

# Amyloidity is not diagnostic for species in the *Mycena pearsoniana* complex (*Mycena* sectio *Calodontes*)

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**Abstract** In *Mycena* sectio *Calodontes* with otherwise amyloid spores, the inamyloid spores of *Mycena pearsoniana* Dennis ex Singer were a distinguishing feature for this species and its subsection *Violacella*. Although the original concept of this species was European, Singer chose to typify it with material collected in Mexico. The name has since been applied to all European collections with inamyloid spores and decurrent lamellae. Our phylogenetic analysis of 91 ITS sequences from European, North and South American *Calodontes* collections shows that European collections identified as *M. pearsoniana* fall into two well-supported sibling clades together with both inamyloid and weakly amyloid North American collections. Since the holotype of *M. pearsoniana* is in an advanced state of decay, we have selected an epitype from a North American locality with a climate comparable to the Mexican type locality. Our results show weakly and

inamyloid spore reactions to be homoplastic in *Calodontes*, and furthermore that spores of *M. pearsoniana* can show either amyloid or inamyloid reactions interchangeably. This raises doubt about the taxonomic value of this trait in *Mycena* systematics.

**Keywords** *Mycena* · ITS phylogeny · Amyloidity

## Introduction

In the original concept and species description, *Mycena pearsoniana* Dennis ex Singer was distinguished morphologically from other species in *Calodontes* by having inamyloid spores and by lacking pleurocystidia (Singer 1959). This species is small, with pileus and stipe pale lilaceous to brown, has pale arcuate to somewhat decurrent

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lamellae, and is solitary or scattered on both broadleaved and conifer litter. Maas Geesteranus (1980, 1989) erected subsection *Violacellae* based on *M. violacella* and included *M. pearsoniana*. The species has a complicated taxonomic history. Kühner (1938) originally misapplied the name *M. pseudopura* (erected by Cooke 1892) for a fungus similar to *M. pura* but with inamyloid spores. Pearson (1955, or actually, Dennis in Pearson) later discovered that the spores from Cooke's type material of *M. pseudopura* were amyloid, which led him to conclude that the taxon with inamyloid spores discovered by Kühner (until then assigned to "*M. pseudopura*") must be another species, which Dennis invalidly named *M. pearsoniana*. Singer (1959), when validating the name *M. pearsoniana*, unfortunately chose to typify it with brownish specimens collected at high elevation in an *Abies religiosa* forest of Mexico, which he deposited in the Argentine LILOA herbarium rather than using European material. Josseland (1960) also tried to provide a name for this taxon, *M. puroides* Joss., but he discovered that Singer (1959) had already provided a name and added a footnote to this, making *M. puroides* superfluous.

Although *M. pearsoniana* has since been applied to European collections, caution should be used based on such an unfortunate typification. Maas Geesteranus (1989) accepted the name but noted that a request to study the type remained unanswered. He did not otherwise indicate problems in using this name for European material. Although we had better luck with obtaining the holotype for study, we found it to be in an advanced state of decay which prevented both detailed morphological examination and molecular analyses. Specimens from the New World have been reported from Venezuela (Dennis 1970) and the Lesser Antilles (Pegler 1983), but so far not from mainland North America. In a recent publication on the *Calodontes* of northern Europe, Harder et al. (2010) showed the existence of two clearly separate ITS lineages of specimens morphologically identified as *M. pearsoniana* as well as many similarly separate lineages of the morphospecies *M. pura*, which is well known to be morphologically variable (Smith 1947; Maas Geesteranus 1989; Robich 2003). Rexer (1994) lists several morphological *Calodontes* species as having a wide distribution across continents. However, with the advances in molecular analyses, it is now well established that long-distance spore dispersal by fungi is much less common than was originally thought. In *Schizophyllum commune*, actual intercontinental gene flow is limited (James et al. 1999), while *Megacollybia platyphylla*, also formerly thought to be a single species worldwide, has been shown to consist of at least 8 phylogenetic species (Hughes et al. 2007). Many other widely distributed "species" also show geographical partitioning, e.g., *Panellus stypticus* (Jin et

al. 2001), species of *Pleurotus* (Vilgalys and Sun 1994), *Lentinus tigrinus* (Grand et al. 2010), *Marasmius androsaceus* (Gordon and Petersen 1997) and *Marasmius scorodonius* (Gordon and Petersen 1998); for a review, see Taylor et al. (2006). While the determination of intercontinental conspecificity is influenced by the species concepts used in any analysis, the temporal and geographical variability of fungi is clear (for further discussion, see Taylor 2008). In order to examine the intercontinental conspecificity of *M. pearsoniana* and to stabilize the nomenclature, we thus set out to locate a suitable epitype following the tradition of using material from as close to the holotype locality as possible. In the absence of suitable Mexican material, we selected a well-annotated North American collection from a similar climatic zone as the Mexican type locality as epitype.

## Materials and methods

### Outline

Colours of the proposed epitype of *M. pearsoniana* were annotated using Kornerup and Wanscher (1978), and concordant Ridgway (1912) names were derived from the University of Tennessee web page (<http://web.bio.utk.edu/mycology/Color/Color-index.htm>). We added 12 ITS sequences from *Calodontes*, some of which were from selected North American *Mycena* aff. *pura* specimens with morphologically interesting similarities to European *M. pearsoniana* (Table 1), to the 78 ITS sequences analyzed in Harder et al. (2010). Additionally, the ITS sequence of Matheny's *Mycena* aff. *pura* PBM2665 (DQ490643) was downloaded from GENBANK.

### Generation of DNA sequence data

All genomic DNA extractions were from lamellae of freshly dried material or herbarium specimens with a standard CTAB-chloroform-isopropanol procedure (Gardes and Bruns 1993), or the procedure of Lindner and Banik (2009) at the Center for Forest Mycology Research (CFMR) in Madison, Wisconsin. PCR-amplification and sequencing of the internal transcribed spacer (ITS) was performed using the primers ITS1F and ITS4 (Gardes and Bruns 1993). Sequencing was done at MACROGEN, Seoul, South Korea, the University of Wisconsin in Madison, and at the University of Tennessee.

Cloning of PCR products at CFMR and UTK was accomplished using pGEM-T Vector System II kits and JM109 competent cells from Promega following the manufacturer's instructions when direct sequencing did not resolve a sequence.

**Table 1** *M. pearsoniana*, *M. pura* and *Mycena* sp. collections

Species	Coll. no.	Location	Deposited	GenBank
<i>M. pearsoniana</i>	FCME25817 <sup>a</sup>	Great Smoky Mountains National Park, Tennessee, (GRSM, TN), USA	UNAM	JN182198
	13283 <sup>a</sup>	GRSM, TN, USA	TENN61544	JN182199
	12919 <sup>a</sup>	GRSM, TN, USA	TENN61384	JN182200
	CBH068	Schwarzwald, Baden-Württemberg, Germany	BIO-KU	FN394614.1
	JV06890	Paderup Mose, Jutland, Denmark	BIO-KU	FN394612.1
	LK716_1993	Perlbachthal, Bayern, Germany	STU	FN394616.1
	LK880_2002	Seebachthal, Bayern, Germany	STU	FN394613.1
<i>M. pearsoniana</i> 2	DJL05NC47 <sup>a</sup>	GRSM, North Carolina, USA	TENN61865	
	TL3966	Skivum Nørrekrat, Jutland, Denmark	BIO-KU	FN394615.1
<i>M. pura</i> s-l.	11863 <sup>a</sup>	Kedrovaya Reserve, Primorsky region, Far Eastern Russia	TENN60747	EU517506
	12167 <sup>a</sup>	Zhigulevsky Reserve, Samara region, South Russia	TENN60106	EU517504
	12202 <sup>a</sup>	Zhigulevsky Reserve, Samara region, South Russia	TENN60139	EU517505
	DJL06TN78 (TN-206) <sup>a</sup>	GRSM, TN, USA	TENN065043	JN182202
<i>Mycena</i> sp.	DJL-BZ-489 <sup>a</sup>	Blue Hole National Park, Cave's Branch, Belize	BRH	JN182203
Pink North American <i>Mycena</i> sp.	PBM 2665 <sup>a</sup>	Princeton, Mid-State Trail near Redemption Rock, Massachusetts, USA	PBM 2665	DQ490643.1
	DJL06TN36 (TN-159) <sup>a</sup>	GRSM, TN, USA	TENN65046	JN182204
	DJL06TN71 (TN-198) <sup>a</sup>	GRSM, TN, USA (TENN65044)	CUW	JN182206

<sup>a</sup> Collections not included in Harder et al. (2010)

All chromatograms were checked manually and sequences were assembled using BioEdit (Hall 1999) or Sequencher. Ambiguities with clear double-peaks were recorded as heterozygous using the standard IUPAC codes. The sequences were aligned in MAFFT v5.6 (Katoh et al. 2005) using the settings L-INS-I. No manual corrections were performed. We subsequently tested different alignments with GBLOCKS 0.91b (Castresana 2000), ranging from the lenient (without any exclusions) to the very conservative using the strictest settings possible (excluding all blocks shorter than 10 bp, all indels, only allowing 4 contiguous non-conserved blocks and a minimum of 15 sequences for accepting a position as conserved) with bootstrapped neighbor-joining (NJ) trees (1,000 replications) in PAUP 4.0b10 (Swofford 2003) and produced 50% majority rule-consensus trees. Since the NJ analyses failed to show any topological differences between the trees produced from lenient and conservative alignments, we ultimately analyzed the alignment in its entirety.

#### Phylogenetic analyses

The total alignment of 91 ITS sequences was divided into three partitions (ITS1, 5.8S and ITS2) defined using the *Mycena* aff. *pura* PBM 2665 isolate AFTOL-ID 1486

(Matheny et al. 2006), and Bayesian analyses in MrBayes 3.0 (Huelsenbeck and Ronquist 2001) were carried out. We performed tests on the respective partitions of the alignment using MrModeltest2 (Nylander 2004) to determine the appropriate nucleotide substitution model. Using the Akaike Information Criterion (AIC), we found a general time reversible model with a gamma shape parameter to be appropriate for ITS1 and ITS2 (GTR+G), and a Kimura two-parameter model (K80) for 5.8S. Gaps were treated as missing data for all analyses. Two runs of six chains each were run simultaneously with the “heat” set to 0.2. Branch lengths were saved for the purpose of constructing Bayesian majority rule phylogenies. To assure that each run had reached a stationary level and that chains were mixing properly, the relationship between likelihood scores and the number of generations was assayed in Tracer 1.4 (Rambaut and Drummond 2007).

Test analyses of the alignment were performed with between 3 and  $8 \times 10^6$  generations to estimate an appropriate value for burn-in and the approximate generation time needed for the likelihood scores to converge and the standard deviation of split frequencies to approach 0.02, respectively. To check whether the posterior probabilities of all splits of the two MCMC runs converged, the resulting tree files of both runs in all analyses were plotted against

each other in AWTY (Wilgenbusch et al. 2004). By discarding all trees sampled prior to the standard deviation of split frequencies reaching 0.02, a burn-in of 25,000 trees (2,500,000 generations) was used in the ITS set based on the test runs, and the analysis were then run for four times the burn-in value. All trees sampled after the burn-in were combined in a 50% majority rule consensus tree. To show the approximate branch lengths, these values were added to the consensus phylogram. Equally weighted maximum parsimony analyses with 10,000 bootstrap replicates were performed in PAUP (Swofford 2003) using the FastStep search algorithm. Values above 50 for branches also present in the Bayesian phylogeny were superimposed on the Bayesian tree.

### Microscopic analyses

Collections of *M. pearsoniana* sensu lato were examined for amyloid reactions immediately after mounting them in Melzer's reagent and then again after 40 min. An immediate, distinct reaction was classified as strongly amyloid; a weak, variable reaction after 40 min as weakly amyloid; and no reaction after 40 min as inamyloid.

## Results

### Phylogeny

In the Bayesian analyses, the two independent runs reached a standard deviation of split frequencies of 0.02 after  $1.37 \times 10^6$  generations, with log-likelihood scores converging after about 10,000 generations. No differences were found either in topology or BPP values in 50% majority rule cladograms between the trees produced by the two separate runs, and the posterior probabilities of all splits of the two MCMC runs of the respective datasets ultimately converged. A consensus phylogram was constructed (Fig. 1) and morphological features added thereupon.

### Morphology and geography

Collections classified as *M. pearsoniana* appeared in two sibling clades with a mixture of inamyloid and weakly amyloid reactions in both groups. There was no consistent pattern of amyloid reaction with geographical distribution. The major *M. pearsoniana* clade comprised European as well as North American collections with completely inamyloid as well as slowly and very weakly amyloid spores. The epitype and TENN 61544 and 61384 from the Great Smoky Mountains National Park initially had spores that were inamyloid, but they all slowly developed a weak amyloid reaction over time. The sister clade, *M. pearsoni-*

*ana* 2, consists of the North American DJL05NC47 (Fig. 2) with weakly amyloid spores and the North European TL-3966 with completely inamyloid spores. The ITS sequence of DJL05NC47 differed from that of the *M. pearsoniana* epitype (FCME 25817) by less than 3% (counting insertions/deletions as one). While this could be considered an amphi-Atlantic variety of *M. pearsoniana*, there do not appear to be any consistent morphological characters on which to base a formal description. Upon cloning and sequencing of 4 individual clones from collection DJLTN0641 (TN167) and enumerating clear SNPs in duplicated forward and back reads of four other collections (DJL06TN36, DJL06TN71, BZ489 and PR6746, the latter not included here) we found that heterozygosity was in the range of 0–2.5%, suggesting that 3% difference in ITS is a reasonable cutoff value for species in Calodontes (Hughes et al. 2009).

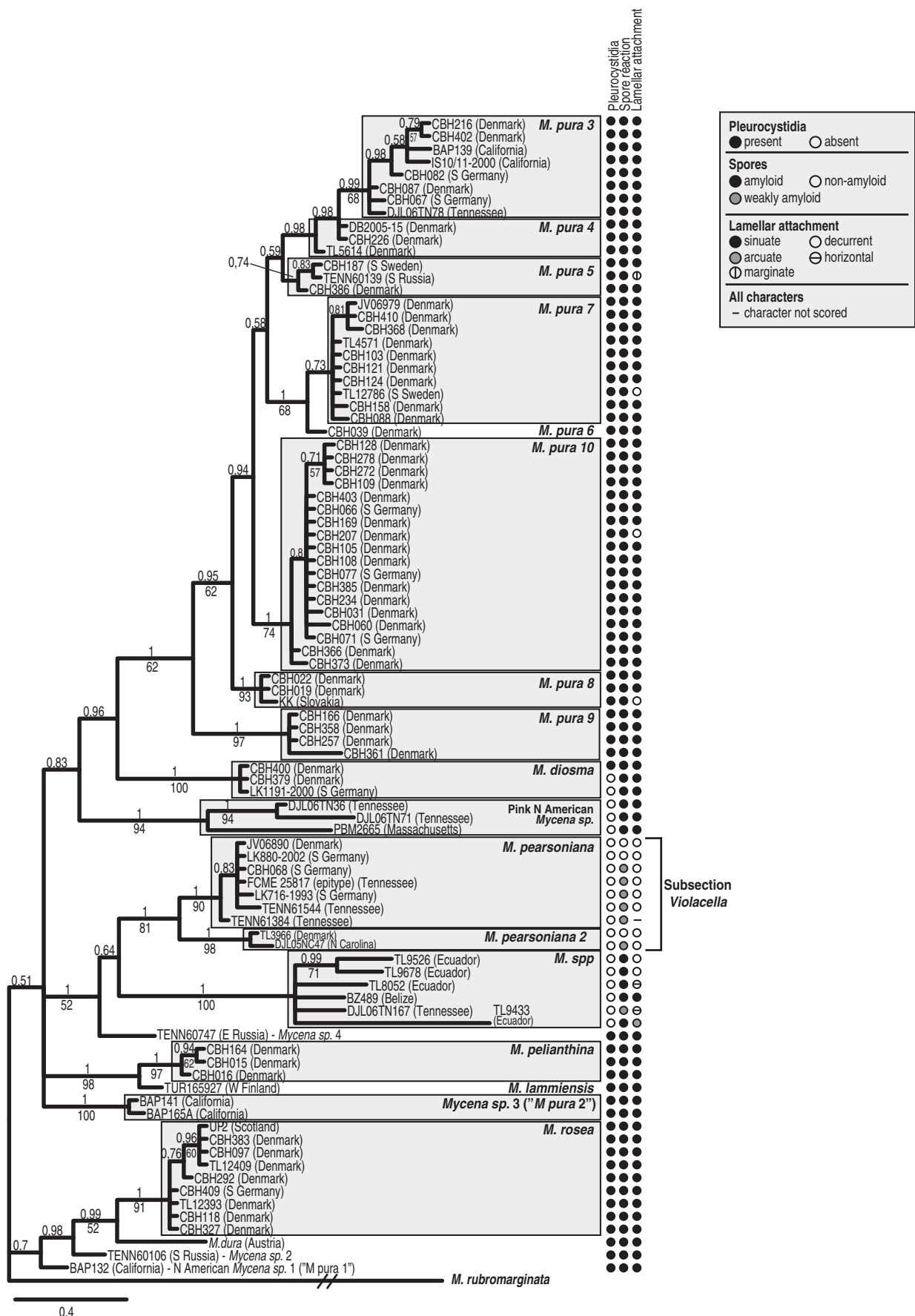
A third, well-supported clade comprising North and South American collections was basal to the *M. pearsoniana* sister clades. ITS sequences of the basal clade in the *Violacellae* subsection differ from the *M. pearsoniana* epitype sequence by 6–7%, which together with the 100% bootstrap support justify recognition at species rank. The un-named species in this clade differ from *M. pearsoniana* in moderate versus close lamellar spacing (1–2/mm vs. 2–3/mm) and a stipe that turns dark brown. We defer description of this new species in the *Violacellae* subsection to another publication on American species.

Epitypification of *Mycena pearsoniana* Dennis ex Singer, 1958, Sydowia 12(1–6): 233

FCME 25817 (Fig. 3) has been shown to closely match Singer's original morphological description and was collected at comparable geographical, ecological and climatic conditions, and is here selected to epitypify *M. pearsoniana*.

**Pileus** hygrophanous, smooth, striate 3/4 in from margin, not sulcate or tessellated, pallid orangish vinaceous (closest to Vinaceous Russet and Light Vinaceous Russet) (9–10 E-F3) when moist, paler at margin (near Vinaceous Buff, 9B2), discolouring to 6D5-4 to 7D4-3 (near Vinaceous Cinnamon), pileipellis not separable; **lamellae** close, narrow, adnate, horizontal or subdecurrent, somewhat intervenose, Vinaceous Buff (9 C2), paler on edge; **stipe** 35–65 × 2–4 mm, cylindrical or slightly enlarged toward base, smooth, moist, villose at base, Vinaceous Buff (9B2) or near Light Cinnamon Drab (9 C2) at apex, Pale

**Fig. 1** Bayesian consensus phylogram of the ITS dataset. Morphological features are indicated to the right of the tree. BPP values and MP bootstrap values above 50 are indicated above and below branches, respectively





**Fig. 2** DJL05NC47 from the sister clade to the core *Mycena pearsoniana*

Ochraceous-Buff at base and with age or bruising (4A2). **Odor** raphanoid. Basidia mostly 4-spored, but some 1- and 2-spored seen as well, clamped. **Basidiospores** (4.8–)5.0–6.3(–7.0) × (3.1–)3.5–3.8 (–4.2)  $\mu\text{m}$  (av. 5.8 × 3.7  $\mu\text{m}$ ;  $n=20$ ; from lamellae fragments), Q 1.30–1.81  $\mu\text{m}$  (Q av. 1.56  $\mu\text{m}$ ), broadly ellipsoid, initially inamyloid, weakly amyloid after 40 min, pleurocystidia absent; cheilocystidia forming a dense, somewhat interwoven band making the edge sterile but also with some on the sides near the edge, subcylindrical to clavate, rounded or more typically constricted-attenuated above, 35–43 × 5–11  $\mu\text{m}$ .



**Fig. 3** FCME 25817, the new epitype of *Mycena pearsoniana*

**Lamellar trama** with rather faint, but vinaceous reaction to Melzer's reagent. Growing in leaf duff of mixed conifer and dicotyledonous trees.

USA, Tennessee, Blount County, Townsend, Schoolhouse Gap Trail, near Laural Gap Road, coll. J. Cifuentes Blanco, on humus, 10 Oct. 2005, Cifuentes 2005/344, FCME 25817.

The epitype resembles what European mycologists would call *M. pearsoniana* in having spores that lack a distinct amyloid reaction, no or very few pleurocystidia, lamellae clearly decurrent and colours that are relatively pale. The spores, however, are smaller than for European collections (6–9 × 3.5–4.5  $\mu\text{m}$ , qav=1.6) as recorded by Maas Geesteranus (1989) and Aronsen (2011).

## Discussion

Despite the limited number of collections examined, this analysis gives further indications both to a true species overlap in *Calodontes* between Europe and North America, the presence of several unrecognised species in North America and Europe, and to unexpected morphological evolution in the section. *Mycena pearsoniana* is a species complex containing two phylogenetically clear but morphologically cryptic groups, which, in contrast to previously mentioned examples (Vilgalys and Sun 1994; Gordon and Petersen 1997; Gordon and Petersen 1998; Jin et al. 2001; Hughes et al. 2007; Grand et al. 2010), shows no geographical pattern. Though a few studies have made a case for long-distance dispersal (Liang et al. 2009), especially for the rust and smuts (Nagarajan and Singh 1990; Brown and Hovmøller 2002), species shared between continents have most often been shown to be due to recent human introductions rather than to true intercontinental wind dispersal, e.g., in many ectomycorrhizal species (Vellinga et al. 2009), in *Amanita phalloides* (Pringle et al. 2009), or the *Acephala applanata* species complex (Queloz et al. 2011). While only thorough phylogeographical analyses can tell whether this is also the case for *M. pearsoniana*, we consider it likely since a saprotrophic generalist should be more easily introduced and established than more host-specific ectomycorrhizal symbionts.

The commonly applied phylogenetic species identification method based on genealogical concordance (GCPSR; Taylor et al. 2000) could shed further light upon the existence or absence of recombination between the two *M. pearsoniana* groups. However, ITS has been shown to be a reliable predictor of major groups identified by GCPSR (e.g., Frøslev et al. 2007; Jargeat et al. 2010), and analyses of partial elongation factor 1-alpha data for a subset of the collections (unpublished data) also show the same major

groups, so we consider it unlikely that other sequence data will challenge these findings significantly.

Morphologically, there is an indication that, in *Calodontes*, abundant pleurocystidia might be a trait which has been lost several times independently, and that the systematic value is more quantitative than qualitative. Inamyloidity (or perhaps very weak amyloidity) appears to be a derived character in this section. While presence/absence of an amyloid reaction is clearly homoplastic and cannot be used in species delimitation in this section, all the species in the two clades (that are well supported by both Bayesian posterior probabilities and parsimonious bootstrap) that comprise the *Violacellae* subsection have spores that are either inamyloid or slowly and weakly amyloid as compared to other species in *Calodontes* that have spores which are rapidly and distinctly amyloid. However, the North American DJLTN167, which is well outside this subsection, also only shows a weak amyloid reaction. Thus, amyloidity can no longer be considered a reliable criterion for delimitation of this subsection, though weakly amyloid/inamyloid spores and decurrent lamellae applied together still appear diagnostic. Exceptions in amyloid reactions are also known in other species (e.g., *Marasmius pseudoniveus* Singer var. *amylocystis* and var. *pseudoniveus*), and genera [e.g., *Boletus amylosporus* (A.H. Sm.) Wolfe, and *Sericeomyces amylosporus* (Malençon) Heinem.]. Taken together, these findings raise questions about the reliability of the widespread use of strictly dichotomic use of amyloid reactions for delimitating species and genera.

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