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RESEARCH ARTICLE

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Preliminary notes on dual relevance of ITS sequences and pigments in *Hygrocybe* taxonomy

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ITS region
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muscaflavin pigments

Abstract The relationships based on ITS sequences of 48 *Hygrocybe* s.l. specimens were studied and compared with previously described taxonomic groups. Our specimens formed two well separated genetic groups. The first one includes the species characterized by vivid yellow and red colours, while species belonging to other clades were pallid or pale brown, and in most cases with pink or olive tones. This separation is supported by the presence of muscaflavin pigments among some species referred to *Hygrocybe* (Bresinsky & Kronawitter 1986). The subgenera distinguished by morphological features can be relatively well recognized on phylogenetic trees, however, the majority of sections were not supported. Variability in the ITS region of *Hygrocybe* species is unusually high. In some cases sequences differed by more than 25 %, and the lengths of ITS regions also showed large differences. Taxa that were considered as closely related, e.g. the *H. conica* aggregate, were found to have identical or highly similar sequences. Our results seem to confirm the taxonomic concept of Bresinsky (2008) who proposed the division of the genus *Hygrocybe*. Hence *H. calyptriformis* and all examined members of subg. *Gliophorus* (*H. irrigata*, *H. laeta*, *H. nitrata*, *H. psittacina*) and subg. *Cuphophyllus* could be excluded from the genus *Hygrocybe* s.str. Based on these results further research using DNA markers at the intergeneric level is suggested to revaluate the taxonomy of former *Hygrocybe* species.

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INTRODUCTION

The term *Hygrocybe* originates from Fries (1821) who at first recognized the group as a member of the 'tribus' *Clitocybe* ('subtribus' *Hygrocybe*) and subsequently, not earlier than 1838, transferred them as a 'tribus' to the genus *Hygrophorus.* It was raised to the rank of a separate genus by Kummer (1871). According to the opinion of most mycologists, both *Hygrocybe* and *Hygrophorus* should be classified as separate genera in the family *Hygrophoraceae*. Currently about half of the researchers recognize multiple segregate genera while the remainder divides the genus *Hygrocybe* into three subgenera, namely subg. *Hygrocybe* s.str. Bon 1976, subg. *Pseudohygrocybe* Bon 1976 and subg. *Cuphophyllus* Donk 1962 (Boertmann 1995, Candusso 1997, Krieglsteiner 2001).

Some taxa of *Hygrocybe* can be determined unambiguously based on macroscopic characters, (e.g. *H. citrinovirens*, *H. conica*, *H. conicoides*, *H. intermedia*, *H. ovina*, *H. pratensis*, *H. spadicea*). Identification of other *Hygrocybe* species can be moderately improved by considering the results of detailed microscopical examination and macroscopic attributes simultaneously (e.g. determining *H. aurantiosplendens*, *H. coccinea*, *H. constrictospora*, *H. marchii*, *H. phaeococcinea* and *H. reidii*; separating the taxa in the groups *H. punicea* vs *H. splendidissima*, *H. glutinipes* vs *H. vitellina*, *H. laeta* var. *flava* vs *H. helobia*, *H. miniata* or *H. ceracea* vs *H. insipida*, and *H. constrictospora* vs *H. mucronella.*

The most important diagnostic features of the genus *Hygrocybe* on a macroscopic level are the thick, waxy, widely-spaced gills producing white spores, and the stipe without veil remnants. Additional significant microscopic markers common to most members of *Hygrocybe* are as follows: long, narrow basidia (6–9 times longer than its width) with smooth and inamyloid spores. All the taxa lack real pleurocystidia. However, to make a firm distinction between the three subgenera, structure and arrangement of the hyphae of the lamellar trama have been accepted as the most reliable microscopic characteristics (Boertmann 1995). The hyphae of the lamellar trama in the subgenus *Hygrocybe* are especially long (> 1 000 µm) and parallel to each other (regular structure), such as those of *H. conica.* Species of the second subgenus, *Pseudohygrocybe*, have a subregular arrangement of short hyphae that rarely exceed 150 µm. The exceptions in subg. *Pseudohygrocybe* that are classified by others in the genus *Neohygrocybe*, i.e., *H. ingrata*, *H. nitrata and H. ovina*; the trama hyphae of these species are 200–500 µm. The genera *Neohygrocybe* and *Gliophorus* were separated by Herink (1959) based in part on the absence of muscaflavin pigment. The lamellar trama in subg. *Cuphophyllus* (*Camarophyllus*) is composed of short hyphae < 150 µm, that are mostly cylindrically shaped and form a highly interwoven hyphal entanglement (irregular lamellar trama). Bas (1990) recategorized the genus *Hygrocybe* and classified it in the family *Tricholomataceae* together with the genus *Camarophyllopsis* as tribus *Hygrocybeae*. In his opinion genus *Hygrophorus* s.str. should also be placed in the family *Tricholomataceae* as a separate tribus. Candusso (1997) also removed the genus *Hygrocybe* from the family *Hygrophoraceae*, but classified it in the family *Agaricaceae* except for genus *Camarophyllopsis.* In contrast, Bon (1992) proposed to separate the *Hygrophoraceae* from the order *Agaricales* and treat it as a distinct order, o. *Hygrophorales*, due to its unique characters. The present authors consider the family *Hygrophoraceae* a distinct group within the *Agaricales* – a position supported by a multigene analysis by Matheny et al. (2006).

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Currently, the number of *Hygrocybe* taxa recognized in Europe ranges between 60 and 133, depending on the authors and their various opinions on taxonomy. While Moser (1983), and Bon (1976, 1990, 1992) elevated certain taxa to higher taxonomic levels (giving species rank to varieties and forms), former separate species have occasionally been contracted by Boertmann (1995) thus reducing the number of *Hygrocybe* taxa. These two opposing processes are taking place simultaneously. While Bon (1976, 1990, 1992) often publishes new *Hygrocybe* species, Boertmann (1995) unites species as well as genera of *Hygrocybe* and *Camarophyllus* and he annuls certain sections inside the subgenera of *Hygrocybe*. At the same time, he still publishes and introduces new *Hygrocybe* taxa, despite the fact that these are usually constructed by the unification of former taxa. In some rare cases, however, Boertman differentiates, e.g. at *H. laeta* var. *flava* Boertm. var. nov., or at the aggregate of *H. lacmus* (Schumach.) P.D. Orton & Watling with three well separable species (*H. lacmus*, *H. flavipes* (Britzelm.) Arnolds, *H. radiate* Arnolds), even though others (e.g. Krieglsteiner 2000) join the species and refer to as *H. lacmus.* Until now, 35 taxa of genus *Hygrocybe* are known from Hungary on the basis of Boertmann's taxonomy and nomenclature (Zagyva 2003). With respect to the genus *Hygrocybe*, the Őrség National Park is the best studied and explored area in Hungary, where 34 of the 35 taxa were found. The basiphyl *H. conicoides* is the only species that has not been found in this region yet (Zagyva et al. 2003). Hungary seems to be poor in *Hygrocybe* partly due to the dominating continental climate becoming increasingly arid, and partly due to the expansion of intensive agriculture in the past decades.

The presence of above mentioned muscaflavin pigments is a remarkable chemotaxonomical character. Bresinsky & Kronawitter (1986) detected muscaflavins in 42 of 53 studied *Hygrocybe* species. The authors distinguished six groups on the basis of pigment content, of which four comprise muscaflavin-free species. Cibula (1976) found that rhodohygrocybin pigments were present in *Hygrocybe* s.str. (except for *H. andersonii*; Cibula & Weber Smith 1996), but were absent from *H. calyptriformis*, *Gliophorus*, *Neohygrocybe* and *Cuphophyllus* species. Emerging or verifying taxonomic groups based on chemotaxonomical features is a widely applied method for various taxa (e.g. Agerer 1999, Binder & Besl 1999). Beisenherz (2002) used rDNA RFLP (Restriction Fragment Length Polymorphism) analysis and cytofluorometry to characterize the genus at a molecular level. Matheny et al. (2006) analysed several *Hygrocybe* s.l. species on the basis of multigene sequences; in their cladogram *Gliophorus*, *Hygrophorus*, *Humidicutis* and *Camarophyllus* (*Cuphophyllus*) are not close to *Hygrocybe* s.str. although still within the Hygrophoroid-clade. Recently, in spite of few results in molecular taxonomy, Bresinsky (2008) divided the European species of genus *Hygrocybe* s.l. into four genera: *Hygrocybe* s.str., *Gliophorus*, *Neohygrocybe* and *Porpolomopsis* (for *H. calyptriformis*). Brock et al. (2009) published a number of ITS sequences of *Hygrocybe* materials without taxonomic discussion. Binder et al. (2010) developed a six-locus nuclear dataset including two non-ribosomal protein coding genes, RPB1 and RPB2 plus the translation elongation factor (1-alpha tef1).

The goal of our studies was to give additional data for the identification, classification and distinction of ambiguously identified *Hygrocybe* species including a larger number of European species than previous analyses (Mattheny et al. 2006, Brock et al. 2009, Binder et al. 2010). We also aimed to find agreement between the phylogeny and previous studies based on chemotaxonomy. Sequence analyses of total ITS (internal transcribed spacer) regions supporting recent taxonomic investigations and a preliminary revision of the genus *Hygrocybe* s.l. were performed using materials from the Carpatho-Pannon region.

MATERIALS AND METHODS

Fungal specimens for ITS sequence analyses were obtained from selected exsiccates of mainly Hungarian herbaria (Fig. 1). The majority of specimens originated from the area of the Őrség National Park (Apátistvánfalva, Farkasfa, Felsőszölnök, Kétvölgy). We sequenced several specimens of a species when possible.

For taxonomic determinations based on morphological characters, the keys of Boertmann (1995), Candusso (1997) and Krieglsteiner (2001) were used separately. EMBL (European Molecular Biology Laboratory) Nucleotide Sequence Database

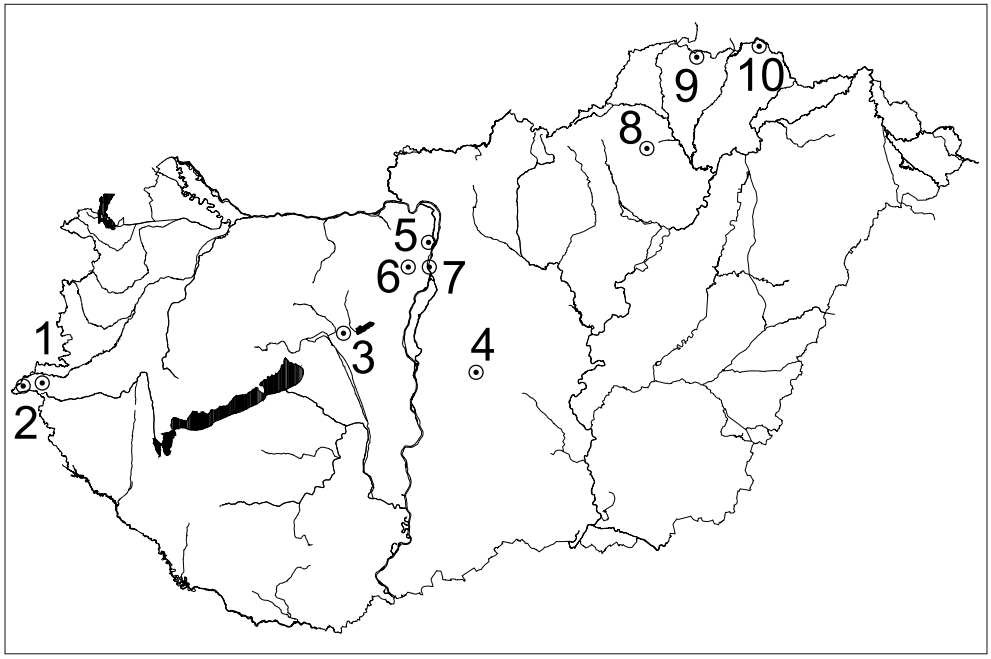

Fig. 1 Map of Hungary with collection locations: I. Transdanubian Hills: Őrség Hills: Farkasfa (1); Vendvidék Hills: Felsőszölnök (2), Kétvölgy (2), Apátistvánfalva (2); II. Great Hungarian Plain: Mezőföld: Székesfehérvár-Sóstó (3); Kiskunság: Kunbaracs (4); III. Transdanubian Medium Mountains: Pilis Hills: Budakalász (5); Buda Hills: Budakeszi (6), Budapest (7); IV. Northern Medium Mountains: Bükk Mountains: Bükkszentkereszt (8); Cserehát: Perecse (9); Zemplén Mountains: Lászlótanya (10). Further collection sites are: Austria, Niederösterreich: Puchberg; Steiermark: Feldbach.

Fig. 2 Evolutionary relationships of *Hygrocybe* s.l. species. The evolutionary history was inferred using the Neighbour-joining method. The bootstrap consensus tree inferred from 1 000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates are collapsed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 .

Accession Number and locality of the specimens used for molecular analysis are enumerated in Table 1. Basidiocarps were collected over the years 1997–2002, between June and October. EMBL Accession Numbers of other *Hygrocybe* sequences are represented below.

DNA extractions and PCR reactions were carried out according to Gardes et al. (1991). The universal primers ITS1 and ITS4 (White et al. 1990) and the fungal specific ITS1F primer (Gardes & Bruns 1993) were used for amplifications. PCR products were cleaned using a Montage-PCR (Millipore) microfilter. Sequencing reactions were carried out using BigDye™ Terminator Cycle 3.1 Sequencing Kit (Applied Biosystems). PCR products were sequenced with ITS1 and ITS4 primers, on ABI PRISM 3100 Genetic Analyser.

CLUSTALW2 (Larkin et al. 2007) program was applied to generate alignments and phylogenetic and molecular evolutionary analyses were conducted using MEGA4 (Tamura et al. 2007).

RESULTS AND DISCUSSION

Total ITS1 + 5.8S rDNA + ITS2 regions were sequenced successfully in most cases. The number of nucleotides were between 348 (*H. nitrate*, but probably not a whole ITS sequence) and 665 (*H. flavipes*).

At first sight, numerous clades are distinguishable in the phylograms in Fig. 2–4. Large differences were noted among sequences, some with only 24 % sequence homology. Few nodes are marked with high bootstrap values, and the topology of the sections or aggregates was rather variable. Every model, however, showed two major clades (A and B) separated in the phylograms comprising all sequences (Fig. 2). The branch supporting the separation of the two major groups had a bootstrap value of 89 %.

The species of clade B (Fig. 3) are characterizable by the presence of vivid yellow, orange and red colours, whereas clade A (Fig. 4) is represented by dull or pale coloured basidiocarps, some with pink, purple or olive tints. Since our phylograms lacked well circumscribed additional groups, our remarks are discussed by sections according to Boertmann's (1995) and Candusso's (1997) taxonomic system. Clade A contains

Table 1 The investigated herbarium specimens. Species names are given according to distinct morphological keys of Boertmann (1995), Candusso (1997) and Krieglsteiner (2001). Based on recent molecular results H16 and H38 does not corresponds to *H. cantharellus*, which was described from the southern Appalachian Mountains in North Carolina, USA, therefore we treated them as *H. lepida* Arnolds (Deborah Jean Lodge, pers. comm.). (The specimen marked with * was identified by Prof. David Boertmann.)

	Boertmann (1995)	Candusso (1997)	Krieglsteiner (2001)	Locality	In Herbaria	Acc. number
H1	*persistens* var. *persistens* (Britzelm.) Singer (1940)	*acutoconica* (Clem.) Singer (1951)	*persistens*(Britzelm.) Singer (1940)	Kétvölgy	T. Zagyva	FM208852
H2	*citrinovirens* (J.E. Lange) Jul. Schäff. (1947)	*citrinovirens* (J.E. Lange) Jul. Schäff. (1947)	*citrinovirens* (J.E. Lange) Jul. Schäff. (1947)	Felsőszölnök	T. Zagyva	FM208853
H3	*calyptriformis* (Berk.) Fayod (1889)	*calyptriformis* (Berk.) Fayod (1889)	*calyptriformis* (Berk.) Fayod (1889)	Kétvölgy	T. Zagyva	FM208854
H4	*chlorophana* (Fr.) Wünsche (1877)	*chloraphana* (Fr.) Wünsche (1877)	*chlorophana* (Fr.) Wünsche (1877)	Felsőszölnök	T. Zagyva	FM208855
H5	*chlorophana* (Fr.) Wünsche (1877)	*chlorophana* (Fr.) Wünsche (1877)	*chlorophana* (Fr.) Wünsche (1877)	Felsőszölnök	T. Zagyva	FM208856
H6	*chlorophana* (Fr.) Wünsche (1877)	*chlorophana* (Fr.) Wünsche (1877)	*chlorophana* (Fr.) Wünsche (1877)	Kétvölgy	T. Zagyva	FM208857
H7	*chlorophana* (Fr.) Wünsche (1877)	*chlorophana* (Fr.) Wünsche (1877)	*chlorophana* (Fr.) Wünsche (1877)	Kétvölgy	T. Zagyva	FM208858
H8	*coccinea* (Schaeff.) P. Kumm. (1871)	*coccinea* var. *coccinea* (Schaeff.) P. Kumm. (1871)	*coccinea* var. *coccinea* (Schaeff.) P. Kumm. (1871)	Kétvölgy	T. Zagyva	FM208859
H9	*conica* var. *conicoides* (P.D. Orton) Boertm. (1995)	*conicoides* (P.D. Orton) P.D. Orton & Watling (1969)	*conica* var. *conicoides* (P.D. Orton) Boertm. (1995)	Székesfehérvár	T. Zagyva	FM208860
H10	*conica* var. *conicoides* (P.D. Orton) Boertm. (1995)	*conicoides* (P.D. Orton) P.D. Orton & Watling (1969)	*conica* var. *conicoides* (P.D. Orton) Boertm. (1995)	Székesfehérvár	T. Zagyva	FM208861
H11	*conica* var. *conicoides* (P.D. Orton) Boertm. (1995)	*conicoides* (P.D. Orton) P.D. Orton & Watling (1969)	*conica* var. *conicoides* (P.D. Orton) Boertm. (1995)	Székesfehérvár	T. Zagyva	FM208862
H12	*coccinea* (Schaeff.) P. Kumm. (1871)	*coccinea* var. *coccinea* (Schaeff.) P. Kumm. (1871)	*coccinea* var. *coccinea* (Schaeff.) P. Kumm. (1871)	Felsőszölnök	T. Zagyva	FM208863
H13	*quieta* (Kühner) Singer (1951)	*obrussea* (Fr.) Wünsche (1877)	*obrussea* (Fr.) Wünsche (1877)	Felsőszölnök	T. Zagyva	FM208864
H16	*cantharellus* (Schwein.) Murrill (1911)	*cantharellus* (Schwein.) Murrill (1911)	*cantharellus* (Schwein.) Murrill (1911)	Farkasfa	T. Zagyva	FM208865
H17	*miniata* (Fr.) P. Kumm.	*miniata* (Fr.) P. Kumm.	*miniata* (Fr.) P. Kumm.	Pilisszentkereszt	T. Zagyva	FM208866
H18	*miniata* (Fr.) P. Kumm.	*miniata* (Fr.) P. Kumm.	*miniata* (Fr.) P. Kumm.	Budakeszi	T. Zagyva	FM208867
H19	*virginea* var. *fuscescens* (Bres.) Arnolds (1986)	*fuscescens* (Bres.) P.D. Orton & Watling (1969)	*virginea* var. *fuscencens* (Bres.) Arnolds (1986)	Székesfehérvár	T. Zagyva	FM208868
H20	*virginea* var. *virginea* (Wulfen) P.D. Orton & Watling (1969)	*virginea* (Wulfen) P.D. Orton & Watling (1969)	*virginea* (Wulfen) P.D. Orton & Watling (1969)	Székesfehérvár	T. Zagyva	FM208869
H21	*virginea* var. *ochraceopallida* (P.D. Orton) Boertm. (1995)	*virginea* var. *ochraceopallida* (P.D. Orton) Boertm. (1995)	*virginea* var. *fuscencens* (Bres.) Arnolds (1986)	Budapest	T. Zagyva	FM208870
H22	*persistens* var. *persistens* (Britzelm.) Singer (1940)	*acutoconica* (Clem.) Singer (1951)	*persistens* (Britzelm.) Singer (1940)	Székesfehérvár	T. Zagyva	FM208871
H23	*persistens* var. *persistens* (Britzelm.) Singer (1940)	*acutoconica* (Clem.) Singer (1951)	*persistens* (Britzelm.) Singer (1940)	Kétvölgy	T. Zagyva	FM208872
H24	*virginea* var. *ochraceopallida* (P.D. Orton) Boertm. (1995)	*virginea* var. *ochraceopallida* (P.D. Orton) Boertm. (1995)	*virginea* var. *fuscencens* (Bres.) Arnolds (1986)	Kétvölgy	T. Zagyva	FM208873
H26	*pratensis* var. *pratensis* (Pers.) Bon (1976)	*pratensis* var. *pratensis* (Pers.) Bon (1976)	*pratensis* (Pers.) Bon (1976)	Kétvölgy	T. Zagyva	FM208874
H27	*psittacina* var. *psittacina* (Schaeff.) P. Kumm. (1871)	*psittacina* (Schaeff.) P. Kumm. (1871)	*psittacina* var. *psittacina* (Schaeff.) P. Kumm. (1871)	Kétvölgy	T. Zagyva	FM208875
H29	*punicea* (Fr.) P. Kumm. (1871)	*punicea* (Fr.) P. Kumm. (1871)	*punicea* var. *punicea* (Fr.) P. Kumm. (1871)	Apátistvánfalva	T. Zagyva	FM208876
H30	*quieta* (Kühner) Singer (1951)	*obrussea* (Fr.) Wünsche (1877)	*obrussea* (Fr.) Wünsche (1877)	Bükkszentkereszt	T. Zagyva	FM208877
H31	*conica* var. *conica* (Scop.) P. Kumm. (1871)	*conica* var. *conica* (Scop.) P. Kumm. (1871)	*conica* var. *conica* (Scop.) P. Kumm. (1871)	Székesfehérvár	T. Zagyva	FM208878
H32	*spadicea* var. *spadicea* (Scop.) P. Karst. (1879)	*spadicea* (Scop.) P. Karst. (1879)	*spadicea* (Scop.) P. Karst. (1879)	Kétvölgy	T. Zagyva	FM208879
H34	*conica* var. *conica* (Scop.) P. Kumm. (1871)	*conica* var. *conica* (Scop.) P. Kumm. (1871)	*conica* var. *conica* (Scop.) P. Kumm. (1871)	Kétvölgy	T. Zagyva	FM208880
H35	*irrigata* (Pers.) Bon (1976)	*irrigata* (Pers.) Bon (1976)	*irrigata* (Pers.) Bon (1976)	Kétvölgy	T. Zagyva	FM208881
H36	*conica* var. *conica* (Scop.) P. Kumm. (1871)	*conica* var. *conica* (Scop.) P. Kumm. (1871)	*conica* var. *conica* (Scop.) P. Kumm. (1871)	Székesfehérvár	T. Zagyva	FM208882
H37	*ceracea* (Wulfen) P. Kumm. (1871)	*ceracea* (Wulfen) P. Kumm. (1871)	*ceracea* (Wulfen) P. Kumm. (1871)	Budakalász	L. Albert	FM208883
H38	*cantharellus* (Schwein.) Murrill (1911)	*cantharellus* (Schwein.) Murrill (1911)	*cantharellus* (Schwein.) Murrill (1911)	Lászlótanya	L. Albert	FM208884
H39	*nitrata* (Pers.) Wünsche (1877)	*murinacea* (Bull.) M.M. Moser (1967)	*nitrata* (Pers.) Wünsche (1877)	Lászlótanya	L. Albert	FM208885
H40	*miniata* (Fr.) P. Kumm.	*miniata* (Fr.) P. Kumm.	*miniata* (Fr.) P. Kumm.	Budakalász	L. Albert	FM208886
H41	*laeta* var. *laeta* (Pers.) P. Kumm. (1871)	*laeta* var. *laeta* (Pers.) P. Kumm. (1871)	*laeta* (Pers.) P. Kumm. (1871)	Budakalász	L. Albert	FM208887

H43	*intermedia* (Pass.) Fayod (1889)	*intermedia* (Pass.) Fayod (1889)	*intermedia* (Pass.) Fayod (1889)	Perecse	L. Albert	FM208888
H44	*citrinovirens* (J.E. Lange) Jul. Schäff. (1947)	*citrinovirens* (J.E. Lange) Jul. Schäff. (1947)	*citrinovirens* (J.E. Lange) Jul. Schäff. (1947)	Perecse	L. Albert	FM208889
H47	*laeta* var. *laeta* (Pers.) P. Kumm. (1871)	*laeta* var. *laeta* (Pers.) P. Kumm. (1871)	*laeta* (Pers.) P. Kumm. (1871)	Feldbach	L. Albert	FM208890
H48	*lacmus* (Schumach.) P.D. Orton & Watling (1969)	*lacmus* (Schumach.) P.D. Orton & Watling (1969)	*lacmus* (Schumach.) P.D. Orton & Watling (1969)	Feldbach	L. Albert	FM208891
H49*	*splendidissima* (P.D. Orton) M.M. Moser (1967)	*splendidissima* (P.D. Orton) M.M. Moser (1967)	*punicea* var. *splendidissima* (P.D. Orton) Krieglst. (1992)	Felsőszölnök	L. Albert	FM208892
H51	*persistens* var. *konradii* (R. Haller Aar.) Boertm. (1995)	*konradii* R. Haller Aar. (1955)	*persistens* (Britzelm.) Singer (1940)	Kunbaracs	K. Halász	FM208893
H52	*pratensis* var. *pallida* (Cooke) Arnolds (1985)	*berkeleyi* P.D. Orton & Watling (1969)	*pratensis* (Pers.) Bon (1976)	Kétvölgy	T. Zagyva	FM208894
H53	*psittacina* var. *psittacina* (Schaeff.) P. Kumm. (1871)	*psittacina* (Schaeff.) P. Kumm. (1871)	*psittacina* var. *psittacina* (Schaeff.) P. Kumm. (1871)	Apátistvánfalva	T. Zagyva	FM208895
H54	*flavipes* (Britzelm.) Arnolds (1989)	*flavipes* (Britzelm.) Arnolds (1989)	*lacmus* (Schumach.) P.D. Orton & Watling (1969)	Puchberg	H. Pidlich-Aigener	FM208896
H55	*colemanniana* (A. Bloxam) P.D. Orton & Watling (1969)	*colemanniana* (A. Bloxam) P.D. Orton & Watling (1969)	*lacmus* (Schumach.) P.D. Orton & Watling (1969)	Puchberg	H. Pidlich-Aigener	FM208897
H57	*punicea* (Fr.) P. Kumm. (1871)	*punicea* (Fr.) P. Kumm. (1871)	*punicea* var. *punicea* (Fr.) P. Kumm. (1871)	Felsőszölnök	T. Zagyva	FM208898
H58	*turunda* sensu Lange [Fl. Ag. Dan. 5: 27 & pl. 168H (1940)]	*turunda* sensu Lange [Fl. Ag. Dan. 5: 27 & pl. 168H (1940)]	*coccineocrenata* (P.D. Orton) M.M. Moser (1967)	Farkasfa	Á. Zöld-Balogh	FM208899
	Hygrocybe canescens (A.H. Sm. & Hesler) P.D. Orton			GenBank		DQ486685
	Humidicutis marginata (Peck) Singer			GenBank		DQ490625
outg.	*Lactarius semisanguifluus* R. Heim & Leclair			GenBank		AF140268
outg.	*Lactarius scrobiculatus* (Scop.) Fr.			GenBank		AF140263
outg.	*Lactarius quieticolor* Romagn.			GenBank		AF140269
outg.	*Pleurotus ostreatus* (Jacq.) P. Kumm.			GenBank		EF514248

Cuphophyllus species, plus species of *Gliophorus* (*G. irrigatus*, *G. laetus* and *G. psittacinus*) and *Neohygrocybe* (*N. nitrata*) presented here as genus *Cuphophyllus*, sections *Neohygrocybe* and *Glutinosae*, and the species *H. calyptriformis*. In a comprehensive multilocus analysis of *Agaricales*, Matheny et. al (2006) demonstrated a monophyletic origin of *Hygrophoraceae* if certain genera traditionally treated in *Hygrophoraceae* (e.g., *Camarophyllopsis* and *Neohygrophorus*) were excluded, while other genera previously considered to belong to the *Tricholomataceae* were included (e.g., *Pseudoarmillariella*). They suggested the rehabilitation of genus names *Camarophyllus*, *Gliophorus* and *Humidicutis*. Clade B includes section *Coccineae* and subgenus *Hygrocybe* except for *H. calyptriformis*.

Especially in the taxa of subg. *Hygrocybe*, differences in the microscopic properties alone cannot be considered as sufficient for the distinction among the taxa. Moreover, attempts that were based solely on microscopic features of dried sporophores (e.g. between *H. ceracea*, *H. constrictospora* and *H. insipida*) often failed. The only hope for unambiguous identification in these cases is if certain features of the fresh sporophores had already been fixed, noted, recorded, or registered at the habitat. There are many taxa at the same time, that could be safely separated based on their macro- and/or microscopic features after decades of storage. They were questioned as separate species, or taxa only in the last decade, primarily due to Boertmann's examinations (Boertmann 1995).

OVERVIEW OF PROBLEMS OF CLASSIFICATION

While there are distinct circumscriptions of the genus *Hygrocybe* (subgenera and sections), some species are difficult to define and often not determined unambiguously. This is caused not only by weather related variations (changes in colour), but also by the lack of molecular taxonomical investigations (Bresinsky 2008). Considering the multidimensional taxonomic problems typical of the genus, below we compare and analyze the results based on the previous taxonomic notions, within the genus, by taxa.

Subgenus *Cuphophyllus* Donk

Section Cuphophyllus

Resolving clade A, section *Cuphophyllus* proves to be a mono- or polyphyletic group, depending on the applied method. The two subsections within section *Cuphophyllus* do not seem to segregate, and bootstrap support for basal branches was weak. Differentiation of subsections *Cuphophyllus* and *Virginei* on the basis of morphological features often raises difficulties.

Subsection *Cuphophyllus*

Although subsection *Cuphophyllus* has a dry pileus surface and shorter spores (5–6 µm) than in subsection *Virginei*, misidentifications occur in numerous cases, as among *H. virginea* and certain specimens of *H. pratensis* var. *pallida*.

Subsection *Virginei* Bataille

Flavipes aggregate: *flavipes*, *lacmus*

Hygrocybe lacmus, *H. flavipes* and *H. colemanniana* do not vary much microscopically, but DNA sequence comparisons reveal large sequence differences among them. According to Boertmann (1995), they should appear as separate species in the future, primarily based on macromorphological features, and secondly due to their minimal and inconstant spore size differences. Krieglsteiner (2000) claimed that neither macro- nor microscopic properties can be used for the reliable differentiation among the three above mentioned taxa. On our phylogenetic trees *H. lacmus* and *H. flavipes*

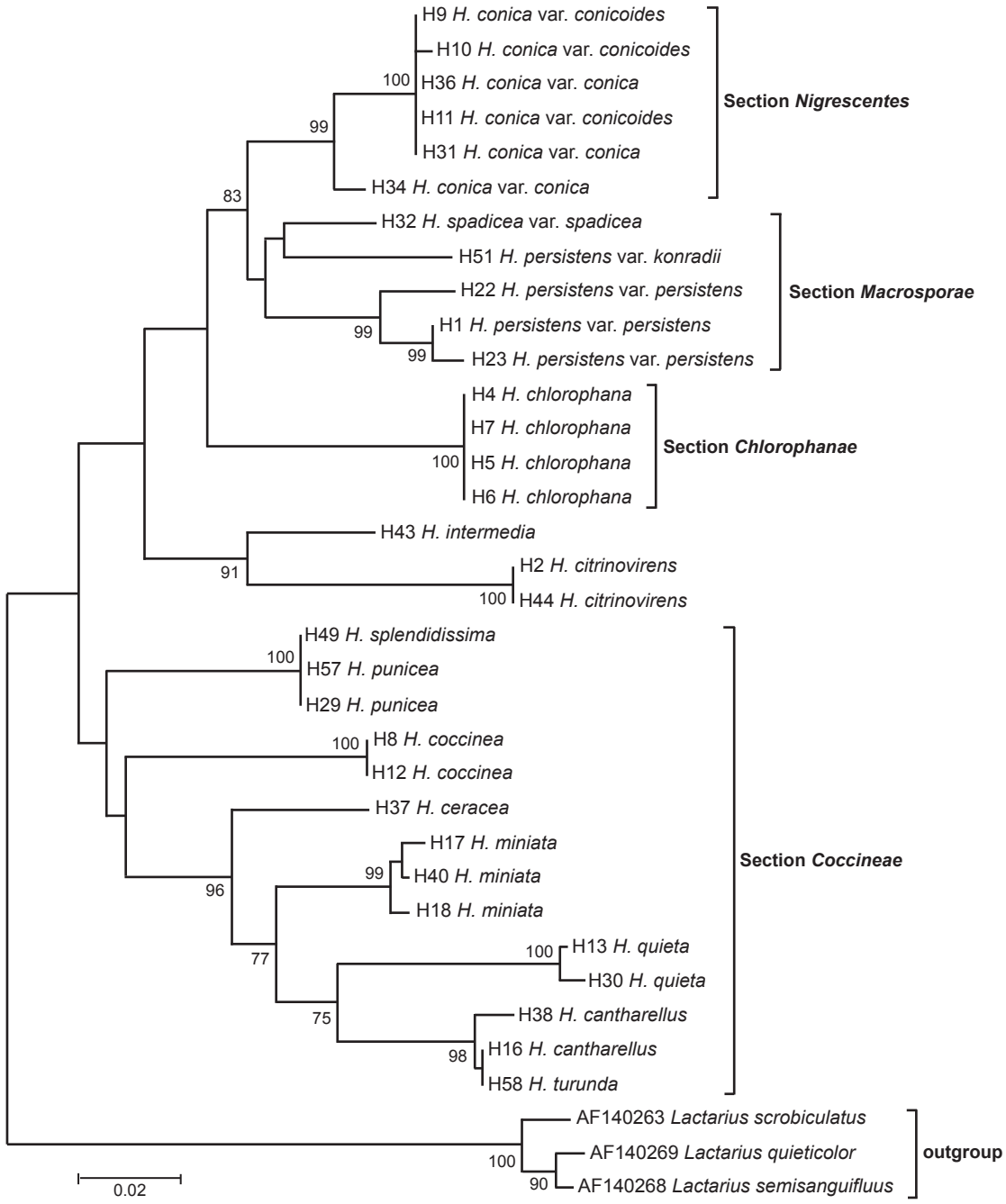

Fig. 3 Evolutionary relationships of 35 taxa of clade B. Neighbour-joining consensus tree inferred from 1 000 replicates. Branches corresponding to partitions reproduced in less than 70 % bootstrap replicates are collapsed. Bootstrap values (% of 1 000 replications) are given for selected nodes. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Outgroup was three species of genus *Lactarius*.

constitute a tight monophyletic group (bootstrap value, bt = 74%), which may be characterized by blue tints in the sporophore.

Virginei aggregate: *C. colemanniana*, *C. fuscescens*, *C. ochraceopallida*, *C. virginea*

While the branch containing *Cuphophyllus* species is rather homogeneous, the *C. pratensis* and *C. virginea* groups are separate, but with low bootstrap values. The distal group in this clade (H19, H20, H21, H24; Fig. 4) comprises three taxa (*C. virginea*, and two taxa identified as *C. ochraceopallida*), which together form a well-supported clade (bt = 77 %). *Cuphophyllus colemanniana* fell in the *Virginei* clade, but without bootstrap support. Boertmann (1995) recommended taxonomic revision of this group (*H. colemanniana*, *H. fuscescens*, *H. ochraceopallida*, *H. russocoriacea*, *H. virginea)*. Candusso (1997) discussed three sections within subg. *Cuphophyllus*. Sect. *Virginei* comprises *H. virginea* and its varieties with *H. canescens* and *H. berkeleyi*, sect. *Cuphophyllus* incorporates *H. pratensis* and *Flavipes* aggregate among others. Though bootstrap support was lacking (Fig. 4), the *C. flavipes* aggregate does not appear to belong to the same clade as *C. pratensis*, nor does *C. canescens* fall within the *Virginei* clade. None of the examined species belongs to the third section *Oreocybe*.

Subgenus *Pseudohygrocybe* Bon

Section *Neohygrocybe* Herink

The uniquely short ITS region of *H. nitrata* significantly separates this species from any other taxa, but additional sequences would be needed to confirm this result and for

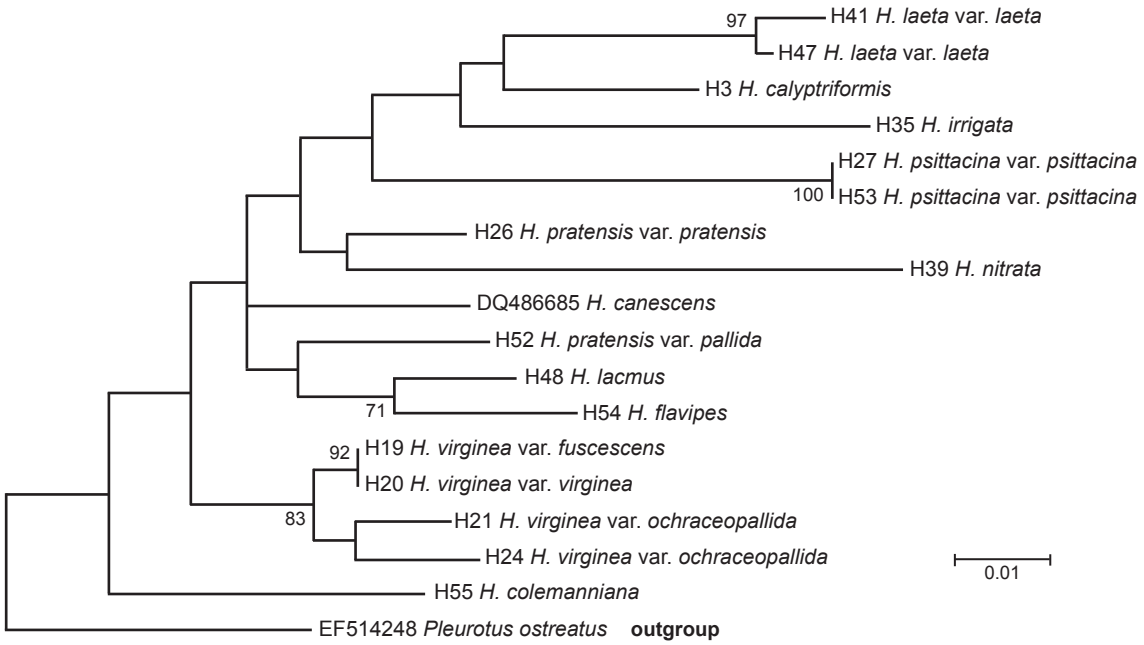

Fig. 4 Evolutionary relationships of 17 taxa of clade A. Neighbour-joining consensus tree constructed using MEGA4. The scale bar indicates the number of base substitutions per site. Bootstrap support values from 1 000 replicates are shown at the nodes. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). The tree was rooted to *Pleurotus ostreatus* (GenBank EF514248).

group definition. Bresinsky & Kronawitter (1986) revealed the correspondence of sect. *Neohygrocybe* with their pigment group 3.0, which lacks muscaflavins. This result seems to confirm the separation of *Neohygrocybe* genus by Bresinsky (2008).

Section *Glutinosae* Kühner

All three examined species (*Gliophorus laetus*, *Gliophorus unguinosus* and *G. psittacinus*) are strictly monophyletic with high bootstrap values. In contrast, nucleotide homology among the species is relatively low. These three taxa can be differentiated morphologically without difficulties. The common features of the taxa are the glutinosity of the sporophore and the medallion clamp connections at the base of the basidia. Matheny et al. (2006), based on their phylogenetic study, proposed to resurrect the *Gliophorus laetus* (Pers.: Fr.) Herink as the valid name of this taxon instead of *H. laeta*. *Hygrocybe psittacina* was recombined in *Gliophorus* by Herink (*G. psittacinus* (Schaeff.) Herink). Recently, *H. irrigata* was also proposed to be transferred to *Gliophorus* (Bresinsky 2008), though *H. unguinosa*, which Boertmann considers a synonym, has already been combined in *Gliophorus*. The dilemma with the combination *Gliophorus unguinosus* (Fr.) Kovalenko is that there are several species in Europe, according to their ITS sequences, they are nearly identical morphologically, and we don't know which ones correspond to which name (or don't correspond to any name).

***Psittacina* aggregate**: *laeta*, *psittacina*

Greenish colour appears near the apex of the stem of both taxa. *Hygrocybe psittacina* has green, yellowish green colouring, *H. laeta* var. *laeta* can show somewhat olive green shade at the same part sometimes (Candusso 1997, Beisenherz 2002). Beisenherz (2002: 53) claimed that red pigment masks the blue and green pigments. Bresinsky & Kronawitter (1986) demonstrated that *H. psittacina*, *H. laeta*, moreover *H. sciophana* (Fr.) Wünsche do not contain any muscaflavin pigments. They are believed to have caretenoid pigments, readily apparent in dried specimens. Accordingly, all our examined species of sect. *Glutinosae* are integrated in clade A, among the muscaflavin-free species. The feature of *H. psittacina* is a disappearing green colour affected by solar radiation or drying. Moreover, Boertmann (1995) described a variety without any green colour on page 81. He affirms that the wide colour palette may have evolved either due to the effect of edaphic factors, like scrubby type on the fixed sand dunes, or by unique variations. Inside the section, only *H. laeta* has decurrent trama and ixocheilocystidia, as well as pale rose-coloured exsiccata. The others have adnexed gills and turn bright orange when dried. Although *H. laeta* samples H41 and H47 represent very distant populations, they showed a minimal difference in ITS regions. *Hygrocybe laeta* f. *pseudopsittacina* (H41) differing in few morphological features from *H. laeta* f. *laeta*, has an ITS region practically identical with that of the type variety. Candusso's sect. *Glutinosae* incorporates *H. insipida*, while Boertmann does not. Our molecular phylogeny is in agreement with Boertmann on this point. *Hygrocybe psittacina*, *H. sciophana* and *H. sciophanoides* cannot be distinguished based on their microscopical properties; Boertmann (1995) accepted only three valid variants of *H. psittacina*.

Section *Coccineae* Fayod

This section seems to be monophyletic according to our molecular examinations. The species of the three subsections (*Coccineae*, *Siccae* and *Squamulosae*) do not form a separated genetic group. Matheny et al. (2006) as well as Bresinsky (2008) discussed all species of this section among *Hygrocybe* s.str.

Subsection *Coccineae* (Bataille) Singer

This subsection is consistent with Boertmann's classification, though it represents a grade rather than a clade (Fig. 3). The two *H. coccinea* samples had identical sequences (bt = 100 %). *Hygrocybe punicea* and *H. splendidissima* proved to be identical based on ITS sequences. According to Krieglsteiner (2001), *H. splendidissima* seems to be a variety of *H. punicea*. *Hygrocybe ceracea* was a well-supported species, having higher ITS homology with *H. miniata* than with other species.

Subsection *Siccae* Boertm.

Subsection *Siccae* proved to be a polyphyletic group, although, only *H. splendidissima* and *H. quieta* were

examined. Two samples of *H. quieta* constitute a branch (bt = 80 %) together with species with decurrent lamellae. According to Moser (1983), *H. konradii* and *H. obrusseus* ss. Cooke, Konrad and Maubl. are synonyms, whereas, Candusso (1997) and Krieglsteiner (2001) considered *H. quieta* to be a synonym of *H. obrussea*. However, our *H. splendidissima* (H49) ITS sequence was identical to *H. punicea* (H29 and H57).

Subsection *Squamulosae* (Bataille) Singer

In the phylogenetic trees, the two aggregates separate into distant clades. One of them incorporates the species *H. cantharellus* and *H. turunda* with decurrent lamellae, the other contains *H. miniata* with adnexed lamellae.

Two controversial aggregates are discussed:

1. **Lepida aggregate**: *H. coccineocrenata*, *H. lepida*, *H. turunda*

Hygrocybe lepida (H16 and H38) and *H. turunda* (H58) formed a well-supported clade characterized by decurrent lamellae and a minutely squamulose pileus. Macroscopically, *H. coccineocrenata* and *H. turunda* caps are covered with dense black scale, while *H. lepida* is not more scaled than *H. calciphila*, *H. lepida* and *H. turunda*, have been distinguished by macroscopic characters, and they remain distinguishable in storage. However, nobody succeeded in observing significant microscopic difference among them.

2. **Miniata aggregate**: *miniata* (adnate or free lamellae)

Hygrocybe miniata samples (H17, H18 and H40) were almost identical genetically and form a well-separable isolated branch (bt = 98).

Subgenus *Hygrocybe*

Subgenus *Hygrocybe* proved to be a monophyletic group based on our molecular examinations. A well-supported clade (bt = 82 %) unites all samples of sections *Hygrocybe* and *Chlorophanae*, though *H. intermedia* appears with *H. citrinovirens* on a separate, basal branch within clade B (bt = 89 %). *Hygrocybe calyptriformis* appeared in sect. *Glutinosae* in clade A rather than clade B where it has traditionally been placed because of the long lamellar trama hyphae exceeding 1 000 µm and the conical pileus shape. Bresinsky (2008) showed that this species and other members of sect. *Glutinosae* lack the water soluble vivid coloured muscaflavin pigments of *Hygrocybe* s.str. in clade A, so he established a new genus, *Porpolomopsis*, for *H. calyptriformis*.

Section *Microsporae* Boertm.

Two samples of *H. citrinovirens* form a well supported branch (bt = 100 %) that is far separated from all other species except *H. intermedia* in clade B. All four *H. chlorophana* samples were genetically identical and formed a well-separable supported branch (bt = 100 %). In the course of our macroscopic and microscopic examinations sample H7 can be determined as *H. chlorophana* var. *aurantiaca*. It was identical with other *H. chlorophana* samples.

Section *Nigrescentes* (Bat.) Candusso

Hygrocybe conica (Matheny et al. 2006), then later all other members (Bresinsky 2008) were discussed among *Hygrocybe* s.s. Boertmann (1995) uses a wider species concept than Bon (1990) in the black-staining group, so he does not recognize *H. riparia*, *H. tristis* and *H. veselskyi* as valid taxa.

***Conica* aggregate**: *H. conica*, *H. conicoides*, *H. olivaceonigra*, *H. riparia*, *H. tristis*, *H. veselskyi*

Three samples each of *H. conica* var. *conica*, and var. *conicoides* formed a monophyletic group. Five of the six ITS sequences were identical. This corresponds with Boertmann's (1995) findings, who considered blackening wax caps as types of one species without microscopical differences. The sequence of H34, identified as *H. conica* var. *conica*, however, represents a separate species. Except *H. olivaceonigra*, Boertmann acknowledges only *H. conica* as a separate species, primarily on the basis of the characteristic of spores, considering the rest as synonyms or forms of *H. conica*.

Section *Macrosporae* Haller ex Bon

H. acutoconica, *H. aurantiolutescens*, *H. konradii*, *H. persistens*, *H. subglobispora*

According to Boertmann the first two taxa may be only synonyms, and he treats *H. konradii* as a variant of *H. persistens* and *H. subglobispora* as a form of *H. persistens* var. *konradii* under the name *H. persistens* var. *konradii* f. *subglobispora*. Three of our *H. persistens* samples formed a monophyletic group (bt = 93 %), while *H. spadicea* and *H. konradii* proved to be separated taxa. The appearance of *H. spadicia* among the non-staining species was somewhat surprising. Arnolds (1980), Bon (1990) (*H. aurantiolutescens* var. *parapersistens*, *H. persistens*) and Moser (1983) (*H. persistens*) recognize *H. persistens* as a collective species, which includes at least two species and several varieties and forms.

Summarizing our results, it can be stated that the subgenera based on morphological differences can be well distinguished in the phylogenetic tree. It is especially relevant to subg. *Hygrocybe.* Identity or high level similarity were demonstrated in cases of species that were considered as closely related taxa, e.g. *H. conica* aggregate.

Our clearly separable groups based on full ITS sequences support Bresinsky's (2008) view that genus *Hygrocybe* s.l. should be narrowed, and that *H. calyptriformis*, all examined members of subg. *Gliophorus* (*H. irrigata*, *H. laeta*, *H. nitrata*, *H. psittacina*) and subg. *Cuphophyllus* should be excluded from the genus *Hygrocybe*. Based on these results we suggest further research using additional DNA markers that are useful at the intergeneric level to re-evaluate the taxonomy of former *Hygrocybe* species due to the limitations of ITS at higher taxonomic levels. A second marker with much less variation among the species also needs the corrobation regarding Bresinsky's concept of the reclassification of the genus.

In the future it would be necessary to extend the molecular examinations to as many species as possible so that the relationships among *Hygrocybe* taxa can be more precisely described. As the environmental burden on *Hygrocybe* habitat is gradually becoming heavier and heavier, the chance is diminishing to gain enough samples to conduct comprehensive surveys covering the whole range of diversity with satisfying number of samples. Several authors (Boertmann & Rald 1991, Boertman 2000) consider *Hygrocybe* species as indicator organisms whose abundance and diversity indicate undisturbed habitats. The improved taxonomic knowledge of the genus *Hygrocybe* could considerably contribute to conservation biology research on these natural grasslands of great importance.

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