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RESEARCH PAPER

Orthotrichum cupulatum Hoffm. ex Brid. var. *lithophilum*, a new variety of epilithic bristle moss from Tasmania

Vítězslav Plášek ^{1,2*}, Jakub Sawicki ³, Rodney D. Seppelt^{4,5},
Lynette H. Cave ⁴

¹ Department of Biology and Ecology, University of Ostrava, Chittussiho 10, 710 00 Ostrava, Czech Republic

² Institute of Biology, University of Opole, Oleska 48, 45-052 Opole, Poland

³ Department of Botany and Nature Protection, University of Warmia and Mazury in Olsztyn, Plac Łódzki 1, 10-719 Olsztyn, Poland

⁴ Tasmanian Herbarium, PO Box 5058, UTAS LPO, Sandy Bay, TAS 7005, Hobart, Tasmania, Australia

⁵ School of Biological Sciences, Macquarie University, Campus Macquarie Park, NSW 2109, New South Wales, Australia

* Corresponding author. Email: vitezslav.plasek@osu.cz

Abstract

Orthotrichum cupulatum var. *lithophilum* is described as a new variety from Tasmania, Australia. The species is primarily characterized by its exerted capsule with cryptoporous stomata, a single peristome with 16 exostome teeth, a bistratose lamina of stem leaves, and a densely hairy vaginula. Molecular data and a brief discussion comparing the newly described variety with the most closely related taxa are also provided. Molecular analyses based on complete plastome sequences clearly separated var. *lithophilum* from the typical form by 272 molecular diagnostic characters specific for *Orthotrichum cupulatum* var. *lithophilum*. The distinctiveness of the new taxon was also confirmed by plastome phylogenomics, which confirmed the monophyly of the new variety and its sister relationship to *Orthotrichum cupulatum* var. *cupulatum*.

Keywords

Australia; new taxa; bryophytes; Orthotrichaceae; taxonomy

1. Introduction

Orthotrichum Hedw. is a large genus mainly with temperate distribution in both the northern and southern hemispheres. However, as is the case with most of such moss genera, *Orthotrichum* has been recognized as a heterogeneous taxon. Extensive taxonomic and molecular investigations have confirmed its polyphyly, resulting in the separation of three distinct segregates from the genus: *Nyholmiella* Holmen & E. Warncke, *Pulviger* Plášek, Sawicki & Ochyra and *Lewinskya* F. Lara, Garilleti & Goffinet (Lara et al., 2016; Sawicki et al., 2009, 2010, 2017).

Species belonging to the *Orthotrichum* genus are primarily found in temperate regions, with fewer occurrences in tropical areas where they are replaced by *Macromitrium* Brid., *Schlotheimia* Brid. and related genera. In Australia, the genus displays a widespread presence, primarily within the forests of the southeastern temperate regions. These mosses predominantly thrive as epiphytes on trees and shrubs, rarely as epilithic species on boulders and rocks (Lewinsky-Haapasaari & Ramsay, 2006). Lewinsky (1984a) provided

detailed distribution maps and an overview of substrate preferences for all taxa, revealing that introduced woody plants, such as those within the genera *Fraxinus* L., *Populus* L., and *Salix* L., support the large diversity of *Orthotrichum* species.

Taxonomic revision of *Orthotrichum* genus (in a broadly taxonomic sense) in Australia was comprehensively treated by Lewinsky (1984a), and subsequently, the recent distribution was also documented by Lewinsky-Haapasaari and Ramsay (2006). According to these studies, the *Orthotrichum* s.l. genus in Australia is represented solely by two non-endemic species, *O. cupulatum* Hoffm. ex Brid. and *O. assimile* Müll. Hal. In neighboring New Zealand its diversity is one more species greater (moreover, endemic *Orthotrichum calvum* Hook. f. & Wilson grows here). It should be noted that in the past, *O. calvum* was also reported from Australia (Scott & Stone, 1976; Watts & Whitelegge, 1906); however, these reports were solely based on collection from Victoria, and no such specimen has been found during the revision process (cf. Lewinsky, 1984a). Consequently, *O. calvum* has been excluded from the Australian flora.

While identifying material collected during a bryofloristic survey in the Vale of Belvoir Conservation Area in northern Tasmania, a noteworthy epilithic moss from the genus *Orthotrichum* was discovered. Upon closer examination, it was determined that two specimens represented a new variety previously unknown to science. To confirm the distinctiveness and taxonomic status of the newly described variety, the complete chloroplast genomes were assembled using the latest 3rd generation sequencing technology.

2. Material and methods

2.1. Plant material

Specimens were collected on two separate occasions: as part of a Bush Blitz survey of the Vale of Belvoir and on a separate visit to the area. Specimens were collected into packets, air dried and returned to the Tasmanian Herbarium for identification and incorporation into the herbarium collections. Plants were subsequently drawn dry and wet, dissected and sectioned for illustration of morphological features.

2.2. Morphological evaluation

Leaves, sections and sporophytes were mounted on glass microscope slides, cleared using lactic acid, and all components drawn using an Olympus SMZ10 dissecting microscope and Zeiss Axioskop compound microscope, both fitted with drawing attachments.

An Olympus SZ61 trinocular microscope was used to take macrophotographs of plants. Detailed SEM photo-documentation of leaves and peristome structures was prepared using a Jeol SEM microscope.

2.3. Molecular analyses

Total genomic DNA from a single individual was extracted using Qiagen Mini Spin Plant Kit (Qiagen, Germany). Details concerning ptDNA enrichment, library construction and nanopore sequencing are identical to those in the previous study (Sawicki et al., 2023). Obtained raw reads were trimmed using porechop 0.2.4 and assembled using Flye 2.91 assembler (Kolmogorov et al., 2019) which produced complete, circularized plastome contigs. Complete chloroplast genomes were annotated using previously published *Orthotrichum* Hedw. sequences (Frangedakis et al., 2021; Mizia et al., 2019) as references in Geneious Prime 2023.2.1 software (Biomatters, Auckland, New Zealand).

The newly sequenced plastomes were aligned with those available in GenBank Orthotrichaceae (Supplementary material, Table S1) plastomes using MAFFT 7.52 (Kato & Standley, 2013). The second copy of IR was removed from subsequent analyses and ambiguously aligned regions were trimmed by Gblocks 0.91 (Talavera & Castresana, 2007).

Chloroplast sequences of 21 specimens of Orthotrichaceae, including seven from *Orthotrichum* were used for phylogenetic analysis. *Macrocoma tenuis* (Hook. & Grev.) Vitt (MT591413) was selected as an outgroup based on earlier studies (Draper et al., 2022).

Phylogenetic analysis was carried out using the Bayesian inference (BI). The optimal model for the plastome dataset was identified as GTR + F + I + G4 by ModelFinder based on the Bayesian information criterion (BIC). Bayesian analysis (BA) was conducted using MrBayes 3.2.7 (Huelsenbeck & Ronquist, 2001), and the MCMC algorithm was run for 20,000,000 generations (sampling every 1,000) with four incrementally heated chains (starting from random trees). The visual inspection of Tracer 1.7 (Rambaut et al., 2018) plots was used to examine the parameters and to determine the number of generations needed to reach stationarity, which occurred at approximately 400,000 generations. Therefore, the first 600 trees were discarded as burn-in, and the remaining trees were used to develop a Bayesian consensus tree.

The discrete molecular diagnostic characters (MDCs) for each species of *Orthotrichum*, *Lewinskya* and *Ulota* D. Mohr (genera with more than two known plastome sequences) were calculated according to the Jörger and Schrödl (2013) approach using FASTACHAR 0.2.4 (Merckelbach & Borges, 2020).

3. Results

3.1. Taxonomic treatment

Orthotrichum cupulatum Hoffm. ex Brid. var. *lithophilum* Plášek & Sawicki, var. nov.

Diagnosis: *Plantae olivacea, obscure viridis vel ferrugineae, usque ad 20 mm altae. Foliis erecto-adpressis, lanceolatis vel ovato-lanceolatis, carinatis, apicibus acuminatis. Lamina foliorum saepius bipartita. Cellulae superiores cum (1–) 3 papillis ramosis; basales glabris. Capsulae exserta, ovoideae-cylindrica. Stomata cryptopora. Vaginula dense pilosa cum capilli longi. Peristomium simplicibus, dentes exostomi 16. Calyptra conico-oblonga, dense pilosa. Sporae 15–19 µm, leniter papilloasae.*

Type: Australia, Tasmania: Middlesex, Vale of Belvoir Conservation Area, on exposed limestone outcrop in grazed pasture, 800 m a.s.l., GSP = 41° 32' 51.27" S, 145° 52' 59.98" E, leg. L.H. Cave #1124, 14 Mar 2010, holotype (HO #556429); isotype (OSTR #7129).

Additional specimen examined (Paratype): Australia, Tasmania: Middlesex, Vale of Belvoir Conservation Area, on limestone rocks, 800 m a.s.l., GSP = 41° 33' 12" S, 145° 52' 48" E, leg. J. Jarman, 25 May 2000 (HO #505285).

Description: Plants olive green, dark green to rusty brown, up to 20 mm tall. Stem moderately branched, branches up to 5 mm long. Rhizoids well-developed, reddish brown, densely distributed mainly at the base of the stem. Stem leaves erect-appressed when dry, erect-spreading when moist, lanceolate to ovate-lanceolate in shape, 1.5–2.0 × 0.3–0.6 mm, keeled, acuminate, sharply acute; lamina rather bistratose (sometimes unistratose), margins entire, recurved from base to near apex, at least on one side (Figure 1). Upper laminal cells isodiametric to short elongate, (7-)10–12 × 8–11 µm, fairly thick-walled, with 1-2(-3) branched papillae; basal laminal cells elongate rectangular to rhomboidal, thick-walled, (18-)20–45 × 10–13 µm, smooth. Alar cells slightly differentiated, forming small auricles with a row of almost rounded cells along the margin. Costa ending below the apex. Sexual condition gonioautoicous. Seta 1.2–1.5 mm long, ochrea up to 1/5 of the

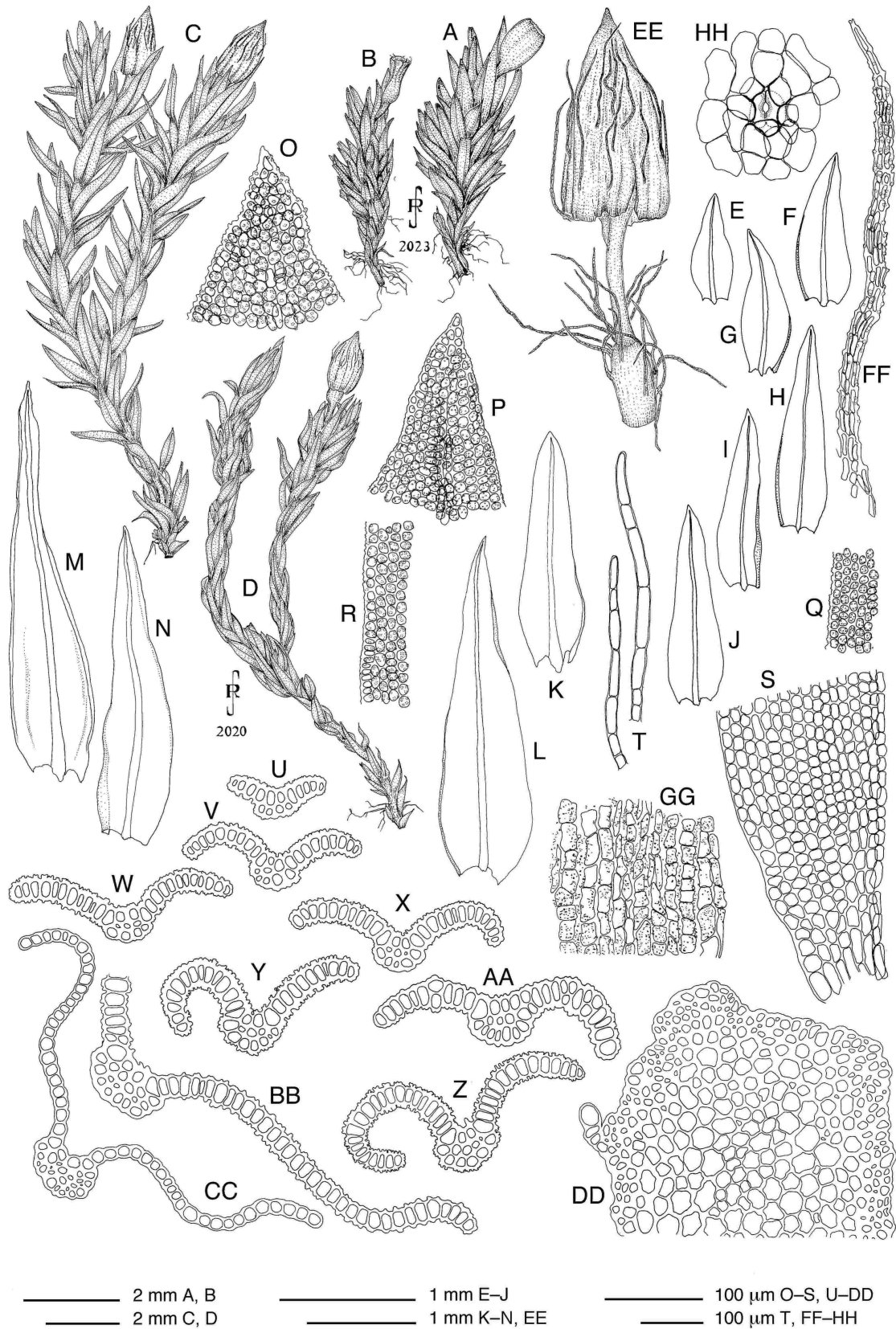


Figure 1 Drawing of *Orthotrichum cupulatum* var. *lithophilum*. A–D – shoots with sporophytes (B, D dry), E–L – stem leaves, M, N – perichaetial leaves, O–S – leaf cells: apex (O, P), margin, mid lamina (Q, R), basal angle (S), T – axillary hairs. U–CC – leaf sections, DD – part TS of stem, EE – sporophyte with hairs on the vaginula and calyptra, FF – cells of a calyptra hair, GG – Exothecial cells below capsule mouth, HH – stoma (immersed) on capsule urn. Figures A, B, E–J, P, Q, S, U–Z were drawn based on the holotype (HO556429), figures C, D, K–O, R, T, AA–HH were drawn based on the paratype (HO505285).

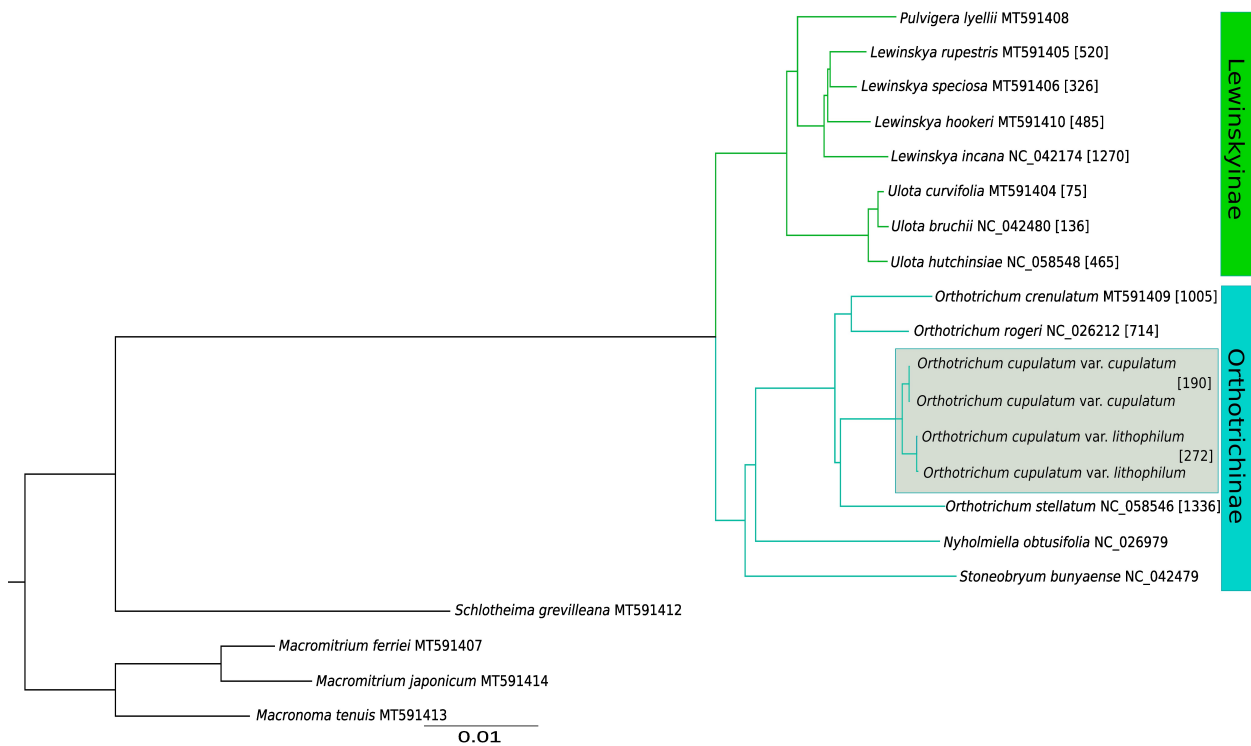


Figure 2 The Bayesian inference tree based on complete chloroplast genomes. All clades have maximum PP values (1.0). The numbers in square brackets indicate the number of MDCs.

seta, vaginula densely hairy with 0.8–1.4 mm long, papillose hairs, which usually reach the base of the urn. Capsule exserted; cylindrical ovoid to oblong-ovoid, about 1.2–1.4 mm long, yellowish brown to red brown, not constricted below the mouth when dry. Exothecial cells differentiated mainly in the upper part of capsule, urn grooved alternately with 8 long and 8 shorter furrows. Stomata cryptopore, scattered throughout the length of the capsule, half covered by subsidiary cells. Peristome single. Exostome teeth 16, orange-yellow to light brown, erect-spreading when dry, OPL and PPL covered with vermicular or net-like lines, slightly ornamented also with low papillae. Endostome segments completely absent. Preperistome present, covering the outer base of the teeth. Calyptra conic-oblong, light brown with red-brown apex, densely hairy with long, yellowish, strongly papillose hairs. Lid with a short beak. Spores 15–19 μm , finely papillose. Asexual reproduction not observed.

Name of the new variety (*lithophilum*) indicates its ecological characteristic – it was found growing on rocks.

3.2. Molecular survey

3.2.1. Chloroplast genome characteristics

The circular plastomes of the four newly assembled specimens have a typical quadripartite structure with one small single-copy (SSC), one large single-copy (LSC), and two inverted repeats (IR). Application of the 3rd generation sequencing did not reveal structural heteroplasmy connected with SSC region inversion. The total length of the plastomes ranged from 122,895 bp to 123,536 bp. The plastome contains 82 protein-coding genes (including the hypothetical chloroplast

reading frames: *ycf1*, 2, 3, 4, 12, and 66), 32 tRNA, and four rRNA. The *rps12* gene is divided into two independent transcription units (5'-*rps12* and 3'-*rps12*) whose transcripts are trans-spliced. The gene structure and order are identical to those of other *Orthotrichum sensu stricto* plastomes (Frangedakis et al., 2021; Mizia et al., 2019).

3.2.2. Phylogeny and molecular diagnostics characters

Phylogenetic analysis based on complete plastome sequences resolved all clades with maximum values of posterior probabilities (Figure 2). The species of Orthotrichaceae formed two distinct clades, one grouping genera belonging to Lewinskyinae (*Lewinskya*, *Pulviger* and *Ulota*) and the second formed by members of Orthotrichinae (*Nyholmiella*, *Stoneobryum* D.H. Norris & H. Rob. and *Orthotrichum s.str.*). Analyzed genera were revealed as being monophyletic, with *Ulota* sister to the *Pulviger/Lewinskya* clade and *Nyholmiella* as sister to *Orthotrichum*.

The number of detected MDCs ranged from 75 for *Ulota curvifolia* (Wahlenb.) Sw. to 1,336 for *Orthotrichum stellatum* Brid. (Figure 2). The remaining *Ulota* species revealed 135 (*U. bruchii* Hornsch. ex Brid.) and 465 (*U. hutchinsiae* (Sm.) Hammar) MDCs. Among species of *Orthotrichum s.str.* the lowest number of MDCs at the species level was found for *O. rogeri* Brid.

(714). The similar number of MDCs was detected for the *O. cupulatum* complex (1,002) and *O. crenulatum* Mitt. (1,005). The numbers were lower for the variety range of *O. cupulatum*: 190 MDCs were found for var. *cupulatum* and 272 for the newly described var. *lithophilum*. The lower

Table 1 Comparison of different characters of individual varieties occur in Australasia. Relevant different characters of the new variety are highlighted in bold.

Features	<i>O. cupulatum</i> var. <i>cupulatum</i>	<i>O. cupulatum</i> var. <i>austro-cupulatum</i>	<i>O. cupulatum</i> var. <i>lithophilum</i>
<i>Lamina</i>	Unistratose	Unistratose	Often bistratose
<i>Capsule position</i>	Immersed to immersed-emergent	Exserted	Exserted
<i>Capsule shape</i>	Ovoid-urceolate	Cylindric ovoid	Cylindric ovoid to oblong-ovoid
<i>Endostome</i>	Rudimentary	Well-developed (as tall as exostome)	Absent
<i>Vaginula</i>	Naked	Naked	Covered by long and papillose hairs
<i>Spores</i>	19–22.5 µm	Not specified	15–19 µm

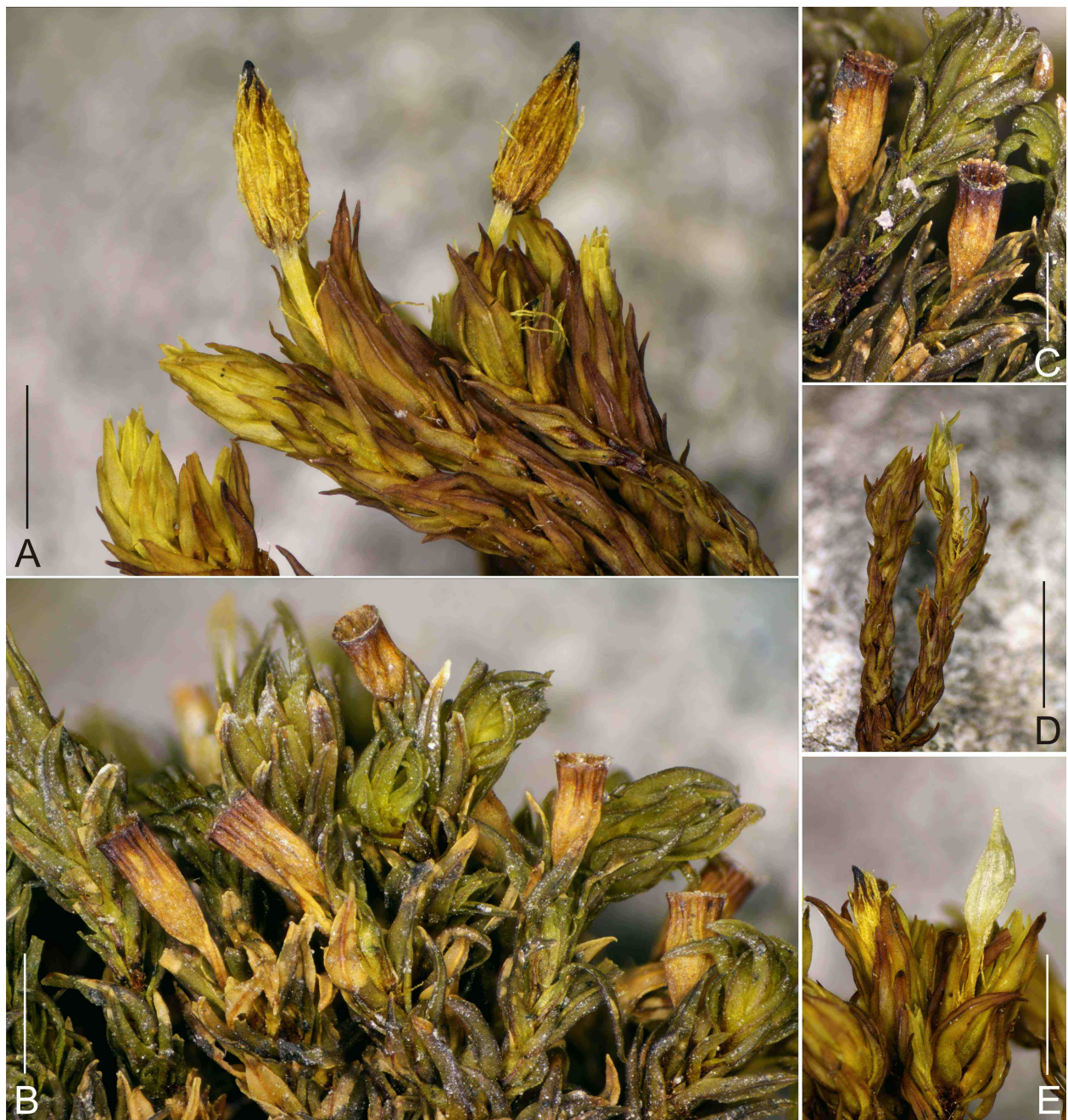


Figure 3 Macro photographs of *Orthotrichum cupulatum* var. *lithophilum*. **A, B** – habit, **C** – mature capsules, **D, E** – fertile plants with uncovered long vaginula hairs. Scale bars: **A–C, E** – 1 mm, **D** – 1 cm. Photographs were taken from the holotype (HO556429).

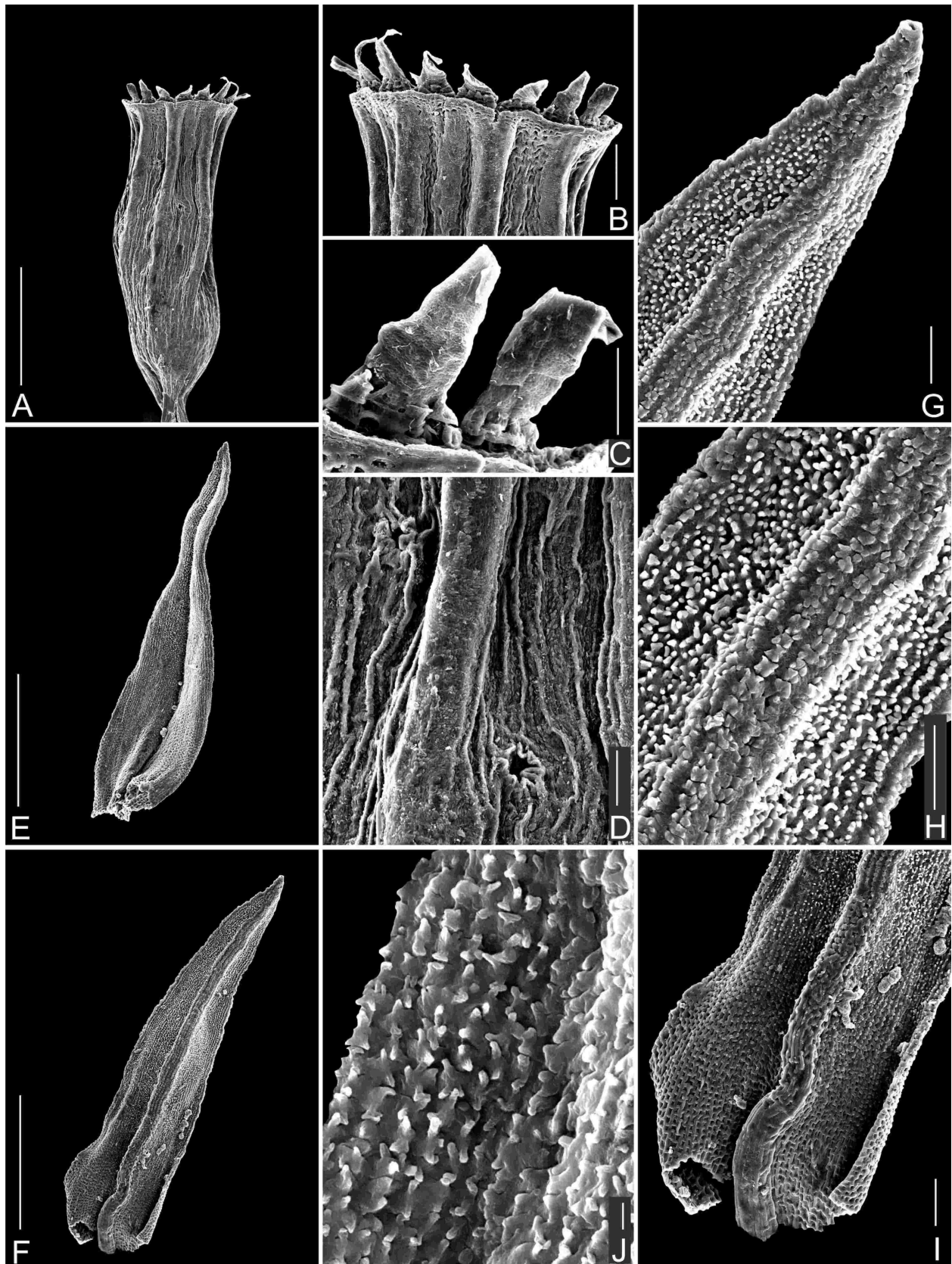


Figure 4 SEM photographs of *Orthotrichum cupulatum* var. *lithophilum*. A – capsule, B – single peristome, C – detailed OPL view on exostome teeth and one preperistome fragment covering the outer base of the tooth (left edge), D – cryptoporous stomata, E – adaxial view of stem leaf, F – abaxial view on stem leaf, G – I – detail views of laminar cells, abaxial views (G - upper part, H - middle part, and I - base of leaf), J – branched papillae on laminar cells. Scale bars: A – 0.5 mm, B – 100 μ m, C, D – 50 μ m, E, F – 0.5 mm, G–I – 50 μ m, J – 10 μ m. Photographs were taken from the holotype (HO556429).

number of MDCs were found for species of *Lewinskya*. With the exception of *L. incana* (Müll. Hal.) F. Lara, Garilleti & Goffinet (1,270), the analyzed species have at least a twofold lower number including 326 for *L. speciosa* (Nees) F. Lara, Garilleti & Goffinet, 485 for *L. hookeri* (Wilson ex Mitt.) F. Lara, Garilleti & Goffinet and 520 for *L. rupestris* (Schleich. ex Schwägr.) F. Lara, Garilleti & Goffinet.

4. Discussion

Three species of the genus *Lewinskya* (*L. hortensis* (Bosw.) F. Lara, Garilleti & Goffinet, *L. rupestris*, and *L. tasmanica* (Hook.f. & Wilson) F. Lara, Garilleti & Goffinet) and two from *Orthotrichum* (*O. cupulatum* and *O. longithecum* R. Br. bis., subsequently synonymized with *O. assimile* Müll. Hal.) have previously been recorded from Tasmania (Lewinsky, 1984a,b). The differences in characteristics among the cryptoporus species are significant. *Orthotrichum cupulatum* typically forms cushions on rock surfaces, while *O. assimile* (syn. *O. longithecum*) thrives on tree trunks. In the former species, the endostome is mostly rudimentary or entirely absent (with the exception of var. *austro-cupulatum*), whereas the latter species possesses eight well-developed segments in its endostome. Additionally, the newly described variety differs from these aforementioned taxa due to the presence of a bistratose lamina and a notably hairy vaginula.

The presence of intraspecific taxa that distinguish *Lewinskya tasmanica* and *Orthotrichum cupulatum* is also known in the region. For the former, two varieties have been described: *L. tasmanica* var. *tasmanica* (Hook.f. & Wilson) F. Lara, Garilleti & Goffinet and *L. tasmanica* var. *parvithecum* (R.Br.bis.) F. Lara, Garilleti & Goffinet. For the latter, they are *O. cupulatum* var. *cupulatum* and *O. cupulatum* var. *austro-cupulatum*. Now, a third variety is newly introduced, *O. cupulatum* var. *lithophilum*.

All three above-mentioned varieties share the same ecological requirements, as they are epilithic species that grow on limestone rocks or stones in exposed habitats. Morphologically, however, they exhibit distinct differences and are, therefore, easily recognizable. For a quick assessment of their distinguishing characteristics, refer to Table 1, which compares individual critical traits. The unique differences that are typical of the newly described variety are highlighted in bold. Even at a macroscopic level, the prominently hairy vaginula is clearly visible, with papillose hairs extending to the base of the capsule (Figure 3A, Figure 3D–E). The vaginula, whether hairy or naked, serves as a commonly used diagnostic feature across the broadly understood genus *Orthotrichum* s.l. A comprehensive analysis discussing the presence or absence of the vaginula in selected species, as well as its genetic determination, was conducted by Plášek and Sawicki (2010).

Under examination with a microscope, a simple peristome, occasionally accompanied by fragments of the preperistome (Figure 4C) can be seen. Furthermore, the new variety is characterized by the presence of a bistratose leaf lamina primarily occurring in the upper half of the leaves. The new variety also exhibits differences in spore size, particularly when compared to *O. cupulatum* var. *cupulatum*. However, the spore size for *O. cupulatum* var. *austro-cupulatum* was not indicated in the description (cf. Lewinsky, 1984a).

For the sake of completeness, it should be noted that a fourth variety, *O. cupulatum* Brid. var. *austro-americanum* Lewinsky, grows in the southern hemisphere. This taxon was described from Peru (Lewinsky, 1984b) but is also present in mountainous regions of Colombia and Argentina. It is distinguished from all other varieties by the exostome teeth being smooth, never striolate or papillose (Lewinsky, 1984b).

The obtained phylogenetic tree is mostly congruent with previous studies on Orthotricheae phylogeny, which clearly separate Lewinskyinae and Orthotrichinae clades (Draper et al., 2021, 2022; Sawicki et al., 2009, 2017). The main difference between the plastid and mitogenomics datasets is in the relationships of *Nyholmiella* and *Stoneobryum* to *Orthotrichum* s.str. The phylogenomics analysis based on complete mitogenomes poorly supported the common clade of *Stoneobryum* and *Orthotrichum* in ML analysis and unresolved Orthotrichinae intergeneric relationships in the case of the BI method (Sawicki et al., 2017), while the plastomic dataset relates *Nyholmiella* as a sister to *Orthotrichum* and *Stoneobryum* as basal for Orthotrichinae with maximal node support (Figure 2).

The number of detected MDCs for Orthotricheae taxa is hard to compare, since none of the other genera have been analyzed to date. Studies on plastome-based delimitation of liverworts revealed comparable to Orthotrichinae the number of MDCs in the leafy genus *Calypogeia* (Šlipiko et al., 2020, 2022), which ranged from 159 (*C. muelleriana*) to 1,369 (*C. neesiana*), but lower than those detected in thalloid genera like *Conocephalum* (over 2,300) and *Pellia* in which the plastome revealed the presence of over 4,700 MDCs between *P. epiphylla* and *P. neesiana* (Paukszto et al., 2023; Sawicki et al., 2020).

5. Supplementary material

The following supplementary material is available for this article:

Table S1. Species used for phylogenetic and barcoding analyses.

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