Phytotaxa 307 (2): 113–122
http://www.mapress.com/j/pt/

Article

ISSN 1179-3155 (print edition)
PHYTOTAXA
ISSN 1179-3163 (online edition)

https://doi.org/10.11646/phytotaxa.307.2.2

Calycina alstrupii sp. nov. (Pezizellaceae, Helotiales), a new lichenicolous fungus from Norway

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Abstract

A new species of *Calycina*, *C. alstrupii* Suija & Motiejūnaitė, is described based on both morphological and molecular characteristics. The new fungus inhabits thalli of *Lobaria pulmonaria* (L.) Hoffm. and is the first proven lichenicolous species of the genus. The new species is compared with closely related taxa of Hyaloscyphaceae *s. lato.*

Key words: Peltigerales, phylogeny, taxonomy

Introduction

During the Nordic Lichen Society (NLS) meeting in 2015 in Nord-Trøndelag (Norway), in Atlantic rainforest with dominant *Picea abies* (Holien *et al*. 2016), we each independently collected thalli of *Lobaria pulmonaria* (L.) Hoffm. (Peltigerales, Lecanoromycetes) infected with an unknown helotialean fungus. Species of *Lobaria* as well as other members of Peltigerales are known to host a large suite of lichenicolous fungi (Hawksworth 1980, Etayo & Diederich 1996, Martínez & Hafellner 1998), including some assigned to Helotiales (Lawrey & Diederich 2016). However, no published descriptions of any known helotialean lichenicolous fungi (Kondratyuk & Galloway 1995, Etayo & Diederich 1996, Huhtinen & Santesson 1997, Huhtinen *et al*. 2008) corresponded to our specimens. To clarify the systematic position of the unknown fungus, we analyzed its morphology as well as fast- and slow-evolving ribosomal and protein-coding markers, and describe a new species based on the results.

Materials and methods

Morphology

We examined dried herbarium specimens by standard techniques, using a Leica S4E stereomicroscope and a Leica DM750 light microscope. Ascomatal microstructures were examined using razor-blade-cut sections mounted in tap water, in a 10% aqueous solution of potassium hydroxide (KOH; K), in Cresyl Blue (CRB), in Congo red and in Lugol's solution (I). The apical apparatus was observed in Lugols solution (I) pretreated with K (denoted as K/I). Measurements were made in water and the sizes are presented as (minimum–)average(–maximum) value.

DNA extraction, PCR amplification and DNA sequencing

Genomic DNA was extracted from the ascomata of specimens (three ascomata per reaction) using High pure PCR Template Preparation Kit (Roche Applied Science®) and following the protocol provided by the manufacturer with minor modifications. We amplified four gene loci—internal transcribed spacer (rDNA ITS) using primer pairs ITS0F (Tedersoo *et al*. 2008) and ITS4 (White *et al*. 1990), large subunit nuclear ribosomal RNA gene (nuLSU) with LROR and LR7 (Vilgalys & Hester 1990), mitochondrial small subunit ribosomal RNA gene (mtSSU) with mrSSU1 and mrSSU3R (Zoller *et al*. 1999) and RNA polymerase II largest subunit gene (RPB1) with primers RPB1-Afasc and

Accepted by Steven Leavitt: 20 Apr. 2017; published: 23 May 2017

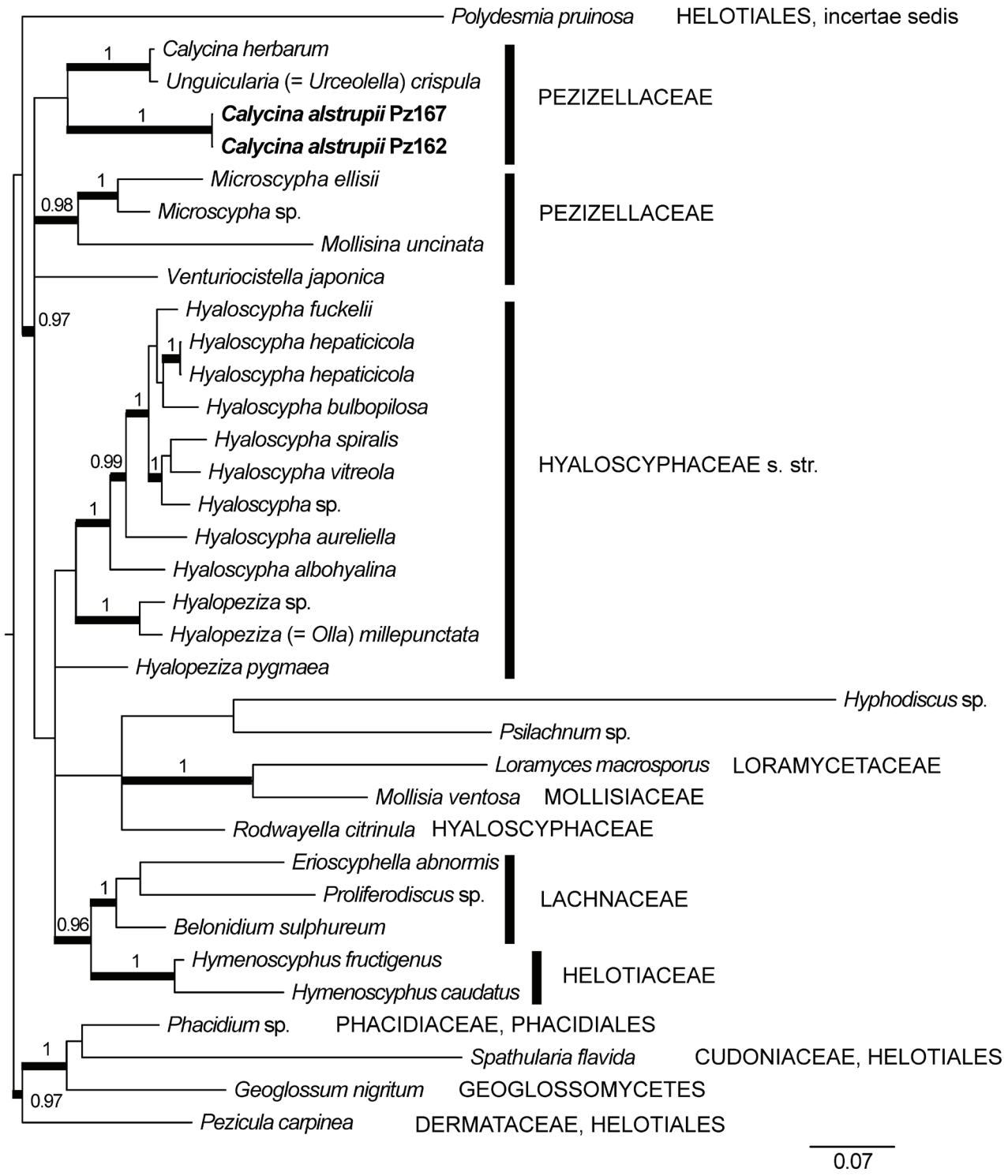

FIGURE 1. Two locus (nuLSU+mtSSU) 50% majority rule consensus tree of selected members of Helotiales based on Bayesian approach and showing the position of *Calycina alstrupii*. The branches with posterior probabilities (PP) ≥ 0.95 are considered as supported. The abbreviations Pz162 and Pz167 at the tips of *C. alstrupii* denote lab codes of holo- and paratype respectively.

RPB1-6Rasc (Hofstetter *et al*. 2007). The PCR reaction mix (25 µl) consisted of 5 µl 5x HOT FIREPol Blend Master Mix (Solis BioDyne, Tartu, Estonia), 0.5 µl of both primers (all 20 µM), 1–8 µl of target-DNA and the remainder distilled water. The PCR products were visualized on a 1% agarose gel stained with ethidium bromide. 1 µl of FastAP and 0.5 µl of Exonuclease I (Thermo Scientific, Waltham, Massachusetts, USA) were added to each tube per 20 µl of the product for the purification of PCR products. Both complementary strands were sequenced in Macrogen Inc. (Amsterdam, the Netherlands) with the same primers as used for amplification except that instead of ITS0F and LROR, ITS5

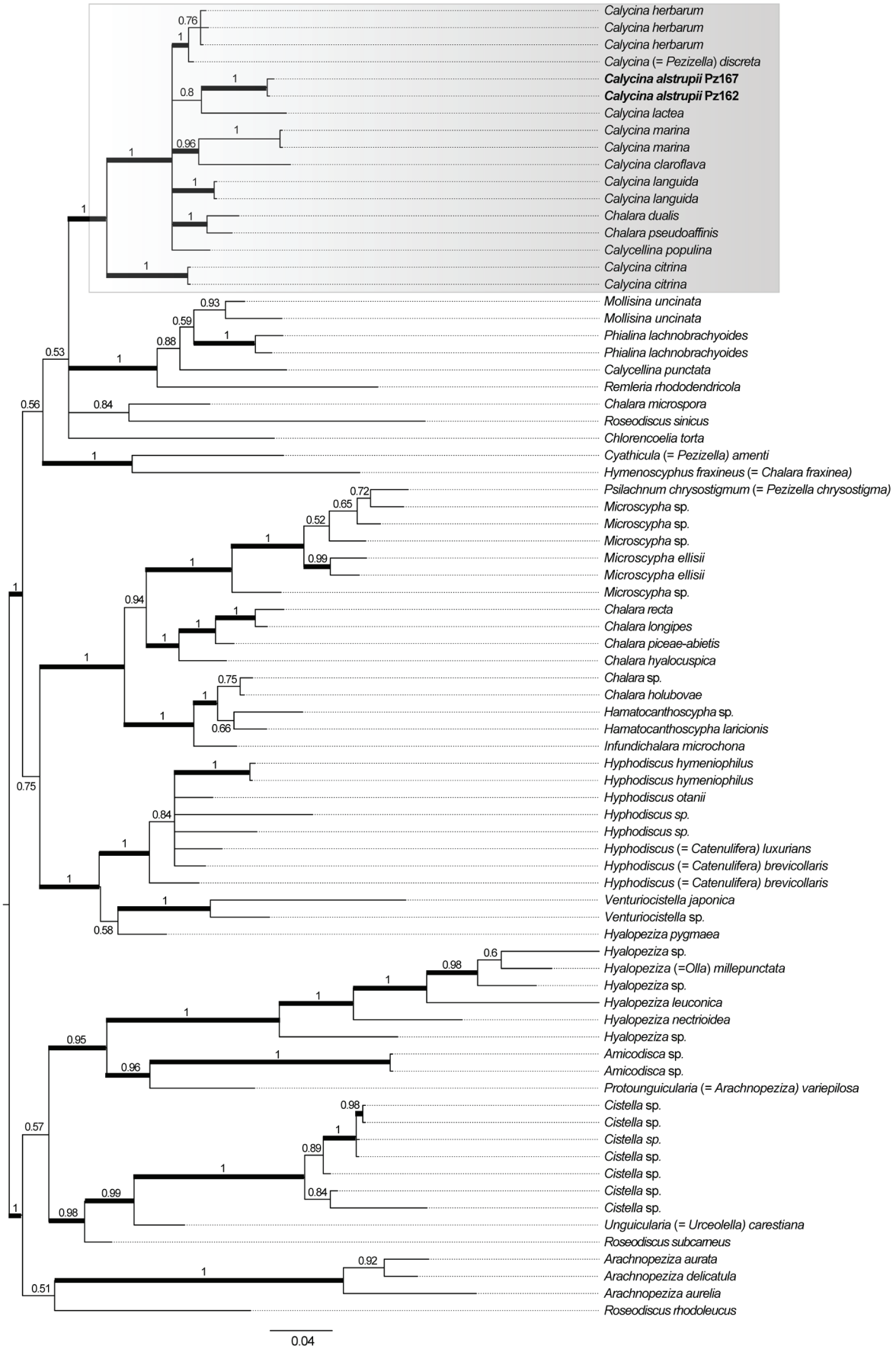

FIGURE 2. A Bayesian phylogeny based on rDNA ITS sequences of selected members of Hyaloscyphaceae *s. lato* and showing *Calycina alstrupii* within *Calycina*-clade. The branches with posterior probabilities (PP) ≥ 0.95 are considered as supported. The abbreviations Pz162 and Pz167 at the tips of *C. alstrupii* denote lab codes of holo- and paratype respectively.

(White *et al*. 1990) and CTB6 (Garbelotto *et al.* 1997) were used respectively. Sequencher 4.10.1. (GeneCodes Corp.®, Ann Arbor, MI, USA) was used to check, assemble and manually adjust the resulting sequence fragments. The consensus sequences were compared with those freely available in NCBI (https://www.ncbi.nlm.nih.gov/) using a BLAST search to confirm their identity. We did not use the RPB1 dataset because none of the sequences retrieved from GenBank after BLAST searches exceeded 80% similarity.

The phylogenetic analyses

The newly generated sequences and sequences downloaded from the GenBank (Table 1), based on the top-scoring match, were aligned with MUSCLE (Edgar 2004) and manually checked and trimmed with SeaView v. 4.6 (Gouy *et al*. 2010). For each gene, the best-fit nucleotide substitution model was calculated and selected, based on the lowest value of AIC (and AICc) criterion with jModeltest v. 2.1.6. (Darriba *et al*. 2012). For mtSSU, the best-fit model was TPMuF+I+G; for nuLSU, it was TrN+I+G; and for ITS, SYM+I+G. Each gene locus was aligned and analysed separately with a Maximum Likelihood (ML) approach using PhyML (Guindon *et al*. 2010) and, as no topological conflict was detected in supported clades (bootstrapping over 100 replicates) by visual inspection (data not shown), mtSSU and nuLSU sequences were then concatenated. Bayesian analyses of the concatenated nuLSU+mtSSU and the rDNA ITS alignments were run separately using the Markov Chain Monte Carlo (MCMC) approach implemented in MrBayes v. 3.2.1. (Ronquist *et al*. 2012). For each analysis, two parallel simultaneous runs were applied with four-chain runs over 600,000 generations for rDNA ITS, and over 850,000 generations for nuLSU+mtSSU datasets, starting with a random tree until the convergence of the chains was confirmed by the standard deviation of split frequencies reaching 0.01. Sampling was done after 500 steps; the first 25% of saved data was discarded as 'burn in'; the 50% majority-rule consensus tree and posterior probabilities (PP) were calculated from the rest of trees. For the first analysis, *Geoglossum nigritum* (Pers.) Cooke (Geoglossomycetes), *Phacidium* sp. (Phacidiales, Leotiomycetes), together with *Spathularia flavida* Pers. (Cudoniaceae, Helotiales, Leotiomycetes) and *Pezicula carpinea* (Pers.) Tul. ex Fuckel (Dermataceae, Helotiales, Leotiomycetes) were chosen to root the tree.

Phylogenetic trees were visualized in FigTree v. 1.4.2 (http://tree.bio.ed.ac.uk/) and Adobe Illustrator CS3® was used for artwork.

TABLE 1. Names of fungal taxa and NBCI accession numbers of rDNA nuLSU and ITS, and mtSSU sequences used in this study.

Taxon name	nuLSU	mtSSU	ITS
Amicodisca sp.	-	-	JN033389
Amicodisca sp.	-	-	JN033411
Arachnopeziza aurata	-	-	JN086847
Arachnopeziza aurelia	-	-	JN086785
Arachnopeziza delicatula	-	-	JN086877
Arachnopeziza variepilosa	-	-	EU940163
Catenulifera brevicollaris	-	-	NR_121471
Calycellina populina	-	-	JN033382
Calycellina punctata	-	-	U57494
Calycina citrina	-	-	KC412005
Calycina citrina	-	-	KC412004
Calycina claroflava	-	-	KC412006
Calycina herbarum	-	-	AY348594
Calycina herbarum	-	-	JN033390
Calycina herbarum	JN086710	JN086783	JN033407
Calycina lactea	-	-	KC412007
Calycina languida	-	-	KC412002
Calycina languida	-	-	KC412003
Calycina marina	-	-	KT185677
Calycina marina	-	-	KT185675
Catenulifera brevicollaris	-	-	AB190385
Catenulifera luxurians	-	-	NR_121470
Chalara dualis	-	-	EF029209
Chalara fraxinea	-	-	HM140838
Chalara holubovae	-	-	FR667223
Chalara hyalocuspica	-	-	FR667221

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TABLE 1. (Continued)

Taxon name	nuLSU	mtSSU	ITS
Chalara longipes	-	-	FR667214
Chalara microspora	-	-	FR667226
Chalara piceae-abietis	-	-	FR667231
Chalara pseudoaffinis	-	-	FR667224
Chalara recta	-	-	FR667210
Chalara sp.	-	-	JX967099
Chlorencoelia torta	-	-	JN033400
Cistella sp.	-	-	KT268435
Cistella sp.	-	-	KT268837
Cistella sp.	-	-	KT269536
Cistella sp.	-	-	KT269719
Cistella sp.	-	-	KT269975
Cistella sp.	-	-	KT270040
Cistella sp.	-	-	KT270225
Geoglossum nigritum	AY54465	AY544740	-
Hamatocanthoscypha laricionis	-	-	JN033441
Hyalopeziza leuconica	-	-	JN033416
Hyalopeziza nectrioides	-	-	JN033381
Hyalopeziza pygmaea	JN086748	JN086819	JN033448
Hyalopeziza sp.	-	-	JN033439
Hyalopeziza sp.	JN086743	JN086812	JN033442
Hyalopeziza sp.	-	-	JN033449
Hyaloscypha albohyalina var. *albohyalina*	JN086734	JN086799	-
Hyaloscypha albohyalina var. *apiralis*	JN086729	JN086795	-
Hyaloscypha aureliella	JN086697	JN086771	-
Hyaloscypha fuckelii	EU940154	EU940294	-
Hyaloscypha hepaticola	EU940118	EU940266	-
Hyaloscypha hepaticola	EU940150	EU940290	-
Hyaloscypha leuconica var. *bulbopilosa*	JN086726	JN086793	-
Hyaloscypha sp.	JN086735	JN086801	-
Hyaloscypha vitreola	JN086681	JN086758	-
Hymenoscyphus caudatus	JN086705	JN086778	-
Hymenoscyphus fructigenus	EU940157	EU940297	-
Hyphodiscus hymeniophilus	-	-	AB546951
Hyphodiscus hymeniophilus	-	-	AB546948
Hyphodiscus otanii	-	-	AB546949
Hyphodiscus sp.	-	-	JX852362
Hyphodiscus sp.	JN086724	JN086791	JN033421
Infundichalara microchona	-	-	HM036588
Lachnum abnorme	JN086698	JN086772	-
Loramyces macrosporus	JN086686	JN086763	-
Microscypha ellisii	-	-	JN033418
Microscypha ellisii	JN086731	JN086797	JN033428
Microscypha sp.	JN086706	JN086779	JN033420
Microscypha sp.	-	-	JN033425
Microscypha sp.	-	-	KJ735022
Microscypha sp.	-	-	JN033444
Mollisia ventosa	JN086700	JN086774	-
Mollisina uncinata	JN086707	JN086780	JN033404
Mollisina uncinata	-	-	JN033457
Olla millepunctata	JN086683	JN086760	JN033380
Pezicula carpinea	JN086691	JN086765	-
Pezizella amenti	-	-	AJ430398
Pezizella chrysostigma	-	-	JF908572
Pezizella discreta	-	-	JF908571
Phacidium coniferarum	JN086687	JN086764	-
Phialina lachnobrachyoides	-	-	JN033380

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TABLE 1. (Continued)

Taxon name	nuLSU	mtSSU	ITS
Phialina lachnobrachyoides	-	-	JN033424
Polydesmia pruinosa	JN086753	JN086824	-
Proliferodiscus sp.	JN086752	JN086823	-
Psilachnum sp.	JN086718	JN086788	-
Remleria rhododendricola	-	-	KT876986
Rodwayella citrinula	JN086717	JN086787	-
Roseodiscus rhodoleucus	-	-	KT972704
Roseodiscus sinicus	-	-	JX260730
Roseodiscus subcarneus	-	-	KT972714
Spathularia flavida	JN086708	JN086781	-
Trichopeziza sulphurea	JN086701	JN086775	-
Hamatocanthoscypha (uncultured)	-	-	KP109912
Urceolella carestiana	-	-	JN033443
Urceolella crispula	JN086682	JN086759	-
Venturiocistella japonica	AB546954	JN086818	JN033447
Venturiocistella sp.	-	-	JN033391

Results

Phylogenetic analyses

The single-gene and the combined nuLSU+mtSSU based analyses confirmed the relationship of the specimens studied with Helotiales, and indicated relationships with Hyaloscyphaceae *s. lato* (PP=0.97; Fig. 1). The extended rDNA ITS dataset with the most representative set of sequences of Hyaloscyphaceae *s. lato* positioned the newly generated sequences into a well-supported (PP=1) though internally unresolved *Calycina*-clade (Fig. 2), being sister to *Calycina lactea* (Ellis & Everh.) Baral, R. Galán & G. Platas (syn. *Bisporella lactea* (Sacc.) Stadelman). However, this relationship remains unsupported.

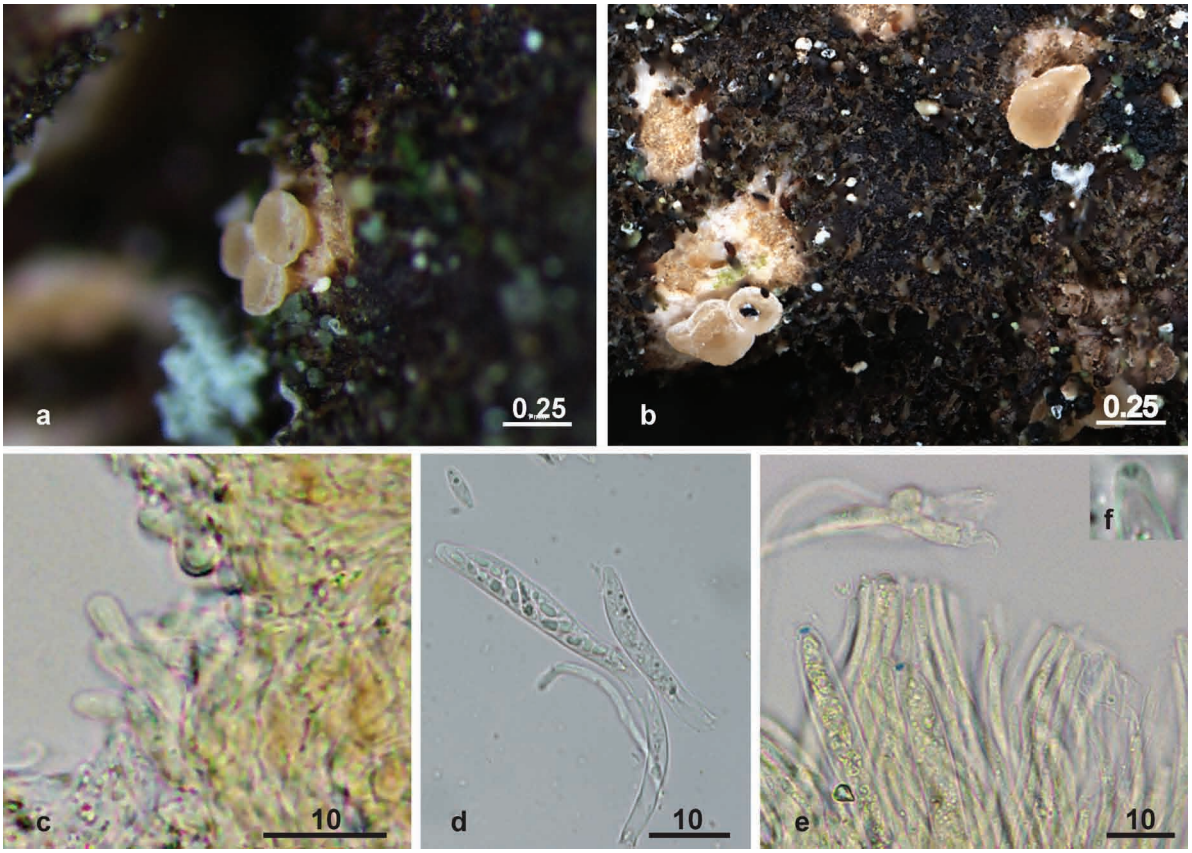

FIGURE 3. *Calycina alstrupii* (holotype, BILAS 10761). **a, b** ascomata on the underside of *Lobaria pulmonaria* thallus (scale in mm); **c** hyphoid-type excipular hyphae in K/I; **d** single ascus with ascospores and an ascospore in water; **e** asci and paraphyses pre-treated with K/I (c–e scale in μm); **f** *Calycina*-type apical apparatus observed in K/I.

Taxonomy

Calycina alstrupii Suija & Motiejūnaitė, *sp. nov.*

MycoBank: MB#819298; Fig. 3

Diagnosis:—*Calycina alstrupii* differs from all known *Calycina* species by being lichenicolous on *Lobaria pulmonaria* and by the combination of 8-spored asci and comparatively small, (5–)5.8(–7) × (1.5–)2.03(–2.5) µm, ascospores.

Type:—NORWAY, Nord-Trøndelag county, Flatanger municipality, Dale Nature Reserve, 64°26'31.9"N, 10°58'14.901"E, elev. 20–200 m, on *Lobaria pulmonaria* growing on trunk of *Alnus incana*, 5 Aug 2015, *J. Motiejūnaitė* (holotype, BILAS-10761!); ibid, on *Alnus*, *A. Suija* (paratype, TU76273!)

GenBank accession nos. holotype (lab code Pz162): KY305095 (rDNA ITS), KY305097 (nuLSU), KY305099 (mtSSU); paratype (lab code Pz167): KY305096 (rDNA ITS), KY305098 (nuLSU), KY305100 (mtSSU)

Ascomata superficial, solitary or gregarious (two to five ascomata in clusters), sessile, growing mostly on the underside, rarely on the upper side (some ascomata of paratype) of *Lobaria pulmonaria* thalli, cream to yellowish to light orange (when young), translucent, slightly gelatinous; disc concave to plane, (0.24–)0.34(–0.47) mm (n=18), roundish to somewhat elongated and irregular in shape; receptacle cupulate, with margin distinct, becoming indistinct in mature ascomata, somewhat pubescent but without setae (Fig. 3a, b). **Apothecial structures** hyaline to slightly yellowish under microscope, inspersed with oil droplets. **Ectal exciple** distinct, hyaline, with some yellowish pigmentation, c. 25 µm wide, with elongated-prismatic cells (*textura prismatica*), the cells in outer part shorter, angular (*textura angularis*), hairs at the margin scattered, short, straight, obtuse at tips (hyphoid), I–, K/I–; without granules on the surface (Fig. 3c). **Medullary exciple** (hypothecium) hyaline, with elongated interwoven cells (*textura prismatica-porrecta*). **Hymenium** hyaline (upper part) to yellowish (lower part), c. 60 µm tall. **Asci** eight-spored, cylindrical to subcylindrical (Fig. 3d), narrowed towards base, arising from single septa without basal protuberances (observed in Congo red), (44–)55.3(–67) × (5–)5.7(–7) µm (n=13), *pars sporifera* (23–)28.6(–35) µm; ascus apex roundish, with apical thickening; ascus apical ring structure of the *Calycina*-type, turning blue in K/I (Fig. 3e, f); ascus wall surface CRB– or only slightly CRB+ blue in dead state. **Ascospores** uniseriate to biseriate in the ascus, hyaline, aseptate, ellipsoid, with apices attenuated at one or both ends (Fig. 3d), or obtuse, 2–3-guttulate, (5–)5.8(–7) × (1.5–)2.03(–2.5) µm (n=23), l/w=2–3.5; wall CRB+ blue in dead state. **Paraphyses** aseptate, simple, unbranched to bifurcate, apically not widened (Fig. 3e), c. 1–1.5 µm wide, not taller than asci, I–, K/I–, containing hyaline vacuolar bodies which are CRB+ blue. **Anamorph** unknown.

Etymology:—named in the memory of Vagn Alstrup, a dedicated Danish researcher of lichenicolous fungi.

Habitat and host:—found in *Picea abies*-dominated boreal rainforest. The ascomata are located on the convex, naked and non-tomentose parts, mainly on the underside, of *Lobaria pulmonaria* thallus. Both the upper- and undersides of the host thallus remain healthy and relatively undamaged at the site of infection. However, other parts of the same thalli show signs of senescence, are discolored and are covered by aggregations of green algae and goniocyst-type thalli of an unindentified lichen. These observations suggest that *Calycina alstrupii* is a parasymbiotic species.

Known distribution:—reported from only a single locality in Nord-Trøndelag county, Norway. However, taking into account the overall distribution area of the host, we would expect the new species to be more widespread, and suspect that it has been overlooked due to the fact that the ascomata develop mainly on the underside of the host thallus and there are no specific signs of infection on the upper side.

Discussion

The genus *Calycina* Nees belongs to a heterogeneous group of Hyaloscyphaceae *s. lato*, whose taxonomic delimitation and infrafamilial classification has remained ambiguous until recently. Jaklitsch *et al*. (2016), following the phylogeny generated by Han *et al*. (2014), treated the genus *Calycina* in the family Pezizellaceae, which differs from Hyaloscyphaceae *s. str.* by the frequent presence of vacuolar bodies and a *Chalara*-type anamorph. However, the delineation of the genera inside Pezizellaceae and the position of individual species remain unresolved. The genus *Calycina* is characterized by having pale ascomata with short, obtuse and smooth (hyphoid) excipular hairs which do not react with iodine, asci with a *Calycina*-type apical apparatus, and ellipsoid to fusiform, hyaline ascospores (Baral & Krieglsteiner 1985, Raitviir 2004, Han *et al*. 2014). The morphologically similar genus *Calycellina* Höhn. also has

hyphoid excipular hairs (Lowen & Dumont 1984) which turn golden brown to reddish brown with iodine solutions (Baral & Krieglsteiner 1985, Raitviir 2004). However, in our analysis as well as in the study by Han *et al*. (2014), *Calycellina populina* (Fuckel) Höhn. was nested within the well-supported *Calycina*-clade. *Psilocistella* Svrček, which is morphologically hardly distinguishable from *Calycina*, differs from the latter by having vacuolar bodies in the tips of the paraphyses and excipular hairs (Raitviir 2004). Unfortunately, the phylogenetic position of *Psilocistella* remains unclear, as sequences for this genus could not be obtained.

All hitherto known species of *Calycina* are saprobes on dead wood and tree stems (Raitviir 2004, Baral *et al*. 2013) or on fucoid algae (Baral & Rämä 2015). The lichenicolous *Calycina ucrainica* (S.Y. Kondr.) S.Y. Kondr., inhabiting *Cladonia* spp. (Kondratyuk & Galloway 1995, as *Pezizella ucrainica* S.Y. Kondr.) most probably does not belong to this genus (Suija *et al*., unpubl.).

Calycina alstrupii fits well with the current circumscription of the genus (Baral & Krieglsteiner 1985, Raitviir 2004, Han *et al*. 2014), but differs from the other known species by several characteristics. Most of these have longer and/or wider spores (Dennis 1963, Raitviir 2004, Baral & Rämä 2015), although in the literature, it is not always clear whether the ascospores were described by examining specimens in the dead or living state, which may significantly influence the measurements. Spores of a similar size to those of the new species are characteristic for *Calycina venceslai* (Velen.) Rait. (6–7.5 × 2.8–3.2 µm) and *C. languida* (P. Karst.) Baral, R. Galán & G. Platas (6–7.5 × 2 µm), but these two species inhabit tree bark (Svrček & Kubícka 1964, Raitviir 2004, Baral *et al*. 2013). Furthermore, the ascomata of *Calycina alstrupii* tend to be somewhat darker (orange) when young, but not pale buff to reddish in the dry state as in *C. venceslai* (Raitviir 2004) or white as in *C. languida* (Svrček & Kubícka 1964). According to the rDNA ITS phylogeny, the species most closely related to *C. alstrupii* is *C. lactea,* which has four-spored asci and grows on decorticated wood (Dennis 1963). The two other lichenicolous species of Pezizellaceae, also inhabiting Peltigerales hosts, *Pezizella epithallina* (W. Phillips & Plowr.) Sacc. and *P. stictae* Etayo, have a different morphology and host (Hawksworth 1980, Etayo 2002), and the first species at least has no close relationships within Hyaloscyphaceae *s. lato* (Suija *et al*. 2015).

To conclude, morphological and molecular features as well as lichenicolous life-style all support *Calycina alstrupii* constituting a species new to science.

Acknowledgements

Rasmus Puusepp is thanked for lab work. Ernestas Kutorga (Vilnius) and Kadri Pärtel (Tartu) are thanked for pointing out important literature and for comments on the manuscript, and Gintaras Kantvilas (Hobart) is thanked for linguistic help. The comments of two reviewers significantly improved the text. The financial support of AS was provided by IUT 20–30 and by the European Regional Development Fund (Centre of Excellence EcolChange).

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