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The first survey of Cystobasidiomycete yeasts in the lichen genus *Cladonia*; with the description of *Lichenozyma pisutiana* gen. nov., sp. nov.

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ABSTRACT

The view of lichens as a symbiosis only between a mycobiont and a photobiont has been challenged by discoveries of diverse associated organisms. Specific basidiomycete yeasts in the cortex of a range of macrolichens were hypothesized to influence the lichens' phenotype. The present study explores the occurrence and diversity of cystobasidiomycete yeasts in the lichen genus *Cladonia*. We obtained seven cultures and 56 additional sequences using specific primers from 27 *Cladonia* species from all over Europe and performed phylogenetic analyses based on ITS, LSU and SSU rDNA loci. We revealed yeast diversity distinct from any previously reported. Representatives of Cyphobasidiales, Microsporomycetaceae and of an unknown group related to *Symmetrospora* have been found. We present evidence that the Microsporomycetaceae contains mainly lichen-associated yeasts. *Lichenozyma pisutiana* is circumscribed here as a new genus and species. We report the first known associations between cystobasidiomycete yeasts and *Cladonia* (both corticate and ecorticate), and find that the association is geographically widespread in various habitats. Our results also suggest that a great diversity of lichen associated yeasts remains to be discovered.

1. Introduction

All lichen thalli host a community of cryptic fungi (e.g. Arnold et al., 2009), which are commonly compared to plant endophytes and have been termed endothallic or endolichenic. These fungi are distinguished from lichenicolous fungi by the fact, that the later fruit or are otherwise symptomatic on thalli (U'Ren et al., 2010). However, many lichenicolous fungi are endothallic, i.e. form their mycelium inside the thallus, before the fruiting-body appears (e.g. *Abrothallus parmotrematis*, Diederich, 2011). In addition, for fungi associated to the thallus surface, the distinction between endothallic and exothallic is problematic since lichens have no structure analogous to the plant cuticle to separate the interior of a thallus sharply from its outside. Thus, instead of endothallic, lichen-associated fungi might be a more suitable term for the fungi living on or within the thallus without having any visible effect.

Most of the known lichen-associated fungi are filamentous ascomycetes, predominantly belonging to the subphylum *Pezizomycotina* (*Ascomycota*). Lichen-inhabiting yeasts and/or basidiomycetes have only rarely been isolated or were neglected or overlooked (e.g. Girlanda et al., 1997; Muggia et al., 2016; Petrini et al., 1990; U'Ren et al., 2012; but see Prillinger et al., 1997). However, Ekman (1999) stated that lichen-associated basidiomycetes are a common source of PCR errors in lichens. Zhang et al. (2015, 2016) identified up to 18 % of endolichenic taxa as representatives of *Basidiomycota* and Fernández-Mendoza et al. (2017) even showed that basidiomycetes are the dominant lichen-associated fungi in some thalli. In addition, many teleomorphic filamentous basidiomycetes are parasites of lichens (Diederich, 1996). Most of them belong to the *Tremellomycetes* (Millanes et al., 2011). Parallel classification of yeasts and filamentous forms of *Tremellomycete* fungi has caused a lot of confusion and the first integrated phylogeny was published only recently (Liu et al., 2016). It might be expected that many of these lichen-associated fungi have an endothallic yeast stage, as also demonstrated by Tuovinen et al. (2019).

Attention has been drawn to basidiomycete yeasts associated with lichens by Spribille et al. (2016). They detected yeasts of the class *Cystobasiodiomycetes* (*Basidiomycota*, *Pucciniomycotina*) in the

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cortex of a great taxonomic range of macrolichens. The authors suggested that these yeasts may play a role in the lichens' phenotype and hypothesized that the yeasts may represent yet another obligatory constituent of the lichen symbiosis (Spribille et al., 2016). Although not yet properly tested, the hypothesis has already been adopted by many authors (eg. Palmqvist et al., 2017; Suryanarayanan and Thirunavukkarasu, 2017; Zúñiga et al., 2017). On the contrary, it has also received a telling critique (Oberwinkler, 2017). Later on, Spribille (2018) discussed superficial biofilms of fungi and bacteria that influence the lichen phenotype.

So far, few cystobasidiomycetes were reported as lichen-associated: cultures of *Cystobasidium laryngis* (*Cystobasidiales*) were obtained from *Usnea antarctica*, *U. aurantiaco-atra* and *Ramalina terebrata* collected from Antarctic islands (Duarte et al., 2016; Santiago et al., 2015), and from *Umbilicaria arctica* collected from Svalbard (Zhang et al., 2016). *Cystobasidium psychroaquaticum* was cultured from *Cladonia pocillum* also from Svalbard (Zhang et al., 2016). An undescribed *Rhodotorula* species was detected in *U snea antarctica* from the South Shetland Islands, Antarctica (Duarte et al., 2016). Park et al. (2015) reported sequences corresponding to two unspecified *Cystobasidiomycete* taxa from environmental samples in *Cladonia borealis* and *C. gracilis* collected from King George Island, Antarctica but they were not deposited in GenBank, so this claim cannot be tested. Except for one lineage, all the yeasts found by Spribille et al. (2016) isolated from various macrolichens from all over the world grouped into the newly described order *Cyphobasidiales* (Spribille et al., 2016). However, no living material was acquired. The genus *Cyphobasidium*, which gives the order its name, is parasitic and produces galls on *Hypogymnia* and *Usnea*.

In our study of lichen symbiosis, we sampled *Cladonia* species from all over Europe. We screened the lichens for *Cystobasidiomycetes* using specific primers and also succeeded in culturing a few strains of these yeasts. The aim of the present paper was to give the first-ever report on occurrence, diversity and morphology of *Cystobasidiomycete* yeasts in the lichen genus *Cladonia*, leading to description of a new genus of these fungi. We focused on both corticate and ecorticate *Cladonia* species to verify whether the yeasts are strictly cortex-inhabiting.

2. Materials and methods

2.1. Sampling

Terricolous *Cladonia* species were collected all over Europe from diverse vegetation types, different bedrocks and soil types and various climatic conditions from April to November 2017. Lichen species, their taxonomic position within the genus *Cladonia* and locality details for specimens from which *Cystobasidiomycete* sequences or cultures were obtained are given in Table 1. The lichen specimens are deposited in the Herbarium PRC (Department of Botany, Charles University, Prague, Czech Republic).

2.2. Isolation, culturing and characterization of the yeasts

The lichen thalli were air-dried and processed within three weeks of collection. Two isolation methods were used: 1) the thalli were washed with Tween and rinsed with water thoroughly several times in a magnetic stirrer, then using a mortar and pestle they were ground into small pieces (isolates Pol13-14 CKV, Pol14-2 CKV, Pol 14-13 CKV). 2) The upper part of a thallus was removed with a sterile razorblade and minute pieces of alga-free tissue were extracted with a sterile preparation needle (isolates CSA5A CKV1, LNV4A CKV1, SNI4A CKV1, SSB6A CKV). While in method 2) the isolates were derived from the medulla, in method 1) their origin cannot be given with certainty. However, the rinsing should eliminate the epithallic biota and thus, all the isolates are considered endothallic. In both cases the thallus fragments were placed onto cultivation media. The media used were malt-yeast agar (MYA), Sabouraud 2 % agar (SAB) or Bold's Basal Medium (BBM) enriched with 1 % glucose (Stocker-Wörgötter and Hager, 2008). Yeasts grown from the lichen tissue were isolated into axenic cultures and kept at 16.5 °C in dark. Morphological characterization was noted from colonies grown on YM agar (yeast extract-malt extract-peptone-glucose agar) following Kurtzman et al. (2011). Tests for ballistoconidia, hyphae or pseudohyphae formation were performed on YM, MYA, potato-dextrose agar (PDA) and corn meal agar (CMA) according to Kurtzman et al. (2011) at 4 °C, 12 °C, 17 °C and 24 °C.

2.3. DNA isolation, amplification and sequencing

DNA from the lichens was isolated following the modified CTAB protocol (Cubero et al., 1999) with minor adjustments. DNA from the cultures was isolated using Chelex following Ferencova et al. (2017). The yeast ITS rDNA from the lichen DNA was amplified using the Cystobasidiomycete-specific primers ITS_symrho_2F and LR0_symrho_R, designed by Spribille et al. (2016). PCR amplification began with denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 1 min and elongation at 72 °C for 1 min, and finished with extension at 72 °C for 2 min. ITS of the yeast cultures was amplified using the primers ITS1F and ITS4 (Gardes and Bruns, 1993; White et al., 1990) with 30 cycles of 94 °C denaturation for 45 s, 54 °C annealing for 1 min and 72 °C elongation for 1 min. The ITS rDNA of the host *Cladonia* species was amplified using the same primers and the same PCR conditions, except the elongation was for 2 min and the final extension for 10 min. LSU rDNA of the cultures was amplified with the LR0R and LR6 primers (Vilgalys and Hester, 1990) with 35 cycles of 95 °C denaturation for 30 s, 55 °C annealing for 30 s and 72 °C elongation for 1 min. SSU rDNA from both cultures and overall lichen DNA was amplified using the *Cystobasidiomycete*-specific primers SSU_symrho_2F and NS6 (Spribille et al., 2016) with 30 cycles of 95 °C denaturation for 30 s, 56 °C annealing for 30 s and 72 °C elongation for 45 s. The PCR products were sequenced by Macrogen Europe, Amsterdam, the Netherlands. The obtained sequences are deposited in GenBank under the accession numbers MK491194 – MK491271 (yeasts, Table 3) and MK508912 – MK508974 (host species, Table 1).

2.4. Sequence analyses

To infer the phylogenetic position of our sequences within the class *Cystobasidiomycetes*, we first performed the multigene phylogenetic analysis using the dataset of Wang et al. (2015a), which is the basis of the currently accepted classification (Oberwinkler, 2017; Wang et al., 2015b). Additionally, we included i) sequences from the type material of both currently recognized *Cyphobasidium* species (Millanes et al., 2016), ii) two representatives of each lineage of the order *Cyphobasidiales*, and iii) two representatives of clade I sensu Spribille et al. (2016). These sequences retrieved from GenBank (Table 2) together with the newly obtained sequences (Table 3) were aligned using MAFFT v.7 (Katoh et al., 2017) using the Q-INS-I method, aligning each locus separately. Ambiguously aligned regions were identified using the program Gblocks v. 0.91b (Castresana, 2000) and eliminated. The final concatenated alignment comprised of 88 unique sequences and 866 SSU rDNA, 313 ITS rDNA, 419 LSU rDNA, 658 RPB1, 1033 RPB2, 925 TEF1 and 392 CYTB alignment positions. Substitution models were estimated with Bayesian Information Criterion using JModelTest v. 2.1.4 (Darriba et al., 2012) as follows: TrN++IG for SSU

Table 1

List of specimens from which Cystobasidiomycetes yeasts were obtained. *Cladonia* host species identification, GenBank accession numbers, and group to which the host species belong (clades sensu Stenroos et al., 2018), PRC accession number and locality details are given. Sequence origin states the source (total lichen DNA or from cultures).

Yeast strain	Sequence origin	Yeast identity	Host Cladonia sp.	Host GenBank accession	Clade	PRC	Locality	Locality type	GPS coordinates	Altitude (m)	Collection date
CSA5A_CKV1	Culture	*Lichenozyma pisutiana*	*C. rei*	MK508912	*cladonia*	4314	Hungary, Csákberény	Abandoned limestone quarry	N47.329388 E18.313593	169.5	4 Jun 2017
EBP4BY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. furcata*	MK508913	*cladonia*	–	Spain, Barranc de la Pegunta	Calcareous soil	N40.246528 W0.351806	1300	18 Aug 2017
EBP6BY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. rangiformis*	MK508914	*cladonia*	–	Spain, Barranc de la Pegunta	Calcareous soil	N40.246528 W0.351806	1300	18 Aug 2017
ECS3DY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. rangiformis*	MK508915	*cladonia*	–	Spain, Camarena de la Sierra	Triassic gypsum	N40.132404 W1.043856	1300	10 Aug 2017
EJA2BY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. chlorophaea gr.*	MK508916	*cladonia*	–	Spain, near Javalambre peak	Calcareous soil	N40.161043 W1.007792	1500	10 Aug 2017
EXV1EY	Total DNA	Uncultured *Lichenozyma pisutiana*	*Cladonia* sp.	MK508917	*cladonia*	–	Spain, between Xodos and Vistabella	Calcareous soil	N40.250517 W0.317483	1300	18 Aug 2017
KAL3CY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. polycarpoides*	MK508918	*cladonia*	4264	Czech Republic, Kalvárie u Motole	Diabase grassland with rock outcrops	N50.065824 E14.323209	323.5	17 May 2017
KAL7AY	Total DNA	Uncultured Microsporomycetaceae	*C. humilis*	MK508919	*cladonia*	4254	Czech Republic, Kalvárie u Motole	Diabase grassland with rock outcrops	N50.065824 E14.323209	323.5	17 May 2017
LNV4A_CKV1	Culture	*Lichenozyma pisutiana*	*C. phyllophora*	MK508920	*cladonia*	4257	Slovakia, Lakšárska Nová Ves	Sand dune	N48.582857 E17.176843	225	5 Jun 2017
NAG1CY	Total DNA	Uncultured Microsporomycetaceae	*C. subulata*	MK508921	*cladonia*	4320	Hungary, Nagytevel	Grassland on sand	N47.269014 E17.600788	238	4 Jun 2017
NAG5EY	Total DNA	Uncultured Microsporomycetaceae	*C. rangiformis*	MK508922	*cladonia*	4305	Hungary, Nagytevel	Grassland on sand	N47.269014 E17.600788	238	4 Jun 2017
NEU1Y	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. verticillata*	MK508923	*cladonia*	4150	Germany, Neuhausen	Early successional sands	N51.671095 E14.387173	72	11 Apr 2017
NEU3BY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. deformis*	MK508924	*erythrocarpae*	4182	Germany, Neuhausen	Early successional sands	N51.671095 E14.387173	72	11 Apr 2017
NEU5CY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. diversa*	MK508925	*erythrocarpae*	4184	Germany, Neuhausen	Early successional sands	N51.671095 E14.387173	72	11 Apr 2017
NEU6AY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. merochlorophaea*	MK508926	*cladonia*	4255	Germany, Neuhausen	Early successional sands	N51.671095 E14.387173	72	11 Apr 2017
NEU7BY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. cf. subulata*	MK508927	*cladonia*	4151	Germany, Neuhausen	Early successional sands	N51.671095 E14.387173	72	11 Apr 2017
NEU8CY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. floerkeana*	MK508928	*erythrocarpae*	4185	Germany, Neuhausen	Early successional sands	N51.671095 E14.387173	72	11 Apr 2017
NFJ10AY	Total DNA	Uncultured Cystobasidiomycetes	*C. cf. rangiferina*	MK508929	*implexae*	4265	Norway, Fjellfrøsvatnet	Mica/schist boulder scree	N69.101452 E19.344055	130	10 Jul 2017
NFJ14AY	Total DNA	Uncultured Cystobasidiomycetes	*C. bellidiflora*	MK508930	*erythrocarpae*	4142	Norway, Fjellfrøsvatnet	Mica/schist boulder scree	N69.101452 E19.344055	130	10 Jul 2017
NFJ16AY	Total DNA	Uncultured Cystobasidiomycetes	*C. cornuta*	MK508931	*cladonia*	4158	Norway, Fjellfrøsvatnet	Mica/schist boulder scree	N69.101452 E19.344055	130	10 Jul 2017
NFJ17AY	Total DNA	Uncultured Cystobasidiomycetes	*C. sulphurina*	MK508932	*erythrocarpae*	4325	Norway, Fjellfrøsvatnet	Mica/schist boulder scree	N69.101452 E19.344055	130	10 Jul 2017
NFJ3AY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. gracilis*	MK508933	*cladonia*	4242	Norway, Fjellfrøsvatnet	Mica/schist boulder scree	N69.101452 E19.344055	130	10 Jul 2017
NKA2AY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. pyxidata*	MK508934	*cladonia*	4266	Norway, Karnes	Limestone outcrops	N69.545138 E20.269084	1.5	12 Jul 2017
NKA3BY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. pocillum*	MK508935	*cladonia*	4261	Norway, Karnes	Limestone outcrops	N69.545138 E20.269084	1.5	12 Jul 2017
NKA4AY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. arbuscula*	MK508936	*arbuscula*	4141	Norway, Karnes	Limestone outcrops	N69.545138 E20.269084	1.5	12 Jul 2017
NKA5AY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. furcata*	MK508937	*cladonia*	4227	Norway, Karnes	Limestone outcrops	N69.545138 E20.269084	1.5	12 Jul 2017

(*continued on next page*)

Table 1 (*continued*)

Yeast strain	Sequence origin	Yeast identity	Host Cladonia sp.	Host GenBank accession	Clade	PRC	Locality	Locality type	GPS coordinates	Altitude (m)	Collection date
NKA6AY	Total DNA	Uncultured Microsporomycetaceae	*C. cf. macroceras*	MK508938	*cladonia*	4152	Norway, Karnes	Limestone outcrops	N69.545138 E20.269084	1.5	12 Jul 2017
NTN1BY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. pocillum*	MK508939	*cladonia*	4262	Norway, Trøsen	Limestone outcrops	N68.569387 E16.649329	4	8 Jul 2017
Pol12-14_CKV	Culture	Microsporomycetaceae isolate	*C. foliacea*	MK508940	*cladonia*	4186	Czech Republic, Kalvárie u Motole	Diabase grassland with rock outcrops	N50.065824 E14.323209	323.5	27 Jul 2017
Pol14-13_CKV	Culture	Microsporomycetaceae isolate	*C. subulata*	MK508941	*cladonia*	4321	Slovakia, Sitno	Andesite rock outcrops in a forest	N48.404301 E18.874294	929	29 Jul 2017
Pol14-3_CKV	Culture	*Lichenozyma pisutiana*	*C. subulata*	MK508942	*cladonia*	4321	Slovakia, Sitno	Andesite rock outcrops in a forest	N48.404301 E18.874294	929	29 Jul 2017
SAL5DY	Total DNA	Uncultured Microsporomycetaceae	*C. furcata*	MK508943	*cladonia*	4217	Hungary, Salfőld	Early succession stages of sand quarry	N46.834682 E17.562669	149	3 Jun 2017
SCK3BY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. rangiferina*	MK508944	*crustaceae*	4278	Sweden, Siljan impact crater	Early stages of heath vegetation on a clear-cut	N61.056844 E15.049959	326.5	30 Aug 2017
SCK4BY	Total DNA	Uncultured Cystobasidiomycetes	*C. deformis*	MK508945	*erythrocarpae*	4183	Sweden, Siljan impact crater	Early stages of heath vegetation on a clear-cut	N61.056844 E15.049959	326.5	30 Aug 2017
SCK7BY	Total DNA	Uncultured Cystobasidiomycetes	*C. gracilis*	MK508946	*cladonia*	4246	Sweden, Siljan impact crater	Early stages of heath vegetation on a clear-cut	N61.056844 E15.049959	326.5	30 Aug 2017
SCK8BY	Total DNA	Uncultured Cystobasidiomycetes	*C. deformis*	MK508947	*erythrocarpae*	4258	Sweden, Siljan impact crater	Early stages of heath vegetation on a clear-cut	N61.056844 E15.049959	326.5	30 Aug 2017
SDA1BY	Total DNA	Uncultured Cystobasidiomycetes	*C. cariosa*	MK508948	*cladonia*	4147	Sweden, Dalhalla	Edge of limestone quarry	N60.949853 E15.104766	254.5	29 Aug 2017
SDA3BY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. pocillum*	MK508949	*cladonia*	4263	Sweden, Dalhalla	Edge of limestone quarry	N60.949853 E15.104766	254.5	29 Aug 2017
SDA8AY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. furcata*	MK508950	*perviae*	4172	Sweden, Dalhalla	Edge of limestone quarry	N60.949853 E15.104766	254.5	29 Aug 2017
SDJ13AY	Total DNA	Uncultured Cystobasidiomycetes	*C. rangiferina*	MK508951	*crustaceae*	4272	Sweden, Djurmo Klack	Granite boulder scree	N60.556239 E15.181526	351	31 Aug 2017
SEP12AY	Total DNA	Uncultured Cystobasidiomycetes	*C. rangiferina*	MK508952	*crustaceae*	4276	Sweden, Paktajåkaluobbalah	Tundra on rock outcrops	N68.439601 E18.631060	352	7 Jul 2017
SEP8AY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. coccifera/borealis*	MK508953	*erythrocarpae*	4155	Sweden, Paktajåkaluobbalah	Tundra on rock outcrops	N68.439601 E18.631060	352	7 Jul 2017
SGA2AY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. subulata*	MK508954	*cladonia*	4324	Sweden, Garpenbergs gård	Metavolcanic boulders	N60.285947 E16.203372	136	24 Aug 2017
SLI2AY	Total DNA	Uncultured Microsporomycetaceae	*C. pocillum*	MK508955	*cladonia*	4153	Sweden, Lindbastmora	Open site in a forest	N60.344481 E15.045676	322	25 Aug 2017
SLI3AY	Total DNA	Uncultured Cystobasidiomycetes	*C. cariosa*	MK508956	*cladonia*	4148	Sweden, Lindbastmora	Open site in a forest	N60.344481 E15.045676	322	25 Aug 2017
SLI5AY	Total DNA	Uncultured *Lichenozyma pisutiana*	*Cladonia* sp.	MK508957	*perviae*	4175	Sweden, Lindbastmora	Open site in a forest	N60.344481 E15.045676	322	25 Aug 2017
SLI6BY	Total DNA	Uncultured Microsporomycetaceae	*C. furcata*	MK508958	*cladonia*	4234	Sweden, Lindbastmora	Open site in a forest	N60.344481 E15.045676	322	25 Aug 2017
SNI4A_CKV1	Culture	*Lichenozyma pisutiana*	*C. cornuta*	MK508959	*cladonia*	4160	Sweden, Nittsjö	Clear-cut in pine forests	N60,926643 E15,064329	223.5	28 Aug 2017
SNI4BY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. cornuta*	MK508960	*cladonia*	4160	Sweden, Nittsjö	Clear-cut in pine forests	N60.926643 E15.064329	223.5	28 Aug 2017
SSB4BY	Total DNA	Uncultured Cyphobasidiales	*C. rangiferina*	MK508961	*crustaceae*	4270	Sweden, Solberga kalkbrott	Limestone gravel	N60.983492 E15.212700	211	27 Aug 2017
SSB6A_CKV	Culture	*Lichenozyma pisutiana*	*C. cariosa*	MK508962	*cladonia*	4149	Sweden, Solberga kalkbrott	Limestone gravel	N60.983492 E15.212700	211	27 Aug 2017
SSB6AY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. cariosa*	MK508963	*cladonia*	4149	Sweden, Solberga kalkbrott	Limestone gravel	N60.983492 E15.212700	211	27 Aug 2017

SSO5AY	Total DNA	Uncultured Microsporomycetaceae	*C. cornuta*	MK508964	*cladonia*	4164	Sweden, Sollerön	A former Viking burial heap	N60.977837 E14.613716	185	26 Aug 2017
SUS5BY	Total DNA	Uncultured Microsporomycetaceae	*C. rei*	MK508965	*cladonia*	4319	Czech Republic, Sušice	Former limestone quarry	N49.2547483 E13.5522144	467	19 May 2017
SYT3BY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. furcata*	MK508966	*cladonia*	4191	Czech Republic, Sytno	Mine spoil heap	N49.738951 E13.027498	450	3 Nov 2017
TIH1AY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. rangiformis*	MK508967	*cladonia*	4312	Hungary, Tihany	Basalt outcrops with dry grassland	46.918950N E17870927	128.5	2 Jun 2017
TIH1BY	Total DNA	Uncultured Microsporomycetaceae	*C. rangiformis*	MK508968	*cladonia*	4311	Hungary, Tihany	Basalt outcrops with dry grassland	46.918950N E17870927	128.5	2 Jun 2017
WLT2EY	Total DNA	Uncultured Microsporomycetaceae	*C. rangiformis*	MK508969	*cladonia*	4291	Wales, Little Tor	Limestone grassland with rock outcrops	N51.57034244 W4.12931286	101	14 Oct 2017
WLT4CY	Total DNA	Uncultured Microsporomycetaceae	*C. pocillum*	MK508970	*cladonia*	4260	Wales, Little Tor	Limestone grassland with rock outcrops	N51.57034244 W4.12931286	101	14 Oct 2017
WST4HY	Total DNA	Uncultured Cyphobasidiales	*C. rangiformis*	MK508971	*cladonia*	4295	Wales, Stackpole	Limestone sand dune	N51.61052960 W4.91996599	74	16 Oct 2017
ZAV2CY	Total DNA	Uncultured Microsporomycetaceae	*C. cf. pocillum*	MK508972	*cladonia*	4259	Czech Republic, Na Závěrce	Grassland with limestone outcrops	N49.9335394 E14.1369492	262	12 Nov 2017
ZAV3BY	Total DNA	Uncultured Microsporomycetaceae	*C. rangiformis*	MK508973	*cladonia*	4282	Czech Republic, Na Závěrce	Grassland with limestone outcrops	N49.9335394 E14.1369492	262	12 Nov 2017
ZAV5BY	Total DNA	Uncultured *Lichenozyma pisutiana*	*C. cf. pocillum*	MK508974	*cladonia*	4267	Czech Republic, Na Závěrce	Grassland with limestone outcrops	N49.9335394 E14.1369492	262	12 Nov 2017

rDNA (gamma shape 0.0.787), TPM1+G for ITS1 rDNA (gamma shape 1.241), K80 + G for 5.8S rDNA (gamma shape 0.178), SYM + G for ITS2 rDNA (gamma shape 0.467), TrN + G for LSU rDNA (gamma shape 0.296); SYM + I + G (gamma shape 0.661), TrN + I + G (gamma shape 0.565) and HKY + I + G (gamma shape 0.652) for the first, second and third codon positions of RPB1, respectively; HKY + I + G (gamma shape 0.343), GTR + I + G (gamma shape 1.299) and SYM + I + G (gamma shape 0.514)) for the first, second and third codon positions of RPB2, respectively; GTR + G (gamma shape 0.463), K80 + I + G (gamma shape 0.487) and GTR + G (gamma shape 0.338) for the first, second and third codon positions of TEF1 respectively; and finally GTR + I + G (gamma shape 0.690), TPM1uf + I + G (gamma shape 0.655) and GTR + I + G (gamma shape 0.544) for the first, second and third codon positions of CYTB, respectively.

All our cultures grouped within the family *Microsporomycetaceae*. Accordingly, in the second analysis we reconstructed its phylogeny based on three rDNA loci. In addition to sequences of the type material of the five currently accepted species (Nakase et al., 2003; Pohl et al., 2011; Bai et al., 2016; Wang et al., 2015b), we also included sequences of the eight uncultured *Cyphobasidiomycete* clones that form clade I in Spribille et al. (2016) and one sequence of a *Rhodotorula* sp. isolated by Duarte et al. (2016) from *U. antarctica*, all of which are apparently closely related to *Microsporomyces* (Tables 2 and 3). *Erythrobasidium elongatum* was selected as the outgroup. The sequences were processed as described above. The final concatenated alignment was composed of 51 sequences and 396 ITS rDNA, 468 LSU rDNA and 635 SSU rDNA positions. Estimated substitution models were: K80 + I for SSU rDNA, HKY + G (gamma shape 0.643) for ITS1 rDNA, K80 for 5.8S rDNA, SYM + G (gamma shape 0.528) for ITS2 rDNA and K80 + G (gamma shape 0.122) for LSU rDNA.

The phylogenetic trees were inferred by Bayesian Inference (BI) using MrBayes v. 3.2.6 (Ronquist et al., 2012), using the 17 and 5 partitions for *Cystobasidiomycetes* and *Microsporomycetaceae*, respectively. Two parallel MCMC runs, with one cold and three heated chains, were carried out for 50 and 10 million generations for *Cystobasidiomycetes* and *Microsporomycetaceae*, respectively. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). The SDSF value between simultaneous runs was 0.002 and 0.001 for *Cystobasidiomycetes* and *Microsporomycetaceae*, respectively. Finally, the burn-in values were determined using the 'sump' command. Bootstrap analyses were also performed by maximum likelihood (ML) using GARLI v. 2.0 (Zwickl, 2006) for *Cystobasidiomycetes* and RAxML v. 8.0.0 (Stamatakis, 2014) for *Microsporomycetaceae* on partitioned datasets. ML analysis consisted of 1000 rapid bootstrap inferences with automatic termination. RAxML analysis was run on the CIPRES Science Gateway v.3.3 web portal (Miller et al., 2010). The resulting trees were visualized using FigTree v. 1.4.3 (Rambaut, 2016). The final visualization was done in the free software R v. 3.4.3 (R Core Team, 2017) using the packages ape (Paradis et al., 2004) and phytools (Revell, 2012).

3. Results

Using the specific primers, we successfully obtained 56 ITS sequences matching Cystobasidiomycetes from 104 *Cladonia* samples. They were apparent in other 43 specimens but we could not obtain legible sequences due to technical reasons (data not shown). In addition to the ITS rDNA region, SSU rDNA was amplified only in three cases (Table 3). We further successfully cultured seven strains of *Cystobasidiomycete* yeasts from six lichen specimens. The cultures were identified by sequencing the ITS, LSU and SSU rDNA

Table 2
List of sequences downloaded from GenBank used for phylogenetic analyses. The last column indicates if they were used in phylogeny of the order *Cystobasidiomycetes* (C) or the family Microsporomycetaceae (M).

Taxon	Strain/voucher	ITS	LSU	SSU	RPB1	RPB2	TEF1	CYTB	Analysis
Bannoa bischofiae	JCM 10338	AB035721	AB082572	AB035721	KJ708018	KJ708292	KJ707777	KJ707684	C
Bannoa hahjimensis	JCM 10336	AB035897	AB082571	AB035897	KJ708014	KJ708146	KJ707750	KJ707682	C
Bannoa ogasawarensis	JCM 10326	AB035713	AB082570	AB035713	KJ708017	KJ708323	KJ707781	KJ707681	C
Bannoa syzygii	JCM 10337	AB035720	AB082573	AB035720	KJ708011	KJ708338	KJ707778	KJ707683	C
Buckleyzyma armeniaca	JCM 8977	AF444523	AF189920	AB126644	KP216521	KJ708211	KJ707762	AB040615	C
Buckleyzyma aurantiaca	JCM 3771	AF444538	AF189921	KJ708436	KJ707970	KJ708212	KJ707757	AB040616	C
Buckleyzyma kluyveri-nielii	JCM 6356	AF444544	AF189988	AB021674	KJ707977	KJ708310	KJ707760	–	C
Buckleyzyma phyllomatis	JCM 7549	AF444515	AF189991	AB021685	KJ707976	KJ708328	KJ707761	KJ707728	C
Buckleyzyma salicina	JCM 2959	AF444511	AF189995	AB021687	–	–	KJ707758	KJ707703	C
Cyphobasidium hypogymniicola	S-F264671	KU587700	KU587694	KU587705	–	–	–	–	C
Cyphobasidium usneicola	S-F264675	KU587704	KU587699	KU587706	–	–	–	–	C
Cyrenella elegans	CBS 274.82	KJ778626	KJ708454	KJ708360	KJ708080	KJ708168	KJ707830	KJ707620	C
Cystobasidium benthicum	JCM 10901	AB026001	AB026001	AB126647	KJ708081	KJ708214	KJ707842	KJ707691	C
Cystobasidium calyptogenae	JCM 10899	AB025996	AB025996	AB126648	KJ708075	KJ708218	KJ707840	KJ707690	C
Cystobasidium fimentarium	DB1489	–	AY512843	AY124479	–	–	LM644071	–	C
Cystobasidium laryngis	JCM 10953	AB078500	AB078500	AB126649	KJ708055	KJ708240	KJ707824	KJ707619	C
Cystobasidium lysinophilum	JCM 5951	AB078501	AB078501	AB126650	KJ708074	KJ708243	KJ707845	KJ707721	C
Cystobasidium minutum	AS 2.1516	AF190011	AF189945	D45367	KJ708059	KJ708246	KJ707825	KJ707562	C
Cystobasidium oligophagum	KM1106	AB702968	AB702967	–	–	–	–	–	C
Cystobasidium pallidum	JCM 3780	AB078492	AF189962	AB126651	KJ708056	KJ708253	KJ707826	KJ707621	C
Cystobasidium pinicola	AS 2.2193	AF444292	AF444293	AB126652	KJ708057	KJ708257	KJ707827	KJ707579	C
Cystobasidium portillonense	071209-Pi2-frotapiedra-7-lev	JQ769323	JQ769312	–	–	–	–	–	C
Cystobasidium psychroaquaticum	CBS:11769	KY103148	KY107444	LM644062	–	–	LM644068	–	C
Cystobasidium ritchiei	CBS:12324	KY103149	KY107445	LM644063	–	–	LM644069	–	C
Cystobasidium slooffiae	JCM 10954	AF444627	AF444722	AB126653	KJ708058	KJ708266	KJ707828	KJ707629	C
Erythrobasidium elongatum	AS 2.1949	AF444561	AF189983	AB021669	KJ708012	KJ708300	KJ707782	KJ707570	C, M
Erythrobasidium hasegawianum	AS 2.1923	AF444522	AF189899	D12803	KF706506	KF706534	KJ707776	KJ707563	C
Erythrobasidium yunnanensis	AS 2.2090	AB030353	AY335162	AF229176	KJ708015	KJ708344	KJ707779	KJ707576	C
Hasegawazyma lactosa	CBS 5826	NR_073295	NG_057668	D45366	KJ708016	KJ708239	AB127098	AB040633	C
Microsporomyces bloemfonteinensis	CBS 8598	EU075189	EU075187	KJ708359	KJ708082	KJ708215	–	KJ707657	C
Microsporomyces hainanensis	CICC 33066	KU296948	KU296947	–	–	–	–	–	C
Microsporomyces magnisporus	JCM 11898	AB112078	AB111954	KJ708428	KJ708013	KJ708317	KJ707780	KJ707695	C
Microsporomyces orientis	CBS 8594	HM559719	HM559718	KJ708358	KJ708078	KJ708249	KJ707843	KJ707656	C
Microsporomyces pini	CBS 107345	EU075190	EU075188	KJ708357	KJ708084	KJ708258	KJ707832	KJ707601	C
Naohidea sebacea	CBS 8477	DQ911616	DQ831020	KP216515	KF706508	KF706535	KF706487	KJ707654	C
Occultifur brasiliensis	UFMG-CM-Y376	KM248526	KM248525	–	–	–	–	–	C
Occultifur externus	JCM 10725	AF444567	AF189910	AB055193	KJ708060	KJ708199	KJ707829	KJ707689	C
Occultifur kilbournensis	NRRL Y-63695	NR_155564	KP413160	–	–	–	–	–	C
Occultifur tropicalis	DMKU SE59	NR_148062	–	–	–	–	–	–	C
Rhodotorula sp.	10.10.L31	KU057818	KT970781	–	–	–	–	–	M
Sakaguchia cladiensis	CBS 10878	FJ008055	FJ008049	KJ708354	–	KJ708219	KJ707847	KJ707603	C
Sakaguchia dacryoidea	JCM 3795	AF444597	AF189972	D13459	KJ708102	KJ708348	KP216514	KJ707709	C
Sakaguchia lamellibrachii	CBS 9598	AB025999	AB025999	AB126646	KJ708098	KJ708314	KJ707876	KJ707667	C
Sakaguchia meli	CBS 10797	FJ807683	KJ708452	KJ708355	KJ708085	KJ708245	KJ707855	KJ707602	C
Sakaguchia oryzae	AS2.2363	AY335160	AY335161	KJ708352	KJ708100	KJ708250	KJ707853	KJ707587	C
Symmetrospora coprosmae	JCM 8772	AF444577	AF189980	D66880	KJ707966	KJ708296	KJ707798	KJ707742	C
Symmetrospora foliicola	AS 2.2527	AF444521	AF189984	AB021671	KJ707969	KJ708302	KJ707797	KJ707589	C
Symmetrospora gracilis	JCM 2963	AF444578	AF189985	KJ708433	KJ707968	KJ708304	KJ707799	KJ707705	C
Symmetrospora marina	JCM 3776	AF444504	AF189944	AB126645	KJ707973	KJ708244	KJ707795	AB040635	C
Symmetrospora symmetrica	AS 2.2299	AY364836	AY364836	KJ708350	KJ707975	KJ708337	KJ707800	KJ707582	C
Symmetrospora vermiculatus	JCM 10224	AB030335	AF460176	AB030322	KJ707967	KJ708342	KJ707801	KJ707675	C
Symmetrospora oryzicola	JCM 5299	AF444546	AF189990	AB021677	KJ707974	KJ708324	KJ707955	KJ707712	C
Uncultured *Cyphobasidiales*	T1433	KU948752	KU948880	KU948829	–	–	–	–	C
Uncultured *Cyphobasidiales*	T1385	KU948738	KU948871	KU948820	–	–	–	–	C
Uncultured *Cyphobasidiales*	T1390	KU948743	–	KU948825	–	–	–	–	C
Uncultured *Cyphobasidiales*	T1587	KU948731	KU948890	KU948834	–	–	–	–	C
Uncultured *Cyphobasidiales*	T1645	KU948778	KU948917	KU948855	–	–	–	–	C
Uncultured *Cyphobasidiales*	T1397	KU948744	KU948912	–	–	–	–	–	C
Uncultured *Cyphobasidiales*	T1630	KU948770	KU948924	KU948845	–	–	–	–	C
Uncultured *Cystobasidiomycetes*	T1613	KU948765	–	KU948843	–	–	–	–	M
Uncultured *Cystobasidiomycetes*	T1402	KU948747	–	–	–	–	–	–	M
Uncultured *Cystobasidiomycetes*	T1400	KU948746	–	–	–	–	–	–	M
Uncultured *Cystobasidiomycetes*	T770	KU948735	–	–	–	–	–	–	M
Uncultured *Cystobasidiomycetes*	T1388	KU948741	–	KU948823	–	–	–	–	M
Uncultured *Cystobasidiomycetes*	T1667	KU948788	–	KU948865	–	–	–	–	C, M
Uncultured *Cystobasidiomycetes*	T1646	KU948779	–	KU948856	–	–	–	–	C, M
Uncultured *Cystobasidiomycetes*	T1615	KU948766	–	–	–	–	–	–	M

Table 3

List of newly obtained sequences and their GenBank accession numbers. The last column shows if they were used in phylogeny of the order *Cystobasidiomycetes* (C) or the family *Microsporomycetaceae* (M). Cultures are in bold.

Strain/voucher	Taxon	ITS	LSU	SSU	Analysis
CSA5A_CKV1	*Lichenozyma pisutiana*	MK491194	MK491265	MK491257	C, M
EBP4BY	Uncultured *Lichenozyma pisutiana*	MK491201	–	–	C, M
EBP6BY	Uncultured *Lichenozyma pisutiana*	MK491202	–	–	C, M
ECS3DY	Uncultured *Lichenozyma pisutiana*	MK491203	–	–	C, M
EJA2BY	Uncultured *Lichenozyma pisutiana*	MK491204	–	–	C, M
EXV1EY	Uncultured *Lichenozyma pisutiana*	MK491205	–	–	C, M
KAL3CY	Uncultured *Lichenozyma pisutiana*	MK491206	–	–	C, M
KAL7AY	Uncultured *Microsporomycetaceae*	MK491207	–	–	C, M
LNV4A_CKV1	*Lichenozyma pisutiana*	MK491196	MK491266	–	C, M
NAG1CY	Uncultured *Microsporomycetaceae*	MK491208	–	–	C, M
NAG5EY	Uncultured *Microsporomycetaceae*	MK491209	–	–	C, M
NEU1Y	Uncultured *Lichenozyma pisutiana*	MK491210	–	–	C, M
NEU3BY	Uncultured *Lichenozyma pisutiana*	MK491211	–	MK491258	C, M
NEU5CY	Uncultured *Lichenozyma pisutiana*	MK491212	–	–	C, M
NEU6AY	Uncultured *Lichenozyma pisutiana*	MK491213	–	–	C, M
NEU7BY	Uncultured *Lichenozyma pisutiana*	MK491214	–	MK491259	C, M
NEU8CY	Uncultured *Lichenozyma pisutiana*	MK491215	–	–	C, M
NFJ10AY	Uncultured *Cystobasidiomycetes*	MK491216	–	–	C
NFJ14AY	Uncultured *Cystobasidiomycetes*	MK491217	–	–	C
NFJ16AY	Uncultured *Cystobasidiomycetes*	MK491218	–	–	C
NFJ17AY	Uncultured *Cystobasidiomycetes*	MK491219	–	–	C
NFJ3A	Uncultured *Lichenozyma pisutiana*	MK491220	–	–	C, M
NKA2AY	Uncultured *Lichenozyma pisutiana*	MK491221	–	–	C, M
NKA3BY	Uncultured *Lichenozyma pisutiana*	MK491222	–	–	C, M
NKA4AY	Uncultured *Lichenozyma pisutiana*	MK491223	–	–	C, M
NKA5AY	Uncultured *Lichenozyma pisutiana*	MK491224	–	–	C, M
NKA6AY	Uncultured *Microsporomycetaceae*	MK491225	–	–	C, M
NTN1BY	Uncultured *Lichenozyma pisutiana*	MK491226	–	–	C, M
Pol12–14_CKV	*Microsporomycetaceae* isolate	MK491199	MK491267	–	C, M
Pol14–13_CKV	*Microsporomycetaceae* isolate	MK491200	MK491268	MK491260	C, M
Pol14–3_CKV	*Lichenozyma pisutiana*	MK491198	MK491269	–	C, M
SAL5DY	Uncultured *Microsporomycetaceae*	MK491227	–	–	C, M
SCK3BY	Uncultured *Lichenozyma pisutiana*	MK491228	–	–	C, M
SCK4BY	Uncultured *Cystobasidiomycetes*	MK491229	–	–	C
SCK7BY	Uncultured *Cystobasidiomycetes*	MK491230	–	–	C
SCK8BY	Uncultured *Cystobasidiomycetes*	MK491231	–	–	C
SDA1BY	Uncultured *Cystobasidiomycetes*	MK491232	–	–	C
SDA3BY	Uncultured *Lichenozyma pisutiana*	MK491233	–	–	C, M
SDA8AY	*Lichenozyma pisutiana*	MK491234	–	–	C, M
SDJ13AY	Uncultured *Cystobasidiomycetes*	MK491235	–	–	C
SEP12AY	Uncultured *Cystobasidiomycetes*	MK491236	–	MK491261	C
SEP8AY	Uncultured *Lichenozyma pisutiana*	MK491237	–	–	C, M
SGA2AY	Uncultured *Lichenozyma pisutiana*	MK491238	–	–	C, M
SLI2AY	Uncultured *Microsporomycetaceae*	MK491239	–	–	C, M
SLI3AY	Uncultured *Cystobasidiomycetes*	MK491240	–	–	C
SLI5AY	Uncultured *Lichenozyma pisutiana*	MK491241	–	–	C, M
SLI6BY	Uncultured *Microsporomycetaceae*	MK491242	–	–	C, M
SNI4A_CKV1	*Lichenozyma pisutiana*	MK491197	MK491270	MK491262	C, M
SNI4BY	Uncultured *Lichenozyma pisutiana*	MK491243	–	–	C, M
SSB4BY	Uncultured Cyphobasidiales	MK491244	–	–	C
SSB6A_CKV	*Lichenozyma pisutiana*	MK491195	MK491271	MK491263	C, M
SSB6AY	*Uncultured Lichenozyma pisutiana*	MK491245	–	–	C, M
SSO5AY	Uncultured *Microsporomycetaceae*	MK491246	–	MK491264	C, M
SUS5BY	Uncultured *Microsporomycetaceae*	MK491247	–	–	C, M
SYT3BY	Uncultured *Lichenozyma pisutiana*	MK491248	–	–	C, M
TIH1AY	Uncultured *Lichenozyma pisutiana*	MK491249	–	–	C, M
TIH1BY	Uncultured *Microsporomycetaceae*	MK491250	–	–	C, M
WLT2EY	Uncultured *Microsporomycetaceae*	MK491251	–	–	C, M
WLT4CY	Uncultured *Microsporomycetaceae*	MK491252	–	–	C, M
WST4HY	Uncultured Cyphobasidiales	MK491253	–	–	C
ZAV2CY	Uncultured *Microsporomycetaceae*	MK491254	–	–	C, M
ZAV3BY	Uncultured *Microsporomycetaceae*	MK491255	–	–	C, M
ZAV5BY	Uncultured *Lichenozyma pisutiana*	MK491256	–	–	C, M

(Table 3). Despite the effort (combinations of various cultivation media and temperatures), only the yeast stage was observed; no conidia, pseudohyphae or hyphae were formed.

BI and ML analyses of *Cystobasidiomycetes* gave identical topologies. Our phylogeny (Fig. 1) supports most of the major groups described by Wang et al. (2015a, 2015b). However, their relationships differ and the analysis found no support for the clustering of genera *Bannoa* and *Erythrobasidium*, nor did it resolve the genus *Occultifur* as monophyletic.

The newly obtained sequences grouped into three distinct lineages within the *Cystobasidiomycetes* (Fig. 1). First, two sequences (SSB4BY and WST4HY) grouped within the lichen-associated order

Fig. 1. Phylogeny of the *Cystobasidiomycetes* obtained by Bayesian inference of concatenated seven-locus dataset. Values at nodes indicate statistical support calculated by MrBayes posterior-node probability/maximum likelihood bootstrap. Values at lower taxonomic rank not shown. Newly obtained sequences are marked in bold. Scale bar represents the expected number of substitutions per site. nc = not calculated.

Cyphobasidiales. Second, a group of eleven sequences grouped into a lineage that appears to be related to *Symmetrospora*. Finally, all the remaining sequences, including those obtained from the cultures, grouped into a monophyletic lineage including the genus *Microsporomyces*. These were further treated in the second analysis.

The phylogeny of *Microsporomycetaceae* (Fig. 2) suggests that the genus *Microsporomyces*, as defined by Wang et al. (2015b) and Bai et al. (2016), is polyphyletic. At least four monophyletic groups were defined within the family: i) *Microsporomyces magnisporus* together with *M. orientalis* and *Microsporomyces pini*, ii)

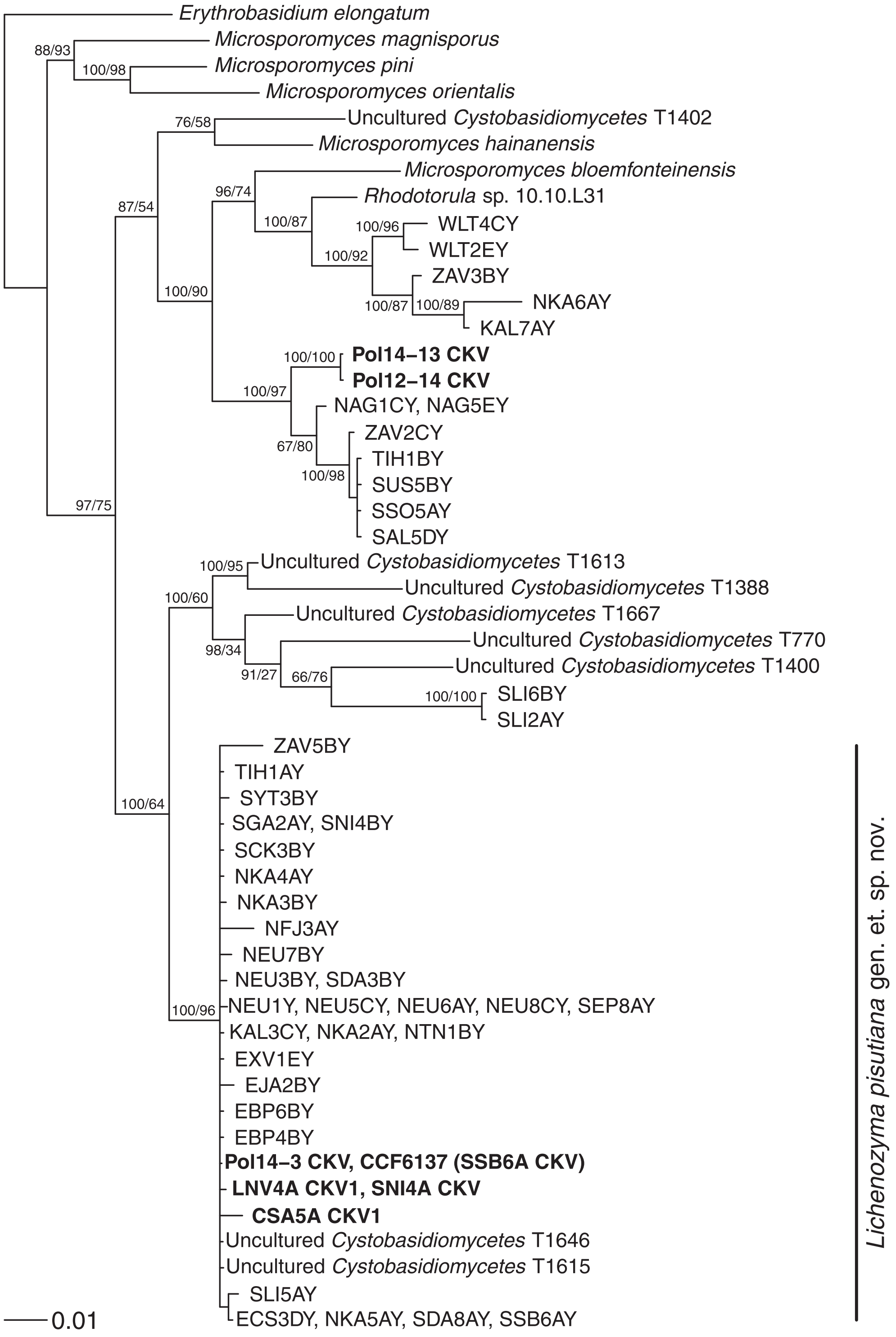

Fig. 2. Phylogeny of the *Microsporomycetaceae* obtained by Bayesian inference of concatenated SSU, ITS and LSU rDNA. Values at nodes indicate statistical support calculated by MrBayes posterior-node probability/maximum likelihood bootstrap. Newly obtained sequences are named by codes only. Cultures are in bold. Scale bar represents the expected number of substitutions per site. nc = not calculated.

Microsporomyces bloemfonteinensis and *Microsporomyces hainanensis* together with a *Rhodotorula* sp. (Duarte et al., 2016), uncultured *Cystobasidiomycetes* T1402 (Spribille et al., 2016), two of our cultures (Pol12-14_CKV and Pol14-13_CKV) and twelve of the sequences obtained from lichen DNA, iii) five uncultured *Cystobasidiomycetes* clones (Spribille et al., 2016) along with two of ours, SLI2AY and SLI6BY, iv) five of our cultures (Fig. 3), 29 clones, and two uncultured clones sequences by Spribille et al. (2016). We propose a new genus, *Lichenozyma*, with a single species *L. pisutiana* for this lineage. The descriptions follow.

4. Taxonomy

Lichenozyma gen. nov.

MycoBank No.: MB 829658

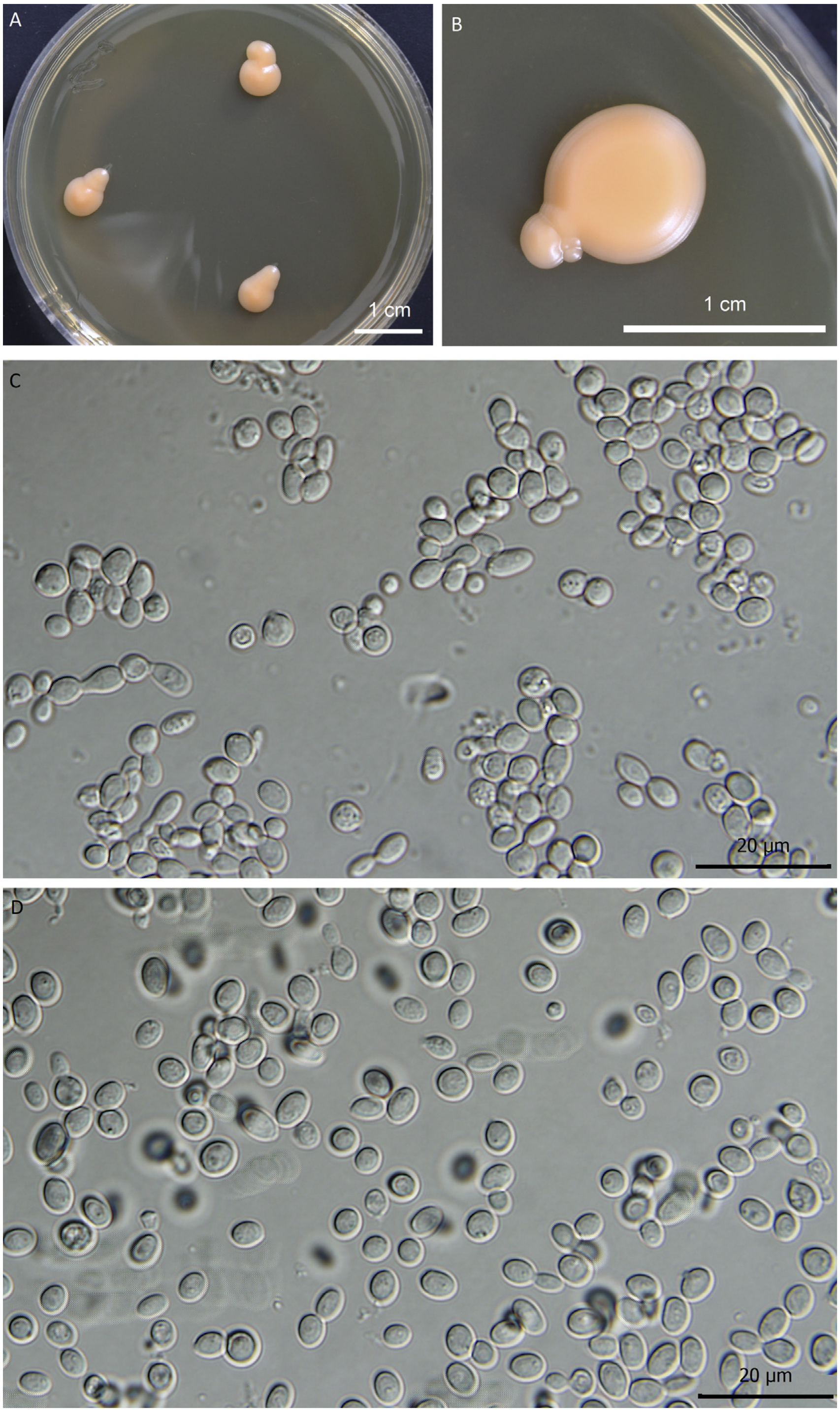

Fig. 3. *Lichenozyma pisutiana* sp. nov. (A) colonies of CCF6137 on SAB after two weeks, (B) colony of strain Pol14-3 CKV on SAB after two weeks, (C) budding cells of strain Pol14-3 CKV on YM after seven days, (D) cells of strain CCF6137 on YM after seven days.

Etym.: referring to its yeast form and its first known occurrence in association with lichens.

The genus is described based on rDNA-derived phylogenic results, which show *Lichenozyma* as a close relative of the genus *Microsporomyces* Q.M. Wang, F.Y. Bai, M. Groenew. and Boekhout.

Colonies ochraceous to pale salmon coloured; sexual reproduction unknown; budding cells present; formation of ballistoconidia, pseudohyphae or hyphae not observed.

The genus is associated to lichens. It has been reported from various *Cladonia* species and *Cetraria ericetorum* collected from diverse habitats and widely separated geographic regions.

Type species: *L. pisutiana*.

Lichenozyma pisutiana sp. nov.

MycoBank No.: MB 829659 Fig. 3.

Etym.: In memory of Slovak lichenologist Ivan Pišút (1935–2017).

Type: Sweden, Dalarna Province, Rättvik Municipality, Solberga, N60.983492, E15.212700, abandoned limestone quarry, 211 m a.s.l., 27 August 2017, J. Steinová and I. Černajová SSB6A; isolated as strain SSB6A_CKV, 5 September 2017 by I. Černajová from *Cladonia cariosa* PRC4149 (holotype PRC4294 dried culture, isotype CCF6137 stored in liquid nitrogen).

Molecular characteristics: SSU rDNA, ITS rDNA (including 5.8S exon) and LSU rDNA sequences of the type are deposited in NCBI/EMBL (GenBank) under the accession numbers MK491270, MK491195 and MK491263, respectively.

Morphological description: Colonies small, up to 4–7 mm in diameter after ten days, even older cultures <1 cm in diameter; ochraceous to pale salmon coloured, smooth, margin entire (Fig. 3a,b); on YM after 10 d at room temperature cells ellipsoidal 5.3–6.6 × 3.2–3.7 μm (Fig. 3c,d), budding polar (Fig. 3c); production of ballistoconidia, pseudohyphae or hyphae not observed (neither on MYA, YM, CMA nor PDA, at 4 °C, 12 °C, 17 °C nor 24 °C).

Ecology: Associated with lichens, not producing visible symptoms on the thalli.

Host range: Various *Cladonia* species listed in Table 1, also reported from *C. multiformis* and *Cetraria ericetorum* (in Spribille et al., 2016).

Distribution: Here reported from Norway, Sweden, Germany, Czech Republic, Slovakia, Hungary, Wales (UK), Spain (Table 1). Also reported from Montana, USA (Spribille et al., 2016).

Additional material examined: Strain CSA5A_CKV1 isolated from *Cladonia rei* (PRC4314), SNI4A_CKV1 from *C. cornuta* (PRC4160), LNV4A_CKV1 from *C. phyllophora* (PRC4257) and Pol14-3_CKV from *C. subulata* (PRC4321), see Tables 1 and 3.

5. Discussion

In this study we focused on *Cystobasidiomycete* yeasts in lichens. Our results show that these yeasts are common associates/inhabitants of the lichen genus *Cladonia*, as we detected them in 95 % of the studied specimens collected in various climatic conditions and habitats (Table 1). Additionally, seven strains were obtained in culture, so the fungi are culturable and can be studied further to detect any function they may have in the lichen symbiosis.

Cystobasidiomycete yeasts had been detected in the upper cortex of macrolichens (Spribille et al., 2016) and were hypothesized to play a role in the lichens' phenotype. However, we found them in corticate (e. g. *Cladonia furcata*), partly corticate (e. g. *C. pocillum*), and ecorticate (e. g. *C. rangiferina*) species. It is thus likely that in *Cladonia* these fungi are rather either constituents of a superficial biofilm (as suggested by Spribille, 2018) or live within the thallus without association with the cortex.

Studies of endolichenic fungi have been intensive in the last decade (e.g. Arnold et al., 2009; Banchi et al., 2018; Fernández-Mendoza et al., 2017; Muggia et al., 2016; Peršoh and Rambold, 2012; U'Ren et al., 2010, 2012, 2014). Despite this, there are only few reports of cystobasidiomycete fungi (see introduction). Culture-dependent studies mostly concentrated on filamentous ascomycetes, thus neglecting basidiomycetes or yeasts in general. Metabarcoding using ITS1 and ITS2 is biased against the detection of basidiomycetes (Banchi et al., 2018). Nevertheless studies of endolichenic fungi based on metabarcoding (Banchi et al., 2018; Fernández-Mendoza et al., 2017) did reveal basidiomycetes (but not *Cystobasidiomycetes*). Interestingly, Banchi et al. (2018) and Fernández-Mendoza et al. (2017) did not surface-sterilize the lichens prior to DNA sampling for legitimate reasons explained in Fernández-Mendoza et al. (2017). Thus, the other tenable explanation of the rarity of basidiomycetes detection is that these fungi are killed by surface sterilization showing that lichen-associated fungi should not be approached with the same methods as plant endophytes. This supports the hypothesis that cystobasidiomycete yeasts are associated with the surfaces of lichens. The hypothesis is further supported in the case of *Bryoria capillaris*. While in some lichens (e. g. *Bryoria fremontii*, *Usnea hirta*, *Hypogymnia tubulosa*) the cystobasidiomycete yeasts embedded in the cortex are scattered, in *B. capillaris* they are actually what we are looking at when looking at the lichen. They have been shown to form an entire layer above the layers of the mycobiont hyphae (Spribille et al., 2016). The fact that we isolated cultures from the medulla is contradictory. We suggest that at least some of the lichen-associated yeasts are not exclusively limited to the surface.

Generally, our knowledge of basidiomycete yeasts is still poor, although taxa with a yeast stage occur in all three subphyla of *Basidiomycota* (Boekhout et al., 2011). The class *Cystobasidiomycetes* includes asexual yeast species and dimorphic species. Their life strategies are diverse, including mycoparasites, endophytes, saprophytes, lichen-associates and fungi adapted to aquatic environment, both marine and freshwater. It may be assumed that a large portion of species diversity in this class remains to be discovered.

We recovered a distinct diversity of yeasts compared to that reported by Spribille et al. (2016) who sampled mainly parmelioid lichens. First, *Cyphobasidiales* were found in two thalli only. Second, we report a previously unknown phylogenetic clade within the class, that appears to be related to *Symmetrospora* (Fig. 1). Its representatives were found in various *Cladonia* species, all collected in Scandinavia, suggesting that these fungi might prefer cold environments. Finally, most of our sequences, however, belong to *Microsporomycetaceae*. These have a broader distribution range, including Scandinavia, central Europe and Spain. Also, two out of three *Cladonia* specimens studied by Spribille et al. (2016) contained yeasts belonging to the same family (as clade I therein). These data suggest a certain degree of specificity of the cystobasidiomycete yeast lineages to their hosts at higher taxonomic ranks. Although no species specificity was found, the lichen-inhabiting *Microsporomycetaceae* might be specific to the genus *Cladonia*. Likely, *Cyphobasidiales* might be specific to *Parmeliaceae*.

The family *Microsporomycetaceae* was described based on molecular data by Wang et al. (2015b) as monogeneric, containing five species. However, according to our analysis, novel and undescribed taxa should be included in the family. The clade that includes the type species of *Microsporomyces*, *M. magnisporus*, also includes only *M. orientalis* and *M. pini*. The other two species - *M. bloemfonteinensis*, a saprophyte of pine needles from South Africa (Pohl et al., 2011) and *M. hainanensis*, isolated from rice seeds from China (Bai et al., 2016) - form a distinct, unrelated lineage. It also includes a *Rhodotorula* strain isolated from *U. antarctica* by Duarte et al. (2016) and uncultured *Cystobasidiomycete* clone T1402 detected in *Thamnolia vermicularis* by Spribille et al. (2016) and sequences obtained from *Cladonia* samples in this study. Another lineage within the family is composed of the reduced clade I in Spribille et al. (2016) and two sequences obtained in this study. According to our data, it is a sister lineage to the genus *Lichenozyma*. Given that most of the representatives of the clade we found to correspond with the *Microsporomycetaceae* are lichen-associated, we can infer that is the common ecological setting of the family, though most of the previously known species are not found in lichens.

Here we propose a new monotypic genus *Lichenozyma*, with the newly described species *L. pisutiana*. Phylogenetic analyses showed that their closest known relatives are the species of *Microsporomyces* and supported the recognition of this new lineage as a novel genus. It is thus defined phylogenetically and ecologically as associated with lichens, mainly of the genus *Cladonia*. It was cultured from five *Cladonia* species, and using specific primers it was further detected in 27 specimens belonging to 17 other *Cladonia* species in this study (Table 1). Uncultured *Cystobasidiomycete* clones T1615 and T1646 from *C. multiformis* and *Cetraria ericetorum*, respectively (Spribille et al., 2016) also belong to the species. The yeasts could be suspected to be an anamorphic form of a known lichenicolous fungus which might be a common case as suggested by Fernández-Mendoza et al. (2017) and recently shown in the case of *Tremella* by Tuovinen et al. (2019). However, our sequence data show that this is not possible, as the only known teleomorphic lichenicolous fungi in *Cystobasidiomycetes* are *Cyphobasidium hypogymniicola* and *Cyphobasidium usneicola*. The only yeasts previously circumscribed from lichens are the species of *Fellomyces* (Prillinger et al., 1997) of the *Tremellales*. Thus, it is not likely that *L. pisutiana* is conspecific with any taxon described in the past and its circumscription as a new taxon is justified. The teleomorph might be discovered in the future.

In conclusion, the present study shows that *Cystobasidiomycete* yeasts are commonly associated with the lichen genus *Cladonia*. Notably, they occur in both corticate and ecorticate species. Any biological relationship to the host still remains unknown and their diversity can be expected to be remarkable.

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