

Integrative Morphological and Molecular Insights into *Rhynchostegium celebicum* (Brachytheciaceae), a moss from the Eastern Himalaya

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Research Article

Keywords: Bryophyta, Darjeeling Hills, *Rhynchostegium celebicum*, SEM, rbcL, Molecular Phylogeny

Posted Date: January 13th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-8194045/v1>

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Additional Declarations: No competing interests reported.

Abstract

This study provides a detailed morphological, ultrastructural, and phylogenetic analysis of the moss *Rhynchostegium celebicum* (Brachytheciaceae), from Darjeeling Hills, a key region within the Eastern Himalayan biodiversity hotspot. Scanning Electron Microscopy (SEM) was the first to document the sporophytic ultrastructure, revealing fine papillose spores and distinct ornamentation on the peristome teeth, indicating differential hygroscopic roles. Molecular phylogenetic analysis employing the plastid *rbcL* sequence strongly supported a monophyletic *Rhynchostegium* clade and showed a close relationship between *R. celebicum* and *R. megapolitanum*. This study substantiates the identity and phylogenetic placement of the species, providing essential insights into the biogeographic distribution and evolutionary relationships within *Rhynchostegium* in the region.

Introduction

Darjeeling, situated in the northern part of West Bengal, lies within the Eastern Himalayan biodiversity hotspot and is recognized as one of India's richest repositories of flora and fauna. This region has notably abundant unique and endemic plant species, including bryophytes, owing to its distinctive climatic conditions [1]. Several new taxa have been reported over the past few decades, primarily based on their morphological architecture [2–7].

Globally, approximately 221 species of *Rhynchostegium* Schimper have been documented (www.worldfloraonline.org). These species belong to the family Brachytheciaceae and constitute one of the three largest genera in this family [8]. Among these, 140 species are accepted (<https://www.bryonames.org/>), with only 11 species reported in India [3]. Among mosses, the family Brachytheciaceae is the second-largest pleurocarpous group in India, comprising 84 species belonging to 10 genera [3]. The genus *Rhynchostegium* Schimper is widespread in the Himalayan region [9, 10]. The introduction of new species and the presence of existing species in novel locations are common occurrences within this genus [11, 12]. One of its species, *Rhynchostegium celebicum* (Sande Lac.) A. Jaeger, is broadly distributed in tropical to subtropical, often mountainous or forested regions across Southeast Asia and the Pacific Islands, including various phytogeographic zones of the Indian subcontinent [2, 13, 14, 15], and has recently been added to the flora of the Darjeeling Hills [9]. It is morphologically characterized by ovate to lanceolate, acuminate, and serrulate leaves on both stems and branches, a weak costa, smooth setae, and a long-rostrate operculum [16, 17], which typically form small to large, loose tufts or extensive mats.

This species was first collected from the Darjeeling Hills in April 2017 during an ongoing project on the moss flora of Darjeeling Hills, and was recollected from the same locality in June 2024. This repeated occurrence suggests that the species may be well-acclimated to this region. The present study provides a comprehensive morphological illustration of sporophytic structures using scanning electron microscopy (SEM) and detailed measurements.

To confirm its taxonomic placement, molecular phylogenetic analysis was conducted using *rbcL* sequence data. The chloroplast gene *rbcL* is highly conserved yet exhibits sufficient variation to discriminate among closely related taxa at the species and genus levels [18]. Its relatively slow evolutionary rate also makes it valuable for resolving relationships at higher taxonomic ranks, such as families and orders, thereby providing a stable framework for phylogenetic inference [19]. The phylogeny of *Rhynchostegium* has been investigated using a combination of three DNA sequence regions: nuclear ITS1-5.8S-ITS2 and plastid *trnL-F* and *trnD-T* [20]. Notably, phylogeny based on the plastidial *rbcL* was conducted for the first time in this genus. Future studies incorporating additional markers are necessary to elucidate the complex phylogeny of Brachytheciaceae.

Here, we provide an extended morphological description of *R. celebicum*, supplemented with illustrations, molecular data, and ecological observations of the Darjeeling Hills. The broad biogeographic significance of this species has also been discussed.

Materials and methods

Collection of specimens

Moss specimens were collected from wild using detailed field records, including precise habitat descriptions and ecological details of the collection site. The distribution map of the moss samples was generated using *QGIS* version 3.44.3 Solothurn (Figs. 1). The specimens were carefully dried, preserved, and deposited in the Lloyd Botanical Garden in Darjeeling, West Bengal, India. Species identification was carried out through extensive examination of morphological and anatomical features, comparing these observations with descriptions in the “Mosses of Eastern India and Adjacent Regions” [2] and “Taxonomy of Indian Mosses” [21]. *Rhynchostegium celebicum* was documented in 2017 at Bhutia Busty, Darjeeling Hills, (N27°03'19.19", E088°15'38.02", 1989 m) growing with liverworts on moist soil over rock. The humid, undisturbed foothill habitat supports rich bryophyte diversity. The species was again collected from the same site in 2024 (N27°03'19.44", E088°15'37.71", 2002 m), this time also occurring epiphytically on *Alnus nepalensis*. Both collections (Supplementary Fig. a,b) have been deposited in the Lloyd Botanical Garden Herbarium, Darjeeling.

Morphological Studies

The morphological characteristics of the moss specimens were analyzed using a stereomicroscopy (Stereomaster-Plus, Dewinter, India), binocular light microscopy (Excel, Dewinter, India), and scanning electron microscopy (EVO 10, Zeiss, Germany). To prepare specimens for microscopy, moss samples were rehydrated in double-distilled water for 10–15 min to facilitate the rapid hydration and removal of surface debris. For the SEM analysis, the specimens were dehydrated using a graded ethanol series (60%, 70%, 80%, 90%, and absolute ethanol), mounted on aluminium stubs with carbon tape, and sputter-coated with gold-palladium for 120 s using an SC7620 Mini Sputter Coater/Glow Discharge System

(Quorum Technologies). High-resolution photomicrographs were obtained at an acceleration voltage of 30 eV. Quantitative analyses included measurements of leaf length, width, leaf cell dimensions, peristome teeth features, and spore diameter, recorded using an ocular micrometer calibrated against a stage micrometer and analyzed using ImageJ software [22].

DNA Extraction, Amplification, and Phylogenetic Analysis

Genomic DNA was extracted, amplified by PCR, and sequenced according to the method outlined by Bera [23]. PCR amplification was performed using primers *rbcLa*-F (ATGTCACCACAAACAGAGACTAAAGC; [24] and *rbcLa*-R (GTAAAATCAAGTCCACCRG; [25]. The sequences were aligned using MEGA11 [26]. For phylogenetic analysis, *rbcL* sequences from other members of the Brachytheciaceae family, available in GenBank, were used to construct a phylogenetic tree. The tree was generated using the maximum parsimony (MP) method with 1000 bootstrap replicates in Mega11 software, utilizing the Tree-Bisection-Regrafting (TBR) algorithm [27]. *Hypnum plumaeforme* (AB accession 029384.1) and *Entodon rubicundus* (AB accession 029386.1), from the Hypnaceae family, were selected as outgroups for tree rooting.

Taxonomic Treatment

Basionym

Hypnum celebicum Sande Lac., Bryol. Jav. 2: 159. 258. 1866.

Etymology

The specific epithet, *celebicum*, refers to 'Celebes (Sulawesi)' in Latin, the county name of the type locality.

Range

Borneo, Cambodia, Hawaii, India, Java, Myanmar, New Caledonia, New Guinea, Philippines, Sulawesi, Sumatra, Taiwan, Thailand, Vietnam.

Distribution in India

Eastern Himalaya: Assam, Darjeeling; Meghalaya: Khasi Hills, and Arunachal Pradesh. Western Himalayas: Uttarakhand; Western Ghats: Tamil Nadu

Ecology and Distribution

In April 2017, *Rhynchostegium celebicum* was identified in association with liverworts on the thin, moist soil layer covering rocks in Bhutia Busty, located in the Darjeeling Hills (N27°03'19.19", E088°15'38.02", 1989 m). This region, a remote part of the Himalayan foothills, is rich in ferns and bryophytes owing to its moist conditions and limited human activity, with steep slopes contributing to the lush environment. In 2024, the same species was collected from the same locality (N27°03'19.44", E088°15'37.71", 2002 m), which grows epiphytically on the bark of *Alnus nepalensis*. These species were found growing on both rock surfaces and tree trunk specimens (Supplementary figures a and b) from both collections were submitted to the Lloyd Botanical Garden Herbarium, Darjeeling (Accession no LB014433 for 2017 and LB014686 for 2024).

Morphological & ultrastructural details

The plants were creeping, approximately ± 5 cm in length, with pinnate or bipinnate branches reaching up to 2 cm in length (Figs. 2a and 2b). They are yellowish-green to light-green, glossy, and form tufts. The leaves are erecto-patent, spreading, and have an ovate to acuminate shape, measuring 1.29 ± 0.11 mm in length and 0.35 ± 0.04 mm in width (Fig. 3a). Leaf margins were denticulated from the middle to the apex. The cells at the base of the leaves measure $37.79 \pm 1.49 \times 5.27 \pm 0.51$ μ m, smooth, while the upper cells are $33.85 \pm 7.14 \times 6.23 \pm 1.12$ μ m. The costa is single, covering approximately two-thirds of the leaf (Supplementary figure c), and measures 0.71 ± 0.10 mm in length and 0.03 ± 0.01 mm in width on average. The laminal cells were thick-walled and lax. The apical single terminal cell was $37.12 \pm 2.65 \times 6.55 \pm 1.16$ μ m, while the median and upper laminal cells were linear and sub-rectangular, measuring $40.05 \pm 6.63 \times 6.25 \pm 0.87$ μ m. The lower laminal cells are rectangular to quadrate, measuring $40.32 \pm 7.25 \times 6.14 \pm 1.22$ μ m (Supplementary figure f). The seta is erect and smooth (Fig. 2c), measuring 1.87 ± 0.15 mm in length. The capsule is cylindrical, horizontal (Fig. 2e) and measures 2.23 ± 0.24 mm in height and 0.73 ± 0.09 mm in width. The operculum is conical with a long rostrum, measuring 1.14 mm in length, and the calyptra is $2.69 \pm 0.09 \times 0.36 \pm 0.02$ mm (Fig. 2d). The peristome was diplolepidous (Fig. 2f, 4a, 4b and 4c). The spores were yellowish-green.

The leaf base exhibited distinctly corrugated, wavy patterns, with epidermal cells arranged in pronounced, parallel undulations (Fig. 3b). The leaf cells were elongated, narrowly elliptical to convex in shape, and interconnected by bifurcated cell wall ends (Fig. 3c and 3d). The leaf apex was elongated and pointed (Fig. 3e, 3f, and Supplementary figure d), bearing clear serrations along the margins (Fig. 3g, 3h and Supplementary figure e). The upper laminal and marginal cells appear to be comparatively more contracted. The leaf surface was smooth and lacked papillae.

The exostome was measured at 256.67 ± 33.55 μ m in length and 45.68 ± 3.33 μ m in width (figures a-c). The dorsal region of the exostomes is characterized by robust, regularly spaced transverse stripes (5.10 ± 1.12 μ m) arranged in parallel, with papillae-like ornamentation present between the stripes (Fig. 4e). The ventral surface of the exostome was narrowly flattened, smooth, and lamellated, with ridges (figure d). The endostome was well-developed, being slightly longer but narrower than the exostome, measuring 361.27 ± 37.85 μ m in length and 35.08 ± 2.34 μ m in width (figures a-c). The dorsal region of the endostomes is characterized by faint ridges that are more widely spaced (13.10 ± 3.11 μ m) compared to

those of the exostome. Additionally, the surface was embellished with irregular papillae, imparting a rough, spiny texture (Figs. 4g and 4h). Smooth, thin-walled cells were located in the basal region of the ventral position of the endostome (Fig. 4f).

The operculum was conical to hat-shaped, with a broad basal portion ($641.05 \pm 68.96 \mu\text{m}$) tapering into a slender, stalk-like apex ($1145.54 \pm 42.62 \mu\text{m}$) (Fig. 5a). The surface appeared relatively smooth compared with that of the teeth, although fine reticulate ornamentation was occasionally restricted to the basal region (Fig. 5a). Spores were predominantly spherical to sub-spherical (roundness index: 0.9 ± 0.02), with a mean diameter of $21.71 \pm 0.93 \mu\text{m}$. Their surfaces were covered with numerous, evenly distributed, small, rounded papillae ($0.9 \pm 0.02 \mu\text{m}$ in diameter), imparting a finely papillose texture (Fig. 5b).

Phylogenetic Relationships of *Rhynchostegium* and Related Genera

Phylogenetic reconstruction demonstrated that all examined *Rhynchostegium* species—including *R. celebicum*, *R. megapolitanum*, *R. riparioides*, and *R. pallidifolium* formed a strongly supported monophyletic clade. Within this lineage, *R. celebicum* (Accession no PP663260.1) showed a particularly close relationship with *R. megapolitanum* (Accession no OZ207752.1), which is a rare bryophyte found in Europe and North America.

The semi-submerged *Platyhypnidium riparioides* (synonym: *R. riparioides*, accession number AB029385.1) was nested within this clade (Fig. 6). Interestingly, the entire *Rhynchostegium* clade was recovered as a sister group to *Palamocladium leskeoides* (LC accession 744569.1). At a broader level, the phylogeny revealed a large and diverse assemblage of *Brachythecium* species, together with allied genera, such as *Sciuro-hypnum*, *Pseudokinbergia*, *Helicodontium*, *Myuroclada*, *Homalothecium*, and *Eurhynchium*.

Discussion

Leaf morphology, particularly the smooth surface of the leaves, distinguishes *Rhynchostegium* from other genera such as *Brachythecium*, *Homalothecium*, and *Eurhynchium*, which are characterized by plicate leaves [28]. Furthermore, *Rhynchostegium* exhibited significant similarities to *Eurhynchium*, except for differences in plant colouration and the terminal cells of the costa. Specifically, in *Rhynchostegium*, plants are whitish in colour, and the terminal cell of the costa lacks a spine, whereas in *Eurhynchium*, it is green and possesses a spine. The leaf length of the studied species was slightly shorter than that reported by Saha et al. [9]. This discrepancy may be attributed to a conflict between parent and offspring dynamics and the morphology of gametophytes and sporophytes [29]. Nevertheless, it remains unclear whether this reduction is a consequence of the nutritional dependence of sporophytes on gametophytes.

Because the ultrastructural details have been documented for the first time, they may be comparable to those of other species. Microscopic leaf morphology contrasted significantly with the features reported by Robinson, although the species were from different families [33]. The spore diameter and shape in *R. celebicum* were slightly different from those observed in *E. praelongum* (approximately 12µm; subglobose) [23]. Additionally, large spores (> 20µm) have been identified in various epiphytic lineages [30, 31]. The ultrastructural characteristics of the peristome are similar to those of *Hypopterygium fauriei* and *Aulacomnium heterostichum*, although these taxa are not closely related [32, 33]. The plates and ridges present on exostomes play crucial roles in determining both the direction and extent of hygroscopic movement [32, 34]. In contrast, endostomes, that lack distinct ridges are unlikely to participate in this movement.

In the present study, plastid *rbcL* gene analysis was performed to provide additional support for the identification of the Eastern Himalayan specimen. However, nuclear data are often required for complete resolution in Brachytheciaceae. Huttunen & Ignatov successfully resolved the complex relationships within the *Rhynchostegium* clade using the ITS region, which has a higher substitution rate, crucial for resolving recent divergence [20, 35]. In this study phylogenetic analysis suggested a close relationship between *R. megapolitanum* and *R. celebicum*, likely because of their shared gametophytic morphology (loosely branched shoots with erect, smooth setae) and ecological plasticity. Their capacity for habitat expansion may also be enhanced by environmental drivers such as climate change and nitrogen deposition [36, 37]. However, both species were placed in a separate clade within the phylogenetic tree based on nuclear ribosomal ITS1-5.8S-ITS2 sequence data [12]. The affinity between *R. riparioides* and *P. leskeoides* further corroborates the phylogenetic placement of *Rhynchostegium* as proposed by Ignatov and Huttunen [38], which is likely attributable to similarities in their gametophytic structures.

However, studies of aquatic species (e.g., *Platyhypnidium*) have revealed multiple independent evolutionary shifts from terrestrial to aquatic habitats, resulting in geographically defined clades of aquatic species [20, 39]. Consequently, morphological similarities among aquatic species are often homoplastic and driven by adaptation to similar ecological pressures rather than shared ancestry. In 2010, Huttunen and Ignatov observed that the Asiatic species *R. celebicum* and *R. javanicum* possessed distinct tandem repeats within the plastid *trnT-E* spacer region [20]. This finding underscores the potential of utilizing specific molecular signatures to trace the biogeographic history of *Rhynchostegium* in the Asian tropics.

The phylogenetic framework further suggests complex relationships between *Rhynchostegium* and *Brachythecium* within Brachytheciaceae, with the latter appearing to be polyphyletic or paraphyletic. Despite this, *Brachythecium* remains morphologically distinct, characterized by a rough seta, conical to shortly beaked operculum, elongate-ovoid capsule, and variable costa that may reach the leaf apex. Although *Rhynchostegium* is recovered as a separate lineage, the dispersed position of *Brachythecium* across the tree indicates a complicated evolutionary history that warrants further investigation combining molecular and morphological evidence. Moreover, molecular markers can be used to elucidate species-specific characteristics.

Declarations

Acknowledgement

The second author is grateful to the Science and Engineering Research Board, Govt. of India, for funding a project on Climate change and providing her fellowship. The authors also extend their gratitude to the HOD, Department of Life Sciences, Presidency University, Kolkata, for providing infrastructural facilities to carry out this research. We appreciate the access to scientific resources facilitated by the Information and Library Network and the e-Library Consortium through the Department of Life Sciences, Presidency University. The authors also convey their sincere thanks to Officer-in-Charge, Lloyd Botanic Garden, Darjeeling, for extending his kind help in the issuance of the accession number.

Funding

This work was supported by the Science and Engineering Research Board (SERB), Govt. of India (Gol), under research grant number: SCP/2022/000716 dated 18.07.2023 (Special Call Project on Climate change). Department of Biotechnology, Gol, under research grant number BT/INF/SP45088/2022 dated 17.02.2022 for Instrumental facilities and the Council of Scientific and Industrial Research (CSIR) under research grant number: 09/028(0992)/2017-EMR-I dated 04.05.2017 for fellowship of the first author.

Ethics declaration: The collection of plant/plant parts used in our study were complied with the local or national guidelines. Plants were collected from wild and were used for only academic purposes and not for any commercial utilization. The relevant permission for collection of this moss specimen was taken from forest authority.

Consent to Participate declaration: not applicable

Consent to Publish declaration: not applicable

Competing interests: No potential conflict of interest was reported by the author(s).

Data availability statement

Plant Specimens were deposited in the Lloyd Botanical Garden Herbarium, Darjeeling (Accession no LB014433 for 2017 and LB014686 for 2024). The datasets generated and/or analysed during the current study are available in the GenBank repository, Accession number: *rbcL* = PP663260.1 (Holotype; <https://www.ncbi.nlm.nih.gov/nuccore/PP663260.1/>).

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Figures

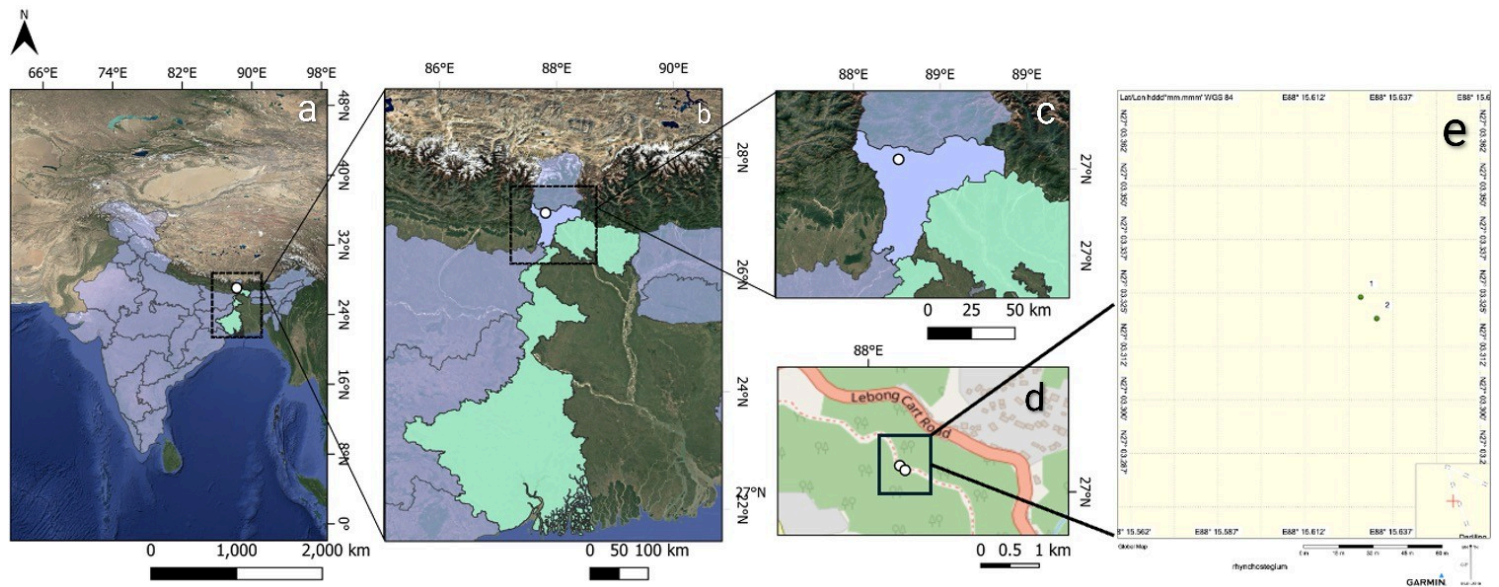


Figure 1

Distribution map of *Rhynchostegium celebicum* **a-d**. Distribution map prepared using *QGIS* software (version 3.44.3 Solothurn), **e**. Zoomed-in view of the distribution area generated using Garmin BaseCamp.

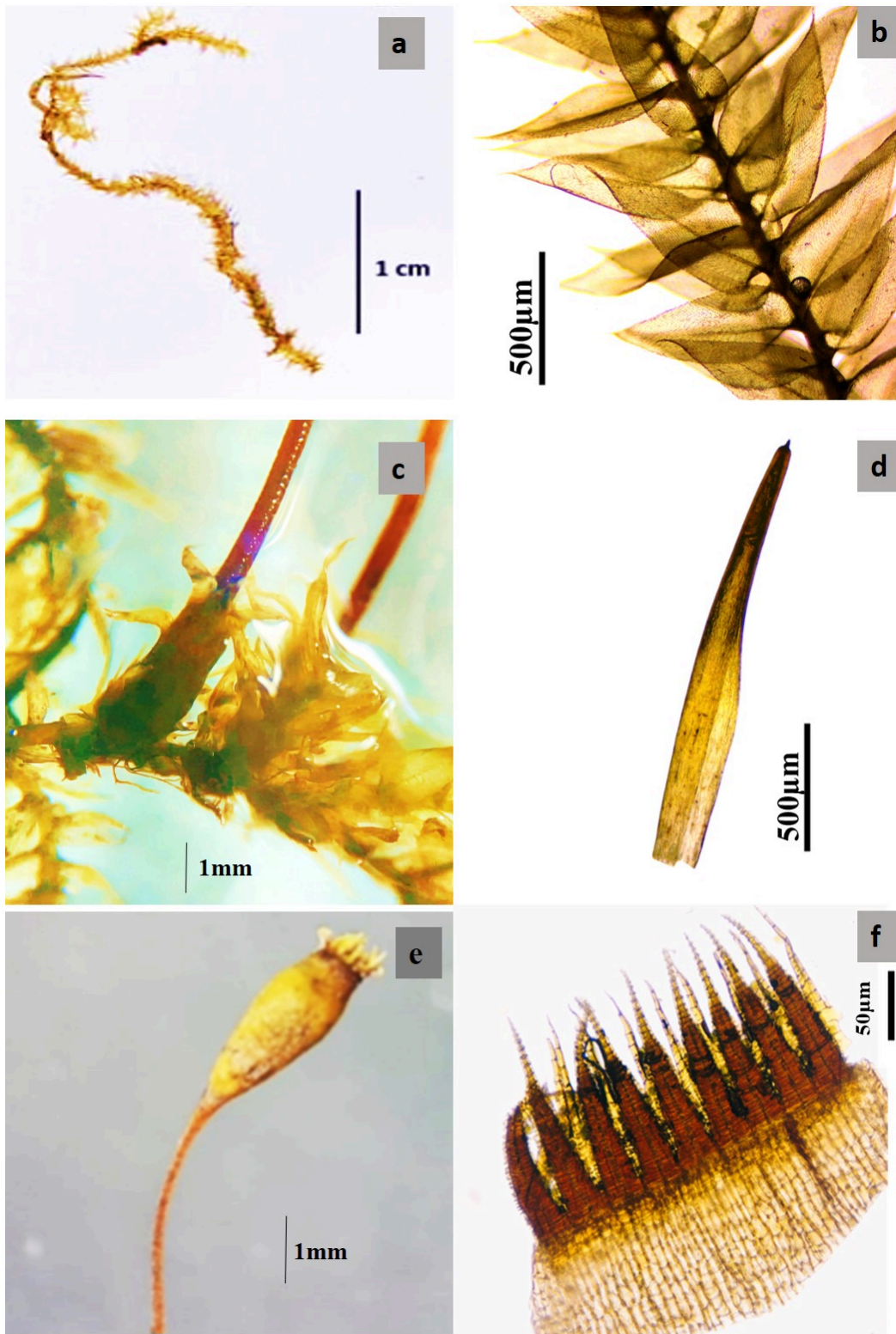


Figure 2

Morphological features of *Rhynchostegium celebicum*. **a** and **b**. Gametophytic plant body with pinnate or bipinnate branching pattern, **c**. Seta with smooth surface arising from main branch of gametophyte, **d**. Calyptra, **e**. Capsule, **f**. Peristome.

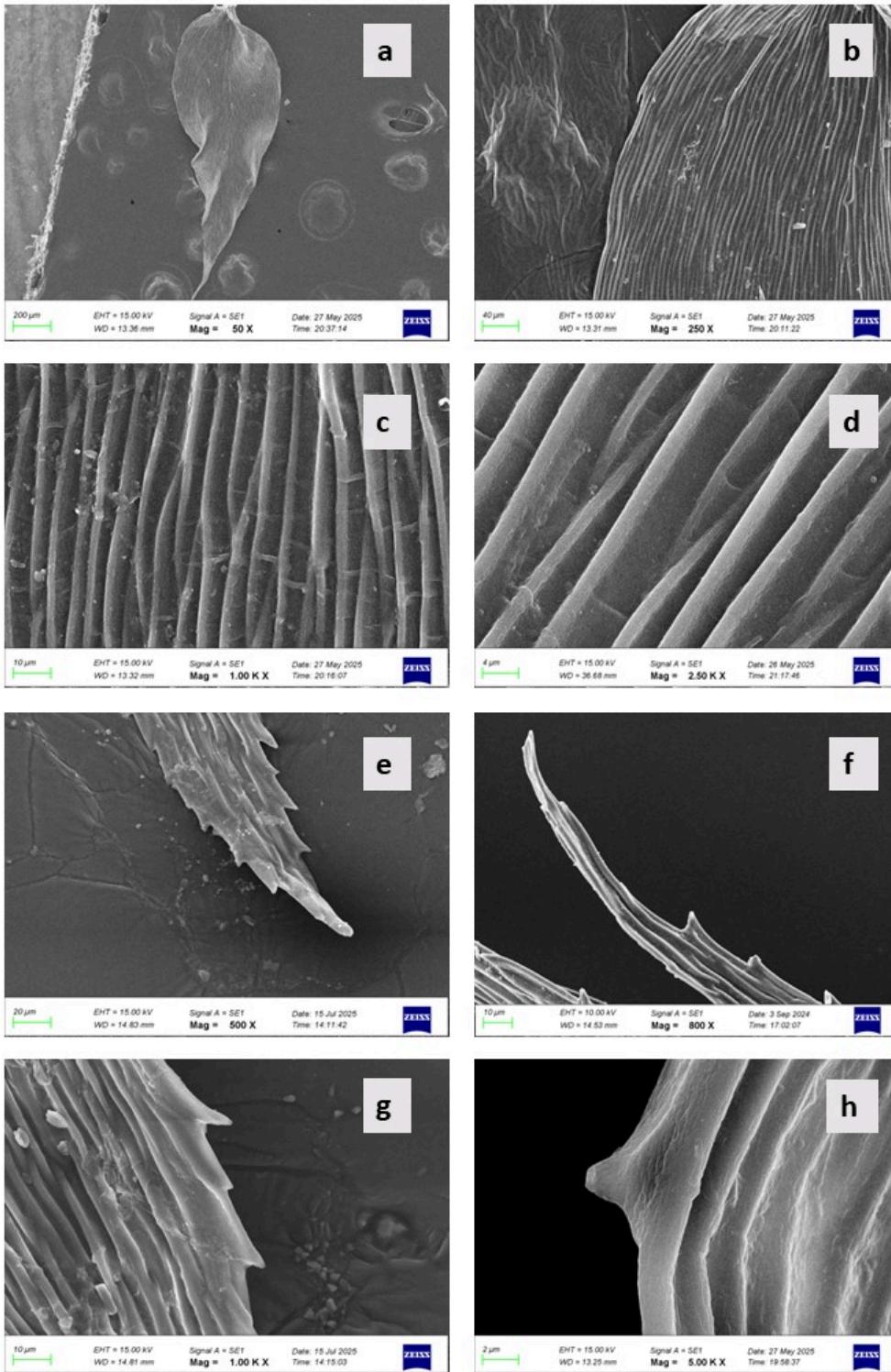


Figure 3

Scanning electron micrographs of *Rhynchoszegium celebicum* leaf surfaces showing ultrastructural details. **a.** Whole leaf, **b.** Basal lamina region, **c** and **d.** Narrow elongated leaf cells interconnected by bifurcated cell wall ends, **e** and **f.** Leaf apex with an acute to acuminate tip, **g** and **h.** Leaf margin showing dentation.

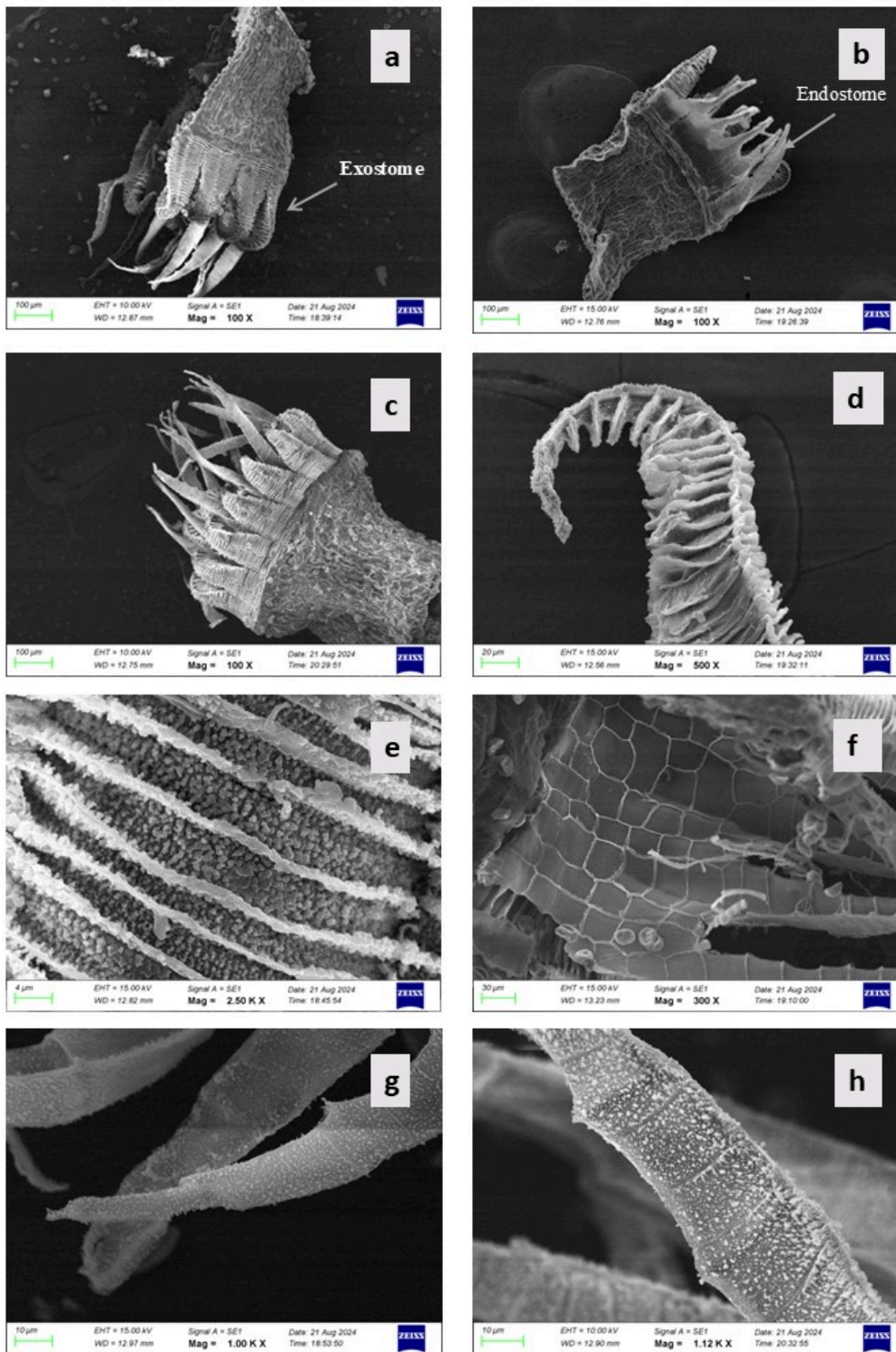


Figure 4

Scanning electron micrographs of *Rhynchostegium celebicum* capsule showing peristome teeth. **a.** dorsal surface of the exostome and **b.** ventral surface of the endostome, **c.** Peristome with exostome and endostome, **d.** ventral surface of exostome **e.** Dorsal view of exostome with spiral striations, ornamentations with horizontal ridges, **f.** Ventral view of endostome showing cellular details, **g** and **h.** dorsal view of endostome with papillae and spiny texture.

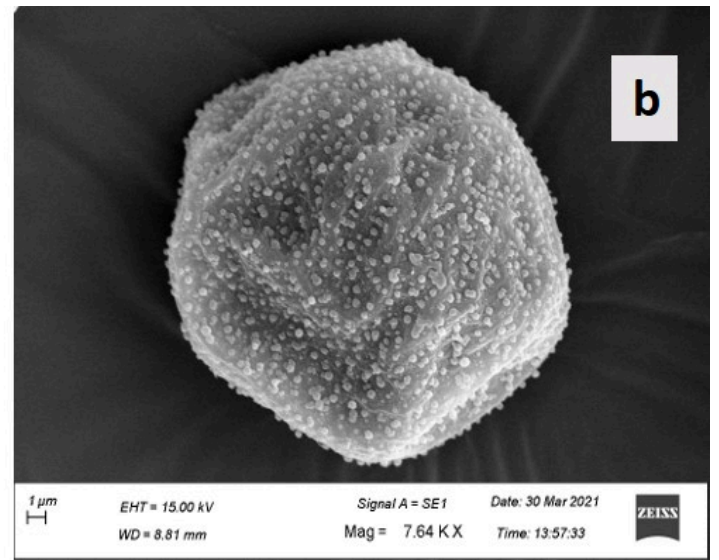
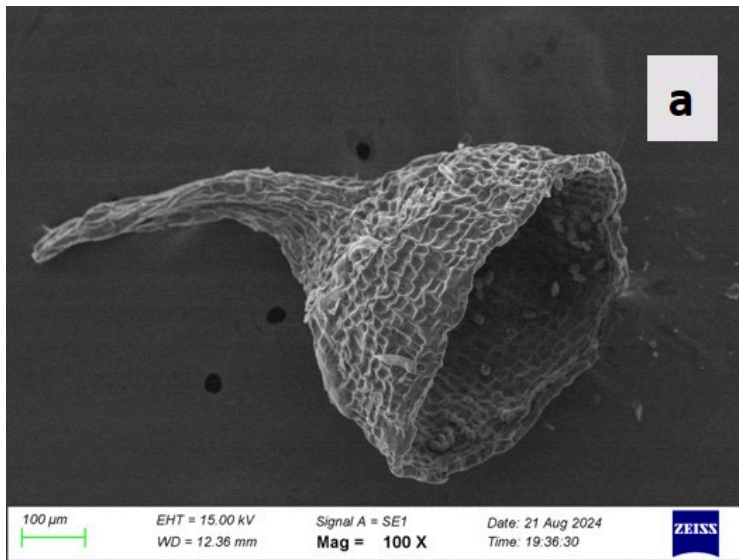


Figure 5

Scanning electron micrographs of *Rhynchostegium celebicum* operculum and spore. **a.**Operculum showing broad base and narrow apical region,**b.** Spore with spherical morphology and surface ornamentation.

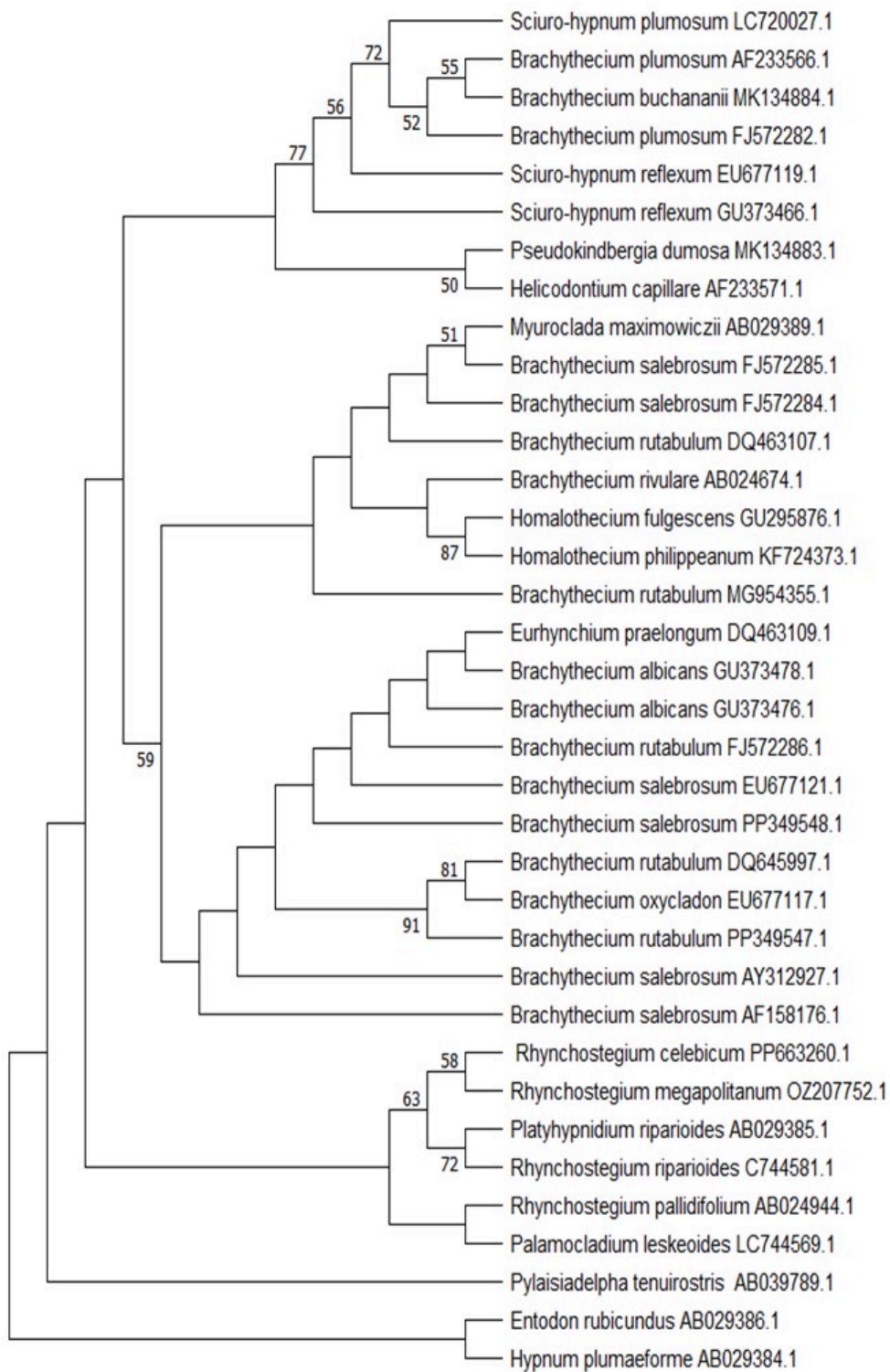


Figure 6

Phylogenetic tree based on maximum parsimony on the plastid *rbcL* gene. Bootstrap values (≥50%) are given above the branches. *Hypnum plumaforme* (AB accession 029384.1) and *Entodon rubicundus* (AB accession 029386.1) from the Hypnaceae family were selected as outgroups for tree rooting.

Supplementary Files

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