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RESEARCH ARTICLE

Phylogenetic and chemical studies in the potential psychotropic species complex of *Psilocybe atrobrunnea* with taxonomic and nomenclatural notes

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Key words

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Abstract Five *Psilocybe* species with unresolved systematic position (*P. atrobrunnea*, *P. laetissima*, *P. medullosa*, *P. pelliculosa*, and *P. silvatica*) were investigated using four molecular markers (EF1-α, ITS, LSU, and IGS). Phylogenetic analysis revealed that with the exception of *P. laetissima*, which is now rightfully classified in the genus *Leratiomyces*, all investigated species belong to *Psilocybe* sect. *Psilocybe*. For the first time, psychotropic compounds psilocin and psilocybin were detected in *P. medullosa* using gas chromatography-mass spectrometry. On the contrary, neither psilocin, nor psilocybin was detected in *P. atrobrunnea* and negative results were also obtained from mycelia grown in vitro on tryptamine/tryptophan-amended media. These results strongly suggest that biosynthesis of these alkaloids was lost in *P. atrobrunnea*. With the exception of minor differences detected in EF1-α marker, all sequences of American and European collections of *P. atrobrunnea* were identical. On the other hand, a thorough nomenclatural study revealed that the name *P. atrobrunnea* must be considered dubious; the oldest available candidate name, *P. fuscofulva*, was therefore adopted. The molecular data suggests that morphologically identical American *P. silvatica* and European *P. medullosa* likely represent distinct species; epitypes of both taxa were therefore designated.

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INTRODUCTION

As recently demonstrated, the European wood-rotting psychotropic *Psilocybe* species (*P. cyanescens*-complex) include *P. cyanescens*, *P. azurescens*, and the highly variable *P. serbica* (Borovička et al. 2011). Furthermore, in Europe there are another related and possibly psychotropic *Psilocybe* species with unresolved systematic position: *P. atrobrunnea* (Lasch: Fr.) Gillet and *P. medullosa* (Bres.) Borov.

Psilocybe atrobrunnea was originally described from Europe by Lasch (1828; as *Agaricus atrobrunneus*) from material collected in a region named 'Marchiae Brandenburgicae', an area currently corresponding to Eastern Germany and Western Poland. Collections of this species have also been reported from other European countries (Singer 1986, Borovička 2006, Noordeloos 2011), Canada, and the USA (Guzmán 1983, Borovička 2006). *Psilocybe atrobrunnea* is found in peat bogs growing in *Sphagnum*, rarely also on moist, decaying wood in peaty habitats. No bluing reaction, typical for psychotropic species, has been reported for this species but according to Stamets (1996), it is 'possibly active'. Various European authors (Noordeloos 1999, Horak 2005, Knudsen & Vesterholt 2008) have preferred the name *Psilocybe turficola* J. Favre (Favre 1939). However, despite being cited as legitimate by Mycobank (MB255229), *P. turficola* was invalidly published (Art. 39.1, no Latin diagnosis). Noordeloos (2011) has therefore adopted the name *P. atrobrunnea*, which has been used by both American and European mycologists (Guzmán 1983, 1995, Singer 1986, Smith 1940, Borovička 2006).

Psilocybe medullosa (Borovička 2007) was originally described from Italy (Bresadola 1898, as *Naucoria medullosa*). In recent publications (Knudsen & Vesterholt 2008, Borovička 2011), it was synonymized with *Psilocybe silvatica* (Peck) Singer & A.H. Sm. described from North America (Peck 1889, as *Psathyra silvatica*). It grows on woody debris and detritus (mostly under conifers – usually *Picea*, but also under *Fagus*) and is apparently rare. According to Borovička (2011), the microcharacters of *P. medullosa* observed in the holotype and recent European collections match well with those in the holotype of *P. silvatica*. But molecular data, now included in this paper, was not available in order to clarify the relationship of both taxa. According to Stamets (1996), *P. silvatica* is a psychotropic species ('weakly to moderately active') but to our best knowledge, chemical analyses have never been published. However, a very similar American species, *P. pelliculosa* (A.H. Sm.) Singer & A.H. Sm., is known to contain psychotropic compounds (Repke et al. 1977, Beug & Bigwood 1982). Both, *P. silvatica* and *P. pelliculosa* were placed in *Psilocybe* sect. *Semilanceatae* by Guzmán (1983, 1995).

Psilocybe laetissima Hauskn. & Singer, only known from Europe, is another species with unclear systematic placement. Described from Austria (Hausknecht & Singer 1986), it grows in dry grasslands and has never been considered to contain

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Table 1 Species under molecular study.

ID	Species	Collection	Origin	EMBL-Bank			
				LSU	ITS	EF1-α	IGS
P 23	*Psilocybe atrobrunnea*	PRM 922256	Czech Rep.	HF678214	–	–	–
P 25	*Psilocybe atrobrunnea*	PRM 860905	Sweden	HF678216	HF912348	–	–
P 28	*Psilocybe atrobrunnea*	UBC (F) 4260	Canada	HF678226	HF912349	–	–
P 32	*Psilocybe atrobrunnea*	PRM 905465	Czech Rep.	HF678220	HF912350	HF912336	HG423578
P 36	*Psilocybe atrobrunnea*	PRM 922257	USA	HF678223	HF912351	HF912337	HG423579
P 54	*Psilocybe atrobrunnea*	PRM 922536	Canada	HG423577	HG423575	HG423576	HG423580
P 30	*Psilocybe cubensis*	PRM 909585	unknown	HF678218	HF912352	HF912338	–
P 11	*Psilocybe medullosa*	PRM 909584	Czech Rep.	HF678212	HF912353	HF912339	HF912364, HF912365
P 17	*Psilocybe medullosa*	PRM 861054	Czech Rep.	HF912334	HF912354	–	–
P 18	*Psilocybe medullosa**	IB 81/470	Austria	–	HF912355	–	–
P 20	*Psilocybe medullosa*	PRM 909586	Czech Rep.	HF912335	HF912356	–	–
P 37	*Psilocybe medullosa*	PRM 922258	Czech Rep.	HF678224	HF912357	HF912340	–
P 19	*Psilocybe pelliculosa*	PRM 909710	USA	HF678213	HF912358	HF912341	–
P 31	*Psilocybe semilanceata*	PRM 921860	Czech Rep.	HF678219	HF912359	HF912342	–
P 24	*Psilocybe silvatica*	DAOM 187848	Canada	HF678215	HF912360	HF912343	HF912366, HF912367
P 34	*Psilocybe weraroa*	PRM 899226	New Zealand	HF678222	HF912361	HF912344	–
P 27	*Leratiomyces laetissimus*	BRNM 710313	Czech Rep.	HF678217	HF912362	HF912345	–
P 38	*Leratiomyces squamosus*	PRM 921883	Czech Rep.	HF678225	HF912363	HF912346	–
P 33	*Hypholoma marginatum*	PRM 921867	Czech Rep.	HF678221	HE994445	HF912347	–

* '*Psilocybe tenax*' sensu M.M. Moser (Moser & Jülich 1995).

psychotropic compounds. Whereas the original authors considered it related to *P. merdaria* (Fr.: Fr.) Ricken in *Psilocybe* sect. *Merdariae* (Hausknecht & Singer 1986), Guzmán (1995) and Noordeloos (1999) classified it in *Psilocybe* sect. *Atrobrunneae*.

The aim of our study was:

i. to explore the relationships among *P. atrobrunnea*, *P. laetissima*, *P. medullosa*, *P. pelliculosa*, and *P. silvatica* by the use of molecular markers;
ii. to verify possible occurrence of the psychotropic compounds psilocin and psilocybin in *P. atrobrunnea* and *P. medullosa*;
iii. to inspect the assumed conspecifity of the European and American collections (*P. atrobrunnea*; *P. silvatica* vs *P. medullosa*).

MATERIALS AND METHODS

Collections used

Collections of *Psilocybe* species used for the molecular study are listed in Table 1. Available sequences of related species, including those from our previous studies (Borovička et al. 2011, 2012) were downloaded from public databases. Collections of *P. atrobrunnea*, *P. pelliculosa*, and *P. silvatica* were identified according to Guzmán (1983, 1995), Noordeloos (1999), Borovička (2006, 2011), and Guzmán et al. (2008). Canadian specimens of *P. silvatica* kept at DAOM could not be used for DNA extraction, but template DNA extracted from a strain (isolated from the collection DAOM 187848) was kindly donated by Scott A. Redhead. The sequenced collection of *P. laetissima* was identified by its original co-author Anton Hausknecht (conf. J. Borovička); a detailed description of this collection was reported by Antonín & Dvořák (2010). Herbarium specimens of sequenced collections are available at BRNM, DAOM, IB, PRM, and UBC (Table 1); herbarium acronyms are used according to Thiers (2012). Samples used for testing of psychotropic compounds were collected and identified by J. Borovička (Table 2); the sample of *P. atrobrunnea* from NY, USA was kindly donated by Eric Smith.

Table 2 Samples for analysis of psilocin and psilocybin.

Species	Collection
*Hypholoma marginatum***	PRM 921867
*Inocybe corydalina**	PRM 899248
Psilocybe atrobrunnea (Czech Rep.)	PRM 922256
Psilocybe atrobrunnea (USA)	PRM 922257
Psilocybe medullosa (Czech Rep.)	PRM 909630
Psilocybe medullosa (Czech Rep.)	PRM 922258
Psilocybe serbica var. *arcana**	PRM 899282
*Stropharia caerulea***	PRM 921876
*Stropharia aeruginosa***	PRM 858114

* positive controls
** negative controls

In-vitro production of psychotropic compounds

Isolates of *P. atrobrunnea* and *P. serbica* were obtained from explants of basidiomata and their identity was verified using the comparison of their ITS rDNA sequences with our data. The production of psilocin (PS) and psilocybin (PSB) was then tested in liquid cultures. The mycelium was first pre-cultivated on potato dextrose agar. The colony margin was cut to 5×5×3 mm blocks, which were used as inoculum for stationary liquid cultures (50 ml liquid medium per 250 ml Erlenmeyer flask). Three inoculum blocks were used per flask. The cultivation medium contained 20 g glucose, 20 g fructose, 9 g glycine, 1 g ammonium succinate, 1 g yeast extract, 500 mg $MgSO_4.7H_2O$, 100 mg KH_2PO_4, 2.5 mg $ZnSO_4.7H_2O$ and 5 mg $FeSO_4.5H_2O$ per litre (pH 5.5, before autoclaving). After autoclaving, a volume of 0.5 ml filter-sterilized solution of tryptamine hydrochloride (246557 Aldrich) and DL-tryptophan (T3300 Sigma) in dimethylsulfoxide was added to 50 ml medium to reach 2 mM final concentration of both compounds. Control treatments received 0.5 ml of pure solvent.

Eight flasks were established in total: 2 fungal species × 2 replicates × 2 treatments (control medium and the medium with tryptamine and tryptophan). Inoculated flasks were incubated for 4 wk at 25 °C in the dark. The pelleted mycelium was shortly dried using paper towel, lyophilized, and subjected to analysis of PS/PSB as described below.

Determination of psilocin and psilocybin

Basidiomata of specimens selected for determination of PS and PSB (Table 2) were cleaned of substrate debris, frozen shortly after harvest and later lyophilized. Based on previously published analytical approaches (Keller et al. 1999, Stříbrný et al. 2003), dry basidiomata were then ground in a mortar and 10 mg of the powdered biomass were extracted with 0.5 ml of methanol (puriss p.a.) in ultrasonic bath for 30 min. The mixture was then centrifuged; 100 µl of the supernatant was separated into a glass vial and evaporated under a stream of nitrogen at room temperature. The dry residue was silylated by 50 µl of the

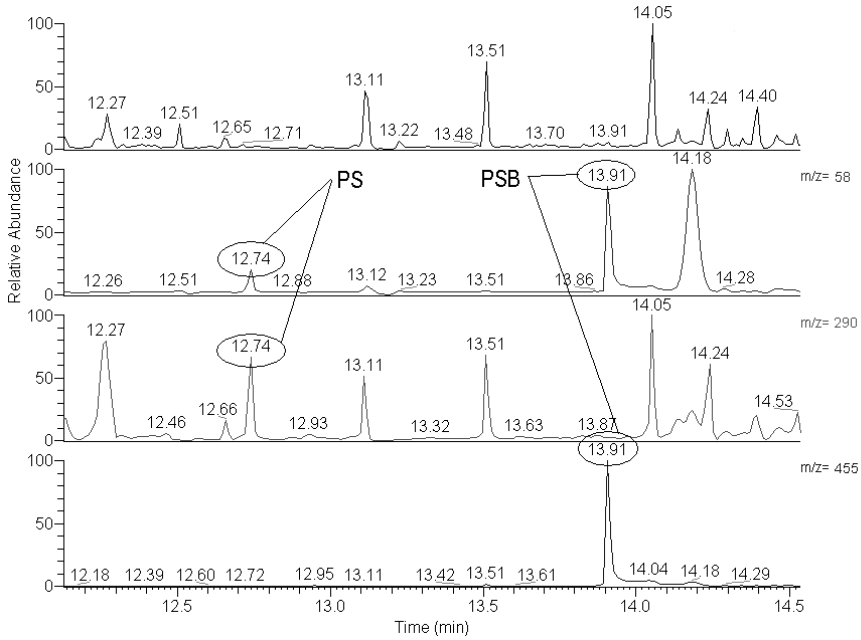

Fig. 1 Total (first line) and single ion (second, third, and fourth line) GC–MS chromatograms of the silylated extract of *Psilocybe medullosa* (PRM 909630). Ions typical for silylated psilocin are m/z 58 and 290, ions typical for silylated psilocybin are m/z 58 and 455. Retention time of silylated psilocin (PS) is 12:74, retention time of silylated psilocybin (PSB) is 13:91.

derivatization agent N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA, puriss p.a., for GC, Fluka) at 100 °C for 30 min. After cooling to room temperature, 1 µl of the reaction mixture was analysed by gas chromatography-mass spectrometry (GC-MS). The lyophilized mycelium was processed in a similar way.

GC-MS analysis was performed with Thermo DSQ equipped with a fused-silica capillary column SLB-5ms (15 m, 0.25 mm, 0.25 µm). The temperature of the injector was 220 °C, splitless time 30 s, the temperature of the transfer line was 250 °C. The oven temperature program: 70 °C for 1 min, then ramp 10 °C/min to 170 °C and final ramp 30 °C/min to 300 °C. Helium was used as carrier gas (1.5 ml/min). The mass spectrometer operated in full scan mode (FS, *m/z* 40-530) together with selected ions monitoring mode (SIM, *m/z* 58, *m/z* 290 and *m/z* 455 – characteristic ions of silylated PS and PSB). The proof of the presence of PS/PSB in the tested samples was carried out by comparing the mass spectra and retention times (RT) of the relevant peaks with the mass spectra and RT of the PS and PSB standards (QUICK-CHEK Solution, Alltech). The retention time of PS was 12:74 and that of PSB 13:91 min.

In order to verify the obtained results, species already known to contain PS/PSB, *Inocybe corydalina* and *P. serbica* var. *arcana* (Stijve et al. 1985, Stříbrný et al. 2003), and negative controls (*Stropharia aeruginosa*, *S. caerulea*, and *Hypholoma marginatum*), were analysed and compared with the tested samples. We were not able to quantify the amounts of PS/PSB in the biomass since only a few micrograms of PS/PSB standards were available. GC-MS chromatogram of silylated extract of *P. medullosa* with SIM chromatograms of ions m/z 58, 290 and 455 are in Fig. 1. Mass spectra of silylated PS and PSB are given in Fig. 2, 3.

Molecular phylogeny

Nuclear DNA was extracted from a small piece of dried or frozen fungal biomass (a piece of basidiome) using the NucleoSpin® Plant II extraction kit (Macherey-Nagel) according to the manufacturer's instructions. Three distinct molecular markers were used to study phylogeny of selected *Psilocybe* species: partial sequences of the divergent D1-D2 domain at the 5' end of the nuclear LSU rRNA gene (Maruyama et al. 2003, 2006), ITS rDNA region containing ITS1, 5.8S, and ITS2 sequences (Pornpakakul et al. 2009, Kallifatidis et al. 2014), and part of the elongation factor 1-α (EF1-α) (Antonín et al. 2009, 2013, Tomšovský et al. 2010). Oligonucleotide primers and PCR conditions were used as published previously (Borovička et al. 2011). Furthermore, partial intergenic spacer (IGS) sequences were obtained for *P. atrobrunnea*, *P. medullosa*, and *P. silvatica* using the primer pair NL11-CNS1 according to Aoki et al. (2003); IGS region was used to assess the similarity among the sequences of these species. Obtained amplicons were purified by isopropanol precipitation and both strands were sequenced at Macrogen Inc., Korea and sequences edited in BioEdit (Hall 1999).

Newly obtained sequences of EF1-α, ITS, and LSU rRNA gene were aligned together with appropriate homologues downloaded from public databases (see Table 1 for details): nucleotide sequences coding for rRNA genes (LSU and ITS rDNA) were aligned using Kalign (Lassmann & Sonnhammer 2005) at http://www.ebi.ac.uk/Tools/msa/kalign/; sequences coding for EF1-α were translated to amino acids, aligned using ClustalW algorithm as implemented in BioEdit, and retranslated back to nucleotides. Introns were aligned separately in nucleotides and were included into the dataset; nucleotide alignment of EF1-α was used to infer the tree. Alignments were manually edited in BioEdit, gaps and ambiguously aligned positions (2 positions from the primary LSU alignment, 61 positions from the EF-1α, and 80 positions from the ITS) were excluded from further analysis; final alignments used for computing trees consisted of 477 nt (EF-1α), 586 nt (ITS), and 575 nt (LSU). Alignments were analysed separately as well as in a combined dataset (1601 nt).

Maximum likelihood (ML), maximum parsimony (MP), and Bayesian inference were used to estimate phylogenetic relationships among investigated fungi using individual gene datasets and the combined dataset. ML analysis was performed using the PhyML program (Guindon & Gascuel 2003) with a gamma corrected GTR model of nucleotide substitution with estimated proportion of invariable sites (GTR+I+G). MP trees were computed using PAUP* 4b10 (Swofford 2000). Robustness of MP and ML trees was calculated by bootstrap analyses in 1 000 replicates. MrBayes 3.2 (Ronquist et al. 2012) was used to assess Bayesian topologies and posterior probabilities (PP) using the gamma corrected GTR substitutional matrix with estimated

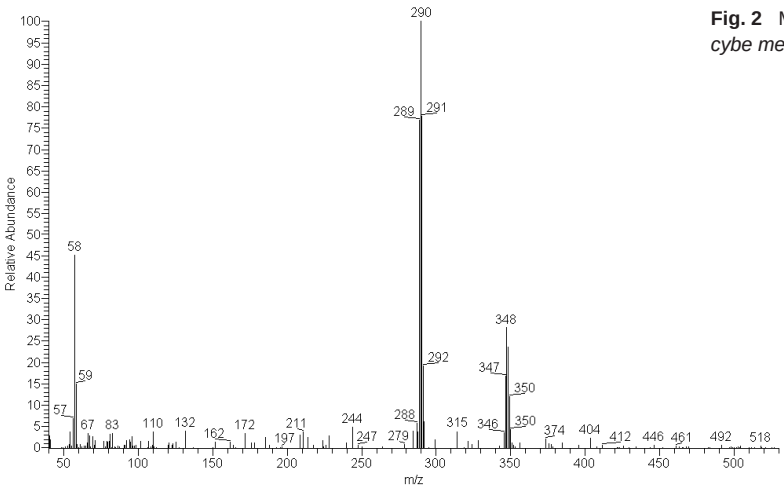

Fig. 2 Mass spectrum of the silylated psilocin found in the extract of *Psilocybe medullosa* (PRM 909630).

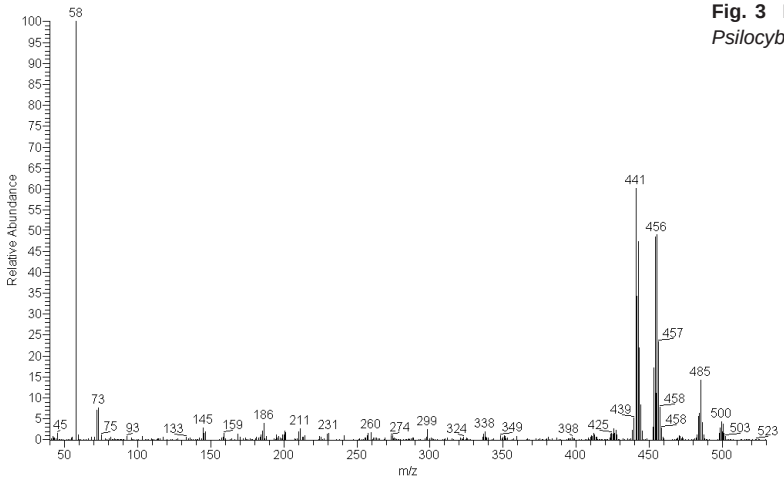

Fig. 3 Mass spectrum of the silylated psilocybin found in the extract of *Psilocybe medullosa* (PRM 909630).

proportion of invariable sites (GTR+I+G). Two independent Monte-Carlo Markov chains were run (under the default settings) for 3 million generations; due to the low complexity of datasets, the chains reached steady state within the first few thousands of generations. Therefore, we used 10 000 generations as a burn-in and omitted them from topology reconstruction and posterior probability (PP) calculation. The trees were rooted using *Hypholoma marginatum* sequences as outgroup. Sequences were accessioned into EMBL-Bank (see Table 1 for numbers) and the alignments made available in TreeBASE (study ID: S15446).

RESULTS

Psychotropic compounds

PS and PSB were detected in positive controls of *Inocybe corydalina* and *P. serbica* var. *arcana* (including mycelia from the in vitro experiment), but also in all tested collections of *P. medullosa* (Fig. 1–3). On the other hand, collections of *P. atrobrunnea* were PS/PSB negative, similar to negative controls (data not shown). Furthermore, negative results were obtained also from cultivated mycelia of *P. atrobrunnea*, both from control and tryptamine/tryptophan-amended media (data not shown). *Psilocybe laetissima* was not tested due to the lack of material; however, its systematic position (see below) and the lack of the bluing reaction indicate PS/PSB absence.

Molecular phylogeny

We have performed phylogenetic analyses based on three different molecular markers: EF1-α, ITS, and LSU rDNA sequences. We analysed those genes individually and also in a combined dataset. Separate analyses of individual genes produced different topologies, likely due to different informational content of particular markers and gene specific taxon sampling.

Maximum likelihood (ML) and maximum parsimony (MP) phylogenetic analysis based on the EF1-α nucleotide sequences (Fig. 4a) shows *P. semilanceata* forming together with *P. pelliculosa* the most basal lineage in the frame of the studied species of the genus *Psilocybe*. The rest are divided into two clades: the first clade is composed of *P. atrobrunnea* and its sister group containing *P. medullosa* and *P. silvatica*. The second clade contains *P. cubensis* and *P. serbica* paraphyletically branching on its root complemented by sequentially almost identical isolates classified as *P. allenii*, *P. azurescens*, *P. cyanescens*, and *P. weraroa*. It should be noted that not all the nodes of the tree are well supported. Also, the positions of *P. serbica* and *P. cubensis* are opposite in ML and MP trees. In MP analysis, six equally parsimonious trees were obtained; however, they show only variation in the polytomic clade of the very closely related species *P. allenii*, *P. azurescens*, *P. cyanescens*, and *P. weraroa* (Borovička et al. 2011). Bayesian tree shows different but unsupported topology placing *P. pelliculosa* and *P. semilanceata* on the root of the clade containing *P. atrobrunnea*, *P. medullosa*, and *P. silvatica*.

The tree based on ITS rDNA sequences (Fig. 4b) divides the *Psilocybe* isolates into two clades. The first one shows *P. atrobrunnea* at the root of the clade composed of *P. allenii*, *P. cubensis*, *P. cyanescens*, *P. ovoideocystidiata*, *P. serbica*, and *P. weraroa*. Similarly to EF1-α, *P. allenii*, *P. cyanescens*, and *P. weraroa* show very low mutual diversity in the sequence of the used marker. The second clade contains *P. caerulescens*, *P. fasciata*, *P. medullosa*, *P. mexicana*, *P. pelliculosa*, *P. semilanceata*, and *P. silvatica*. Also, contrary with the EF-1α tree, *P. semilanceata* together with *P. pelliculosa* do not form an

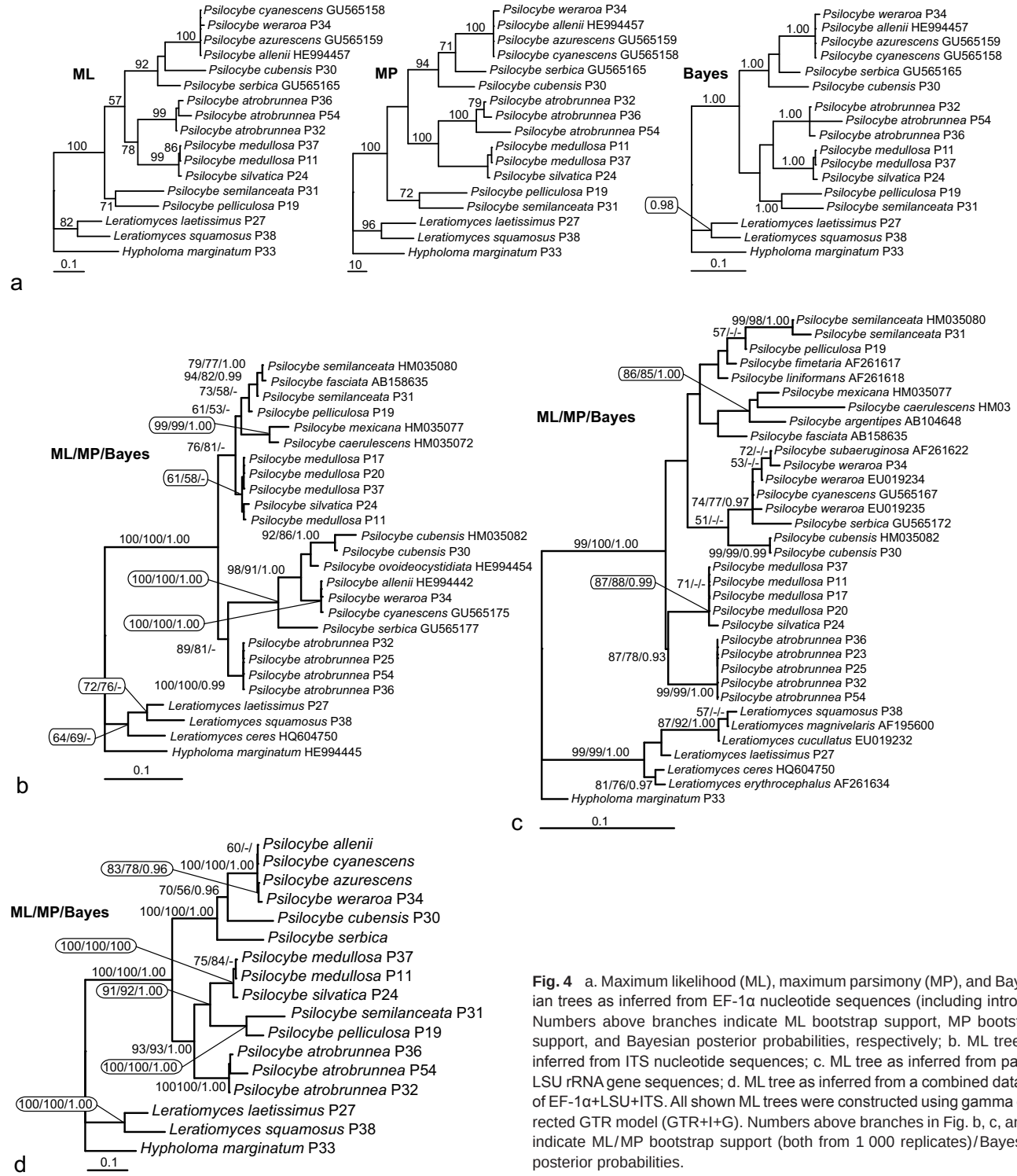

Fig. 4 a. Maximum likelihood (ML), maximum parsimony (MP), and Bayesian trees as inferred from EF-1α nucleotide sequences (including introns). Numbers above branches indicate ML bootstrap support, MP bootstrap support, and Bayesian posterior probabilities, respectively; b. ML tree as inferred from ITS nucleotide sequences; c. ML tree as inferred from partial LSU rRNA gene sequences; d. ML tree as inferred from a combined dataset of EF-1α+LSU+ITS. All shown ML trees were constructed using gamma corrected GTR model (GTR+I+G). Numbers above branches in Fig. b, c, and d indicate ML/MP bootstrap support (both from 1 000 replicates)/Bayesian posterior probabilities.

early branch but appear in an advanced position within the clade containing *P. caerulescens*, *P. fasciata*, *P. medullosa*, *P. mexicana*, and *P. silvatica*.

A phylogeny based on a part of the nuclear LSU rRNA gene (Fig. 4c) shows, in contrast with the ITS rDNA and similarly to EF-1α trees, a relationship of *P. atrobrunnea*, *P. medulosa*, and *P. silvatica*. The rest of the *Psilocybe* species are placed in an unsupported clade. Within the first subclade, *P. fasciata* is not related to *P. semilanceata* as in the ITS tree, but branches sister to *P. argentipes*, *P. caerulescens*, and *P. mexicana*. This entire lineage is in a sister position to the clade composed of *P. fimetaria*, *P. liniformans*, *P. pelicullosa*, and *P. semilanceata* but with no bootstrap support. The second subclade contains *P. cubensis* on the root, with relatively closely related species in the crown: *P. cyanescens*, *P. serbica*, *P. subaeruginosa*, and *P. weraroa*. According to this analysis, *P. weraroa* appears to be non-monophyletic. The LSU tree is generally mostly unsupported, obviously due to the low informational content of the DNA marker (from 576 characters, 61 characters are variable and only 48 parsimony informative).

Analysis of the combined dataset (Fig. 4d) shows a well-resolved and robust phylogeny (bootstrap supports of the main clades were between 91 and 100 %). We decided to build our taxonomic suggestions (see below) based on this tree. *Psilocybe* isolates grouped into two well-defined lineages: the first lineage is composed of *P. serbica* in the most basal position, *P. cubensis*, and almost identical sequences classified as *P. allenii*, *P. azurescens*, *P. cyanescens*, and *P. weraroa*. The second

lineage contains *P. atrobrunnea*, *P. medullosa*, *P. pelliculosa*, *P. semilanceata*, and *P. silvatica*. Within this lineage, *P. atrobrunnea* forms the earliest branch, *P. semilanceata* clusters with *P. pelliculosa* with high support and these two species form a sister group to *P. medullosa* – *P. silvatica* subclade. Although the combined tree is well-supported, trees constructed from single genes (EF-1α, ITS, and LSU) based on the limited taxon sampling of the combined tree (Fig. 4d) were not mutually fully congruent. However, the only difference lies in the positions of *P. pelliculosa* and *P. semilanceata* in the particular trees. In MP analyses, the EF-1α placed both species on the early branching position shown in Fig. 4a (MP). ITS topology (not shown) was identical with the combined tree with *P. pelliculosa* and *P. semilanceata* appearing on the root of the *P. medullosa* – *P. silvatica* clade (Fig. 4d), while a weakly supported (bootstrap 52 %) position of *P. semilanceata* and *P. pelliculosa* on the root of the *P. atrobrunnea*, *P. medullosa*, and *P. silvatica* clade was obtained for the LSU tree (not shown). It is also obvious that the tree topology is highly affected by taxon sampling, as demonstrated on the case of ITS, which displays different topologies when limited dataset for the combined tree and the larger dataset shown in Fig. 4b is computed.

DISCUSSION

Phylogenetic studies have recently shown that the genus *Psilocybe* (Fr.: Fr.) P. Kumm. in the sense of Guzmán (1983) and Singer (1986) is polyphyletic (Moncalvo et al. 2002, Ramírez-Cruz et al. 2013). In order to keep the well-known and widely used name *Psilocybe* for the psychoactive species, it was recently conserved with *P. semilanceata* (Fr.) P. Kumm. as the conserved type (Redhead et al. 2007), and *Psilocybe* is now applied to the clade of psychotropic species: *Psilocybe* sensu stricto (s.str.) (Ramírez-Cruz et al. 2013).

According to our results (Fig. 4d, combined dataset), the phylogenetic placement of *P. atrobrunnea* clearly indicates its close relationship with *P. semilanceata* and other psychotropic species. But neither PS nor PSB was detected in its basidiomata. A question has arisen whether this absence pertains to the capability of the fungus to produce those compounds or to environmental stimuli. It could be hypothesized that the absence of PS/PSB might have been induced by the occurrence in peat habitats with limited sources of available nitrogen (Bobbink et al. 1998). In that case, the biosynthesis of PS/PSB can be artificially elicited in axenic culture (Agurell & Nilsson 1968). However, our in vitro experiment, where mycelium of *P. atrobrunnea* was grown on medium rich in PS/PSB precursors tryptamine and tryptophan, did not lead to detection of PS/PSB in the tissue. This would indicate that the capability of PS/PSB synthesis was lost in *P. atrobrunnea* and therefore the genus *Psilocybe* s.str. does not exclusively contain psychotropic species. This is, nevertheless, not a surprise since a similar phenomenon has been recently reported for the family *Inocybaceae*, where biosynthesis of the alkaloids muscarine and PSB has evolved independently on several occasions, together with several losses (Kosentka et al. 2013). Our negative results confirm the earlier negative report published by Leung et al. (1965). Høiland (1978) reported the occurrence of PSB in *P. atrobrunnea* from Norway; however, recent revision of his collection revealed it had been misidentified and actually represents *P. medullosa* (Borovička 2011).

Psychotropic *Psilocybe* species are well known for their characteristic bluing (or greenish) reaction on stipe/pileus surface including mycelial strands and mycelium – especially when bruised or in age. Despite the fact that *P. medullosa* has been proven to contain PS/PSB, we have never noted any kind of this reaction on basidiomata in the field, even when damaging the mycelial strands at the stipe base, where the reaction is easily observable in most *Psilocybe* species. In *P. silvatica*, which is believed to be psychotropic and very close to *P. medullosa* (see below), the bluing reaction has not been mentioned by American authors (Smith 1940, Singer & Smith 1958, Stamets 1996). In contrast, Guzmán (1983) reported a slight bluing reaction in the stipe context (on cut) and stipe surface near the base. Judging from our analysis (when compared to our positive controls), it could be hypothesized that the PSB/PS content was very low in the tested specimens. As the bluish product is a consequence of oxidation of PS (Horita & Weber 1961, Levine 1967), we speculate that low PS content might lead to the lack of the characteristic bluing reaction in *P. medullosa*, similarly as in *Panaeolus cinctulus* (Stijve 1985, Gerhardt 1996).

According to Ramírez-Cruz et al. (2013), the species formerly classified in *Psilocybe* sect. *Semilanceateae* (Guzmán 1995) are not monophyletic and this was also shown in our analysis (Fig. 4d); the wood rotting species of the so-called *P. cyanescens* complex are included in a separate lineage together with *P. cubensis* (*Psilocybe* sect. *Cubensae* according to Guzmán 1995) and the secotioid *P. weraroa*.

While *P. silvatica* has been traditionally considered to be related to *P. semilanceata* (Guzmán 1983, 1995), the type species of *Psilocybe* sect. *Psilocybe* (Redhead et al. 2007, Norvell 2010), and this has been confirmed by our molecular data, *P. atrobrunnea* is the type of *Psilocybe* sect. *Atrobrunneae* (Singer 1948), which includes 11 non blue staining species (Guzmán 1995). As our phylogenetic analysis shows (Fig. 4d), *P. atrobrunnea* clearly fits in *Psilocybe* sect. *Psilocybe*. We therefore suggest that the *Psilocybe* sect. *Atrobrunnea* could be a synonym of the *Psilocybe* sect. *Psilocybe*, but this is yet to be tested as *P. atrobrunnea* was the only species of the section included in our phylogenetic analysis. However not only molecular, but also morphological characters of *P. atrobrunnea* (cap shape in young basidiomata, shape of basidiospores and cheilocystidia) would suggest close relationship with *P. semilanceata*; in fact, the only feature not matching *Psilocybe* sect. *Psilocybe* (Guzmán 1995, as *Psilocybe* sect. *Semilanceatae*) is the lack of the blue staining reaction.

Psilocybe laetissima, also classified in *Psilocybe* sect. *Atrobrunneae*, was excluded from *Psilocybe* s.str., and transferred to *Leratiomyces laetissimus* (Hauskn. & Singer) Borov. et al. (Noordeloos 2011) and this is confirmed here with the molecular data (Fig. 4d).

Psilocybe medullosa has an interesting taxonomic and nomenclatural history. As shown by Borovička (2011), it used to be classified within various genera by different European authors (*Galera*, *Galerina*, *Phaeogalera*, *Psilocybe*); it is known from at least 8 European countries. As we demonstrate, it undoubtedly belongs to the genus *Psilocybe*. Furthermore, *P. medullosa* has been classified under various names in Europe (Stridvall & Stridvall 1990, Borovička 2011). *Agaricus tenax* Fr.: Fr. (Fries 1815, Observ. Mycol. 1: 54) and *A. temulentus* Fr.: Fr. (Fries 1821, Syst. Mycol. 1: 268) which would have priority over *Naucoria medullosa* Bres. (1898) must be considered ambiguous since they have been interpreted in different senses in specialized literature, very likely due to their equivocal original descriptions and the lack of type or authentic specimens or illustrations.

The microcharacters of the holotype of *N. medullosa* are practically identical with those in the holotype of *Psathyra silvatica* (Borovička 2011); both taxa are characterized by relatively small subellipsoid basidiospores and fusiform cheilocystidia with a relatively long neck (more than 10 µm). The only two available illustrations of *P. silvatica* (Smith 1940, Stamets 1996) known to the authors depict basidiomata very similar

to *P. medullosa*. Despite being sought in various herbaria and within the 'online community', no recent collections of *P. silvatica* have been found so we were unable to investigate genetic variation among American collections as only one collection from the New World could have been sequenced. As revealed from our analysis (Fig. 4d), *P. medullosa* is closely related to *P. silvatica*. However, there are appreciable differences in all four investigated regions (Table 3). Consequently, we recommend keeping the European *P. medullosa* and the American *P. silvatica* as distinct species. An analogous problem arises in the group of *P. cyanescens* where only minor differences have been found within *P. allenii*, *P. azurescens*, *P. cyanescens*, *P. subaeruginosa*, and *P. weraroa* (Borovička et al. 2011, 2012). On the other hand, DNA regions of European and American collections of *P. atrobrunnea* were nearly identical, indicating their probable conspecifity (Table 3).

Our LSU sequence of *P. silvatica* (HF678215) is slightly different from that already published in GenBank for the same isolate (AY129383) by Nugent & Saville (2004). This discrepancy is very likely caused by sloppy editing of the AY129383 sequence since our data is 100 % identical with the unpublished sequences obtained at DAOM (kindly sent by Scott A. Redhead along with the isolate). With regard to the indicated polyphyly in *P. weraroa* (Fig. 4c), this should be taken with care since this result is based just on GenBank data. According to our experience in our *P. weraroa* collections (P34 and one unpublished), sequencing of LSU often fails and generates poor data; further investigation of this species is needed.

The problem of Psilocybe atrobrunnea

Agaricus atrobrunneus Lasch, Linnaea 3: 423. 1828: Fr., Syst. Mycol. 3, Index: 8. 1832 (basion.). MycoBank 461744

≡ *Psilocybe atrobrunnea* (Lasch: Fr.) Gillet, Hyménomycètes: 586. 1878. MycoBank 198092.

Holotype. To our best knowledge, there is neither an original collection nor illustration. Guzmán (1983) did not report any holotype and according to our recent communication with curators of public herbaria (B, L, MSTR, MW, PAV, WRSL) supposed to contain Lasch's collections (Stafleu & Cowan 1979) and others (BREM, FR, GFW, GOET, HAL, JE, M, REG, TUB, WI), no type specimens of *Agaricus atrobrunneus* exist.

Neotype. Singer's (1986: 571) statement "*P. atrobrunnea* (Lasch) Gillet (*P. dichroa* Karst.; *P. fuscofulva* Peck; *P. turficola* Favre – synonymy according to A.H. Smith), the type (sensu Lasch) which I recollected has raphanaceous odor and slightly hexagonal sublentiform spores (LE)." is here considered as an effective neotypification: even if Singer's designation is not a standard one, it matches all requirements for an effective choice according to the current International Code of Nomenclature for Algae, Fungi, and Plants of Melbourne (McNeill et al. 2012, hereinafter 'Code'). From Singer's statement we interpret that Singer collected again a collection of *P. atrobrunnea* in the original sense of Lasch, and he deposited it in LE. We have consulted with other mycologists and nomenclaturists regarding Singer's statement, and they let us know that:

a. Lasch's type (or a duplicate) of *A. atrobrunneus* could be deposited at LE, and therefore Singer examined the type and not a collection collected by him;
b. Singer collected his collection in the same place where Lasch's type was collected and therefore the word 'type' is referred to the original collection and not to Singer's one;
c. Singer's word 'recollected' could mean 'remembered' and not 'collected again', and therefore again Singer's sentence referred to the original type; or
d. Singer's neotypification was a not effective one as he didn't mention a collecting number.

So, we must elucidate all above points before considering designation of a neotype. Our first logical step was to request to the curator of Komarov Botanical Institute (LE) information about any Singer's collection of *P. atrobrunnea*. Fortunately, according to the curator Olga Morozova (2012, pers. com.), Singer deposited a collection of *P. atrobrunnea* in LE, which is now lost in the post or destroyed by suspicious customs agents when it was sent to Mexico for examination. Interestingly, Morozova provided us with the whole label data as follows: "*Psilocybe atrobrunnea* (Lasch: Fr.) Gillet, Russia, Leningrad region, Sovkhozy (st. Neva), in Pineto, inter muscos, IX-1936, coll. et det. R. Singer (LE 11847)", and she pointed out to us that surely Singer (1986) had in mind this lost collection since no other collection of this taxon collected by Singer has been located at LE. On the other hand, Mueller (1995: 146, right column, second paragraph) in a paper honouring Singer's work wrote the following statement: "Rolf consistently made it his practice to deposit type material in the institution where he was working". In fact, the type of *Janauaria amazonica* also typified by Singer in 1986 (page 495, footnote) was deposited at F (Chicago). Thus, we also contacted Robert Lücking, curator at F, where Singer worked in 1986, and he informed us that "we do not have any collection under that name [*P. atrobrunnea*], neither type or non-type".

Now, we unambiguously can state that:

a. the type of *Agaricus atrobrunneus* Lasch was not deposited at LE;
b. Singer's collection is not a topotype, as Leningrad is more than 1 000 km far from 'Marchiae Brandenburgicae' where Lasch collected the original specimen of *A. atrobrunneus*; and
c. Singer's word 'recollected' can only mean collected again, as it was confirmed that Singer's collection from Leningrad area was extant in LE.

Concerning if Singer's neotypification was or was not an effective one, despite his statement being very brief, he gave us much information as:

a. he used the word 'type' as prescribed by the Art. 7.10 of the Code;
b. he pointed out that his sense is Lasch's original one ('sensu Lasch'), and not in the sense of whatever synonym that he accepted;
c. he mentioned that he was the collector ('I recollected') and in the label of the specimen at LE we can read "coll. & det. R. Singer";
d. he stated that his specimen has 'raphanaceous odor', a character mentioned by Lasch ("sapor odorque fere Rafani");
e. he cited a herbarium ('LE') in which his specimen was deposited (but lost), and we have verified that this is true.

Taking into consideration that the information provided by Singer meets all requirements for an effective publication of a neotype, we consider that Singer's neotypification effective.

Table 3 Numbers of nucleotide changes in particular DNA regions of specimens of *P. atrobrunnea* and *P. medullosa* vs *P. silvatica* are indicated.

Species	LSU	ITS	EF1-α	IGS
P. silvatica (P24)*	ref.	ref.	ref.	ref.
P. medullosa (P11)*	1	5	4	8
P. medullosa (P37)	1	6	4	n.a.
P. medullosa (P17)	1	4	n.a.	n.a.
P. medullosa (P20)	1	6	n.a.	n.a.
P. atrobrunnea (P32)	ref.	ref.	ref.	ref.
P. atrobrunnea (P36)	0	0	5	0
P. atrobrunnea (P54)	0	0	1	0

Heterozygous and indel positions were not considered.
* epitypes, designated in this study.
ref. – reference sample; n.a. – not analysed.

Some colleagues have emphasized that Singer did not mention a collection number or enough data to allow a precise identification of the type. They claim that in Art. 7.10 the second condition "if the type element is clearly indicated by direct citation including the term 'type' (typus) or an equivalent", direct citation refers to a collecting number. However, this interpretation is not possible since:

i. there is no comma after direct citation and therefore the condition only can be referred to the use of the term 'type' or an equivalent; and since
ii. many designations of neotypes, lectotypes, and epitypes are based on old illustrations in which no indication to a collecting number are available.

Also the definition of a neotype (Art. 9.6) states that it can be a specimen or a illustration selected to serve as nomenclatural type, but there is no mention that a precise collecting number must be provided. In this case, Singer also met this article as he used a specimen, which was deposited in LE; Singer probably did not provide more collection data because the specimen had been collected 50 years prior. We have also noted that Singer's typifications based on taxa not described by him were more informal than those based on new taxa, as for example the typification of *Psalliota* sect. *Bivelares* Kauffman and *Psalliota* sect. *Univelares* Kauffman (Singer 1975).

So, our first reaction was to designate a new neotype for *A. atrobrunneus*, but once we studied the Code we realized that this was not possible because no specific provision to replace a lost neotype exists in the Code (as it exists in the Zoological Code; Art. 75.4.1)! We asked W. Greuter for advice, who along with N. Turland and J. McNeill agreed that the Code does not foresee the possibility that a neotype might get lost or be destroyed. Fortunately, as pointed out by W. Greuter, the Code (Art. 9.8) provides, however, designation of an epitype in case that a neotype "cannot be critically identified for purposes of the precise application of the name to a taxon". Therefore, as a lost specimen cannot be used, all three of them proposed us to designate an epitype as an interim solution.

Nevertheless, it must be stressed that the combination of characteristics provided by Singer ("slightly hexagonal sublentiform spores") does not match that observed in collections of *P. atrobrunnea* in the widely accepted concept of Guzmán (1983), Borovička (2006), and Noordeloos (2011): both American and European collections are characterized by subellipsoid basidiospores. Therefore, as the name *A. atrobrunneus* was published without any illustration and lacking important microscopic characters necessary to precisely identify species in the genus *Psilocybe*, and the fact that this name has been interpreted in two different ways in modern literature, we consider *P. atrobrunnea* a dubious and ambiguous name.

According to Smith (fide Singer 1986) and Guzmán (1983), the American species *P. fuscofulva* Peck, Bull. New York State Mus. Nat. Hist. 1, 2: 7. 1887 (MycoBank 222041) was considered synonymous with *P. atrobrunnea* s.auct., non Singer (1986). Both protologue, macroscopic appearance of the exsiccatae, and the microcharacters (shape and size of basidiospores and cheilocystidia) observed in the holotype (NYS 1310, studied by the authors) match well our fungus and we have therefore decided to adopt the name *P. fuscofulva* for both American and European collections representing the taxon previously known in the literature (except for Singer) as *P. atrobrunnea* or *P. turficola*; no designation of an epitype is necessary at this time for *P. fuscofulva*.

TYPE DESIGNATIONS

Psathyra silvatica Peck, Rep. State Bot. New York State Mus. 42: 116. 1889. (basion.). MycoBank 179953

≡ *Psilocybe silvatica* (Peck) Singer & A.H. Sm., Mycologia 50: 277.1958. MycoBank 304494.

Holotype. NYS 2788 (USA; leg. H. Peck).

Epitype (designated here, MBT177347) that supports the holotype cited above: (specimen) Canada, Newfoundland, Gros Morne National Park, Berry Head Pond, gregarious on fir debris, 21 Sept. 1983, leg. & det. Scott A. Redhead, DAOM 187848. EMBL-Bank: HF678215, HF912360, HF912343, HF912366, HF912367.

An interpretative type is necessary for a precise application of the name for American collections.

Naucoria medullosa Bres., Fungi Trident. 2: 53. 1898. (basion.). MycoBank 188182

≡ *Psilocybe medullosa* (Bres.) Borov., Mykol. Sborn. 84, 4: 114. 2007. MycoBank 532147.

Holotype. S F-16066 (Italy; leg. G. Bresadola).

Epitype (designated here, MBT177348) that supports the holotype cited above: (specimen) Czech Republic, Silesia, Bruntál district, Krnov-Ježník, gregarious on wood chips and spruce needles (*Picea*) in underbrush of *Urtica* and *Rubus*, 13 Oct. 2007, leg. J. Borovička, J. Kuba & F. Kuba, PRM 909630. The epitype collection comes directly from the same site as the sequenced specimen P11 (PRM 909584; EMBL-Bank: HF678212, HF912353, HF912339, HF912364, HF912365). The epitype collection is depicted in Noordeloos (2011) on p. 559 (bottom figure), p. 560 (bottom figure) and p. 561 (top figure).

Despite the fact that the protologue and the microcharacters of the holotype enable morphological identification of European collections, our results indicate that the American *P. silvatica* is a morphologically similar, but molecularly distinct species. Therefore, an interpretative type is necessary for a precise application of the name.

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