Microcalicium loraasii, a new calicioid fungus from old-growth boreal forest in Norway

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Microcalicium loraasii is described as new to science from old-growth boreal forest in Grane municipality, Northern Norway. It is characterized by its sessile to short stalked, cylindrical ascomata with a greyish white pruina, non-protruding mazaedium and 0–4-septate ascospores. Maximum Likelihood and Bayesian Inference phylogenetic analyses of mtSSU, nrLSU and nrITS sequence data confirm its placement in *Microcalicium*. The species is so far only known from the type locality where it was growing on a decorticated snag of *Pinus sylvestris*. A key to *Microcalicium* worldwide is provided.

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Introduction

The genus *Microcalicium* comprises four known species worldwide, *M. ahlneri* Tibell, *M. arenarium* (Hampe ex Massal.) Tibell, *M. conversum* Tibell and *M. disseminatum* (Ach.) Vain. (Tibell 1978, 1987, 1999). The species of *Microcalicium* share the characteristics of having a well-developed aeruginose mazaedium, ascospores with distinct ornamentation of spirally arranged ridges and an aeruginose pigment in the excipulum that reacts K+ orange. *Microcalicium ahlneri, M. arenarium* and *M. disseminatum* are widely distributed in the Northern Hemisphere (Selva 2014, Tibell 1999, Titov 2001), whereas *M. conversum* is widespread in North-America (McCune 2017, McMullin 2018, McMullin & Arsenault 2016, Selva 2014, Tibell & Ryan 2004). *Microcalicium arenarium*, *M. conversum* and *M. disseminatum* also occur in the Southern Hemisphere (Tibell 1987, 1998).

Microcalicium is the only genus in the Microcaliciaceae (Tibell 1984). Microcaliciaceae is currently classified as a *family incertae sedis* in the Ascomycota (Wijayawardene et al. 2018), while the only phylogenetic studies including *Microcalicium* suggest a sister group relationship with the genus *Varicellaria* in Pertusariales (Prieto et al. 2013) or place the family in a non-resolved Pertusariales polytomy (Beimforde et al. 2020).

Recently two specimens of *Microcalicium* were collected in Nordland, northern Norway which did not fit any of the previously described species. Molecular studies proved that it represented a distinct lineage within the genus. The aim of this paper is to describe this species as new to science.

Material and Methods

This study is based on two separate collections from the same *Pinus sylvestris* snag that are deposited in herbarium TRH. For detailed study of internal structures, we have used a Zeiss Axio Scope A.1 and a Leica DM 1000 Led light microscope with 10×, 40× and 100× magnification. The habitus photos were taken with a Leica M165c Stereo microscope. Spore measurements were made in water. Spore size is given as minimum value-maximum value and as the arithmetic mean of a total of 25 spores from 2 different ascomata. GPS positional data for the specimens are according to the World Geodetic System (WGS84) datum.

Direct PCR amplification and sequencing: Approximate 50 × 50 µm large hand sections of the capitulum were used for direct PCR amplification with the *Thermo Scientific™ Phire™ Plant Direct PCR Kit* following the protocol of the manufacturer. The following primers were used for PCR amplification of the mtSSU (mtSSU1 and mtSSU3R; Zoller et al. 1999), nrLSU (LIC24R and LSU-hypR2; Bendiksby & Timdal 2013, Miadlikowska & Lutzoni 2000) and nrITS (ITS4 and ITS1f; White et al. 1990). Annealing temperatures were 59°C for mtSSU and nrITS, and 57°C for nrLSU. The PCR products were cleaned using the *ExoSAP-IT™ PCR Product Cleanup Reagent* (Applied Biosystems™). Sequencing was done at Eurofins Genomics GmbH, Germany.

Additional sequences of the mtSSU, nrLSU, nrITS were obtained from Genbank for *Microcalicium* spp. and 12 outgroup species in Pertusariales (Table 1).

Alignment: Sequences were aligned with MAFFT as implemented in the Guidance Web Server (Penn et al. 2010). The E-INS-i accurate executable was used and the single-gene alignments were manually corrected for obvious aligning errors. Longer insertions and ambiguously aligned regions were removed prior to the analysis. The resulting three single-gene alignments were tested for conflicting tree topologies using Maximum Likelihood (ML) and Bayesian Inference (BI) analyses with the same settings as for the final analyses. Serious conflict was assumed when deviant tree topologies were supported by ≥ 70% bootstrap values (ML BS) and ≥ 0.95 posterior probabilities (BPP). A partitioned dataset was used for the phylogenetic analyses to enable independent parameter estimation for the three gene loci.

ML and Bayesian analyses: Icmadophilaceae (*Icmadophila ericetorum* and *Dibaeis baeomyces*) were used as operational outgroup for all analyses. Maximum Likelihood and Bayesian inference (Holder & Lewis 2003, Huelsenbeck et al. 2001) were used for inferring phylogenetic hypotheses.

Maximum likelihood was performed with the RAxML-HPC black box v. 8.2.10 implemented in the CIPRES Science Gateway (Miller et al. 2010) using rapid bootstrapping and full ML analysis under the GTR+GAMMA approximation allowing for a proportion of invariable sites. The analysis was stopped after 1000 bootstrap replicates using the bootstopping option implemented in RAxML (Pattengale et al. 2009).

Bayesian analysis was performed with MrBayes 3.2.6 (Ronquist & Huelsenbeck 2003) implemented in the CIPRES Science Gateway (Miller et al. 2010). Models of sequence evolution for the different gene partitions were estimated under the Bayesian Information Criterion (BIC; Stone 1979) implemented in MEGAX (Kumar & al. 2018). Models not supported by MrBayes were substituted with the nearest, less complex supported model. Models set for each partition were: HKY+G (mtSSU), GTR+G+I (nrLSU), K2+G (nrITS1, nr5.8S, nrITS2). Two independent runs were performed for 2.000.000 generations and every 100th generation was sampled. Average SDSF

Table 1. Specimens used for the phylogenetic analyses and their GenBank accession numbers. Newly generated sequences are indicated in bold.

Taxon	Voucher	mtSSU	nrLSU	nrITS
Aspicilia cinerea	USA, Lumbsch 19190c (F)	DQ780272	DQ780304	HQ650637
Coccotrema cucurbitula	Argentina, Vobis s.n. (ESS -20862	AF329161	AF274092	AF329162
C. maritimum	Canada, Brodo 30130 (CAN)	AF329163	AF329164	AF329165
Dibaeis baeomyces	USA, Spribille 38948 (GZU)	KJ462397	KJ462342	KJ462265
Icmadophila ericetorum	USA, Spribille 36042 (GZU)	KJ462399	KJ462344	KJ462267
Lepra albescens	Czech Republic, Schmitt s.n. (ESS-20967)	AF329175	AF329176	AF329177
L. amara	Germany, AFTOL-ID 1067 (F)	AY300900	AF274101	HQ650677
L. scaberula	Australia, Archer s.n. (ESS-20867)	AF431959	AF274099	–
Lobothallia radiosa	Switzerland, Lumbsch s.n. (F)	DQ780274	DQ780306	JF703124
Microcalicium ahlneri	–, Wedin s.n. (S)	JX000126	–	–
M. arenarium	–, Wedin s.n. (S)	JX000127	JX000091	JX000107
M. disseminatum 1	–, Wedin 6353 (UPS)	JX000128	JX000092	–
***M. disseminatum* 2**	Norway, Holien 16273c (TRH-L-19885)	**OM955644**	**OM955630**	**OM955634**
***M. disseminatum* 3**	Norway, Frisch 21/No307 (TRH-L-24755)	**OM955645**	**OM955631**	**OM955635**
M. loraasii	Norway, Lorås s.n. (TRH-L-18239)	–	**OM955632**	**OM955636**
Ochrolechia parella	France, Feige s.n. (ESS-20864)	GU980977	AF274097	AF332123
Varicellaria hemisphaerica	Germany, AFTOL-ID 959 (F)	DQ973000	AF381556	HQ650676
V. velata	Japan, Kashiwadani 45750 (S-F76497)	GU980981	AY300855	JX000109

had fallen to 0.001784 at termination, and the 50% of trees were discarded as burn-in. The remaining trees were summarized in a Bayesian 50% majority rule consensus tree.

Results

Phylogeny: New nrLSU and nrITS sequences were generated for *Microcalicium loraasii*, and mtSSU, nrLSU and nrITS sequences for two specimens of *M. disseminatum*. A single conflict was observed between the nrLSU and the nrITS gene loci with respect to the sister group relationship of *Microcalicium*. In the nrLSU, *Varicellaria* is sister to *Microcalicium* (ML BS 84, BPP 0.94), while *Lepra* is sister to *Microcalicium* in the nrITS (ML BS 87, BPP 0.94). This conflict does neither affect the position of *M. loorasii* within *Microcalicium* nor the placement of the genus in Pertusariaceae, and the three single-gene alignments were analysed together. The final concatenated alignment comprised 2286 nucleotide positions (mtSSU 720, nrLSU 918, nrITS 648). Of these, 845 nucleotide positions were variable (mtSSU 265, nrLSU 228, nrITS 352) and 607 phylogeny-informative (mtSSU 175, nrLSU 167, nrITS 265).

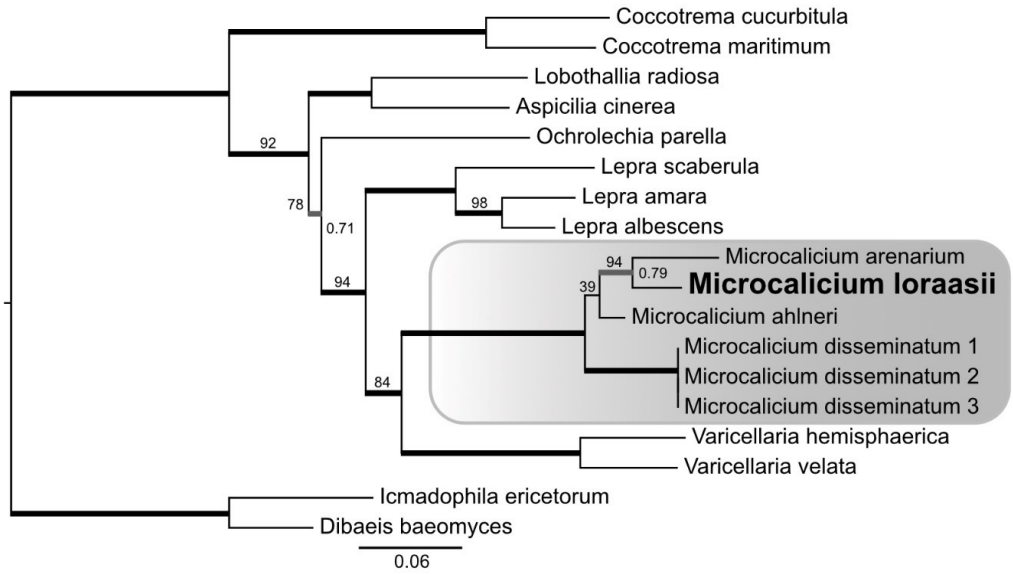

Figure 1. Maximum likelihood tree of selected species of Pertusariales. Branches supported by ML BS ≥ 70 and BPP ≥ 0.95 are indicated by bold black lines. Branches supported by ML BS ≥ 70 only are indicated by bold grey lines. ML BS values < 100 and and BPP values < 1 are stated for each node. *Microcalicium loraasii* is indicated in bold. The family Icmadophilaceae is the operational outgroup.

Microcalicium loraasii falls in the *Microcalicium* clade (Fig. 1). The sister relationship with *M. arenarium* is supported by the RaxML analysis (ML BS 94) but not by the Bayesian analysis (BPP 0.79). *Microcalicium* is sister to *Varicellaria* in Pertusariaceae (ML BS 84, PBB 1) and nested within Pertusariales.

Taxonomy

***Microcalicium loraasii* Holien & Frisch sp. nov.** Figs 2, 3

Mycobank: MB 843264.

Diagnosis: Resembling *Microcalicium conversum* and *M. disseminatum* but differs by having more cylindrical ascomata with a distinct greyish white pruina, non-protruding mazaedium, up to 4-septate, often somewhat curved ascospores and by growing on hard pine wood.

Type: Norway, Nordland, Grane, Danielåsen W, 65.54868°N, 13.60418°E, alt. ca 340 m, on dead wood on a snag of *Pinus sylvestris* in an old-growth coniferous forest, 27.05.2021, H. Holien 16188 & J. Lorås (TRH holotype L-19863; UPS isotype).

Etymology: *Loraasii* – named in honour of Mr. Jostein Lorås, Nesna for his tremendous effort in mapping old-growth forests in the southern part of Nordland county, Grane municipality in particular.

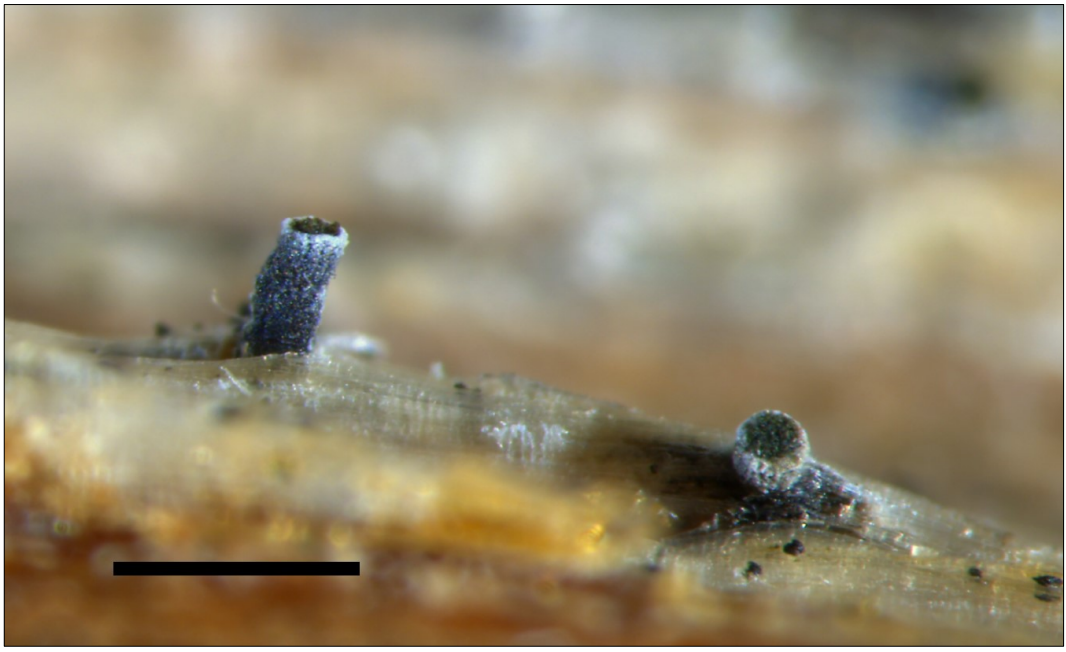

Figure 2. Ascomata of *Microcalicium loraasii* (TRH L-18239). Scale = 0.5 mm. Photo: A. Frisch.

Description: Thallus apparently saprobic, indistinct, no algae observed. Hyphae immersed in the substrate. Ascomata sparse to numerous, sessile to short-stalked (stalk to 0.15 mm), cylindrical, 0.1–0.4 mm high. Capitulum 0.08–0.16 mm diameter (n = 10). Excipulum aeruginose, outer wall with a distinct greyish white pruina. Mazaedium greenish black, slightly convex but not protruding. Paraphyses slightly sclerotized, persistent. Asci in chains, ellipsoidal, 16–21 × 5–7 µm. Ascospores aeruginose, non-septate, with distinct ornamentation of spirally arranged ridges, sometimes slightly curved, 7–13.5 × 3–5 µm, mean 10.74 × 4.12 (n = 25); ageing spores up to 4-septate and up to 18 µm long. Pycnidia numerous, black, somewhat irregular in shape, ca 60–85 µm in diameter. Wall 7–10 µm wide, with aeruginose pigment. Conidiogenous cells 5–7 × 2–2.5 µm. Conidia colourless, broadly ellipsoid with rounded ends, ca 3–4 × ca 2 µm.

Chemistry: Aeruginose pigment in the excipulum and pycnidial wall reacting K+ orange brown.

Distribution and habitat: *Microcalicium loraasii* is only known from the type locality just outside the newly established Danielåsen nature reserve in Grane municipality, Nordland county, Norway (Holien et al. 2018, Lovdata 2017). The forest can be characterized as old-growth mixed coniferous forest with *Picea abies* and *Pinus sylvestris*. The vegetation is quite poor and dominated by heather, *Calluna vulgaris,* in the pine areas and bilberry, *Vaccinium myrtillus*, in the spruce areas. The forest is naturally regenerated after a large fire in 1831 (Holien et al. 2018). *Microcalicium loraasii* was growing on hard wood of a decorticated snag of *P. sylvestris* (see Fig. 4). Among interesting associated lichens on the snag were the redlisted *Acolium inquinans*, *Calicium denigratum* and *Ramboldia elabens*, as well as a presumably undescribed species of the genus *Chaenothecopsis*.

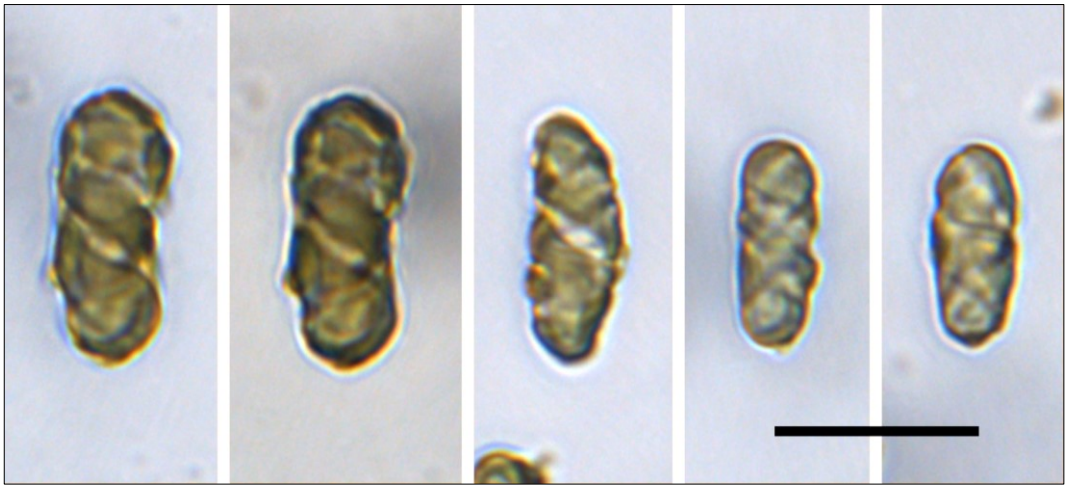

Figure 3. Ascospores of *Microcalicium loraasii* (TRH L-18239). Scale = 10 µm. Photo: A. Frisch.

Additional specimen examined (paratype): Norway, Nordland, Grane, Danielåsen W, 65.5487°N, 13.6042°E, alt. ca 340 m, on dead wood, snag of *Pinus sylvestris*, in old-growth coniferous forest, 13.04.2018, J. Lorås (TRH L-18239).

Key to Microcalicium

1. Ascomata with a distinct stalk 2
– Ascomata mostly sessile or short stalked 3

2. Ascomata with stalks, 0.6–1.8 mm high, mazaedium without sclerotized hyphae . . *M. arenarium*
– Ascomata with stalks, 0.4–1.1 mm high, mazaedium with sclerotized hyphae . . . . . *M. ahlneri*

3. Ascomata pruinose, mazaedium non-protruding . . . . . *M. loraasii*
– Ascomata non-pruinose, mazaedium protruding 4

4. Mature ascospores mostly 1–3(-7)-septate . . . . . *M. disseminatum*
– Mature ascospores mostly 1-septate . . . . . *M. conversum*

Discussion

Microcalicium loraasii is a rather distinct species both habitually and microscopically. It is easily identified, even in the field, based on its sessile to short stalked, cylindrical ascomata with a distinct greyish white pruina on the excipulum and non-protruding greenish black mazaedium. It seems to belong to a community on quite hard wood of pine snags with several other interesting species. As the amount of old pine snags has declined substantially during the last 150 years (Holien et al. 2018 & 2020) and is still declining, the species is most certainly rare and should be considered for the Norwegian Red List. It cannot be excluded, however, that the species may also occur in the anamorphic stage only and is overlooked. This should be studied further.

The phylogenetic placement of *Microcalicium* in Pertusariales (Fig. 1) is in line with previous phylogenetic studies (Beimforde et al. 2020, Prieto et al. 2013), as is the placement of the genus as

Figure 4. The locality for *Microcalicium loraasii* in Grane municipality, Nordland county, showing the *Pinus sylvestris* snag where the holotype was collected. Photo: H. Holien.

sister to *Varicellaria* (Prieto et al. 2013). In our analyses, *M. loraasii* is sister to *M. arenarium* (Fig. 1). This placement is supported by RAxML only and the phylogenetic relationships within *Microcalicium* in general are poorly resolved.

Microcalicium arenarium is easily distinguished from *M. loraasii* by the distinctly stalked apothecia, reddish brown excipular pigments, and the smaller (6–8 × 2–3 µm), persistently 1-septate ascospores (Tibell 1978). In its morphology, *M. loraasii* most closely resembles *M. disseminatum*. This species shares the sessile to only shortly stalked apothecia with an aeruginose pigmentation in the exciple, sclerotized and persistent paraphyses, and ascospores with more than 1 transverse septum at least when old. In addition, the two species are known to produce numerous pycnidia, a character that is further known from *M. conversum* but unknown in *M. ahlneri* and *M. arenarium* (Tibell 1978).

Microcalicium loraasii can be separated from *M. disseminatum* by apothecia with a distinct whitish pruina along the edge of the exciple, a non-protruding mazaedium and slightly smaller ascospores (7–13.5 × 3–5 µm, 0-septate vs. 11–16 × 4–6 µm, (1–) 3-septate); occasional old ascospores have been observed in *M. loraasii* that are up to 18 µm long and up to 4-septate, while ageing ascospores up to 28 µm long and 4–5-septate have been reported for *M. disseminatum* (Tibell 1978). The only other described species in *Microcalicium* having sessile to shortly stalked apothecia, *M. conversum*, differs by the reddish brown exciple, 1-septate, smaller ascospores of 8.9–11.1 × 2.8–3.4 µm, the reddish brown, K+ aeruginose pigment of the pycnidial wall and narrowly elliptical conidia of 2–3 × 1 µm (Tibell 1978).

Microcalicium conversum was synonymized with *M. disseminatum* by Tibell (1998), but as *M. conversum* has been treated at species level in North America recently (McCune 2017, McMullin & Arsenault 2016, McMullin 2018, Selva 2014, Tibell & Ryan 2004) we followed their treatment. If *M. conversum* is a species different from *M. disseminatum* needs further study.

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