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Full Length Article

Morpho-Molecular Study on some Taxa of Apocynaceae *Sensu Lato*

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Abstract

Apocynaceae *sensu lato* (*s.l.*) is treated as distinct family in some taxonomic systems while in others is treated as two separated families *viz.* Apocynaceae *sensu stricto* (*s.s.*) and Asclepiadaceae. So the main objective of the present study was to adopt one of the two views. The morphological characters *viz.* whole plant, lamina vein architecture and lamina epidermal characters of 20 wild and ornamental species were examined using LM and SEM. The data were numerically analyzed to detect the phenetic relationship among the studied species. DNA barcoding based on the sequencing of *rbcL* gene was cladistically analyzed to detect the phylogenetic relationship among the studied species. The generated phenogram showed a clear separation of two subseries; one of them belonged to Apocynaceae and the other with the remaining taxa belonging to Asclepiadaceae. The obtained cladogram showed that all of the studied species were divided into four lineages. It is concluded that the phenetic analysis supports the treatment of Apocynaceae *s.l.* as two distinct families; Apocynaceae *s.s.* Asclepiadaceae contrary of the phylogenetic one that supports the treatment of Apocynaceae *s.s.* and Asclepiadaceae as one family (Apocynaceae *s.l.*) with four subfamilies *viz.* Apocynoideae, Rauvolfiodeae, Asclepiadoideae and Periplocoideae. © 2021 Friends Science Publishers

Keywords: Apocynoideae; Asclepiadoideae; Cladistics; Phenetics; Periplocoideae; Rauvolfiodeae

Introduction

Apocynaceae and Asclepiadaceae constitute the most diverse lineage of Gentianales and regarded as two closely related families (Civeyrel *et al.* 1998). Endress *et al.* (1996) and Sennblad and Bremer (2002) reported that Apocynaceae *sensu lato* (*s.l.*) includes the former Asclepiadaceae, and this merge is based on both morphological and molecular similarities. Melchior (1964), Dahlgren (1983) and Jeffrey and Cronquist (1984) separated Asclepiadaceae from Apocynaceae and divided Apocynaceae *s.l.* into two distinct families recognizing two subfamilies per each; Plumieroideae and Echitoideae within Apocynaceae, while Cynanchoideae and Periplocoideae within Asclepiadaceae.

It has long been known that some taxa of Apocynaceae *s.l.* are more morphlogically similar to Asclepiadaceae than they are to their own family, a series of morphology-based papers focused on pollen have proved to be the most important morphological character (Endress *et al.* 2014; Dabbub *et al.* 2020). The importance of stomatography and leaf venation as a helpful taxonomic tool needs no emphasis, as these characters have immense value in identifying species of Apocynaceae *s.l.* (Chandra *et al* 1968). Foliar epidermal characters are considered as very important diagnostic tool to differentiate among the species of the family (Ayaz *et al.* 2020). Light microscopy (LM) and Scanning electron microscopy (SEM) on leaf of Apocynaceae were believed to have a pivotal role in the classification and delimitation of its species (Bashir *et al.* 2020).

A wide range of molecular approaches has been used by numerous research groups to find the natural relationship within various groups of Apocynaceae (Potgieter and Albert 2001; Rapini *et al.* 2003; Rapini *et al.* 2007; Simões *et al.* 2007; Livshultz 2010). Fishbein (2001) used chloroplast matk gene and confirmed the monophyly of subfamilies and assumed a closer relationship of Periplocoideae to Apocynaceae *s.s.* than to the remainder of Asclepiadaceae. Sennblad and Bremer (1996) used the sequence data for *rbcL* gene of 24 species of Apocynaceae and Asclepiadaceae to evaluate the existing familial and subfamial classification, and concluded that Asclepiadaceae is nested into Apocynaceae. Judd *et al.* (1994) and Kunze (1996) reported that *rbcL* gene region did not support the monophyly of Asclepiadaceae and Periplocaceae as separate families, rather indicated a close relationship among Periplocaceae and Apocynaceae *s.s.*

A controversy over the taxonomic relationship between the Apocynaceae and Asclepiadaceae persisted. Apocynaceae *s.l.* has been divided into five subfamilies *viz.* Apocynoideae, Rauvolfioideae, Asclepiadoideae, Secamonoideae and Periplocoideae. The former two subfamilies belong to Apocynaceae *s.s.*, while the latter three belonging to Asclepiadaceae (Endress *et al.* 2000; Endress *et al.* 2007; Angiosperm Phylogeny Group 2016). The principle target of the current investigation was to embrace whether Apocynaceae should be treated as distinct entity or two separated taxonomic entities through the use of some diagnostic morphological characters in phenetic analysis, as well as DNA barcoding based on the sequencing of *rbcL* gene that is used in phylogenetic analysis.

Materials and Methods

Twenty species representing four sub-families and 19 genera of Apocynaceae *s.l.* were collected from natural habitats (wild species) and different botanical gardens (cultivated species) in Egypt and Libya (Table 1). The identification and Synonyms were authenticated based on previous infornation (Bailey 1949; Tackholm 1974; Boulos 2000; IPNI 2012).

Macromorphological characters investigation

The macromorphological characters of the whole plant *viz.* habit, stem, leaf and flower were examined, described from the fresh specimens and with the aid of text books (Bailey 1949; Hutchinson and Dalziel 1954; Boulos 2000; Endress and Bruyns 2000; Pandey and Pandey 2006). *Huernia andreaeana* is with rudimentary leaves, so the lamina vein architecture is not dealt with. Lamina vein architecture is examined from fresh leaves in *Nerium oleander, Cynanchum acutum* and *Carissa carandas,* while in other species, leaves were cleaned and soaked in a strong household bleach solution (sodium hypochloride less than 5%, sodium hydroxide less than 5% and water) until they turn white. The leaves were removed from bleach and rinsed with water then stained with 1% safranine (Johansen 1940).

Lamina epidermal strips were prepared from fresh leaves of the studied species (leaves rudimentary in *H. andreaeana*). The epidermal strips were prepared by mechanically stripping (Johansen 1940) or by chemical methods (Pohl 1967) by taking fragment of 5-10 mm^2 from median portion of leaf, soaked in nitric acid and hydrogen peroxide solution (1:1) for a period (2 h for two days) depending on the leaf thickness. The epidermal strips were stained with 1% safranine, mounted on slides. Examination and photomicrographs were taken using Canon power-shot A720, 8.0 mega pixels. For SEM investigation, small pieces (7 mm^2) of the fresh leaf material were fixed on SEM stubs with double-sided tape, coated with gold in SPI-Module sputter coater. Only abaxial surface was observed and photographed by Scanning Electron Microscope (JSM-5500 LV; JEOL Ltd-Japan) by using high vacuum mode. Terminology of epidermal characteristics was performed with the help of previous information (Metcalfe and Chalk 1950; Ash *et al.* 1999; Prabhakar 2004; Stearn 2005).

DNA extraction and PCR amplification with rbcL primers

The fresh leaves of the studied species were collected and subjected to molecular analysis, *Solenostemma argel* material was practically unavailable and loaded from gene bank (ID HG530567). Leaves (100 mg) of the available material were ground to a powder using liquid nitrogen in Eppendorf tube then DNA were isolated using CTAB (Cetyl-trimethyl ammonium bromide) protocol of (Doyle and Doyle 1987). The purified DNA was used in PCR amplification of *rbcL* region using universal primers as the following:

Forward primer: 5´-ATG TCA ACA CAA ACA GAG ACT AAA GC-3´

Reverse primer: 5´-GAA ACG GTC TAT CCA ACG CAT-3´

The amplification reactions were performed in 25 µL as follow; 5x GoTaq® Flexi buffer 5µL, $MgCl_2$ (25 mM) 2.5 µL, dNTPs (10 mM each) 0.5 µL, forward primer (10 µM) 1.2 µL, reverse primer (10 µM) 1.2 µL, Go Taq™ (5 U/µL) 5 µL, DNA Stock 2 µL, H2O 7.6 µL up to make 25 µL total volume. The reaction conditions were as the following:

Initial denaturation at 95°C for 5 min., 40 cycles at 94°C for 30 s., 58°C for 30 s., 72°C for 45 s. and 72°C for 10 min. All positive PCR amplicons were prepared for the cleanup step (purification) from other undesired substances as dimers, RNA, free nucleotides and unamplified DNA fragments by using PCR product purification Kit (Thermo PCR purification kit, USA). It is an essential step before automated DNA-sequencing. The purified DNA was submitted for sequencing to Macrogen, Korea; 6F, 172, Dolma-ro, Bundang-gu, Seongnam-si, Gyeonggi-do (Jeongja-dong, Seoul National University Bundang Hospital Healthcare Innovation Park).

Phenetic analysis

The morphological data of 20 species were subjected to phenetic analysis based on 176 morphological characters states. Prior to analysis the presence for character states were indicated as numerical values in order to make comparison during similarity estimation feasible. Thus, a uniform convention was used for all characters; “1” used for present and “0” for absent. All computations were carried out using NTSYS-PC version 2.02 software program (Rohlf 2000).

Phylogenetic analysis

Crucianella angustifolia (family Rubiaceae) was designed as an out group for phylogenetic analysis and loaded also from gene bank (ID X81094). The sequences were prepared for alignment to get the best trees. The chromatograms in sequences were compiled using BIOEDIT V3 program (Hall 1999). Sequence service chromatogram (fasta file) of sample was imported into a new alignment file created in BIOEDIT. The MEGA7 a phylogenetic program was used for assembling data and constructing the tree. Alignment fasta file was entered into the program for constructing the phylogenetic tree. Tree was constructed according to Neighbor Joining model, which is based on the number of distances between sequences. The number of bootstrap tests was performed using 1000 replicates.

Results

Phenetic analysis

A summary of morphological characters states and its codes as revealed by LM & SEM is presented in Table 2 and some of the most specific structures were illustrated in Fig. 1.

The obtained phenogram (Fig. 2) resulting from the cluster analysis of 176 morphological characters states of whole plant, lamina architecture, stomatography (LM and SEM) showed that *H. andreaeana* basally segregated with unresolved relationship due to the lack of many character states (102 leaf character states). The remaining 19 species were separated into two series; I and II. Series I comprised *Acokanthera oblongifolia*, *Alstonia scholaris*, *Adenium obesum*, *C. carandas*, *C. macrocarpa*, *Cerbera odollam*, *Tabernaemontana divaricate*, *Wrightia coccinea*, *Kopsia arborea*, *Plumeria obtusa*, *Cascabela thevetia*, *N. oleander*, *Asclepias curassavica*, *Cryptostegia grandiflora*, *Calotropis procera*, *S. argel*, *Gomphocarpus sinaicus* and *C. acutum*, at a taxonomic distance 0.38 based on sharing of simple leaf, paracytic or anomocytic stomata, smooth surface anticlinal wall, pinnate primary vein category and regular polygonal reticulate quaternary vein category. Series II included *Catharanthus roseus* as a single entity at a taxonomic distance 0.38. Owing to annual herb, amphistomatic leaf type, eucamptodromus secondary veins, opposite percurrent tertiary veins with straight course, wrinkled anticlinal wall of epidermal cells.

Series I was divided into two sub-series; (A and B) at a taxonomic distance 0.45. Sub-series A was divided into two clusters; C1 and C2 at a taxonomic distance 0.47. Cluster 1 was divided into two groups; G1 and G2 at a taxonomic distance 0.51. Group 1 included *A. oblongifolia* and *A. scholaris* as one group due to presence fissured layers wax with ill-defned sculpture of lamina abaxial epidermal cells and elliptic leaf. Group 2 included nine studied species sharing the presence of narrow, depressed anticlinal walls with smooth surface, and raised periclinal walls.

Cluster 2 included *N. oleander* at a taxonomic distance 0.47 owing to cladodromous secondary veins categoty, sunken stomata and curved anticlinal wall adaxially, incomplete looped marginal veins, one-branched freely ending ultimate veins, granulate crystalloid epicuticular wax and colliculate sculpture of lamina abaxially.

Sub-series B was divided into two clusters; C3 and C4 at a taxonomic distance 0.55. C 3 was divided into two groups; G3 and G4 at a taxonomic distance 0.60. Group 3 included *A. curassavica* and *C. grandiflora* grouped together as one group. The latter group 4 included *C. procera*, *G. sinaicus* and *S. argel* based on sharing morphological characters *viz.* ad medially ramified tertiary veins, amphistomatic leaf type and sunken or leveled stomata elevation abaxially. The latter cluster 4 included *C. acutum* due to presence climbing stem, cordate leaves with actinodromus primary vein category, ill-defned sculpture of lamina abaxially with hight density of non-entire platlets epicuticular.

Phylogenetic analysis

The obtained sequences were uploaded to Genbank and gained accession numbers as follow; BankIt2396104 *A. oblongifolia* MW208824, BankIt2396104 *A. obesum* MW208825, BankIt2396104 *A. scholaris* MW208826, BankIt2396104 *A. curassavica* MW208827, BankIt2396104 *C. procera* MW208828, BankIt2396104 *C. carandas* MW208829, BankIt2396104 *C. macrocarpa* MW208830, BankIt2396104 *C. thevetia* MW208831, BankIt2396104 *C. roseus* MW208832, BankIt2396104 *C. odollam* MW208833, BankIt2396104 *C. grandiflora* MW208834, BankIt2396104 *C. acutum* MW208835, BankIt2396104 *G. sinaicus* MW208836, BankIt2396104 *H. andreaeana* MW208837, BankIt2396104 *K. arborea* MW208838, BankIt2396104 *N. oleander* MW208839, BankIt2396104 *P. obtusa* MW208840, BankIt2396104 *T. divaricata* MW208841, BankIt2396104 *W. coccinea* MW208842.

The generated cladogram (Fig. 3), which was rooted by *C. angustifolia* as an outgroup, supported the monophylly of the taxa under investigation, which can be differentiated into four lineages. The first one included *P. obtusa* and *K. arborea* as sister taxa due to sharing of intermarginal secondary veins, well developed areolation, two or more branched freely ending ultimate veins, straight anticlinal wall of ab/ adaxial epidermal cells and leveled stomata (synapomorphic characters). The second lineage included *A. oblongifolia*, *C. carandas*, *C. macrocarpa*, *A. scholaris*, *C. odollam* and *C. thevetia*. All taxa are nested together at 0.771 bootstrap value after separation of *C. roseus* and *T. divaricata* at earlier basal level. *A. oblongifolia*, *C. carandas* and *C. macrocarpa* are nested together at 0.787 bootstrap value based on synapomorphic characters *viz.* shrub habitat, salver corolla tube and union of carpels (ovary, style and stigma) throughout. In the third lineage, *C. grandiflora* and *W. coccinea* were nested

Table 1: Data of collection of the studied species

No.	Taxa	Locality/source
1.	*Acokanthera oblongifolia* Benth. & Hook.f. - Gen. Pl. [Bentham & Hooker f.] 2(2): 696. 1876 [May 1876]; nom. inval. (IK) = *A. spectabilis* (Sond.) Hook.f.	BG
2.	*Adenium obesum* Roem. & Schult. - Syst. Veg., ed. 15 bis [Roemer & Schultes] 4: 411. 1819 (IK) = *A. arabicum* Balf.f	OBG
3.	*Alstonia scholaris* (L.) R.Br. - Mem. Wern. Nat. Hist. Soc. 1: 75. 1811 [dt. 1809; issued in 1811] (IK) = *Echites scholaris* L.	GZ
4.	*Asclepias curassavica* L. - Sp. Pl. 1: 215. 1753 [1 May 1753] (IK) = *A. margaritacea* Hoffmanns. ex Schult.	OBG
5.	*Calotropis procera* W.T.Aiton - Hort. Kew., ed. 2 [W.T. Aiton] 2: 78. 1811 (IK) = *C.* persica Gand.	WML
6.	*Carissa carandas* L. -- Mant. Pl. 52. 1767 [15-31 Oct 1767] (IK) = *C. carandas* var. congesta (Wight) Bedd.	BG
7.	*Carissa macrocarpa* (Eckl.) A. DC. - Prodr. [A. P. de Candolle] 8: 336. 1844 [mid Mar 1844] (IK) = *Arduina grandiflora* E.Mey.	OBG
8.	*Cascabela thevetia* (L.) Lippold. - Feddes Repert. 91: 52. 1980 (GCI) = *Thevetia peruviana* (Pers.) K.Schum.	BG
9.	*Catharanthus roseus* (L.) G.Don. - Gen. Hist. 4(1): 95. 1837 (IK) =*Vinca rosea* L.	AUG
10	*Cerbera odollam* Gaertn. - Fruct. Sem. Pl. 2: 193. 1791 (IK) = *Odollamia malabarica* Raf.	AG
11.	*Cryptostegia grandiflora* R.Br. - Bot. Reg. 5: t. 435. 1820 [1819 publ. 1820] (IK) = *C. grandiflora* Roxb. ex R.B.	AMG
12.	*Cynanchum acutum* L. - Sp. Pl. 1: 212. 1753 [1 May 1753] (IK) = *C. excelsum* Desf.	AU
13.	*Gomphocarpus sinaicus* Boiss. - Diagn. Pl. Orient. ser. 1, 11: 80. 1849 [Mar-Apr 1849] (IK) = *Asclepias sinaica* (Boiss.) Muschl = *Gomphocarpus schimperi* C.Presl	SK
14.	*Huernia andreaeana* (Rauh) L.C.Leach - J. S. African Bot. 40(1): 21. 1974 (IK) = *H.* appendiculata Berger.	WML
15.	*Kopsia arborea Blume. - Cat. Gew. Buitenzorg (Blume) 13. 1823; et Bijdr. Fl. Ned. Ind. 16: 1030 [Oct 1826-Nov 1827]. (IK) = K. jasminiflora Pit.*	BG
16.	*Nerium oleander* L. - Sp. Pl. 1: 209. 1753 [1 May 1753] (IK) = *N. carneum* Dum.Cours.	AUG
17.	*Plumeria obtusa* L. - Sp. Pl. 1: 210. 1753(IK) = *P. apiculata* Urb.	AUG
18.	*Solenostemma argel* Hayne - Getreue Darstell. Gew. ix. t.38. 1825. (IK) = *Cynanchum arghel* Delile.	A
19.	*Tabernaemontana divaricata* (L.) R.Br. ex Roem. & Schult. - Syst. Veg., ed. 15 bis [Roemer & Schultes] 4: 427. 1819 (IK) = *Taberna discolor* (Sw.) Miers	BG
20.	*Wrightia coccinea* Sims - Bot. Mag. 53: t. 2696. 1826 (IK) = *Nerium coccineum* Roxb. ex Hornem. = *N. coccineum Lodd.*	AG

BG: Botanical Garden, Botany Department, Faculty of Science, Ain Shams University. AU: Al-Azhar University Garden. A: Asswan. OBG: Orman Botanical Garden, Giza. AUG: Al-Zawia University Garden in Libya. WML: Western Mountain in Libya. AG: Al-Zohriya Garden, Gizzira. SK: Saint Katherine, South Sinai. AMG: Agriculture Museum Garden. GZ: Giza Zoo

together at a high bootstrap value (0.792) due to the presence of opposite leaves, weak brochidodromous secondary veins and paracytic lens-shaped stomata (synapomorphic characters). In the last lineage, eight studied species were nested at 0.772 bootstrap value. *A. obesum* and *N. oleander* were separated consequently at basal level, while *A. curassavica, G. sinaicus, C. procera, C. acutum, S. argel* and *H. andreaeana* were nested together at 0.993 bootstrap value. The latter six species shared many synapomrphic characters such as campanulate corolla tube, presence of gynostegium, two carpels (free below and united above) with true pollinia. These species were separated consequently from *H. andreaeana* at basal level due to autapomorphic characters *viz.* succulent stem, redumintary leaves features in addition to winged pollinium. *C. acutum* was separated based on autapomorphic characters *viz*. climbing stem, cordate leaf and erect orientation of pollinium. *C. acutum* was delimited from all the studied species of Asclepiadeae on the basis of rhizome stem and presence of opposite petiolated leaves, reaching *G. sinaicus* and *A. curassavica* that forming sister species based on synapomorphic characters *viz.* globose head stigma, oblong-ovate shape of pollinium sac and yellow colour pollinium. *S. argel* is considered a sister group to *C. procera, A. curassavica* and *G. sinaicus*.

Discussion

The generated phenogram supports the treatment of Apocynaceae *s.l.* as two distinct families, Apocynaceae *s.s.* and Asclepiadaceae where there is a clear separation of series I into two subseries; one of them belongs to Apocynaceae and the other with the remaining taxa belongs to Asclepiadaceae (Brown 1810; Bessey 1915; Hutchinson 1959; Takhtajan 1980; Goldberg 1986; Dabbub *et al.* 2020). Within Apocynaceae *A. oblongifolia* and *A. scholaris* were nested in one group and this is in accordance with (Byng *et al*. 2016) and (Endress and Bruyns 2000) where they included them within subfamily Rauvolfioideae. Group 2 contained two species from Apocynoideae (*A. obesum* and *W. coccinea*) and seven species from Rauvolfioideae according to (Byng *et al.* 2016) and (Endress and Bruyns 2000). Sub-series B was divided into two clusters; C3 and C4. C 3 was divided into two groups; G3 and G4. Group 3 included *A. curassavica* and *C. grandiflora* grouped kept together as one group. The latter group 4 included *C. procera, G. sinaicus* and *S. argel*. This group is comparable with tribe Asclepiadeae according to (Melchior 1964; Endress *et al.* 2014; Byng *et al.* 2016; El-gazzar *et al.* 2018).

Phylogentically, *P. obtusa* and *K. arborea* showed a sister relationship both belonging to Rauvolfioideae, this is comparable with Melchior (1964) and Sennblad and Bremer (1996) who gathered them under subfamily Plumeroideae (Apocynacese *s.s.*), while Endress and Bruyns (2000) and Byng *et al.* (2016) put them in subfamily Rauvolfioideae (Apocynacese *s.l.*). *A. oblongifolia*, *C. carandas*, *C. macrocarpa, A. scholaris*, *C. odollam* and *C. thevetia* were nested together (Rauvolfioideae). While *C. roseus* and *T. divaricata* were separated at earlier basal level; the pattern of separation clarified that Rauvolfioideae is not monophyletic group (paraphyletic). (Simões *et al*. 2007) reported six monophyletic tribes within Rauvolfioideae *viz.*

Table 2: Morphological character, states (176) and its codes of the studied species used for phenetic analysis. (0 = Absent; 1 = Present)

Organ	Character		Species / Character states	*Acokanthera oblongifolia*	*Adenium obesum*	*Alstonia scholaris*	*Asclepias curassavica*	*Calotropis procera*	*Carissa carandas*	*Carissa macrocarpa*	*Cascabela thevetia*	*Catharanthus roseus*	*Cerbera odollam*	*Cryptostegia grandiflora*	*Cynanchum acutum*	*Gomphocarpus sinaicus*	*Huernia andreaeana*	**Kopsia arborea*	*Nerium oleander*	*Plumeria obtusa*	*Solenostemma argel*	*Tabernaemont-ana ivaricata*	*Wrightia coccinea*
Whole plant	Duration		Perennial	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1
			Annual	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	Habit		Herb	0	0	0	1	0	0	0	0	1	0	0	1	0	1	0	0	0	1	0	0
			Shrub	1	1	0	0	1	1	1	1	0	0	1	0	1	0	1	1	1	0	1	0
			Succulent herb	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
			Tree	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
Stem	Stem latex		Milky	1	0	1	1	1	1	1	1	0	1	1	1	1	0	1	0	1	1	1	1
			Watery	0	1	0	0	0	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0
	Stem strength		Erect	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1
			Weak	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0
	Stem texture		Glabrous	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	1
			Spiny	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Leaf	Occurrence		Rudimentary	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
			Foliage	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
	Arrangement		Alternate	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	1	0	0	0
			Opposite	1	0	0	1	1	1	1	0	1	0	1	1	1	0	1	0	0	1	1	1
			Whorled	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
	Leaf petiole		Sessile	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
			Petiolate	1	0	1	1	0	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
	Lamina shape		Ovate	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	1
			Ob-ovate	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
			Oblong-ovate	0	0	0	0	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0
			Elliptic	1	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
			Lanceolate	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	1	1	1	0	0
			Cordate	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	Lamina apex		Acute	1	0	0	1	1	0	0	1	0	0	1	1	1	0	1	1	0	1	0	0
			Obtuse	0	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
			Acuminate	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	1	1
Flower	Inflorescence		Cymose	1	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	0	1	1
			Racemose	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0
	Sepal texture		Hairy	0	1	1	1	1	1	1	0	0	0	1	1	1	0	0	1	0	1	0	0
			Glabrous	1	0	0	0	0	0	0	1	1	1	0	0	0	1	1	0	1	0	1	1
	Corolla tube shape		Salver-like	1	1	1	0	0	1	1	0	1	0	0	0	0	0	1	0	0	0	0	0
			Campanulate	0	0	0	1	1	0	0	0	0	0	0	1	1	1	0	0	0	1	0	0
			Funnel	0	0	0	0	0	0	0	1	0	1	1	0	0	0	1	1	0	1	1	1
			Rotate	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	Petal color		White	1	0	1	0	0	1	1	0	0	1	0	0	0	0	1	1	0	1	1	0
			Violetish-white	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
			Pink	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
			Red	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
			Yellow	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0
			Pinkish- white	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	Petal apex		Acut	1	1	1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1
			Acuminate	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
			Obtuse	0	0	0	0	0	0	1	1	1	1	0	0	0	0	0	0	0	0	0	0
	Petal texture		Hairy	1	0	1	0	0	1	1	1	1	0	1	0	1	0	1	0	0	0	0	0
			Glabrous	0	1	0	1	1	0	0	0	0	1	0	1	0	1	0	1	1	1	1	1
	Androecium	Stamens adhesion	Epipetalous	1	1	1	0	0	1	1	1	1	1	0	0	0	0	1	1	1	0	1	1
			Gynostegium	0	0	0	1	1	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0
		Stamens Union	Adhere to stigma	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
			Adhere to style& stigma	0	0	0	1	1	0	0	0	0	0	0	1	1	1	0	0	0	1	0	0
			Not adhere to style nor stigma	1	1	1	0	0	1	1	1	1	1	0	0	0	0	1	1	1	0	1	1
	Gynoecium	Ovary: Carpels	Free below and united above	0	0	0	1	1	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0
			United throughout	1	1	1	0	0	1	1	1	1	1	0	0	0	0	1	1	1	0	1	1
		Ovary: Texture	Hairy	0	1	1	0	0	1	0	0	1	0	0	0	0	1	0	1	0	0	0	0
			Glabrous	1	0	0	1	1	0	1	1	0	1	1	1	1	0	1	0	1	1	1	1
		Style: Number	One	1	1	1	0	0	1	1	1	1	1	0	0	0	0	1	1	1	0	1	1
			Two	0	0	0	1	1	0	0	0	0	0	1	1	1	1	0	0	0	1	0	0
		Stigma: Shape/ head	Globose head	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0
			Pentagonal head	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	1	0	0
			Armed	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0
			Cylindrical	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
			Bilobed	1	1	1	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	1	1
	Nectary disc		Present	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0
			Absent	1	1	0	1	1	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1

Table 2 Continued

Table 2: Continued

Lamina architecture	1° vein category	Pinnate	1	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1
		Basal actinodromous	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	2°vein category	Weak brochidodromou	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	1
		Festooned brochidodromous	1	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
		Brochidodromous	0	1	0	1	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0
		Eucamptodromous	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
		Intramarginal vein	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	0	1	0	0	0
		Cladodromous	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
	2° vein spacing	Decreasing toward base	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
		Increasing toward base	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
		Irregular	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	1	0	0
		Uniform	0	1	1	1	1	0	0	1	1	1	0	0	1	0	1	1	1	0	0	1
	2° vein angle	Uniform	0	1	1	0	1	0	0	1	1	0	0	1	0	0	1	1	1	1	0	1
		Smoothly decrease toward base	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
		Smoothly increase toward base	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0	0	0	0	0	0
	Inter-2° vein	Absent	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
		Weak	0	0	0	1	0	1	0	1	0	0	1	1	1	0	1	1	0	1	0	0
		Strong	1	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	1	0	0	0
	3°vein category	Regular polygonal reticulate	0	1	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
		Random reticulate	1	0	0	0	0	0	1	1	0	1	0	0	0	0	0	0	0	1	0	0
		Alternate percurrent	0	0	1	0	1	0	0	0	0	0	0	1	1	0	1	1	0	0	0	0
		Opposite percurrent	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
		Mixed opp/alt	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1
	3°vein course	Exmedially	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
		Admedially ramified	0	1	1	1	1	1	1	1	0	1	0	1	1	0	1	1	1	1	1	0
		Straight	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
		Sinuous	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
	4° vein category	Ill-developed	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0
		Regular polygonal reticulate	1	1	1	1	1	1	1	1	0	1	1	1	1	0	1	1	1	0	1	1
	5° vein category	Ill-developed	1	0	0	0	1	0	0	1	1	0	0	0	1	0	0	1	0	1	0	0
		Regular polygonal	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	0	1	0
		Dichotomizing	0	1	1	1	0	1	0	0	0	1	0	1	0	0	1	0	0	0	0	1
	Aereolation	Lacking	1	1	1	1	1	0	0	0	1	1	1	1	1	0	0	0	0	1	0	0
		Well-developed	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	1	1	0	0	1
		Poorly developed	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0
	Free ending ultimate vein	Absent	1	0	0	0	1	0	1	1	1	0	0	0	1	0	0	0	0	1	0	0
		1- branched	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0
		2- or more branched	0	1	1	1	0	0	0	0	0	1	0	1	0	0	1	0	1	0	1	1
	Marginal ultimate venation	Looped	1	1	0	1	1	1	1	1	1	1	1	0	0	1	0	1	1	1	1	1
		Incomplete loop	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0
Lamina epidermal characters (LM)	Leaf type	Ill-defined	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
		Hypostomatic	1	1	1	1	0	1	1	1	0	1	1	1	0	0	1	0	1	0	1	1
		Amphistomatic	0	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0
	Abaxial cell shape	Polygonal	1	1	1	0	1	0	0	0	0	0	1	0	1	0	1	0	1	1	0	0
		Irregular	0	0	0	1	0	1	1	1	1	1	0	1	0	0	0	1	0	0	1	1
	Abaxial anticlinal walls	Straight	0	1	1	0	1	0	0	0	0	0	1	0	1	0	1	0	1	1	0	0
		Curved	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	1	0	0	0	0
		Undulate	0	0	0	1	0	0	0	1	1	1	0	1	0	0	0	0	0	0	1	1
	Adaxial cell shape	Polygonal	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	0	1	1	1	0
		Irregular	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	0	1
	Adaxial anticlinal walls	Straight	1	1	1	1	1	1	1	0	1	1	1	1	1	0	1	0	1	1	1	0
		Curved	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
		Undulate	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
	Abaxial stomatal type	Wanting	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Paracytic	1	1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	1	1
		Anomocytic	0	0	0	1	1	0	1	0	1	0	0	1	1	0	0	0	0	1	0	0
		Anisocytic	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0
		Sunkun	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
		Paracytic& Anisocytic	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
	Adaxial stomatal type	Wanting	1	1	1	1	0	1	1	1	0	1	1	1	0	0	1	1	1	0	1	1
		Anomocytic	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0
		Anisocytic	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	Trichomes	Wanting	1	0	1	1	1	1	1	1	0	1	1	1	0	0	1	0	1	0	1	1
		Eglandular	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0
		Glandular (clavate)	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Lamina epidermal characters (SEM)	Epicuticular wax	Striated	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
		Fissured layers	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Smooth film	0	0	0	0	0	1	1	1	0	1	0	0	1	0	1	0	0	0	0	0
		Granulate crystalloid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
		Crystalloid threads	0	1	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	1	1	1
		Non entire platelets	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
	Sculpture	Rugose	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
		Ruminate	0	1	0	1	1	1	1	1	1	1	1	0	1	0	0	0	1	1	1	1
		Ill-defined	1	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
		Reticulate-foveate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
		Colliculate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
	Abaxial anticlinal walls width	Not obvious	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
		Narrow	0	1	0	1	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1
		Broad	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
	Abaxial anticlinal walls elevation	Not obvious	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
		Raised	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Depressed	0	0	0	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1

Table 2: Continued

Table 2: Continued

Abaxial anticlinal wall surface		Ill-defined	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
		Smooth	0	1	0	1	1	1	1	1	0	1	1	0	1	0	1	1	1	1	1	1	1
		Wrinkled	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Abaxial periclinal wall elevation		Ill-defined	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
		Raised	0	0	0	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	1	1
		Depressed	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Abaxial periclinal wall surface		Ill-defined	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
		Smooth	0	1	0	1	1	1	1	1	1	1	0	0	1	0	1	0	0	0	1	1	
		Wrinkled	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	
		Granulated	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	
Stomata	Shape	Circular	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	
		Lens-shaped	0	1	0	0	0	0	0	0	1	0	1	1	1	0	0	0	0	0	0	1	
		Elliptic	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0		
		Ovate	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0		
		Semi-circular	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	1	0	
		Sunken in groove	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	
	Elevation	Leveled	1	0	0	0	0	1	0	0	1	1	0	0	0	0	1	0	1	1	1	1	
		Sunken	0	0	0	0	1	0	1	0	0	0	0	1	1	0	0	1	0	0	0	0	
		Raised	0	1	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	

Alyxieae, Carisseae, Hunterieae, Plumerieae, Tabernaemontaneae and Willughbeieae. On the other hand, phylogenetic studies reported the non-monophyletic groups in Rauvolfioideae (Potgieter and Albert 2001). The subfamily Plumerioideae of the Apocynaceae has often been interpreted as a paraphyletic group, from which the other subfamilies of the Apocynaceae have evolved (Fallen 1986; Endress *et al*. 2014). *A. oblongifolia*, *C. carandas* and *C. macrocarpa* are nested together (tribe Carissae) at 0.787 bootstrap value, which is inaccordance with the previous reports (Pichon *et al.* 1950; Endress and Bruyns 2000; Sennblad and Bremer 2002; Endress *et al.* 2014; Byng *et al.* 2016). Likwise, *C. grandiflora* (Priplocoideae) and *W. coccinea* (Apocynoideae) were nested together contrary of Melchior (1964), Endress *et al*. (2014) and El-gazzar *et al.* (2018) whom separated them; *W. coccinea* in Apocynoideae and *C. grandiflora* in Priplocoideae.

A. obesum and *N. oleander* (Apocynoideae) were separated at basal level, while *A. curassavica, G. sinaicus, C. procera, C. acutum, S. argel* and *H. andreaeana* (Asclepiadoideae) were clustered together. This clade is corresponding to tribe Stapeliae passing with *C. acutum* as a separate clade (Chase *et al*. 2016), *C. acutum* is delimited from all the studied species of Asclepiadeae, reaching *G. sinaicus* and *A. curassavica* that forming sister species (Sennblad and Bremer 1996). *S. argel* (Glossonematinae) is considered a sister group to *C. procera, A. curassavica* and *G. sinaicus* (Asclepiadinae) (Melchior 1964). From the pattern of the fourth lineage, there is a strong support to the monophylly of tribe Asclepiadoideae (Chase *et al.* 2016; El-gazzar *et al*. 2018).

Melchior (1964) divided Apocynaceae *s. l.* into two distinct families and two sub-families for each; Apocynaceae (Plumieroideae and Echitoideae) and Asclepiadaceae (Cynanchoideae and Periplocoideae). Apocynaceae and Asclepiadaceae have been treated as one family and are recognized five sub-families; three of Asclepiadaceae (Periplocoideae, Asclepiadoideae and Secamonoideae) and two of Apocynaceae *s.s.* (Rauvolfiodeae and Apocynoideae) (Endress 2004; Endress *et al.* 2007; Endress *et al.* 2014; Chase *et al.* 2016). The obtained cladogram nested studied species of Asclepiadoideae in a separate lineage and the remaining studied species of Periplocoideae, Apocynoideae and Rauvolfioideae into three lineages. The phylogenetic analysis in the present study supports the treatment of Apocynaceae and Asclepiadaceae as one family (Apocynaceae *s.l.*) with four subfamilies, which is accirding to previous reports (Endress 2004; Endress *et al.* 2014; Chase *et al.* 2016).

Conclusion

The phenetic analysis in the present study supports the treatment of Apocynaceae *s.l.* as two distinct families Apocynaceae *s.s.* and Asclepiadaceae contrary of the phylogenetic one that supports the treatment of Apocynaceae *s.s.* and Asclepiadaceae as one family (Apocynaceae *s.l.*) with four subfamilies *viz.* Apocynoideae, Rauvolfiodeae, Asclepiadoideae and Periplocoideae.

Author Contributions

MS drafted the manuscript and statistically analyzed the data, AM analyzed and interpreted the results, MT planned the study conception and design and made critical revision, HD acquainted the data and UA made illustrations, made the write up and responded to journal reviewers.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability Declaration

Authors declare that all data are available upon request from corresponding author

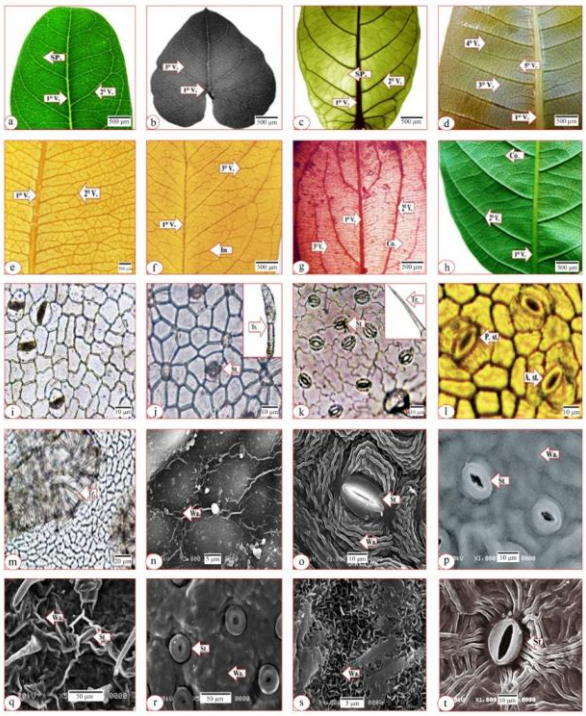

Fig. 1: (a- h): Major categories of lamina vein architecture. a. *C. carandus*, b. *C. acutum*, c. *C. procera*, d. *P. obtusa*, e. *K. arborea*, f. *A. oblongifolia*, g. *C. roseus*, h. *C. grandiflora*; (i-m): Major aspects of lamina epidermal characteristics (LM). i. *A. oblongifolia*, j. *G, sinaicus*, k. *C. roseus*, l. *P. obtusa*, m. *N. oleander*; (n- t): Major aspects of lamina epidermal characteristics (SEM). n. *N. oleander*, o. *P. obtusa*, p. *C. carandus*, q. *A. obesum*, r. *A. oblongifolia*, s. *C. acutum*, t. *C. procera*. 1° V.; Primary vein, 2° V.; Secondary vein, 3° V.; Tertiary vein, 4° V.; Quaternary vein, 5° V.; Quinary vein, A.St.; Anisocytic stomata, P.St.; Paracytic stomata, Co.; Course; In.; Intersecondaries, Sp.; Space, St.; Stomata. Tr.; Trichome, Wa.; Wax

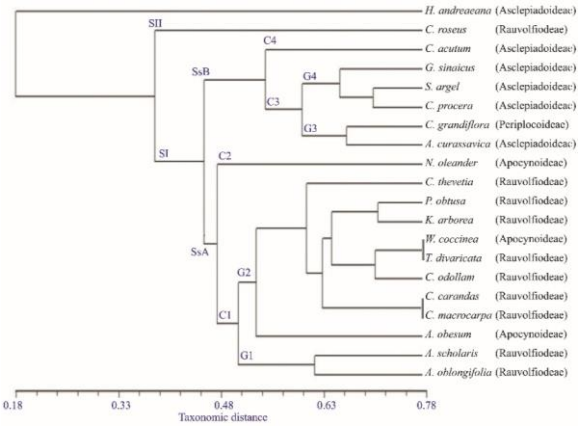

Fig. 2: Phenogram based on 167 morphological characters of the studied taxa of Apocynacaeae *s.s.* S; series, Ss; subseries, C; cluster, G; group

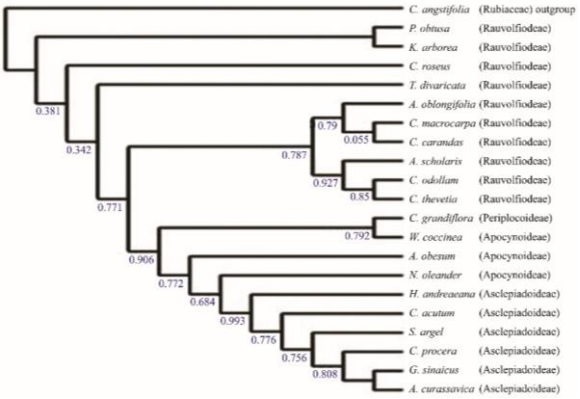

Fig. 3: Neighbor joining phylogenetic tree of the studied taxa of Apocynacaeae *s.s.* based on chloroplast *rbc*L sequence

Conformation to Ethical Guidelines

All material that were used in the current research do not need to ethically approved, human or animal materials not included

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