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Standard Paper

A new *Calicium* on *Ramboldia*

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Abstract

A lichenicolous species, *Calicium ramboldiicola*, growing on *Ramboldia elabens* is described. In phylogenetic analyses with 22 *Calicium* taxa, based on 121 sequences from five DNA regions (mtSSU, *Mcm7*, nuITS, nuLSU, β-tubulin), the new species formed a strongly supported clade with *C. abietinum* and *C. verrucosum*. Although the ascomata of the new species resemble miniatures of those of *C. abietinum*, no morphological synapomorphies for this clade were found. *Calicium ramboldiicola* is known from boreal and hemiboreal areas of northern Europe and north-eastern North America. It is the second known lichenicolous species of *Calicium* and the first found on a lichen in the *Lecanorales*.

Keywords: Ascomycota; biodiversity; ecology; lichen; lichenicolous; phylogeny; taxonomy

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Introduction

The genus *Calicium* Ach. in *Caliciaceae* Chevall. includes mazaediate fungi (Prieto *et al.* 2013) with stalked ascomata and mostly 1-septate, dark, thick-walled spores with distinctive surface ornamentation. A recent molecular study supports a wider circumscription of the genus to also include some species with sessile ascomata that were previously placed in *Cyphelium* Ach. (Prieto & Wedin 2017). However, *Cyphelium sessile* (Pers.) Trevis. and *C. marcianum* B. de Lesd., which are lichenicolous on species of *Pertusaria* and *Lepra*, were transferred to *Acolium* (Prieto & Wedin 2017).

Regional revisions of *Calicium* species in Australasia (Tibell 1987), Europe (Tibell 1999*a*), North America (Selva *et al.* 2023*a*, *b*), South America (Tibell 1996, 1998) and the Himalayas (Tibell 2006) have contributed descriptions of numerous species, and *Calicium* has thus been rather well investigated on a global scale. With the new circumscription and the recent description of additional species, it is known to include 36 species (Tibell & Knutsson 2016; Prieto & Wedin 2017; Selva *et al.* 2023*a*). Among these is the recently described *Calicium episcalaris* Tibell & Knutsson, parasitic on *Hypocenomyce scalaris* (Ach. ex Lilj.) M. Choisy (Tibell & Knutsson 2016; Selva *et al.* 2023*b*). All other described species of *Calicium* have a distinct, autonomous thallus that varies from thick and verrucose in some species to immersed in others.

Three of the authors were therefore surprised to encounter, independently of each other, what looked like a perfectly 'classical' small *Calicium* growing on the thallus of *Ramboldia elabens* (Fr.) Kantvilas & Elix in boreal and hemiboreal areas of northern Europe and north-eastern North America. The first collection was made by one of us (LT) in 1977 in Sweden. Since then, we have found the species at additional localities in Sweden, Norway and Canada, and we have also detected several other specimens in herbaria.

Material and Methods

Microscopy

Squash preparations and hand-cut sections of apothecia were mounted in water, 10% KOH (K), 1.5% Lugol's solution (I), and lactophenol cotton blue (LCB), and examined with light microscopy (LM). Measurements of ascospores and anatomical characters were made in water and are reported as (minimum–)a–b(–maximum) (n = number of measurements), where 'a' is the arithmetic mean minus 1 standard deviation (SD) and 'b' is the arithmetic mean plus 1 SD. Apothecial dimensions were measured in air-dried specimens examined with a stereomicroscope. For scanning electron microscopy (SEM), air-dried specimens were mounted on stubs, coated with gold in an Edwards S150A sputter coater, and examined at 15 kV in a JEOL2 JSM 6400 instrument at the University of New Brunswick.

Taxon sampling

Five specimens of the new species and a wide selection of species of the genus *Calicium* were included in the phylogenetic analysis (Table 1). Based on previous studies (Prieto & Wedin 2017), three

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members of the *Caliciaceae* (*Diplotomma alboatrum* (Hoffm.) Flot., *Pyxine cocoes* (Sw.) Nyl. and *Pyxine sorediata* (Ach.) Mont.) were used as outgroups.

Molecular techniques

Sequences of *Calicium* were either downloaded from GenBank or produced in the laboratory for the new species. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instructions. Five regions were amplified: nuITS, nuLSU, mtSSU, β-tubulin and *Mcm7*. The nuITS was amplified with the primers ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990), and the nuLSU with LR0R (Rehner & Samuels 1994), LR3, LR3R, LR6 and LR7 (Vilgalys & Hester 1990). We used the primers mtSSU1 and mtSSU3R for amplification of the mtSSU region (Zoller *et al.* 1999), Mcm7-709for and Mcm7-1348rev (Schmitt *et al.* 2009) for amplification of the *Mcm7* region and Bt3-LM and Bt10-LM for the protein coding β-tubulin (Myllys *et al.* 2001).

PCR amplifications were carried out using Illustra™ Hot Start Mix RTG PCR beads (GE Healthcare, UK) in 25 µl, adding 3–6 µl of diluted genomic DNA, 10 µM of each primer and distilled water. The following amplification programme was used: initial denaturation at 95 °C for 15 min, 35 cycles of 95 °C for 45 s, 54–56 °C for 50 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. PCR products were purified with ExoSAP-IT (USB Corporation, Santa Clara, California, USA) and sequenced using the same amplification primers.

Sequences were assembled and edited using Sequencher v. 4.10.1. (Genes Codes Corporation, Ann Arbor, Michigan, USA) and subsequently aligned using MacClade v. 4.01 (Maddison & Maddison 2001). Ambiguous regions (*sensu* Lutzoni *et al.* 2000) and introns were delimited manually and excluded from phylogenetic analyses.

Phylogenetic analyses

Each individual gene region was analyzed using maximum likelihood (ML) implemented in RAxML v. 8.2.12 (Stamatakis 2014) with a GTRGAMMA model for tree inference and bootstrapping with a GTRCAT model and 1000 replicates. Gene-tree incongruence was checked by comparing maximum likelihood bootstrap values (ML-BS) between the individual gene trees. After checking there was no conflict among clades, data were combined into a single concatenated data matrix. The combined maximum likelihood (ML) analysis was run with seven distinct partitions (nuITS, nuLSU, mtSSU, first and second codon positions of the *Mcm7* and β-tubulin and the third codon position of the *Mcm7* and β-tubulin) as individual analyses. All analyses were run on the CIPRES Science Gateway v. 3.3 (Miller *et al.* 2010). The resulting trees were visualized and edited in Figtree v. 1.4.4. (Rambaut 2010).

Results

The concatenated data matrix consisted of 25 taxa, 22 being *Calicium* species, and three outgroups. *Calicium* was represented by 109 sequences, of which 15 are new. In the analysis (Fig. 1), *Calicium* was strongly supported, and within the genus two major clades are apparent. *Calicium ramboldiicola*, together with *C. abietinum* and *C. verrucosum* Tibell, belongs to a strongly supported subclade. The other lichenicolous *Calicium* species, *C. episcalaris*, belongs to the same major clade as *C. ramboldiicola*.

Taxonomy

Calicium ramboldiicola Tibell, S. R. Clayden & Wedin

MycoBank No.: MB 856766

Resembling *Calicium abietinum* but occurring on *Ramboldia elabens*, lacking a distinct thallus, and with smaller apothecia, shorter asci, and ascospores with more coarsely and irregularly cracked ornamentation.

Type: Sweden, Lule Lappmark, Jokkmokk parish, River Pärlälven, W of Lake Nårvejaure, valley of the brook Laddonbäcken, on decorticated fallen *Pinus*, 66°36′22″N, 19°16′54″E, alt. *c.* 360 m, 2021, *Wedin* 9898 (S—holotype; CANL—isotype). The type has a full representation of all five DNA regions used in this study, and was chosen for this reason.

(Fig. 2)

Thallus not evident, immersed in the thallus of *Ramboldia elabens*, not causing obvious necrosis or discoloration of the host lichen.

Apothecia on the areoles and rarely the apothecia of *R. elabens*, stalked to nearly sessile, with a black capitulum and dark olive-brown stalk, shiny except for the black mazaedium, lacking pruina, (0.25–)0.30–0.44(–0.55) mm tall ($n = 30$), all parts K–, I–. *Stalk* 0.08–0.20 mm diam., consisting of densely interwoven thick-walled hyphae with lumina 0.7–2 µm wide, central part dark olive-brown, grading to pale brown to hyaline in outer part, with an outermost clear gelatinous layer 5–10 µm wide. *Capitulum* initially globose to obovoid, becoming flat-topped-cupulate, (0.15–)0.17–0.27(–0.35) mm diam. ($n = 30$). *Excipulum* to *c.* 50 µm thick at base, narrowing to *c.* 20 µm laterally, structure continuous with that of the stalk, of ±isodiametric to somewhat elongated cells with strongly thickened walls, inner part dark olive-brown, outermost hyaline gelatinous layer thinner than on the stalk. *Hypothecium c.* 30–60 µm tall, slightly concave at top, upper part concolorous with inner part of exciple and stalk, lower part hyaline to very pale brown. *Asci* cylindrical to narrowly clavate, (34–)35.8–41.8(–43) × (4–)4.2–5.4(–6) µm ($n = 10$), with uniseriate but somewhat irregularly orientated spores. *Ascospores* initially greenish grey, soon becoming dark brown, ellipsoid, 1-septate, constricted or not at septum, surface initially smooth to finely roughened, developing coarse irregular cracks, (9.5–)10.8–13.9(–16.5) × (5–)5.7–7.2(–8) µm ($n = 73$).

Etymology. Named after the genus of the host lichen, *Ramboldia elabens.*

Distribution. Calicium ramboldiicola is currently known from Norway and Sweden in northern Europe, and from the eastern Canadian provinces of New Brunswick and Québec in northeastern North America. The occurrences in Canada are in intermediate oceanic–continental and continental (OC and C_1) sectors of the hemiboreal bioclimatic zone (*sensu* Tuhkanen 1984; Clayden 2010). In Scandinavia, *C. ramboldiicola* has a more distinctly boreal distribution. To date, it has been found at eight localities, spanning *c.* 10° of latitude, from the southern to northern sub-zones of the boreal zone and their elevational counterparts as defined by Ahti *et al.* (1968). These occurrences are in areas with intermediate oceanic–continental (OC, O_1) climates, not the more highly oceanic sectors (O_2, O_3) of westernmost Scandinavia.

Further study is needed to determine whether the distribution of *C. ramboldiicola* conforms more fully to that of its host lichen.

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Table 1. Details on taxa used in the phylogenetic analyses, including voucher information and GenBank Accession numbers. New species and sequences are in bold.

		GenBank Accession number				
Species name	Voucher information	mtSSU	*Mcm7*	nuITS	nuLSU	β-tubulin
Calicium abietinum	*Tibell* 25061 (UPS)	KX512971	KX529041	KX512905	KX512872	KX529003
C. adspersum	*Prieto* 3037 (S)	KX512949	KX529055	KX512907	KX512895	KX529022
C. chlorosporum 1	*Tibell* 25012 (UPS)	KX512956	KX529059	-	KX512892	-
C. chlorosporum 2	*Thor* 20859 (UPS)	KX512955	-	-	-	-
C. corynellum	*Prieto* CC1 (S)	KX512985	KX529048	KX512908	KX512855	KX528989
C. denigratum	*Prieto* (S) F209596	KX512965	KX529044	KX512909	KX512878	-
C. episcalaris 1	*Knutsson* (UPS)	-	-	KX228552	-	-
C. episcalaris 2	*Soun* 1013 (ROK)	-	-	OQ717343	-	-
C. episcalaris 3	*Vondrák* 24110 (PRA)	OQ646151	-	OQ717763	-	-
C. glaucellum	*Wedin* 8563 (S)	KX512980	KX529032	KX512910	KX512864	-
C. lecideinum	*Prieto* (S) F473050	KX512961	KX529046	KX512911	KX512882	KX529009
C. lenticulare	*Tibell* 23284 (UPS)	KX512979	KX529033	KX512912	-	KX528997
C. montanum	*van den Boom* 23445 (UPS)	-	-	KX529069	KX512853	-
C. nobile 1	*Tibell* 21968 (UPS)	KX512988	KX529060	KX512913	KX529070	-
C. nobile 2	*Tibell* 23396 (UPS)	KX512987	KX529061	KX512914	KX529071	-
C. notarisii	*Prieto* 3007 (S)	KX512960	KX529047	KX512915	KX512883	KX529011
C. parvum 1	*Vondrák* 26019a (PRA)	OQ682893	-	OQ717345	-	-
C. parvum 2	*Vondrák* 23767 (PRA)	OQ646152	-	OQ717765	-	-
C. pinastri	*Vondrák* 21874/ *Vondrák* 24287 (PRA)	OQ682894	-	OL396663	-	-
C. pinicola 1	*Lendemer & Knudsen* 14982 (UPS)	KX512972	KX529040	KX512916	KX512871	KX529015
C. pinicola 2	*Thor* 19856 (UPS)	KX512991	KX529066	KX512917	KX512887	KX529014
C. quercinum	*Tibell* 22287 (UPS)	-	-	KX512918	KX512854	-
***C. ramboldiicola* 1**	*Wedin* 9898 (S)	**PQ308401**	**PQ161417**	**PQ304346**	**PQ304346**	**PQ150070**
***C. ramboldiicola* 2**	*Johannesson & Wadstein* (UPS)	**PQ308402**	**-**	**PQ304347**	-	**PQ150071**
***C. ramboldiicola* 3**	*Clayden* 28675 (NBM)	**PQ308403**	**PQ161418**	-	-	-
***C. ramboldiicola* 4**	*Wedin* 7234 (UPS)	**PQ308405**	**-**	**PQ304348**	-	-
***C. ramboldiicola* 5**	*Hermansson* 13315 (UPS)	**PQ308404**	**PQ161419**	**PQ304349**	-	-
C. salicinum	*Prieto* (S) F209764	KX512982	KX529027	KX512919	KX512861	KX528991
C. tigillare	*Prieto* 3038 (S)	JX000123	JX000162	JX000104	JX000088	KX529002
C. trabinellum	*Wedin* 8517 (S)	-	KX529026	KX512920	KX512858	KX528995
C. verrucosum	*Tibell* 23198 (UPS)	-	KX529030	-	-	-
C. viride	*Wedin* 24/4 2000	AY584696	JX000153	HQ650703	AY340538	KX529013
Diplotomma alboatrum	*Prieto* 3034 (S)	KX512966	KX529043	KX512924	KX512877	KX529007
Pyxine coccoes	*Prieto* (S) F473049	KX512964	-	KX512936	-	KX529010
P. sorediata	*Wetmore* 91254 (S)	KX512973	KX529039	KX512937	KX512870	KX529001

Ramboldia elabens occurs widely in boreal and temperate-montane areas of the Northern Hemisphere (Kantvilas & Elix 2007). In North America, it has a mainly Appalachian–Great Lakes distribution, with outliers as far west as the Black Hills of South Dakota (Wetmore 1967). Reports of *R. elabens* from areas of North America west of the Black Hills are based on mis-identifications of other lichens (Pérez-Ortega *et al.* 2010). In Eurasia, it occurs discontinuously from Scandinavia and sub-alpine western and central Europe (Hafellner 1993) to Japan (Inoue 1982) and the Russian Far East (e.g. Konoreva *et al.* 2018).

Ecology. *Calicium ramboldiicola* has been found exclusively on the crustose lichen *Ramboldia elabens* growing on hard exposed wood ('lignum') of conifers, including standing snags and fallen

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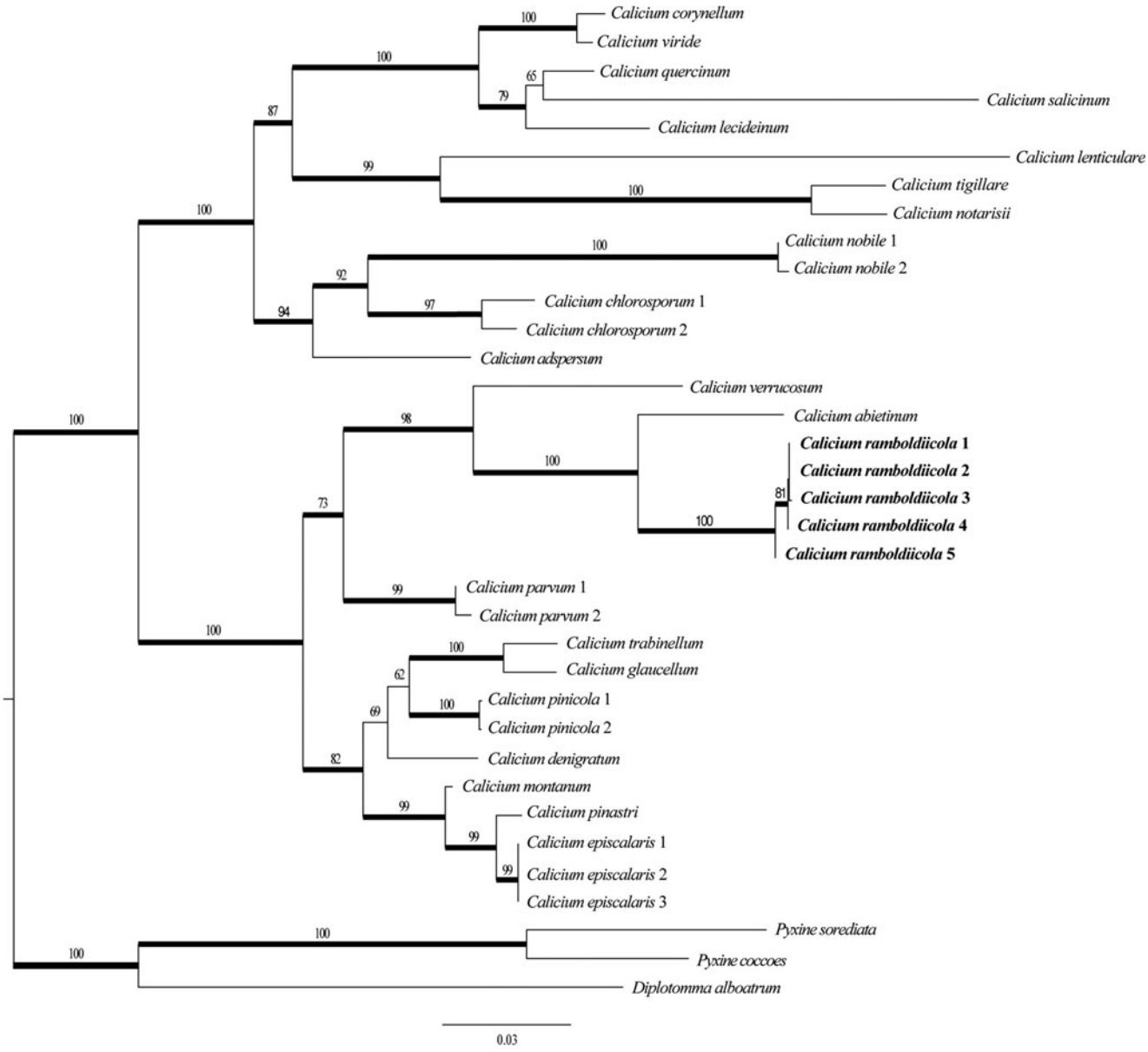

Figure 1. Phylogeny of *Calicium* using a combination of five loci (nuITS, nuLSU, mtSSU, *Mcm7* and β-tubulin) resulting from maximum likelihood (ML) in RAxML, with bootstrap values shown above nodes. Strongly supported branches (ML ≥ 70) and *C. ramboldiicola* are shown in bold.

trees. *Ramboldia elabens* also occurs less frequently on hard, weathered wood of built structures such as old barns or fences, but no occurrences of *C. ramboldiicola* are known on host thalli occupying such substrata. In Scandinavia, all samples are from very old *Pinus sylvestris* L. snags in old-growth forest localities. In New Brunswick, the habitat at one of the localities is an open, south-west-facing, granitic boulder talus slope with patches of *Picea rubens* Sarg. and *Pinus strobus* L. At the other, the habitat is an open, wet, ombrotrophic bog, with scattered low thickets of *Larix laricina* (Du Roi) K. Koch and *Picea mariana* (Mill.) Britton *et al.* Associated lignicolous lichens at one or both localities in New Brunswick include *Imshaugia aleurites* (Ach.) S. L. F. Mey., *Mycoblastus alpinus* (Fr.) Kernst., *M. sanguinarius* (L.) Norman, *Ochrolechia mahluensis* Räsänen, *O. pseudopallescens* Brodo, *Pertusaria sulcata* Dibben, *Xylographa disseminata* Willey, and *X. vitiligo* (Ach.) J. R. Laundon.

Although the thallus of *R. elabens* is often moderately thick, with contiguous, somewhat lumpy areoles contrasting with the black, shiny apothecia, it can also be thin and even to mostly endoxylic towards the thallus margin or more extensively. Apothecia of *Calicium ramboldiicola* are present mainly on well-developed areoles of *R. elabens* (Fig. 2A), but also on more or less endoxylic portions of some thalli (Fig. 2C), and less commonly on the host apothecia. Because of the small amount or age of the material available for study, we have not examined areoles histologically to assess whether the hyphae of *C. ramboldiicola* are distinguishable from those of *R. elabens*, or whether there are differences in the abundance or appearance of photobiont cells in areoles of *R. elabens* with or without apothecia of *C. ramboldiicola.* Older apothecia of *R. elabens* sometimes develop uneven or wrinkled discs, but these changes occur in thalli with or without *C. ramboldiicola.* Apothecia of *C. ramboldiicola* are sporadically present on

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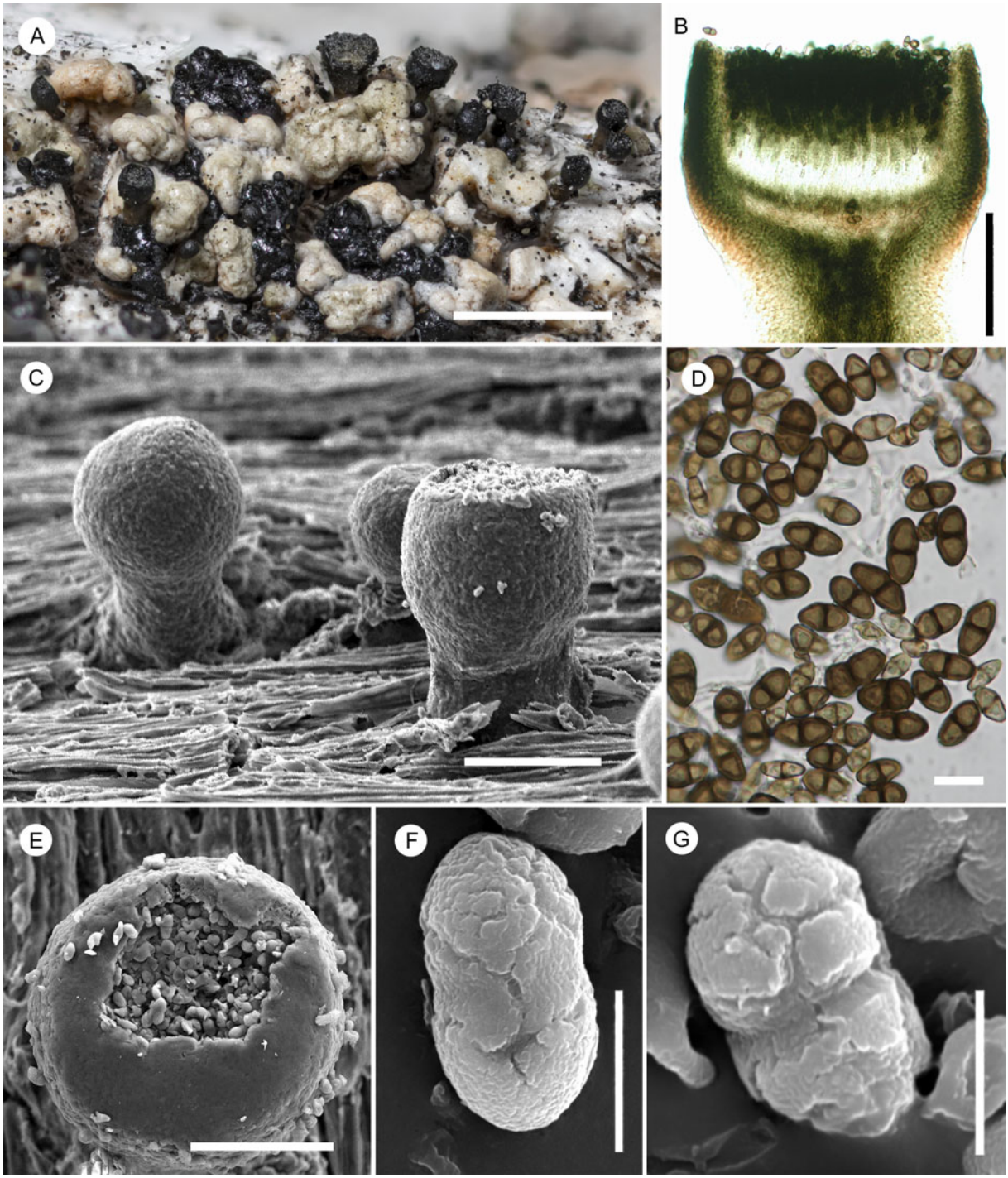

Figure 2. *Calicium ramboldiicola*. A, apothecia arising from thallus and apothecia of *Ramboldia elabens* (*Clayden* 21343). B, longitudinal section of mature apothecium, LM (*Clayden* 21343). C, immature and mature apothecia, arising from thin to endoxylic area of thallus of *R. elabens*, SEM (*Clayden* 24619). D, mature ascospores, liberated from asci, LM (*Clayden* 28675). E, capitulum in oblique view, with wall rupturing apically and exposing the mazaedium, SEM (*Clayden* 24619). F, mature ascospore with surface ornamentation formed by irregular cracking, SEM (*Clayden* 21343). G, mature ascospore constricted at septum and with coarsely ornamented surface, SEM (*Clayden* 21343). LM = light microscopy; SEM = scanning electron microscopy. Scales: A = 1 mm; B & C = 100 μm; D = 10 μm; E = 50 μm; F & G = 5 μm. In colour online.

eroded or senescent areoles of the host lichen, but these occurrences may be accidental. That is, the deterioration of such areoles could have been caused by agents other than the *Calicium*.

Additional specimens examined. **Canada:** *New Brunswick*: Kings Co., Canadian Forces Base Gagetown, Nerepis Hills, 1 km NE of confluence of Askwith Brook with Nerepis River, 45.442°N, 66.298°W, alt. 140 m, on decorticated snag of *Picea rubens*, 2010, *Clayden* 21343 (NBM); *ibid*., 2022, *Clayden* 28675 (NBM); Sunbury Co., Bull Pasture Bog Protected Natural Area, 46.0524°N, 66.3113°W, alt. 100 m, lignicolous on snag of *Larix laricina*, 2014, *Clayden* 24619 (NBM). *Québec*: Parc de la

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Vérendrye, Réservoir Cabonga, Îles Bruyères, 47°14′30″N, 76° 34′30″W, sur un vieux tronc de conifère en bordure du réservoir, 1991, *Bastien* 789 (QFA).—**Norway:** *Troms*: Storfjord, 13.5 km SSE of Skibotn, along Skibotnelva, above Gustavsvingen, 69°16′N, 20°28′E, alt. 120–200 m, 2003, *Wedin* 7234 (UPS). *Buskerud*: Nore of Uvdal, Viken, Godfarfoss S, 60.4312°N, 8.605°E, 2021, *Reiso* (O L-2289081).—**Sweden:** *Dalarna*: Los parish, Gönhammaren, on dry, still standing *Pinus*, *Forsslund* (UPS); Ore parish, Jässelåsen, Rärmyren, 61° 14′N, 15°16′E, alt. 370 m, on dry, still standing *Pinus* snags, 2003, *Hermansson* 13315 (UPS); Svärdsjö parish, E of Orrtjärnen, on dry snag in a bog, 2024, *Skog* (S). *Jämtland*: Undersåker parish *c.* 15.5 km S of Undersåker church, south side of Lake Håckrenmagasinet, *c.* 570 m E of Furubergskojan, 63.19080°N, 13.42355°E, 2020, *Johannesson* & *Wadstein* (UPS). *Lule Lappmark*: Gällivare parish, Linaåive Nature Reserve, E of Parajaure, 67°16′N, 20°26′E, alt. 490 m, on decorticated branches of fallen, dead *Pinus*, 1977, *Tibell* 6916 (UPS); *ibid*., Jokkmokk par., Karats area, SW of Mt Luspevarasj, 66.62713°N, 18.81745° E, elev. 440 m, 2021, *Westberg* SCNB150 (UPS).

Discussion

Fungi with stalked ascomata resembling those of *Calicium* are a heterogeneous group from a phylogenetic standpoint, but they are often studied together and thus form a defined research area (Temu *et al.* 2024). Although lichenicolous species occur in many calicioid genera, especially *Chaenothecopsis* and *Sphinctrina* (both in the *Mycocaliciales*; Tibell & Wedin 2000), *Calicium ramboldiicola* is one of rather few lichenicolous species discovered in *Caliciaceae*. In this family, *Acolium marcianum* (B. de Lesd.) M. Prieto & Wedin and *A. sessile* (Pers.) Arnold are associated with *Pertusaria* and *Lepra* species (*Pertusariaceae* and *Variolariaceae*, respectively; both *Pertusariales*), whereas the species described here is found with *Ramboldia* (*Ramboldiaceae*, *Lecanorales*; Miadlikowska *et al.* 2014). This is only the second *Calicium* species known to occur on *Lecanoromycetes*, the other being *Calicium episcalaris*, on *Hypocenomyce* (*Ophioparmaceae*, *Umbilicariales*). Other genera of *Caliciaceae* including small numbers of lichenicolous species are *Diplotomma* and *Tetramelas* (Cannon *et al.* 2021).

The ascomata of *C. ramboldiicola* are small but otherwise similar to those of other species in *Calicium*, such as *C. glaucellum*. In our analysis (Fig. 1), *C. ramboldiicola* is sister to *C. abietinum*, and indeed resembles a miniature of that species, with much smaller ascomata but different spore ornamentation: *C. ramboldiicola* has an irregularly cracked spore surface, while that of *C. abietinum* presents with minute, distinct warts. The clade including *C. abietinum*, *C. ramboldiicola* and *C. verrucosum* has high support (Fig. 1), but apart from a general similarity in ascoma morphology, we have not been able to find any specific morphological synapomorphies.

Calicium ramboldiicola also resembles *C. pinastri* in its small size, lack of pruina, short cylindrical asci, and spore ornamentation. However, the apothecial stalks in *C. pinastri* are black, lacking the brown hue that is usually present in *C. ramboldiicola*. Also, *C. pinastri* is a mainly corticolous species, in Europe occurring especially on trunks of *Pinus sylvestris* (Tibell 1999*a*, *b*). Our phylogenetic analysis (Fig. 1) indicates that these two species are not closely related to one another within *Calicium*.

Ramboldia elabens is currently red-listed in Sweden and categorized as near-threatened (SLU Artdatabanken 2020). As *C. ramboldiicola* is known only from this host, it makes the new lichenicolous species extremely rare *per se*. Similar to *R. elabens*, it probably occurs only in localities of very high conservation value. Thus, it should be given consideration as a threatened species and probably placed in a higher category than its host.

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