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ORIGINAL ARTICLE

Molecular phylogeny of *Psilocybe cyanescens* complex in Europe, with reference to the position of the secotioid *Weraroa novae-zelandiae*

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Abstract A phylogenetic analysis with three molecular markers was undertaken to test the hypothesis that the complex of *Psilocybe cyanescens* in Europe consists of several, morphologically distinct species. The results support the existence of two molecularly well-supported morphological groups, that of *Psilocybe cyanescens* and *P. azurescens* on the one hand, and the complex of *P. serbica* on the other. However, in the last group, no sequence variability within the three molecular markers from *P. serbica* and related taxa *P. bohemica, P. arcana,* and *P. moravica* was found. It was decided, therefore, to merge these taxa into *P. serbica*, and to distinguish them below species level. It was also demonstrated that the secotioid *Weraroa novae-zelandiae* belongs to the *P. cyanescens* species complex. Accordingly, it was transferred to *Psilocybe* as *P. weraroa*, nomen novum.

Keywords Strophariaceae · *Psilocybe serbica* · *Psilocybe cyanescens* · *Weraroa novae-zelandiae* · DNA markers · Phylogeny

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Introduction

The discovery of the Mexican cult of "magic mushrooms" in the 1950s has started an intensive investigation of taxonomy, ecology and biochemistry of psychoactive fungi throughout the world (Heim and Wasson 1958). The major part of the known psychoactive species belongs to the genus *Psilocybe* (Basidiomycota, Agaricales, Strophariaceae) (for reviews, see Courtecuisse and Deveaux 2004; Anderson et al. 2008; Guzmán 2009). *Psilocybe* species grow throughout most of the world and more than 200 taxa have been described to date (Guzmán 1983, 1995); more than 25 species are reported from Europe (Horak 2005; Noordeloos 1999; Knudsen and Vesterholt 2008).

Based on their ecology, the European psychoactive species, which are sometimes used as recreational drugs (Hillebrand et al. 2006), are divided into two main groups: (1) species growing in pastures and meadows, often on dung, and (2) species growing on plant debris in forests, parks and gardens. The latter is known as the *Psilocybe cyanescens* complex. During the 1980s, the European

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Psilocybe species of the *P. cyanescens* complex were studied by the German mycologist, G.J. Krieglsteiner, who proposed a rather wide species concept of *Psilocybe cyanescens* Wakef., including as synonyms *P. serbica* M.M. Moser & E. Horak, *P. mairei* Singer and *P. bohemica* Šebek ex Šebek (Krieglsteiner 1984, 1986); this concept has been widely accepted in Europe (Ludwig 2001; Bon and Roux 2003; Horak 2005; Knudsen and Vesterholt 2008). Guzmán (1983, 1995), however, recognizes *P. serbica* and *P. cyanescens* as distinct species, but makes no reference to the works of Krieglsteiner.

However, as a result of a thorough morphological and ecological study of this species complex in the Czech Republic (Borovička and Hlaváček 2001a; b; Borovička 2003, 2005, 2006, 2008), seven taxa have been distinguished. These are divided over two stirpes (groups): (1) stirps *Cyanescens*, including *P. cyanescens* Wakef. (Dennis and Wakefield 1946) and *P. azurescens* Stamets and Gartz (1995), and (2) stirps *Serbica* including *P. serbica* Moser and Horak (1968), *P. bohemica* Šebek ex Šebek (1983), *P. arcana* Borovička and Hlaváček (2001a), *P. moravica* Borovička (2003), *P. moravica* var. *sternberkiana* Borovička (2006), and possibly *P. mairei* Singer (1973).

The aim of this study was to explore the relationships within the proposed *P. cyanescens* species complex by use of molecular markers and to compare that to the analysis of morphological, phenological and ecological data. Furthermore, the secotioid genus *Weraroa* was shown to be polyphyletic, as its species were shown to be distributed in different subclades of the Strophariod clade (Bridge et al. 2008; Moncalvo et al. 2002). The type species, *Weraroa novae-zelandiae*, appeared in the clade "Psychedelia" close to *P. cyanescens*. For that reason, this species has been included in our study.

Materials and methods

Collections used

This study includes material of all taxa of the stirpes *Cyanescens* and *Serbica* except for *Psilocybe mairei* Singer. This last species was described from North Africa as *Hypholoma cyanescens* Maire (1928), but no recent collections were available. All of the collections used for DNA extraction fit well with the morphological and ecological concept by Borovička (2008) (Table 1). When possible, holotype collections were used for sequencing (*P. arcana*, *P. moravica*, *P. moravica* var. *sternberkiana*) or the collections were sampled in original localities (*P. bohemica*, locality of epitype; *P. arcana*, topotype). We were not successful in obtaining DNA data from the paratype of *P. serbica* (herb. E. Horak 63/2536), so we used a collection donated from Croatia whose microcharacters (spore size, cheilocystidia shape and pleurocystidia occurrence) agree well to those observed in the original collection. Available sequences of related species were downloaded from GeneBank™ (Electronic supplementary material, ESM, Table S1). The secotioid fungus *Weraroa novae-zelandiae* was also included in this study because earlier reports show that this species might well be very closely related to members of the *P. cyanescens* complex (Bridge et al. 2008).

Molecular phylogeny

Nuclear DNA was extracted from a small piece of dried or frozen fungal biomass (a piece of basidiocarp) using the MoBio Power Soil DNA isolation kit (Mo-Bio Laboratories, Solana Beach, CA, USA) according to the manufacturer's instructions. We have used three molecular markers in particular: partial sequences of the nuclear LSU rRNA gene (Moncalvo et al. 2000, 2002; Maruyama et al. 2003, 2006; Nugent and Saville 2004; Garnica et al. 2007) and ITS1+5.8S+ITS2 region (Guzmán-Dávalos et al. 2003; Watling and Martin 2003; Nugent and Saville 2004; Matheny et al. 2006; Pornpakakul et al. 2009), which have previously been used to infer phylogenetic relationship of the genus *Psilocybe*; in addition to those, we have sequenced part of the elongation factor 1-α (EF1α) which has not previously been used for phylogenetic studies in *Psilocybe*, but appeared useful in other groups of fungi (Kauserud and Schumacher 2001; Antonín et al. 2009).

Primer pair NL-1 and NL-4 was used to amplify the part of 28 S rDNA. To amplify the complete sequence of ITS1+5.8S+ITS2 region and a part of 18S rDNA, the forward, fungal-specific ITS-1F primer and universal reverse primer ITS-4 or basidiomycete-specific reverse primer ITS-4B were used as recommended (White et al. 1990; Gardes and Bruns 1993). The partial elongation factor EF1α sequence was amplified using the forward primer EF-595F and reverse primer EF-1160R. The EF1α sequence included two partial and one complete exon and two introns (Kauserud and Schumacher 2001).

The reaction to amplify both rRNA genes was performed in 50 μl volume with 1 μl undiluted DNA preparation (approx. 5 ng μl^{-1}) as a template, PPP Master Mix (*Taq*-Purple DNA polymerase PCR Master) supplying reaction with 2.5 U of *Taq* DNA polymerase, deoxyribonucleotides (200 μM each) and 2.5 mM $MgCl_2$, and 200 nM of each primer. The reaction was, following the initial denaturation (5 min at 94°C), subjected to 34 cycles of 1 min at 94°C, 52°C for 1 min and 2 min at 72°C. To complete the amplicons, the reaction was further incubated for 10 min at 72°C. The elongation factor EF1α was amplified in total volume of 50 μl and amplification program consisted of pre-denaturation for 4 min at 95°C, which was followed by

Table 1 Psilocybe species under study

ID	Species	Collection	Origin	GeneBank™		
				ITS	LSU	EF1-a
Stirps Cyanescens (Borovička 2005)						
P 02	*Psilocybe cyanescens* Wakef.	PRM 902040	Belgium	GU565176	-	GU565161
P 21	*Psilocybe cyanescens* Wakef.	PRM 901481	Germany	GU565175	GU565167	GU565158
P 01*	*Psilocybe azurescens* Stamets & Gartz	PRM 901020	Oregon, USA	GU565173	GU565166	GU565159
Stirps Serbica (Borovička 2005)						
P 15	*Psilocybe serbica* M.M. Moser E. Horak	PRM 903176	Croatia	GU565177	GU565168	GU565165
P 14	*Psilocybe bohemica* Šebek ex Šebek	L 0342813	Czech Rep.	GU565178	GU565169	GU565162
P 05	*Psilocybe arcana* Borov. & Hlaváček	PRM 895093, holotype	Czech Rep.	GU565180	-	-
P 05*	*Psilocybe arcana* Borov. & Hlaváček	PRM 915262, topotype	Czech Rep.	GU565181	GU565172	GU565160
P 06	*Psilocybe moravica* Borov.	PRM 900455, holotype	Czech Rep.	GU565182	GU565171	GU565164
P 07	*P. moravica* var. *sternberkiana* Borov.	PRM 901650, holotype	Czech Rep.	GU565179	GU565170	GU565163

PRM Herbarium of the Mycological Department, National Museum, Prague; *L* Nationaal Herbarium Nederland, Leiden University branch

37 cycles containing temperature profile 94°C for 30 s, 55°C for 35 s and 72°C for 40 s. The program was terminated by final extension at 72°C for 10 min. Obtained amplicons were purified using the MoBio UltraClean PCR Clean-up Kit and both strands were directly sequenced with ABI Prism 3130XL sequence analyzer.

The sequences coding for rRNA genes were aligned to their homologues downloaded from GeneBank™ using Kalign program (Lassmann and Sonnhammer 2005) (available at http://www.ebi.ac.uk/Tools/kalign/index.html). Gaps and ambiguously aligned regions were excluded from further analysis. Nucleotide sequences coding for homologues of elongation factor EF1α were downloaded from GeneBank™ and they were in the form of deduced amino acid sequences aligned to the genes from *Psilocybe* spp. using ClustalW algorithm. The alignment was retranslated back to nucleotides (BioEdit; see Hall 1999). Both introns were identified and their phylogenetic signal was explored separately from the mature protein coding sequence. Both nucleotide and aminoacid alignments were subsequently used to construct phylogenetic trees by maximum parsimony (MP) and maximum likelihood (ML) methods. Models for ML were selected using Modeltest program (GTR+Γ+I) (Posada and Crandall 1998). All parameters such as gamma shape parameter and proportion of invariant sites were estimated from particular datasets. Robustness of constructed trees was tested by bootstrap analysis in 1,000 and 300 replicates for MP and ML, respectively.

To compare molecular analysis with that using phenotypic data, we have analyzed 34 morphological, ecological and phenological characters and computed a dendrogram based on them. We have used both approaches to test the validity of the proposed morphospecies with a phylogenetic analysis using molecular markers and to elucidate the evolution of this group. The morphological matrix was constructed from 34 morphological, ecological and phenological data (ESM, Table S2) including, e.g., habitus, fruitbody size, shape of pileus, coloration of pileus when moist and in drying, occurrence of olive-green tinges, shape of stipe, character and occurrence of velar remnants, smell, size and shape of spores, shape and occurrence of pleuro- and cheilocystidia, distribution in Europe, ecology (preferred substrate, habitat), and fruiting maximum in Central Europe. The tree based on this matrix was computed using maximum parsimony and minimum evolution as implemented in PAUP*4b10 (Swofford 1998). Robustness of the tree was tested by bootstrap analysis from 1,000 replicates for both methods.

Results and discussion

A computational matrix reflecting phenotypic characters (morphology, ecological and phenological features, 34 countable characters) was established for *P. cyanescens, P. azurescens*, *P. serbica, P. arcana, P. bohemica, P. moravica*, and *P. moravica* var. *sternberkiana,* and was used to infer phylogeny of species of interest. Distant *P. cubensis* was used as outgroup (ESM, Table S2). Analysis of phenotypic characters gave a tree showing two distinct stirpes *Cyanescens* and *Serbica*. Within the stirps *Serbica*, *P. moravica* appeared as a sister to *P. moravica* var. *stenberkiana* with *P. bohemica* as the most basal species. The remaining species of this stirps, *P. serbica* and *P. arcana*, form a separated but related cluster (Fig. 1a).

Molecular phylogeny shows two distinct groups within the species of the *P. cyanescens* complex in Europe (Fig. 1b–e): group 1 encompassing *P. cyanescens* and *P.*

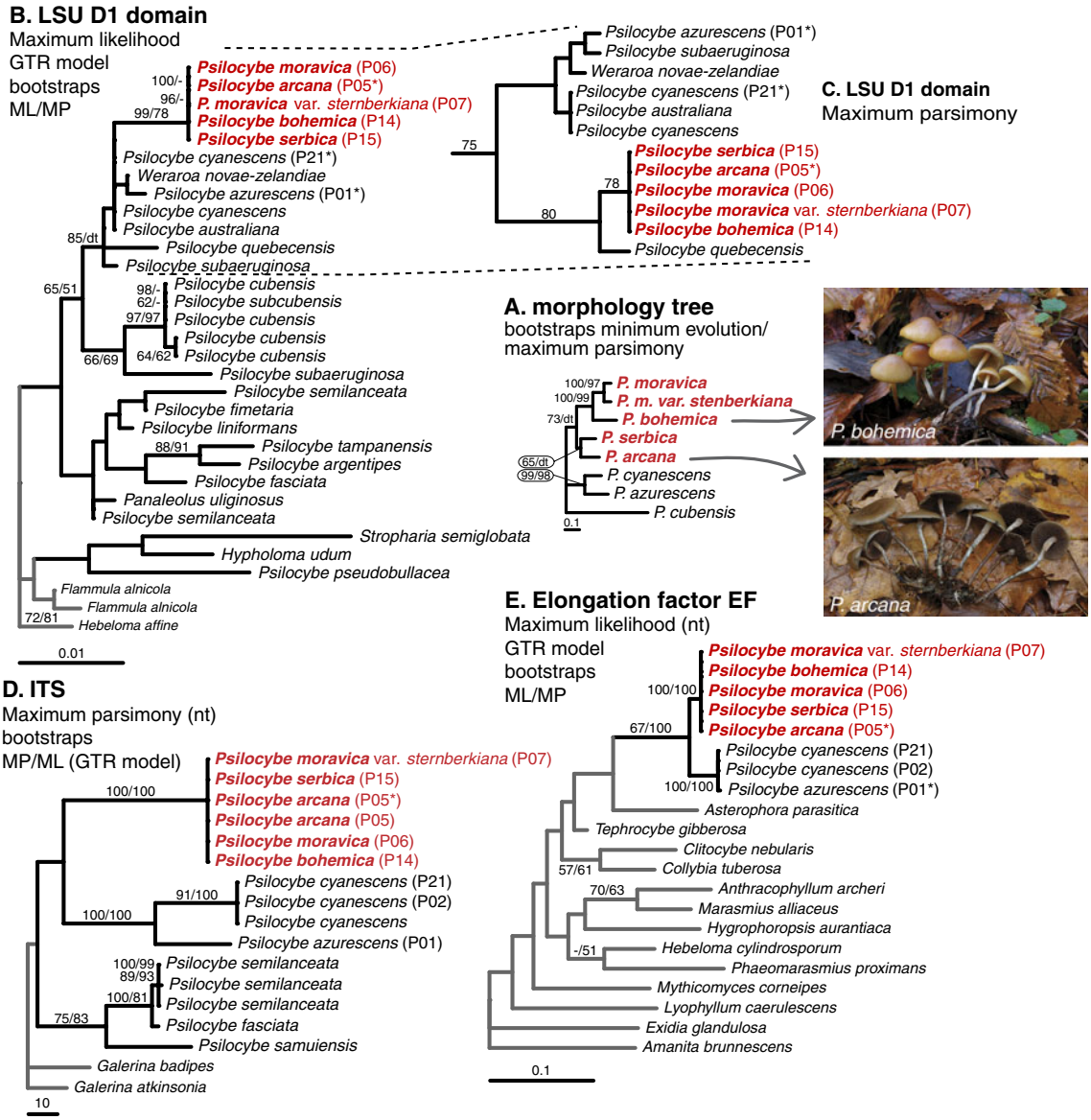

Fig. 1 Phylogenetic trees as inferred from various molecular and morphological data. **a** Minimum evolution tree based on morphological, phenological and ecological data (bootstraps minimum evolution/maximum parsimony; both 1,000 replicates). **b** Maximum likelihood tree (loglk=−2707.37145; gamma shape 0.015; proportion of invariant sites 0.000) based on partial LSU rDNA sequences. **c** Topology of species of interest as inferred by maximum parsimony method. **d** Maximum likelihood tree (loglk = −1938.37061; gamma shape 0.496; proportion of invariant sites 0.386) as inferred from ITS1+5.8 S+ITS2 sequences. **e** Maximum likelihood tree (loglk=−1693.32043; gamma shape 1.026; proportion of invariant sites 0.509) as inferred from nucleotide sequences coding for elongation factor 1−α (EF1α). Numbers above branches indicate ML/MP bootstraps (number of replicates 300/1,000)

azurescens, and group 2 encompassing *P. serbica*, *P. bohemica*, *P. arcana* and *P. moravica* (including its var. *sternbekiana*). The latter group is very well supported by bootstrap values. Furthermore, the taxa of the second group do not differ among each other in the sequence of all three DNA markers. We have also analyzed concatenated introns from EF1α and have obtained the same topology as from the coding sequence (not shown). The absence of DNA polymorphism among the taxa of the *P. serbica* complex in studied markers indicates that these taxa represent, in fact, a single species despite their phenotypic variability.

In general, molecular markers can successfully uncover the existence of cryptic species. Such species do not display morphological variation but can be well distinguished thanks to the polymorphism of DNA markers (Geiser et al. 1998; Roy et al. 1998; Cruse et al. 2002). In our case, however, the opposite situation seems to be at hand: a group of morphologically distinguishable fungal taxa that

display no sequence variation in three widely used DNA markers. In this particular case, the Morphological Species Concepts (MSC) and Ecological Species Concept (ESC) are in conflict with the Phylogenetic Species Concept (Giraud et al. 2008).

When only morphological and ecological characters are used for classification, *P. serbica* complex is very variable and this variability has led to description of several species within it in the past. However, when the DNA sequence characters are used, the previously described taxa within the stirps *Serbica* appear to be identical. This is surprising particularly in the case of ITS, which represents a highly polymorphic marker and consequently a powerful tool for taxonomic purposes at species level. It was suggested, based on analysis of 1,373 ITS2 sequences, that in 93% of cases where two taxa differed in any property of the ITS2 (sequential or secondary structural) they represented different species (Müller et al. 2007). Furthermore, this is contrasting with, for example, the results of Nugent and Saville (2004) and Maruyama et al. (2003) who successfully used partial sequences of LSU rRNA gene to infer phylogeny of closely related hallucinogenic species of the genus *Psilocybe*. Another example can be found in the *Armillaria* study of Antonín et al. (2009).

We therefore conclude that the *P. cyanescens* complex in Europe consists of three well-defined species, *P. cyanescens*, *P. azurescens* and *P. serbica*. The last species is, furthermore, very variable in macro- and microcharacters. The most distinct morphological entities, so far described at species level, namely *P. arcana*, *P. bohemica* and *P. moravica*, need to get a formal rank below species level. Consequently, we propose to treat *P. moravica* var. *sternberkiana* on the level of forma.

We have to note that our analysis led to the taxonomic suggestions also concerning other *Psilocybe* species. A secotioid fungus traditionally classified as *Weraroa novae-zelandiae* (G. Cunn.) Singer is obviously closely related to the investigated bluing species of the *Psilocybe cyanescens* complex (as already suggested by Bridge et al. 2008) and belongs to the genus *Psilocybe*. A similar phenomenon has also been observed in the case of other secotioid fungi, e.g., *Gyrophragmium dunalii* (Fr.) Zeller and *Endoptychum agaricoides* Czern., which are currently classified as *Agaricus aridicola* Geml, Geiser & Royse (Geml et al. 2004) and *Chlorophyllum agaricoides* (Czern.) Vellinga (Vellinga 2002), respectively.

Conclusion

Our analysis does not support the widely accepted view that there are only two wood-rotting bluing *Psilocybe* species (*P. cyanescens* and *P. azurescens*) in Europe. Molecular analysis recognizes two sibling groups within the *P. cyanescens* complex and three distinct species: *P. cyanescens*, *P. azurescens* and *P. serbica*. The recently described morphospecies *P. bohemica, P. arcana,* and *P. moravica* apparently represent just morphological and ecological varieties of *P. serbica*, and should accordingly be treated below species level. The species of the two groups can be unambiguously recognized microscopically by occurrence and shape of pleurocystidia (Borovička 2008). *Psilocybe serbica* now appears as a highly variable species characterized, e.g., by various robustness, shape and coloration of fruit-bodies, and microscopically by a large range of spore size and varying abundance/shape of pleuro- and cheilocystidia. The phylogeny also shows that the secotioid *Weraroa novae-zelandiae* fits well in the agaricoid *P. cyanescens* group.

Taxonomic changes

Psilocybe serbica* var. *bohemica (Šebek ex Šebek) Borov., Oborník & Noordel., comb. & stat. nov.

Mycobank: MB 515725

Basionym: *Psilocybe bohemica* Šebek ex Šebek, Česká Mykol. 37(3): 177 (1983).

Psilocybe serbica* var. *arcana (Borov. & Hlaváček) Borov., Oborník & Noordel., comb. & stat. nov.

Mycobank: MB 515724

Basionym: *Psilocybe arcana* Borov. & Hlaváček, Mykol. Sborn. 78(1): 3 (2001a).

Psilocybe serbica* var. *moravica (Borov.) Borov., Oborník & Noordel., comb. & stat. nov.

Mycobank: MB 515726

Basionym: *Psilocybe moravica* Borov., Mykol. Sborn. 80(4): 127 (2003).

Psilocybe serbica* f. *sternberkiana (Borov.) Borov., Oborník & Noordel., comb. & stat. nov.

Mycobank: MB 518345

Basionym: *Psilocybe moravica* var. *sternberkiana* Borov., Czech Mycol. 58(1-2): 76 (2006).

Psilocybe weraroa Borov., Oborník & Noordel., nom. nov.

Mycobank: MB 515728

Replaced synonym: *Secotium novae-zelandiae* G. Cunn., Proc. Linn. Soc. N.S.W. 49(2): 107 (1924).

Etymology This species has been known as *Weraroa novae-zelandiae* (G. Cunn.) Singer; the epithet of the replacement name (nomen novum) refers to the former generic name. It was necessary to create the replacement name because the binomial *Psilocybe novae-zelandiae* was preoccupied by *P. novae-zelandiae* Guzmán & E. Horak, Sydowia 31(1-6): 51 (1979) [1978]; see also Redhead et al. (2007).

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