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Phylogenetic placement of the lichenicolous, anamorphic genus *Lichenodiplis* and its connection to *Muellerella*-like teleomorphs

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ABSTRACT

Lichenicolous fungi are a specialized group of taxa which inhabit lichens and develop diverse degrees of specificity and parasitic behaviour towards their hosts. They are recognized only by their phenotypic symptoms and sexual or asexual spore-producing structures on the lichen thalli. Only recently, molecular data and culture dependent approaches have helped in uncovering the species diversity and in verifying the phylogenetic position and anamorph–teleomorph relationships of some taxa. Here, we studied the phylogenetic placement of representative taxa of two lichenicolous genera, the coelomycete *Lichenodiplis* and the ascomycete *Muellerella*. We obtained molecular data for three nuclear and mitochondrial loci (28S, 18S, and 16S), both from fresh collected specimens and culture isolates. Our multilocus phylogeny places *Lichenodiplis* and *Muellerella* samples in one monophyletic, fully supported clade, sister to *Epibryon* (Epibryaceae) in Chaetothyriales (Eurotiomycetes). Morphological analyses of axenically cultured fungi show the formation of conidiomata and conidiospores in both *Lichenodiplis* and *Muellerella* isolates. We suggest that the species *Lichenodiplis lecanorae* and *Muellerella atricola* represent, respectively, the anamorphic and teleomorphic stages of the same fungus and discuss their relationships with the other fungal families in Chaetothyriomycetidae.

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Introduction

Morphological similarities between dothidealean and chaetothyrialean fungi have in the past led to the systematic misplacement of several groups. More recently, molecular data have helped verify the phylogenetic position of many taxa, and these have been transferred between the two classes Dothideomycetes and Eurotiomycetes (Gueidan *et al.* 2014; Wijayawardene *et al.* 2014). The availability of molecular data have also made possible the assemblage of multiple loci datasets, resulting in the reappraisal of phylogenetic placements and relationships for a great number of species at different taxonomic levels (e.g. Gueidan *et al.* 2008, 2011, 2014; Schoch *et al.* 2009; Hyde *et al.* 2013), including anamorphic fungi. These fungi have also been studied by culture isolation approaches, which has improved our understanding of

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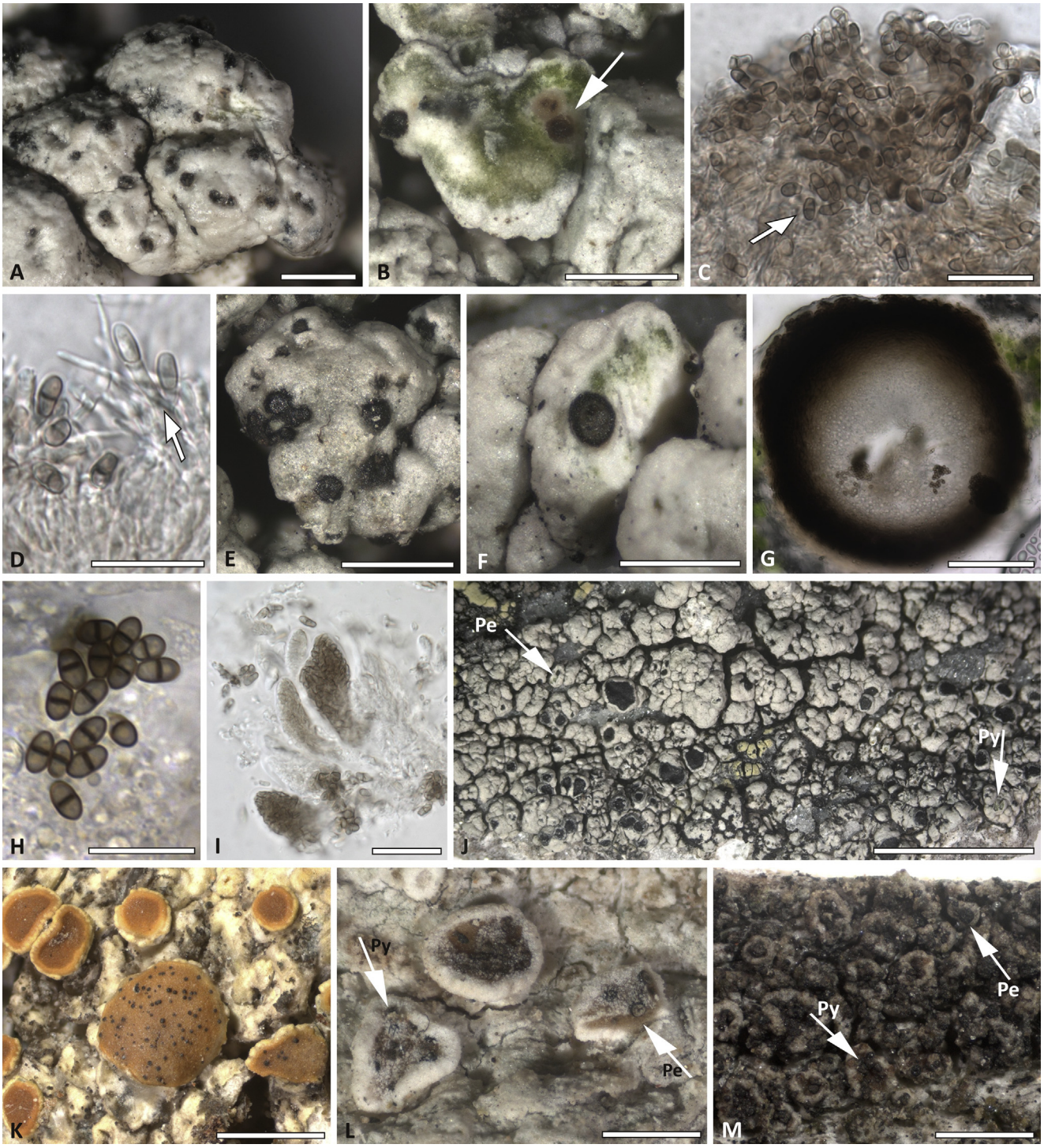

Fig 1 – **Habitus of the lichenicolous *Lichenodiplis lecanorae* (A–D) and *Muellerella atricola* (E–I) on the host *Tephromela atra* and co-occurence of *Lichenodiplis* pycnidia (Py) and *Muellerella* perithecia (Pe) on diverse lichen hosts (J–M); sample ID are reported in square brackets '[ ]'. –*Lichenodiplis lecanorae*: (A) pycnidia on host thallus [Muggia 0297-13], (B) section of a pycnidium in thallus [Muggia 002-13], (C) pycnidium section and conidiospores [Muggia 002-13], (D) conidiospores and conidiogenous hyphae [Muggia 002-13]. –*Muellerella atricola*: (E) perithecia on host thallus [Muggia 001-13], (F) section of a perithecium on the thallus [Muggia 001-13], (G) perithecium section [Muggia 002-13], (H) ascospores, (I) asci and ascospores, squash section [Muggia 002-13]. (J) perithecia of *M. atricola* and pycnidia of *L. lecanorae* on *T. atra* [Muggia 001-13]. (K) infection of *L. lecanorae* on *Caloplaca flavorubescens* [GZU 34-2012], black pycnidia on the apothecium disk (the rare perithecia not shown here). (L) infection of *L. lecanorae* on *Lecanora* sp. and rare perithecia of *Muellerella* in the same hymenium [Ertz 13635, BR]. (M) infection of *L. lecanorae* on *Lecanora* sp. and perithecia of *M. lichenicola* on adjacent apothecia [Ertz 8852, BR]. Scale bars: K = 1 mm; A, B, E, F, L, M = 0.5 mm; G = 60 μm; C, D, I = 20 μm; H = 10 μm.**

Table 1 – List of environmental samples (white) and culture isolates (grey) used in the molecular analyses. The DNA extraction numbers, voucher numbers, culture isolates ID, the origin of the environmental samples and NCBI accessions for the new sequences are reported.

DNA extractron N.	Specimen type – voucher#	Origin of the environmental samples *[culture colelction ID]*	Loci sequenced		
			28S	18S	16S
L1858	Culture form *Lichenodiplis lecanorae* on *Tephromela atra* (inoculum 1)	Austria, Styria, Koralpe Mts, Handalm, 46°50′38″N/15°01′10″E, ca. 1800 m a.s.l., 27.IV.2012.	KT263086	KT263100	KT263118
L1860	Culture form *Lichenodiplis lecanorae* on *Tephromela atra* (inoculum 2)	Austria, Styria, Koralpe Mts, Handalm, 46°50′38″N/15°01′10″E, ca. 1800 m a.s.l., 27.IV.2012. *[LMCC0513]*	KT263087	KT263101	KT263119
L1992	Culture from *Muellerella atricola* on *Tephromela atra* – Muggia 0633-13 (inoculum 1)	Isolated from sample L2006 *[LMCC0066]*	KT263083	–	KT263120
L1993	Culture from *Muellerella atricola* on *Tephromela atra* – Muggia 0633-13 (inoculum 2)	Isolated from sample L2006	KT263084	KT263102	KT263121
L1994	Culture from *Muellerella atricola* on *Tephromela atra* – Muggia 0633-13 (inoculum 3)	Isolated from sample L2006 *[LMCC0515]*	KT263085	KT263103	KT263122
L2206	*Lichenodiplis lecanorae* on *Tephromela atra* – specimen Muggia 002-13	Switzerland, Canton Ticino, Adula Alps, Luzzone Lake, 21.VIII.2012. (also infected by *Muellerella atricola*)	KT285901	KT285921	KT285910
L2207	*Lichenodiplis lecanorae* on *Tephromela atra* – Muggia 0297-13	Austria Styria, Rottermanner Tauern, Seekoppe, 2150 m a.s.l., on big siliceous rocks on the top, 11.VIII.2013.	KT285902	KT285922	KT285911
L2208	Culture from *Lichenodiplis lecanorae* L2207 (first inoculum)	Isolated from sample L2007 *[LMCC0507]*	KT285903	KT285923	KT285912
L2263	Culture from *Lichenodiplis lecanorae* L2207 (second inoculum)	Isolated from sample L2007	KT285905X	KT285928	KT285916
L2209	Culture from *Muellerella lichenicola* – specimen Ertz D. 16261	Canary Islands, Gomera, Arure, trail N of Mirador Ermita del Santo, 810 m alt., twigs of *Pinus*, on *Caloplaca* sp., 2011.	KT285904	KT285924	KT285913
A333	*Muellerella atricola* (perithecia) on thallus of *Tephromela atra*	Austria, Styria, Koralpe Mts, Glitzfelsen, 46°46′50″N/15°01′35″E, ca. 1800 m a.s.l., on *Tephromela atra*, 18.VI.2012.	KT285906	KT285929	KT285917
A440	*Muellerella atricola* (perithecia) on thallus of *Tephromela atra*	Austria, Styria, Koralpe Mts, Krakaberg, 46°46′43″N/14°58′13″E, on *Tephromela atra*, 21.VI.2012.	KT285907	KT285930	KT285918
A528	Cultured fungus from thallus of *Tephromela atra*	Austria, Styria, Koralpe Mts, Glashüttenkogel, 46°50′20″N/15°02′35″, on *Tephromela atra*, 26.IV.2012. *[LMCC0148]*	KT263088	KT263104	KT263123
A663	*Muellerella atricola* (perithecia) on thallus of *Tephromela atra*	Austria, Styria, Koralpe Mts, Sprungkogel, 46°48′54″N/14°58′14″E, ca. 1860 m a.s.l., on *Tephromela atra*, 18.07.2012.	KT285908	KT285931	KT285919
EZ19202 (A)	Culture from *Lichenodiplis lecanorae* – specimen Ertz D. 19202	Isolated from sample EZ19202	KT285909	KT285932	–
EZ19202 (B)	*Lichenodiplis lecanorae* – specimen Ertz D. 19202	Belgium, Brabant district, Meise, Botanical Garden, 42 m a.s.l., on *Ginkgo biloba*, on *Lecanora saligna*, 13.VIII.2014.	–	–	KT285920
L2254	*Lichenodiplis lecanorae* – specimen Brackel W. 6411	c, on dead *Quercus*, 715 m a.s.l., on *Caloplaca cerellinoides*, 20.VIII.2011.	–	KT285925	KT285914
L2256	*Lichenodiplis lecanorae* – specimen Brackel W. 6713	Bayern, Oberpfalz, Kreis-Amberg-Sulzbach, Klosterberg E Kastl, on *Sambucus*, 485 m a.s.l., on *Lecanora sambuci*, 12.XI.2013.	–	KT285926	–
L2257	*Lichenodiplis lecanorae* – specimen Brackel W. 6945*	Italy, Toscana,Prov. Massa Carrara, below Passo Cirone, on *Tephromela atra*, ca. 1000 m a.s.l., 5.X.2013.	–	KT285927	KT285915

species diversity and anamorph–teleomorph relationships (e.g. Crous et al. 2001, 2004, 2006; Lizel et al. 2003; Réblová et al. 2004; Ertz et al. 2014). Such discoveries have impacted taxonomic revisions, with suppression or introduction of new names (Hawksworth 2011; Taylor 2011; Hyde et al. 2013; Kirk et al. 2013; Wijayawardene et al. 2014). Most recent phylogenetic studies focused on the diversity of Dothideomycetes and Eurotiomycetes also demonstrated shared ancestry (Gueidan et al. 2008, 2011) of fungi placed either in one or the other class. Furthermore, evolutionary relationships among taxa with different life styles and from different ecological niches were also highlighted (Diederich et al. 2013; Muggia et al. 2013; Ertz et al. 2014; Ertz & Diederich 2015). However, due to difficulties encountered in extracting and amplifying DNA from inconspicuous and often melanized fungal samples, as well as in isolating and growing certain fungi in axenic cultures, the phylogenetic placement of several fungal groups still remains poorly investigated or completely unknown.

A particularly interesting group of fungi, for which we still have too little genetic information, is that represented by lichenicolous taxa. Lichenicolous fungi are species with inconspicuous and often melanized mycelia that inhabit lichen thalli and develop diverse degrees of specificity and parasitic behaviour towards their hosts (Lawrey & Diederich 2003). They are usually recognized by phenotypic symptoms and their sexual or asexual spore-producing structures on the lichen hosts. Lichenicolous fungi have been classified according to morpho-anatomical characters, but molecular data are still lacking for the majority of the described species, estimated at over 1800 worldwide (Lawrey & Diederich 2015). It is likely that the very narrow host ranges of certain species and their strict dependence on their host are responsible for the failure of many lichenicolous fungi to grow in axenic cultures. Attempts to isolate lichenicolous fungi are usually performed by inoculating spores or tiny fragments of the fruiting bodies (apothecia, perithecia or conidiomata) on media. However, the production of reproductive structures in culture has only been observed for the two successfully isolated genera *Lichenoconium* (Lawrey et al. 2011) and *Phoma* (Lawrey et al. 2012).

Recently, molecular data obtained from the few available culture isolates and environmental samples were combined to confirmed the placement of some most common and collected genera of lichenicolous ascomycetes within Dothideomyceta (Lawrey et al. 2011, 2012; Ertz et al. 2014; Frisch et al. 2014; Ertz & Diederich 2015), Eurotiomycetes (Untereiner et al. 2011; Diederich et al. 2013; Pérez-Ortega et al. 2014), and Helotiales (Lawrey et al. 2015; Suija et al. 2015). Further, molecular and phylogenetic results suggested also anamorph–teleomorph relationship among lichenicolous fungi: the DGGE technique was used to prove that the genus *Vouauxiomyces* is the anamorphic state of the otherwise apotheciate genus Abrothallus (Pérez-Ortega et al. 2011), and phylogenetic analyses showed the genera *Phaeosporobolus* and *Lichenostigma* to be monophyletic, with *Phaeosporobolus usneae* the asexual stage of *Lichenostigma maureri* (Ertz et al. 2014). However, the biology of lichenicolous fungi, either on their host or axenically grown in culture, remains largely unknown.

As a part of a wider study on the diversity of lichenicolous fungi (Fleischhacker et al. 2015), two taxa, the coelomycete *Lichenodiplis lecanorae* (Fig 1A–D) and the ascomycete *Muellerella atricola* (Fig 1E–I), gained our interest as they co-occurred multiple times on thalli of the host lichen *Tephromela atra* (Fig 1J). A further screen of herbarium collections revealed the co-presence of *Lichenodiplis* and *Muellerella* species also on other lichen hosts (Fig 1K–M). *Lichenodiplis* (Fig 1A–D) is the genus introduced by Hawksworth & Dyko (1979) to circumscribe species with dark-brown, 1-septate conidiospores with apex obtuse and base truncated. The genus currently includes 12 species (MycoBank April 2015) that are lichenicolous on different hosts. These fungi usually invade the apothecia of the hosts, but they can also produce pycnidia on the thalli if the apothecia are already heavily infected (Hawksworth & Dyko 1979).

The genus *Muellerella* is, alternatively, one of the most widespread and frequently collected lichenicolous fungi. It is easily recognizable due to the conspicuous black, sometimes slightly shiny, perithecioid, ostiolate ascomata, with multispore asci containing 1-septate, ellipsoid, brown ascospores (Fig 1E–I), that can be immersed or sessile on the thallus and/or on the apothecia of the host lichens. The genus has been classified in the family Verrucariaceae (Triebel & Kainz 2004) and it currently comprises 33 species, including eight varieties (MycoBank, April 2015). *Muellerella* species can be bryophilous, lichenicolous or saprophytic (Triebel & Kainz 2004) and have been placed in close relationship with *Epibryon* and *Dactylospora* species infecting mosses and hepatics (Döbbeler & Triebel 1985). On lichens, *Muellerella* species present a continuum of morphological variation and subtle character diversity (e.g. variation in ascospore size and number), which has been correlated with its host specificity. Many taxa have therefore been described according to their occurrence on different lichen hosts, but the genetic diversity of this complex of species has never been explored, and the genus is in need of revision.

In the present study we performed molecular and morphological analyses on freshly collected samples, culture isolates and herbarium specimens from different geographic origins of *Lichenodiplis lecanorae*, including the type species of the genus, and of *M. atricola* and *Muellerella lichenicola*. We attempt to assess *i)* their phylogenetic placement and *ii)* whether they represent, due to their often co-occurrence, a case of anamorph–teleomorph relationship.

Materials and methods

Sampling

Fresh samples and herbarium vouchers of *Lichenodiplis* and *Muellerella* were used for morphological and molecular analyses; only freshly collected sample were used for culture isolation (Table 1). Herbarium material from BR, GZU and the private collection of Josef Hafellner were examined and a total of 94 specimens of *Lichenodiplis hawksworthii*, *L. lecanorae*, *L. lichenicola*, *L. pertusariicola*, *Muellerella atricola*, and *M. lichenicola* infecting 33 different lichen hosts were included (Table S1).

Culture isolation

Five specimens were selected for the isolation of *Lichenodiplis* and *Muellerella* fungi: *i)* one sample of *Caloplaca* sp. infected

Table 2 – New primers designed in this study and specific for *Lichenodiplis lecanorae* and *Muellerella atricola*. The primer names, their sequences and their melting temperatures are reported.

Locus	Direction	Primer name	Primer sequence	Melting T (°C)
Partial ITS and 28S	Forward	Mu_ITS1008f	5′-TCGGGGTCGACTTATAGCCC-3′	59.5 °C
	Reverse	Mu_LR729r	5′-GTTCGACCCGGGGTCACC-3′	61.1 °C
18S	Forward	Mu_ns2f	5′-GCACTTATACCGTGAAACTGCG-3′	56.4 °C
	Reverse	Mu_ns3r	5′-CCCAGTGAAGGACATCGGGC-3′	60.6 °C
16S	Forward	Mu_mtSSU27f	5′-CAAATTACGTGCCAGCAGTCG-3′	56.9 °C
	Reverse	Mu_mtSSU651r	5′-ATAGCCCACACTATTAAGGCC-3′	54.3 °C

only by *Muellerella lichenicola*, *ii)* two specimens of *Tephromela atra* infected by both *Muellerella atricola* and *Lichenodiplis lecanorae*, *iii)* one specimen of *T. atra* infected only by *L. lecanorae* and *iv)* one specimen of *Lecanora saligna* infected only by *L. lecanorae* (Table 1). The axenic cultures were prepared from freshly collected samples (up to one month old) using hand-cut sections of pycnidia and perithecia. Where pycnidia and perithecia were present on the same thallus, we sampled both by selecting thallus parts where they were at least 2 cm from each other (Fig 1J). The thallus areoles were washed by pipetting once with sterile bi-distilled sterile water and three times with Tween80 to remove any external bacteria and yeast (Bubrick & Galun 1986). Pycnidia and perithecia were then carefully sliced with a sterile razor blade and tiny fragments of the hymenial and conidiomata tissue were inoculated on agar plates. Up to six fragments were inoculated on one agar plate and up to three agar plates were prepared for each sample. The agar plates were sealed with parafilm to avoid desiccation of the medium and were incubated in a growth chamber at 20 °C, with a light–dark regime of 14:10 h with light intensity of 60–100 μmol photons $m^{-2}s^{-1}$ and 60 % humidity. Bold's basal medium (BBM; Bold 1949; Bishoff & Bold 1963), with added ampicillin to reduce contaminant bacterial growth, was used for the first inoculation. For the samples *i)* and *iv)* thin sections were made through perithecia or pycnidia and the outer wall was removed with a sterile razor blade to expose ascospores or conidia, which were spread directly on petri plates. The cultures were kept at room temperature in the laboratory of the Botanic Garden Meise and exposed to a natural daylight regime. No culture chambers were used to test whether different light or temperature conditions could improve the growth rate. The inocula were checked weekly for contamination. After three to five months, cultures obtained from the thallus fragments and spores which reached about 1–3 mm in diameter were subcultured and prepared for DNA extraction and sequencing. The subcultures were made on malt yeast media (MY, Ahmadjian 1967), Lilly-Barnett's (LBM, Lilly & Barnett 1951), and *Trebouxia* (TM, Ahmadjian 1967). The cultured strains are deposited at the University of Graz and at the Botanic Garden Meise in the culture collection of the first (LM) and last (DE) authors.

The identity of the cultures was checked by sequencing the same nuclear (28S and 18S) and mitochondrial (16S) loci selected for the original environmental samples. The DNA extraction protocol followed Cubero *et al.* (1999); PCR amplification, sequencing, and the morphological analyses were carried out as reported below.

DNA extraction, amplification, and sequencing

Pycnidia and perithecia were carefully dissected under the stereo-microscope and prepared for DNA extraction. The fungal material was always taken from a single area of the thallus and transferred to a 1.5 ml tube. Similarly, a small part of each culture, both from the original inocula and the mature isolates, was taken and transferred to a 1.5 ml tube. The material was first frozen and then pulverized with metal beads using a TissueLyserII (Retsch). The DNA was extracted according to the protocol of Cubero *et al.* (1999). The phylogenetic relationships of the *Lichenodiplis* and *Muellerella* samples and the cultured strains were studied with sequences of the nuclear large and partial nuclear small ribosomal subunits (28S and 18S) and the mitochondrial small ribosomal subunit (16S). The loci were amplified using both already published and newly designed primers (Table 2). The new primers were designed using sequences obtained from the first sequenced *Lichenodiplis* and *Muellerella* isolates (L1858, L1860 and L1992, L1993, L1994; Table 1). The nuclear 28S fragment was obtained in two pieces using primers SR6R (http://www.botany.duke.edu/fungi/mycolab) and LR5 for the first part, and LR3R, and LR7 (Vilgalys & Hester 1990) for the second part (http://www.biology.duke.edu/fungi/mycolab/primers.htm) and the new Mu_ITS1008f and Mu_LR729r. The region ITS was amplified only in three samples and is therefore not included in the phylogenetic analysis. The nuclear 18S locus was amplified using the primers nuSSU0072, nuSSU0852 (Gargas & Taylor 1992) or NS1 (White *et al.* 1990), and the new Mu_ns2f and Mu_ns3r (Table 2). The mitochondrial 16S locus was amplified using the primers mtSSU1 and mtSSU3r (Zoller *et al.* 1999) or MSU7 (Zhou & Stanosz 2001), and the new Mu_mtSSU27f and Mu_mtSSU651r (Table 2). The PCR amplifications carried out with the newly designed primers were performed using the proofreading Phusion polymerase (BioLabs) under the following conditions: an initial denaturation step at 98 °C for 5 min, followed by 35 cycles of denaturation at 98 °C, annealing at 57 °C, both for 30 s, a 1 min elongation step at 72 °C. The final elongation step was 7 min at 72 °C. The PCR conditions applied for the previous published primers were: an initial denaturation step at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min and elongation at 72 °C for 2 min; the final elongation step was at 72 °C for 7 min. Both complementary strands were always sequenced and sequencing was run by Microsynth (Vienna, Austria). The sequences were assembled and edited in BioEdit (Hall 1999).

Table 3 – List of taxa retrieved from GenBank and used in the phylogenetic analysis of Fig 4 and Fig S1.

Taxon	Sample ID	28S	18S	16S
Agonimia allobata	L467	FJ455771	–	GU121589
Agonimia tristicula	L469 (Hafellner 66664)	FJ455772	–	
Anthracothecium nanum	AFTOL 1649	FJ358271	FJ358339	FJ225773
Arachniotus littoralis	CBS 454.73	FJ358272	FJ358340	FJ225773
Arachnomyces glareosum	CBS 116.129	FJ358273	FJ358341	FJ225785
Byssoonygena ceratinophila	IMI370021	AB075353	AJ315176	–
Caliciopsis orientalis	AFTOL 1911	DQ470987	DQ471039	FJ190654
Caliciopsis pinea	AFTOL 1869	DQ678097	DQ678043	FJ190653
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
Celothelium aciculiferum	F16591	DQ329019	–	DQ328992
Celothelium cinchonarum	F17105f	DQ329020	–	DQ328993
Ceramothyrium carniolicum (1)	CBS 175.95	FJ358232	FJ358300	–
Ceramothyrium carniolicum (2)	AFTOL 1063	EF413628	EF413627	–
*Chaenothecopsis savonica**		AY796000	U86691	–
Cladophialophora arxii		AB100683	AJ232948	–
Cladophialophora devriesii		AJ972912	AJ232947	–
Cladophialophora minourae	CBS 556.83	FJ358235	FJ358303	–
Cladophialophora parmeliae (1)	Ertz 16591	JX081671	–	JX081675
Cladophialophora parmeliae (2)	CBS 293.37	JQ342182	–	JQ342181
Coniosporium uncinatum (1)	CBS 100.212	–	GU250922	GU250913
Coniosporium uncinatum (2)	CBS 100.219	–	GU250923	GU250914
Dactylospora imperfecta	AFTOL 5006	FJ176896	FJ176841	–
Dactylospora lobariella	AFTOL 2137	FJ176891	FJ176837	–
Endocarpon pallidum	AFTOL 661	DQ823097	DQ823104	FJ225674
Epibryon bryophilum	M2	EU940090	EU940017	EU940242
Epibryon hepaticola	M10	EU940091	EU940018	EU940243
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	–	FJ225681
Lithothelium septemseptatum	AFTOL 12	AY584638	AY584662	AY584620
Neocatapyrenium rhizinosum	AFTOL 2282	EF643757	EF689840	FJ225683
Normandina pulchella	L530	GU121566	GU121584	GU121610
Norrlinia peltigericola		AY300845	AY779280	AY300896
Onygena corvina	CBS 281.48	FJ358287	FJ358352	FJ225792
Onygenaceae sp.	NFCCI2185	JQ048938	JQ048939	–
Parabagliettoa dufourii	AFTOL 2254	EF643792	EF689868	FJ225684
Phialophora europaea	CBS 129.96	FJ358248	FJ358317	FJ225750
Placocarpus schaereri	AFTOL 2289	EF643766	EF689850	–
Placopyrenium bucekii	AFTOL 2238	EF643768	EF689852	FJ225693
Pyrenula aspistea (1)	GW1044	JQ927470	–	JQ927462
Pyrenula aspistea (2)	AFTOL 2012	EF411063	EF411060	–
Pyrenula cruenta		AF279407	AF279406	AY584719
Pyrenula macrospora	CG1520a	JQ927473	–	JQ927466
Pyrenula pseudobufonia		AY640962	AY641001	AY584720
Pyrgillus javanicus	AFTOL 342	DQ823103	DQ823110	FJ225774
Schanorella spirotricha	CBS 304.56	FJ358288	FJ358353	FJ225793
Sclerococcum sphaerale (1)	Diederich 17283	JX081673	–	JX081678
Sclerococcum sphaerale (2)	Diederich 17279	JX081672	–	JX081677
Sclerococcum sphaerale (3)	Ertz 17425	JX081674	–	JX081676
*Sphinctrina turbinata**		EF413632	EF413631	FJ713611
Staurothele areolata	AFTOL 2291	EF643772	EF689856	FJ225699
*Stenocybe pullatula**		AY796008	SPU86692	–
Thelidium papulare	AFTOL 2249	EF643781	EF689861	DQ329005
Verrucaria viridula	AFTOL 2299	EF643814	EF689884	FJ225712
Verrucula inconnexaria	AFTOL 307	EF643821	EF689892	FJ225718

Table 3 – (*continued*)

Taxon	Sample ID	28S	18S	16S
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
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

Alignment and phylogenetic analyses

The identity of the new generated sequences was compared with sequences available in the NCBI GenBank database. The taxa which closest matched our sequences were selected for the phylogenetic analyses. As our sequences showed the closest matches with representatives of the order Chaetothyriales, we included in our dataset taxa representatives of the class Eurotiomycetes, selecting representatives of the orders Chaetothyriales, Onygenales, Pyrenulales, and Verrucariales (Table 3), and based our selection on previous phylogenetic inferences of Gueidan *et al.* (2008, 2011, 2014), and Diederich *et al.* (2013). Three species of Mycocaliciales were chosen as outgroups: *Chaenotheca savonica*, *Sphinctrina turbinata*, and *Stenocybe pullatula*. The sequence alignments were prepared manually in BioEdit (Hall 1999) and individually for the three loci. Introns and SNPs were removed from the alignments. For six specimens we were unable to generate sequences for all the selected loci and for other taxa sequences were not available in GenBank.

Combined data of different loci, whether fully or partially congruent, have been commonly considered by inferring organismal phylogeny (Dettman *et al.* 2003). We therefore performed, as in previous studies (Kauff & Lutzoni 2002; Miadlikowska *et al.* 2006; Muggia *et al.* 2014), both single locus and combined datasets. We analysed the single locus datasets with a Maximum Likelihood (ML) approach (Mason-Gamer & Kellogg 1996; Reeb *et al.* 2004) and the combined dataset using ML and Bayesian approaches. The combined dataset was treated in partition by genes nuclear 28S and 18S and mitochondrial 16S in both ML and Bayesian approaches. The ML analyses were performed using the program RA×ML v. 7.0.3 (Stamatakis *et al.* 2005). As only a single model of molecular evolution can be used across gene partitions in RA×ML, the ML analyses (for single loci and combined datasets) were performed with the GTRMIX model and 1000 bootstrap replicates were run. The Bayesian Markov Chain Monte Carlo (B/MCMC) analyses were run in MrBayes v. 3.1.2 (Huelsenbeck & Ronquist 2003; Ronquist *et al.* 2005). The model of molecular evolution applied to each gene partition in the Bayesian analysis, GTR + I + G, was estimated in JModeltest v. 2.1.4 (Darriba *et al.* 2012) using the Akaike Information Criterion (Posada & Crandall 1998). The B/MCMC analysis was run with six chains simultaneously, each initiated with a random tree, for ten million generations; trees were sampled every 100 generations for a total sample of 100 000 trees. Log-likelihood scores against generation time were plotted using Tracer 1.4 (Rambaut & Drummond 2007) to determine when the stationarity of likelihood values had been reached as a guide for where to set the burn-in stage (Ronquist *et al.* 2005). Burn-in was set at five million generations (the first 50 000 sampled trees) and a majority rule consensus tree was calculated from the posterior sample of 50 001 trees. The convergence of the chains was confirmed by the convergent diagnostic of the Potential Scale Reduction Factor (PSRF), which approached 1 (Ronquist *et al.* 2005). The phylogenetic trees were visualized in TreeView (Page 1996).

Morphological analyses

Morphological and anatomical characters of both environmental samples and cultured strains were analysed using standard microscopic and photographic techniques. The analysed lichen thalli infected by *Lichenodiplis* and/or *Muellerella* included the same specimens selected for molecular analyses (Table 1) and the 94 additional herbarium samples (Table S1). Pycnidia and perithecia were hand sectioned and analysed wet-mounted using light microscopy. The morphological analyses of the cultured strains were performed on six to 18 m old subcultures and considered the following characters: form of growth, branching, and pigmentation of the hyphae, formation of conidiogenous structures and conidia. Small fragments of the culture mycelium and conidiogenous structures were taken and squashed sections mounted in water. Images were acquired with a ZeissAxioCam MRc5 digital camera fitted to the microscopes. Both images of growth habit and hyphae structure were digitally optimized using the CombineZM software (open source image processing software available at www.hadleyweb.pwp.blueyonder.co.uk/). The photos were further refined with Adobe Photoshop 7.0 and the figure were prepared with CorelDRAW ×4.

Results

Culture isolation

Five inoculations of *Lichenodiplis*, coming from three different samples, and four of *Muellerella*, coming from two different

samples, grew successfully in culture (Table 1). About 75 % of the cultures were discarded due to contaminations by bacteria, yeast or black fungi. The successful axenic isolates were derived from inocula of *i)* perithecia fragments of *Muellerella atricola* present on the thallus of *Tephromela atra* also infected by *Lichenodiplis lecanorae*, *ii)* pycnidia fragments of *L. lecanorae* recovered alone on the two different samples of *T. atra* Muggia 0297-13 and Muggia 002–13, *iii)* conidiospores culture of *L. lecanorae* sample Ertz 19202, *iv)* ascospores culture of *Muellerella lichenicola* specimen Ertz 16261 (Table 1).

One culture isolate, A528 (Table 1), is included here because our analyses showed it to correspond morphologically and genetically to the samples listed above. However, this culture was isolated – luckily but unintentionally – from the thallus of *T. atra* A528, which has been included in the study of Fleischhacker *et al.* (2015) and Muggia *et al.* (in press).

Morphological analysis

The axenically isolated *Lichenodiplis* and *Muellerella* fungi were analysed at different growth stages and on three different growth media, LM, MY, and TM (Figs 2 and 3). Cultures initially inoculated on BBM developed a glossy mass of cells and very thin, pale hyphae which spread in the medium (Fig 2A, B). The inocula, subcultured on MY and TM, developed a dense mycelium of pale brown hyphae which grew compactly and partially inside the medium (Figs 2C, D and 3A, J, L). The inocula grown on LBM medium and their

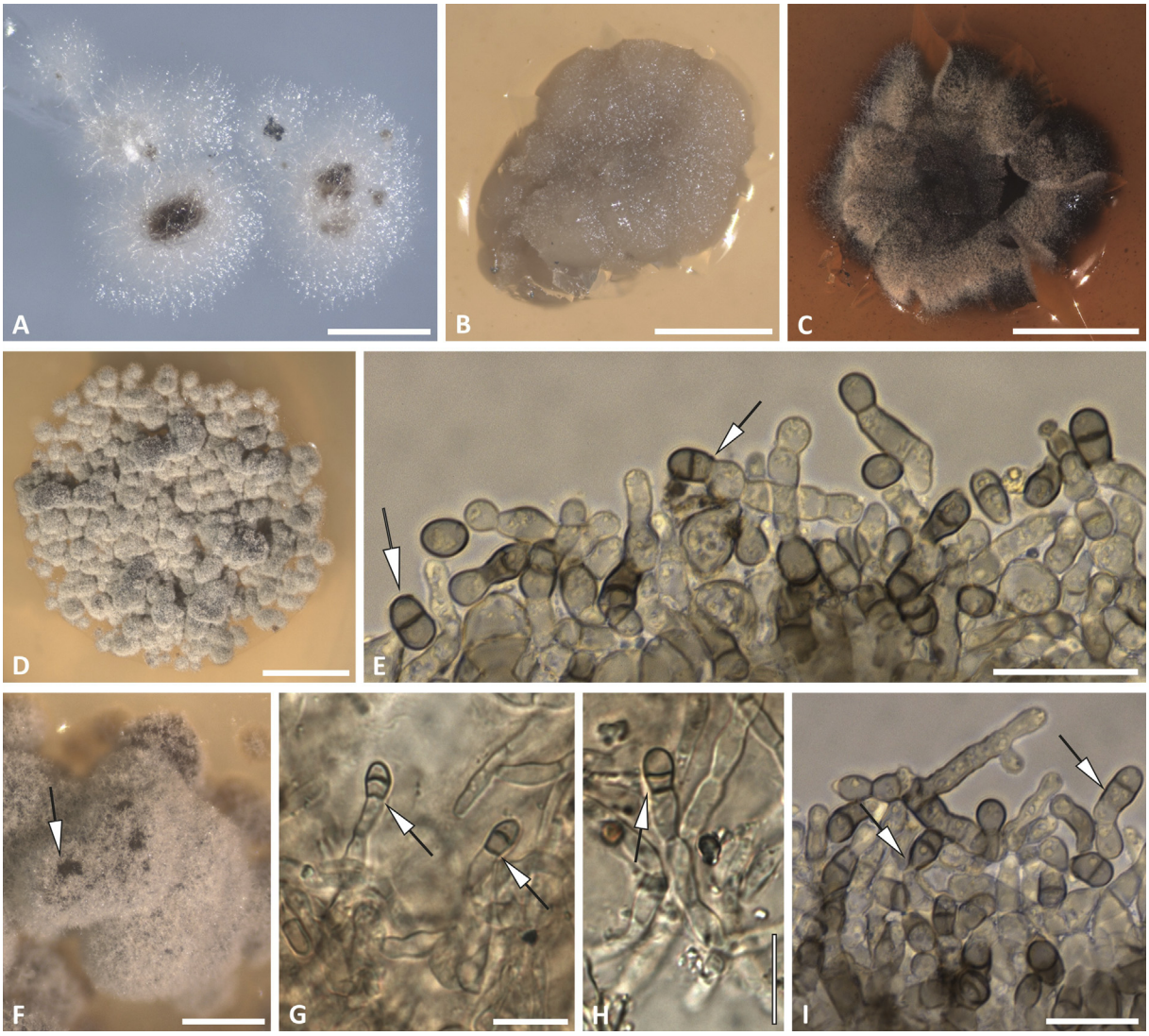

Fig 2 – Habitus of cultured *Lichenodiplis lecanorae*. Sample ID and/or corresponding extraction number (as in Table 1) are reported in square brackets '[ ]'. (A) two month old inoculum on LBM medium [Muggia 0297-13]; (B) five month old inoculum on TM medium [Muggia 0297-13/L2208]; (C) one year old culture on MY medium [Muggia 0297-13/L2208], (D) six month old subculture [Muggia 0297-13/L2208]; (E) eight month old subculture on MY [Muggia 0297-13/L2208]; (F) 16 month old subculture with pycnidia (arrows) on TM [Muggia 0297-13]; (E, I) conidia and conidiogenous hyphae (arrows) [L1858]; (G, H) conidia and conidiogenous hyphae (arrows) [Muggia 0297-13/L2208]. Scale bars: A, F = 0.5 mm; B = 1 mm; C = 2.5 mm; D = 5 mm; E = 20 μm; G-I = 10 μm.

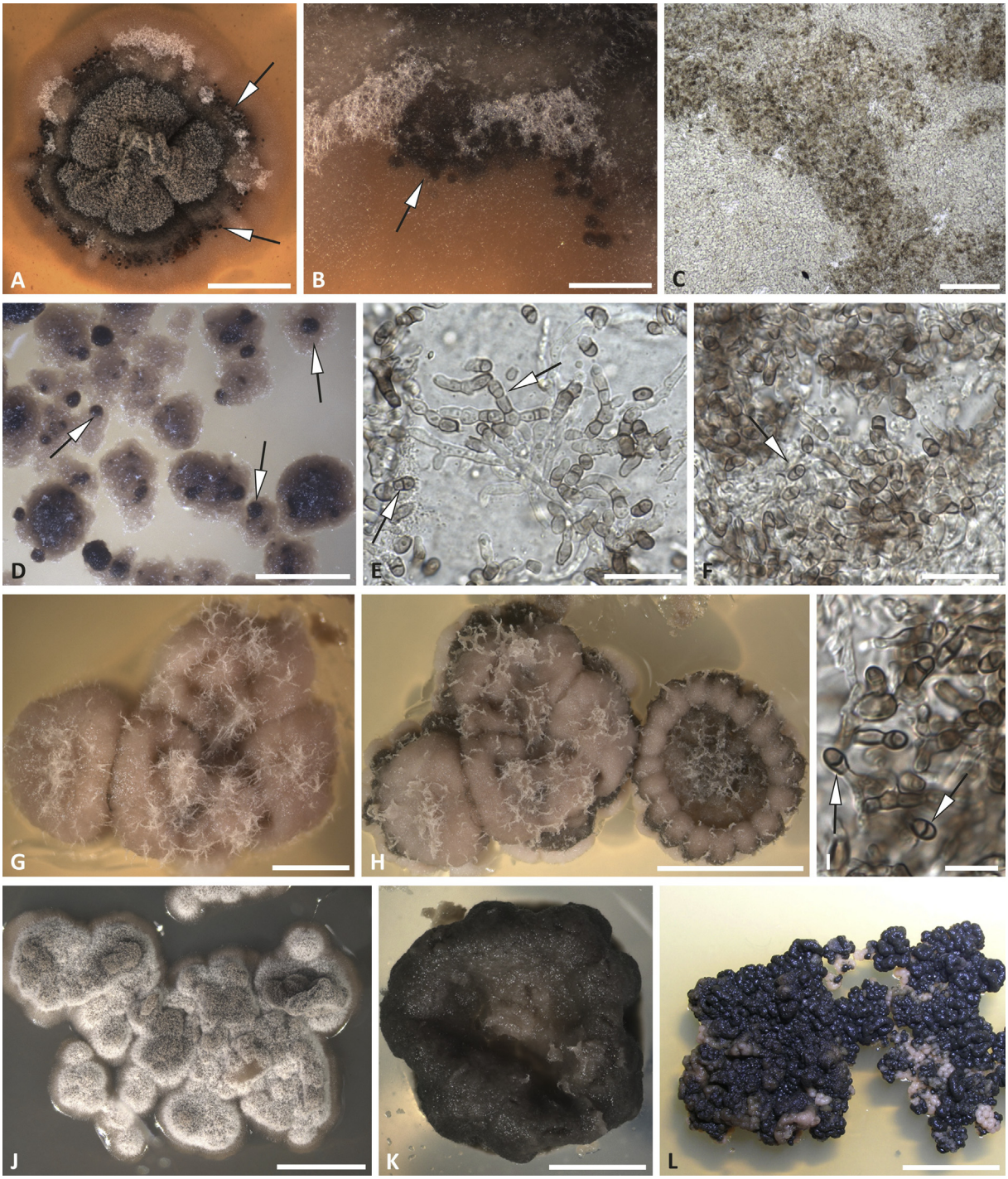

Fig 3 – Habitus of cultured *Muellerella* spp. (A–K) and *Lichenodiplis lecanorae* (L). Sample ID and/or corresponding extraction number (as in Table 1) are reported in square brackets '[ ]'. (A) one year old culture of *M. atricola* on MY medium [L1993]; (B) detail of (A), mycelium margin with pycnidia (arrows) [L1993]; (C) detail of (B), squashed section of pycnidia, the darker part is conidiocells and conidiogenous hyphae [L1993]; (D) eight month old subcultures with pycnidia [L1993]; (E, F) conidia and conidiogenous hyphae (arrows) [L1994]; (G, H) subculture of *M. lichenicola* [Ertz 16261/L2209] three month (G) and five month (H) old culture on MY, developing a dark margin with pycnidia; (I) conidia and conidiogenous hyphae (arrows) [L1992]; (J) one year old subculture on LBM medium; (K) subculture of *M. lichenicola* on LBM medium [Ertz 16261/L2209]. (L) culture of *Lichenodiplis lecanorae* [Ertz 19202] on MY. Scale bars: H, J, L = 5 mm; A = 2 mm; D, G, K = 1 mm; B = 0.4 mm; C = 100 μm; E, F = 20 μm; I = 10 μm.

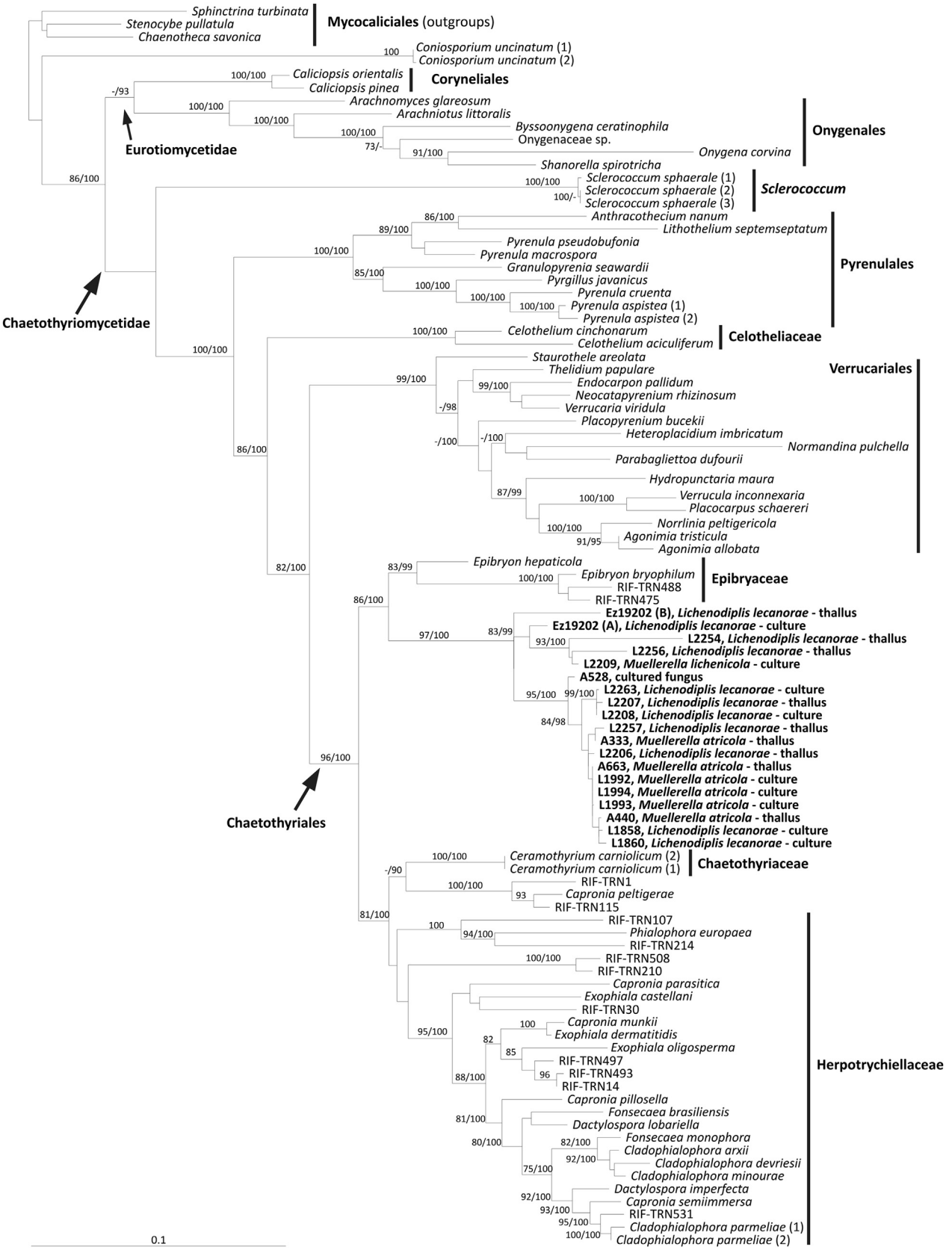

Fig 4 – Multilocus phylogenetic inference of *Lichenodiplis* and *Muellerella* taxa. The ML and Bayesian phylogenetic hypotheses were inferred from the combined dataset of the nuclear 28S and 18S, and the mitochondrial 16S. ML and Bayesian topologies corresponded, the ML analysis is shown; ML bootstrap support values (>70 %) and Bayesian posterior probabilities (PP > 95 %) are reported above branches (bootstrap value/PP). The newly sequenced samples are highlighted in bold.

further subcultures have maintained a gelatinous mycelium which developed more or less accentuated dark brown areas and only scattered aerial hyphae (Fig 3G, H, K). After almost 18 m, mature subcultures derived from both *Lichenodiplis* and *Muellerella* inocula and grown on the three different media produced slightly melanized, dot-like structures containing conidiogenous cells (Figs 2E–I; 3B–F, I). These structures were either densely localized at the margins of the mycelium or scattered in the central parts among the compact hyphae.

The screening of the herbarium specimens revealed the co-occurrence of pycnidia and perithecia in 12 samples of *Lichenidiplis lecanorae* and in two samples of *Muellerella lichenicola* (Fig 1K–M).

Phylogenetic analysis

We obtained 49 new sequences (15 for the nuclear 28S, 17 for the nuclear 18S and 17 for the mitochondrial 16S loci); only two samples are here represented by a single sequence and three samples by two sequences (Table 1). The new sequence data include nine environmental samples, six of *Lichenodiplis lecanorae* and three of *Muellerella atricola*, and ten cultured isolates, five from *L. lecanorae*, four from *M. atricola* and one of the isolated fungus A528.

Our phylogenetic results recovered relationships among the families and the orders of Eurotiomycetes which were congruent with previous studies (Gueidan *et al.* 2008, 2014; Diederich *et al.* 2013). The single locus analyses and the multilocus analysis were topologically congruent (Fig 4, Fig S1). All newly sequenced samples, both in the single locus and in the multilocus analyses, form a monophyletic, fully supported clade, where sequences from cultures and thalli are intermixed and constitute two subclades and one branch, which are unresolved among each other. The major subclade groups the environmental samples and the culture isolates (14 in total) of *L. lecanorae* and *M. atricola* deriving only from the host thalli of *Tephromela atra*. The second subclade includes three *Lichenodiplis* and *Muellerella* samples deriving from hosts other than *T. atra*, such as *Caloplaca* and *Lecanora*. The single branch holds the 16S sequence of the environmental sample of *L. lecanorae* Ez19202(B).

This monophyletic '*Lichenodiplis–Muellerella*' clade was evident in the multilocus and in the mitochondrial 16S analyses, sister group of Epibryaceae, which included two samples of *Epibryon* and two rock inhabiting fungi. Alternatively, in the nuclear 28S and 18S analyses it is the unsupported sister group of Chaetothyriaceae and Herpotrychiellaceae (Fig S1).

Discussion

Phylogenetic placement of Lichenodiplis *and* Muellerella

Our phylogenetic inferences based on single locus and combined datasets show for the first time the placement of the two lichenicolous fungal genera *Lichenodiplis* and *Muellerella* within the subclass Chaetothyriomycetidae. The phylogenetic reconstructions place in a single, monophyletic and fully supported clade sequences derived both from environmental samples and culture isolates of *Lichenodiplis lecanorae*, *Muellerella atricola*, and *Muellerella lichenicola*. This '*Lichenodiplis–Muellerella*' clade that we recognize is nested in Chaetothyriales and is the sister group of the family Epibryaceae.

Lichenodiplis was originally assigned to the Sphaeropsidales by Hawksworth & Dyko (1979), an order traditionally used for anamorphic fungi with pycnidial conidiomata. *Muellerella* was previously assigned to Verrucariales on the base of morphological characters of the ascus structure and interascal filaments (Triebel & Kainz 2004; Eriksson 2005; Gueidan *et al.* 2007). Its placement in a new clade as sister to Epibryaceae raises, therefore, some considerations. Though the genus is mainly represented by species parasitic on lichens, *Muellerella* species parasitizing mosses were also described and their relationship with the lichenicolous ones hypothesized (Döbbeler & Triebel 1985; Matzer 1996). Fungi related to Epibryaceae have been recently isolated from lichens (Muggia *et al.* accepted) and this would further support the close relationship between members of Epibryaceae and the '*Lichenodiplis–Muellerella*' clades recovered here.

Though the monophyly of the '*Lichenodiplis–Muellerella*' clade is evident, we are not confident in delimiting a new family or introducing any taxonomic changes on the basis of our results, since they were focused on just two species of each genus. It is possible that a wider taxon sampling might give different results and place in paraphyly or polyphyly taxa assigned so far to *Lichenodiplis* and *Muellerella*. Both genera are indeed known from a wide host range and from diverse habitats, so that morphological studies suggesting high host specificity lead to the description of a high number of species. For example, Pérez-Vargas *et al.* (2013) described the new species *Lichenodiplis anomalus* Etayo & Pérez-Vargas for specimens previously identified as *L. lecanorae* growing on *Ochrolechia*; up to now 33 *Muellerella* species have been reported (Mycobank April 2015). Alternatively, Fleischhacker *et al.* (2015) recognized *Muellerella* 'strains' according to their lichen hosts instead of splitting them in different taxa. Whether these complexes of species form monophyletic evolutionary units still needs study.

Furthermore, some authors (Diederich 2003; Hafellner 2007; Pérez-Vargas *et al.* 2013) pointed out the difficulties in distinguishing the genera *Minutoexcipula* and *Laeviomyces* from *Lichenodiplis*. *Minutoexcipula* was distinguished from *Lichenodiplis* by the plane to convex, sporodochia-like conidiomata, which are superficial and arise from the upper cortex of the host thallus (Atienza & Hawksworth 1994). In contrast, *Lichenodiplis* has immersed, unilocular, pycnidial conidiomata, which may become erumpent. According to Atienza *et al.* (2009) both genera, *Lichenodiplis* and *Minutoexcipula*, should be maintained because of the differences in the complexity of the conidiogenous cells, presence of conidiophores and in the structure of the exciple. However, it has not been investigated whether these traits vary according to the biology of the species when growing on different hosts. Interestingly, those samples included in our analyses and occurring on the thalli of *Tephromela atra* have pycnidial conidiomata and are therefore determined as *L. lecanorae*. However, *Minutoexcipula tephromelae* is the species described as occurring specifically on thalli – and not on apothecia – of *T. atra* (Atienza *et al.*

2009). Future molecular studies might show that *Minutoexcipula* also belong to the '*Lichenodiplis–Muellerella* clade'. The genus *Laeviomyces* D. Hawksw. was described for two lichenicolous coelomycetes very similar to *Lichenodiplis* but differing in non-septate conidia. Diederich (2003) could not find other morphological differences; he considered the conidial septation insufficient for distinguishing genera and thus treated both genera as synonyms. Species of *Lichenodiplis* having simple conidia should therefore be added in future phylogenetic studies to test the taxonomic value of the conidial septation.

The relationship of *Lichenodiplis* and *Muellerella* to other verrucarialean genera of lichenicolous fungi deserves further investigations. Lichenicolous fungi currently assigned to Verrucariales are *Adelococcus*, *Bellemerella*, *Clauzadella*, *Endococcus*, *Gemmaspora*, *Haleomyces*, *Halospora*, *Merismatium*, *Muellerella*, *Norrlinia*, *Phaeospora*, *Pseudostigmidium*, *Sagediopsis*, *Sarcopyrenia*, *Stigmidium*, and *Telogalla*. These were assigned to three families: Adelococcaceae, including *Adelococcus* and *Sagediopsis* (Triebel 1993), Sarcopyreniaceae, including *Sarcopyrenia* (Navarro-Rosinés *et al.* 1998) and Verrucariaceae, including the remaining genera listed above, though with the exception of *Gemmaspora*, *Merismatium*, *Pseudostigmidium*, and *Stigmidium*, which were considered as Verrucariales incertae sedis (Lawrey & Diederich 2015 and references therein). Among them, sequences were available only for *Norrlinia* which was shown to belong to the Verrucariaceae (Lumbsch *et al.* 2004; Muggia *et al.* 2010), and only one sequence was available for *Endococcus fusigera*, which is insufficient for assessing its phylogenetic relationship. Thus, considerable work remains to be done before the verrucarialean lichenicolous fungi can be re-appraised.

Anamorph–teleomorph relationships

The repeated co-occurrence of the perithecia of *Muellerella atricola* and the conidiomata of *Lichenodiplis lecanorae* on the same host thalli of *Tephromela atra*, the congruence of sequences obtained from both taxa, the morphological traits and the growth type observed in the culture isolates are all suggestive of the anamorph–teleomorph relationship for the two fungi. The formation of pycnidia-like structures and conidiogenous cells was reported in almost all isolates after one and a half years of culturing. However, the density and the localization of these conidiomata were variable and likely dependent on the growth medium. Slightly diverse morphologies are, however, commonly observed in fungal isolates when cultured on different media (Muggia, pers. comm.). This is the first time that conidiomata and conidiocells are reported for cultured chaetothyrialean lichenicolous fungi. Only two studies previously showed the formation of conidiogenous cells and conidia in cultured lichenicolous fungi belonging to Dothideomycetes, such as the genera *Lichenoconium* (Lawrey *et al.* 2011) and *Phoma* (Lawrey *et al.* 2012).

Further support for the anamorph–teleomorph relationship between *Lichenodiplis* and *Muellerella* is given by 12 samples of *L. lecanorae* and two samples of *Muellerella lichenicola* screened among herbarium specimens. The 14 samples revealed the co-presence of pycnidia and perithecia on lichen hosts other than *T. atra*, such as *Caloplaca* and *Lecanora*. Interestingly, only a very careful inspection of these samples revealed the co-presence of the two reproductive structures. We observed that in specimens recorded as *L. lecanorae*, perithecia were extremely rare and sometimes poorly developed. In specimens recorded as *M. lichenicola*, conidiomata were localized on few apothecia not inspected by previous workers. It is likely that when the more conspicuous *Muellerella* perithecia were detected, the presence of additional structures was not further considered or searched for. The potential anamorph–teleomorph relationship between *L. lecanorae* and *M. lichenicola* is also suggested by the second and less represented subclade that includes only four sequences coming from samples on thalli of *Caloplaca* and *Lecanora* and their cultures. This will likely be even more strongly supported by sequence data gained from pycnidia and perithecia in further sampling.

The original description of conidiocells (hyaline, short bacilliform) in *Muellerella* (Triebel & Kainz 2004), should be revisited, as it does not correspond to our observations and conclusions. Unfortunately, the co-occurrence of the two morphs is uncommon and therefore difficult to obtain in quantity. Future studies including also species of *Minutoexcipula* might show additional examples of anamorphs having *Muellerella* as teleomorphs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2015.08.011.

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