Floral Structure and Gynostemium Ultrastructural Features of Two Habenaria Species and Their Taxonomic Significance: Postprint

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Date: 2023-07-13T00:00:00+00:00

Abstract

The floral morphology of Habenaria exhibits considerable variation, presenting complex taxonomic challenges. To identify key taxonomic characters for this genus, this study examined Habenaria dentata and Habenaria glaucifolia using stereomicroscope and scanning electron microscope to observe and compare their floral structures and gynostemium ultrastructural features. The results demonstrated: (1) Significant differences in floral structure between the two species, evident in the presence/absence of lateral petal lobes, morphology of the three labellum lobes, and spur-to-ovary length ratio. (2) Marked interspecific differences in gynostemium ultrastructure, manifested in the morphology of the staminode, anther chamber wall cells, 'anther-tube' wall cells, 'stigmatic branch', and papillae at its junction with the style. (3) Divergent pollinium exine ornamentation, with H. dentata displaying reticulate ornamentation versus bacculate→reticulate ornamentation in H. glaucifolia. This study preliminarily proposes that presence/absence of lateral petal lobes, spur morphology, spur-to-ovary length ratio, and pollen exine ornamentation type constitute key taxonomic characters for species delimitation in Habenaria. Building upon previous research, this paper supplements the floral structural and gynostemium ultrastructural data for H. glaucifolia, and does not support its segregation from the genus Habenaria. These findings provide novel data for the classification of Asian Habenaria species.

Full Text

Floral Structure and Column Ultrastructure of Two *Habenaria* Species with Their Taxonomic Implications

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Abstract: The genus *Habenaria* exhibits highly diverse floral morphology and presents complex taxonomic challenges. To identify key taxonomic features for this genus, we investigated the floral structure and column ultrastructure of *Habenaria dentata* and *Habenaria glaucifolia* using stereomicroscopy and scanning electron microscopy (SEM). Our results reveal: (1) Significant differences in floral architecture between the two species, specifically in the presence or absence of lateral petal lobes, the morphology of the three labellum lobes, and the spur-to-ovary length ratio. (2) Distinct column ultramicroscopic characteristics, including variations in staminode morphology, anther chamber exine cells, "anther-canal" exine cells, and the papillae structure of the elongated stigma and its junction with the style. (3) Divergent pollen massula exine sculptures, with *H. dentata* displaying reticulate ornamentation and *H. glaucifolia* showing a baculate→reticulate transition pattern. We propose that the presence/absence of lateral petal lobes, spur morphology, spur-to-ovary length ratio, and pollen exine sculpture type represent critical diagnostic characters for species delimitation in *Habenaria*. Building upon previous research, this study provides supplementary analysis of the floral structure and column ultrastructure of *H. glaucifolia* and does not support its recognition as a genus separate from *Habenaria*. These findings offer new data for the classification of Asian *Habenaria* species.

Key words: *Habenaria*, floral structure, column, elongated stigma, exine sculpture, scanning electron microscope

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Habenaria Willd. is the largest terrestrial orchid group within the subfamily Orchidoideae (family Orchidaceae), comprising approximately 876 species distributed across temperate regions of the Northern Hemisphere and tropical areas of both the Old and New Worlds. The genus reaches its greatest diversity in central and southern Africa and eastern Asia, which represent major centers of species richness (Dressler, 1993; Pridgeon et al., 2001). China harbors 58 species, including 22 endemics, concentrated primarily in the mountainous southwestern region, particularly the Hengduan Mountains (Lang et al., 1999). *Habenaria* species are perennial herbs with well-developed tubers; they bear terminal racemes with resupinate flowers, where the dorsal sepal and lateral petals often converge to form a hood. The petals are typically trilobed, and a spur may be present or absent. The column is short with two staminodes; the anther is bilocular, containing a pair of loosely arranged pollinia, each with a narrow caudicle and a naked viscidium. The stigma is prominently raised and elongated into two "stigma arms," while the rostellum is well-developed and extends to form an "anther-canal" that adheres to the caudicle (Dressler, 1993; Lang et al., 1999; Pridgeon et al., 2001).

Floral characters such as petal lobing, spur presence, labellum division, connective width, pollinia morphology, "anther-canal" structure, and stigma arm configuration have traditionally served as taxonomic criteria for *Habenaria* and its relatives (Dressler, 1981; Singer, 2001; Pedron et al., 2012; Suetsugu & Tanaka, 2014; Ikeuchi et al., 2015). However, the genus's extensive geographic distribution, high species diversity, and complex floral variation have generated considerable controversy regarding its systematic position, generic boundaries, and infrageneric classification (Dressler & Dodson, 1960; Bateman et al., 2003, 2009; Inda et al., 2012; Batista et al., 2013).

Traditionally, *Habenaria* has been placed in subtribe Habenariinae, which together with subtribe Orchidinae constitutes tribe Orchideae (Dressler, 1993; Pridgeon et al., 2001). These two subtribes exhibit markedly different stigma structures: Orchidinae possess a single, unforked, concave stigmatic cavity, whereas Habenariinae have two protruding, elongated stigma arms. This classification has been contentious, however, lacking support from both morphological and molecular evidence (Kurzweil & Weber, 1991, 1992; Pridgeon et al., 2001; Batista et al., 2013; Ngugi et al., 2020; Rewicz et al., 2022). Furthermore, the elongated stigma arm character is not exclusive to *Habenaria* but also occurs in other Habenariinae genera, including *Herminium*, *Peristylus*, *Gymnadenia*, and *Neottianthe*, making it inadequate for delimiting *Habenaria* (Kurzweil & Weber, 1992; Pridgeon et al., 2001). Current distinctions between *Habenaria* and its relatives rely primarily on whether the bifid petals are fused with other floral structures, the degree of labellum division, the absence of calli, and whether stigmas are entire (Pridgeon et al., 2001; Lang et al., 1999; Chen et al., 2009). Several species formerly elevated to independent genera have since been synonymized under *Habenaria* (POWO, 2023), including *Podandriella*, *Pseudoperistylus*, *Centrostigma*, *Platycoryne*, and *Roeperocharis*. Based on floral morphology, these are more appropriately treated as morphologically distinct sections within *Habenaria* (Kurzweil & Weber, 1992). Conversely, some taxa previously included in *Habenaria*, such as *Platanthera* and *Coeloglossum*, have been removed and recognized as separate genera (Dressler, 1993). For instance, *Habenaria bakeriana* was transferred to *Platanthera* as *P. bakeriana* based on its lack of an "anther-canal" and its unlobed, ligulate labellum—a move strongly supported by molecular phylogenetic evidence (Bateman et al., 2009). Similarly, *Habenaria purpureopunctata* was reclassified from the newly erected genus *Hemipiliopsis* (Luo & Chen, 2003; Luo et al., 2005) to *Hemipilia purpureopunctata* based on molecular data (Jin et al., 2014). Current molecular studies indicate that *Habenaria*, like most genera in subtribe Orchidinae, is non-monophyletic, forming two distantly related clades with other allied genera as sister to subtribe Orchidinae, necessitating additional evidence to clarify its classification (Batista et al., 2013; Jin et al., 2014; Ngugi et al., 2020; Rewicz et al., 2022).

Floral structural morphology and ultramicroscopic characters play crucial roles in delimiting subfamilies, tribes, and genera within Orchidaceae (Dressler, 1981, 1986; Freudenstein & Rasmussen, 1997, 1999; Bateman & Rudall, 2006; Singer et al., 2007). These features—including petals, labellum, column, anther, pollen, anther cap, and stigma—have been studied across Apostasioideae and Cypripedioideae (Newton & Williams, 1978; Burns-Balogh & Hesse, 1988), subtribe Orchidinae (Kurzweil & Weber, 1991; Luo & Chen, 2003; Luo et al., 2005; Lumaga et al., 2006; Bell et al., 2009; Passarelli & Rolleri, 2010), and genera such as *Bulbophyllum* (Nunes et al., 2014, 2017) and *Dendrobium* (Wang et al., 2021a, 2021b). Early investigations of floral morphology and column ultrastructure in African Orchidinae and Habenariinae suggested that characters such as erect anthers and elongated stigma arms overlap between *Habenaria* and related genera, limiting their utility for subtribal delimitation (Kurzweil & Weber, 1991, 1992). Studies on pollen ultrastructure in South American *Habenaria* species using SEM have demonstrated significant interspecific variation in pollinium morphology, exine sculpture, tetrad arrangement, and adhesive threads, highlighting the taxonomic potential of these features (Passarelli & Rolleri, 2010; Liu, 2015, 2016).

Habenaria glaucifolia is a Chinese endemic terrestrial orchid restricted to Shaanxi, Gansu, Sichuan, Guizhou, Yunnan, and Tibet, growing in forest understories, thickets, and grasslands at elevations of 2,000–4,300 m (Lang et al., 1999). Its distinctive floral morphology includes a trilobed labellum with linear, revolute lobes, a broadly horseshoe- or U-shaped connective, and two elongated, lanceolate stigma arms. Based on these features, the species was once assigned to the independent genus *Senghasiella* (Szlachetko, 2001). However, ontogenetic studies of column development have retained *H. glaucifolia* within *Habenaria*, interpreting its unique floral morphology as a derived condition within the genus's evolutionary trajectory (Luo et al., 2005). *Habenaria dentata* is widely distributed across tropical and subtropical regions of southeastern Asia, occurring in forest understories and along streambanks at 190–2,300 m throughout southern China (Lang et al., 1999). Its tubers contain bioactive compounds and are used in traditional Chinese medicine (Chen, 2004). Historically placed in *Orchis* (Grefing et al., 1800) and *Platanthera* (Lindley, 1835), the species has since been reinstated in *Habenaria* (Govaerts, 2003). Given the limited ultrastructural data on Asian *Habenaria* species (Luo et al., 2005; Liu, 2015, 2016), this study examines Chinese populations of *H. dentata* and *H. glaucifolia* using light microscopy and SEM to compare their floral structure, column morphology, and pollinium ultrastructure. We address two primary questions: (1) Which floral characters serve as key taxonomic features within the genus? (2) What is the taxonomic significance of column and pollinium ultrastructure in *Habenaria*?

Materials and Methods

Plant material was collected in Yunnan Province, China: *Habenaria dentata* from Wuliang Mountain, Jingdong County, and *Habenaria glaucifolia* from Yulong Snow Mountain, Lijiang City, during 2020–2021. Fresh flowers were dissected and photographed under a stereomicroscope (Leica M165 FC) to document mature floral morphology. Additional fresh material was fixed in 50% ethanol for 24 h, then transferred to 70% ethanol for long-term storage at room temperature. Fixed anthers and stigmas were isolated under a stereomicroscope, dehydrated through an ethanol gradient, and critical-point dried using liquid CO_2. Dried anthers were pierced with dissecting needles to release pollen. Samples were mounted on conductive adhesive tape, sputter-coated with platinum, and examined under a scanning electron microscope (ZEISS Sigma 300). Images were processed using Adobe Photoshop CC 2019. Orchid structural terminology follows *The Manual of Chinese Orchid Identification* (Chen & Cribb, 2009), and pollen ultrastructure descriptions follow *Pollen Flora of Seed Plants* (Wei, 2003).

Results

2.1 Comparison of Floral Structure Between the Two *Habenaria* Species

Under the stereomicroscope, *Habenaria dentata* displays racemes 5–12 cm long, glabrous, with white flowers (Plate 1:1). The dorsal sepal and petals form a hood, while lateral sepals are reflexed (Plate 1:3). Lateral petals are entire, lanceolate (Plate 1:7). The labellum is shallowly trilobed, with a ligulate-lanceolate mid-lobe and subrhombic lateral lobes bearing serrated margins (Plate 1:5). The spur is markedly longer than the ovary (Plate 1:9). The erect anther bears one staminode on each side (Plate 2:5). Two locules each contain a granular pollinium with a slender caudicle and naked viscidium (Plate 2:7). The locule base extends into a narrow "anther-canal" enveloping the caudicle (Plate 2:1,2,5). Two stigma arms arise basally on the column and remain separate (Plate 2:1,2).

Habenaria glaucifolia produces racemes 5–20 cm long, pubescent, with greenish-white flowers (Plate 1:2). The dorsal sepal and petals also form a hood, with reflexed lateral sepals (Plate 1:4). Lateral petals are deeply bifid: the upper lobe equals the dorsal sepal in length and is spatulate-oblong, while the lower lobe is lanceolate and much smaller (Plate 1:8). The labellum is deeply trilobed above the base, with a linear mid-lobe slightly broader than the lateral lobes, which are coiled apically (Plate 1:6). The spur is subequal in length to the ovary (Plate 1:10). The erect anther bears one staminode per side (Plate 2:6). Two locules each contain a granular pollinium with a slender caudicle and naked viscidium (Plate 2:8). An "anther-canal" also envelops the caudicle. Two stigma arms arise basally but are fused into a tongue-like structure (Plate 2:3,4).

2.2 Comparison of Column Ultrastructure Between the Two Species

SEM examination reveals similar anther ultrastructure in both species: a prominent auriculate staminode adaxial to each anther chamber; tightly arranged anther wall cells with distinct intercellular boundaries; and quadrangular, regularly arranged exine cells on the anther-canal surface (Plate 3:5,6). However, significant differences occur in staminode shape and wall cell morphology. *Habenaria dentata* possesses club-shaped staminodes (Plate 3:1), whereas *H. glaucifolia* has triangular staminodes (Plate 3:2). Anther wall cells are more regular in *H. dentata*, bearing dense wavy striations, while those of *H. glaucifolia* appear relatively smooth with surface protrusions (Plate 3:3,4). The anther-canal exine cells of *H. dentata* exhibit rugulate ornamentation, contrasting with the smoother surface in *H. glaucifolia* (Plate 3:7,8).

Both species possess elongated stigma arms with papillae and receptive regions. In *H. dentata*, the receptive area occurs along the thickened, smooth stigma margin (Plate 4:1,3). Papillae at the stigma-style junction differ between species: *H. dentata* has smaller papillae with densely arranged wavy striations (Plate 4:5,7), while *H. glaucifolia* exhibits larger papillae with coarser, ridge-like striations (Plate 4:6,8).

2.3 Comparison of Pollinium Ultrastructure

Pollinia in both species are subquadrangular or flattened-cuneate (Plate 5:1,2), with surface layers of tectum or residual tectum and filamentous material (Plate 5:3,4). In *H. dentata*, a central septum divides each locule, with tightly packed, erect pollinia on either side (Plate 5:1). Transverse sections reveal bicellular pollen with generative and vegetative cells (Plate 5:7). *Habenaria glaucifolia* shows more distinct tetrad arrangements, predominantly tetrahedral and cruciate, with occasional T-shaped tetrads (Plate 5:2).

The primary difference lies in exine sculpture. *Habenaria dentata* displays irregular reticulate ornamentation with prominent muri, irregular lumina, and granular sculpturing within lumina (Plate 5:5). *Habenaria glaucifolia* exhibits two exine types: reticulate sculpture at the pollinium ends with high muri, variable lumina, and granular infilling; and short-baculate sculpture centrally (Plate 5:6,8).

Discussion

3.1 Floral Structural Characters and Taxonomic Significance

Lateral petal morphology, labellum configuration, and spur characteristics show marked interspecific variation in *Habenaria* and constitute key features for species identification (Lang et al., 1999; Pridgeon et al., 2001; Chen et al., 2009). Both study species share typical *Habenaria* traits—paired pollinia, elongated caudicles, naked viscidia, and two stigma arms—but differ significantly in labellum, lateral petal, and spur morphology.

The presence or absence of lateral petal lobes represents an important taxonomic character. Most *Habenaria* species have bifid petals whose anterior lobes often fuse with rostellum side lobes and the labellum (Kurzweil & Weber, 1992). This yields two morphological types: entire lateral petals (e.g., *H. delavayi*, *H. diplonema*) and bifid lateral petals (e.g., *H. aitchisonii*, *H. wolongensis*) (Lang et al., 1999; Chen et al., 2009). Our study confirms this distinction, with *H.*

dentata having entire petals and *H. glaucifolia* bearing bifid petals, underscoring the taxonomic value of this character.

Labellum lobing also serves as a diagnostic feature. While most *Habenaria* species possess trilobed labella with deeply divided filiform or lanceolate segments, some taxa and related genera like *Platycoryne* have entire labella; quadrilobed or quinquelobed labella are rare, occurring only in the allied genus *Cynorkis* (Pridgeon et al., 2001; Chen et al., 2009). For example, *H. idua* differs from *H. tonkinensis* primarily in its green labellum with distinctly upturned lateral lobes (Zhang et al., 2015). Although both study species have trilobed labella, their lobe morphology differs substantially: *H. dentata* has a ligulate-lanceolate mid-lobe and subrhombic, serrate lateral lobes, whereas *H. glaucifolia* has a linear mid-lobe and apically coiled lateral lobes. These differences warrant careful taxonomic consideration.

Spur morphology also varies considerably. In most *Habenaria* species, the labellum base fuses with the column to form a spur, typically slender and elongate with a tapered or clavate apex. This trait reaches an extreme in *H. cataphysema*, whose spur apex forms a massive spherical structure (Williamson, 1977). While most species have straight or slightly curved spurs, some exhibit geniculate bending (e.g., *H. tentaculigera*) or spiral twisting, and *H. anaphysema* uniquely forms a ring-shaped spur (Kurzweil & Weber, 1992). Both study species have subclavate spurs, but differ in details: *H. dentata* has an apically enlarged spur equal in width to the spur mouth, while *H. glaucifolia* shows a medially bent spur with a narrower apex. Furthermore, the spur-to-ovary length ratio varies significantly among species, allowing classification into three types: spur much longer than ovary (e.g., *H. commelinifolia*, *H. davidii*), spur subequal to ovary (e.g., *H. wolongensis*, *H. schindleri*), and spur shorter than ovary (e.g., *H. siamensis*, *H. pectinata*) (Lang et al., 1999; Chen et al., 2009). In our study, *H. dentata* has a spur longer than the ovary, while *H. glaucifolia* shows subequal lengths, confirming the taxonomic importance of spur characters.

3.2 Column Ultrastructure and Taxonomic Significance

Compared to the more primitive subfamilies Apostasioideae and Cypripedioideae, which possess two or three fertile anthers and lack a rostellum, Orchidoideae represents a more derived lineage characterized by one fertile anther, two staminodes, and a well-developed rostellum (Dressler & Dodson, 1960).

Our observations confirm that both study species possess a single fertile anther with lateral staminodes and an anther-canal enveloping the caudicle, placing them in a relatively derived group. However, they differ in staminode shape and anther-canal cell morphology: *H. dentata* has club-shaped staminodes and rugulate anther-canal cells, whereas *H. glaucifolia* has triangular staminodes and smoother canal cells. These features merit further investigation.

Both species exhibit elongated stigma arms with papillae and receptive regions.

Within Orchidaceae, only Orchidinae genera such as *Habenaria*, *Platanthera*, and *Herminium* possess two stigma arms, but *Habenaria* shows particularly prominent elongation (Dressler & Dodson, 1960; Dressler, 1993; Chase et al., 2015). While the column is typically glabrous, exceptions exist (e.g., *H. hirsutitrunci*; Williamson, 1980). Our study found no conspicuous trichomes on either species, but documented differences in stigma arm fusion (*H. dentata*: separate; *H. glaucifolia*: fused) and papilla size/surface ornamentation, consistent with previous SEM work on *H. glaucifolia* (Luo et al., 2005). The receptive region in *H. dentata* occurs along the thickened stigma margin, matching observations in *H. glaucifolia* and *Platanthera chlorantha* (Stpiczyn, 2003; Luo et al., 2005). In some species, receptive areas appear as extended, flattened lobes on the stigma arms (Kurzweil & Weber, 1992). These significant column differences underscore the importance of ultrastructural characters for species delimitation in *Habenaria*.

3.3 Pollen Exine Sculpture and Taxonomic Significance

Pollen sculpture correlates with pollination syndrome: anemophilous pollen tends to be smooth, while entomophilous pollen is typically ornate. *Habenaria* species are specialized entomophilous orchids with complex floral structures and correspondingly intricate pollen exine patterns (Singer, 2001; Suetsugu & Tanaka, 2014; Xiong et al., 2015).

Within the five-subfamily classification of Orchidaceae (Chase et al., 2015), Apostasioideae species such as *Apostasia wallichii* and *Neuwiedia zollingeri* var. *singapureana* have reticulate exine (Newton & Williams, 1978). Most Cypripedioideae lack complex sculptures, with *Cypripedium* and *Paphiopedilum* showing smooth, unsculptured pollen (Williams & Broome, 1976). In Epidendroideae, *Cephalanthera* exhibits three sculpture types (smooth, rugulate, baculate), *Limodorum* shows rugulate-baculate patterns, and *Aphyllorchis* and *Epipactis* are predominantly reticulate (Ackerman & Williams, 1980). Orchidoideae displays rich diversity: *Anacamptis* has smooth-rugulate or rugulate-striate patterns; *Dactylorhiza* shows smooth, smooth-rugulate, and reticulate types; and *Orchis* exhibits four types (smooth, reticulate, perforate-striate, baculate) (Lumaga et al., 2006).

Habenaria pollen sculpture is even more diverse, including smooth, rugulate, striate, baculate (e.g., *H. schindleri*), verrucate, and reticulate types, with numerous transitional forms (Schill & Pfeiffer, 1977; Liu, 2015, 2016; Hesse & Burns-Balogh, 1984; Lumaga et al., 2006). Phylogenetic trends in Orchidinae suggest two evolutionary pathways: (1) smooth → rugulate/striate (in clades comprising *Anacamptis*, *Neotinea*, *Ophrys*, and *Serapias*), and (2) smooth → baculate → reticulate (in clades including *Chamorchis*, *Dactylorhiza*, *Gymnadenia*, *Orchis*, and *Platanthera*) (Lumaga et al., 2006). Our study shows *H. dentata* with reticulate sculpture and *H. glaucifolia* with a baculate→reticulate transition, suggesting the latter represents a more derived condition within Orchidinae.

In summary, the presence/absence of lateral petal lobes, labellum lobe morphology, spur characters, spur-to-ovary ratio, and pollen exine sculpture type exhibit species-level variation suitable for infrageneric classification in *Habenaria*. Additionally, *H. dentata* and *H. glaucifolia* share several traits consistent with *Habenaria*—including convergent dorsal sepals and lateral petals, protruding stigmas, receptive region position, staminode placement, granular pollinia, and exine patterns—providing no support for recognizing *H. glaucifolia* as a separate genus (Szlachetko, 2001; Luo et al., 2005).

Acknowledgments

We thank the Yunnan Biodiversity Research Institute of Southwest Forestry University for providing experimental facilities, and Mr. Xu Gu for technical assistance. We also acknowledge the Plant Multidimensional Imaging and Diversity Analysis Platform of the Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, for SEM services, and Ms. Zhijia Gu for her help with electron microscopy.

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