The yeast lichenosphere: high diversity of basidiomycetes from the lichens Tephromela atra and Rhizoplaca melanophthalma

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ABSTRACT

Lichens are well-known examples of complex symbiotic associations between organisms from different Kingdoms. Microfungi in particular, establish diverse associations with the hosting lichen thallus, as species-specific parasites or transient co-inhabitants. The whole community of lichen-associated fungi constitute the 'lichen mycobiome' comprising both ascomycetes and basidiomycetes, including filamentous and yeast taxa. Metabarcoding results and microscopy analyses show that in some thalli, basidiomycetes are frequent lichen-associated fungi but still only a few species could be axenically isolated and morphologically characterized. Within a broad project aiming at characterizing the mycobiome diversity by culture-dependent and independent approaches in two lichen species selected as reference models – *Rhizoplaca melanophthalma* and *Tephromela atra*, we succeed in isolating and culturing 76 new strains of basidiomycetous yeasts. The lichen thalli were collected in different mountain regions worldwide and at relatively high elevation. The yeast strains were isolated on different growth media and were studied for their morphological and genetic diversity. Nuclear internal transcribed spacer (ITS) and ribosomal large subunit (LSU) sequence analyses identified them to belong to ten families within the orders Agaricostilbomycetes, Cystobasidiomycetes, Microbotryomycetes, Tremellomycetes and Ustilaginomycetes. The yeasts here detected showed patterns of host-preference in a few cases and they are potentially related to the ecological conditions.

1. Introduction

Recent discoveries have promoted the reconsideration of lichens, expanding our understanding beyond a simple 'two-partner-symbiosis'. These symbioses are now considered self-sustaining ecosystems derived from mutualistic association of a biotrophic fungus (mycobiont) and phototrophic microorganisms (photobionts, e.g. chlorophytes and/or cyanobacteria), along with an indeterminate number of other microscopic organisms (Hawksworth and Grube, 2020). The 'lichen' resulting from these interactions can be considered as the symbiotic phenotype of the lichen-forming fungus, i.e., the mycobiont (Honegger, 2012), although in a few documented cases the thallus phenotype may be determined by the biologically relevant photobiont (e.g. Sanders and Lücking, 2002). The multiplicity of microorganisms associated with the lichen thalli spans from prokaryotes, microalgae to microfungi (e.g., Grube et al., 2009; Aschenbrenner et al., 2017; Moya et al., 2017; Muggia and Grube, 2018 and references therein). Some of these microbes may grow independently of lichen systems under certain conditions in nature and in axenic cultures (Arnold et al., 2009; Muggia et al., 2016, 2017). However, knowledge on their diversity and potential role(s) that they can play in the lichen symbioses is still incomplete (Spribille, 2018; Muggia and Grube, 2018; Tagirdzhanova et al., 2021).

Among lichen-associated microorganisms, fungi in particular engage in diverse associations with the hosting lichen thallus, often

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as species-specific parasites or transient generalist co-inhabitants within the thalli (Fernández-Mendoza et al., 2017; Muggia and Grube, 2018). The presence of accessory fungi in lichen thalli has attracted interest for many years. Fungi recognized to be specific parasites of different lichen species have been well studied and are formally recognized as 'lichenicolous fungi' (Crittenden et al., 1995; Lawrey and Diederich, 2003; Diederich et al., 2018). Lichenicolous fungi have traditionally been considered to develop symptomatically on the lichens and build their reproductive structures on the surface of the thalli, or semi-immersed within (Lawrey and Diederich, 2003, 2011; Hawksworth, 1982; Muggia et al., 2015; Rambold and Triebel, 1992). Most of the lichenicolous fungi are very slow growing in axenic culture, and the majority of the taxa have been studied only from the environmental lichen samples (Ertz et al., 2014; Muggia et al., 2015, 2019). In contrast, fungi which develop cryptically within lichen thalli and which mycelia are hardly detectable by standard microscopy techniques have been known since 1990 by the application of culture-dependent approaches (Petrini et al., 1990; Girlanda et al., 1997). Because their lifestyle in lichens resembles that of endophytic fungi in plants, these taxa have been commonly termed 'endolichenic fungi' (Arnold et al., 2009; U'Ren et al., 2012, 2014; Muggia et al., 2016, 2017; Fernandez–Mendoza et al., 2017; Banchi et al., 2018; Muggia and Grube, 2018). Endolichenic fungi grow relatively quickly *in vitro*, especially when their culture isolation is performed from thallus fragments (Muggia et al., 2016, 2017; Muggia and Grube, 2018). However, high throughput sequencing (HTS) and metabarcoding analyses demonstrated that also the lichenicolous fungi can be cryptically present in thalli that do not correspond to their specific lichen host on which they produce symptoms, behaving as endolichenic fungi (Fernández-Mendoza et al., 2017; Banchi et al., 2018; Tuovinen et al., 2021). Thus, it is often difficult to make a clear distinction between the two fungal groups. Both lichenicolous and endolichenic fungi are now regarded as 'lichen-associated fungi' and constitute the 'lichen mycobiome' (Fernández-Mendoza et al., 2017; Banchi et al., 2018; Muggia and Grube, 2018). Similar mycobiomes (in terms of species composition) may be present in thalli of closely related mycobionts (Fernández-Mendoza et al., 2017; Smith et al., 2020).

Lichen mycobiomes comprise both ascomycetes and basidiomycetes, either filamentous (mycelium consisting of hyphae) or yeast (unicellular) taxa (Millanes et al., 2011, Fernández-Mendoza et al., 2017; Diederich, 1996; Diederic et al., 2018). Spribille et al. (2016) suggested that basidiomycetous yeasts in the Cyphobasidiales (Cystobasidiomycetes) could be a potential third, biologically relevant partner in the lichen symbioses. Since then, the presence of yeasts in lichens — belonging to Cystobasidiomycetes and Tremellomycetes — has been documented very specifically by fluorescent *in situ* hybridization (FISH), coupled with confocal laser scanning microscopy (CLSM; Spribille et al., 2016; Tuovinen et al., 2019, 2021). Early metabarcoding molecular data showed that up to 18% of endolichenic taxa are representatives of Basidiomycota (Zhang et al., 2015, 2016) and in some thalli basidiomycetes can even be the dominant lichen-associated fungi (Fernández-Mendoza et al., 2017). On the contrary, some subsequent analyses hardly detected Cystobasidiomycetes yeasts using metabarcode sequencing (Lendemer et al., 2019; Smith et al., 2020).

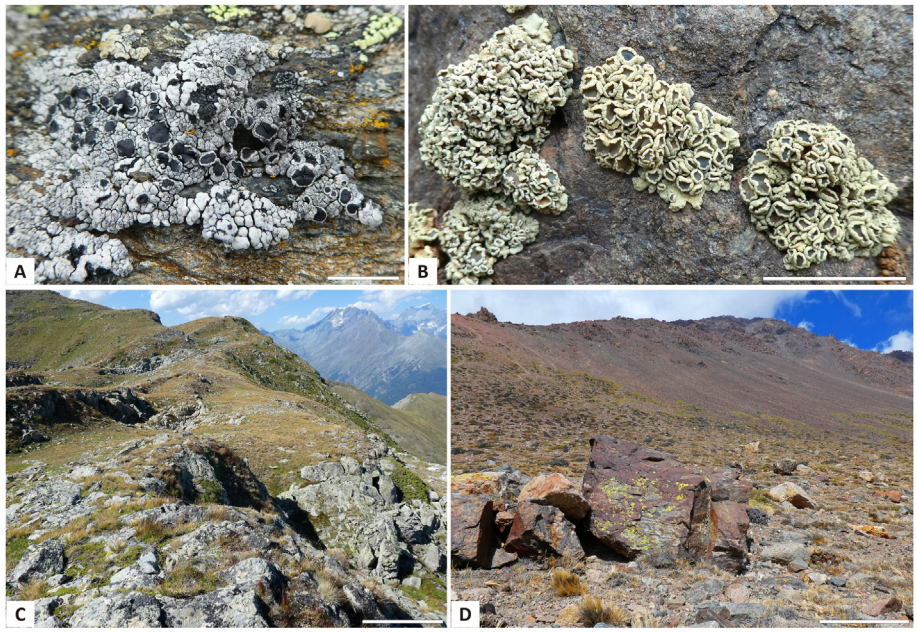
Dimorphism, i.e., the alternating formation of both a haploid unicellular yeast phase and a dikaryotic filamentous mycelium during their life cycle, is common in basidiomycetes (Bandoni, 1995; Boekhout et al., 2011; Oberwinkler, 1987; Sampaio, 2004; Millanes et al., 2021). In his revision about Pucciniomycotina yeasts, Oberwinkler (2017) suggested that, as is the case for other basidiomycete yeasts, the Cyphobasidiales yeasts in lichens are a part of the lifecycle of these basidiomycetes growing and forming large colonies inside the lichen thalli. It is also generally assumed that lichenicolous species in the Tremellomycetes also have a yeast stage. Although less frequently detected by light microscopy, basidiospores germinating by budding have been observed and documented in the basidiomata of lichenicolous *Tremella* and in isolated and cultivated *Fellomyces* yeasts from lichens (Diederich, 1996; Prillinger et al., 1997; Zamora et al., 2011, 2016). Tuovinen et al. (2019, 2021) demonstrated that the yeast stage of several lichen-associated *Tremella* is common and widespread within the lichen thalli. Dimorphism has been also confirmed for other lichenicolous species of mycelial basidiomycetes, such as *Cyphobasidium* species (Spribille et al., 2016).

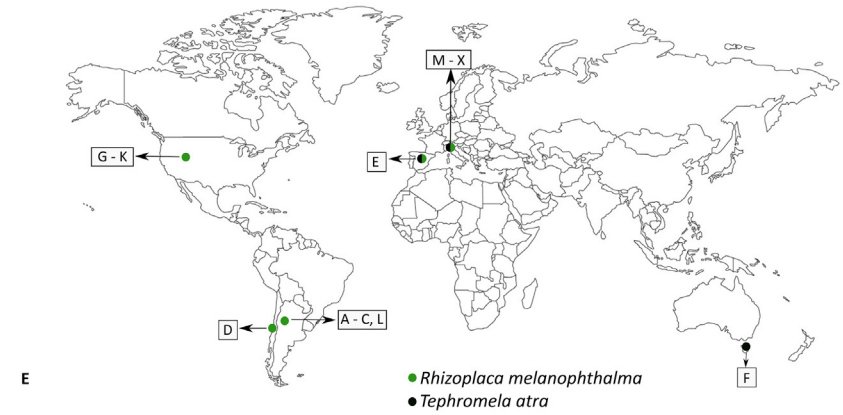
Despite the potential importance of basiomycetous yeasts in lichens, host–yeast association are still incompletely understood and the yeast diversity in these symbiotic systems remains largely unknown. Extreme habitats — as the cold Arctic and Antarctica — where lichens dominate (Bridge and Spooner, 2012; Santiago et al., 2015; Duarte et al., 2016; Pankratov et al., 2017), would be particularly interesting to study the diversity of lichen-inhabiting yeasts. Psychrophilic yeasts in lichens were detected in subfossils of glacier-preserved thalli and were identified to belong to Cystofilobasidiales (DePriest et al., 2000). More recent studies highlighted the presence of new basidiomycetous species in the genera *Fellomyces*, *Mrakia*, *Naganishia*, *Piskurozyma* and *Vishniacozyma* exclusively from lichens (Pankratov et al., 2017). Also, a geographically widespread association between *Cladonia* lichen species and the recently discovered Cystobasidiomycetes yeast *Lichenozyma pisutiana* has been reported (Černajová and Škaloud, 2019). Although some previous research investigated the presence of basidiomycetous yeasts in lichen species of the family Parmeliaceae and in the genera *Lecanor*a and *Cladonia* (Spribille et al., 2016; Tuovinen et al., 2019, 2021; Černajová and Škaloud, 2019), and strengthened the perception of a great diversity of lichen-associated yeasts, other lichens have been comparatively less studied.

Thus, in the frame of a wider project investigating the mycobiomes of two cosmopolitan lichens selected as study models — *Rhizoplaca melanophthalma* and *Tephromela atra*, we applied a targeted culture-dependent approach to better understand the range of basidiomycetous yeasts associating with lichens. We aimed at investigating if the diversity of cultivable yeasts is related to *i)* the lichen species or *ii)* to their respective geographic origin (the two species were sometimes collected in the same locality side by side). We also investigated whether the isolated Cystobasidiomycetes and *Tremella macrobasidiata* yeasts (for which species-specific primers were already available; Millanes et al., 2011; Spribille et al., 2016; Tuovinen et al., 2021) were detectable in the lichen thalli by PCR amplification. The isolated strains were studied in their morphological and phylogenetic diversity, and were recognized to belong to ten families among Agaricostilbomycetes, Cystobasidiomycetes, Microbotryomycetes, Tremellomycetes and Ustilaginomycetes.

2. Material and methods

Sampling — The two lichen species *R. melanophthalma* and *T. atra* were chosen as study systems because of their worldwide distribution under diverse ecological conditions and because their symbioses have been abundantly investigated in the past (Leavitt et al., 2011, 2016a, b; Muggia et al., 2008, 2010, 2014a,b). *R. melanophthalma* is characterized by an umbilicate thallus (attached at a single point), whereas *T. atra* builds a crustose thallus composed of adjacent areoles (Fig. 1a and b). Lichen thalli of both species were collected in different localities trying to cover as much as possible their ecological (type of substrates and climate) and





E

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
Chionosphaeraceae							■				■		■			■		■			■	■		
Agaricostilbaceae																				■	■			
Cystobasidiomycetes													■											
Microsporomycetaceae					■			■																
Yunzhangia																							■	
Colacogloea																				■		■		
Naganishia		■	■	■																				
Yeast Lineage II	■	■	■	■	■		■		■															
Fibulobasidium										■														
Lichenicolous Clade III	■				■		■					■					■		■					
Pseudotremella													■	■										■
Yeast Lineage I					■	■							■		■								■	
Vishniacozyma																					■			
Tranzscheliella	■						■	■				■												

F

Fig. 1. Lichen species (A,B) and the environments (C,D) in which basidiomycetes diversity was investigated: A) *Tephromela atra* (collected in the Alps (Italy); B) *Rhizoplaca melanophthalma* (collected in the Cordillera de los Andes (Argentina); C) Italian Alps (locality P, Table 1); D), Cordillera de los Andes (locality B, Table 1); E) Map of the localities (letters as in Table 1) from which we successfully isolated yeast strains from *R. melanophthalma* (green) and *T. atra* (black); F) Presence-absence matrix of isolated yeast strains (indicated according to their phylogenetic placement) from *R. melanophthalma* (green) and *T. atra* (black), localities are identified by letters (Table 1). Scale bars: A) 1 cm, B) 2 cm, C,D) 1 m. (For interpretation of the references to colour/colour in this figure legend, the reader is referred to the Web version of this article.)

geographical distributional ranges worldwide (Table 1, Fig. 1e). The sampling was performed in boreal, alpine, temperate, humid and arid habitats in Europe (Alps and Spain), North America (Rocky Mountains), South America (Andes) and Tasmania (Three Thumbs) and covered diverse elevations from 500 m up to 5100 m above sea levels (m a.s.l.; Fig. 1a–e). Lichen thalli growing on different rock types, such as acidic, granitic, schist-arenaria and siliceous rocks, were collected (Table 1). In total, 136 thalli of *R. melanophthalma* coming from 34 populations, and 84 thalli of *T. atra* from 21 distinct populations were used for fungal isolation. All the lichen samples were deposited at the herbarium of the University of Trieste (TSB).

Culture isolation – Fungal isolation from *R. melanophthalma* and *T. atra* thalli followed the protocol of Yamamoto et al. (2002). Approximately 2 mm^2 fragments of lichen thalli were dissected with a sterile razor blade. For *R. melanophthalma*, one marginal lobe and one apothecium were taken, while for *T. atra*, one marginal areole and one apothecium. The fragments were washed three times for 15 min with sterile water, followed by 30 min of washing

Table 1
Samples of *Rhizoplaca melanophtalma* and *Tephromela atra* are reported with their thallus ID and the geographic origins; localities are identified by alphabetic letters (A-X).

Lichen host	Thallus ID	Altitude (m a.s.l.)	Substrate	Geographic origin	Locality ID
Rhizoplaca melanophthalma	(L2383-L2391)	1450	basaltic boulders	Argentina, prov. Mendoza, dep. Malargue, Laguna de Llancanelo, RP186, 20 km after the crossroad with RN40; S/SW exposed, scattered in dry pampa vegetation, ca. 35°42′50″ S/69°27′18″ W (*L. Muggia*).	A
Rhizoplaca melanophthalma	(L2436-L2455)	3550	granitic boulders	Argentina, prov. Mendoza, Tunuyan, Cordillera del los Andes (E side), road 94 towards portillo Argentino, camp 'Yareta', 3550 m a.s.l., on acid big boulder, E–S exposed (*L. Muggia*).	B
Rhizoplaca melanophthalma	(L2457-L2470)	3330	acid rocks	Argentina, prov. Mendoza, Cordillera de los Andes (E side), Las Cuevas, lowest border of Mt. Tolosa glacier, S–W exposed (*L. Muggia*).	C
Rhizoplaca melanophthalma	(L2526-L2544)	3300	on acid rock	Chile, prov. Santiago de Chile, Valle (valley) del Yeso, Cordillera del Los Andes (W side), on the path going from the Bano del Plomo to the Laguna de los Patos, S-exposed (*J. Orlando & D. Leiva*).	D
Tephromela atra	(L2583-L2584)	1900	siliceous-granitic boulders	Europe, Spain, prov. Madrid, Miraflores del la Sierra, Puerto de la Morquera, towards Pico Najarra, about 150 m above Puerto de la Morquera, ca. 40°49′22″ N/3°49′49″ W (*L. Muggia & S. Perez-Ortega*).	E
Rhizoplaca melanophthalma	(L2585-L2594)				
Tephromela atra	(L2595-L2603)	545	dolorite boulders	Australia, Tasmania, three Thumbs, summit area, 42°36′S/147°52′E, Grid; 570752828/Grid. Sq.: 5728; in dry sclerophyll forest (*G. Kantvilas*).	F
Rhizoplaca melanophthalma	(L2635-L2667)	1700	quartzite	USA, Utah, Utah Co., Rock Canyon, ca. 2 km from trailhead, on exposed quartzite outcrop on north-facing side of canyon; 40.2649, −111.6179 (*S.D. Leavitt 19–303*).	G
Rhizoplaca melanophthalma	(L2668-L2685)	1665	sandstone boulders	USA, Utah, Emery County, vic. of Horse Canyon Rest Area along US Highway 6, on sandstone in Pinyon/Juiper woodland; 39.4123, −110.4320 (*S.D. Leavitt 19–235*).	H
Rhizoplaca melanophthalma	(L2686-L2703)	2020	Wasatch Formation	USA, Utah, Rich Co., southeast of Bear Lake along Highway 30 and west of Sage Creek Junction, on rock in sage-steppe habitat; 41.7905, −111.2129 (*S.D. Leavitt 19–157*).	I
Rhizoplaca melanophthalma	(L2704-L2721)	2490	sandstone boulder	USA, Utah, Duchesne Co., Ashley National Forest, South Unit, on Nutter's Ridge, on sandstone outcrup north-east of exclusure site (*S.D. Leavitt*).	J
Rhizoplaca melanophthalma	(L2722-L2731)	1845	basalt/volcanic rocks	USA, Idaho, Owyhee Co. Along Mud Flat Rd, 27.7 miles from Highway 78. 42.704228–166.3832 (*S.D. Leavitt 19.233*).	K
Rhizoplaca melanophthalma	(L2786-L2799)	2700	acidic rocks	Argentina, prov. Mendoza, road RP52, near to Paramillo, ca. 30 m above the road, ca. 32°30′13″ S/69°03′18″ W (*L. Muggia*).	L
Tephromela atra	(L3274-L3286)	2150	siliceous rocks/cliffs	Italy, Trentino Alto Adige, prov. Trento, Pergine Valsugana, Val dei Mocheni, Passo La Portella, S-exposed, ca. 46°05′38″ N/11°21′57″ E (*L. Muggia & A. Cometto*).	M
Rhizoplaca melanophthalma	(L3333-L3350)	2100	siliceous rocks	Italy, Trentino Alto Adige, prov. Bolzano, Mazia Valley (Matschertal), path to Tartscher Kreuz, on boulders in open meadow, S-exposed, ca. 46°41′33″ N/10°35′49″ E (*L. Muggia & A. Cometto*).	N
Tephromela atra	(L3351-L3361)				
Tephromela atra	(L3396-L3403)	1650	siliceous/shists tiles	Italy, Piemonte, prov. Verbania-Cusio-Ossola, Val Vigezzo, Alpe Villasco, on roof tile, N-exposed (*L. Muggia & A. Cometto*).	O
Tephromela atra	(L3404-L3418)	2300	granitic boulders	Italy, Aosta Valley, saddle below Mt. Chaligne S/E side, alpine vegetation, ca. 45°46′08″ N/7°14′52″ E (*L. Muggia A. Cometto*).	P
Rhizoplaca melanophthalma	(L3419-L3437)				
Tephromela atra	(L3472-L3480)	1950	silecous bricks/rocks	Italy, Aosta Valley, prov. Aosta, Gressoney Valley, path to Colle Pinter, Alta Via n. 1, about 100 height meter above Alm Alpenzu, N/W/S-exposed, ca. 45°48′13″ N/7°48′50″ E (*L. Muggia & A. Cometto*).	Q
Rhizoplaca melanophthalma	(L3481-L3495)	2800	granitic-siliceous cliff	Italy, Aosta Valley, prov. Aosta, Gressoney Valley, Colle Pinter, Alta Via n. 1 (AV1, path n. 6), big cliffs right above the pass, S/W-exposed, 45°49′12″ N/7°47′14″ E (*L. Muggia & A. Cometto*).	R
Tephromela atra	(L3520-L3527)	1550	siliceous rocks/cliffs	Italy, Aosta Valley, prov. Aosta, Gressoney Valley, Alta Via n. 1 (AV1, path n. 6), path from Gressoney to Alpe Alpenzu, S/E-exposed, ca. 45°48′263″ N/7°48′11″ E (*L. Muggia & A. Cometto*).	S
Tephromela atra	(L3528-L3536)	1750	granitic boulders	Italy, Piemonte, prov. Turin, Valley D′ Ala (Lanzo Valley), Ala di Stura, loc. Balme, path n. 228 to Lago Ru, open Larix vegetation on broad bankings, S-exposed (*L. Muggia & A. Cometto*).	T
Rhizoplaca melanophthalma	(L3537-L3553)				
Tephromela atra	(L3554-L3562)	1500	granitic rocks	Italy, Piemonte, prov. Turin, Valley D′ Ala (Lanzo Valley), Ala di Stura, loc. Balme, path n. 228 to Lago Ru, at bifurcation with the path to climbing crag "Le Ginevre", 100 height m above Balme, shadowed, 45°18′11″ N/7°12′56″ E (*L. Muggia & A. Cometto*).	U
Rhizoplaca melanophthalma	(L3563-L3575)				
Rhizoplaca melanophthalma	(L3616-L3639)	2250	siliceous rocks/boulders	Italy, Piemonte, prov. Cuneo (Alpi Cozie), Val Varaita-Val Maira, Colle di Sampeyre, W of the pass, 44°33′06″ N/7°07′05″ E (*L. Muggia & A. Cometto*).	V
Tephromela atra	(L3640-L3651)				
Tephromela atra	(L3694-L3707)	2100	schist-arenaria rocks	Italy, Piemonte, prov. Cuneo (Alpi Marittime), Mt. Ventoso, below the summit, W-exposed, ca. 44°04′56″ N/7°42′58″ E (*L. Muggia & A. Cometto*).	W
Tephromela atra	(L3720-L3722)	2150	schist-arenaria rocks	Italy, Piemonte, prov. Cuneo (Alpi Marittime), Mt. Saccarello, few meters S/E of the summit, S-exposed, ca. 43°03′40″ N/7°42′46″ E (*L. Muggia & A. Cometto*).	X
Rhizoplaca melanophthalma	(L3723-L3729)				

with 500 μl of Tween80 diluted 1:10. A final washing step was performed rinsing the thallus fragments three times for 15 min with sterile water. The clean fragments were ground in sterile water under the hood and tiny thallus fragments were picked with a sterile bamboo stick and transferred into agar tubes. Six different media were used to promote the growth of as many different fungi as possible: Trebouxia medium (TM, Ahmadjian, 1987), Lilly and Barnett (LB, Lilly and Barnett, 1951), Sabouraud (SAB, Pagano et al., 1958), Potato Dextrose agar (PDA, ApplChem A5828), Dichloran/Glycerol agar (DG18, Hocking and Pitt, 1980) and Malt Yeast-extract (MY, Lilly and Barnett, 1951). We inoculated two tubes of the same medium for each sample for a total of 12 inocula from each lichen individual. The tubes were incubated in growing chamber under the following conditions: 17 °C, 20 μmol × photons $m^{-2} \times s^{-1}$, with

a light/dark cycle of 14/10 h. After three to six months, the inocula had reached a diameter of about 1–3 mm and were checked for the yeast morphology using light microscopy to exclude any -similarly looking-contamination by bacteria. The confirmed yeast strains were then further processed to set subcultures on Petri plates using the same growth medium where the inocula were isolated successfully. Three subcultures were prepared for each strain.

Once the strains were taxonomically identified (see below), accumulation curves for each lichen species were built to assess if the sampling effort was sufficient. Moreover, a Venn diagram was generated to compare the yeast taxa shared between *R. malanophthalma* and *T. atra*.

Morphological analysis — Cell morphological traits of the cultured yeast strains were analyzed using light microscopy. Part of the colony was removed with a sterile loop, diluted in a drop of water and the cells were mounted in water or were additionally stained with 1% Phloxin B after pre-treatment with 5% KOH (Diederich, 1996). Digital photos were taken with a Zeiss AXIO Imager A2 coupled to a Thorlabs digital camera and were slightly improved for colour saturation and sharpness with Adobe Photoshop 7.0 (Adobe System Incorporated, San Jose, CA, USA) and photo-tables were assembled using CorelDRAW X7 (Corel Corporation, Ottawa, Canada).

Molecular analyses: DNA extraction, PCR amplification and sequencing — Small parts of the cultured yeast colonies were taken with a sterile inoculation loop and transferred into 1.5 ml reaction tubes, containing three sterile tungsten beads for homogenization, frozen and ground using a TissueLyserII (Retsch). The DNA extractions were performed following the C-TAB protocol of Cubero et al. (1999), with minor adjustments. The identity of the cultured strains was checked with sequences of the nuclear internal transcribed spacers (nucITS) and 5.8S rDNA ribosomal gene and the D1/D2 domain of the 28S nuclear large ribosomal subunit (nucLSU). The nucITS fragment was amplified with the primers ITS1F (Bruns and Gardes, 1993) and ITS4 (White et al., 1990), while the nucLSU was amplified with the primers LR0R and LR5 (Vilgalys and Hester, 1990; http://www.biology.duke.edu/fungi/mycolab/primers.htm). All strains were sequenced for their ITS locus; if the ITS sequences were identical (99%–100% identity) for strains sharing the same origin -i.e. isolated from the same lichen host thallus, or from thalli coming from the same population - the LSU locus was further sequenced only for a subset of the strains. Polymerase chain reactions (PCR) were prepared for a 25 µl final volume containing 5 µl DNA, 12,5 µl of AccuStart II PCR ToughMix, 0,4 µl for each of the 10 µM primers. PCR amplifications were performed under the following conditions: one initial heating step of 3 min at 94 °C linked to 35 cycles of 45 s at 94 °C, 45 s at 55 °C, 1 min at 72 °C, and one final extension step of 5 min at 72 °C after which the samples were kept at 4 °C. A negative control was used to verify the absence of non-specific amplification products.

PCR amplifications were also performed on the DNA extracts of those lichen thalli from which yeast strains corresponding to Cystobasisdiomycetes and *Tremella macrobasidiata* were isolated in culture. Doing so we aimed at inferring the presence of the yeasts inside the thalli. This additional PCR analysis was possible for Cystobasisdiomycetes and *Tremella macrobasidiata* because specific primers for only these two taxa have proven to work well to amplify the DNA of asymptomatic yeasts isolated from lichen thalli (Spribille et al., 2016; Tuovinen et al., 2021). DNA extractions from the thalli were performed using approximately 2 mm^2 fragments of lobes and areolas of *R. melanophthalma* and *T. atra*, respectively, of the same lichen thalli used for culture isolation. The thallus fragments were previously washed (as described for culture isolations) and DNA extraction followed the C-TAB protocol (Cubero et al., 1999). Thalli of *R. melanophathalma* L2590 and L2668 and *T. atra* L3276 were checked for Cystobasidiomycetes using the Cystobasidiomycete-specific primers ITS_symrho_2F and LR0_symrho_R (Spribille et al., 2016). PCR amplifications were performed under the following conditions: one initial heating step of 3 min at 94 °C linked to 30 cycles of 30 s at 94 °C, 1 min at 48 °C, 1 min at 72 °C, and one final extension step of 2 min at 72 °C after which the samples were kept at 4 °C. Thalli of *R. melanophthalma* L2589, L2636, L2637 and L2786 and *T. atra* L3472 and L3523 were checked for *T. macrobasidiata* using *Tremella* specific primers TmM_ITS_970F (Tuovinen et al., 2021) and Basid-LSU3-3 (Millanes et al., 2011). PCR amplifications were performed with touch down PCR conditions, i.e., one initial heating step of 3 min at 94 °C linked to 4 cycles of 40 s at 94 °C, 40 s at 64 °C, 90 s at 72 °C, 4 cycles of 30 s at 94 °C, 30 s at 62 °C, 90 s at 72 °C, 32 cycles of 30 s at 94 °C, 30 s at 60 °C, 90 s at 72 °C, and one final extension step of 7 min at 72 °C after which the samples were kept at 4 °C. A positive (derived from the culture yeast DNA extraction) and a negative control were used.

All the amplicons were checked for their quality and size by 1% agarose gel electrophoresis stained with Green Safe Gel and purified using Mag-Bind® Normalizer Kit (Omega bio-tek). Clean amplicons were sent for Sanger sequencing to Macrogen Europe (The Netherlands).

Phylogenetic analysis — A first approximation of the identity of the newly generated nucITS and nucLSU sequences was checked with BLAST similarity search (Altschul et al., 1990) using sequences available in Genbank database. As our sequences showed high similarity with representatives of the classes Agaricostilbomycetes, Cystobasidiomycetes, Microbotryomycetes, Tremellomycetes and Ustilaginomycetes, we prepared individual multiple sequence alignments (MSA) for each of these major basidiomycetous classes and for each sequenced locus. The taxon sampling for each analysis was constructed based on the blast similarity sequence results and on the groups and sequences retrieved from previous phylogenetic studies; sequences of type materials were included when available (Supplementary Tables S1–S6). The taxon sampling of Agaricostilbomycetes was based on Millanes et al. (2021) and Diederich et al. (2022), that of Cystobasidiomycetes on Černajová and Škaloud (2019) and Millanes et al. (2016a,b), that of Microbotryomycetes on Kachalkin et al. (2019) and Yurkov et al. (2016), and that of Ustilaginomycetes on Li et al. (2017) and Wang et al. (2015a). For the class Tremellomycetes, two separate MSAs were prepared, one for the order Tremellales and the other for Filobasidiales. Representative taxa were selected from the phylogenetic studies of Duarte et al. (2016), Millanes et al. (2011), Scorzetti et al. (2002) and Zamora et al. (2016). A further MSAs was specifically prepared for a reduced group in the Tremellales, using *Phaeotremella* as outgroup (Supplementary Table S7).

The MSAs were prepared in Bioedit v7.2.5 (Hall, 1999) and initially aligned in MAFFT v.7 (Katoh et al., 2002) using the g-ins-I substitution model. We manually removed ambiguous single nucleotide polymorphisms (SNPs) and introns from the alignment. We analyzed single locus datasets using Maximum Likelihood (ML) and Bayesian Inference (BI) approaches running the analyses on CIPRES Science Gateway v.3.3 web portal (Miller et al., 2011). RAxML v.8.2 (Stamatakis, 2014) was used for the ML analysis applying GTRGAMMA substitution model and 1000 bootstrap pseudoreplicates. The BI analysis was performed with the program MrBayes v.3.2 (Ronquist et al., 2012) running 5 million generations with 6 chains starting from a random tree. Every 100th tree was sampled, and the first 25% of data were discarded as burn-in. The distribution of log-likelihood scores was examined using the program Tracer v1.5 (Rambaut and Drummond, 2007) to determine that stationary phase for each search was reached and chains had achieved convergence. The first 25% of the sampled topologies were discarded as part of a burn-in procedure, while the remaining trees

were used for calculating the posterior probabilities in the majority rule consensus tree. The convergence of the chains was also confirmed by the Potential Scale Reduction Factor (PSRF), which approached 1 (Ronquist et al., 2011). After checking the phylogenetic concordance between the nucITS and the nucLSU datasets for each of the six taxonomic groups (Agaricostilbomycetes, Cystobasidiomycetes, Microbotryomycetes, Filobasidiales, Tremellales and Ustilaginomycetes) we concatenated the two loci, using the program SequenceMatrix v.1.9 (Vaidya et al., 2011), for the final class level analyses. The combined datasets were analysed with both RAxML and MrBayes programs following the same conditions previously described. The phylogenetic trees were visualized in TreeView v.1.6.6 (Page, 1996).

3. Results

Culture isolation — Starting from 136 thalli belonging to 34 populations of *R. melanophthalma* and 84 thalli belonging to 21 population of *T. atra*, a total 76 basidiomycetous yeast strains grew from 29 thalli belonging to 18 populations of *R. melanophthalma* and 18 thalli belonging to 13 populations of *T. atra*, collected in 24 different localities (Table 1). In particular, we isolated 51 basidiomycetes yeast strains from *R. melanophthalma* and 25 from *T. atra*. Because of the relatively low success rate of the isolation, it was not possible to homogeneously isolated a certain number of species from different lichen samples. The Venn diagram (Supplementary Fig. S1) shows that there are only three taxa found in both lichen species (Chionosphaeraceae, Lichenicolous clade III of Tremellales, and Agaricostilbaceae; Supplementary Fig. S1a). While the accumulation curves show that there is an extremely low increase of yeast species diversity among the analyzed thalli (Supplementary Fig. S1b). The pattern of isolated yeasts was very uneven among localities, as a single isolate could be obtained for six localities (i.e., J, K, P, Q, R and S; Table S8). The 76 basidiomycetous yeast strains belonged to five different classes (Table 2) as follow: ten strains belonged to the class Agaricostilbomycetes, isolated from thalli of both lichen species collected between 2500 and 1500 m a.s.l of North and South America and in the Alps; five strains belonged to the class Cystobasidiomycetes, isolated from thalli of both lichen species collected between 2150 and 1600 m a.s.l in North America, Spain and the Alps; three strains belonged to the class Microbotryomycetes, isolated from thalli of both lichen species collected between 2250 and 1740 m a.s.l in the Alps; 54 strains belonged to the class Tremellomycetes, isolated from the thalli of both lichen species collected in wide range of habitats below 3500 m a.s.l., worldwide; four strains belonged to the class Ustilaginomycetes, isolated only from thalli of *R. melanophthalma* between 2700 and 1540 m a.s.l in North and South America. Sites G (Utah, USA) and M (Italy) are the sites from which the highest diversity of yeast taxa was isolated from thalli of *R. melanophthalma* and *T. atra*, respectively. Here from either lichen species up to four different yeast species could be identified (Fig. 1f; Table S8), while from the other sites only one or up to three different yeast species could be isolated (Table S8). Furthermore, from site G three different yeast species could be isolated from the lichen sample *R. melanophthalma* L2637, while in site M two yeast species could be isolated from the sample *T. atra* L3276.

Calacogloea sp., *Naganishia* sp., *Tranzscheliella* sp., *Vishniacozyma* sp., the Yeast lineage II in Tremellales, *Fibulobasidium* sp. and a Microsporomycetaceae sp. were isolated only from *R. melanophthalma* thalli coming from different localities and growing mainly on granitic boulders (Table 1; Fig. 1f; Supplementary Table S8, Fig. S1a). Yeast Lineage I in Tremellales, *Pseudotremella* sp., *Yunzhangia* sp. and an unknown Cystobasidiomycetes were isolated only from *T. atra* thalli growing on schistous-siliceous rocks (Table 1; Supplementary Table S8, Fig. S1a). Instead, Chionosphaeraceae sp., Lichenicolous Clade III in Tremellales and Agaricostilbaceae sp. were isolated from both lichen species collected in different localities (Supplementary Fig. S1a). Moreover, in only four cases yeast strains were successfully isolated from both *R. melanophthalma* and *T. atra* collected in the same locality: i.g from the locality E (Spain) *Tremella macrobasidiata,* Yeast lineage II, and *L. pisutiana* derived from *R. melanophthalma* while Yeast Lineage I from *T. atra*; from the locality T (Italy) *Colacogloea* sp. was isolated from *R. melanophthalma* while Agaricostilbaceae sp. from *T. atra*; from the locality U (Italy) Agaricostilbaceae sp. and *Vishniacozyma* sp. were isolated from *R. melanophthalma* while Chionosphaeraceae sp. from *T. atra*; from the locality V (Italy) *Colacogloea* sp. was isolated from *R. melanophthalma* while Chionosphaeraceae sp. from *T. atra* (Fig. 1f).

Many other fungal strains belonging to the classes of Eurotiomycetes, Dothideomycetes, Sordariomycetes, Lecanoromycetes and Leotiomycetes were isolated (a total of 1652 additional isolates) and identified by ITS sequences during the screening of the yeast strains, but they will be analyzed in detail in another study.

Phylogenetic analysis — A total of 76 new nucITS and 37 new nucLSU sequences were obtained for the cultured yeasts (Table 2). The combined nucITS-nucLSU phylogenetic trees are presented in Figs. 2–7. We performed six separate phylogenetic analyses corresponding to Agaricostilbomycetes, Cystobasidiomycetes, Microbotryomycetes, Tremellales and Filobasidiales (Tremellomycetes) and Ustilaginomycetes. In general, our phylogenetic reconstructions are well-supported and topologically congruent with the phylogenies we used as references, i.e., the studies of Liu et al. (2015a,b), Li et al. (2017, 2020), Wang et al. (2015a,b) and Zamora et al. (2016), Černajová and Škaloud (2019), Millanes et al. (2011, 2016). Also, the phylogenetic trees inferred by ML and Bayesian analyses do not report topological incongruence, neither between the two single loci nucITS and nucLSU individually, nor in the combined nucITS-nucLSU analyses.

Agaricostilbomycetes (Fig. 2) — Two strains, L4069 and L4105, isolated from thalli of *Rhizoplaca melanophtalma* and *T. atra*, respectively, sampled on granitic rocks from the Alps (in two closely located sites), belonged to the family Agaricostilbaceae, in which they were closely related to *Sterigmatomyces* spp. and *Pseudobensingtonia* spp. (Wang et al., 2015b). We refer to them as still unnamed Agaricostilbaceae sp. Eight other strains, four isolated from thalli of *T. atra* collected in four different localities of the Alps (L4046, L4051, L4100 and L4101), and four strains isolated from thalli of *R. melanophthalma* collected in two different localities of the Alps and two in North America (L3034, L3044, L3045, L4089), were grouped into the family of Chionosphaeraceae and were very closely related to *Kurtzmanomyces nectairei*; we refer to them as still unnamed *Kurtzmanomyces* sp.

Cystobasidiomycetes (Fig. 3) — Although the backbone phylogeny was not supported, all the major family- and order-level lineages were fully supported. Here, two strains isolated from one thallus of *T. atra* from the Alps (L4045 and L4050), grouped into a still unnamed lineage (likely of still *incertae sedis* in Cystobasidiomycetes) with three samples of uncultured Cystobasidiomycetes detected previously by Černajová and Škaloud (2019) and Mark et al. (2020). Two other strains, isolated from thalli of *R. melanophthalma* collected in North America (L3243 and L3244), are nested within Microsporomycetaceae. A fifth strain isolated from a *R. melanophthalma* collected in Spain (L3041), also in Microsporomycetaceae, was closely related to the recently described species *L. pisutiana* isolated from *Cladonia* lichen thalli by Černajová and Škaloud (2019).

Table 2
Origin data and sequence accession numbers of Basidiomycetes strains newly isolated in culture: culture ID, the original lichen host (thalli of *Rhizoplaca melanophthalma* and *Tephromela atra* and their ID), the phylogenetic placement, the geographic origin of the original lichen samples (letter-code as in Table 1), and the new corresponding NCBI accession numbers are reported.

ID culture	Lichen host	Phylogenetic placement	ID localities	ITS	nucLSU
L3034	*R. melanophthalma* L2637	Agaricostilbomycetes/Agaricostilbales/Chionosphaeraceae	G	OP045981	OP045800
L3045	*R. melanophthalma* L2638	Agaricostilbomycetes/Agaricostilbales/Chionosphaeraceae	G	OP045982	–
L3044	*R. melanophthalma* L2725	Agaricostilbomycetes/Agaricostilbales/Chionosphaeraceae	K	OP045983	OP045801
L4046	*T. atra* L3275	Agaricostilbomycetes/Agaricostilbales/Chionosphaeraceae	M	OP045974	OP045802
L4051	*T. atra* L3407	Agaricostilbomycetes/Agaricostilbales/Chionosphaeraceae	P	OP045975	OP045803
L4089	*R. melanophthalma* L3483	Agaricostilbomycetes/Agaricostilbales/Chionosphaeraceae	R	OP045976	–
L4101	*T. atra* L3556	Agaricostilbomycetes/Agaricostilbales/Chionosphaeraceae	U	OP045978	–
L4100	*T. atra* L3643	Agaricostilbomycetes/Agaricostilbales/Chionosphaeraceae	V	OP045977	OP045804
L4105	*T. atra* L3530	Agaricostilbomycetes/Agaricostilbales/Agaricostilbaceae	T	OP045980	–
L4069	*R. melanophthalma* L3565	Agaricostilbomycetes/Agaricostilbales/Agaricostilbaceae	U	OP045979	OP045836
L4045	*T. atra* L3276	Cystobasidiomycetes	M	OP045984	OP045807
L4050	*T. atra* L3276	Cystobasidiomycetes	M	OP045985	–
L3243	*R. melanophthalma* L2668	Cystobasidiomycetes/Cystobasidiales/Microsporomycetaceae	H	OP046047	OP045806
L3244	*R. melanophthalma* L2668	Cystobasidiomycetes/Cystobasidiales/Microsporomycetaceae	H	OP046048	–
L3041	*R. melanophthalma* L2590	Cystobasidiomycetes/Cystobasidiales/Microsporomycetaceae	E	OP045986	OP045805
L4063	*T. atra* L3695	Microbotryomycetes/*Yunzhangia*	W	OP045987	OP045808
L4070	*R. melanophthalma* L3541	Microbotryomycetes/*Colacogloea*	T	OP045988	OP045809
L4072	*R. melanophthalma* L3617	Microbotryomycetes/*Colacogloea*	V	OP045989	OP045810
L2767	*R. melanophthalma* L2454	Tremellomycetes/Filobasidiales/*Naganishia*	B	OP045990	OP045811
L2770	*R. melanophthalma* L2468	Tremellomycetes/Filobasidiales/*Naganishia*	C	OP045991	OP045812
L2774	*R. melanophthalma* L2469	Tremellomycetes/Filobasidiales/*Naganishia*	C	OP045992	OP045813
L2781	*R. melanophthalma* L2543	Tremellomycetes/Filobasidiales/*Naganishia*	D	OP045993	OP045814
L2615	*R. melanophthalma* L2390	Tremellomycetes/Tremellales/Yeast Lineage II	A	OP045995	–
L2766	*R. melanophthalma* L2454	Tremellomycetes/Tremellales/Yeast Lineage II	B	OP045996	–
L2776B	*R. melanophthalma* L2469	Tremellomycetes/Tremellales/Yeast Lineage II	C	OP045997	–
L2867	*R. melanophthalma* L2460	Tremellomycetes/Tremellales/Yeast Lineage II	C	OP045994	–
L3738	*R. melanophthalma* L2460	Tremellomycetes/Tremellales/Yeast Lineage II	C	OP046000	OP045818
L2779	*R. melanophthalma* L2543	Tremellomycetes/Tremellales/Yeast Lineage II	D	OP045999	–
L2885	*R. melanophthalma* L2528	Tremellomycetes/Tremellales/Yeast Lineage II	D	OP045998	–
L2887	*R. melanophthalma* L2528	Tremellomycetes/Tremellales/Yeast Lineage II	D	OP046004	OP045815
L2860	*R. melanophthalma* L2590	Tremellomycetes/Tremellales/Yeast Lineage II	E	OP046001	–
L2895	*R. melanophthalma* L2637	Tremellomycetes/Tremellales/Yeast Lineage II	G	OP046005	OP045817
L2894	*R. melanophthalma* L2686	Tremellomycetes/Tremellales/Yeast Lineage II	I	OP046002	OP045816
L3022	*R. melanophthalma* L2686	Tremellomycetes/Tremellales/Yeast Lineage II	I	OP046003	–
L3038	*R. melanophthalma* L2686	Tremellomycetes/Tremellales/Yeast Lineage II	I	OP046007	–
L3039	*R. melanophthalma* L2686	Tremellomycetes/Tremellales/Yeast Lineage II	I	OP046006	–
L3046	*R. melanophthalma* L2686	Tremellomycetes/Tremellales/Yeast Lineage II	I	OP046009	–
L3743	*R. melanophthalma* L2686	Tremellomycetes/Tremellales/Yeast Lineage II	I	OP046015	–
L3051	*R. melanophthalma* L2687	Tremellomycetes/Tremellales/Yeast Lineage II	I	OP046010	OP045835
L3080	*R. melanophthalma* L2687	Tremellomycetes/Tremellales/Yeast Lineage II	I	OP046013	–
L3740	*R. melanophthalma* L2687	Tremellomycetes/Tremellales/Yeast Lineage II	I	OP046014	–
L3744	*R. melanophthalma* L2687	Tremellomycetes/Tremellales/Yeast Lineage II	I	OP046016	–
L3026	*R. melanophthalma* L2688	Tremellomycetes/Tremellales/Yeast Lineage II	I	OP046012	–
L3052	*R. melanophthalma* L2688	Tremellomycetes/Tremellales/Yeast Lineage II	I	OP046011	–
L3742	*R. melanophthalma* L2688	Tremellomycetes/Tremellales/Yeast Lineage II	I	OP046017	–
L3029	*R. melanophthalma* L2689	Tremellomycetes/Tremellales/Yeast Lineage II	I	OP046008	–
L3025	*R. melanophthalma* L2704	Tremellomycetes/Tremellales/*Fibulobasidium*	J	OP046025	OP045819
L3024	*R. melanophthalma* L2387	Tremellomycetes/Tremellales/Lichenicolous Clade III	A	OP046022	–
L2892	*R. melanophthalma* L2589	Tremellomycetes/Tremellales/Lichenicolous Clade III	E	OP046021	–
L3023	*R. melanophthalma* L2636	Tremellomycetes/Tremellales/Lichenicolous Clade III	G	OP046019	–
L3027	*R. melanophthalma* L2636	Tremellomycetes/Tremellales/Lichenicolous Clade III	G	OP046020	OP045820
L3741	*R. melanophthalma* L2637	Tremellomycetes/Tremellales/Lichenicolous Clade III	G	OP046023	–
L2898	*R. melanophthalma* L2638	Tremellomycetes/Tremellales/Lichenicolous Clade III	G	OP046018	–
L3785	*R. melanophthalma* L2786	Tremellomycetes/Tremellales/Lichenicolous Clade III	L	OP046024	OP045821
L4075	*T. atra* L3472	Tremellomycetes/Tremellales/Lichenicolous Clade III	Q	OP046026	–
L4085	*T. atra* L3523	Tremellomycetes/Tremellales/Lichenicolous Clade III	S	OP046027	OP045822
L4044	*T. atra* L3274	Tremellomycetes/Tremellales/*Pseudotremella*	M	OP046028	OP045823
L4066	*T. atra* L3353	Tremellomycetes/Tremellales/*Pseudotremella*	N	OP046029	–
L4067	*T. atra* L3353	Tremellomycetes/Tremellales/*Pseudotremella*	N	OP046030	OP045824
L4074	*T. atra* L3354	Tremellomycetes/Tremellales/*Pseudotremella*	N	OP046031	–
L4077	*T. atra* L3354	Tremellomycetes/Tremellales/*Pseudotremella*	N	OP046033	–
L4076	*T. atra* L3721	Tremellomycetes/Tremellales/*Pseudotremella*	X	OP046032	–
L4080	*T. atra* L3721	Tremellomycetes/Tremellales/*Pseudotremella*	X	OP046034	OP045825
L4091	*T. atra* L3720	Tremellomycetes/Tremellales/*Pseudotremella*	X	OP046035	–
L2889	*T. atra* L2583	Tremellomycetes/Tremellales/Yeast Lineage I	E	OP046037	OP045827
L2878	*T. atra* L2596	Tremellomycetes/Tremellales/Yeast Lineage I	F	OP046036	OP045826
L4049	*T. atra* L3276	Tremellomycetes/Tremellales/Yeast Lineage I	M	OP046038	OP045828

(*continued on next page*)

Table 2 (*continued*)

ID culture	Lichen host	Phylogenetic placement	ID localities	ITS	nucLSU
L4087	*T. atra* L3276	Tremellomycetes/Tremellales/Yeast Lineage I	M	OP046040	–
L4093	*T. atra* L3396	Tremellomycetes/Tremellales/Yeast Lineage I	O	OP046041	–
L4065	*T. atra* L3398	Tremellomycetes/Tremellales/Yeast Lineage I	O	OP046039	OP045829
L4102	*T. atra* L3695	Tremellomycetes/Tremellales/Yeast Lineage I	W	OP046042	OP045830
L4103	*R. melanophthalma* L3566	Tremellomycetes/Tremellales/*Vishniacozyma*	U	OP046043	OP045831
L2609	*R. melanophthalma* L2390	Ustilaginomycetes/Ustilaginales/*Tranzscheliella*	A	OP046044	OP045832
L3062	*R. melanophthalma* L2635	Ustilaginomycetes/Ustilaginales/*Tranzscheliella*	G	OP046046	–
L2891	*R. melanophthalma* L2669	Ustilaginomycetes/Ustilaginales/*Tranzscheliella*	H	OP046045	OP045833
L2900	*R. melanophthalma* L2788	Ustilaginomycetes/Ustilaginales/*Tranzscheliella*	L	OP046049	OP045834

The detection of Cystobasidiomycetes within thalli of *R. melanophathalma* L2590 and L2668 and *T. atra* L3276 performing PCR amplifications using the Cystobasidiomycetes specific primers failed, as no PCR products were obtained.

Microbotryomycetes (Fig. 4) – Two strains isolated from the thalli of *R. melanophthalma* collected in two localities of the Alps (L4070 and L4072) were found in the lineage of *Colacogloea* spp., a yeast genus isolated from soil (Yurkov et al., 2016; Kachalkin et al., 2019). A third strain isolated from a thallus of *T. atra* sampled on the

Fig. 2. Phylogenetic inference of Agaricostilbomycetes: Bayesian analysis based on the concatenated nuclear ITS-LSU dataset. Bayesian posterior probabilities ≥0,8 are reported above branches; branches in bold denote RAxML bootstrap support >75%. Newly obtained sequences are in bold and highlighted in yellow; in parenthesis the original lichen species and the sampling locality (according to Table 1) are reported. Agaricostilbomycetes clades are named according to the phylogenetic study of Millanes et al. (2021). (For interpretation of the references to colour/colour in this figure legend, the reader is referred to the Web version of this article.)

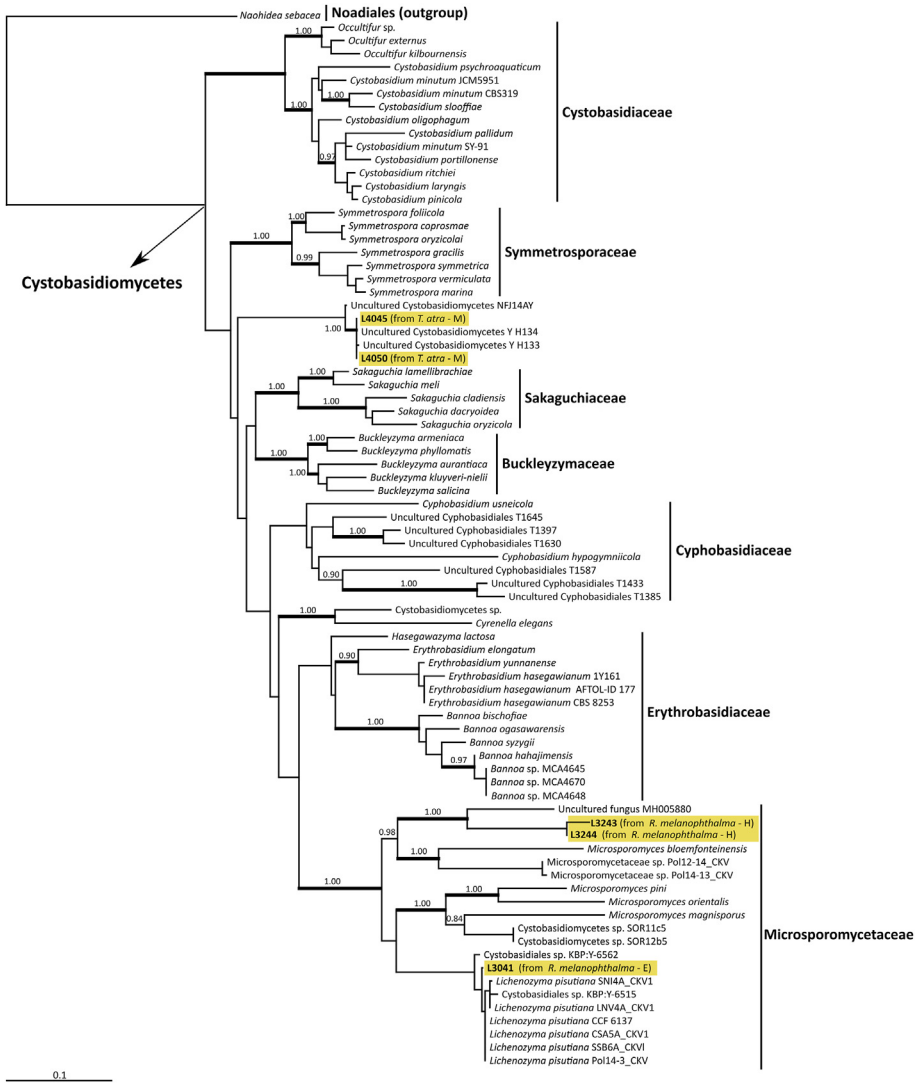


Fig. 3. Phylogenetic inference of Cystobasidiomycetes: Bayesian analysis based on the concatenated nuclear ITS-LSU dataset. Bayesian posterior probabilities ≥0,8 are reported above branches; branches in bold denote RAxML bootstrap support >75%. Newly obtained sequences are in bold and highlighted in yellow; in parenthesis the original lichen species and the sampling locality (according to Table 1) are reported. Cystobasidiomycetes clades are named according to the phylogenetic studies of Černajová and Škaloud (2019) and Millanes et al. (2016a,b). (For interpretation of the references to colour/colour in this figure legend, the reader is referred to the Web version of this article.)

Alps (L4063), formed a small clade with *Yunzhangia auriculariae*; however, this relationship receives no support.

Tremellomycetes (Figs. 5 and 6) — The phylogenetic inference of Filobasidiales (Fig. 5) recovered five well supported clades — 'aerius' (*Solicoccozyma*), 'cylindricus' (*Piskurozyma*), 'gastricus' (*Goffeauzyma*), *Filobasidium* and 'albidus' (*Naganishia*) in accordance to Liu et al. (2015a,b) and Boekhout et al. (2011). Four strains isolated from thalli of *Rhizoplaca melanophthlama* collected in three localities of South America (L2767, L2770, L2774 and L2781) were placed in Filobasidiales, all of them are nested in the genus *Naganisha* (Fig. 5).

Within the order Tremellales (Fig. 6), the newly sequenced strains belonged to six different lineages. Seven strains isolated from thalli of *T. atra* sampled in five localities of the Alps, Spain and Tasmania (L2878, L2889, L4049, L4065, L4087, L4093 and L4102) grouped within a clade named 'Yeast Lineage I' and were closely related to another yeast (GB accession number KBPY6612) isolated for the first time from the lichen *Cladonia rangiferina* by Kachalkin and Pankratov (unpublished work). The strain L4103 isolated from *R. melanophthalma* from the Alps is nested within *Vishniacozyma*. The single strain L3025 is within Fibulobasidium clade, closely related to *Fibulobasidium* spp. and *Sirobasidium* spp. Twenty-three strains isolated from thalli of *R. melanophthalma* of seven populations sampled in North and South America, Spain and the Alps (L2615, L2776B, L2779, L2860, L2867, L2885, L2887, L2894, L2895, L3022, L3026, L3029, L3038, L3039, L3046, L3051, L3052, L3738, L3740, L3742, L3743 and L3744) formed a distinct lineage (named 'Yeast Lineage II', Fig. 6) along with four samples of uncertain position. Closely related to the clade named 'Lichenicolous Clade III' by Millanes et al. (2011) we found two isolates from thalli of *T. atra* collected in two localities in the Alps (L4075 and L4085) and seven isolates coming from *R. melanophthalma* collected in four different localities of North and South America and Spain (L2892, L2898, L3023, L3024, L3027, L3741 and L3785).

The phylogenetic placement of these strains was further confirmed by the analyses performed only with species belonging to Tremellales and including the sequences successfully obtained from the thallus PCR amplification with the *Tremella* specific primers (Supplementary Fig. S2). Indeed, the sequences obtained from thalli of *R. melanophthalma* L2636, L2637, L2589 and L2786,

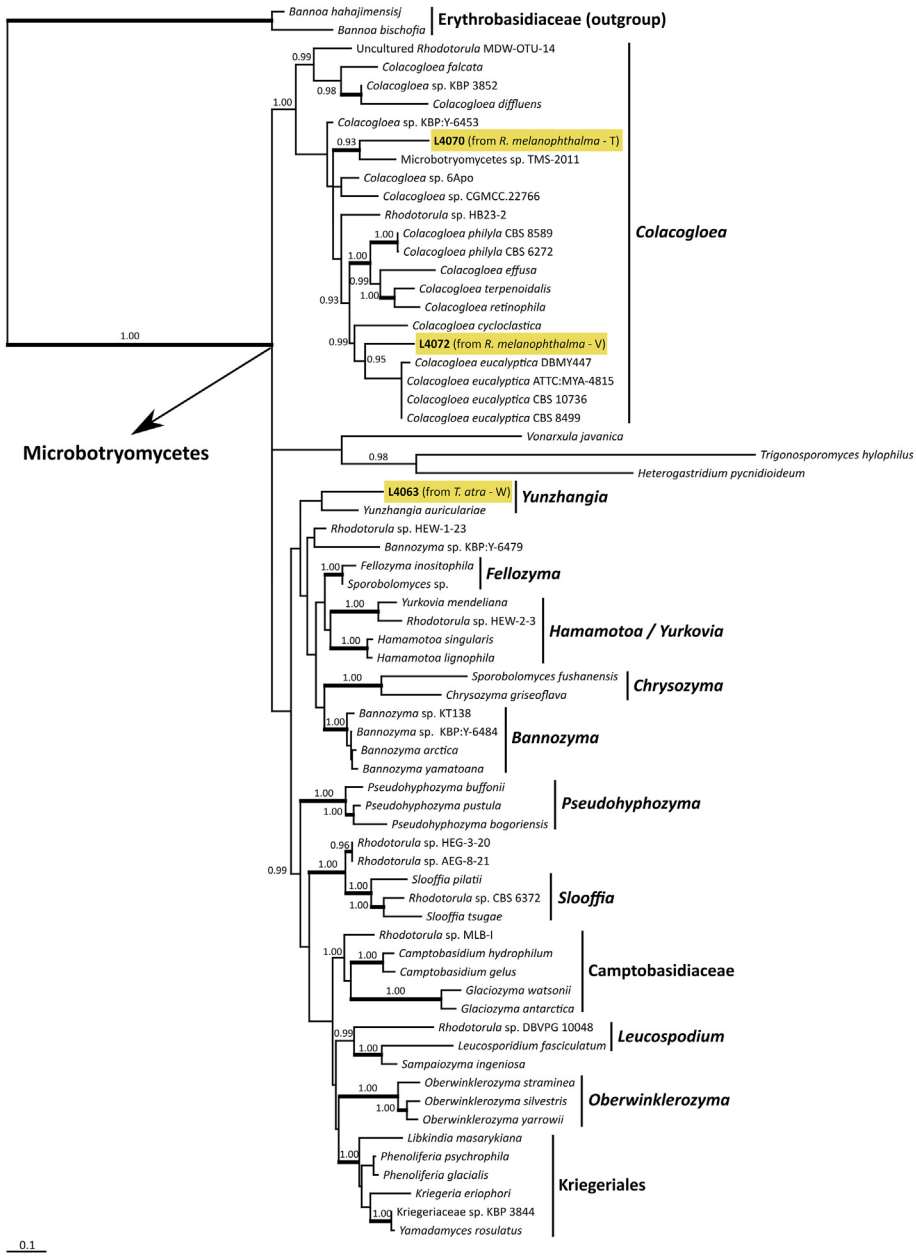


Fig. 4. Phylogenetic inference of Microbotryomycetes: Bayesian analysis based on the concatenated nuclear ITS-LSU dataset. Bayesian posterior probabilities ≥0,8 are reported above branches; branches in bold denote RAxML bootstrap support >75%. Newly obtained sequences are in bold and highlighted in yellow; in parenthesis the original lichen species and the sampling locality (according to Table 1) are reported. Microbotryomycetes clades are named according to the previous phylogenetic studies of Yurkov et al. (2016) and Kachalkin et al. (2019). (For interpretation of the references to colour/colour in this figure legend, the reader is referred to the Web version of this article.)

and from *T. atra* L3472 and L3523 correspond to *T. macrobasidiata* and were grouped together with the respective sequences derived from the isolated strains L2892, L2898, L3023, L3024 L3027, L3741, L3785, L4075 and L4085. Eight strains isolated from thalli of *T. atra* of three populations collected in the Alps (L4044, L4066, L4067, L4074, L4076, L4077, L4080, L4091 and L4085) correspond to *Pseudotremella.*

Ustilaginomycetes (Fig. 7) — Four strains isolated from thalli of *R. melanophthalma* collected in three localities in North and South America (L2609, L2891, L2900 and L3062) were nested within a large lineage including *Tranzscheliella* spp.

3.1. Morphological analysis

Agaricostilbomycetes (Fig. 8) — The strains belonging Chionosphaeraceae (L3034, L3044, L3045, L4046, L4051, L4089, L4100 and L4101) were characterized by colonies of 1,5 cm in diameter and pinkish coloured (Fig. 8a–e). Most of the cells were isodiametric to ellipsoid (5 × 4 μm; Fig. 8f, h-k); budding polar cell are present (Fig. 8f, h-k). Germination cells were observed only in a single strain (L4046; Fig. 8i and j). The strains recovered within the family Agaricostilbaceae (L4069 and L4105) were characterized by colonies of 1,5 cm in diameter and orange coloured. Cells were usually ellipsoidal (7 × 4 μm) and budding cells were present (Fig. 8g, l).

Cystobasidiomycetes (Fig. 9) — The two strains L4045 and L4050, forming a still unnamed lineage with other three uncultured

Fig. 5. Phylogenetic inference of Filobasidiales: Bayesian analysis based on the concatenated nuclear ITS-LSU dataset. Bayesian posterior probabilities ≥0,8 are reported above branches; branches in bold denote RAxML bootstrap support >75%. Newly obtained sequences are in bold and highlighted in yellow; in parenthesis the original lichen species and the sampling locality (according to Table 1) are reported. Filobasidiales clades are named according to the previous phylogenetic studies of Boekhout et al. (2011) and Liu et al. (2015a,b). (For interpretation of the references to colour/colour in this figure legend, the reader is referred to the Web version of this article.)

Cystobasidiomycetes, were characterized by orange colonies (Fig. 9a). Their cells were isodiametric (5 μm) to ellipsoid (8 × 4 μm; Fig. 9b–f); budding polar cell were present (Fig. 9c–e). The two strains (L3243 and L3244) belonging to *Microsporomyces* spp., grow as orange colonies of about 1,5 cm diameter (Fig. 9g). Their cells were usually isodiametric (5–8 μm diameter; Fig. 9l); budding cells were not observed. The strain L3041, nested within *L. pisutiana,* was characterized by ochraceous to pale orange colony (Fig. 9h) and ellipsoid cells (7 × 4 μm) often presenting budding polar cells (Fig. 9i and j).

Microbotryomycetes (Fig. 10) – The two strains (L4070 and L4072) in the lineage of *Colacoglea* spp., grew as pale orange colonies (Fig. 10a and b). Their cells are ellipsoid (6 × 3 μm) and budding polar cell were present (Fig. 10c–e). The strain L4063, which is phylogenetically placed as sister of *Y. auriculariae,* was characterized by pale orange colonies. Mostly of the cells were ellipsoid (4 × 3 μm) to isodiametric (8 μm; Fig. 10f, h, i, k); budding polar cell were present (Fig. 10f, h-k). Germination cells were observed (Fig. 10g).

Tremellomycetes (Figs. 11 and 12) – The four strains (L2767, L2770, L2774 and L2781) recognized as *Naganishia albidosimilis*, grew as rosa- orange colonies of about 1,5 cm in diameter (Fig. 11a–d). The cells are isodiametric (6 μm) to ellipsoid (3–6 × 6–9 μm) and budding polar cells are present (Fig. 11e).

The seven strains (L2878, L2889, L4049, L4065, L4087, L4093 and L4102) grouped within the clade of 'Yeast Lineage I', in the order of Tremellales, and closely related to a yeast isolated from lichens (KBPY6612), were characterized by pale orange colonies (Fig. 11g, j). Mostly of the cells are isodiametric (4–7 μm diameter) often with budding polar cells (Fig. 11f, h, i). Only the strains L4065 presents germination cells (Fig. 11h). The strain L4103 nested within *Vishniacozyma*, was characterized by colonies salmon coloured. The strain L3025, closely related to *Fibulobasidium*, was characterized by colony of 1 cm in diameter, pink-orange coloured.

Fig. 6. Phylogenetic inference of Tremellales: Bayesian analysis based on the concatenated nuclear ITS-LSU dataset. Bayesian posterior probabilities ≥0,8 are reported above branches; branches in bold denote RAxML bootstrap support >75%. Newly obtained sequences are in bold and highlighted in yellow; in parenthesis the original lichen species and the sampling locality (according to Table 1) are reported. Tremellales clades are named according to the previous phylogenetic studies of Duarte et al. (2016), Millanes et al. (2011) and Scorzetti et al. (2000), Zamora et al. (2016). (For interpretation of the references to colour/colour in this figure legend, the reader is referred to the Web version of this article.)

Mostly of the cells were oval (from 5–7 × 3–4 μm) often with budding polar cells (Fig. 11k-m).

The 23 strains (L2615, L2776B, L2779, L2860, L2867, L2885, L2887, L2894, L2895, L3022, L3026, L3029, L3038, L3039, L3046, L3051, L3052, L3738, L3740, L3742, L3743 and L3744) forming the clade 'Yeast Lineage II', grew as whitish to orange colonies of about 1,5 cm in diameter (Fig. 11n, q). Most of the cells were isodiametric (5–8 μm diameter) to oval (7 × 5 μm; Fig. 11p, r-u); polar budding cells were often present; and only the strain L2878 was characterized by multiple budding cells on the mother cell (Fig. 11o).

The isolates (L2892, L2898, L3023, L3024 L3027, L3741, L3785, L4075 and L4085) closely related to *T. macrobasidiata* grew as pale pink to orange colonies (Fig. 12a). The cells were isodiametric (4–8 μm diameter) to ellipsoidal (8 × 5 μm; Fig. 12b, k-m); polar, bipolar and multipolar budding cells were presents (Fig. 12b–d, g, k-m). Moreover, many germination cells were observed (Fig. 12e, f, h-k). The eight strains (L4044, L4066, L4067, L4074, L4076, L4077, L4080 and L4091) grouped within *Pseudotremella* were characterized by colony of 2 cm in diameter and creamy to orange (Fig. 12n-p). These strains are dimorphic with both the unicellular and filamentous stages produced in culture. The yeast cells were isodiametric (4–8 μm diameter; Fig. 12q, s-v); polar budding cells are present (Fig. 12s–v); in the filamentous morphology the hyphae had a diameter of about 4 μm (Fig. 12r).

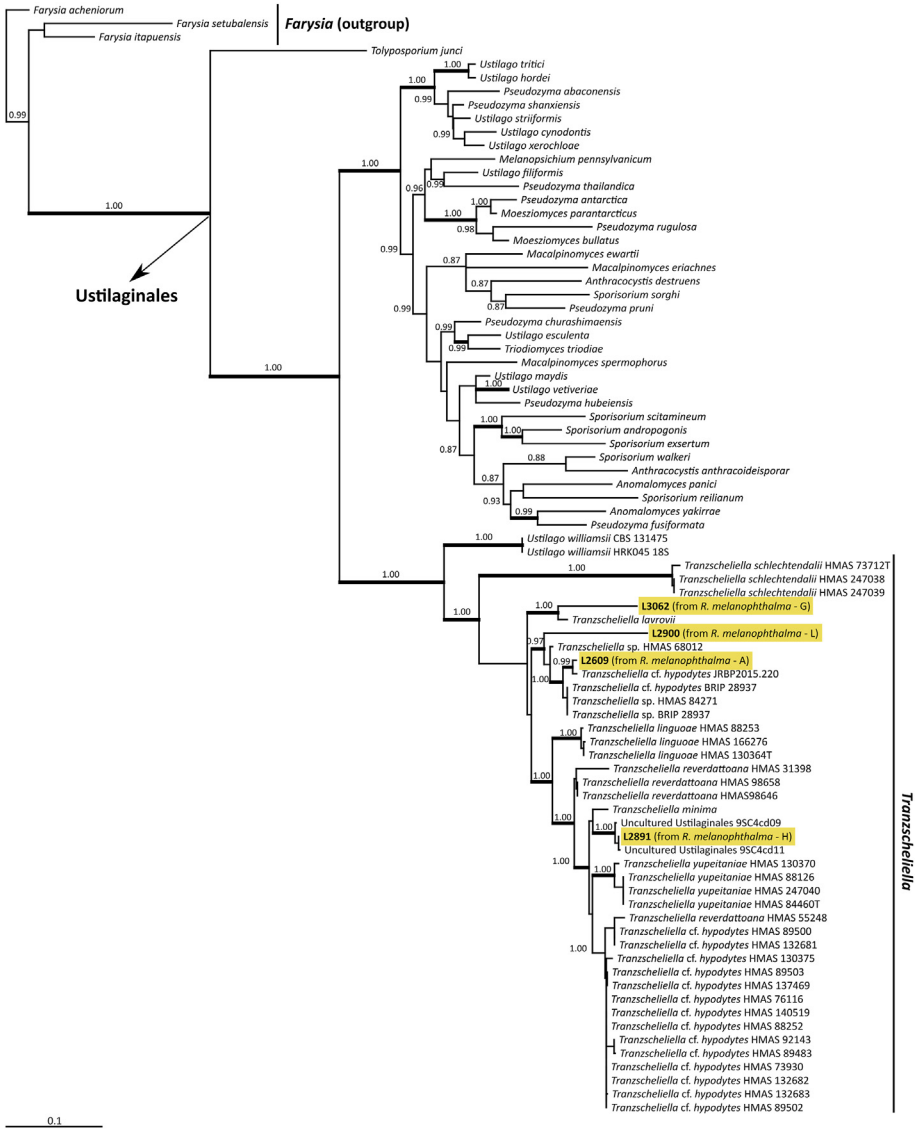


Fig. 7. Phylogenetic inference of Ustilaginomycetes: Bayesian analysis based on the concatenated nuclear ITS-LSU dataset. Bayesian posterior probabilities ≥0,8 are reported above branches; branches in bold denote RAxML bootstrap support >75%. Newly obtained sequences are in bold and highlighted in yellow; in parenthesis the original lichen species and the sampling locality (according to Table 1) are reported. Ustilaginomycetes clades are named according to the previous phylogenetic studies of Li et al. (2017) and Wang et al. (2015a). (For interpretation of the references to colour/colour in this figure legend, the reader is referred to the Web version of this article.)

Ustilaginomycetes (Fig. 13) – Four strains recovered within the big lineage of *Tranzschelliella* spp. (L2609, L2891, L2900 and L3062) grew as white to orange colonies of 3 cm in diameter (Fig. 13a–c). These strains were characterized by both the unicellular and filamentous stages (Fig. 13d–i). The yeast cells were isodiametric (3–6 μm diameter) to ellipsoidal (7 × 4 μm); polar and bipolar budding cells are presents (Fig. 13e); germination cells were observed (Fig. 13d–i). The hyphae are 2–4 μm thick and sometimes generate ramifications (Fig. 13e, g, i).

4. Discussion

In lichens, the major fraction of the mycobiome is composed by ascomycetes belonging to the subphylum Pezizomycotina, while only a minor fraction is represented by basidiomycetes (Zhang et al., 2015, 2016; Fernàndez-Mendoza et al., 2017; Banchi et al., 2018). Endolichenic ascomycetes were also frequently isolated in cultures (Arnold et al., 2009; Muggia et al., 2016, 2017, 2021) in contrast to basidiomycetes, which are still poorly represented as axenic isolates (Santiago et al., 2015; Zhang et al., 2016; Černajová; Škaloud, 2019; 2020). Recent study by Lendemer et al. (2019) applying metagenomics analyses, suggested that Cystobasidiomycete yeasts represent only a minor fraction of lichen-associated fungi across a comprehensive sampling of lichens (as Cystobasidiomycete yeasts reads were detected only in 2.7% of the analyzed thalli). Similarly, Smith et al. (2020) found little evidence supporting that Cystobasidiales yeasts would always be present in macrolichens, reporting low abundance reads of basidiomycetes only in samples of *Bryoria* lichens. However, highly specific, *ad hoc* targeted microscopic inspections using fluorescent probes and confocal microscopy consistently identify a high frequency of basidiomycetes yeasts in lichen thalli (Tuovinen et al., 2019, 2021).

Here, we provide a wider perspective into the range of basidiomycete yeasts isolated from lichens and able to grow in axenic culture. The application of HTS and metabarcoding analyses have facilitated a more expansive view into the range of lichen-

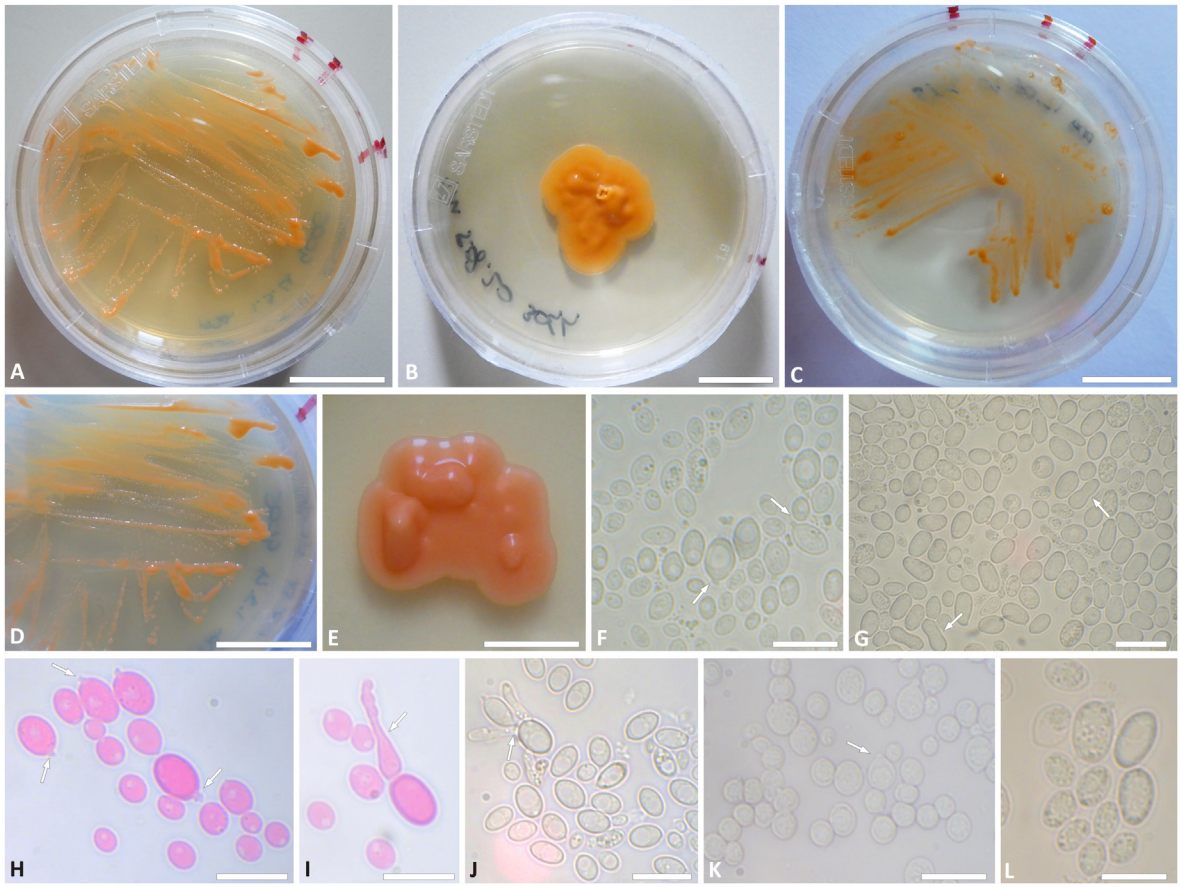


Fig. 8. Morphology of six-month old representative cultured fungal strains belonging to Agaricostilbomycetes and included in the phylogenetic analysis of Fig. 2. Colony shape of *Kurtzmanomyces* sp. L4046 (A), L3044 (B), L3045 (C), L4046 (D) and L3034 (E); mature cells and polar budding cells of *Kurtzmanomyces* sp. L4046 (F), of Agaricostilbaceae sp. L4069 (G, L); mature cells and polar budding cells strained with Phloxin B of *Kurtzmanomyces* sp. L4046 (H, I), germination cells are visible in (I, J), cells and polar budding cells of *Kurtzmanomyces* sp. L3044 (K). Scale bars: 1,5 cm (A–E), 10 μm (F–L).

associated ascomycetes and basidiomycetes. Our study contributes to this body of knowledge by providing axenic culture isolates with accompanying morphological description and DNA sequence data for 76 strains of basidiomycetous yeasts, belonging to five classes and isolated from *R. melanophthalma* and/or *T. atra* collected in boreal, alpine, temperate, humid and arid habitats worldwide. Most of the identified strains were never isolated from lichens before, and some would deserve taxonomic species description, as they either build new own clades or expand clades with other undescribed taxa. The formal descriptions are beyond the aims of this study, and will be tackled instead in a separate, taxonomic manuscript. In this perspective, indeed, we will address *i)* the common procedure to perform assimilation tests/assays for the precise identification of yeast species and *ii)* a complete characterization of the yeast and filamentous phases of the life cycle, when possible. We refer, therefore, to the previously published phylogenies to recognize and temporary name species-level lineages.

Although our results highlight a great diversity of basidiomycetous yeasts, still within individual lichen thalli this diversity seems to be relatively low, as in general one to three yeast taxa could be detected, whereas only from one thallus of *R. melanophthalma* (L2637) up to four different yeast taxa were isolated. A more comprehensive overview of the basidiomycetous yeasts associated to these two lichen species could be revealed in forthcoming metabarcoding sequencing results, which are under analysis.

Interestingly, the only strains recovered in the class Filobasidiales (Tremellomycetes) and nested within *Naganishia* were isolated from thalli of *R. melanophthalma* growing on acidic rocks in South America between 3300 and 3500 m a.s.l. Yeast strains found only in *R. melanophthalma* form the distinct clade 'Yeast Lineage II' (including also four other unidentified samples Fig. 6) in the Tremellales, and are included in the clades of *Fibulobasidium* and *Vishniacozyma* (Tremellomycetes), *Calacogloea* sp. (Microbotryomycetes) and *Tranzscheliella* sp. (Ustilaginales). In contrast, yeast strains isolated only from thalli of *T. atra* form the distinct clade Yeast Lineage I (Fig. 6) with two other unidentified samples, others belong to *Pseudotremella* (Tremellomycetes), or have an uncertain position in the Microbotryomycetes. On the other hand, we identified strains nested within Agaricostilbaceae and Chionosphaeraceae (Agaricostilbomycetes), and *Tremella macrobasidiata* (Tremellomycetes) from both thalli of *R. melanophthalma* and *T. atra*.

4.1. Agaricomycotina diversity in R. melanophthalma *and* T. atra – *Tremellomycetes*

Our results show that most of the basidiomycetes associated to the two investigated lichen species belong to the class Tremellomycetes (Agaricomycotina), for which so far 72 lichenicolous fungi have been described (Diederic et al., 2018; 2019, 2020; Diederich and Ertz, 2020). As introduced before, many Tremellomycetes are characterized by their dimorphism (Bandoni, 1995)

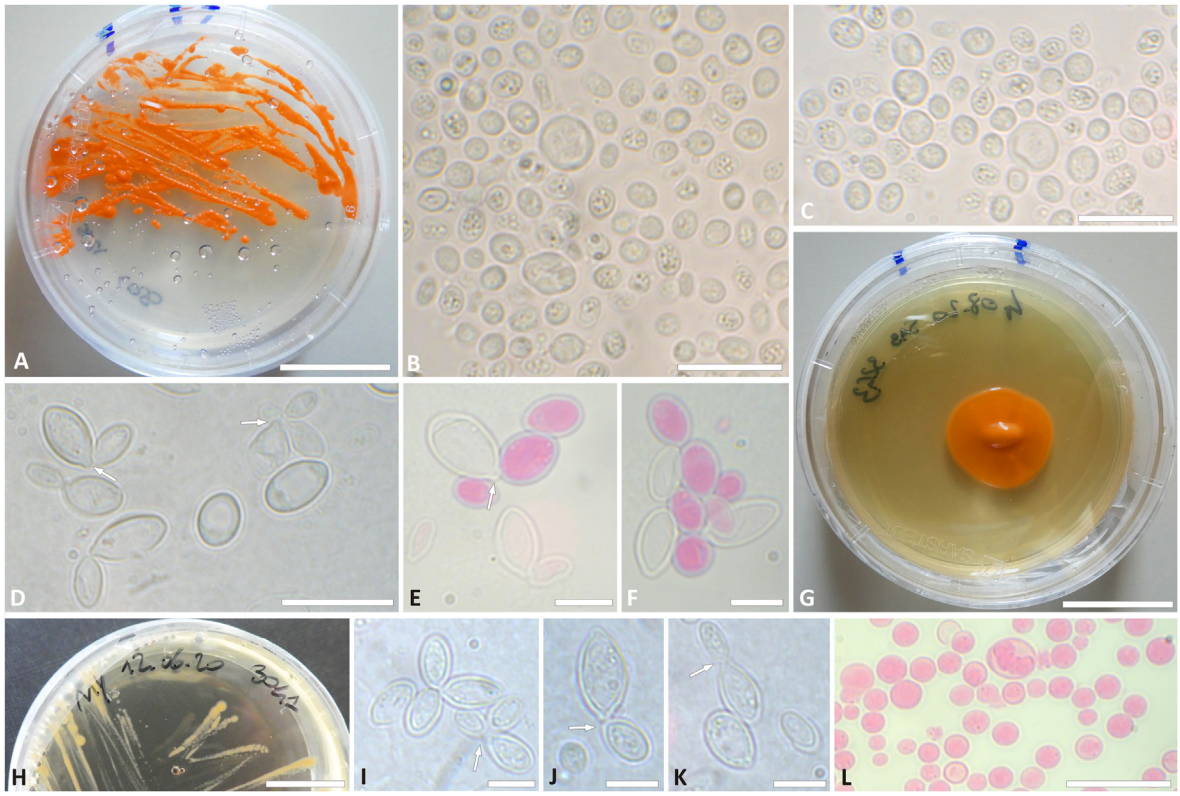


Fig. 9. Morphology of six-month old representative cultured fungal strains belonging to Cystobasidiomycetes and included in the phylogenetic analysis of Fig. 3. Colony shape of uncultured Cystobasidiomycetes L4050 (A), of uncultured Erythrobasidiaceae L3243 (G), of *L. pisutiana* L3041 (H); mature cells and polar budding cells of uncultured Cystobasidiomycetes L4050 (B, C) and L4045 (D, K), of *L. pisutiana* L3041 (I, J); mature cells and polar budding cells strained with Phloxin B of uncultured Cystobasidiomycetes L4045 (E, F), of uncultured Erythrobasidiaceae L3243 (L). Scale bars: 1,5 cm (A, G, H), 10 μm (B-D, L), 5 μm (E, F, I–K).

and this was shown also for some lichen-associated *Tremella* species in thalli of *Lecanora* and *Letharia* (Tuovinen et al., 2019, 2021). Indeed, *Tremella* in lichens are recognizable for the formation of basidiomata which often induce the formation of big deforming galls on the thalli. Only inside the basidiomata, tremelloid haustoria have been observed, and thus the species were considered as mycoparasites of the lichen mycobiont (Spatafora et al., 2017). The interaction between these tremellomycete haustoria and mycobiont hyphae, however, has not been clarified yet (Bauer and Oberwinkler, 2008). Whether as yeast or filamentous state, Tremellomycetes detected by the DNA metabarcoding approach are reported for thalli lacking basidiomata (Lindgren et al., 2015; Zhang et al., 2015; Fernández-Mendoza et al., 2017; Banchi et al., 2018; Smith et al., 2020). We isolated the Tremellomycetes strains from thalli which also did not present any galls or deformation: we detected *Tremella macrobasidiata* strains by PCR amplifications from thalli of *R. melanophthalma* and *T. atra* that did not show any symptom of infection. This result strengthens the assumption that the unicellular yeast stage may be common in lichens.

The classification of the Tremellomycetes has been – and still is – problematic. In this study we used the integrated phylogenies published by Liu et al. (2015a,b) to recognize and name the newly identified strains. To better understand species diversity in Tremellomycetes, we analysed our new sequences using two individual datasets of Tremellales and Filobasidiales. Within both orders the newly isolated fungal strains are very closely related to yeasts isolated and described from other rather cold and extreme environments, such as strains 9.L31, LTSP_EUKA_P5H04 and 112_NA3_P32_B23, *Saitozyma* sp., *Vishniacozyma carnescens* and *N. albidosimilis*. In particular, the uncultured fungus LTSP_EUKA_P5H04 and 112_NA3_P32_B23 were obtained from soil collected respectively in British Columbia (Canada) and North American Arctic Transect (NAAT; Hartmann et al., 2009; Timling et al., 2014). Moreover, strain 9.L31 was identified for the first time by Duarte et al. (2016) who isolated it from a thallus of *Usnea antarctica* collected in the South Shetland Island (Antarctica). Our isolates, instead, come from thalli of *R. melanophthalma* collected in South and North America at high elevation (over 3000 m a.s.l.) and in Spain at 1450 m a.s.l.

Strains forming the distinct clade, here indicated as 'Yeast Lineage I' (Fig. 6), present the highest sequence similarity with a sample of uncertain position (KBPY6612), which is reported from the lichen *C. rangiferina* from the Altai mountains (Russia) by Kachalkin but still indicated as an unpublished work. Our strains derive from thalli of *T. atra* collected in the Italian Alps, Spain and Tasmania, in an altitudinal range from 500 m to 2000 m a.s.l., likely similar to alpine environments of the Altai mountains.

Only one strain, isolated from *R. melanophthalma* collected in the Alps, is nested within *V. carnescens*, and is recognised to be this species. *V. carnescens* was isolated from Antarctic soil (in South Victoria Land, Ross Sea region and Connell et al., 2008; Arenz et al., 2006; Tsuji, 2018) but it is also reported from *Cladonia* lichens collected in Subarctic Russia (Kachalkin et al., 2017), thus justifying its presence also in our lichen sample from a similar environment, such as the Alps.

Tremella macrobasidiata (Zamora et al., 2011; Millanes et al., 2011) was isolated from both *T. atra* and *R. melanophthalma*; while *Pseudotremella* aff. *indecorata* (Millanes et al., 2011; Fan et al., 2021) was isolated from *T. atra* only, from thalli collected in the Alps, on a mountain massif in Spain, and in North and South

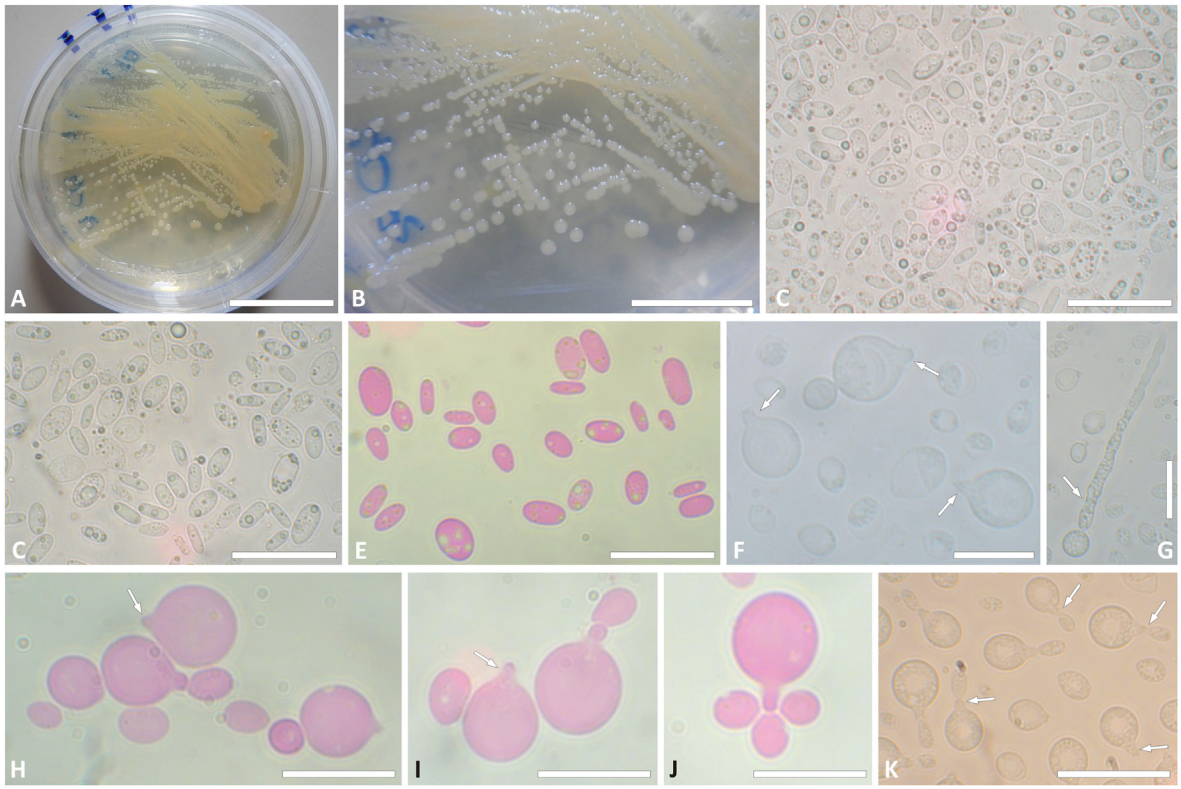


Fig. 10. Morphology of six-month old, representative cultured fungal strains belonging to Microbotryomycetes and included in the phylogenetic analysis of Fig. 4. Colony shape of *Colacogloea* sp. L4072 (A, B); mature cells and polar budding cells of *Colacogloea* sp. L4072 (C–D), of *Yunzhangia auricularia*eL4063 (F, K); germination cell of *Y. auricularia*e L4063 (G); mature cells and polar budding cells strained with Phloxin B of *Colacogloea* sp. L4072 (E), of *Y. auricularia*e L4063 (H–J). Scale bars: 1,5 cm (A, B), 20 μm (C-E, G, K), 10 μm (F, H-J).

America. *T. macrobasidiata* is placed in the phylogenetic clade named as Lichenicolous Clade III by Millanes et al. (2011) -and here-together with other lichenicolous *Tremella* and *Biatoropsis* species, while *Pseudotremella* aff. *indecorata* (not lichenicolous) belongs in *Pseudotremella*. *Tremella macrobasidiata* is characterized by a dimorphic lifestyle: the sexual filamentous (hyphae) stage with the formation of basidiomata was observed specifically on the lichen *Lecanora chlarotera* (Zamora et al., 2011, 2016); the yeast stage (asexual) instead was detected in two *Lecanora* species (Tuovinen et al., 2021). Our microscopy analyses confirmed the dimorphism for both species *T. macrobasidiata* and *Tremella indecorata*, as in the cultured strains we observed either yeasts and budding germination of cells or hyphae growing out of the yeast cells and starting a filamentous mycelium (Fig. 12).

Within the order Filobasidiales, we isolated strains of *N. albidosimilis* from thalli of *R. melanophthalma* collected above 3000 m a.s.l. in Argentina and Chile. *N. albidosimilis* is a psychrophilic basidiomycete isolated from Antarctica (Vishniac and Kurtzman, 1992; Scorzetti et al., 2000; Pavlova et al., 2001; Connell et al., 2008; Arenz et al., 2006). Its presence in lichen thalli form dry, cold extreme habitats, such as the localities visited by us, is reasonable.

4.2. *Pucciniomycotina diversity in* R. melanophthalma *and* T. atra

In our lichen samples, Pucciniomycotina yeasts are represented by the three orders Agaricostilbomycetes, Cystobasidiomycetes and Microbotryomycetes. This phylum is formed by a large group of fungi including plant pathogens (mainly Pucciniales), lichenicolous heterobasidiomycetes and many other remarkably ecologically and biologically diverse fungi (Aime et al., 2006). Instead, lichen-inhabiting species have been recognized only in the three genera *Crittendenia*, *Cyphobasidium* and *Lichenozyma* (Millanes et al., 2021). Within the class Cystobasiodiomycetes, both yeast and dimorphic species having different life strategies are found, such as endophytes, saprophytes, mycoparasites, lichen-associates and fungi adapted to aquatic environments (Boekhout et al., 2011). The lichen-associated yeasts were hypothesized to play a key role in the lichen symbiosis (Spribille et al., 2016), and studies have aimed at uncovering their diversity. Presently, only a few studies have reported on axenically isolated strains of Cystobasidiomycetes. *Cystobasidium psychroaquaticum* was cultured from *Cladonia pocillum* from Svalbard (Zhang et al., 2016); *Cystobasidium* spp. (Cystobasidiales) were isolated from *Usnea aurantiaco-atra*, *U. antarctica*, 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). Here, we successfully isolated *L. pisutiana* (Microsporomycetaceae) from *R. melanophthalma* thalli collected on a mountain massif in central Spain at 1900 m a.s.l. This yeast was described and isolated for the first time by Černajová and Škaloud (2019) from various *Cladonia* species and *Cetraria ericetorum*. Because L. *pisutiana* does not produce any visible symptoms on the lichen thalli, neither in *Cladonia* nor in our samples, Černajová and Škaloud (2019) proposed that it could represent the anamorphic form of a 'still to be discovered' lichenicolous fungus. We isolated L. *pisutiana* from *R. melanophthalma* growing on silicious-granitic boulders, whereas Černajová and Škaloud (2019) isolated it from *Cladonia rei* collected on limestone quarry, suggesting a lack of substrate- and host preference for this yeast species.

We also isolated for the first time in axenic culture two yeast strains whose sequences match most closely with sequences of two

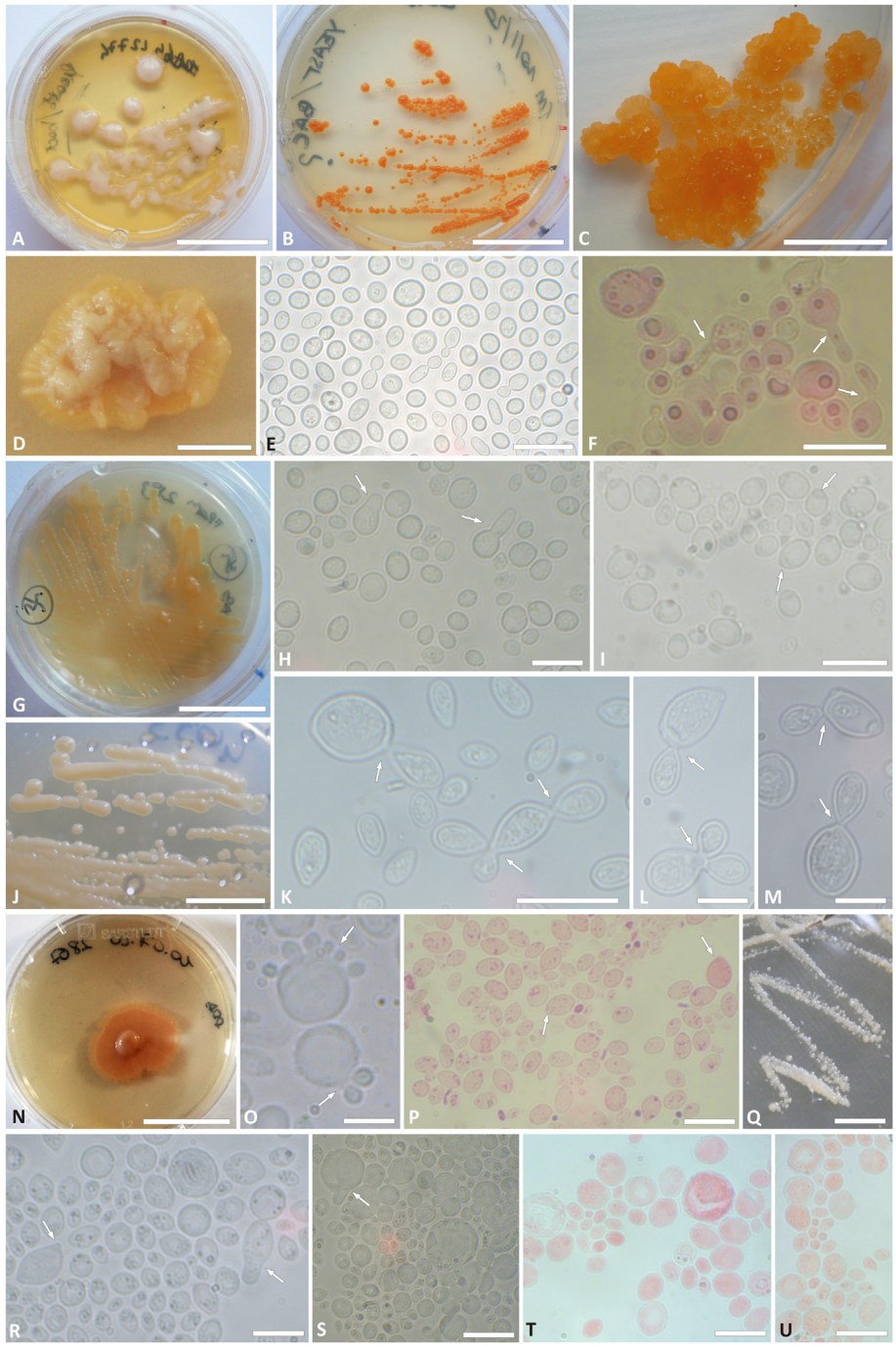


Fig. 11. Morphology of six-month old, representative cultured fungal strains belonging to Tremellomycetes and included in the phylogenetic analysis of Figs. 5 and 6. Colony shape of *Naganishia albidosimilis* L2774 (A), L2781 (B), L2770 (C) and L2767 (D); mature cells and polar budding cells of L2774 are visible in (E). C-olony shape of *Saitozyma* sp. L2889 (G) and L4093 (J); cells and germination cells strained with Phloxin B of L2889 are visible in (F); mature cells, polar budding cells and germination cells of L4065 (H) and L4049 (I). Colony shape of *Cryptococcus* sp. L2867 (N) and L2887 (Q); mature cells and polar budding cells of L3025 (K–M) and L2894 (R, S); multipolar budding cells of L2867 (O); cells and polar budding cells strained with Phloxin B of *Cryptococcus* sp. L3051 (P) and L3738 (T, U). Scale bars: 1,5 cm (A, B, G, N), 1 cm (Q), 0,5 cm (C, D, J), 10 μm (E, F, H, I, K, P, U), 5 μm (L, M, O, R-T).

fungi sequenced from thalli of *Lecanora pulicaris* and *Cladonia bellidiflora* (Mark et al., 2020; Černajová and Škaloud, 2019). The fungus from *L. pulicaris* was detected by Mark et al. (2020) in a metagenomic analysis, that from thalli of *C. bellidiflora* by Černajová and Škaloud (2019) using the specific primers for Cystobasidiomycetes designed by Spribille et al. (2016). As our strains derive from thalli of *T. atra* collected on the Alps, this yeast seems to be rather unspecific for its lichen hosts.

In the class Agaricostilbomycetes we identified two strains within Agaricostilbaceae closely related to *Sterigmatomyces* and *Pseudobensingtonia*, and most of the strains in the family Chionosphearaceae–- closely related to *K. nectairei*. Species of *Chionosphaera* are not known to be lichenicolous, as *Chionosphaera apobasidialis* was found on bark of deciduous trees and as the mycoparasite of *Cladosporium herbarum* (Cox, 1970), while *C. cuniculicola* grows inside bark beetle galleries (Kirshner et al.,

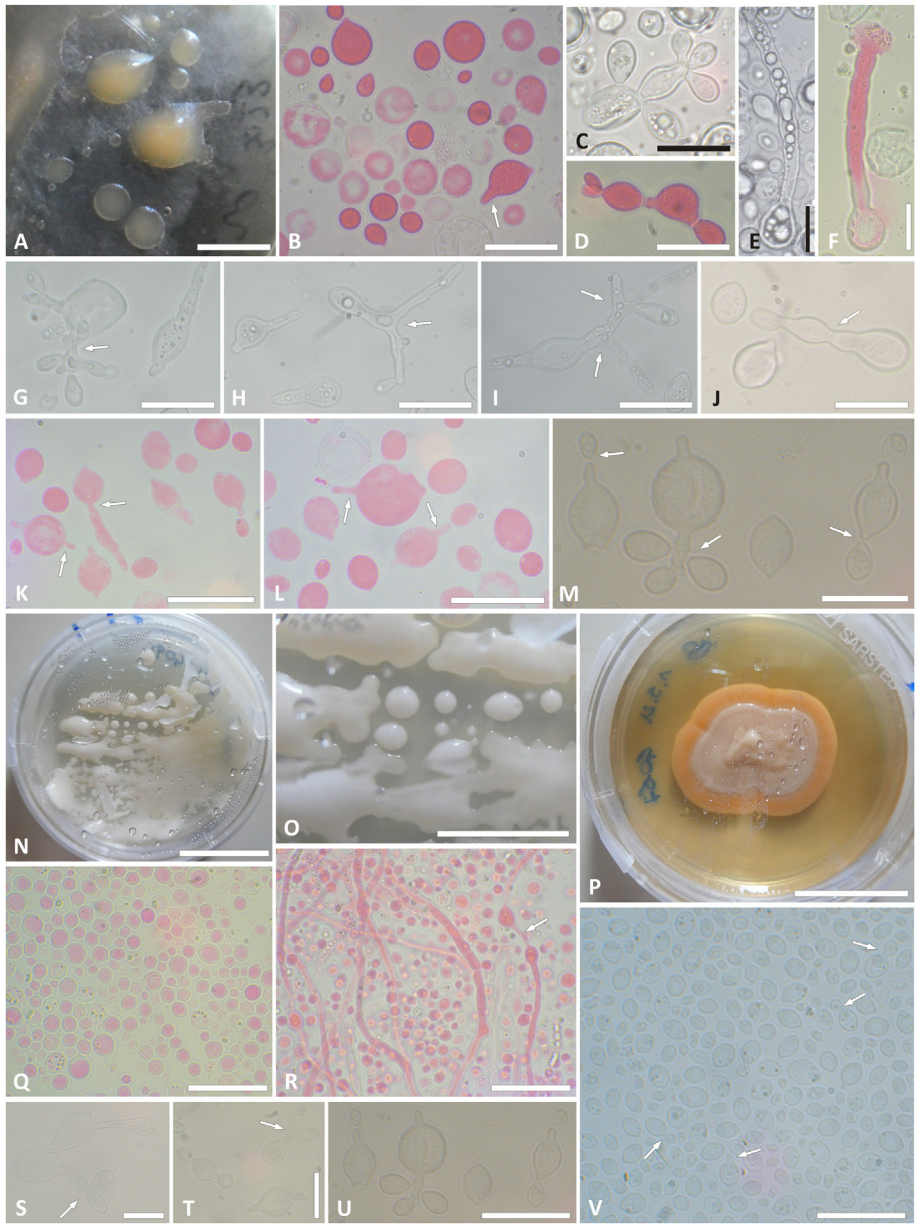


Fig. 12. Morphology of six-month old, representative cultured fungal strains belonging to Tremellomycetes and included in the phylogenetic analysis of Fig. 6. Colony shape of *Tremella macrobasidiata* L3023 (A); multipolar budding cells of L3023 (C), L3785 (G) and L4085 (M); germination cells of L3023 (E) and L3785 (H–J); mature cells and budding polar cells strained with Phloxin B of L3023 (B, D) and L4085 (K, L); germination cells strained with Phloxin B of L3023 (F) and L4085 (K). Colony shape of *T. indecorata* L4091 (N, O) and L4067 (P); mature cells and polar budding cells of L4080 (S–V); mature cells strained with Phloxin B of L4067 (Q); hyphae strained with Phloxin B of L4067 (R). Scale bars: 1,5 cm (N, P), 0,5 cm (A, O), 20 μm (Q, R, V), 10 μm (B-D, G-M, U), 5 μm (E, F, O, S).

2001). *C. apobasidialis* and *C. cuniculicola* life cycles have been studied in detail in axenic culture by Roberts (1997). The suggestion that these species could complete their life cycle within the lichen thalli has not been supported yet. Our strains, obtained from both *R. melanophthalma* and *T. atra*, were placed in a clade with *K. nectairei*, which is evolutionary relatively closely related to the two afore mentioned *Chionosphaera* species (Kwon-Chung, 2011; Millanes et al., 2021). The ecological preferences of *Kurtzmanomyces* in nature is not yet clarified: *Kurtzmanomyces tardus* was isolated from contaminated demineralized water, *K. nectairei* from cheese and *Kurtzmanomyces insolitus* from the fruit body of a heterobasidiomycete (Sampaio et al., 1999). Some years ago, *Kurtzmanomyces* spp. were also described as saprophytic and isolated from desert soils crusts in northwestern China (Zhang et al., 2013) and from the High Arctic Archipelago Svalbard (Mundra et al., 2016).

Instead, the recognized lichenicolous genus in Agaricostilbomycetes is *Crittendenia* (Millanes et al., 2021), which is not present among our samples. *Crittendenia* includes two described lichenicolous species: *C. coppinsii* growing on thalli of *Melanelixia* and *Melanohalea* species (Blanco et al., 2004; Arup and Sandler Berlin, 2011; Divakar et al., 2017), and *C. lichenicola* growing on lichen species of *Micarea* (Millanes et al., 2021). Some other still undescribed *Crittendenia* species parasitize lichen hosts in the families Lecanoraceae, Lobariaceae, Parmeliaceae, Physciaceae, Ramalinaceae and Teloschistaceae (Millanes et al., 2021). Just recently, the new family Crittendeniaceae was established (Diederich et al., 2022).

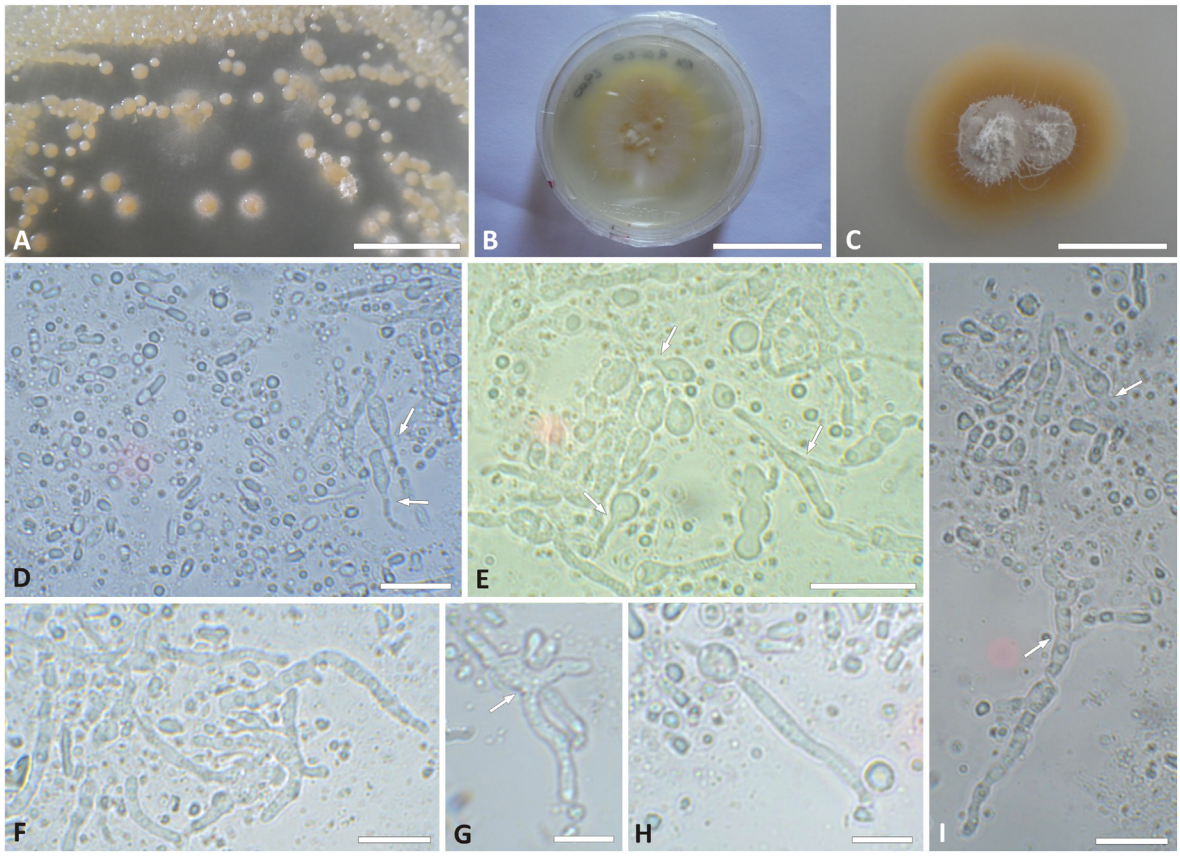


Fig. 13. Morphology of six-month old, representative cultured fungal strains belonging to Ustilagomycetes and included in the phylogenetic analysis of Fig. 7. Colony shape of *Tranzschelliella* sp. L2891 (A), L2900 (B) and L3062) (C); unicellular stages, filamentous stages and germination cells of L2900 are visible in (D–I); polar budding cells of L2900 (E). Ramification of hyphae of L2900 are visible in (E, G, I). Scale bars: 0,5 cm (A), 3 cm (B), 1,5 cm (C), 10 μm (D, E, F, I), 5 μm (G, H).

We found only three strains within the class Microbotryomycetes which are closely related to two genera previously isolated and described from soil, i.e., *Colacogloea* and *Yunzhangia*. The strain isolated from *T. atra* collected on the Alps, appears, although without support, as sister species of *Y. auriculariae*, a yeast isolated from Antarctic soils and detected in several previous studies (Ray et al., 1989; Pavlova et al., 2001; Buzzini et al., 2012; Shivaji and Prasad, 2009). Instead, the two strains coming from *R. melanophthalma* thalli collected on the Alps are nested within *Colacogloea* spp. These yeasts were isolated from soil and plant residues in monoterpene-rich environments (Thanh et al., 2004; Pohl et al., 2011) and beech forest soils (Yurkov et al., 2016; Kachalkin et al., 2019), two environments completely different from those visited by us. However, some *Colacogloea* spp. are mycoparasites characterized by septate basidia and colacosomes (Oberwinkler et al., 1990; Bauer and Oberwinkler, 1991, 2008). This observation, together with the above reported presence of lichenicolous (our strains) and mycoparasitic species (as *C. apobasidialis*) within the same family Chionosphaeraceae seems to be in line with the observation provided by Oberwinkler (2017), who suggested the many yeast taxa in lichens could represent mycoparasites of the lichen mycobiont.

4.3. *Ustilagomycotina diversity in* R. melanophthalma *and* T. atra

Ustilagomycotina are the least represented in lichens. Here, we isolated only four strains nested within *Tranzscheliella* species from thalli of *R. melanophthalma* collected in North and South America. *Tranzscheliella* spp. are known plant pathogen of 33 genera of grasses (Poaceae) widely distributed around the world (Vánky, 2011; Li et al., 2017). Surprisingly, recent metabarcoding analyses report *Tranzscheliella* spp. also from Artic deep-sea sediments (Ogaki et al., 2021).

5. Conclusions

This is the first study in which an extensive sampling of two reference/model species of cosmopolitan lichens – *R. melanophthlama* and *T. atra* – has been performed to widen our knowledge and characterize the diversity of lichen-associated basidiomycetous yeasts. We report on the successful isolation of 76 yeast strains belonging to the three phyla in Basidiomycota. Phylogenetic analyses helped to identify yeast strains both corresponding to already known lichenicolous basidiomycetous fungi (*Tremella macrobasidiata*, *T. indecorata*, *Saitozyma* sp., *L. pisutiana*), and to potentially new taxa previously unknown from lichens. Here, we also highlight the importance of understanding the diversity and symbiotic relationships of lichen-associated yeast and the pressing need of a stable, comprehensive taxonomy to recognized them. Bacon and White (2000) suggested that lichens act as protective niches for other microorganisms, helping them in thriving and dispersing in extreme environments, such as the mountain regions where *T. atra* and *R. melanophthlama* were collected. Later, Santiago et al. (2015) introduced the concept of "lichensphere" referring to the surface and those narrow spaces inside lichen thalli offering favorable natural microhabitats for microorganisms. In recent studies it was speculated whether yeasts potentially synthesize secondary metabolites useful for the

acquisition of nutrients in lichens (Spribille et al., 2016, 2020; Tagirdzhanova et al., 2021), but there is still no experimental evidence. We noticed a few patterns of host and substrate preference, thus the yeasts here detected might be ecologically-constrained and forthcoming analyses will clarify their taxonomy and their potential presence in additional lichen species.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2022.07.004.

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