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Comparative leaf morphology and anatomy on ten taxa of Calycanthaceae Lindl. (Laurales)

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Abstract

The comparative leaf morphology and anatomy of ten species of family Calycanthaceae have been studied. Leaf anatomy is very comparable to each other in cell shape and their arrangement. Collected leaves were preserved in FAA and alcohol series were applied for LM and SEM. The layer of epidermis is two in *Idiospermum* and one in rest of other genera. The structure of vascular bundle is V-shape in *Sinocalycanthus* and *Calycanthus* whereas U-shape in *Idiospermum* and *Chimonanthus*. The density of trichome is higher in *Calycanthus* than other genera. The presence of trichome, stomata, epidermal layer, density of trichome and stomata, and leaf surface are represented the distinction among the genera. The adaxial surface of *Idiospermum* and *Sinocalycanthus* are smooth whereas of *Calycanthus* and *Chimonanthus* are rough. The crystals are present in *Calycanthus*, *Sinocalycanthus* and *Chimonanthus* whereas absent in *Idiospermum*. The shape of the vascular bundle, density of trichome, epidermal layer, and crystals play important role in the phylogenetic relationship of Calycanthaceae.

Key words: Adaxial and abaxial surface, *Calycanthus*, Stomata, Trichomes, Vascular bundle

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Introduction

The Calycanthaceae are a small family of flowering plant under the order Laurales, inhabited in tropical and warm temperate regions and contains only ten known species with the debate in four or three genera (Cheng and Chang, 1964; Nicely, 1965; Blake, 1972; Renner, 1999, 2005; Zhou *et al.*, 2006; Paudel and Heo, 2018a, b, c; 2020a, b). Calycanthaceae are deciduous except *Chimonanthus nitens*, which is an evergreen species (Nicely, 1965). The family consists of famous ornamental small tree, used as medicine and foods, and are mainly distributed in Australia, North America, and China (Xu *et al.*, 2018). In the APG IV system, Calycanthaceae placed in the order Laurales (Chase *et al.*, 2016). Calycanthaceae are unique among Laurales due to differ from the ovule number and placentation in other Laurales, and the seed of *Idiospermum* is one of the largest embryo known in angiosperms (Blake, 1972). In Calycanthaceae, carpels have two ovules per carpel with collateral placentation, positioned one on top and other at an anthesis (Blake, 1972; Endress and Igersheim, 1997). The evolutionary trend of stomata in the three genera was recognized as the guard cell at polar region from having no T-shaped thickening (Li and Li, 2000). The developmental studies are on the gynoecium in *Calycanthus* (Erbar and Leins, 1983; Van Heel, 1984). There is no information about the gynoecium development of *Idiospermum* despite its extremely large seeds (Blake, 1972). Within the Calycanthaceae, authentic gynoecium features are unique to *Idiospermum* which the presence of only one

carpel (Worboys and Jackes, 2005). The morphology of *Idiospermum australiense* is distinctively different from that of the rest of the Calycanthaceae, especially in gynoecium morphology (Staedler *et al.,* 2009). Bennett (1950) found no evidence that the cortical system is a modification of lateral traces of a trilacunar alternatively, multilacunar nodal. The apparent lateral traces of trilacunar or multilacunar nodal are in case some dicot families. Eames (1961) believed that it was an additional independent system, which had four vas ular bundles of The leaves of ten species of Calycanthaceae were collected and fixed with FAA (Table 1). The serial section of 5-6 μm thickness using disposable knives stuck onto glass slides and dried on electrical slide warmer for 24 hrs. The dried slides stained with 0.1% Toluidine blue O for 60-90 sec, rinsed with running water, and dried again on the electrical warmer for more than 6 hrs to remove water. The stained slides were mounted with Entellan (Merck Co., Germany). The slides were observed under unilacunar structure of the primary cylinder. Bennett (1950) also indicated that transverse connections between the cortical strands, which are presented in the nodal region of *Calycanthus*, less well developed than *Chimonanthus*. Black (1972) noted the detail vegetative structure for *Idiospermum*. Furthermore, Wilson (1976) found that nodal anatomy and pattern of leaf trace are very similar between the Calycanthacae and Idiospermaceae.

Materials and Methods

Olympus BX50 light microscope (Olympus Co., Japan). Photographs were taken with digital camera system attached to the microscope, and the multiple image alignments were done using Photoshop CS6. For scanning electron microscopy, the pre-treatment was applied. The preserved leaf samples were passed through the ethyl-alcohol series, then immersed in 100% ethanol after that dried from the critical point dryer (CPD). SEM images were carried out from KBSI, Chuncheon at EHT=3.0kV.

Table 1. Collection information of ten species of Calycanthaceae used in the present study.

Taxa	Collection information
Calycanthus occidentalis HooK. & Arn.	Korea. Cultivated at Kangwon National University, K. Heo & N. Paudel *s.n*. 2016 (KWNU)
Chimonanthus fragrans (Loisel.) Lind.	Korea. Cultivated in Chollipo Arboretum, K. Heo *s.n.* 2009 (KWNU)
Chimonanthus luteus (G.Don) Biel.	Korea. Cultivated in Chollipo Arboretum, K. Heo *s.n.* 2009 (KWNU)
Chimonanthus nitens (Oliv.) Rehder	Korea. Cultivated in Chollipo Arboretum, K. Heo *s.n.* 2009 (KWNU)
Chimonanthus praecox (L.) Link	Korea. Cultivated at Kangwon National University, K. Heo & N. Paudel *s.n.* 2016 (KWNU)
Chimonanthus salicifolius S.Y. Hu	Korea. Cultivated in Chollipo Arboretum, K. Heo *s.n.* 2009 (KWNU)
Chimonanthus yunnanensis (W.W.Sm.) Hu	Korea. Cultivated in Chollipo Arboretum, K. Heo *s.n.* 2009 (KWNU)
Chimonanthus zhejingenensis M.C. Liu.	Korea. Cultivated in Chollipo Arboretum, K. Heo *s.n.* 2009 (KWNU)
Idiospermum australiense S.T. Blake	Australia. Central Coast, Cultivated in Royal Botanical Garden, Sydney, R.G. Coveny *s.n.* 1994 (KWNU)
Sinocalycanthus chinensis W.C.Cheng & S.Y.Chang	Korea. Cultivated at Kangwon National University, K. Heo & N. Paudel *s.n.* 2016 (KWNU)

Results and discussion

Trichome: Trichomes were frequently occurred on the mid rib and secondary vein (Fig. 1). They were unicellular and non-glandular (Figs. 1A-G). The density of trichome was lower in adaxial than that of the abaxial surface in *Calycanthus* (Fig. 1A). Trichomes were originated from the base and arranged horizontally in *Chimonanthus nitens* (Fig. 1D). They were rarely found in adaxial surface in *C. salicifolius* (Fig. 1F). In addition, the density and frequency of trichomes were different among the genera (Table 2). In *Chimonanthus zhenjingenensis*, *Idiospermum australiense* and

Adaxial surface

Sinocalycanthus chinensis, trichomes were not observed on adaxial surface (Figs. 1H-J).

Abaxial surface

Trichome: Trichomes *were* densely occurred on the dorsal and ventral veins (Fig. 2). The density of trichome was lower in *Sinocalycanthus* than that of *Calycanthus* (Fig. 2J). In *Calycanthus occidentalis*, trichomes were densely found on both abaxial and adaxial surfaces (Fig. 2A). In comparison, the density of the trichomes was lower on adaxial than that on abaxial surface. Trichome was found rarely

in adaxial surface in *Chimonanthus salicifolius* (Fig. 2F).

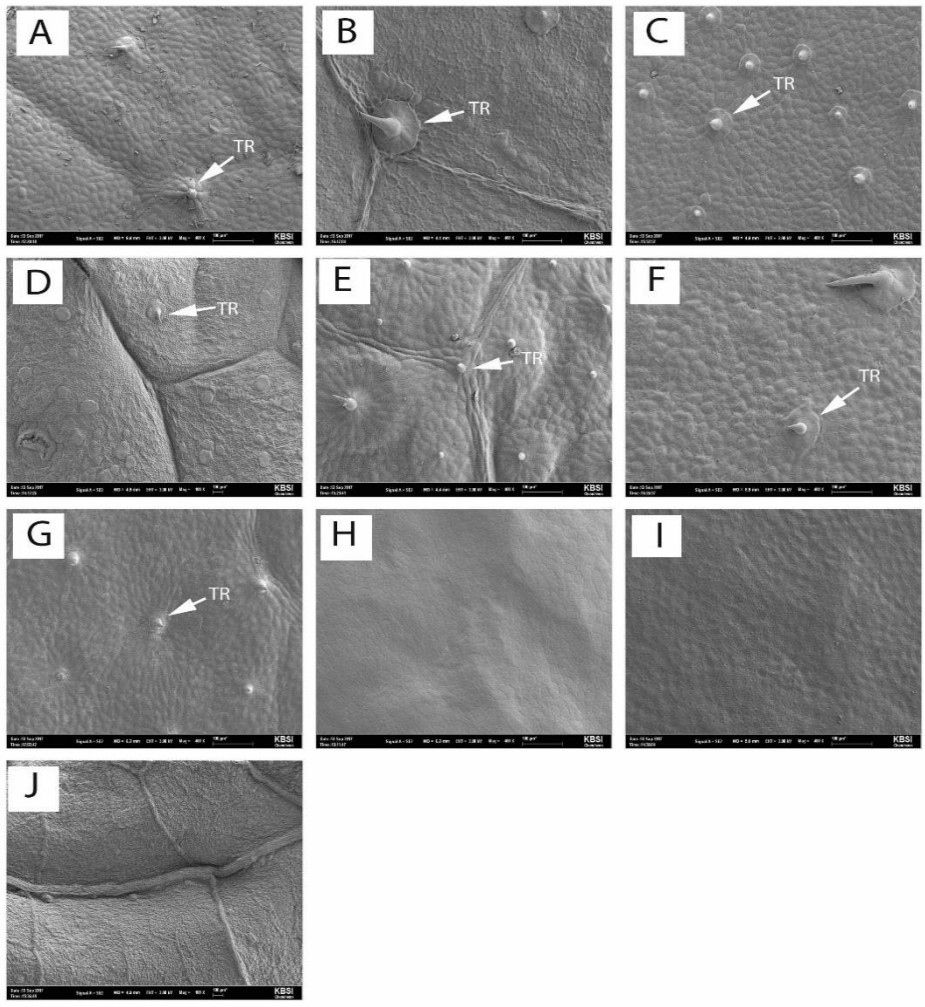

Figure 1. Trichome development on the adaxial leaf surface of Calycanthaceae: A. *Calycanthus occidentalis*, B. *Chimonanthus fragrans*, C. *C. luteus*, D. *C. nitens*, E. *C. praecox*, F. *C. salicifolius*, G. *C. yunnanensis*. H. *C. zhejiangensis*, I. *Idiospermum australiense*, J. *Sinocalycanthus chinensis*. (TR= trichome)

Table 2. Leaf morphology and anatomy of ten species of Calycanthaceae.

Taxa	Trichome		Shape of epidermal cell in CS	Epidermal cell surface	Shape of vascular bundle	Crystal
	Adaxial surface	Abaxial surface				
Calycanthus occidentalis	Present, rarely	Present, densely	Barrel shape	Irregular	V-shape	Absent
Chimonanthus fragrans	Present, rarely	Present, moderately	Barrel shape	Haphazardly irregular	U-shape	Present
Chimonanthus luteus	Present, densely	Present, densely	Rectangular	Hexagonal	U-shape	Present
Chimonanthus nitens	Present, densely	Present, densely	Barrel shape, rectangular	Irregular, rough	U-shape	Absent
Chimonanthu praecox	Present, densely	Present, densely	Rectangular	Hexagonal	U-shape	Present
Chimonanthus salicifolius	Present, densely	Present, moderately	Rectangular	Irregular, rough	U-shape	Present

Chimonanthus yunnanensis	Present, densely	Present, densely	Rectangular	Irregular, rough	U-shape	Absent
Chimonanthus zhejiangenensis	Absent	Present, moderately	Barrel shape, rounded	Irregular, smooth	U-shape	Absent
Idiospermum australiense	Absent	Present, rarely	Barrel shape, rectangular	Irregular, rough	U-shape	Absent
Sinocalycanthus chinensis	Absent	Present, moderately	Rectangular	Irregular	V-shape	Present

Stomata structure: The surface view of stomata showed the key differences among the four genera. In all genera, stomata were distributed in abaxial surface (Fig. 2). The deep seat and protect filament like special band cell were the different characters in *Calycanthus* occidentalis (Fig. 3A). The stomata are generally elongated shape in *Chimonanthus* species but rounded in *Calycanthus* and *Sinocalycanthus chinensis* (Fig. 2). In all species, stomata were paracytic (Figs. 2A-J). The density of stomata was the lowest in *Idiospermum* (Fig. 2J)

Leaf anatomy

Epidermis: The shape of epidermal cells was rectangular. The upper epidermis cell was thicker than lower epidermis. In all genera, the continuity of the epidermis was broken by the presence of stomata in abaxial side (Fig. 3). The shape and size of epidermal cells were varied in all genera. Adaxial and abaxial epidermis were barrel-shaped cells in *Calycanthus occidentalis*, *Chimonanthus fragrans*, *C. zhejiangensis*, and *Idiospermum australiense* (Figs. 3A-B, 3H-I). There were well-developed hypodermal layers in *Idiospermum* (Fig. 3I).

Mesophyll: The palisade was more developed on the adaxial side with radially elongated cells. The number of palisade layer was clearly shown in Calycanthaceae (Fig. 3). All species have single layered of palisade (Figs. 3A-J). Well-developed hypodermis is the distinguished feature of *Idiospermum australiense* (Figs. 3I, L). The spongy parenchyma organized with loosely arranged polygonal cells containing many chloroplasts in all genera. The spongy parenchyma is loosely arranged and occupied more space than the palisade. Crystal was observed in mesophyll in *Chimonanthus fragrans*, *C. luteus*, *C. praecox*, *C. salicifolius* and *Sinocalycanthus chinensis* (Figs. 3B-C, 3E-F, 3J-K).

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Vascular bundle: The vascular bundle was V-shape in *Calycanthus occidentalis* and *Sinocalycanthus chinensis* (Figs. 4A, 4J), whereas it was U-shaped in *Chimonanthus* and *Idiospermum* (Figs. 4B-I). The vascular bundles were scattered in spongy parenchyma (Fig. 4). *C. zhejiangensis* was highly differentiated from the other species and detected the closest relationship with *Chimonanthus salicifolius*, which possibly supported that *C. zhejiangensis* was a distinct species rather than the *C. nitens*. Besides, *C. praecox* was much more closely related with *C. campanulatus* than the other species moreover extensive genetic differentiation existed among *C. praecox* (Zhou *et al.*, 2006). In results, the leaf anatomy is different for the genera of Calycanthaceae. Many researchers focused on their description of the plant for molecular phylogeny from the molecular data (Renner 1999; Qui et al., 2005). Cuticle morphology and cross section of leaf are strongly represented similarities and dissimilarities characters for phylogeny of Calycanthaceae. The floral structure and floral architecture in Calycanthaceae were studied (Staedler *et al.*, 2007, 2009). The presence of well-developed vascular bundle is in *Chimonanthus*

praecox, C. nitens, C. fragrans, C. salicifolius, C. occidentalis. *Chimonanthus zhejiangensis* is unique to the other without a trichome. Stomata size and frequency, the epidermal cell structure, the trichome type and distribution pattern are the diagnostic treatment for the taxa (Oak *et al.*, 2018). The cuticle ornamentation was striate, undulate striate, favolate, granular with cavities and the stomata are the paracytic type (Ruohui *et al.*, 1993).

A vascular bundle appears as the continuous ring of primary xylem and primary phloem. In our results, primary xylem and primary phloem for *Sinocalycanthus chinensis*, *Calycanthus occidentalis* and *Chimonanthus fragrans, C. nitens, C. salicifolius*, *C. yunnanensis*, *C. luteus*, *C. zhejiangenensis*, *C. praecox* were advanced and arranged in compact formed. The bundle sheath and trichome are the characteristics of *Calycanthus zhejiangensis, C. yunnanensis,* and *C. luteus*.

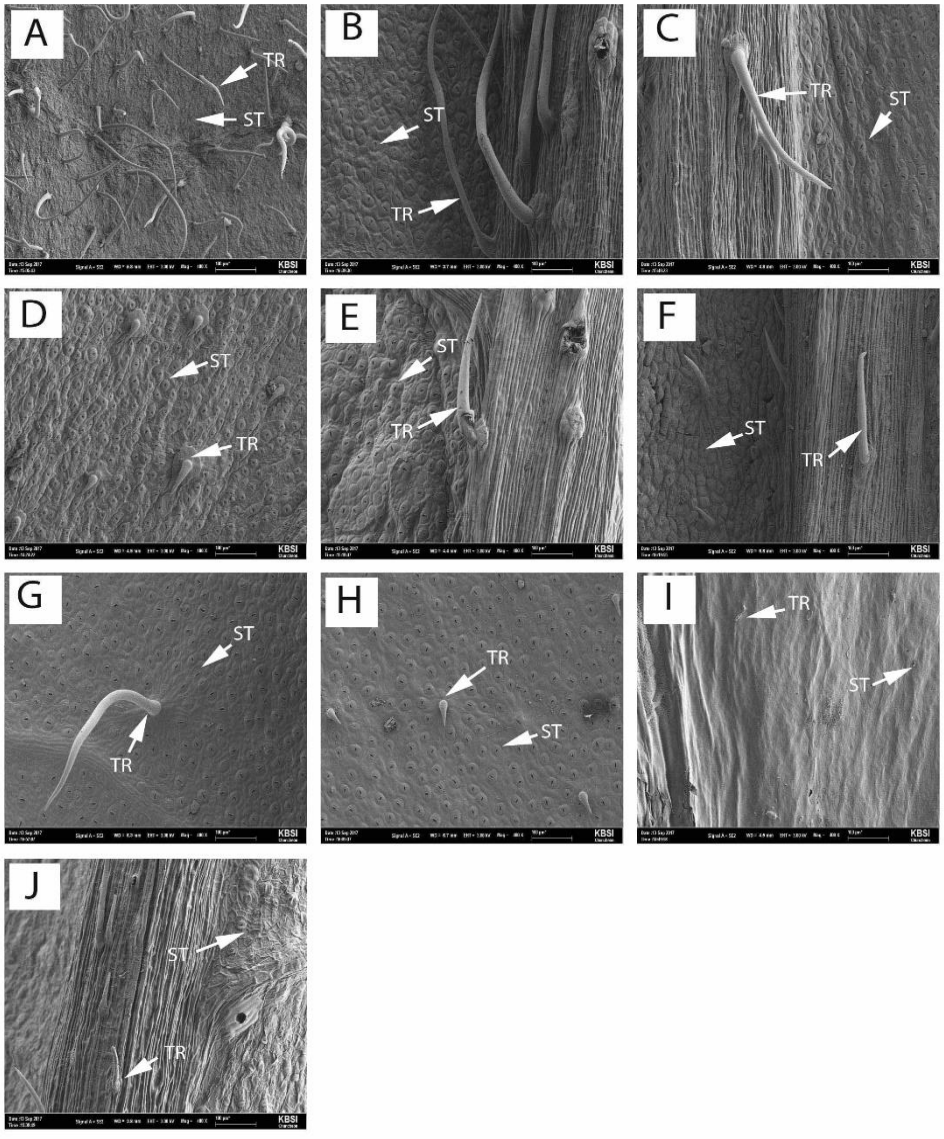

Figure 2. Trichome and stomata on the abaxial surface of Calycanthaceae: A. *Calycanthus occidentalis*, B. *Chimonanthus fragrans*. C. *C. luteus*. D. *C. nitens*, E. *C. praecox*, F. *C. salicifolius*, G. *C. yunnanensis*, H. *C. zhejiangensis*, I. *Idiospermum australiense*, J. *Sinocalycanthus chinensis*. (TR= trichome, ST= stomata)

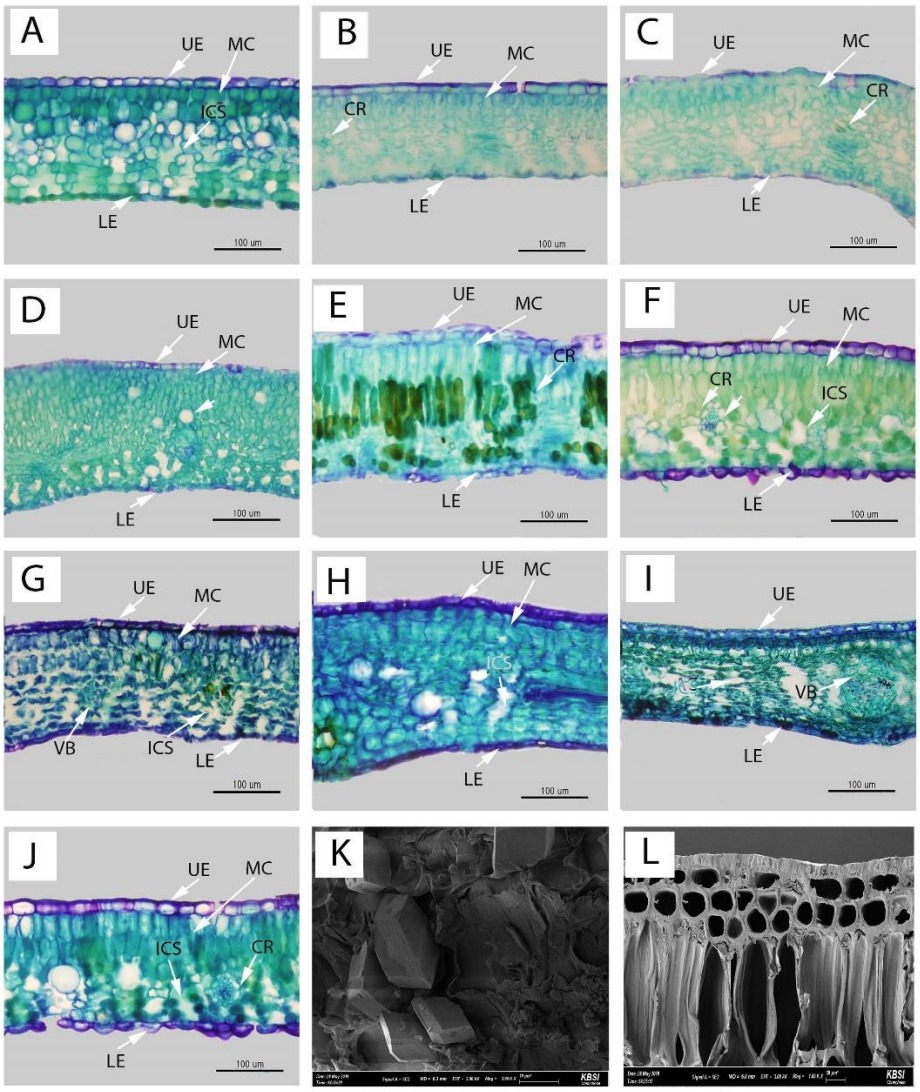

Figure 3. Cross section of leaf blade of Calycanthaceae: A. *Calycanthus occidentalis*, B. *Chimonanthus fragrans*, C. *C. luteus*, D. *C. nitens*, E & K. *C. praecox*, F. *C. salicifolius*, G. *C. yunnanensis*, H. *C. zhejiangensis*, I & L. *Idiospermum australiense*, J. *Sinocalycanthus chinensis*. (UE= upper epidermis, LE= lower epidermis, MC= mesophyll cell, ICS= inter-cellular space, CR= crystal, HP= hypodermis)

Present results are supporting for the paracytic stomata and granular trichome. Stomata density, chloroplast density in palisade and spongy parenchyma were indistinct which are the characteristic features (Leroy *et al.*, 2008). Those characters are similar in case of Calycanthaceae. The present results noted that those characters are represented in the Calycanthaceae. The type of the trichome is unicellular with variable length (Nicely, 1965). The stomata are only on the lower surface as rubiaceous accompanied with either side of more subsidiary cells parallel to the long axis pore of the guard cells (Metcalfe and Chalk, 1950). In all genera, stomata are observed as paracytic. The leaf of the Calycanthaceae has great taxonomic value for the anatomical and morphological point of view. The results are for the density of trichome, stomata character between adaxial and abaxial surfaces, epidermal surface shape. Additionally, structure of vascular bundle, mesophyll tissue layer, spongy mesophyll, and bundle sheath extension are the characters for the phylogeny of Calycanthaceae.

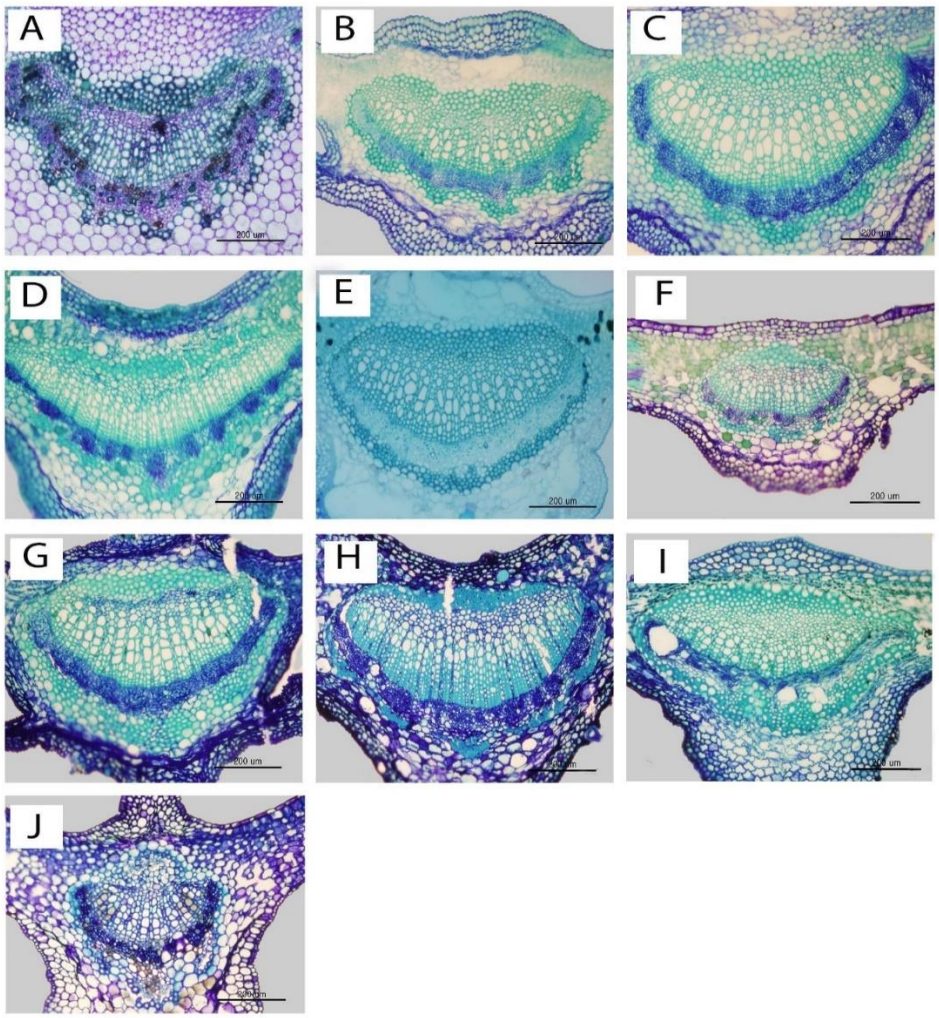

Figure 4. Shape of vascular bundle in mid rib of Calycanthaceae: A. *Calycanthus occidentalis*, B. *Chimonanthus fragrans*, C. *C. luteus*, D. *C. nitens*, E. *C. praecox*, F. *C. salicifolius*, G. *C. yunnanensis*, H. *C. zhejiangensis*, I. *Idiospermum australiense*, J. *Sinocalycanthus chinensis*

The result shows that the density of trichome in abaxial surface is high in *Calycanthus occidentalis* in compare to the *Chimonanthus praecox, C. nitens, C. fragrans, C. salicifolius, C. zhejingenensis*, *C. yunnanensis*, and *C. luteus*. On the other hand, *Idiospermum australiense* trichome is not presented in the abaxial surface.

The stomata density in *Sinocalycanthus* is similar as in *Calycanthus* but, in *Chimonanthus*, it is comparatively slightly lower. Also, noted that the upper epidermal layer is smooth in *Idiospermum australiense* whereas rough in *Sinocalycanthus* and *Chimonanthus* except *C. zhejiangensis.* In anatomical features, result shows there was well-developed hypodermis in *Idiospermum*. The morphological and anatomical characters of the leaves treated for the proper placement of the genus. *Sinocalycanthus* and *Calycanthus* are similar as the following characteristics; epidermis cell is paranchymatous, elongated subsidiary cells; but in *Idiospermum* and *Chimonanthus* epidermal cells are irregular with paranchymatous, and V-shaped vascular bundle. In addition, *Chimonanthus* was rounded stomata with U-shaped vascular bundle in mid rib, which is the common character of all species. In *Idiospermum,* subsidiary cell is not distinguished. The characteristic features of the leaf morphology and anatomy are important for phylogeny of Calycanthaceae.

Conclusion

The presented comparative leaf anatomy and morphology of small family Calycanthacae is very comparable to each other in cell shape and their arrangement. The vascular bundle is noted U and V shaped. Furthermore, the distinct characteristic is between the trichome, stomata, epidermal layer, density of trichome and stomata. The adaxial surface is also notable characteristic for the taxonomy point of view. The crystals are the unique remarkable characteristic for the taxonomy of Calycanthacae. All characters presented here on anatomy and morphology of leaf are remarkable for the phylogenetic relationship of the Calycanthaceae.

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