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A three-gene phylogeny of the *Mycena pura complex* reveals 11 phylogenetic species and shows ITS to be unreliable for species identification

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ABSTRACT

Phylogenetic analyses of *Mycena* sect. *Calodontes* using ITS previously suggested ten cryptic monophyletic ITS lineages within the *Mycena pura* morphospecies. Here, we compare ITS data (645 bp incl. gaps) from 46 different fruit bodies that represent the previously described ITS diversity with partial tEF-1-α (423 bp) and RNA polymerase II (RPB1) (492 bp) sequence data to test the genealogical concordance.

While neither of the markers were in complete topological agreement, the branches differing between the tEF and RPB1 trees had a low bootstrap (<50) support, and the partition homogeneity incongruence length difference (ILD) tests were not significant. ILD tests revealed significant discordances between ITS and the tEF and RPB1 markers in several lineages. And our analyses suggested recombination between ITS1 and ITS2, most pronounced in one phylospecies that was identical in tEF and RPB1. Based on the agreement between tEF and RPB1, we defined 11 mutually concordant terminal clades as phylospecies inside the *M. pura* morphospecies; most of them cryptic. While neither of the markers showed an unequivocal barcoding gap between inter- and intraspecific diversity, the overlap was most pronounced for ITS (intraspecific diversity 0–3.5 %, interspecific diversity 0.4 %–8.8 %). A clustering analysis on tEF separated at a 1.5 % level returned all phylogenetic species as Operational Taxonomic Units (OTUs), while ITS at both a 1.5 % level and at a 3 % threshold level not only underestimated diversity as found by the tEF and RPB1, but also identified an OTU which was not a phylogenetic species. Thus, our investigation does not support the universal suitability of ITS for species recognition in particular, and emphasises the general limitation of single gene analyses combined with single percentage separation values.

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Introduction

The nuclear ITS marker (internal transcribed spacer) has proven useful to identify closely related phylospecies in many fungal groups, often in good agreement with morphological/biological species concepts (Garnica *et al.* 2003; Leonardi *et al.* 2005; AFS Taylor *et al.* 2006; Frøslev *et al.* 2007; Hallenberg *et al.* 2007; Jargeat *et al.* 2010; Eberhardt *et al.* 2012). Furthermore, ITS is and has been used extensively as a barcode in fungal community descriptions for almost two decades (e.g. Gardes & Bruns 1996; Lindahl *et al.* 2007; Jumpponen *et al.* 2010; Tedersoo *et al.* 2012) and has recently been formally proposed as a universal barcode candidate for fungi (Schoch *et al.* 2012) and accepted by the *Consortium* for the *Barcode* of Life.

Mycena[1] sect. *Calodontes* sensu Maas Geesteranus (1992) (i.e. *Mycena pura* and relatives) includes a number of taxa, which are difficult to separate morphologically (Maas Geesteranus 1992; Rexer 1994). Harder *et al.* (2010) showed that ITS analyses support the morphospecies *Mycena pelianthina*, *Mycena diosma*, *Mycena rosea*, *Mycena dura* and *Mycena lammiensis* as monophyletic units, while *Mycena pearsoniana* likely is polyphyletic (Harder *et al.* 2012). However, the remaining *M. pura* morphospecies complex still clearly harbours cryptic variation, as ten monophyletic ITS groups that were not separated by any obvious morphological traits could be identified.

Single gene phylogenies, however, are sensitive to locally high mutation rates and recombination. Therefore, in this paper, we further investigate the cryptic variation within the *M. pura* morphospecies complex using phylogenetic species recognition based on genealogical concordance between loci (genealogical concordance phylogenetic species recognition (GCPSR), Avise & Ball 1990; Taylor *et al.* 2000). This has been used as a species recognition criterion in many groups of organisms (Müller *et al.* 2005; Evans *et al.* 2007; Lorion *et al.* 2010), and particular in fungi (e.g. Fisher *et al.* 2002; Redecker *et al.* 2003; Bidochka *et al.* 2005; Grünig *et al.* 2007; Hirata *et al.* 2007; Jargeat *et al.* 2010).

We combined the previous ITS phylogenies with two other markers that have also been found useful in resolution of the lower level relationships in different fungal groups; translation elongation factor 1-alpha (tEF) (Helgason *et al.* 2003; Kroon *et al.* 2004; Kauserud *et al.* 2007a, 2007b; Jargeat *et al.* 2010; Zhao *et al.* 2011; Voglmayr *et al.* 2012) and the large subunit RNA polymerase II (RPB1) (Matheny *et al.* 2002; Frøslev *et al.* 2005; Matheny 2005). For both markers, we targeted fragments of 'barcode length' (about 4–500 bp). We aimed to see if phylogenetically concordant species yielded a pattern consistent with the morphotaxa, and whether ITS was sufficient to identify such species in *Calodontes*. Unfortunately, cultures were unsuccessful like in earlier attempts (e.g. Boisselier-Dubayle *et al.* 1996), so it was not possible to test the biological species concept on the phylogenetic groups.

[1] The Calodontes section has earlier been placed in the separate genus *Prunulus* Gray by Redhead (lectotype *Prunulus denticulatus* (Bolton) Gray 1821) (Redhead *et al.* 2001). However, a forthcoming phylogeny by Bryan A. Perry *et al.* (unpublished studies) on the entire *Mycena* genus *sensu lato* will uphold the name *Mycena* for Calodontes. Perry kindly analysed our tEF data and found them to fit in what will be the new *Mycena sensu stricto* (pers. com.).

Materials and methods

Primers

For tEF, we initially used the primer pair EF-595f and EF-1160r (Kauserud & Schumacher 2001) to generate a starting sequence using these authors' protocol. The partitioning into exons and introns was determined using the most similar sequence found by BLAST against GENBANK, *Phyllotopsis* sp. MB35 (AFTOL-ID 773). Two new primer pairs (tEFMp_f1 + tEFMp_r1 and tEFMp_f2 + tEFMp_r2, Table 1) were designed with the forward in the 3rd and the reverse in the 5th exon of our starting sequence, respectively. For RPB1, we used the primer pair gRPB1-A-f (Stiller & Hall 1998) and fRPB1C-r (Matheny *et al.* 2002) to obtain a starting sequence using the protocol in Matheny *et al.* (2002). The partitioning into exons and introns was determined from the *Mycena aff. pura* PBM 2665 isolate AFTOL-ID 1486 (Matheny *et al.* 2006). This was subsequently used to design a new primer pair (RPB1Mp_For1 and RPB1Mp_Rev1) comprising the large first intron of *M. pura* and a fragment of the first exon. Primers were designed using Primer3 v 0.4.0 (Rozen & Skaletsky 2000). For ITS, the primer pair ITS1F (Gardes & Bruns 1993) and ITS4 (White *et al.* 1990) was used, and the protocol of Gardes & Bruns (1993). The partitioning of the ITS sequences was determined using the *M. aff. pura* PBM 2665 isolate AFTOL-ID 1486 (Matheny *et al.* 2006). All primers are listed in Table 1.

Collections and outgroup selection

The tEF and RPB1 genes of representatives from all 45 separate lineages in the ITS dataset in Harder *et al.* (2010) were sequenced. For further information on the collections, see Tables 1 and 2 in the Supplementary data. As *Mycena rubromarginata* is apparently the closest known relative of the Calodontes (Moncalvo *et al.* 2002; Matheny *et al.* 2006), it was again chosen as outgroup.

Table 1 – Primers used in the study. Newly synthesised primers in italics.

Locus	Name	Primer sequence
ITS	*ITS1F*	5′-CTTGGTCATTTAGAGGAAGTAA-3′
	ITS4	5′-TCCTCCGCTTATTGATATGC-3′
tEF	*EF595f*	5′-CGTGACTTCATCAAGAACATG-3′
	EF1160r	5′-CCGATCTTGTAGACGTCCTG-3′
	tEFMp_f1	5′-TGG TGG TAC TGG TGA GT-3′
	tEFMp_r1	5′-GGA AGA CGG AGT GGC TTG T-3′
	tEFMp_f2	5′-CTG GTG AGT TCG AAG CTG GT-3′
	tEFMp_r2	5′-ACG TCC TGC AGG GGA AGA C-3′
RPB1	gRPB1A-f	5′-GA(G/T)TGTCC(T/G)GG(A/T)CATTTTGG-3′
	fRPB1C-r	5′-C(A/C/G/T)GC(AT(A/G)TT(A/G)TTCAT (A/G)TCCAT(A/G)TA-3′
	RPB1Mp_f1	5′-AAT TGG GGG AAA CTG AAA GC-3′
	RPB1Mp_r1	5′-TGT CTC GCA GAC CAT CTT TG-3′

DNA extraction, PCR, cloning, and sequencing

DNA extractions were done from lamellae from dried material with a standard CTAB procedure (Gardes & Bruns 1993). PCR was performed on a MJ Research PTC-200 thermocycler. PCR of tEF and RPB1 were done using a touchup protocol: 94 °C for 60 s, ten cycles of (1) 94 °C for 35 s (2) annealing at 53 °C for 45 s, and (3) 72 °C for 45 s, 25 cycles of (1) 94 °C for 35 s (2) annealing at 56 °C for 45 s, and (3) 72 °C for 45 s, and finally 72 °C for 10 min. PCR of ITS was as in Gardes & Bruns (1993). All sequencing were done by MACROGEN, Seoul, South Korea. Sequences are deposited in GenBank.[2] Accession numbers are found in Table 1 in the Supplementary data.

To test if the tEF and RPB1 primers amplified pseudogenes or paralogs, a set of ten tEF and five RPB1 clones from the specimen CBH216 were successfully sequenced. Cloning was done using fresh PCR product and the TOPO TA Cloning Kit from Invitrogen (Invitrogen, Carlsbad, CA). The sequenced clones were identical or differed in only a single nucleotide position, and the reading frames of the exons were uninterrupted, supporting the notion that these markers are single copy genes (data not shown).

To make sure that observed discordance between markers were not due to contamination, we also reextracted DNA and amplified and resequenced ITS of specimens from lineages shown to cause conflict between ITS and the two other markers (BAP132, CBH039, DB2005-152, CBH367, and CBH371). All duplicate sequences were identical to those originally developed.

Alignments

Sequences were assembled in BioEdit (Hall 1999). Ambiguities with clear double peaks were recorded as heterozygous using the standard International Union of Pure and Applied Chemistry (IUPAC) names. All alignments were done with MAFFT v5.6 (Katoh *et al.* 2005) under the settings L-INS-I for ITS and G-INS-I for tEF and RPB1. All sequences aligned unambiguously and no manual corrections were performed. For all datasets, we 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 four contiguous nonconserved blocks and a minimum of 15 sequences for accepting a position as conserved). For both alignment types, we made bootstrapped NJ trees (1000 replications) in PAUP 4.0b10 (Swofford 2003) and produced 50 % majority rule consensus trees. The NJ analyses did not show any topological differences between the trees produced from the lenient and the conservative alignments, and we ultimately analysed all alignments in their entirety. All alignments details are listed in Table 2. Alignments were submitted to TreeBase (http://purl.org/phylo/treebase/).

[2] Accession numbers for the tEF and RPB1 sequences have been requested, but could not be made available by Genbank before publication due to the lapse in US government funding of NCBI in October 2013. They will be made accessible by a later addendum to the article.

Table 2 – Alignment details.

Alignments	ITS	tEF	RPB1
Number of sequences (excl. outgroup)	45	45	45
No. of sites (incl. gaps and. outgroup)	645	423	492
Longest ungapped sequence	616	412	482
Shortest ungapped sequence	593	404	475
No. of segregating sites	156	150	102
No. of unambiguous parsimony informative sites	83	86	46

Partition homogeneity and site saturation tests

To test if the datasets from ITS, tEF, and RPB1 were congruent, partition homogeneity (incongruence length difference (ILD)) tests (Farris *et al.* 1994) in PAUP 4.10b (Swofford 2003) (parsimonious heuristic search, TBR swapping, without the steepest descent algorithm, 1000 replications) were carried out on the total concatenated dataset (without the outgroup), excluding all noninformative sites (Lee 2001). Since the tests on the entire alignment and the ITS–tEF and ITS–RPB1 pairs showed highly significant inhomogeneity (see Results section), we carried out separate phylogenetic analyses of each locus.

As inhomogeneity between markers can be caused by both recombination and site saturation, we used Xia's site saturation test (Xia *et al.* 2003) implemented in DAMBE (Xia & Xie 2001) to test for significant saturation.

To see if there was heterogeneous evolution within the markers, we also measured the saturation index as specified in PhyloBayes (Lartillot *et al.* 2009) using 100 replications with the CAT model. Since there was almost no variation in 5.8S or the first exon of RPB1, we only performed the tests on ITS1, ITS2, tEF introns, tEF exons, and the RPB1 intron.

Phylogenetic trees

All phylogenies were constructed using the maximum likelihood algorithm and the GTRGAMMA model contained in RAxML (Stamatakis 2006) and visualised with FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/) via raxmlGUI (Silvestro & Michalak 2011). Bootstrapping was done on 1000 resampled alignments, using each 5th tree as a starting point for a heuristic search. Equally weighted maximum parsimony analyses with 1000 bootstrap replicates for separate genes and the concatenated alignment were performed in PAUP (Swofford 2003) using the FastStep search function algorithm. Parsimony bootstrap values above 50 for branches present in the RAxML phylogeny trees were added to the trees.

Clustering was done in CLOTU (Kumar *et al.* 2011) via the Bioportal cluster in Oslo on the separate ITS and tEF alignments excluding the outgroup with the single linkage clustering BlastClust algorithm (which makes clusters based on pairwise comparisons of all sequences) ignoring the filtering and trimming step, and using 1.5 and 3 % as Operational Taxonomic Unit (OTU) separation values. Because of the limited variation in RPB1, we did not apply the clustering approach on this marker.

Phylospecies and pairwise nucleotide distance

We used a modified version of the GCPSR concept of Taylor *et al.* (2000) to assign putative phylospecies using the tEF and RPB1 markers. Absolute pairwise nucleotide distance differences (*p* distances) were calculated for all alignments in DAMBE (Xia & Xie 2001) using sitewise deletion of indels and ambiguities.

Results

ML phylogenies and species assignment

For the six named morphospecies outside *Mycena pura* (*Mycena pelianthina*, *Mycena diosma*, *Mycena rosea*, *Mycena dura*, *Mycena lammiensis*, and *Mycena pearsoniana*), and for the Ecuadorian

Fig 1 – Maximum likelihood tree based on ITS (internal transcribed spacer) nucleotide sequences from 45 different collections of *Mycena* sect. *Calodontes* and an outgroup *Mycena rubromarginata*. The outgroup branch is shortened for visual reasons (shown by '//'). Note that only one of the two *M. pearsoniana* lineages from Harder *et al.* (2012) is included here. Collections enclosed in rectangles represent phylospecies, which we established as well supported clades and unique single specimens that occurred in phylogenetic trees of both RPB1 and tEF. Bootstrap values above 50 from maximum parsimony/maximum likelihood are shown at the branches. Phylospecies (well supported terminal clades and unique single specimens retrieved in both RPB1 and tEF) are enclosed in rectangles in Figs 1–3. The Ecuadorian specimens comprise three phylospecies, but they have no formal names and are shown enclosed in a dotted rectangle.

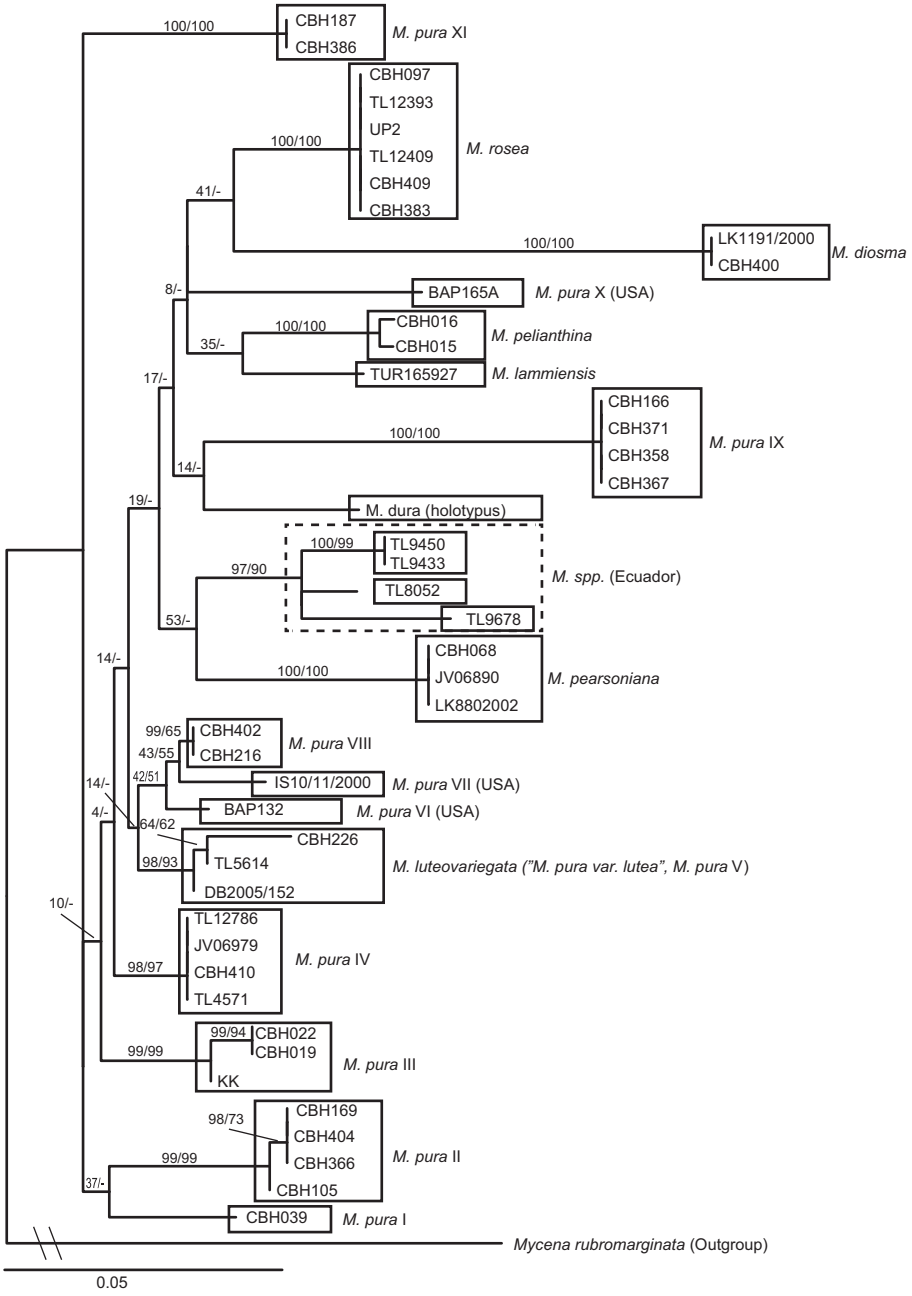

Fig 2 – Maximum likelihood tree based on tEF. Legend as Fig 1.

clade included here (which harbours three separate phylospecies), all three markers show a high degree of consistency (Figs 1–3). Inside the (apparently paraphyletic) morphospecies *M. pura*, however, the tEF and RPB1 trees agree on all clades and single lineages, while the ITS tree shows several topological discordances. The yellow grassland *M. pura* var. *lutea* was paraphyletic in the ITS analysis, but formed a very well supported clade in the tEF and RPB1 trees with bootstrap support above 90 in both maximum likelihood and maximum parsimony. Similarly, the CBH367 + CBH371 and CBH166 + CBH358 form one well supported clade in the tEF and RPB analyses, but two different well supported clades in the ITS analysis. The reason is that while these four collections share most of the same ITS1 domain, the ITS2 of CBH367 + 371 is very different from that of CBH166 + CBH358 (see Fig 4). They had a mutual *p* distance of 3.5 % for the entire ITS region (including the invariable 5.8S), but an ITS1 and ITS distance of 2.3 and 10.9 %, respectively. The ITS2 of CBH367 + 371 is more similar, although not identical, to the ITS2 sequence of the specimens in *M. pura* III.

The partition homogeneity tests of the concatenated alignment of all three markers, and of ITS combined with either tEF or RPB1, all showed significant inhomogeneity ($P = 0.001$). It required the exclusion of five clades or lineages (BAP132, CBH039, the *M. pura* var. *lutea* collections, CBH367 + CBH371 and CBH166 + CBH358) to obtain a homogeneous alignment including ITS. The tests indicated that the tEF and RPB1

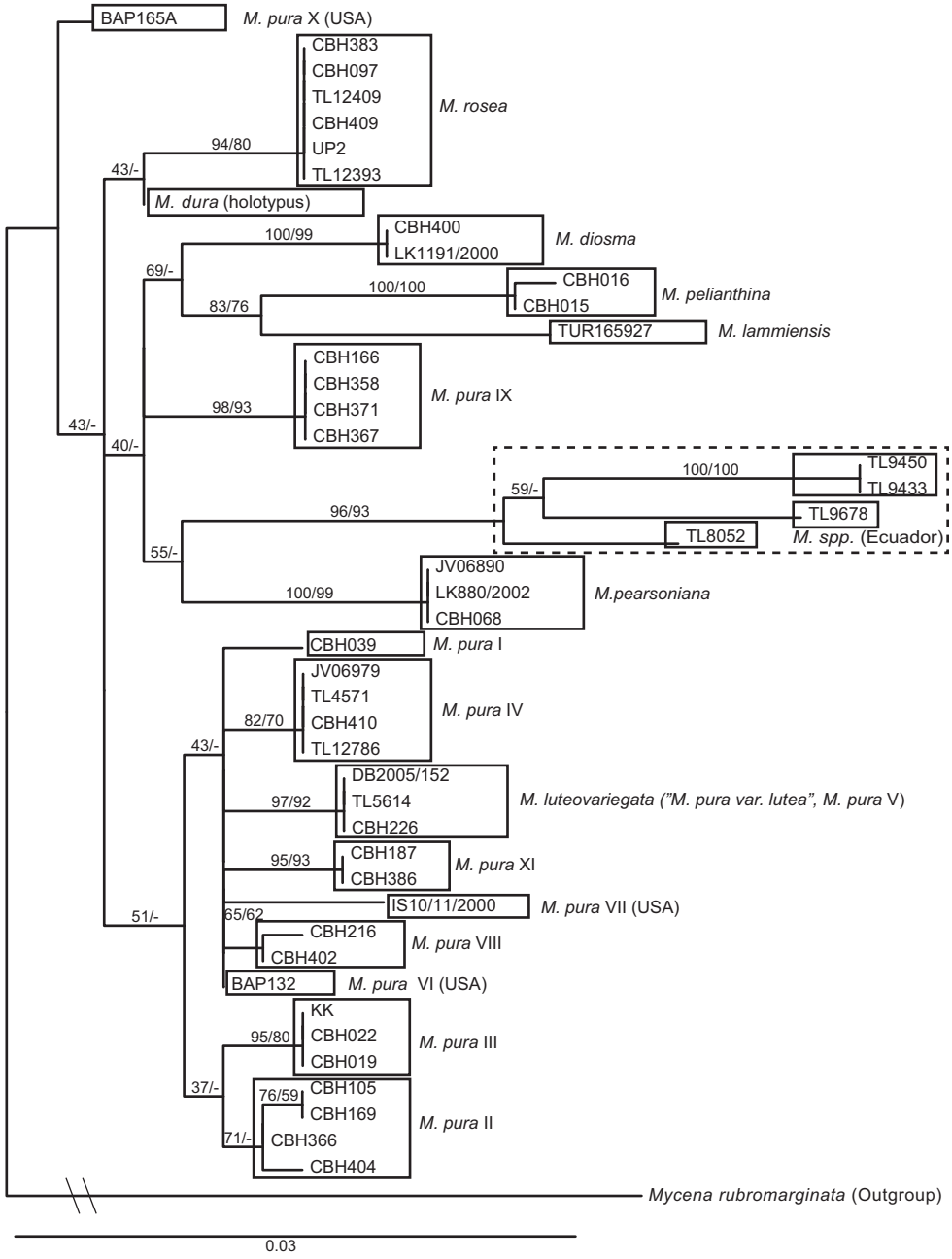

Fig 3 – Maximum likelihood tree based on RPB1. Legend as Fig 1.

markers were congruent ($P = 0.49$). Because of this unreliability of ITS, we used the tEF and RPB1 markers to identify 11 separate lineages in the *M. pura* morphospecies *sensu stricto* (i.e. after the recognition of *M. rosea*, *M. diosma*, and *M. dura*) (labelled with Roman numbers I–XI) as phylogenetic species.

Strictly interpreted, in the GCPSR concept of J Taylor *et al.* (2000, 2006) congruence requires tree topologies to be identical, and several of the basal branches differ between all three markers. However, all these diverging branches have a low bootstrap support in either tEF or RPB1, or both, and the differences would be eliminated if all branches with a support below <50 are collapsed. Lack of resolution in the backbone of phylogenies is often associated with the absence of unsampled intermediate clades (Ebersberger *et al.* 2012), and our sampling is far from exhaustive. By disregarding this and considering all topological incongruences however low their support, almost all ingroup taxa would be recognised as conspecific. This would e.g. lump *M. diosma*, *M. rosea*, and *M. pelianthina* because *M. diosma* appears as sister group to *M. rosea* with a support of 41 % in tEF and not as sister to *M. pelianthina* as in the RPB1 tree. This would be quite wrong, since these taxa form highly supported separate clades, display little if any intraspecific polymorphism, have a mutual sequence difference of up to 10 % in ITS and have distinct morphologies which correlate well with all three DNA

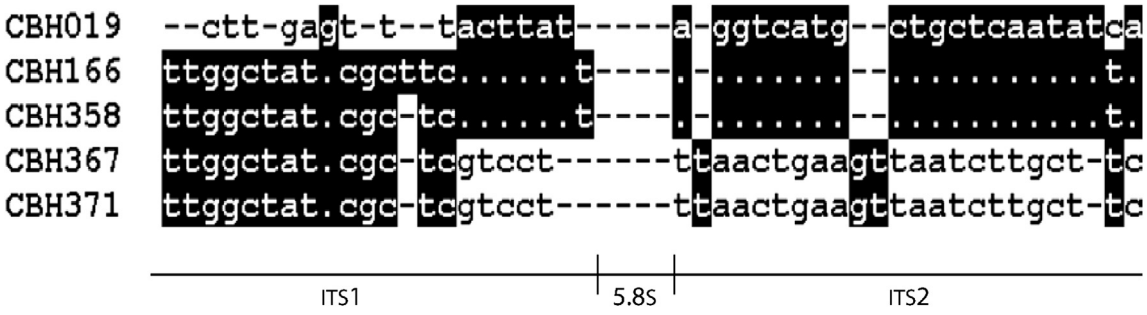

Fig 4 – Alignment of segregating positions ($n = 53$) in *M. pura* IX compared with CBH019 (*M. pura* III). Note that the specimens CBH166 + 358 and CBH367 + 371 are almost identical up until the end of ITS1 (position 22 in this alignment) but markedly divergent in ITS2, suggesting a recombination event in the end of ITS1. All five specimens were identical in 5.8S, which for reasons of visual clarity is illustrated by two '–' here.

markers. We therefore adopted a more relaxed approach, still based on the congruence between tEF and RPB1 shown by the homogeneity test, but where we suggest that well supported (bootstrap >80) terminal clades and unique single specimens occurring in both phylogenetic trees represent phylospecies.

Phylospecies recognised in this manner are enclosed in rectangles in Figs 1–3. Thus, by this definition, we could separate 20 phylospecies in the analysed material, i.e. the six already named non *pura* morphospecies, the 11 European and North American *M. pura* phylospecies and the three Ecuadorian phylospecies (Figs 2 and 3).

The site saturation tests in DAMBE indicated low levels of site saturation in all three markers ($P > 0.001$, where high P values in this test indicate high saturation). Nonetheless, ITS2 showed a markedly higher saturation index than any other region in the three markers (Table 3), indicating generally heterogeneous evolution in ITS1 and ITS2.

Pairwise nucleotide comparisons

The pairwise comparisons (Fig 5, Table 2) showed a slight intra/interspecific overlap in genetic distances in all three markers, though, in tEF there is an indication of a discontinuity in the distribution between 1 % and 2 % sequence difference. Intra- and interspecific distances in ITS were 0–3.5 % and 0.4–8.8 %, respectively, with the introgressed CBH367 + 371 lineage causing the most notable overlap seen at 3.7 % (Fig 5A). Overall, tEF was the most variable marker (Fig 5B), while RPB1 was the most conservative (Fig 5C). See Table 2 for a comparison of key features of all three markers.

Table 3 – Saturation indices.

Gene region	Observed homoplasy index
ITS1	0.304744 ± 0.064488
ITS2	0.723595 ± 0.234375
tEF introns	0.290758 ± 0.0463272
tEF exons	0.410317 ± 0.0993787
RPB1 1st intron	0.054876 ± 0.0083381

Clustering analyses

The indication of a discontinuity in tEF between 1 and 2 % (Fig 5B) suggests that a slight gap between intra- and interspecific diversity (barcode gap) may exist here. Using 1.5 % as OTU separation level on the tEF data assigns all individual collections to 20 OTUs which are identical with the 20 phylospecies identified by the phylogenetic analyses of tEF and RPB1 (For a list see Table 3 in the Supplementary data). The apparent overlap around 1.5 % was due to a single specimen (CBH226) having several unique mutations.

On the contrary, the standard species cut off value of 3 % ITS sequence difference (Hughes *et al.* 2009) commonly used for clustering in fungal next generation sequencing (NGS) studies (Buée *et al.* 2009; Tedersoo *et al.* 2010; Blaalid *et al.* 2012a) could only identify 11 of the 20 phylospecies as OTUs. It lumps *Mycena pelianthina* and *Mycena lammiensis*, and all the *Mycena pura* phylogenetic species except VII and X into just two OTUs. Even the low value of 1.5 % (also used in Blaalid *et al.* 2012a) still underestimates diversity as represented by the phylospecies. Worse still, at both levels the recombinant *M. pura* IX is identified as two separate OTUs.

Discussion

Phylogeny and markers

While 11 mostly cryptic phylospecies in *Mycena pura*, may appear to be a high number, we stress that the RPB1 marker has a very low variation compared to the two other markers. E.g. in *M. pura* species III, specimen KK and its sister group of CBH019 + CBH022 differ in tEF and ITS, but are RPB1 identical. This is likely a slight differential lineage sorting, which apparently is common in recently diverged species with a presumably short time between nodes (Pamilo & Nei 1988), where genes under high purifying selection pressures are combined with faster evolving metabolic or more or less neutral noncoding markers (Carbone & Kohn 2004). We, thus, consider it likely that another compatible and more variable marker might have identified even more concordant species. Furthermore, the lack of resolution in the backbone of phylogenies is commonly associated with the absence of unsampled

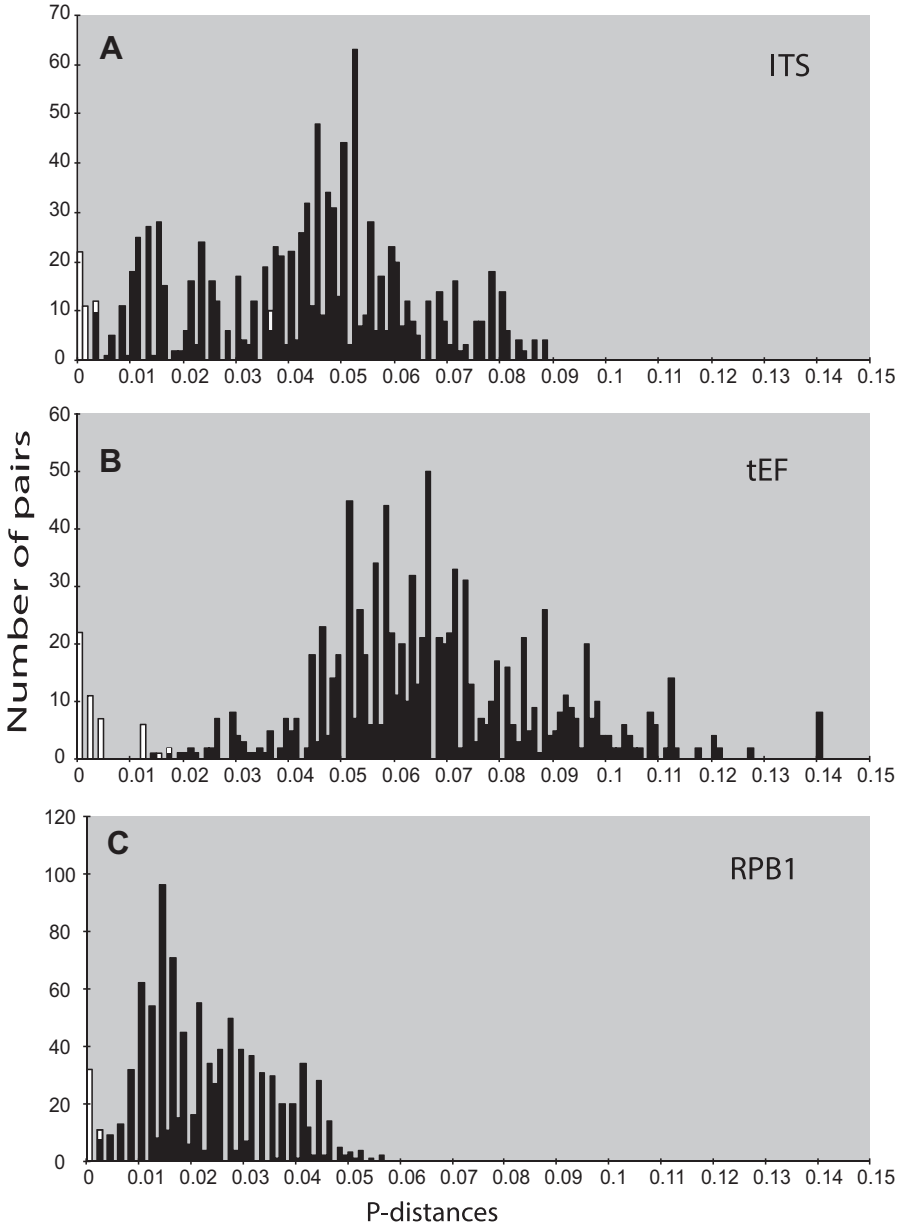

Fig 5 – Pairwise uncorrected *p* distances in (A) ITS (B) tEF, and (C) RPB1 between 45 collections of *Mycena* sect. *Calodontes* (i.e. a total of 990 comparisons). Each column represents the number of pairwise comparisons corresponding to a particular *p* distance. White = intraspecific variation, black = interspecific variation. Columns containing pairs of both types are represented with proportionally black and white columns. Note the *M. pura* IX pairwise comparisons displaying four number of pairs with an intraspecific distance of 3.7 % in ITS.

intermediate clades (Ebersberger *et al.* 2012). Thus, while it is possible that subsequent research may collapse that some of the phylospecies we identify here, it is still very likely that the *M. pura* morphospecies contain many more yet unknown cryptic phylogenetic species.

Most *M. pura* colour varieties (e.g. *multicolor*, *ianthina*, *violacea*) were earlier found to have no phylogenetic basis (Harder *et al.* 2010). However, the addition of tEF and RPB1 suggest that *M. pura* var. *lutea* is a valid phylospecies. Furthermore, it is morphologically recognisable and a grassland specialist, in contrast to the other species in the *Calodontes* section which all typically inhabit forest litter. Thus, we accordingly suggest to raise it to species level under the name *Mycena luteovariegata*, 'the pied yellow'. We propose this name since *M. lutea* is preoccupied, and because its colouration on the pileus is in fact rarely uniform yellow, but often patchily yellow brownish–reddish (as the epitype seen in Fig 1 in the Supplementary data). See the Taxonomy section below.

Mycena pura I is a completely violet specimen with abundant pleurocystidia and without the characteristic pileus zonation of *Mycena diosma*, but since it is only supported by one collection, we are uncertain whether this phenotype is characteristic, especially given the rich colour variation in

this group. Thus, we refrain from giving *M. pura* I a formal scientific name. None of the other *M. pura* phylospecies show any discernible pattern with respect to ecology, timing of the year, morphology etc., and must thus be designated as cryptic at least for the time being. These phylospecies make up most of the peak between 0.4 % and about 3.5 % of the ITS pairwise distance histogram (Fig 5A), thus filling out the space where a barcoding gap is seen in other groups. These ITS interspecific distances are relatively low, but comparable to those found in some other cryptic basidiomycete species, e.g. *Hebeloma crustuliniforme* (Aanen *et al.* 2000a), *Alnicola* spp. (Moreau *et al.* 2006), *Serpula himantioides* (Kauserud *et al.* 2006) or *Amanita muscaria* (Geml *et al.* 2006).

The non *pura* species, *Mycena pelianthina*, *M. diosma*, *Mycena rosea*, *Mycena dura*, *Mycena lammiensis*, and *Mycena pearsoniana*,[3] show virtually no intraspecific variation and interspecific variation between 2.4 and 8.8 %.

The reason for the discordance between the markers is internal conflicts within the ITS region. The comparatively high saturation index in ITS2 compared to ITS1 (Table 3) indicates that some form of heterogeneous evolution is occurring in the gene. Since the saturation levels are low, the sources of inhomogeneity in ITS are likely to be either recent or ongoing recombination events, or shared polymorphic positions between recently separated lineages.

An internal partition homogeneity test dividing the ITS into three domains (ITS1, 5.8S and ITS2) showed a significant inhomogeneity ($P = 0.001$), which disappeared when CBH367 + 371 were excluded ($P = 0.41$). As the difference within CBH166 + 358 and CBH367 + 371 (*M. pura* IX) is unsupported by the other markers, this conflict could represent a relatively recent introgression from a lineage closely related to *M. pura* III and a subsequent physical recombination event between ITS1 and ITS2 (Fig 4). The fact that CBH166 + 358 and CBH367 + 371 also differ by 1.1 % in a 547 bp fragment of the conservative but tightly ITS2 linked LSU (data not shown) provide some indirect support for this, but further specific research is needed to produce direct evidence.

In the other conflicting lineages (BAP132, CBH039, and the *M. pura* var. *lutea* collections), the ITS phylogeny identified the same lineages as the two other markers despite the inhomogeneity. We suggest that the these are true separate phylospecies, and that the remaining inhomogeneity in ITS may be ascribed to shared polymorphic positions within the ITS, confounding the phylogenetic reconstruction.

Similar different evolutionary histories in the ITS1 and ITS2 also occurred in *Flammulina* (Hughes & Petersen 2001), *Coniophora puteana* (Kauserud *et al.* 2007a, b), in *Heterobasidion* (Garbelotto *et al.* 2004), and in the *Tricholoma scalpturatum* complex (Jargeat *et al.* 2010). In the two latter cases, however, the recombinant lineages have been well supported separate phylogenetic groups also in other nonITS linked markers. E.g. in Jargeat *et al.* (2010), *Tricholoma inocybeoides* consistently formed a distinct clade in both ITS, glyceraldehyde-3-phosphate dehydrogenase (GPD), and tEF, but had an ITS1 very similar to *Tricholoma argyraceum* and the ITS2 more similar to *T. scalpturatum*. Hence, the authors concluded that it was likely a recent speciation by hybridisation between the latter two. In the two different ITS lineages of *M. pura* IX, such a speciation might be under way.

With the Ascomycota, it is now widely recognised that ITS is of low reliability for species recognition in many genera (for a good overview see Begerow *et al.* 2010; Vrålstad 2011). Gazis *et al.* (2011) directly showed how ITS underestimated diversity compared to tEF in ascomycote endophytes, and how this obscured ecological patterns clearly seen in tEF and (GPD). In basidiomycetes, ITS has generally been interpreted as a reliable species indicator, and this has been supported by the results from multigene phylogenies showing fine agreement with other markers as well as a clear barcoding gap, e.g. in *Cortinarius* (Frøslev *et al.* 2007), *Tricholoma* (Jargeat *et al.* 2010) or *Hebeloma* (Eberhardt *et al.* 2012). However, our data show that this is not the case in *Mycena* sect. *Calodontes*. Obviously, this raises questions about the applicability of ITS in other basidiomycetes.

If ITS may fail to identify close to 50 % of the 'true' diversity in some groups at the standard OTU separation value of 3 %, then this is clearly a potentially serious bias for NGS studies exploring the diversity. Furthermore, the occurrence of likely recombinant ITS sequences in hitherto five (not particularly closely related) genera (*Flammulina*, *Coniophora*, *Heterobasidion*, *Tricholoma*, and now possibly *Mycena*) suggests that the phenomenon is commonplace. This heterogeneity adds other potential sources of confusion. At the 3 % level, sequences like those of *M. pura* IX would be classified as one OTU by ITS1 and two by ITS2, while the *T. inocybeoides* of Jargeat *et al.* (2010) would likely be lumped with *T. argyraceum* in ITS1, but in *T. scalpturatum* by ITS2. Thus, while Blaalid *et al.* (2012b) found that using either ITS1 or ITS2 as 'metabarcode' does not affect the actual species *number* much at the high 3 % level for a selection of basidiomycetes, it would still likely affect the *abundance* of each species. This emphasises the necessity of direct knowledge on the specific fungal group in question for making qualified interpretations of the diversity of ITS environmental sequencing data.

The tEF fragment, however, showed good resolution and a bimodal *p* distance distribution. Despite its relative short length, the genetic distances it produced were larger and its inter/intraspecific distances more clearly separated. To our knowledge, all studies that used this marker found it to be highly reliable. Therefore, we suggest more focus on development of generally applicable primers to update Rehner (2001) or Kauserud & Schumacher (2001) and a continued effort to build up the tEF database as an alternative to ITS.

Taxonomy

Mycena luteovariegata Bugge Harder & Læssøe nom. nov.
MycoBank No.: MB802679.
Basionym: *Mycena pura* var. *lutea* Gillet, *Hyménomycètes* (Alençon): 283 (1876) non-*M. lutea* Bres. Holotype: Gillet, *Hyménomycètes* (Alençon): 283 (1876), plate 478.
Epitype (here selected): Denmark, Sjælland, Rejnstrup Overdrev, found on grassland, 30. October 2006, E. Rald s.n., CBH226 (C, genbank FN394604.1). Notes on epitype: Pileus (Fig 1A and C in the Supplementary data) conical to flat, 2.5–5 cm wide, hygrophanous, sulphur yellow at centre with

[3] Only one of the two *M. pearsoniana* lineages found in Harder *et al.* (2012) is included in this analysis.

reddish grey towards margin. Lamellae greyish rose, adnate to subdecurrent (Fig 1B in the Supplementary data). Stipe cylindrical, 6.5–8 cm long, reddish grey. Spore data ($n = 20$): Length 5.6–7.3, avg. 6.6, SD 0.55 μm. Width 2.8–3.8, avg. 3.37, SD 0.27 μm. Q value 1.74–2.24, avg. 1.95, SD 0.15 μm. Slightly smaller than generally reported from *M. pura*. Ovoid, amyloid.

Species and speciation

One explanation for the overlaps between intra- and interspecific genetic variation in the *Mycena pura* phylospecies and the apparently recent recombination events within ITS could be that they are a result of relatively recent diversifications. This would also explain the phenotypic plasticity and the historical problems of taxonomic resolution of *M. pura*. Many phylogeographic studies on speciation processes in agarics indicate allopatric/vicariant speciation (Kohn 2005), but sympatric speciation as a consequence of sexual incompatibility has been documented in the *Hebeloma crustuliniforme* morphospecies complex (Aanen *et al.* 2000b). Such reproductive barriers may arise quickly in basidiomycote populations (Bresinsky *et al.* 1987) due to the nature of their multigene mating system (Chase & Ullrich 1990a, 1990b; Casselton & Olesnicky 1998). We hypothesise that many such recent speciation events in the *Calodontes* could have occurred sympatrically.

Due to the apparent uncultivability, we unfortunately do not know if the phylospecies are intersterile. While the existence of closely related but phylogenetically concordant *M. pura* phylospecies with mutual ITS *p* distances of less than 1 % indicates limited gene flow between phylospecies, the recombinant *M. pura* IX (Fig 4) with an intraspecific *p* distance in ITS2 of 10.9 % suggests that even genetically distant strains may retain some interfertility. We do not know whether this is a rare hybridisation event between two lineages not normally in contact thus without having experienced selection for intersterility in sympatry ('reinforcement', Petersen & Ridley 1996; le Gac & Giraud 2008). A full resolution of these questions and of this enigmatic group of fungi will require a broader taxonomic sampling with coalescent analyses and successful cultivation studies.

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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.2013.09.004.

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