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Muellerella, a lichenicolous fungal genus recovered as polyphyletic within *Chaetothyriomycetidae* (*Eurotiomycetes*, *Ascomycota*)

Lucia Muggia[1*], Sergio Pérez-Ortega[2] & Damien Ertz[3,4]

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Abstract. Molecular data and culture-dependent methods have helped to uncover the phylogenetic relationships of numerous species of lichenicolous fungi, a specialized group of taxa that inhabit lichens and have developed diverse degrees of specificity and parasitic behaviors. The majority of lichenicolous fungal taxa are known in either their anamorphic or teleomorphic states, although their anamorph-teleomorph relationships have been resolved in only a few cases. The pycnidium-forming *Lichenodiplis lecanorae* and the perithecioid taxa *Muellerella atricola* and *M. lichenicola* were recently recovered as monophyletic in *Chaetothyriales* (*Eurotiomycetes*). Both genera are lichenicolous on multiple lichen hosts, upon which they show a subtle morphological diversity reflected in the description of 14 species in *Muellerella* (of which 12 are lichenicolous) and 12 in *Lichenodiplis*. Here we focus on the teleomorphic genus *Muellerella* and investigate its monophyly by expanding the taxon sampling to other species occurring on diverse lichen hosts. We generated molecular data for two nuclear and one mitochondrial loci (28S, 18S and 16S) from environmental samples. The present multilocus phylogeny confirms the monophyletic lineage of the teleomorphic *M. atricola* and *M. lichenicola* with their *L. lecanorae*-like anamorphs, but places the rest of the *Muellerella* species studied in two different monophyletic lineages with strong support. The first, *Muellerella* spp. 1, is nested within some new lineages of black fungi isolated from different epilithic lichen thalli, while the second, *Muellerella* spp. 2, is closely related to the *Verrucariales*. Based on these results, we reappraise the phylogenetic placement of *Muellerella* and suggest its polyphyly within *Chaetothyriomycetidae*.

Key words: diversity, multilocus analysis, parasitic, phylogeny, *Verrucariales*.

Introduction

In the past decade, molecular data have increasingly helped to resolve the phylogenetic position of many fungal taxa, filling numerous gaps in our current knowledge of the fungal tree of life. Many genera have been tested for their monophyly, either confirming it (e.g., see review by Tedersoo et al. 2018) or not (e.g., Aveskamp et al. 2009; Rai et al. 2014; Ertz et al. 2015a, b). Additionally, comparisons of anamorphic and teleomorphic states, sometimes complemented by axenic cultures, have allowed researchers to establish the connections between sexual and asexual states in numerous fungi (e.g., Pérez-Ortega et al. 2011; Ertz et al. 2014; Muggia et al. 2015). Together, these findings have led to important taxonomic revisions, including the introduction and invalidation of several species names (Hawksworth 2011). However, fungal taxa characterized by inconspicuous mycelia or specialized ecological niches have often been neglected due to difficulties encountered in obtaining molecular data from their thalli.

Among these poorly investigated fungal groups are the lichenicolous fungi, the majority of which are Ascomycota. They are known to inhabit lichen thalli or the apothecia of the mycobiont, upon which they are detectable by their symptomatic infections and their sexual or asexual spore-producing structures (Lawrey & Diederich 2003; Diederich et al. 2018). Lichenologists distinguish these fungi from those that inhabit the lichen thalli asymptomatically, that is, the 'endolichenic fungi' (Arnold et al. 2009)

[1] Department of Life Sciences, University of Trieste, Via Giorgieri 10, 34127 Trieste, Italy
[2] Real Jardín Botánico, CSIC, Plaza de Murillo, 2, 28014 Madrid, Spain
[3] Meise Botanic Garden, Department Research, Nieuwelaan 38, B-1860 Meise, Belgium
[4] Fédération Wallonie-Bruxelles, Direction Générale de l'Enseignement non obligatoire et de la Recherche scientifique, rue A. Lavallée 1, B-1080 Bruxelles, Belgium
[*] Corresponding author e-mail: lmuggia@units.it, lucia_muggia@hotmail.com

that are detectable only by molecular analyses or culture isolation. The lichenicolous lifestyle has multiple origins in the fungal kingdom, from both lichenized and non-lichenized ancestors (Arnold et al. 2009; Pino-Bodas et al. 2017). Lichenicolous fungi have been reported in seven classes of Ascomycota, but the majority of taxa have been placed in the three big classes *Dothideomycetes*, *Eurotiomycetes* and *Lecanoromycetes* (Pino-Bodas et al. 2017; Diederich et al. 2018; Muggia & Grube 2018). Though 2000 species of lichenicolous fungi are known worldwide (Diederich et al. 2018), only a few taxa have been the focus of molecular analyses, while the majority of the described species are still classified according to morphological or anatomical characters. Lichenicolous fungi have evolved diverse degrees of specificity towards their hosts, ranging from parasites to commensals (Lawrey & Diederich 2003). Many species seem to have a very narrow host range and to be highly dependent on their lichen hosts, which makes it particularly difficult to isolate and grow them in axenic culture (Crittenden et al. 1995) or to retrieve a reasonable number of environmental samples for molecular investigation.

Recently, Muggia et al. (2015) clarified the phylogenetic relationship between two lichenicolous fungi that frequently co-occurred on thalli of the host lichen *Tephromela atra*: the pycnidium-forming *Lichenodiplis lecanorae* and the perithecioid *Muellerella atricola*. Using molecular data obtained from environmental samples and culture isolates, the authors revealed the anamorph-teleomorph relationship of the two species. An in-depth screening of herbarium collections confirmed the co-occurrence of *Lichenodiplis* and *Muellerella* species on other lichen hosts. In particular, the phylogenetic analysis of Muggia et al. (2015) indicates that *M. lichenicola* also has *L. lecanorae* as anamorphic state. These first results of Muggia et al. (2015) hint that *Lichenodiplis lecanorae* represents several cryptic taxa that are the asexual state of at least two *Muellerella* species (viz. *M. atricola* and *M. lichenicola*). Because of this, we use the phrase '*L. lecanorae*-like anamorphic state' to refer to the anamorphic state of *Muellerella* species included in the present study.

The genus *Muellerella* in particular is one of the most widespread and frequently collected lichenicolous fungi. At present, 12 accepted lichenicolous species have been described from a wide range of lichen hosts growing mainly on calcareous and siliceous rocks and on trees (von Brackel 2014; Diederich et al. 2018). *Muellerella* species are easily recognizable due to the conspicuous black, sometimes slightly shiny perithecia that are immersed or sessile on the thallus and/or on the apothecia of the host lichens, polyspored asci usually containing 0–1-septate, ellipsoid, brown ascospores (Fig. 1, 2). Triebel (1989) and Triebel & Kainz (2004) classified the genus in the family *Verrucariaceae*, while the phylogenetic inference of Muggia et al. (2015) suggested that the genus forms a new monophyletic lineage sister to *Epibryaceae* within *Chaetothyriales*. *Muellerella* species can indeed be bryophilous, lichenicolous or saprophytic (Döbbler & Triebel 1985; Triebel 1989; Triebel & Kainz 2004). When occurring on lichens, species of *Muellerella* present a continuum of morphological variation and subtle character diversity (e.g., variation in ascospore size and their number per ascus), which has been correlated with its host specificity. Because of this, some species have been described according to their occurrence on only certain lichen host species or genera (e.g., *M. antarctica* from *Hypogymnia antarctica*, *M. atricola* from *Tephromela atra*, *M. lecanactidis* from *Sigridea californica*, *M. stictinae* from species of the genus *Sticta*, *M. vesicularia* from species of the genus *Toninia*). Their genetic diversity has never been assessed, however.

In this study we extend the original taxon sampling of Muggia et al. (2015) by including *Muellerella* species from different host lichens, and we consider the previous dataset (Muggia et al. 2015) as a framework for testing the monophyly of this genus.

Materials and methods

Sampling

Fresh samples and herbarium vouchers (from BR, TSB and MA-Lichen) of *Muellerella erratica*, *M. ventosicola*, and three specimens not fitting the currently accepted *Muellerella* species were used for molecular and morphological analyses (Table 1, Table S1). The specimens were identified following Triebel (1989) and Hafellner (2007), and are named according to the current nomenclature presented by Diederich et al. (2018).

The final molecular dataset (Table 2) includes (i) the newly sequenced specimens of *Muellerella erratica*, *M. ventosicola*, and three specimens not fitting the current *Muellerella* species concepts, infecting a total of six different lichen host species (Table 1); (ii) sequences of *Muellerella atricola*, *M. lichenicola* and their *Lichenodiplis lecanorae*-like anamorphic state published by Muggia et al. (2015); (iii) representatives of orders of *Chaetothyriomycetidae*, viz. *Chaetothyriales*, *Phaeomoniellales*, *Pyrenulales* and *Verrucariales* (*Verrucariaceae*), and within *Chaetothyriales* the families *Chaetothyriaceae*, *Cyphellophoraceae*, *Epibryaceae*, *Herpotrichiellaceae* and *Trichomeriaceae*, selected from the recent phylogenetic studies of Gueidan et al. (2014) and Teixeira et al. (2017); and (iv) selected isolates of cultured endolichenic fungi obtained from different epilithic lichen thalli and representing new lineages (clade I, clade II, clade IV, clade V, clade VI+VII) in *Chaetothyriomycetidae*, as published by Muggia et al. (2016, 2017). Some of the latter fungal strains were isolated from lichen thalli infected by *Muellerella* species (Muggia et al. 2016, 2017; Table 2). The sequences of these cultured endolichenic fungi were selected to test whether our newly generated sequences correspond to any of these lineages, and thereby to evaluate whether they ought to be included in *Muellerella*.

DNA extraction, amplification and sequencing

Perithecia of *Muellerella* were carefully dissected under a stereomicroscope and prepared for DNA extraction, taking care to remove the lichen thallus and perithecial wall.

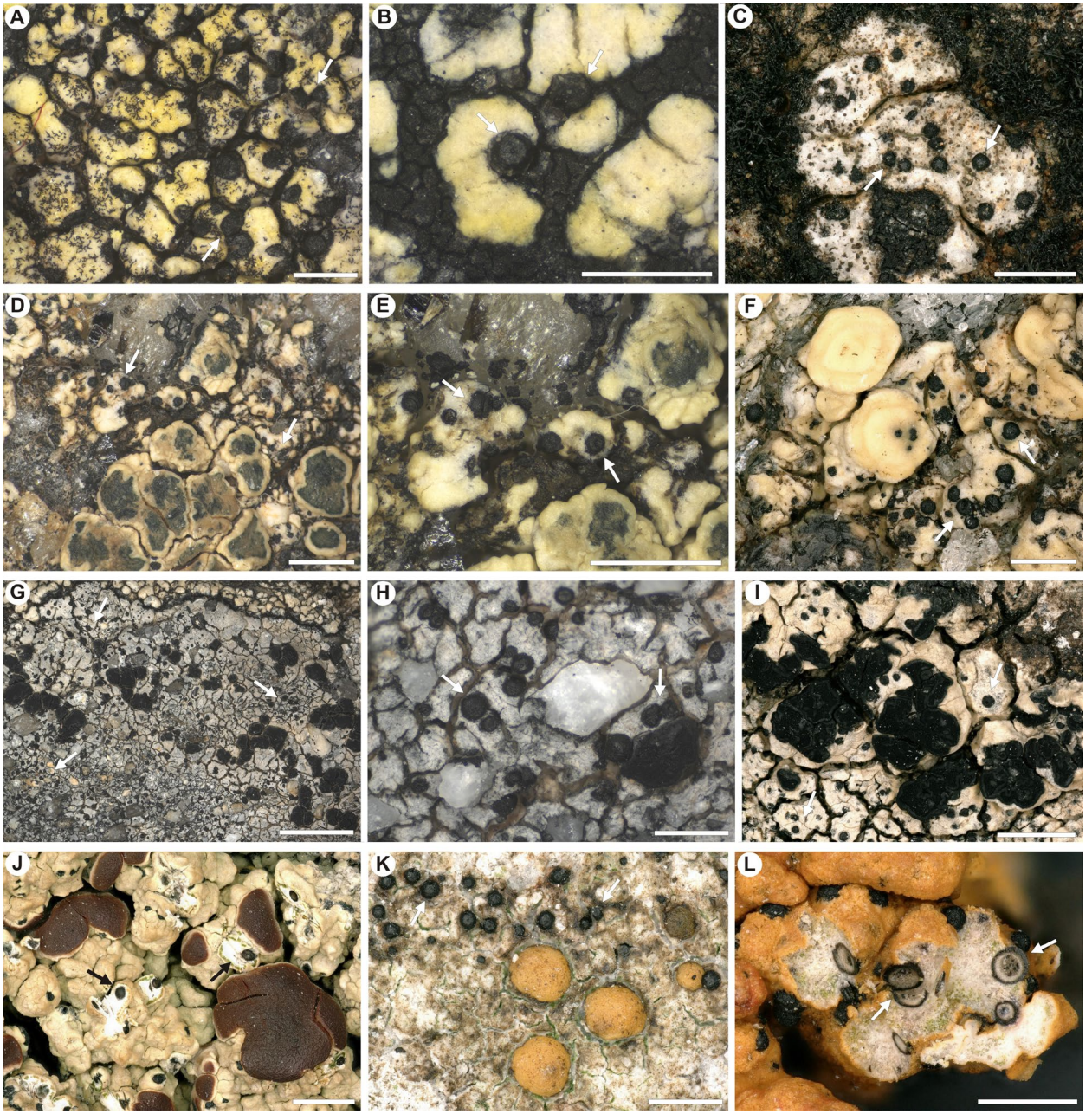

Figure 1. Habitus of lichenicolous species of *Muellerella* spp. on different lichen hosts [specimen ID]. A, B – *M. ventosicola* s.lat. on *Rhizocarpon geographicum* [Muggia L2362 (A), Muggia L2355 (B)]; C – *Muellerella* sp. on *Trapelia* sp. [Ertz 17847]; D, E – *M. erratica* on *Lecanora intricata* [SPO-4576]; F – *M. erratica* on *Lecanora polytropa* [Ertz 20470]; G–I – *M. erratica* on *Lecidea* spp. [(G, H) SPO-4599, (I) Ertz 20487]; J – *M. ventosicola* s.str. on *Ophioparma ventosa* [Reidar 150307]; K – *Muellerella* sp. on *Protoblastenia rupestris* [Ertz 20419]; L – *M. erratica* on *Xanthoria elegans* [Ertz 20485], detail of thallus sectioned transversally in a perithecia-rich area. Arrows indicate perithecia of *Muellerella*. Scales: A, B, F, H, K, L = 0.5 mm; C–E, I, J = 1 mm; G = 4 mm.

A single perithecium was taken per sample and transferred to a 1.5 ml tube. The material was first frozen and then pulverized with metal beads using a TissueLyserII (Retsch) or with an iron pestel. The DNA was extracted using a ZR Fungal/Bacterial DNA MicroPrep™ Kit (Zymo Research) or an EZNA Forensic DNA kit (Omega Bio-Tek), following the manufacturers' instructions (standard protocol). We also used hand-made sections of the perithecia for direct PCR as in Ertz et al. (2015a) at the Meise Botanic Garden. Fragments of the hymenium, rarely also with tiny fragments of the perithecial wall, were placed directly in microtubes with 20 µl H_2O. Amplification reactions were prepared for a 50 µl final volume containing 5 µl 10× DreamTaq Buffer (Thermo Fisher Scientific, Waltham, MA), 1.25 µl of each of the 20 µM primers, 5 µl of 2.5 mg ml^{-1} bovine serum albumin (Thermo Fisher Scientific, Waltham, MA), 4 µl of 2.5 mM each dNTPs (Thermo Fisher Scientific, Waltham, MA), 1.25 U DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA), and the tiny fragments of the lichenicolous fungus.

The phylogenetic placement of *Muellerella* was studied by sequencing the same loci as in Muggia et al. (2015, 2016, 2017) in order to allow comparison of the results and verification of coherency in the extended analysis. We amplified the partial nuclear large (28S) and small (18S) subunits ribosomal DNA and the mitochondrial

Table 1. Newly sequenced specimens of *Muellerella* spp. from different lichen hosts, and NCBI accession numbers for the corresponding new sequences.

DNA extr. N.	Specimen type – voucher no.	Origin of environmental samples	Loci sequenced		
			28S	18S	16S
DP946	*Muellerella erratica* – specimen Ertz 20485	Austria, Carinthia, Glockner-Gruppe, above Hochtor Pass, on *Xanthoria elegans*, 2670 m a.s.l., 12.VII.2015.	MN241079	MN241075	MN241086
DP855	*Muellerella ventosicola* – specimen Reidar 150307	Norway, Sør-Trøndelag, Oppdal, Grønbakken S of Kongsvold fjellstue, on *Ophioparma ventosa*, 960 m a.s.l., 08.V.2015	MN241080	MN241076	MN241087
DP956	*Muellerella erratica* – specimen Ertz 20470	Austria, Carinthia, Glockner-Gruppe, above Hochtor Pass, on *Lecanora polytropa*, 2620 m a.s.l., 12.VII.2015.	MN241081	–	MN241088
DP951	*Muellerella* sp. – specimen Ertz 20419	Austria, Styria, Hochschab-Gruppe, NW of Tragöss-Oberort, N of Hochturm Mt., on *Protoblastenia rupestris*, ~1050 m a.s.l., 06.VII.2015.	MN241082	MN241077	MN241089
DP953	*Muellerella ventosicola* – specimen Ertz 20489	Austria, Carinthia, Glockner-Gruppe, above Hochtor Pass, on *Rhizocarpon geographicum*, 2670 m a.s.l., 12.VII.2015.	MN241083	MN241078	MN241090
DP806	*Muellerella* sp. – specimen Ertz 17847	Reunion Island, Saint-Denis, sentier de la Roche Ecrite, Plaine des Chicots, on cf. *Trapelia*, 1935 m a.s.l., 06.XII.2012.	–	–	MN241091
S6004	*Muellerella* sp. – specimen SPO-8778	Spain, Madrid, Miraflores, Puerto de la Morcuera, on *Lecanora polytropa*, 2001 m a.s.l., 17.II.2019.	MN241084	–	MN241092
S6005	*Muellerella ventosicola* – specimen SPO-8775	Spain, Madrid, Miraflores, Puerto de la Morcuera, on *Rhizocarpon geographicum*, 2001 m a.s.l., 17.II.2019.	MN241085	–	MN241093
A405	*Muellerella ventosicola* – specimen Muggia-A405	Austria, Steiermark, Koralpe massif, Krakaberg, S of summit, on *Rhizocarpon geographicum* 2040 m a.s.l, 17.VII.2012.	–	–	MN241094

small (16S) subunit ribosomal DNA. We used already published primers, including the traditional general fungal primers and those specifically designed for *Muellerella* by Muggia et al. (2015), as follows. The nuclear 28S fragment was obtained with primers LIC15R and LR6 (Vilgalys & Hester 1990; Miadlikowska et al. 2002) and primers Mu_ITS1008f and Mu_LR729r (Muggia et al. 2015). The nuclear 18S fragment was amplified using primers nSSU131 and nSSU1088 (Kauff & Lutzoni 2002) and primers Mu_ns2f and Mu_ns3r (Muggia et al. 2015). The mitochondrial 16S subunit was amplified with primers mrSSU1 and mrSSU3R (Zoller et al. 1999) or MSU7 (Zhou & Stanosz 2001), and Mu_mtSSU27f and Mu_mtSSU651r (Muggia et al. 2015). Whether or not direct PCR was chosen as the amplification method, the applied PCR conditions were those given in Muggia et al. (2015, 2016). The PCR reaction yield was verified by running the products on a 1% agarose gel using ethidium bromide or SYBR® safe DNA stain (Invitrogen). Both strands were sequenced by Macrogen®, and the sequences were assembled using Sequencher 5.4.6. (Gene Codes Corporation, Ann Arbor, MI USA, http://www.genecodes.com) or SeqMan v.14 (Lasergene, DNA Star Inc., WI, USA).

Alignment and phylogenetic analyses

A BLAST search in GenBank was performed for a preliminary taxonomic assignment of each sequence, confirming their matches with taxa of *Chaetothyriomycetidae* (see Results below). First phylogenetic inferences (not shown), based on each individual locus, were performed with a sequence dataset that included members of the class *Eurotiomycetes* representing the orders *Chaetothyriales*, *Coryneliales*, *Onygenales*, *Pyrenulales* and *Verrucariales*; three species of *Mycocaliciales* (*Chaenotheca savonica*, *Sphinctrina turbinata* and *Stenocybe pullatula*) were chosen as outgroups to allow direct comparison with the previous results of Muggia et al. (2015). This first dataset was reduced to the final dataset (Table 2), as all newly obtained sequences were consistently placed within or basal to *Chaetothyriales* or *Verrucariales*. The final dataset therefore included a selection of representatives of *Chaetothyriomycetidae* only, viz. *Pyrenulales* (selected as outgroup), *Phaeomoniellales*, *Verrucariales*, and within *Chaeothyriales* the families *Chaetothyriaceae*, *Cyphellophoraceae*, *Epibryaceae*, *Herpotrichiellaceae* and *Trichomeriaceae* selected from the phylogenetic studies of Gueidan et al. (2014), Muggia et al. (2015, 2016, 2017), Teixeira et al. (2017), Vasse et al. (2017) and from a preliminary dataset of *Eurotiomycetes* in preparation by Muggia et al. (unpublished). The single-locus sequence alignments were prepared manually in BioEdit 7.0 (Hall 1999). Introns and ambiguous aligned regions were removed manually from the alignments.

Combined data of different loci, whether fully or partially congruent, have been commonly considered by inferring organismal phylogeny (Dettman et al. 2003). As in previous studies (Miadlikowska et al. 2006; Muggia et al. 2014, 2016; Pino-Bodas et al. 2017), we also considered both single-locus and combined datasets. Both the

Table 2. List of taxa retrieved from GenBank and used in the phylogenetic analysis of Fig. 3.

Taxon	Sample ID	28S	18S	16S
Agonimia allobata	L467	FJ455771	–	GU121589
Agonimia tristicula	L469 (Hafellner 66664)	FJ455772	–	GU12159
Agonimia sp.	–	AY300845	AY779280	AY300896
Aphanophora eugeniae	CBS 124.105	FJ839652	–	–
Capronia munkii	AFTOL 656	EF413604	EF413603	FJ225723
Capronia parasitica	CBS 123.88	FJ358225	FJ358293	FJ225724
Capronia peltigerae	–	HQ613813	HQ613815	HQ613814
Capronia pillosella	AFTOL 657	DQ823099	DQ823106	FJ225725
Capronia semiimmersa	AFTOL 658	FJ358226	FJ358294	FJ225726
Ceramothyrium carniolicum	AFTOL 1063	EF413628	EF413627	–
Cladophialophora arxii	IFM 52022 / CBS 306.94	AB100683	AJ232948	–
Cladophialophora devriesii	CBS 147.84	AJ972912	AJ232947	–
Cladophialophora minourae	CBS 556.83	FJ358235	FJ358303	FJ225734
Cladophialophora parmeliae	Ertz 16591	JX081671	–	JX081675
Cyphellophora fusarioides	MUCL 44033	KC455252	KC455298	–
Cyphellophora olivacea	CBS 123.74	KC455261	KC455304	–
Cyphellophora oxyspora	CBS 698.73	KC455262	KC455305	–
Dolabra nepheliae	CBS 122.120	GU332517	–	GU332519
Endocarpon pallidum	AFTOL 661	DQ823097	DQ823104	FJ225674
Epibryon bryophilum	M2	EU940090	EU940017	EU940242
Epibryon hepaticola	M10	EU940091	EU940018	EU940243
Epibryon intercapillare	M125	EU940102	EU940029	EU940254
Epibryon turfosorum	M292	EU940145	–	EU940285
Exophiala castellani	CBS15858	FJ358241	JN856014	FJ225739
Exophiala dermatitidis	AFTOL 668	DQ823100	DQ823107	–
Exophiala oligosperma	CBS 725.88	FJ358245	FJ358313	FJ225743
Fonsecaea brasiliensis	CBS 119.710	KF155183	KF155203	–
Fonsecaea monophora	CBS 102.243	FJ358247	FJ358315	FJ225747
Granulopyrenis seawardii[*]	–	EF411062	EF411059	–
Heteroplacidium imbricatum	AFTOL 2281	EF643756	EF689839	FJ225679
Hydropunctaria maura	AFTOL 2263	EF643801	EF689876	FJ225681
Knufia karalitana (1)	CCFEE 5656	KR781069	–	–
Knufia karalitana (2)	CCFEE 6001	KR781073	–	–
Knufia marmoricola	CCFEE 5721	KR781075	–	–
Knufia mediterranea	CCFEE 5768	KR781079.	–	–
Knufia petricola	CBS 101157	FJ358249	FJ358318	–
Neocatapyrenium rhizinosum	AFTOL 2282	EF643757	EF689840	FJ225683
Parabagliettoa dufourii	AFTOL 2254	EF643792	EF689868	FJ225684
Phaeomoniella capensis	CBS 123.535	FJ372408	–	–
Phaeomoniella prunicola	STEU:6119	GQ154615	GQ154636	–
Phialophora europaea	CBS 129.96	FJ358248	FJ358317	FJ225750
Placocarpus schaereri	AFTOL 2289	EF643766	EF689850	–
Placopyrenium bucekii	AFTOL 2238	EF643768	EF689852	FJ225693
Pyrenula aspistea[*]	AFTOL 2012/ GW1044	EF411063	EF411060	JQ927462
Pyrenula cruenta[*]	–	AF279407	AF279406	AY584719
Pyrenula macrospora[*]	CG1520a	JQ927473	–	JQ927466
Pyrenula pseudobufonia[*]	–	AY640962	AY641001	AY584720
Pyrgillus javanicus	AFTOL 342	DQ823103	DQ823110	FJ225774
Staurothele areolata	AFTOL 2291	EF643772	EF689856	FJ225699
Thelidium papulare	AFTOL 2249	EF643781	EF689861	DQ329005
Trichomerium foliicola	MFLUCC10-0054	JX313657	–	–
Trichomerium sp.	LS-2015b	KP174948	KP174898	KP174992
Verrucaria viridula	AFTOL 2299	EF643814	EF689884	FJ225712
Verrucula inconnexaria	AFTOL 307	EF643821	EF689892	FJ225718
Vonarxia vagans	CBS 123533	NG_057821	NG_062869	–
rock isolate TRN1	–	FJ358250	FJ358319	FJ225754
rock isolate TRN14	–	–	FJ358321	FJ225756
rock isolate TRN30	–	FJ358252	FJ358322	FJ225757
rock isolate TRN107	–	FJ358253	FJ358323	FJ225758
rock isolate TRN115	–	FJ358254	–	FJ225759
rock isolate TRN210	–	FJ358255	FJ358325	FJ225760
rock isolate TRN214	–	FJ358256	–	FJ225761

Table 2. Continued.

Taxon	Sample ID	28S	18S	16S
rock isolate TRN475	–	FJ358260	FJ358329	FJ225764
rock isolate TRN488	–	FJ358262	–	FJ225766
rock isolate TRN493	–	FJ358263	FJ358331	FJ225767
rock isolate TRN497	–	–	FJ358332	FJ225768
rock isolate TRN508	–	FJ358265	FJ358333	FJ225770
rock isolate TRN531	–	FJ358267	FJ358335	FJ225772
Cultured fungus from *Tephromela atra* infected by *Taeniolella atricerebrina*	A573	KT263034	KT263047	KT263060
Cultured fungus from *Lecanora polytropa* infected by *Lichenoconium lecanorae*	A859	KT263036	KT263049	KT263062
Cultured fungus from *Lecidea* sp. infected by *Muellerella erratica*	A526	KT263136	KT263180	KT263224
Cultured fungus from *Lecanora polytropa* infected by *Lichenoconium lecanorae*	A529	KT263138	KT263182	KT263226
Cultured fungus from *Lecidea lapicida* infected by *Cecidonia umbonella*	A872	KT270601	KT270689	KT270771
Cultured fungus from *Lecidea* sp. infected by *Muellerella erratica*	A875	KT270604	KT270692	KT270774
Cultured fungus from *Aspicilia* sp. infected by *Endococcus verrucosus*	A926	KT270637	KT270726	KT270806
Cultured fungus from *Lecanora polytropa* infected by *Cercidospora epipolytropa*	A945	KT270649	KT270735	KT270818
Cultured fungus from *Aspicilia* sp. infected by *Endococcus verrucosus*	A952	KT270655	–	KT270824
Cultured fungus from *Aspicilia* sp. infected by *Endococcus verrucosus*	A949	KT270653	KT2707238	KT270822
Cultured fungus from *Lecanora polytropa* infected by *Muellerella erratica*	A974	KT270668	KT270751	KT270837
Cultured fungus from *Tephromela atra* infected by *Taeniolella atricerebrina*	A980	KT270672	KT270754	KT270841
Cultured fungus from *Lecanora intricata* infected by *Muellerella erratica*	A989	KT270678	KT270760	KT270847
Cultured fungus from *Lecanora polytropa* infected by *Lichenoconium lecanorae*	A1161	MF071427	–	MF085488
Cultured fungus from *Aspicilia* sp. infected by *Endococcus verrucosus*	A1125	MF071409	MF071350	MF085468
Cultured fungus from *Rhizocaron geographicum* infected by *Muellerella ventosicola*	A1113	MF071402	MF071345	MF085462
Cultured fungus from *Lecanora polytropa* infected by *Cercidospora epipolytropa*	A1120	MF071405	MF071347	MF085464
Cultured fungus from *Rhizocaron geographicum* (A97) infected by *Muellerella ventosicola*	A944	KT263072	KT263094	KT263110
Cultured fungus from *Rhizocaron geographicum* (A263) infected by *Muellerella ventosicola*	A993	KT263073	KT263095	KT263111
Cultured fungus from *Rhizocaron geographicum* (A385) infected by *Muellerella ventosicola*	A1015	KT263076	KT263096	KT263114
Lichenodiplis lecanorae	L1858	KT263086	KT263100	KT263118
Lichenodiplis lecanorae	L1860	KT263087	KT263101	KT263119
Muellerella atricola	L1992	KT263083	–	KT263120
Muellerella atricola	L1993	KT263084	KT263102	KT263121
Muellerella atricola	L1994	KT263085	KT263103	KT263122
Lichenodiplis lecanorae	L2206	KT285901	KT285921	KT285910
Lichenodiplis lecanorae	L2207	KT285902	KT285922	KT285911
Lichenodiplis lecanorae	L2208	KT285903	KT285923	KT285912
Lichenodiplis lecanorae	L2263	KT285905	KT285928	KT285916
Muellerella atricola	A333	KT285906	KT285929	KT285917
Muellerella atricola	A440	KT285907	KT285930	KT285918
Muellerella atricola	A528	KT263088	KT263104	KT263123
Muellerella atricola	A663	KT285908	KT285931	KT285919
Muellerella lichenicola	L2209	KT285904	KT285924	KT285913
Lichenodiplis lecanorae	DE19202	KT285909	KT285932	KT285920
Lichenodiplis lecanorae	L2254	–	KT285925	KT285914
Lichenodiplis lecanorae	L2256	–	KT285926	–
Lichenodiplis lecanorae	L2257	–	KT285927	KT285915

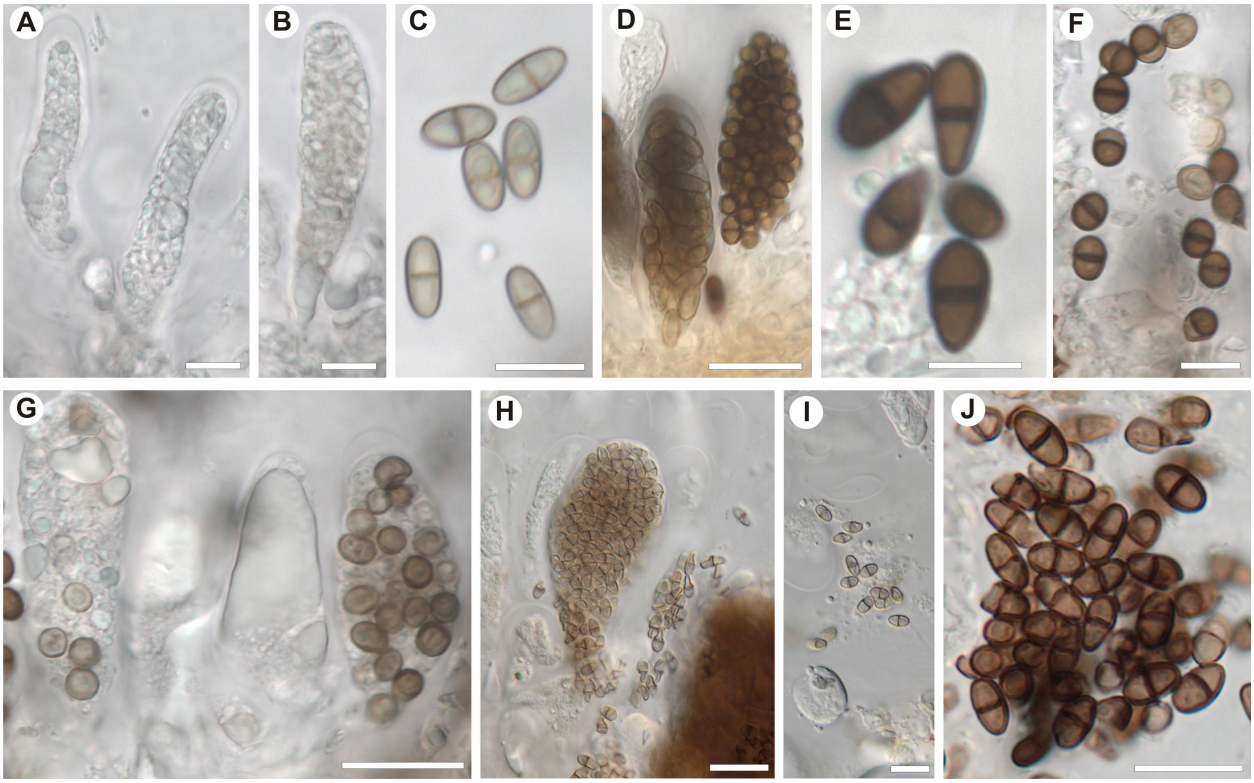

Figure 2. Asci and ascospore variation of *Muellerella* spp. [sample ID]. A–C – asci and ascospores of *M. erratica* on *Lecanora intricata* [SPO-4576]; A, B – polyspored asci in an immature state; C – brown, 2-celled, ellipsoid ascospores; D, E – asci and ascospores of *M. erratica* on *Lecidea* sp. [SPO-5499]; D – polyspored, mature asci; E – dark brown, mature 2-cell, subellipsoid ascospores; F, G – ascospores and asci of *M. ventosicola* s.lat. on *Rhizocarpon geographicum* [Muggia L2363]; F – brown, 2-celled, ellipsoid ascospores; G – mature (right) and immature (left) polyspored asci, empty ascus in center; H, I – mature polyspored ascus (H) and 2-celled subellipsoid ascospores (I) of *M. lichenicola* on *Caloplaca* sp. (Ertz 16261); J – brown, 2-celled, ovoid ascospores of *M. erratica* on *Xanthoria elegans* [Ertz 20485]. Scales: A–C, E, F, H–J = 10 μm; D, G = 20 μm.

single-locus and the combined dataset were analysed with a Maximum Likelihood (ML) approach using RAxML v. 8.2 (Stamatakis 2014) with the user interface. The GTR-GAMMA model was used for both the single-locus and the combined datasets (treating the combined dataset into partition by gene). Node support was assessed by running 1000 bootstrap replicates. We analysed the three single-locus datasets for their topological incongruence by assuming a conflict significant when two different relationships (one monophyletic and the being non-monophyletic) for the same set of taxa were both supported with bootstrap values ≥70% (Mason-Gamer & Kellogg 1996; Reeb et al. 2004). Based on this criterion we detected partial conflict among the three loci (Table S2), so here we show the single-locus and the combined phylogenetic inferences.

Results

Phylogenetic analysis

We obtained 22 new sequences (seven for nuclear 28S, four for nuclear 18S and nine for mitochondrial 16S loci; Table 1). Among the newly sequenced *Muellerella* specimens, four are represented by all three loci and three by two loci, while two specimens are represented by the single mitochondrial 16S sequences (Table 1). We performed DNA extraction and amplification for another 15 *Muellerella* samples also, but due to unsuccessful PCR amplification and/or failure in the sequencing process, we did not obtain molecular data to include here. Also, for the newly sequenced *Muellerella* specimens we included only data from their thalli (environmental samples), as culture isolates prepared for three *Muellerella* samples (SPO-4576, SPO-4598, SPO-4599) turned out to represent the lichen host *Lecidea* spp.

The new sequences showed their closest matches with representatives of the order *Chaetothyriales* and with the three cultured endolichenic fungal strains representing clade II (A944, A993 and A1015), which were isolated from thalli of *Rhizocarpon geographicum* infected by *M. ventosicola* s.lat. (as reported in Muggia et al. 2016, 2017). None of the new sequences matched the previously published sequences of *Muellerella atricola*, *M. lichenicola* and their *Lichenodiplis lecanorae*-like anamorphic state.

Due to the missing data in the taxon samplings of the single-locus alignments, some topological differences have been recovered among the inferred single-locus phylogenies (Fig. 3A–C). The major incongruences are given by (i) the paraphyly of *Herpotrichiellaceae* in the phylogeny based on nuclear 28S (Fig. 3A), (ii) the position of *Cyphellophoraceae* nested in *Herpotrichiellaceae* in the phylogeny based on nuclear 18S (Fig. 3B), and (iii) the position of *Verrucariales* within *Chaetothyriales* and the splitting of *Chaetothyriales* into three paraphyletic lineages in the phylogeny based on mitochondrial 16S (Fig. 3C). *Phaeomoniellaceae* is always monophyletic; it includes

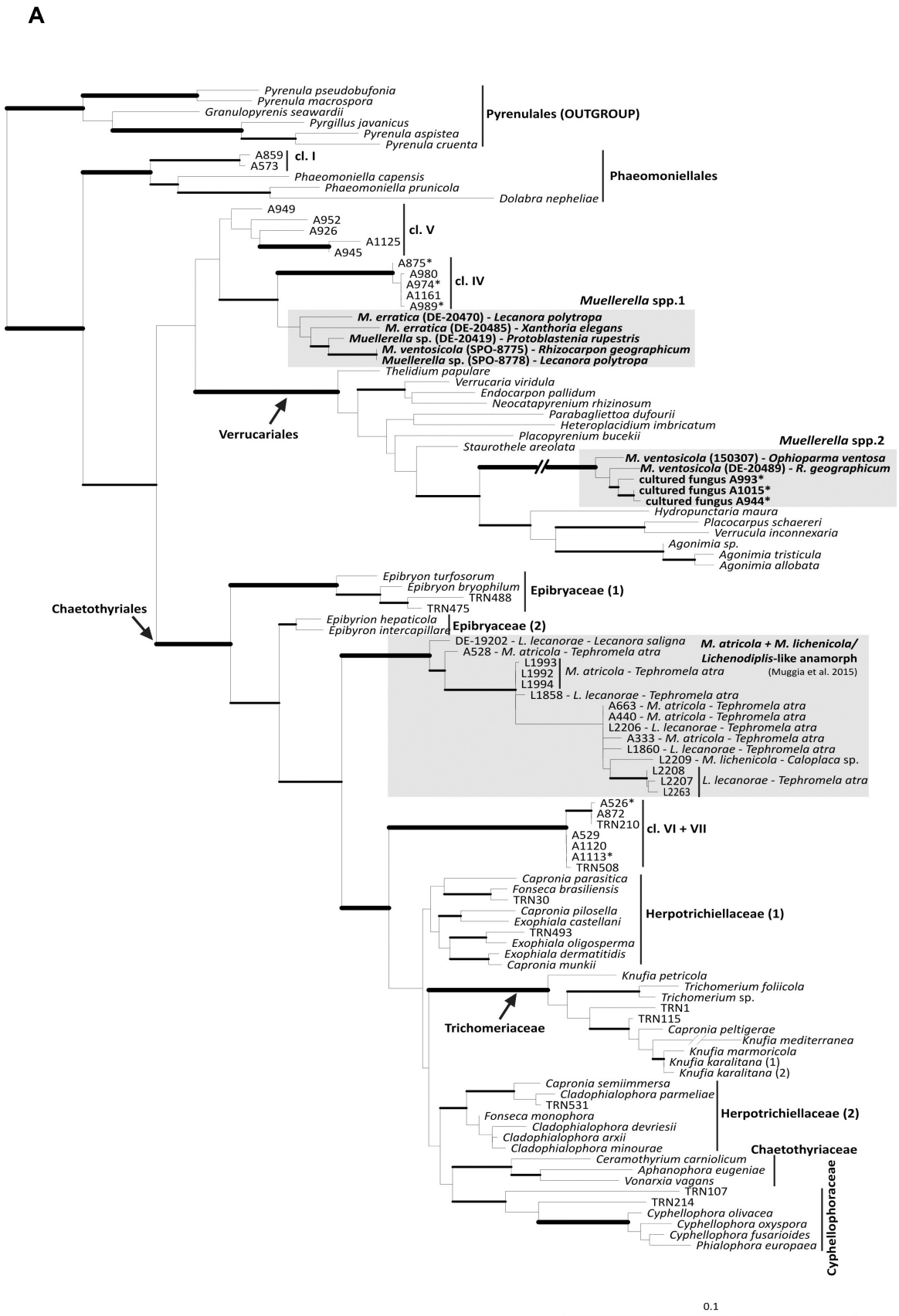

Figure 3. Single-locus (A–C) and multilocus (D) phylogenetic inferences of *Muellerella* taxa. The ML phylogenetic hypotheses were inferred from the individual datasets of the nuclear 28S (A), nuclear 18S (B) and mitochondrial 16S (C) loci and the combined dataset of these three loci (D). Branches supported by ML bootstrap support values >98% and 98%<70% are bolded with two different thicknesses, respectively. The newly sequenced samples are bolded and are reported with the *Muellerella* species names and the lichen hosts. Culture isolates derived from lichen thalli infected by *Muellerella* spp. (Muggia et al. 2016, 2017) are asterisked (*); see Table 2 for further details on these specimens and Table S2 for detailed description of topological congruence/incongruence of phylogenetic inferences A–C.

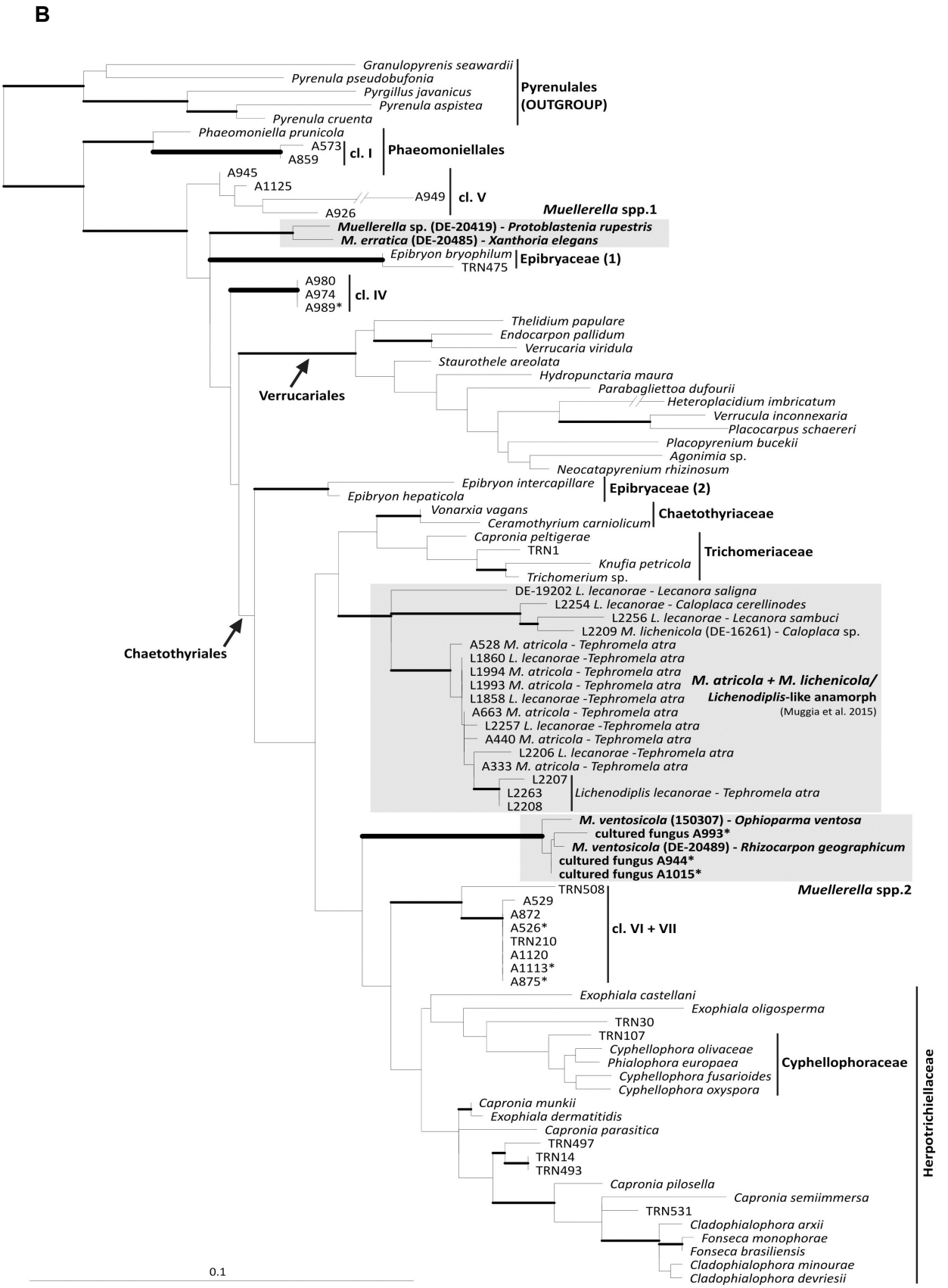

Figure 3. Continued.

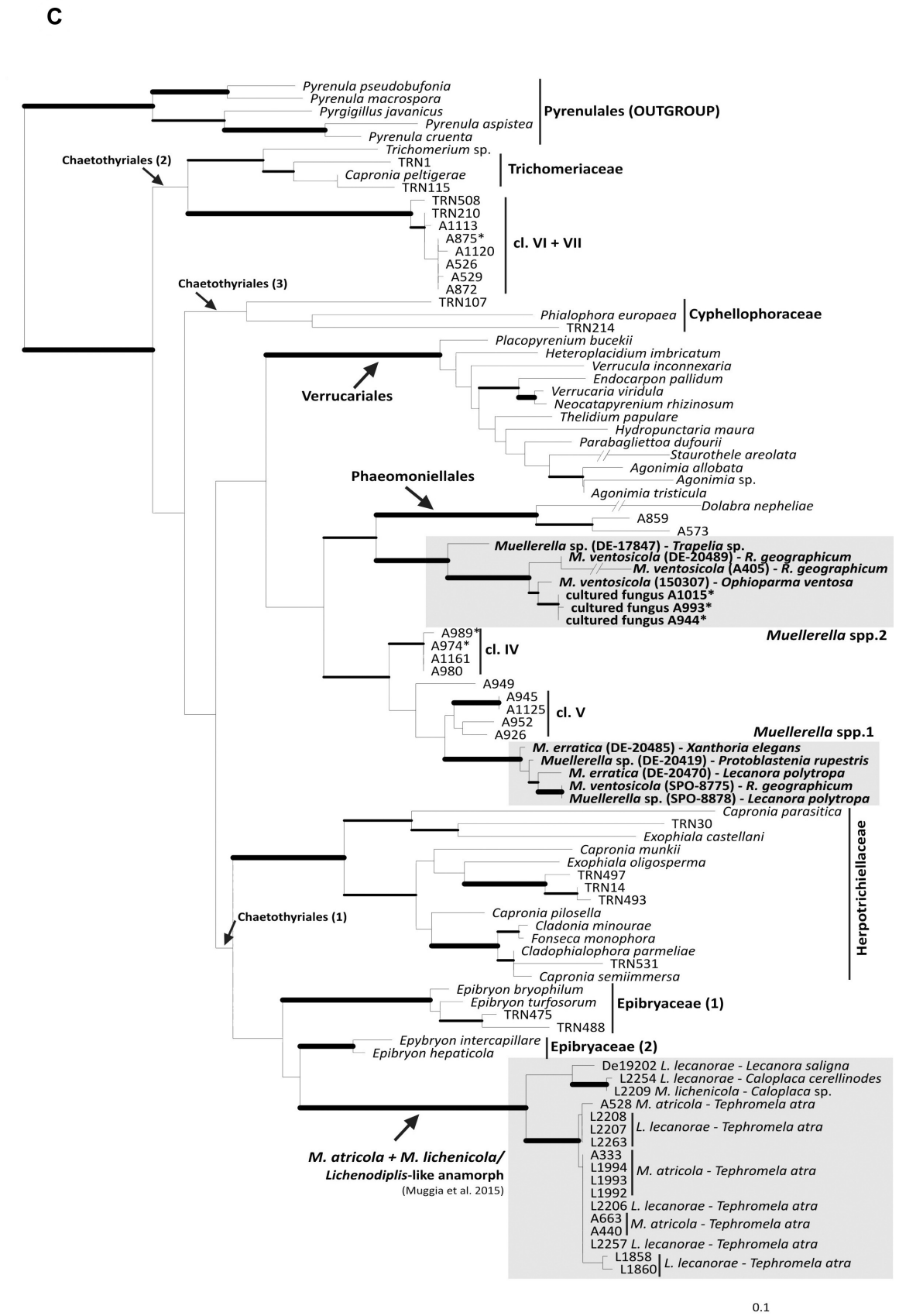

Figure 3. Continued.

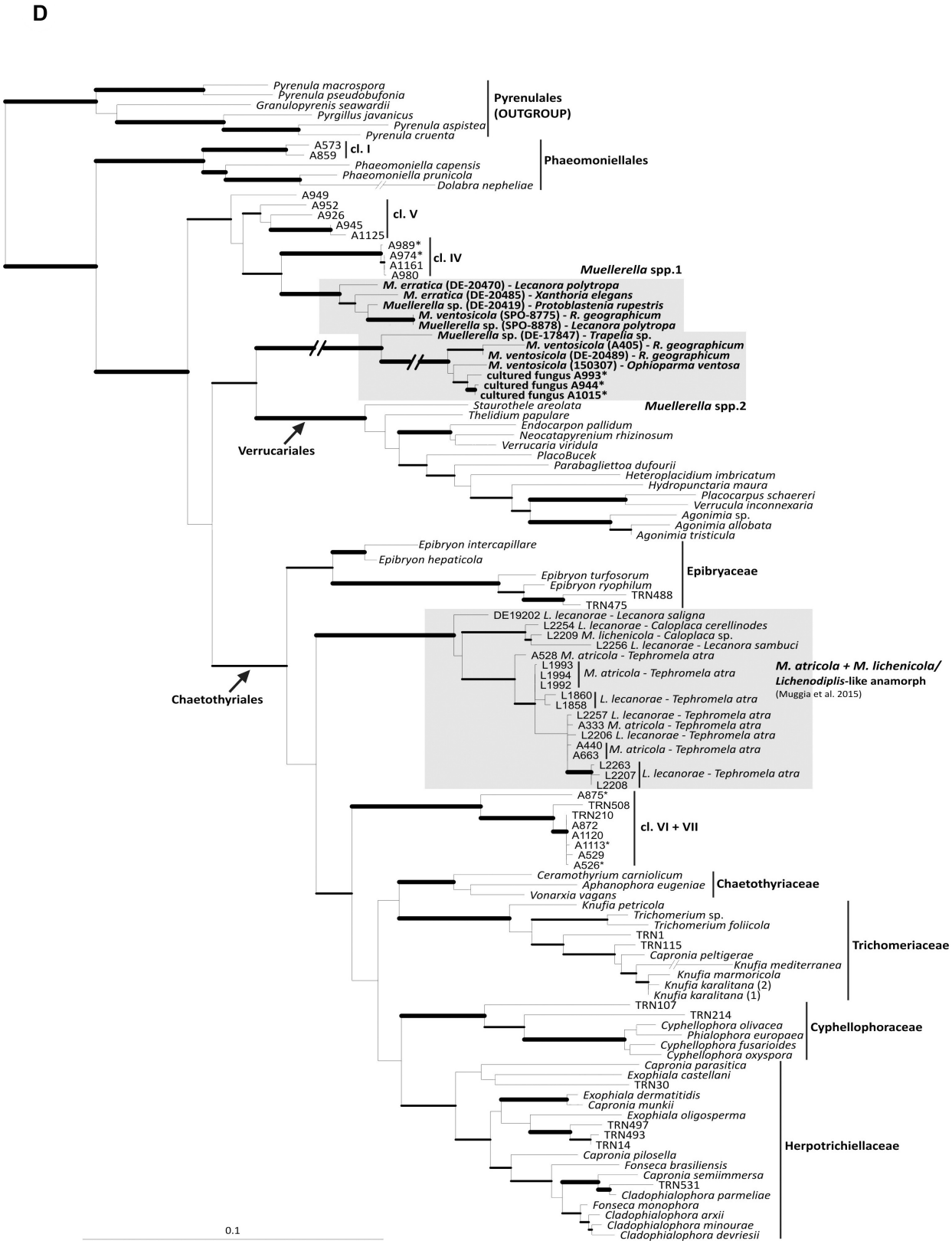

Figure 3. Continued.

clade I of endolichenic fungi and is recovered as basal in whole *Chaetothyriomycetidae*. *Epibryaceae* is always paraphyletic, forming two well-supported lineages [here labeled *Epibryaceae* (1) and (2)] always basal to *Chaetothyriales*. Trichomeriaceae is always monophyletic and within *Chaetothyriales*, representing Chaetothyriales (2) in the phylogeny based on the mitochondrial 16S locus.

The newly generated *Muellerella* sequences belong to two lineages that are labeled *Muellerella* spp. 1 and *Muellerella* spp. 2. The lineage *Muellerella* spp. 1 groups samples of *M. erratica*, *M. ventosicola* and unidentified *Muellerella* species, and is always recovered either as sister lineage of clades IV and V of cultured endolichenic fungi, or nested within them, but these phylogenetic relationships are only partly supported. Lineage *Muellerella* spp. 2, alternatively, groups three specimens of *M. ventosicola* from both *R. geographicum* and *Ophioparma ventosa*, and three cultured strains of clade II of Muggia et al. (2016, 2017; i.e. strains A944, A993, A1015) isolated from thalli of *Rhizocarpon geographicum* infected by *M. ventosicola* s.lat.. The sample *Muellerella* sp. DE17847, obtained from a thallus of *Trapelia* sp., is represented only by the 16S sequence and is recovered as basal in *Muellerella* spp. 2. This *Muellerella* spp. 2 lineage is nested within *Verrucariales* in the 28S phylogeny (Fig. 3A), is nested in *Chaetothyriales* in the 18S phylogeny (Fig. 3B), and is closely related to *Phaeomoniellales* in a supported sister relationship in the 16S phylogeny (Fig. 3C). The previously recognized lineage of *M. atricola+M. lichenicola* and their *L. lecanorae*-like anamorph is recovered as monophyletic, and is fully supported within *Chaetothyriales* in all three single-locus analyses.

Clades IV, V and VI+VII represent black melanized fungi isolated from diverse lichen species; originally these three lineages were recovered inside *Chaetothyriales* by Muggia et al. (2016, 2017). In the present analyses, instead, only clade VI+VII is confirmed to be placed within *Chaetothyriales*, whereas clades IV and V are placed outside *Chaetothyriales* (see above), being closely related to the clades of *Muellerella* spp. 1 and *Verrucariales* (Fig. 3A–D).

The multilocus phylogenetic hypothesis (Fig. 3D) recovered relationships among the families and the orders of *Chaetothyriomycetidae* that were congruent with previous studies (e.g., Diederich et al. 2013; Gueidan et al. 2008, 2014; Muggia et al. 2015, 2016, 2017; Teixeira et al. 2017; Vasse et al. 2017). The backbone phylogeny and the individual families and order lineages received full support. The fully supported monophyly of the clade *M. atricola+M. lichenicola* and their *L. lecanorae*-like anamorph within *Chateothyriales*, as recognized by Muggia et al. (2015), is again confirmed; however, its sister relationships with *Epibryaceae* – as suggested by Muggia et al. (2015) – is not recovered. The new lineages *Muellerella* spp. 1 and *Muellerella* spp. 2 are also recovered with the same groupings of samples identified in the single-locus phylogenies. Here, *Muellerella* spp. 1 is supported as sister lineage of the endolichenic fungal clade IV, and both are sister to four samples forming clade V. *Muellerella* spp. 2 is, instead, the fully supported sister lineage of *Verrucariales*, and the sample *Muellerella* sp. DE17847 is again basal within it.

Discussion

In this study we expanded the taxon sampling of *Muellerella* species to investigate the monophyly of the genus, as speculated in a previous study by Muggia et al. (2015). *Muellerella* samples were selected from a number of localities from Europe and Reunion Island as well as from six different lichen hosts, which are among the most common species to be parasitized by this lichenicolous fungal genus. Further, we could consider in this study *Muellerella* species that are commonly found on lichens: *Muellerella erratica* is indeed one of the best-known lichenicolous fungi reported from more than a hundred host species (Triebel 1989).

The present results suggest that the genus *Muellerella* is not monophyletic, as our sequences belong to three major lineages within *Chaetothyriomycetidae*. The first lineage is represented by the monophyletic *Muellerella atricola+ M.lichenicola* complex (including the asexual *Lichenodiplis*-like states), corroborating previous results by Muggia et al. (2015). The second and the third clades are the newly recovered lineages *Muellerella* spp. 1 and *Muellerella* spp. 2, each of them monophyletic and fully supported.

Muellerella spp. 1 is related to two lineages of melanized fungi isolated from lichen thalli, viz. clades IV and V (Muggia et al. 2016, 2017). The phylogenetic placement of these two melanized fungal lineages is discordant from that originally inferred (Muggia et al. 2016). Indeed, they were originally recovered within *Chaetothyriales*, closely related to clade VI+VII (which is here still recovered within *Chaetothyriales*), but in the present analyses they form together with *Muellerella* spp. 1 a fully supported lineage (Fig. 3D) at the base of *Chaetothyriales* and *Verrucariales*. Although *Muellerella* spp. 1 and clades IV and V are closely related, and clade IV (but also clade VI+VII) contains isolates of endolichenic fungi obtained from lichen thalli infected by *Muellerella* spp., it is unlikely that any of these strains correspond to *Muellerella*. The isolates recovered in clades IV and VI+VII are melanized fungi morphologically very similar to each other (Muggia et al. 2016, 2017) and highly similar to the melanized rock-inhabiting fungi (RIF) isolated from rocks (Ruibal et al. 2009) and lichen thalli from arid Mediterranean habitats (Harutyunyan et al. 2008, Selbmann et al. 2013).

The position of the third clade *Muellerella* spp. 2, nested within *Verrucariales* in the 28S-based phylogeny and sister of this order in the combined analysis, was statistically supported. This placement agrees with the systematic position of *Muellerella* hypothesized by Triebel (1989). The main morphological characters that could support a relationship with the Verrucariales are the interascal filaments disappearing in an early stage of development but with persisting periphysoids. However, these characters are also shared by *Muellerella* spp. 1 and *M. atricola+M. lichenicola*, rendering morphological synapomorphies for these lineages difficult to infer with

the few data currently at hand. The representatives of *Muellerella* spp. 2 are *Muellerella* specimens amplified directly from their hymenium and three fungal strains isolated from different thalli of *R. geographicum* infected by *M. ventosicola* s.lat. These cultured fungi formed clade II in Muggia et al. (2016, 2017), which was already recovered as sister to *Verrucariales*. The present results suggest that these three strains (A944, A993, A1015) likely represent a species of *Muellerella*. However, as we recovered one sample of *M. ventosicola* s.lat. also in clade *Muellerella* spp. 1, we cannot be certain that these strains belong to *M. ventosicola*. To confirm this hypothesis, a careful study of the species *M. ventosicola*, including sequences of its holotype, if possible, will be necessary. These cultured isolates A944, A993 and A1015 are paler than those of *M. atricola*, *M. lichenicola* and their *L. lecanorae*-like anamorph, and so far we have not observed the formation of pycnidia and conidiospores in them, as we did for the cultured *M. atricola*, *M. lichenicola* and their *L. lecanorae*-like anamorph (Muggia et al. 2015). Obtaining further new culture isolates of these new *Muellerella* lineages would be needed to test whether these other *Muellerella* taxa also share an asexual state. An asexual state was not observed in the sequenced specimens of *M. erratica* and *M. ventosicola*, suggesting that it is absent or very rare in this group. Interestingly, *Muellerella atricola* and *M. lichenicola* are characterized by ~100-spored asci, in contrast to *M. erratica* and *M. ventosicola* which have ~64-spored asci (Triebel 1989, Hafellner 2007). The degree of polyspory and the presence of a *Lichenodiplis* asexual state appear to be correlated with our phylogenetic results, supporting the *M. atricola*+*M. lichenicola* group as a lineage distantly related to the *Muellerella* spp. 1 and spp. 2 clades.

The polyphyly of the genus *Muellerella* leaves open the question of its family placement. This placement will be determined by the phylogenetic position of the generic type, *M. polyspora*, a species recorded mainly from the corticolous lichen *Arthonia radiata*. Unfortunately, this species is very rare, and fresh material was not available for sequencing, hampering progress in the taxonomy of the group. *Muellerella polyspora* has simple ascospores, unlike most species of *Muellerella* that have 1-septate ascospores (e.g., Hawksworth 1979; Ihlen & Wedin 2008) as well as all specimens of *Muellerella* that have been sequenced so far.

Interestingly, the close relationship of non-lichenized fungal lineages (clades IV, V and VI+VII of endolichenic fungi) and lichenicolous fungi (*Muellerella* spp. 1 and spp. 2 clades) with a lineage of lichenized fungi (*Verrucariales*), recovered in the phylogenies based on the 28S and the combined datasets, recalls a pattern already observed in other fungal groups. This is observed also for lichenicolous fungi placed in *Polycoccaceae* and recovered as sister to the lichenized family *Trypetheliaceae* (Ertz et al. 2015a) within *Dothideomycetes*, and for the order *Lichenostigmatales* sister group of the lichenized lineage *Arthoniales*/ *Arthoniomycetes* (Ertz et al. 2014).

It is now amply acknowledged that lichens with and without obvious symptoms of fungal infections harbor numerous fungal species in their microbiomes (U'Ren et al. 2010, 2012, 2014; Fleischhacker et al. 2015; Muggia et al. 2016, 2017; Fernández-Mendoza et al. 2017; Banchi et al. 2018) and that their identification is complemented by study of their corresponding axenic isolates. Comparing DNA sequences from the original lichen host sample and from the culture isolates helps determine the identity of these fungi, as found by Muggia et al. (2015) in studies of *M. atricola* and *L. lecanorae*. Unfortunately, we were not able to retrieve culture isolates of the *Muellerella* species for which we obtained sequences from the lichen thalli. The lack of corresponding culture isolates complicates an assessment of the identity of the *Muellerella* fungi amplified from the lichen thalli. However, the cultures we obtained from three of those 15 specimens chosen for molecular analyses that failed (see above) were not affected by fungal contamination, and only the host mycobiont (*Lecidea* sp.) grew axenically after a year and a half. It is likely that the mycobiont grew out from a tiny thallus fragment that remained attached to the perithecial hyphae.

The reduced number of molecular data and the multiple attempts that are usually needed to obtain reliable sequences to be included in phylogenetic analyses represent problems that still have to be overcome in future studies of lichenicolous fungi. The environmental material is usually difficult to find, and morphology-based species identification is usually required before performing molecular analyses. Although perithecia of *Muellerella* are usually abundant in infected lichen thalli, they are nonetheless very tiny structures and the only ones from which DNA extraction and culture isolation can be reliably performed. In general, lichenicolous fungi build inconspicuous reproductive structures (e.g., perithecia, apothecia, pycnidia) on the host thalli, and their removal typically consumes the material while often yielding insufficient DNA for successful amplification. Though PCR biases are well documented and often depend on the level of primer matching in different taxa (Green et al. 2015), to our knowledge there is no report with respect to lichenicolous fungi about bias introduced by direct PCR instead of traditional DNA extraction and amplification. Previously, Muggia et al. (2015) gained their data from environmental samples by performing traditional DNA extraction followed by PCR amplification. In the present study, the new sequences were generated mainly by direct PCR of perithecial material. The single exception is the sample *Lichenodiplis lecanorae* DE19202, of which sequences were obtained by direct PCR and are included in the monophyletic clade *M. atricola*+*M. lichenicola*/ *Lichenodiplis*-like anamorph. We may therefore exclude any amplification bias generated by direct PCR that could have led to the amplification of a species not belonging to *Muellerella* corresponding to the newly recovered *Muellerella* spp. 1 and spp. 2 lineages. In light of these considerations, amplifying *Muellerella atricola* by direct PCR would likely rule out whether amplification biases might be an issue in detecting certain lichenicolous taxa in lichens. For this reason we also used the *Muellerella*-specific primers designed by Muggia et al. (2015) to minimize potential amplification biases. However, when

using these primers for the 28S and 18S regions we did not recover any band, or else the sequencing was unsuccessful. This seems to confirm that the primers are indeed very specific to *M. atricola* + *M. lichenicola* and their *L. lecanorae*-like anamorph lineage and do not work for the other lineages of *Muellerella* recovered here. The new sequences representing the new clades *Muellerella* spp. 1 and spp. 2 will now be used to design additional species-specific primers to target *Muellerella* taxa on their lichen hosts with greater precision.

In the present context, the amount of molecular data is still too small to be correlated with the morphological variation within *Muellerella* species, although the degree of polyspory and the absence/presence of a *Lichenodiplis*-like anamorphic state appear to be congruent with our phylogenetic results. Indeed, the *Lichenodiplis* asexual state may be confined to the *M. atricola*+*M. lichenicola* clade, a lineage including species forming asci with ~100 spores. The clades *Muellerella* spp. 1 and spp. 2 likely represent distinct genera characterized by *Muellerella* species with fewer ascospores per ascus (up to ~64 spores) and by the absence of a *Lichenodiplis* anamorphic state. The results also hint at genetic diversity potentially shaped by host specificity. *Muellerella atricola* and *M. lichenicola*, with their *L. lecanorae*-like anamorphic states, both form fully supported clades. *M. ventosicola* s.lat. appears paraphyletic, with the specimen *M. ventosicola* SPO-8775 nested in the *Muellerella* spp. 1 clade, while all other specimens identified as *M. ventosicola* are part of the *Muellerella* spp. 2 clade. Whether the genetic diversity of *Muellerella* spp. could also depend on geographical differentiation still needs to be tested.

To confirm these hypotheses and to obtain a comprehensive understanding of *Muellerella* species diversity, much wider taxon sampling is required, including multiple samples representing the same *Muellerella*-lichen host combination from both the same and different geographic origins.

Acknowledgements

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Supplementary electronic material

Table S1. Characters analysed in the *Muellerella* spp. specimens included in the molecular analysis of this study. Download file

Table S2. Description of the topological congruence/incongruence of the phylogenetic inferences shown in main Fig. 3A–D. Download file

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