Mycol Progress (2010) 9:395–405
DOI 10.1007/s11557-009-0648-7

ORIGINAL ARTICLE

A comparison between ITS phylogenetic relationships and morphological species recognition within *Mycena* sect. *Calodontes* in Northern Europe

Christoffer Bugge Harder · Thomas Læssøe · Rasmus Kjøller · Tobias G. Frøslev

Received: 12 February 2009 / Revised: 21 November 2009 / Accepted: 4 December 2009 / Published online: 20 January 2010

Abstract The members of *Mycena* sect. *Calodontes* (Tricholomataceae s.l., Basidiomycota) are characterised by a collybioid aspect and more or less purplish to reddish colours and a distinct raphanoid odour. In Europe, nine species have been recognised though some of these based on somewhat dubious morphological differences. Historically, most were assigned to *Mycena pura*. However, since *Mycena pura* displays one of the most striking colour variabilities within European agarics, many attempts have been made to subdivide it into independent entities, and several forms, varieties and species have been split from *Mycena pura* s.l. based largely on differences in colouration, gross macromorphology or other phenetic traits. We developed a large sample of ITS sequences of all species of sect. *Calodontes* known from Europe for which vouchers exist. Furthermore, partial LSU data were developed and additional sequences downloaded from GENBANK to assess the relationship of *Calodontes* with other *Mycena* spp. We show that most *Calodontes* form a monophyletic group including a few North and South American collections, but that this cannot be conclusively shown when an additional North American sequence is added. For all other species than *M. pura* and *M. diosma*, we found morphological species recognition to be in agreement with the ITS data. Several significantly different clades can be recognised within the *M. pura* morphospecies, none of which can be linked to the observed (and described by proxy) colour varieties/forms. Indications of a possible environmental basis of the colour differentiation in the *M. pura* morphospecies are discussed.

Keywords Basidiomycete phylogenetics · Morphological species recognition · Cryptic speciation

Introduction

Mycena sect. *Calodontes* (Fr. ex Berk.) Quél. sensu Maas Geesteranus (1992) (Tricholomataceae s.l.) is a group of saprobic, agaricoid basidiomycotes. The species are often colourful, reddish-violaceous with a collybioid to tricholomatoid habit, prominent voluminous cystidia, mostly amyloid spores and a raphanoid smell. Forms without pigments ("albinistic") are often encountered. The section presently comprises about 30 described species from most parts of the world (Smith 1947; Singer 1969; Corner 1986; Maas Geesteranus 1992; Rexer 1994).

The members of *Calodontes* have been placed in the separate genus *Prunulus* Gray by Redhead (lectotype *Prunulus denticulatus* (Bolton) Gray 1821) (Redhead et al. 2001). However, recent large-scale phylogenetic reconstructions of the Agaricales by Moncalvo et al. (2002) and Matheny et al. (2006) do not conclusively establish either whether *Mycena* s.l. is polyphyletic or if *Calodontes* is monophyletic. Thus, we will for the time being continue to use the generic name *Mycena* for *Calodontes*.

Nine species belonging to *Calodontes* have been reported from Europe: *Mycena pura* (Pers.: Fr.) P. Kumm., *M. pelianthina* (Fr.: Fr.) Quél., *M. diosma* Krieglst. & Schwöbel, *M. rosea* (Schumach.) Gramberg, *M. pearsoniana* Dennis ex

Electronic supplementary material The online version of this article (doi:10.1007/s11557-009-0648-7) contains supplementary material, which is available to authorized users.

C. B. Harder (✉) · T. Læssøe · R. Kjøller · T. G. Frøslev
Department of Biology, University of Copenhagen,
Øster Farimagsgade 2D,
1353 Copenhagen, Denmark
e-mail: cbharder@bio.ku.dk

Singer (ss. auct),[1] *M. dura* Maas Geest. & Hauskn., *M. lammiensis* Harmaja, *M. kuehneriana* A.H. Sm. and *M. sororia* Perr.-Bertr., Boissel. & Lambourd.

Unfortunately, it proved impossible to examine *M. kuehneriana* (from Europe) and *M. sororia* since no vouchers for those species appear to exist. *M. sororia* was described from France as a close relative of *M. rosea* (Perreau-Bertrand et al. 1996). To our knowledge, it has not been recorded by anyone other than the authors themselves, and the type allegedly deposited in PC could not be located (Buyck, personal communication). *Mycena kuehneriana* (Smith 1947) is widely distributed in North America, but only recorded once from Europe (in Norway, Aronsen 1986). The specimen (deposited in Leiden) could not be found (Noordeloos, personal communication).

Two other species, *M. lammiensis* (Harmaja 1985) and *M. dura* (Maas Geesteranus and Hausknecht 1994), are only known from very few collections within their respective type countries, Finland and Austria (plus a single Italian record).

Mycena rosea and *M. diosma* are known from many countries in Europe, and *M. pelianthina*, *M. pura* and *M. pearsoniana* also from other continents (e.g. Maas Geesteranus 1992; Rexer 1994).

Based on micromorphological characters, the species can be quite unequivocally assigned to three subsections: *Violacellae* Sing. ex Maas Geest. with inamyloid spores and no pleurocystidia (*M. pearsoniana*), *Marginatae* J.E. Lange with amyloid spores and pleuro- and cheilocystidia with brownish content (*M. pelianthina*, *M. lammiensis*), and *Purae* (Konrad & Maubl.) Maas Geest. with amyloid spores and colourless pleuro- and cheilocystidia (*M. pura, M. diosma*, *M. rosea* and *M. dura*) (Maas Geesteranus 1992; Arne Aronsen).[2] The species delimitation within these subsections is typological, based largely on macromorphology, colouration and smell.

Mycena pelianthina and *M. lammiensis* can allegedly be told apart by the latter having spores usually broader than 4 μm (Maas Geesteranus 1992). There are no decisive micromorphological traits separating e.g. *M. rosea*, *M. pura* and *M. diosma* (Krieglsteiner and Schwöbel 1982; Maas Geesteranus 1992; Boisselier-Dubayle et al. 1996). *Mycena rosea* is separated from *M. pura* by being generally larger and having a bright pinkish cap and lamellae and a pronounced umbo, and it has further been shown to differ from *M. pura* in enzyme electrophoretic mobility (Perreau et al. 1992). Krieglsteiner and Schwöbel (1982) described *M. diosma* as a new species because it was "unique among all *M. pura*-related species in Europe with its deep purple lamellae and its distinctive cigar box-like smell". *Mycena pura* s. str., which displays a wide array of reddish, whitish, blue or yellow or brown phenotypes, has additionally been divided into a multitude of official and unofficial forms and varieties based on these colouration differences; for an overview, see Maas Geesteranus (1992) or Index Fungorum.[3]

Clearly, there is no consistency in the criteria used for delimitation of species, varieties and forms. Furthermore, it has been well described that colouration can vary considerably due to minor mutations in a few or even single genes (Clegg and Durbin 2000; Badyaev 2006; Vaez et al. 2008) and/or environmental conditions (Turner 1988; Lymbery 1992; Follett and Hilbeck 1996; Chen et al. 1996; Ben-Tal and King 1997; Price 2006). Thus, other lines of evidence as compatibility tests and molecular data are much needed.

In the present work, we tried both, but unfortunately our attempts to culture our collections were almost invariably unsuccessful (unpublished), in agreement with earlier attempts (Boisselier-Dubayle et al. 1996; R.A. Petersen, personal communication) and we are not aware of any reports of successful cultivation of *Calodontes*. Despite the rapidly growing body of phylogenetic research in fungi, few sequence data for *Calodontes* species existed previous to ours. Using molecular data of the ribosomal LSU and ITS regions, we aimed to elucidate primarily whether this section is a true monophyletic group, and yielded a pattern consistent with the morphological species delimitation and recognition (MSR).

Materials and methods

Collections

We compiled a sample of 248 collections comprising all known European species (except *M. sororia* and *M. kuehneriana*) and the varieties/forms *M. rosea* f. *candida* Robich (Robich 2003), *M. pura* f. *violacea* (Gillet) Maas Geest. (Gillet 1874), *M. p.* f. *ianthina* (Gillet) Maas Geest. (Gillet 1874), *M. p.* f. *lutea* (Gillet) Arnolds (Gillet 1874), *M. p.* f. *multicolor* (Bres.) Kühner (Bresadola 1928), *M. p.* var. *luteorosa* Bon (Bon 1999) and *M. p.* f. *alba* (Gillet) Arnolds (Gillet 1874). A bluish specimen known to some German field mycologists as "*Mycena pura* f. *caesio-pura*" (Wölldecke, personal communication) was also included.

[1] Although the species was first discovered in Europe, the type specimen of *M. pearsoniana* is originally described from Mexico (Singer 1959). The Mexican specimen might well turn out to be a different species, hence the "ss. auct" should formally be added to the species epithet for the European taxon; for reasons of clarity and simplicity, we will refrain from this for the rest of this paper.

[2] http://home.online.no/~araronse/Mycenakey/lammiensis.htm

[3] www.indexfungorum.org

Due to the relative obscurity of *M. lammiensis* and *M. dura*, we were only able to gain access to two and a single collection (the holotype) from those, respectively.

Fresh basidiomata were collected mainly in Denmark, Germany and Southern Sweden in the autumn of 2005–2006. Additional specimens were obtained from various herbaria. In total, the European sample consisted of specimens from Denmark, Sweden, Finland, Germany, Slovakia, Austria and the UK. Additionally, five collections identified as *M. pura* or *M. pura* f. *violacea* from the US (California) were included, as were four collections identified as *M.* aff. *pura* or *Prunulus* sp. from Ecuador.

The type specimen (from Mexico) of *M. pearsoniana* was obtained from LIL, but, unfortunately, its condition precluded almost any analysis. The type specimen of *M. diosma* could not be located in SMNS in which it was allegedly deposited (Krieglsteiner and Schwöbel 1982), but material identified as *M. diosma* by G. Krieglsteiner collected close to the type locality was obtained and sequenced; similarly, collections of *M. lammiensis* from close to the type locality were obtained. No holotype specimen exists for *M. pura*, *M. rosea* or *M. pelianthina*, but our sample covered the type localities as mentioned in Maas Geesteranus (1992) quite well.

Morphological characters

Most specimens collected by C.B.H. or T.L. were photographed in fresh condition and subsequently dried. Microscopy was performed a posteriori on selected exsiccata mounted in a solution of Congo Red in ammonia (1 mL 25% ammonia in 1.5 g Congo Red in 50 mL water (as prescribed by Adamcík et al. 2005) or in Melzer´s reagent.

The following characters were scored and mapped on the ITS tree: (1) colour of pileus (the dominant colour outside the centre, as most species are hygrophanous), lamellae and stipe (based on Kornerup and Wanscher 1974), (2) lamellar attachment, (3) ecological association (coniferous forest, frondose forest and grassland), (4) pleuro/cheilocystidia with coloured/colourless content, (5) spores amyloid/inamyloid in Melzer´s reagent, (6) pleurocystidia absent/present. The latter two microscopic characters were applied only to an a posteriori selected subset representative of the phylogenetic/morphological variation. The first four are mapped for all fresh specimens and dried ones for which a description existed. For simplicity, we have transformed the colour codes of (1) into the seven main colour groups, brownish, reddish, yellowish, whitish, blue, violet and rose for pileus, stipe and lamellae alike; for a list of the exact colour codes according to Kornerup and Wanscher (1974) (see electronic supplementary material, ESM, Table 2. Length and width of 20 spores of *M. pelianthina* (specimens CBH015 and CBH164) and *M. lammiensis* (TUR165927) were measured at ×1,000 magnification. The ITS tree is set out in Fig. 2 (see below).

DNA extraction, PCR and sequencing

All genomic DNA extractions were done from lamellae of freshly dried material or herbarium specimens with a standard CTAB-chloroform-isopropanol procedure (Gardes and Bruns 1993). PCR-amplification and sequencing of the internal transcribed spacer (ITS) and the first 700 bp of the nuclear ribosomal large subunit (LSU) was performed using the primers ITS1F (Gardes and Bruns 1993) together with either ITS4 or TW13 (White et al. 1990). Sequencing was done at MACROGEN, Seoul, South Korea.

All chromatograms were checked manually and sequences assembled using BioEdit (Hall 1999). Ambiguities with clear double-peaks were recorded as heterozygous using the standard IUPAC codes. Alignments were done with MAFFT v5.6 (Katoh et al. 2005) using the settings L–INS–I for ITS and E–INS–I for LSU. The LSU alignment was unequivocal except for a small fragment of 10–17 bp at the beginning which failed to amplify in some sequences and was excluded from analysis. In the ITS alignment which contained many indels, a few manual corrections were performed. We subsequently tested two different alignments, one lenient (without any exclusions) and another very conservative produced with GBLOCKS 0.91b (Castresana 2000) 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 neighbour-joining (NJ) trees (1,000 replications) in PAUP 4.0b10 (Swofford 2003) and produced 50% majority rule-consensus trees. For all datasets, the NJ analyses did not show any topological differences between the trees produced from the lenient and the conservative alignments. We ultimately retained most indels but otherwise chose a strict approach excluding alignment ambiguities associated with small blocks, missing data in the beginning and the end and large outgroup indels. More ITS alignments details are listed in ESM Table 3.

Phylogenetic analyses

From all the resulting sequence data, initial NJ trees with 1,000 bootstrap replicates were constructed in PAUP 4.0b10 (Swofford 2003). Based on this NJ tree, the ITS dataset was subsequently trimmed, discarding information which was both genetically and morphologically redundant in order to facilitate the Bayesian and maximum parsimony phylogenetic analyses. Unique sequence groups were identified using DnaSP 4.10.9 (Rozas et al. 2003),

considering gaps but treating ambiguities as missing data. Subsequently, most of the identical sequences from inconspicuous specimens were excluded from further analyses, as were two unique but apparently persistently chimeric sequences. We ultimately analysed a dataset of 77 ITS sequences. The partitioning of the ITS sequences for Bayesian analyses was determined using the *Mycena* aff. *pura* PBM 2665 isolate AFTOL-ID 1486 (Matheny et al. 2006).

For both datasets, Bayesian analyses in MrBayes 3.0 (Huelsenbeck and Ronquist 2001) were carried out. In those, the ITS dataset was assumed to have three partitions (ITS1, 5.8S and ITS2), while the LSU alignment was unpartitioned.

We performed tests on the respective partitions of the ITS and the LSU data using MrModeltest2 (Nylander 2004). Using the Akaike Information Criterion (AIC), we found a HKY model with a certain proportion of invariable sites and a gamma shape parameter to be appropriate for ITS1 (HKY+I+G), a Jukes-Cantor model (JC) for 5.8S, and a general time reversible model with a gamma shape parameter (GTR+G) for both the ITS2 and the LSU data. 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 phylograms. To assure that each run had reached stationarity 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).

Multiple analyses of both datasets were initially performed with between 3 and 8×10^6 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 18,000 trees (1,800,000 generations) was used in the ITS set, and a burn-in of 21,000 trees (2,100,000 generations) for the LSU. The analyses 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 for ITS. For LSU, a consensus cladogram was constructed.

Equally weighted maximum parsimony analyses with 10,000 bootstrap replicates for both genes were performed in PAUP (Swofford 2003) using the FastStep search algorithm. Values above 50 for branches also present in the Bayesian phylogenies were superimposed on the Bayesian trees.

Based on the preliminary ITS dataset, a limited partial LSU sequence dataset was developed from the different morphospecies[4] and from our own collected Danish specimens of *M. galericulata* (Scop.: Fr.) Gray and *M. adonis* (Bull.: Fr.) Gray (Fig. 1). A range of LSU sequences were downloaded from genbank (For accession numbers see supplementary data, Part 3).

Results

Sequence data and phylogenetic analyses

ITS sequence information was obtained for 248 specimens. The number of collections from each morphospecies can be seen in Table 1. The NJ analysis resulted in well-supported monophyletic clades for all morphotaxa except for *M. pura*, which consisted of several different, distinct clades (data not shown). Additional information about all 82 specimens contained in the trimmed ITS alignment and/or the LSU alignment can be seen in Table 1 in the supplementary data.

In the Bayesian analyses of the ITS alignment, the two independent runs reached a standard deviation of split frequencies of 0.02 after just below 1.8×10^6 generations, with log-likelihood scores converging after 8,000 generations. In LSU, the two independent runs reached a standard deviation of split frequencies of 0.02 after about 2.1×10^6 generations, while the log-likelihood values of both independent runs reached stationarity after about 8,000 generations. For all analyses, no differences at all were found either in topology or BPP values in 50% majority rule cladograms between the trees produced by the two separate runs. Likewise, the posterior probabilities of all splits of the two MCMC runs of the respective datasets ultimately converged.

Morphological characters, species delimitation and subsections outside *M. pura* from the ITS data

Morphological species recognition showed agreement with the phylogenetics in *M. rosea* and *M. pelianthina.* The standard macromorphological recognition criteria are reliable, and there is little intraspecific genetic variation in either species. The clades supporting those species are supported by BPP values of 1.00 and MP bootstrap values of 82% and 95%, respectively. The white form *M. rosea* f. *candida* is ITS-identical to the coloured form. *Mycena*

[4] Unfortunately, LSU sequencing of *M. dura* failed despite repeated attempts.

Fig. 1 Bayesian consensus cladogram of 63 basidiomycete LSU sequences. A monophyletic group including almost all *Calodontes* is highlighted; as noted, the collection *Mycena aff. pura* 2665 falls outside this group. BPP values and MP bootstrap values above 50 are indicated above and below branches, respectively

dura does appear to be a separate species most closely related to *M. rosea*, but more evidence is needed and no firm conclusions about MSR can be drawn from an analysis of just the type specimen. *Mycena lammiensis* is a distinct sister species of *M. pelianthina* in the clearly monophyletic subsect. *Marginatae* (BPP 1.00, MP bootstrap 99% support). We found average spore width to be a valid criterion to discriminate between *M. pelianthina* and

Table 1 The distribution of the 248 ITS sequences listed by morphospecies

Morphotaxa	Collections
M. rosea	47
M. diosma	21
M. pelianthina	21
M. lammiensis	1
M. dura	1
M. pearsoniana	7
M. sp. (Ecuador)[a]	5
M. pura	145

[a] The Ecuadorian samples included several different morphotypes which are so far unnamed.

M. lammiensis as claimed by. e.g.. Maas Geesteranus (1992); though not all *M. lammiensis* spores are broader than 4 μm, they are on average, significantly broader than those of *M. pelianthina* (*t* test, unequal variances, $P<0{,}01$). Though the character combination of absence of pleurocystidia and inamyloid spores is unequivocal, the ITS tree indicates that the *M. pearsoniana* morphospecies might harbour two phylogenetic species (Fig. 2). *Mycena diosma* was phylogenetically distinct, but contained several specimens without the published diagnostic characters. Of the 21 ITS sequences identical to the one extracted from collections identified as *M. diosma* by G. Krieglsteiner (Table 1), 15 were from specimens identified by C.B.H. or T.L. as fresh collections; we only identified 11 of these correctly as *M. diosma*. Not all had violet lamellae or cigar box-like smell. The all-white specimen CBH379 which had an indistinct smell and was identified as *M. pura* f. *alba* is likely just an albinistic aberration, but three other collections, like specimen CBH400, had uncharacteristic reddish lamellae (and no distinct smell), and could not, even a posteriori, be discerned from any other *M. pura*. Furthermore, there were also specimens outside this clade with violet lamellae, as seen in specimen CBH039 (ITS group 6). Pictures of CBH039, CBH379 and CBH400 can be found in ESM Fig. 4e f and h).

All collections analysed had basophilic, stellate crystals on top of the stipe (see Clémençon 1997), prominent, voluminous cheilocystidia and lacrymoid to cylindrical spores. The cystidia were fusiform, subcylindrical, clavate, apically attenuated to broadly rounded. It was not possible to group the material into discrete units based on micromorphological characters bar the already well-known *M. pearsoniana* (inamyloid spores) and *M. pelianthina* (coloured cystidia).

A screen for UV active pigments inspired by Bresinsky and Huber (1967) revealed nothing of taxonomic importance since all non-albinistic specimens (i.e. all except CBH379, CBH410 and TL12409) were UV active (purplish-coloured).

Variation within the *Mycena pura* morphospecies

The specimens belonging to the *M. pura* morphospecies contained several distinct clades and single deviating sequences. Based on a combination of phylogenetic support and morphology, we divided our samples into 10 different *M. pura* ITS groups (Fig. 2). Groups 2, 5, 8, 9 and 10 were well- or fairly well-supported clades with BPP supports of 1.00 and MP bootstrap support of at least 72%. Group 3 is poorly supported (BPP 0.87, MP Bootstrap 59%) and is quite diverse with respect to geography, containing specimens from Denmark (CBH402, CBH216, CBH087), Southern Germany (CBH067, CBH082) and southwestern USA (BAP139, IS10-11_2000). Specimen CBH039 (Group 6) is a single separate lineage basal to group 7 which differs from the specimens in this group consistently by four to six nucleotides. For this reason and because of its morphological differences (very distinct violet lamellae similar to those of *M. diosma*), we separated it from group 7, which then has a BPP of 0.88 and a MP Bootstrap of 69%. Group 4 consisted of yellow grassland specimens appearing paraphyletically to but well separated from the clade containing the ITS groups 5 to 10. Specimen BAP132 (Group 1) is a unique sequence apparently not closely related to any other in the ITS tree.

Discussion

Is *Calodontes* monophyletic?

The nuclear ribosomal large subunit (LSU) is alignable across fungal genera and up to order level (Moncalvo et al. 2002; Matheny et al. 2006), and, therefore, LSU is useful in resolving higher level phylogeny in fungi. The LSU tree (Fig. 1) shows that all specimens except for the North American *M.* aff. *pura* 2665 (Matheny et al. 2006) form a well-supported monophyletic clade in the tree, including most North, Central and South American specimens receiving a Bayesian BPP support of 1.00 and a MP bootstrap support of 68%. It also shows that this partial LSU fragment does not give a resolution sufficiently high to recognise species within *Calodontes*.

Mycena aff. *pura* 2665 from North America (Matheny et al. 2006) is surprisingly divergent and constitutes a distinct lineage to the other *Calodontes* specimens. The notion that *Calodontes* should be a monophyletic group thus cannot be clearly supported by this analysis. It is likely that inclusion of specimens from the many unsampled parts of the world

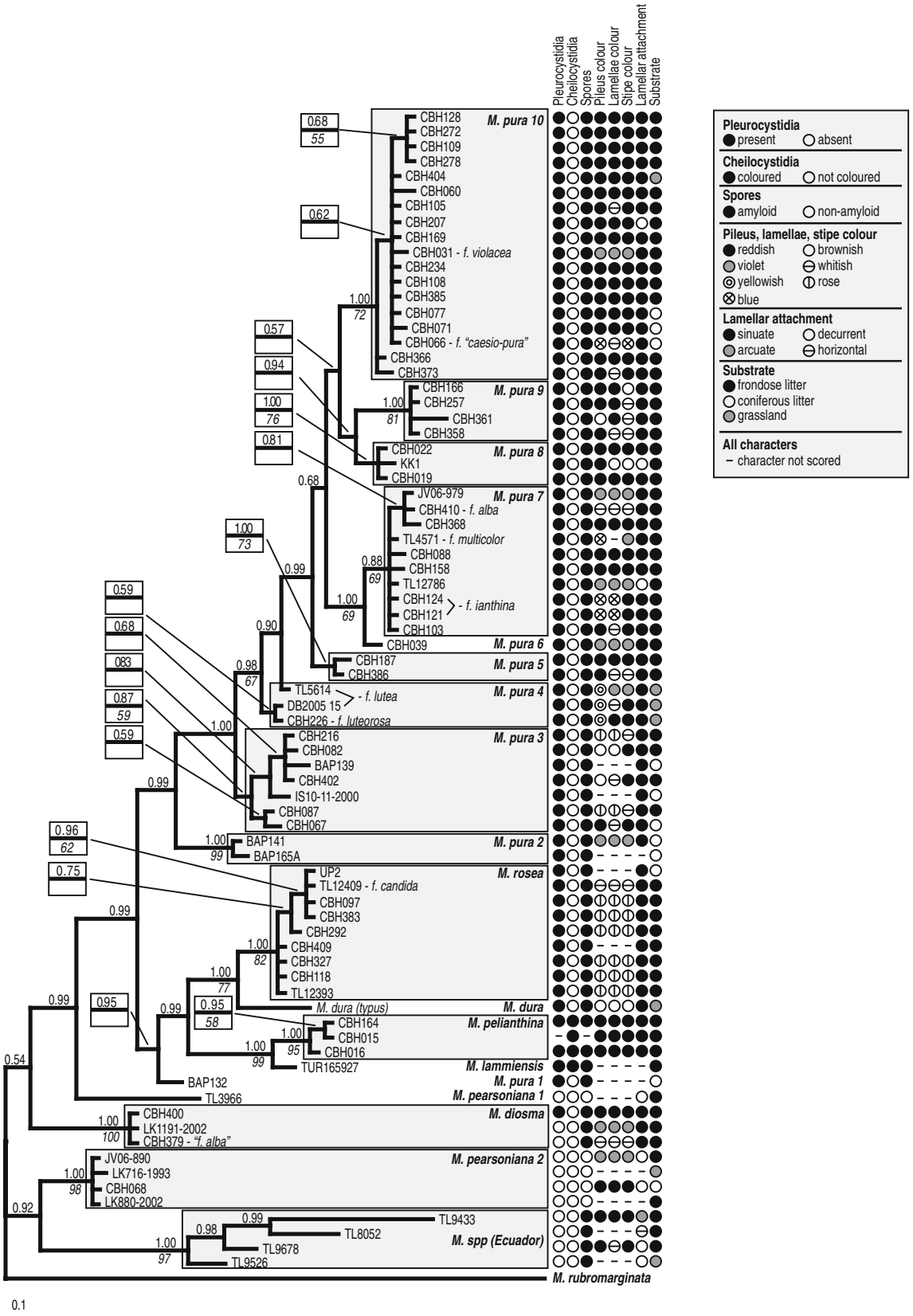

Fig. 2 Bayesian consensus phylogram of the ITS dataset. Based on bootstrap support values and morphological characters, 10 seemingly different *M. pura* and two *M. pearsoniana* groups could be identified. They are indicated to the right of the tree. BPP values and MP bootstrap values above 50 are indicated above and below branches, respectively

would give a clearer result, but we cannot rule out that the section is para- or polyphyletic.

Species delimitation and morphological evolution of *Calodontes*

ITS has proved to be a good method for identifying closely related phylogenetic species across many groups, often in quite good agreement with morphological/biological species concepts (Garnica et al. 2003; Leonardi et al. 2005; A. Taylor et al. 2006a, b; Frøslev et al. 2007; Hallenberg et al. 2007), and ITS is further being investigated as a universal bar code candidate for fungi[5] (Nilsson et al. 2006; Eberhardt, unpublished). In this light, we consider it likely that the well-supported ITS groups 2, 5, 8, 9 and 10 or at least some of them are true phylogenetic species and would be recognised by genealogical concordance recognition (GCPSR; Taylor et al. 2000). While more sequence data are needed to evaluate this closer, several conclusions are clear from Fig. 2. First, neither the well-supported ITS groups of *M. pura* nor the other species show any sign of coarse-level ecological specialisation; thus, we have found nearly all the species discovered here at the same time of the year within the precincts of one Danish *Fagus*/*Quercus*-dominated forest. Second, based on our data, it is evident that neither the named colour varieties/forms, colouration nor lamellar attachment show any discernible patterns which can be linked to the different *M. pura* ITS groups. The forms *M. p.* f. *ianthina, M. p.* f. *alba, M. p.* f. *multicolor* are ITS-identical to ordinary reddish forms in group 7, and the ITS group 10 contains the pastel blue specimen CBH066 ("*M. p.* f. *caesio-pura*") from a South German coniferous forest, the ordinary reddish specimen CBH404 from a Danish meadow as well as specimen CBH031 (det. as *M. p.* f. *violacea*); and while all these collections had sinuate lamellar attachment, specimen CBH207 from the same clade had decurrent lamellae (pictures of CBH066, CBH108, CBH207 and CBH404 can be found in ESM Fig. 4a–d). *Mycena p.* f. *lutea* (specimen DB2005/152) was identical to a specimen determined as *M. p.* f. *luteorosa* (specimen CBH226) although it was found on a calcareous meadow in Denmark, while the original *M. p.* f. *luteorosa* (Bon 1999) was described from a *Dryas* bog in subalpine France. In the light of the systematic value of the other colour-based varieties, there does not seem to be much reason to expect exactly this variety to stand out, and since the group of yellow specimens (ITS group 4) is paraphyletic, it is unlikely to be a separate phylogenetic species. Thus, even when considering the inescapable uncertainties associated with hygrophany, and even if our reduction of the section´s richly facetted colour spectrum into the aforementioned seven colour categories may be perceived as somewhat rough, we consider it very unlikely that much new insight about speciation and relationships inside *Mycena pura* is to be expected from even the most careful studies on colouration.

Third, while the species which Krieglsteiner and Schwöbel (1982) described as *M. diosma* is unequivocally distinct in both the LSU and the ITS trees, their morphological species recognition criteria (dark purple lamellae and cigar box-like smell) are unreliable. For *M. diosma*, the cigar box-like odour appears to be able to change into the ordinary raphanoid-like smell upon even light disturbances, and most experienced field mycologists will know that there is a certain degree of subjectivity between collectors in their opinion on smell nuances. At best, a strict combination of both criteria would recognise about two-thirds of our collected specimens truly belonging to the distinct clade containing the presumably phylogenetic species. Instead, we suggest that the species can be morphologically recognised on a combination of amyloid spores and few, if any, pleurocystidia. The smell and the lamellae colour can be invoked as supportive macromorphological characters. Since *M. diosma* appears not to be closely related either to most *M. pura* clades or to *M. rosea*, the "*M. pura* complex" sensu Krieglsteiner and Schwöbel (1982), Corner (1986) or Boisselier-Dubayle et al. (1996) is not reflected in the phylogentic relationships, and subsection *Purae* appears to be polyphyletic.

In the ITS tree, there is a large clade containing *M. rosea*, all *M. pura*, *M. pelianthina* and *M. lammiensis* with a BPP support of 0.99. Accordingly, *M. diosma* and both *M. pearsoniana* groups appear to be more distantly related to the other species. The two *M. pearsoniana* lineages are not particularly closely related, and their inamyloidy is likely to be a homoplastic trait.

Overall, our data do not give much information as to the sequence of speciation events. Though it cannot be ruled out that another outgroup might have given a better resolution of the ITS tree, the LSU tree gives no clear indications that other non-*Calodontes Mycena* taxa should be more closely related to *Calodontes* than *M. rubromarginata*. More data from other genes will be necessary to elucidate this point further.

Biogeography

The Ecuadorian specimens included here presumably harbour three or four separate phylogenetic species distinct from all other sampled lineages, and a suggested global or pantropical *M. pura* complex like that of Corner (1986) would likely comprise a multitude of quite separate phylogenetic species. Though our sample from outside Northern Europe is very limited, it does indicate

[5] http://www.allfungi.org/

that the *M. pura* morphospecies contains both shared and distinct phylogenetic species between Europe and North America. Specimens BAP141 and BAP165-A are clearly separate from all European specimens, while IS10/11-2000 and BAP139 equally clearly cluster together with European specimens in ITS group 3 (Fig. 2). The divergent *M.* aff. *pura* 2665 in the LSU tree (Fig. 1) is likely to be a separate species. Many alleged biogeographical similarities for fungal distribution patterns between continents are likely to be due to indiscriminate use of MSR criteria founded on names coined for European morphotaxa, which conflicts with biological and phylogenetic species concepts, e.g as shown in *Phellinus* by Fischer and Binder (2004) or more generally by Taylor et al. (2006a, b). Furthermore, as shown by James et al. (1999), in vitro interfertility may not reflect actual gene flow.

Though we consider it likely both that there might be several cryptic species in *M. pura* and that some of those will eventually be unique to specific continents or regions, it will require more data on the *Calodontes* from the rest of the world for phylogeographic and coalescence/molecular clock studies to answer whether the apparent overlap in *M. pura* is real, whether it reflects old common distributions, possible recent anthropogenic introductions or occasional natural long-distance dispersal events between continents (which is presumably rare outside rust and smuts within Basidiomycota; Nagarajan and Singh 1990; Gage et al. 1999; Brown and Hovmøller 2002) or more than one of these possible explanations.

Differentiation in colouration within *Calodontes*?

The distinct pinkish colouration in *M. rosea* has been identified as stemming from pyrroloquinolone alkaloid pigments unique to this species (Peters and Spiteller 2007), named Mycenarubin A and B by the authors. According to Spiteller (unpublished), a blue/bluish pigment, which was structurally distantly related, could be found in *M. pura* (and separate pigments were found in *M. pelianthina*, too). In the light of these findings, *M. diosma* might well be thought to possess a specific pigment, but if such a pigment were common to all the 21 (or the 20 non-albinistic) ITS-identical members of the *M. diosma* clade, the link between chemotype and phenotype would be less pronounced than in *M. rosea*. However, there are indications that the phenotypic colour variation could be due to environmentally induced causes. In *M. haematopus* (Pers.: Fr.) P. Kumm., Peters et al. (2008) found its red pyrroloquinolone alkaloid pigment to be sensitive to colour-altering structural changes, which could be inflicted by drying stress or heating, and according to Spiteller (personal communication) there are indications that this could also apply to the structurally related *M. pura* pigment. This corresponds with our observations of some *M. pura* specimens sometimes getting a dingy yellowish tinge increasing with age and/or drying. Heating or other chemical reactions triggered by the increased exposure to sunlight in open land would also explain why yellow forms are most often found in open land; this argument could be further strengthened by the fact that the yellow colouration in *M. pura* f. *lutea* is normally only found on the pileus (or at least much more pronounced here). Furthermore, we observed that all specimens displaying bluish colours when fresh (like specimen CBH066) became reddish when dried and were then almost indistinguishable from specimens which had been reddish also in fresh condition.

We do not know which of our groups, if any of them, that Spiteller's aforementioned *M. pura* specimens belonged to, but it is clear that colour variation in *M. pura* is not linked to ITS type. While more research is needed, we consider it plausible that most differences in colouration are a result of environmental rather than genetic factors.

Acknowledgements We thank the Staatliches Museum für Naturkunde Stuttgart, Anne Storgaard, Anton Hausknecht, Arne Aronsen, Jan Vesterholt, Erik Rald, Brian Perry, David Boertmann, Slavomir Adamcik, Karen Hansen, Ursula Peintner, Jens H. Petersen, Sigisfredo Garnica, Johannes Schmitt and Klaus Wölldecke for provision and loans of specimens. We are grateful for the comments, informations and suggestions we received from D. Jean Lodge, Patrick B. Matheny, Ron A. Petersen, Bart Byuck, Ernest Emmett, Marie-Catherine Boisselier-Dubayle, Michael Weiß, Scott Redhead and Michael Kuo. We are also indebted to Peter Spiteller and Ursula Eberhardt for their kind sharing of unpublished results and manuscripts, and to Anette Løth, Ulrik Søchting and Søren Rosendahl for practical and theoretical assistance.

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