

Comparative morpho-anatomical studies of *Hoya incrassata* and *Hoya soligamiana* (Apocynaceae) from Mount Hamiguitan, Philippines

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Abstract This study compared the morpho-anatomical characters of two horticulturally important hoyas endemic to the Philippines viz., *Hoya incrassata* and *Hoya soligamiana*. Fresh cuttings were used for morphological and anatomical measurements. Free hand sections were taken from leaves, shoots and roots for determination of structural differences. Leaf pigments were cleared to observe the venation and stomatal arrangement. The results showed that hoyas differ in their leaf shape and texture, flower colour, and the inflorescence shape and position. *H. soligamiana* has glabrous lanceolate leaves, whitish to creamy petals, purplish to pink inner and outer coronal lobes, and whitish to pink fused sepals. *H. incrassata* has coriaceous lance-ovate leaves, whitish to yellowish fused petals, yellow inner coronal lobes, reddish to orange outer coronal lobes, and fused sepals. The measured anatomical traits in the cross sections of roots and stems of the two *Hoya* species did not differ with the exception of stomatal types and arrangement in the leaves. The pigment clearing of the leaves showed that *H. incrassata* has sunken cyclocytic stomata while *H. soligamiana* has actinocytic stomata. Morpho-anatomical information provides taxonomic value for identifying and classifying the two *Hoya* species.

Keywords. Endemic hoyas, morphology, Mindanao, plant diversity, taxonomy, wax plants.

1 Introduction

The Philippines is one of the most biologically diverse countries in the world in terms of unique terrestrial and marine plants and animal species per unit area (Posa *et al.* 2008). One third of more than 9, 250 flowering plant species native to the Philippines are endemic, leading to 45 to 60 % endemism in the

native flora (Madulid 1994). Among the plant groups found in the Philippines that harbors a number of endemic species are some members of the genus *Hoya*. Commonly known as wax plant or hoyas, the genus *Hoya* is notable for its fleshy leaves and flowers covered with a semi-glossy layer of waxy substances or cuticle. It is a semi-woody vine with nearly oval or ovate-shaped leaves 2 to 3 inches long, arranged opposite to each other along the stems (Siar 2005). Most *Hoya* are found as epiphytes and usually hangs on the branches and crown of tall jungle trees seeking for sunlight (Kloppenburg, 2006). This genus comprises approximately 200 species mainly distributed in the Southeast Asian region, particularly, in the Philippines, New Guinea, Western Pacific Islands and Southern Asia including Indian subcontinent (Tsiang and Li 1997; Kleijn and Donkelaar 2001).

In the monograph of Kloppenburg (2009), 79 species of *Hoya* are reported in the Philippines. However, in the more updated checklist, a total of 118 *Hoya* species are already known in the country (Pelser *et al.* 2011), many of which were newly discovered endemic species. Hoyas are commonly used as ornamental plants and commercially sold at high price since they have beautiful and often fragrant flowers (Tran *et al.* 2011) and it is no surprise that some species are already endangered due to over-collection in the wild and illegal trade not just in the Philippines but in Southeast Asia as a whole (Phelps and Webb, 2015). Some hoyas are also collected due to their medicinal properties in different Asian countries (Chuakul 2005; Mollik *et al.* 2010; Gautam 2013; Borlagdan *et al.* 2016). Threatened plants like hoyas are protected by Philippine laws and there are corresponding fines and penalties for the illegal collection and trade (Fernando *et al.* 2008). Despite the regulations provided by Philippine environmental laws, many *Hoya* species are being harvested in the wild due to the demand from private collectors.

Proper identification of biological specimen is crucial in order to protect any endangered and traded species, but in the case of *Hoya*, species identification is almost impossible without the inflorescence. Though biochemical composition of the plants' latex also provide taxonomic value (Baas *et al.* 1981; Heneidak *et al.* 2006), species identification of Asian hoyas to date still relies on the morphological examination of both leaf and floral characters (Li *et al.* 1995; Aurigue *et al.* 2012; Rodda 2012; Averyanov *et al.* 2017). Comparative anatomical studies have been found to be useful in elucidating taxonomic relationships (Standley 1990; Sharifnia and Albouyeh 2002; de Faria *et al.* 2012) as well as in screening some medicinal species (Buenavista and Mateo 2017). In the Philippines, local plant traders offer stem-cuttings of hoyas and some of which are possibly threatened species (Phelps and Webb 2015). As such, this study was carried-out to differentiate the morphological and anatomical features of the different organs of two horticulturally important *Hoya* species.

2 Materials and Methods

2.1 Collection of the *Hoya* specimens

Fresh cuttings of the vegetative and reproductive organs of *Hoya* species were collected from Mount Hamiguitan, Davao Oriental and ex-situ propagated (Fig. 1) in the garden of the Department of Biology, Central Mindanao University, Musuan, Bukidnon, Philippines. Three (3) specimens of each vegetative and reproductive organs were used for morpho-anatomical examinations.



Fig. 1. Habitat of *Hoya soligamiana* (left) and *Hoya incrassata* ex-situ (right).

2.2 Morphological and Anatomical Examinations

Comparison of the different organs of the two *Hoya* species was done by examining the qualitative and quantitative morphological characteristics. Measurements were determined using ruler and vernier caliper. Free-hand technique was then employed to prepare the anatomical sections. Clearing technique and maceration of plant samples were done following the standard procedure of Johansen (1940).

Free-hand technique

Free-hand sectioning technique was employed to examine the anatomical sections of hoyas. Thin sections were taken 10 cm. from the base of the vegetative organs of the plant. A piece of unripe papaya (*Carica papaya*) fruit was cut into small pieces. A groove was made in the center part of the sliced papaya. A portion of the root, stem and leaf of the plant were inserted in the groove of cut unripe papaya and tied with a thread. With the use of sharp

razor blades, thin sections were made and were fixed in 70% ethyl alcohol for 1 hour then washed with water. The thin sections were dehydrated in 85%, 95% and 100% ethyl alcohol for 10 minutes respectively. It was then stained with 1% safranin for 1 hour. The excess stain was removed by washing with 95% ethyl alcohol and was counterstained using 0.5% fast green for 10 seconds and dehydrated with 100% ethyl alcohol for 1 minute. The sections were cleared using 25%, 50%, 75% and 100% xylene. The sections were then mounted in a glass slide with eukitt and covered with a cover slip. The prepared slides were then examined under a compound microscope.

Clearing technique

Clearing technique was employed on leaves of the *Hoya* species to study the venation pattern, stomatal type and the epidermal tissue. Fresh leaves of *Hoya* species were cut into smaller pieces and were fixed in a vial containing 3% aqueous sodium hydroxide and superox (bleaching agent) in 2:1 ratio. The fresh solution was changed daily until most of the pigments will be extracted and was then washed with water gently. The water was decanted and replaced with concentrated chloral hydrate solution. Then, the leaves were placed in 95% ethyl alcohol for 6 to 24 hours and stained with 1% safranin for 12 to 48 hours. Overstained sections of *Hoya* species were destained through washing it several times with 95% ethyl alcohol and transferred in 100% ethyl alcohol, clove oil and 100% xylene (1:1:1) solution for at least 10 minutes. The leaves were counterstained with 0.5 % fast green and dehydrated for at least 10 minutes each in 95% ethyl alcohol and 100 % ethyl alcohol then cleared in 50% xylene, 75% xylene and pure xylene. The cleared leaves were mounted on a glass slide with eukitt and covered with cover slip. The prepared slides were examined under a compound microscope.

Maceration technique

Maceration technique was used to determine the type of vessel and tracheid present in the stem of *Hoya* species. The stem of hoyo was cut into small pieces about 10 mm. on a chopping board and was placed in vials with macerating fluid of equal mixtures of 10% chromic acid and 10% aqueous nitric acid for at least 12 to 24 hours. The macerating fluid was changed daily with freshly prepared ones until the specimen crumbled easily when touched with a needle. It was then washed thoroughly with water and placed in 2% aqueous ferric sulfate for 6 minutes and was washed with water 5 times. The stem was stained with haematoxylin stain for 16 minutes and with 1% aqueous basic fuchsin for an hour and mounted in glycerin jelly. The prepared slides were examined using a compound microscope.

3 Results and Discussion

Morphological and anatomical comparison of *H. soligamiana* and *H. incrassata* shown certain similarities and differences (Table 1).

Table 1. Morphological comparison of the vegetative and reproductive organs of *H. soligamiana* and *H. incrassata*.

Gross Morphology	<i>Hoya soligamiana</i>	<i>Hoya incrassata</i>
Root		
type of root	adventitious root	adventitious root
Texture	rough	rough
Diameter	0.5 to 1.2 mm	0.67 to 1.2 mm
Stem		
Shape when cut	round	round
Color	greenish brown to brown	light brown to brown
Texture	smooth	slightly coarse
Length: internode	7.0 cm to 11.5 cm	8.8 cm to 13.5 cm
Diameter	1.9 mm to 2.6 mm	2.7 mm to 3.8 mm
Leaf		
Color	green	yellowish green
Petioles	present	present
Stipules	absent	absent
Texture	glabrous	leathery/ coriaceous
Type	simple	simple
Venation pattern	pinnately netted; plinerve	pinnately netted
Shape	lanceolate	lance-ovate
Margin	entire	entire
Base	rounded	obtuse
Apex	acuminate	cuspidate
Length	8.7 cm to 15.4 cm	9.0 cm to 19.0 cm
Width	2.8 cm to 4.0 cm	3.7 cm to 6.5 cm
Flower		
Corolla	whitish to creamy white sparsely pubescent	whitish to yellowish; glabrous
Calyx	5; fused; whitish to pink	5; fused
Corona	5; purplish to pink	5
Inner lobe	rounded to obtuse	yellow; rounded
Outer lobe	acute	reddish to orange; acute
Floral Insertion	hypogenous	hypogenous
Ovary	paired	paired
Floral Symmetry	radial	radial
Inflorescence	umbel; 19 to 21 flowers per cluster; convex and positively geotropic	umbel; 40 to 50 flowers per cluster; convex and negatively geotropic

Similarities on the gross morphology of the two *Hoya* species are the following: both species are perennial climbing epiphytes that produce milky sap in all parts which was as also observed by Kloppenburg (2006), Kidyoo and Thaithong (2007) and Wai *et al.* (2008) hence, they are sometimes called milkweed plants. The two species have opposite phyllotaxy where the leaves are borne in pairs facing each other and having an umbel inflorescence. The individual flower of the two species is radial in symmetry wherein the flower parts are arranged on two or more planes. Both also have five fused petals, five fused corona and five fused sepals. Both have hypogenous floral insertion in which the perianth and stamens arises below the ovary, thus the ovary is superior. The stigma is fused and ovaries are paired in both hoyas.

Differences on their gross morphology were also noted. *Hoya soligamiana* has greenish brown to brown stem with smooth texture whereas *Hoya incrassata* has light brown to brown stem with slightly coarse texture. *Hoya incrassata* has the longer internode that measures up to 13.5 cm long and 3.8 mm in diameter stem when compared to *Hoya soligamiana* which is only 7.0 cm to 11.5 cm long. Indeed, though plants' texture is not commonly used (Li *et al.* 1995), it can be added to keys useful in distinguishing hoyas. In terms of leaf morphology, *H. soligamiana* has green, glabrous, lanceolate shaped leaves with three midvein, rounded base and acuminate apex. The leaf size measures 8.7 cm to 15.4 cm long and 2.8 cm to 4.0 cm in width. On the other hand, *H. incrassata* has yellowish green, leathery or coriaceous leaves, lance-ovate in shape with obtuse base and cuspidate apex. It measures 9.0 cm to 19.0 cm long and 3.7 cm to 6.5 cm wide. In terms of floral structures, *H. soligamiana* flower (Fig. 2) has whitish to creamy white corolla which is sparsely pubescent.

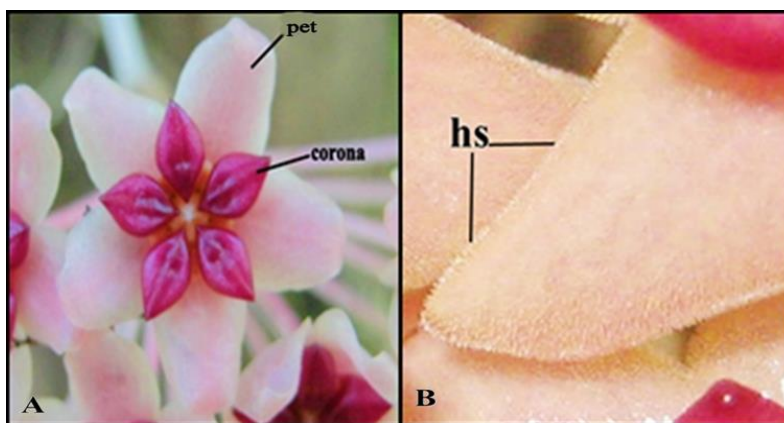


Fig 2. Flower of *Hoya soligamiana*. A) petals (pet), corona (co) and B) hairs (hs).

It has purplish to pink corona and whitish to pink calyx. The inner coronal lobe is rounded to obtuse and acute outer coronal lobe. The inflorescence of *H. soligamiana* is composed of 19 to 21 flowers per clusters that are convex and positively geotropic. The absence or presence of hairs, shape and color of corolla, and leaf size and shape are among the useful characters used in delineating some hoya species (Li *et al.* 1995; Averyanov *et al.* 2017). When compared, the flower of *H. incrassata* is whitish to yellowish corolla which is glabrous. The corona is composed of yellow inner lobe with rounded shape and reddish to orange outer lobe. *Hoya incrassata* has 40 to 50 flowers per cluster that are convex and negatively geotropic.

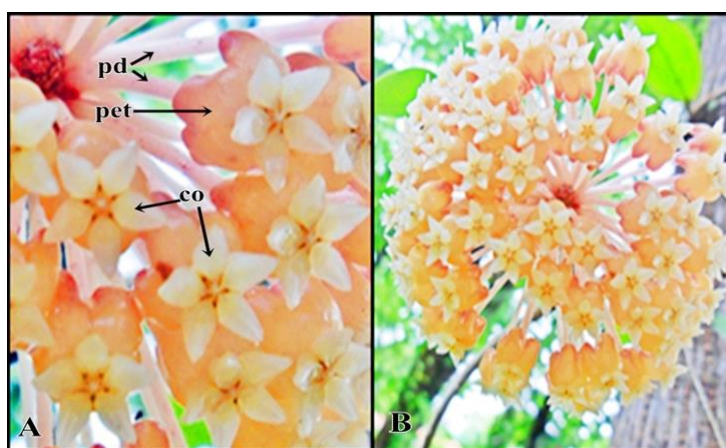


Fig 3. Flowers of *Hoya incrassata*. A) corona (co), pedicel (pd) and petals (pet); B) umbel inflorescence.

As mentioned by Forster *et al.* (1998), the color of the corolla may be due to the pigments dissolved in the fluid of the cell or to anthocyanin pigments contained in the vacuoles and a combination of both in some cells. *Hoya soligamiana* have similar characteristics to *Hoya siariae* on the basis of the leaf morphology which is both glabrous on adaxial and abaxial surfaces, lanceolate, smooth and pinnately net-veined. They are also similar in numbers of flowers composed of 15 to 20 flowers per cluster in an umbel inflorescence. They differ in the some floral characters wherein *Hoya siariae* has pinkish, small and shiny flowers.

The adventitious root and stem of both species undergo secondary growth (Fig. 4). Periderm formation is also an important stage in the development of protective layers near injured or dead tissue whether resulting from the mechanical wounding or invasion or parasites. The stem and root of both

hoyas also have a complex cortex composed of parenchyma and sclerenchyma tissues.

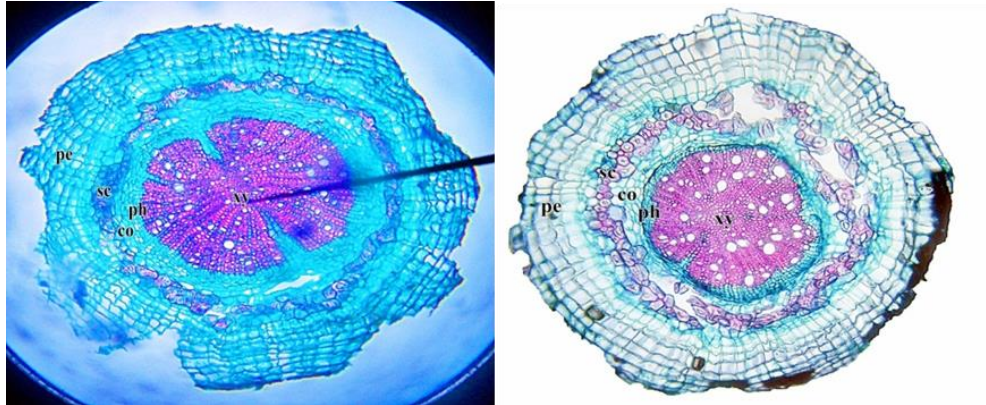


Fig 4. Transsection of the roots of *H. soligamiana* (left) and *H. incrassata* (right). [periderm (pe), cortex (co), sclerenchyma cell (sc) [brachysclereids], phloem (ph) and xylem (xy); Mag. 100x].

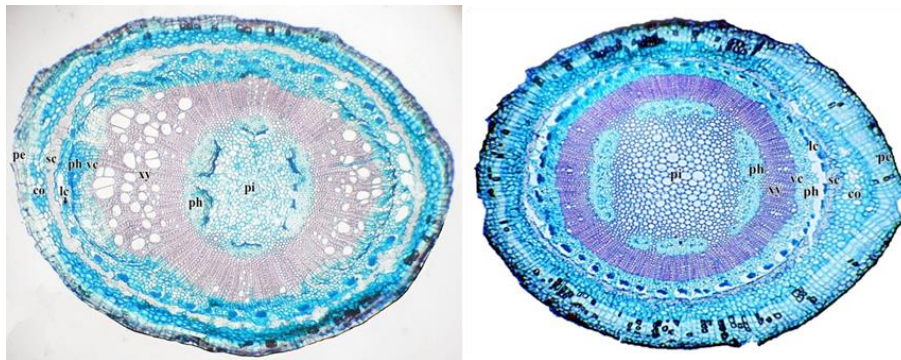


Fig 5. Transsection of the stem of *H. soligamiana* (left) and *H. incrassata* (right). [periderm (pe), cortex (co), sclerenchyma cell (sc) [fibers], laticifers (lc), phloem (ph), vascular cambium (vc), xylem (xy) and pith (pt); Mag. 100x]

The root's stele of the two species (Fig. 4) is haplostelic with solid core of secondary xylem is surrounded by a cylinder of secondary phloem that forms circular in transition whereas their stem stele (Fig. 5) are amphiphloic siphonostele with the presence of phloem tissues on both the outer and inner sides of the xylem cylinder. The roots of both *Hoya* species (Fig. 4) revealed

amphicribal vascular bundle with the secondary phloem surrounding the secondary xylem. Sclereids in the cortex appear to be almost characteristic of the Apocynaceae and Asclepiadaceae and are present throughout all life forms (Schweingruber *et al.* 2011).

Moreover, continuous cylinder of sclereids have also been known to occur on the periphery of the vascular region in the stem of *Hoya* (Esau 1977). The stem cortex (Fig. 5) and leaf (Fig. 6) mesophyll vascular bundle of the two species has the presence of laticifers, a series of connected cells that secretes latex, a fluid of complex composition. Laticifers have been known to be present to members of family Asclepiadaceae (Schweingruber *et al.* 2011).

The two hoyas have endarch type of xylem differentiation in the root (Fig. 4) and stem (Fig. 5) where the protoxylem occupies the innermost portion of the vascular bundle while the metaxylem occurs at the periphery. The stem has bicollateral vascular bundle wherein the secondary phloem occurs both on the outer and inner side of the xylem. The family Asclepiadaceae has internal phloem which was observed on their stem and leaf of the two *Hoya* species (Kloppenburger 2006),

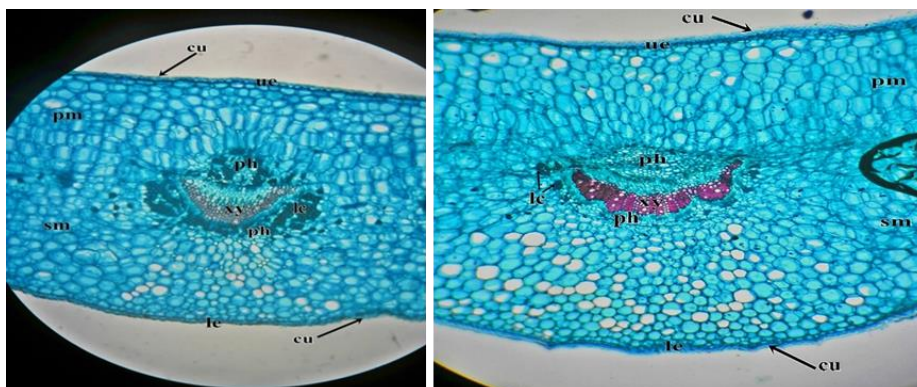


Figure 6. Transection of the leaf of *H. soligamiana* (left) and *H. incrassata* (right). [upper epidermis (ue), phloem (ph), xylem (xylem), laticifers (lc), palisade mesophyll (pm), spongy mesophyll (sm), lower epidermis (le) and cuticle layer (cu); Mag. 150x]

Through maceration technique, it was also observed that the stem of *H. soligamiana* and *H. incrassata* have annular tracheids where the secondary wall forms ring structure and spiral tracheids. Fiber and vessel elements with a simple perforation plate are both present in the macerated stem of the two *Hoya* species. The passageway of water in tracheids from cell to cell occurs mainly through pit pairs, in which pit membranes may be assumed to be highly penetrable to water and dissolved substances (O'Brein 1974). Also, in

vessel members, the water moves freely through perforation walls. The anatomy of the leaf of the two *Hoya* species (Fig. 6) revealed a uniseriate upper and lower epidermis which is made up of closely packed cells and covered with cuticle layer.

Cuticle layer contains cutin that covers the outer wall of the epidermis and serves to reduce loss of water (Esau 1997). The vascular system of the midrib is composed of a simple arc-shaped strand of xylem and phloem. They have amphicribal vascular bundle. The mesophyll of the leaf is composed primarily of a compact palisade layer composed of elongated, cylindrical and bigger cells. Cleared leaf of *H. incrassata* (Fig. 7) revealed the presence of cyclocytic stomata where the stomate's guard cells were surrounded by subsidiary cells forming one or two narrow rings that contain four or more number of ordinary epidermal cells which are confined to the abaxial surface.

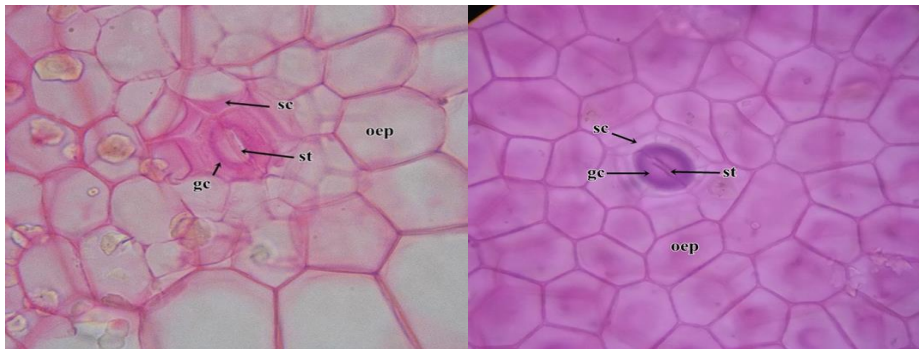


Fig 7. Cleared leaf of *H. soligamiana* (left) and *Hoya incrassata* (right) showing the actinocytic stomata and cyclocytic sunken stomata, respectively. [guard cell (gc), stoma (st), subsidiary cell (sc), and ordinary epidermal cell (oep); Mag. 600x]

The stomata of *H. incrassata* is sunken while *Hoya soligamiana* (Fig. 7) has actinocytic stomata on the same level of the lower epidermal cell. Sunken stomata is often an adaptation for low supply of available water (Esau 1977), which is often scarce for epiphytic plants like hoyas.

4 Conclusion

This study showed that the two *Hoya* species can be differentiated morphologically and anatomically despite of several shared characteristics. *Hoya incrassata* and *Hoya soligamiana* can be segregated based on the characters of the leaf and inflorescence. *H. soligamiana* has glabrous lanceolate plinerved leaves with whitish petals, purplish to pink inner and

outer coronal lobe and whitish to pink fused sepals. On the other hand, *H. incrassata* has coriaceous lance-ovate leaves, whitish to yellowish petals, yellow inner coronal lobe, reddish to orange outer coronal lobe and fused sepals. Anatomically, both species have almost similar anatomical architecture except on the type of stomata. *Hoya incrassata* has sunken cyclocytic stomata while *H. soligamiana* has actinocytic stomata. Morpho-anatomical information evidently provides taxonomic value for identifying and classifying the two *Hoya* species. Since taxonomic accuracy is vital for conservation initiatives, it is hope that more studies will be done on threatened and highly priced species which remains to be poorly studied.

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