Asclepiadoideae (Asclepiadeae, Ceropegieae, and Marsdenieae) were downloaded from GenBank (NCBI) and were assembled also in MacClade.

Table 1. Vouchers, EMBL accession numbers and tribal classification of taxa included in the study.

Tribe and species	**Voucher**	**ITS Accession No.**
OUTGROUP		
Periplocoideae		
Peiploca migrescens	Marrello et al. 1354 (MO)	DQ916859
Raphionacme lobulata	Dold 4461 (GRA)	DQ916862
INGROUP		
Asclepiadeae		
Gomphocarpus fructicosus	Nicholas 2796 (UDW)	AM96906
Gomphocarpus glaucophyllus		AM96907
Pergularia daemia	Masinde 888 (in cult).	AM396877
Tylophora tenuis	Schneidt JS 95-100(ABD)	AJ320468
Tylophora villosa	Schneidt JS 95-39 (ADB)	AJ320469
Tylophora yunnanensis	Schneidt JS 96-134	AJ320471
Marsdenieae		
Dischidia astephana	Wanntorp L. 562 (S)	DQ334459
Dischidia hirsute	Wanntorp L. 563 (S)	DQ334456
Gunnessia pepo	P.F. Foster PIF 6465(BRI)	DQ334446

Hoya camphorfolia	Wanntorp L. 590 (S)	DQ334520
Hoya ciliate	Wanntorp L. 586 (S)	DQ334515
Hoya incrassate	Chase 7136 (K)	DQ334514
Marsdenia carvalhoi	Chase 17115 (K)	DQ334423
Rhyssolobium dumosum	Bryuns 3948 (Bol)	AM233396
Ceropegieae		
Anistoma cordifolia	Nicholas 2811 (UDW)	AJ310780
Brachystelma christianeae	Peckover subspecks 2531 (UBT)	AJ310796
Brachystelma filifolium	Peckover subspecks 2540 (UBT)	AJ310791
Carallum subulata	Mangelsdorff 28	DQ469521
Ceropegia bulbosa	Rowland sub Butler C 726 (UBT)	AJ488783
Echidbopsis squamulata	Mangelsdorff Y27	DQ469550
Echidbopsis urceolata	Vlk s.n	DQ469552
Echidbopsis watsonii	Cole 3176	DQ469553

Parsimony analysis was performed with Phylogenetic Analysis Using Parsimony (PAUP*) version 4.0b (Swofford, 2000) on a Power Macintosh G3 computer via heuristic searches, with the MULTREES option on, tree-bisection-reconnection (TBR) branch swapping, swap on best only in effect, and 5000 random addition sequences. In all analyses, characters were given equal weight and gaps were treated as missing data. The base frequencies for the whole ITS and each region were calculated. The length of the tree (L), consistency index (CI), and retention index (RI) were calculated to estimate homoplasy. Bootstrap (BS) values were performed to assess relative support for identical clades using 5000 replicates, the MULTREES option off, nearest neighbor interchanges (NNI) branch swapping support of 86-100% was treated

as strongly supported, 70-85% as moderately supported and 50-69% as weakly supported (Alejandro et al., 2005)

RESULTS AND DISCUSSION

ITS sequence characteristics

The ITS sequences of the 33 taxa included in the study varied from 614 (*Tylophora vilosa*) to 673 base pairs (bp) (*Gomphocarpus glaucophyllus*). The three *C. mariae* isolates yielded a total of 630 to 642 bp. The average GC content, ranging from 63.21% to 63.86%, corroborates with previous literature (e.g., Hershkovitz and Zimmet, 1996).

The aligned data matrix of the 33 taxa had a total of 742 positions. Due to alignment ambiguities, a total of 102 positions (21-30, 79-84, 195-205, 272-285, 471-501, 506-515, and 708-729) were excluded in the analysis. Of the remaining 640 positions, 328 characters were considered to be phylogenetically informative sites that were included in the analysis. The summary of the variations within the whole ITS region is shown in Table 2.

Parsimony analysis of the ITS data resulted to forty equally parsimonious trees (L = 1212; CI = 0.63; RI = 0.70). The strict consensus tree (SCT) is shown in Figure 3.

Table 2. Summary of variations within the ITS region.

Region	Length	Total of excluded characters	Phylogenetically informative sites
ITS-1	302	39	173
5.8S gene	165	0	16
ITS-2	275	63	139
Whole ITS region	742	102	328

Tribal position, monophyly and closely-related genera of *C. mariae*

Based on the ITS tree (Fig. 3), the placement of *C. mariae* within the tribe Marsdenieae is confirmed corroborating with Schlecter (1915) and Meve et al. (2009) based on morphology. In contrast, our results do not support the suggestion of Omlor (1998). The three isolates formed a monophyletic group with a strong support (BS = 100%) distinct from the rest of the members of Marsdenieae. Moreover, the *C. mariae* isolates were found sister to the clade containing *Dischidia* and *Hoya* with a weak support (BS = 56%). Several

characters agree with the close relation of *C. mariae* with *Dischidia* and *Hoya*. In general, the three genera are epiphytic, with adventitious roots, persistent inflorescences, and valvate corolla lobes (Fig. 4) (Meve et al., 2009). Kloppenburg and Siar (2006) even recognized *C. mariae* as a Hoya species due to similarities in corolla and corona morphology. The main variation of the three genera occurs in the seed morphology. The seeds of both *Dischidia* and *Hoya* are cylindrical without wings (reduced if present) while *Clemensiella* is characterized by obovate seeds with wings and apically attached coma (Meve et al., 2009). The seed features of *Clemensiella* are typical in most members of Marsdenieae.

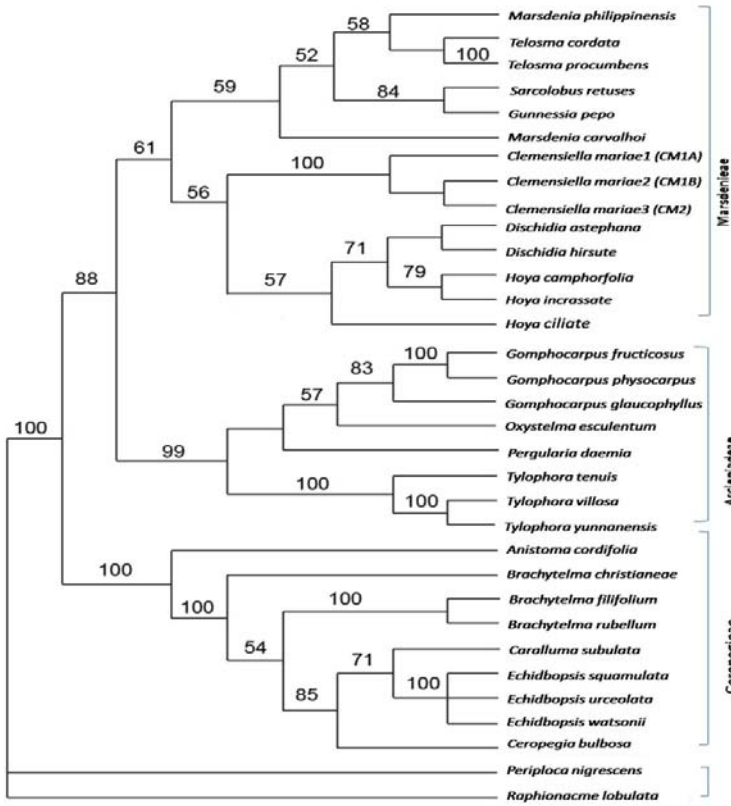

Fig 3. Strict consensus tree derived from forty equally parsimonious trees based on the phylogenetic analysis of the ITS sequence data. Numbers above nodes indicate the bootstrap values >50%. The three tribes of subfamily Asclepiadoideae are labeled in brackets. The two basal taxa with a small bracket are the outgroups.

Fig. 4. Flower buds of *Clemensiella mariae* showing valvate corolla aestivation.

Implications on the tribal position of *Clemensiella*

Some *Hoya* species are known to produce certain compounds such as esters (essential oils), rubber and triterpenes that are derivatives of several anti-bronchitis drugs (Baas, 1983; Warnaar, 1984; Baas and Berkel, 1991; Baas et al., 1992). Likewise, *Dischidia* species contain compounds such as flavonoids, steroids and triterpenoids (Chen et al., 1993). Since *Clemensiella* is closely related with both *Hoya* and *Dischidia*, its potential as a source of essential compounds for medicinal and industrial uses should also be explored.

CONCLUSIONS

The first molecular study of *Clemensiella* established its position in the tribe Marsdenieae based on the ITS sequences data. The genus is monophyletic and closely related to *Dischidia* and *Hoya*. The three genera are similar in terms of habit, presence of adventitious roots, persistence of inflorescence and aestivation of corolla lobes but they differ in terms of seed morphology. Some *Hoya* and *Dischidia* are known for their economic importance. Their close relations to *Clemensiella* indicate that such medicinal as well as industrial uses may also be present in the unexplored genus.

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