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The phylogenetic position of *Haasiella* (Basidiomycota, Agaricomycetes) and the relationships between *H. venustissima* and *H. splendidissima*

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Abstract: Based on a combined ITS-LSU rDNA analysis, the omphalinoid genus *Haasiella* is shown to be part of the Hygrophoroid clade and sister to *Hygrophorus*. *H. venustissima* and *H. splendidissima* are conspecific; *H. splendidissima* is the tetrasporic/clamped and heterothallic form of *H. venustissima*.

Key words: Agaricales, apomictic taxa, *Chrysomphalina*, Hygrophoraceae, *Omphalina*, omphalinoid fungi

INTRODUCTION

In recent years phylogenetic analyses based on rDNA molecular characters have provided fresh conceptual insight into the taxonomy of taxa traditionally assigned to *Omphalina* Qué. s.l. (small agarics with a convex to deeply umbilicate pileus, central stipe, thin context, decurrent lamellae, white spore print and thin-walled unornamented spores (Bigelow 1970; Singer 1986; Norvell et al. 1994; Lutzoni 1997; Redhead et al. 2002a, b). Molecular-phylogenetic research in fact has shown that the genus *Omphalina* s.l. is a polyphyletic and heterogeneous assemblage (the so-called omphalinoid taxa, Redhead 1986; Lutzoni 1997; Redhead et al. 2002a, b), which in turn has spawned the recognition of several generic segregates of *Omphalina*. These taxa are distributed across three major clades: (i) the Hygrophoroid clade (*Arrhenia* Fr., *Chrysomphalina* Cléménçon, *Chromosera* Redhead, Ammirati & Norvell, *Lichenomphalia*

Redhead, Lutzoni, Moncalvo & Vilgalys, *Melanomphalia* M.P. Christ. p.p. [Lutzoni and Vilgalys 1995, Lutzoni 1997; Redhead et al. 2002a; Aime et al. 2005; Matheny et al. 2006; Lawrey et al. 2009; Binder et al. 2010; Ovrebø et al. 2011]); (ii) the Hymenochaetoid clade (*Blasiphalia* Redhead, *Cantharellopsis* Kuyper, *Contumyces* Redhead, Moncalvo, Vilgalys & Lutzoni, *Gyroflexus* Raitheh., *Loreleia* Redhead, Moncalvo, Vilgalys & Lutzoni, *Rickenella* Raitheh. [Moncalvo et al. 2000, 2002; Redhead et al. 2002b; Palice et al. 2005; Larsson et al. 2006; Larsson 2007]); (iii) the Tricholomatoid clade (*Omphalina* s.s. including the type species *O. pyxidata* [Bull.] Qué. [Vizzini et al. 2011a] and *Omphalina giovanellae* [Bres.] Singer [Moreno et al. 2007]).

The rare omphalinoid species belonging to the genus *Haasiella* Kotl. & Pouzar (typified by *H. venustissima* [Fr.] Kotl. & Pouzar) have not been studied molecularly so far. They are characterized by monomitic basidiomes with a pink-orange pigmentation due to an accumulation of carotenoid pigments (Arpin 1966; Arpin and Fiasson 1971; Tyler 1971; Valadon 1976), a lignicolous habitat, a yellow-pinkish spore-print, a gelatinized pileipellis, a thickening hymenium, elongated basidia, a bidirectional lamella trama and metachromatic spores in cresyl blue (Kotlaba and Pouzar 1966; Horak 1968; Cléménçon 1982, 2004; Kost 1986; Kuyper 1986, 1995; Singer 1986; Reijnders and Stalpers 1992; Norvell et al. 1994). *Haasiella* has been reported only from Europe thus far. The only two recognized species, *H. venustissima* and *H. splendidissima* Kotl. & Pouzar, are distinct on the basis of the number of sterigmata and presence/absence of clamp connections.

Based on a combined ITS and LSU rDNA molecular analyses, the aim of the paper was (i) to infer the phylogenetic position of *Haasiella* within the Agaricomycetes Doweld and (ii) to investigate whether the morphologically closely related *H. venustissima* and *H. splendidissima* are conspecific (syntaxic) or distinct taxa.

MATERIALS AND METHODS

Herbarium specimens were checked microscopically to determine whether they were correctly classified (see Consiglio et al. 2012). Herbarium abbreviations are according to Thiers (2011). Authorial citations follow the Index Fungorum Authors of Fungal Names (<http://www.indexfungorum.org/authorsoffungalnames.htm>).

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TABLE I. *Haasiella* and *Omphalina* collections used for molecular analyses

Species	GenBank accession numbers		Source, country, date and collector
	ITS	LSU	
<i>Haasiella venustissima</i>	JN944393	JN944394	E.C. 08191, ITALY, Rio Secco (Mombaroccio, PU), 15/10/2008, leg. G. Consiglio & M. Maletti
<i>Haasiella splendidissima</i> 1	JN944395	JN944396	JVG1071013 - 1, SPAIN, Pardines, 13/10/2007, leg. J. Vila
<i>Haasiella splendidissima</i> 2	JN944397	—	WU 5654, AUSTRIA, Kärnten, Kraig, Wimitzbach-Tal, 26/09/1986, leg. R. Schütz & A. Hausknecht
<i>Haasiella splendidissima</i> 3	JN944398	JN944399	Herbarium Roux n. 3666, MOLDAVIAN REPUBLIC, National Park Codrii, 29/09/2001, leg. S. Manic
<i>Haasiella splendidissima</i> 4	JN944400	JN944401	Herbarium Roux n. 4044, FRANCE, Gresse-en-Vercors (Isère), 6/10/2003, leg. V. Dumas
<i>Haasiella splendidissima</i> 5	—	—	PR 613781 (holotype), SLOVAKIA, Banská Bystrica, 27/09/1965, leg. Z. Pouzar, F. Kotlaba & D.A. Reid
<i>Omphalina pyxidata</i>	JN944402	JN944403	TO AV98, ITALY, Torino, Pinerolo, 10/10/2010, leg. A. Vizzini

DNA extraction, PCR amplification and DNA sequencing.— Genomic DNA was isolated from 1 mg of herbarium specimens (TABLE I) with the DNeasy Plant Mini Kit (QIAGEN, Milan, Italy) according to the manufacturer's instructions. Universal primers ITS1F/ITS4 were used for the ITS region amplification (White et al. 1990; Gardes and Bruns 1993) and primers LR0R/LR7 (Vilgalys and Hester 1990; Vilgalys lab unpubl, <http://www.botany.duke.edu/fungi/mycolab>) for the LSU amplification. Amplification reactions were performed in a PE9700 thermal cycler (Perkin-Elmer, Applied Biosystems) following Vizzini et al. (2011b). PCR products were purified with the AMPure XP kit (Beckman) and sequenced by DiNAMYCODE srl (Turin, Italy) and Macrogen Inc. (Seoul, Republic of Korea). Sequencing reactions were performed in both forward and reverse directions on the PCR products. Sequences were assembled and edited with the phred/phrap/consed software suite. The sequences were submitted to GenBank (accession numbers are reported in TABLE I, FIG. 1), and the alignments and phylogenetic tree are available at TreeBASE (www.treebase.org) under accession number S12079.

Sequence alignment and phylogenetic analysis.—The sequences obtained were checked and assembled with Geneious 5.3 (Drummond et al. 2010) and compared to those available in GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) with the BLASTN algorithm. Based on BLASTN results, sequences were selected according to the outcomes of recent phylogenetic studies on Agaricales Underw. (Matheny et al. 2006; Binder et al. 2010; Ovrebo et al. 2011; Seitzman et al. 2011). A combined analysis of ITS and LSU sequences was carried out with sequences from the same strain or specimen. *Gymnopus dryophilus* (Bull.) Murrill (DQ241781 and AY640619) was used as outgroup taxon. The alignments were generated with MAFFT (Katoh et al. 2002) with default conditions for gap openings and gap extension penalties. The sequences alignment, its manual adjustment and the best-fit models estimation follow Vizzini et al. (2011b). The GTR + G substitution model and a partitioned matrix were used in all analyses.

Molecular-phylogenetic analyses were performed with Bayesian inference (BI), maximum likelihood (ML) and maximum parsimony (MP) approaches. BI using Monte Carlo Markov chains (MCMC) was carried out with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). Four incrementally heated simultaneous MCMCs were run 10 000 000 generations under model assumption. Trees were sampled every 1000 generations resulting in an overall sampling of 10 001 trees. The burn-in was evaluated with Tracer 1.5 (Rambaut and Drummond 2007); the first 25% trees was discarded. For the remaining trees a majority rule consensus tree showing all compatible partitions was computed to obtain estimates for Bayesian posterior probabilities (BPP). Branch lengths were estimated as mean values over the sampled trees. Only BPP values exceeding 50% are reported in the trees. This Bayesian analysis was repeated three times, always with random starting trees and random starting values for model parameters to test the independence of the results from the revisiting of the prior topologies during chain growth (Huelsenbeck et al. 2002). ML estimation was performed through RAxML 7.0.4 (Stamatakis 2006) with 1000 bootstrap replicates (Felsenstein 1985) using the GTRGAMMA (in both analyses) algorithm to perform a tree inference and search for a good topology. Support values from bootstrapping runs (MLB) were mapped on the globally best tree with the -f a option of RAxML and -x 12345 as a random seed to invoke the novel rapid bootstrapping algorithm. MP analysis was performed with PAUP* 4.0b10 (Swofford 2002) using the heuristic search mode with 100 random addition sequence replicates and tree bisection reconnection (TBR) branch swapping but keeping only 10 trees per replicate to discover possible islands of maximum parsimony. All character states were treated as unordered and equally weighted. Gaps were treated as missing data. Branch robustness was estimated by nonparametric bootstrapping (Felsenstein 1985) with 500 replicates with 10 random addition replicates per bootstrap. Only bootstrap values exceeding 50% are visualized in the tree (MPB). Support values for major clades that are supported in BI, ML and MP are visualized in the resulting tree. Pairwise percent identity

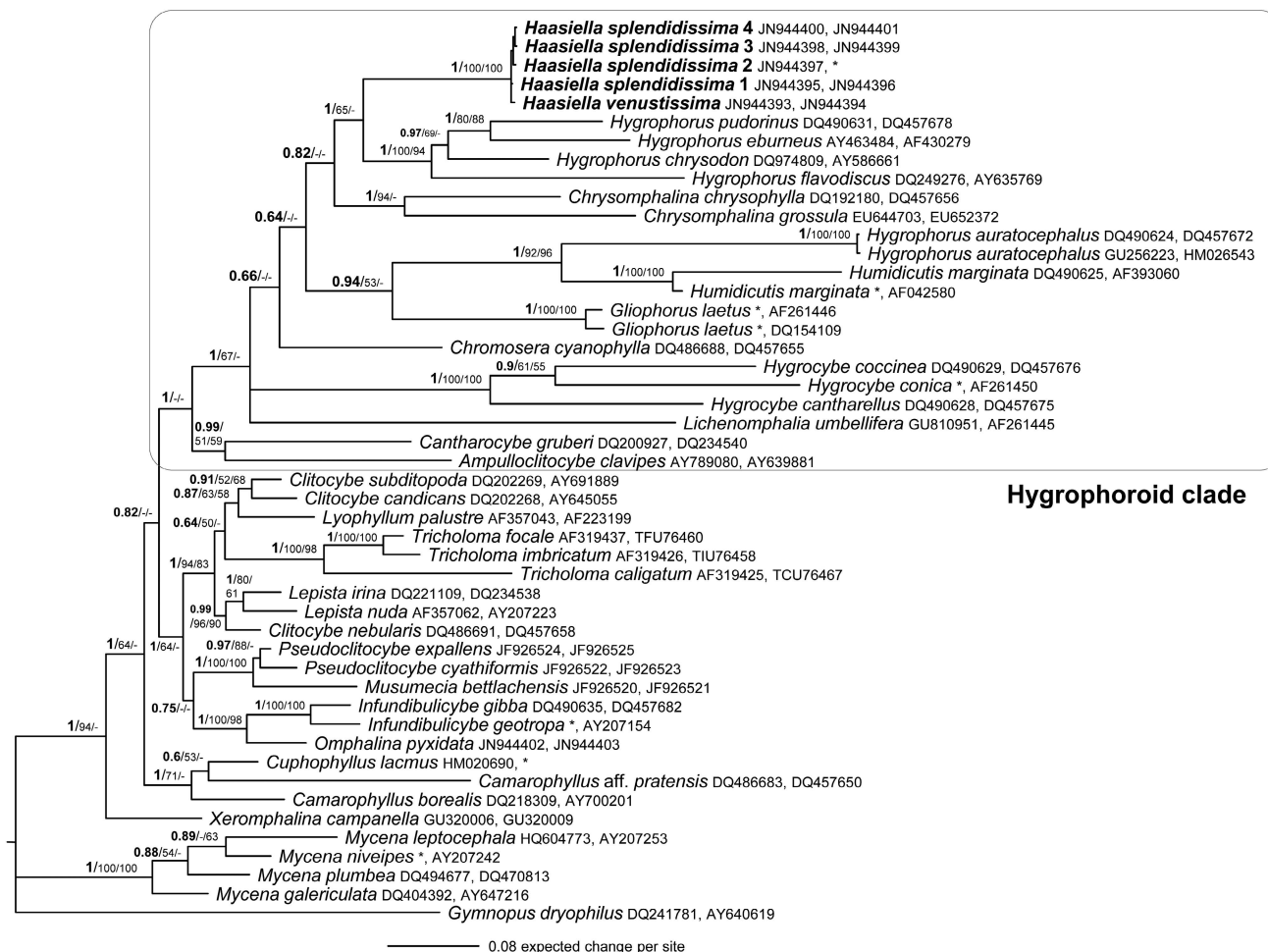


FIG. 1. Bayesian phylogram obtained from the combined ITS-LSU sequence alignment. Support values for clades that are supported in either the Bayesian (posterior probabilities values = BPP), maximum likelihood (ML bootstrap percentage = MLB) and maximum parsimony (MP bootstrap percentage = MPB) analyses are indicated. BPP greater than 0.50 and MLB/MPB greater than 50% are above branches. Numbers (1–5) refer to the *H. splendidissima* collections (TABLE I).

values for the *Haasiella* sequences (see results) were calculated with MEGA 5.0 (Tamura et al. 2011).

RESULTS

Amplification and sequencing of the ITS and LSU rDNA regions were successful for all the specimens selected for molecular study (TABLE I), with the exception of *H. splendidissima* 5 (type specimen was too old). No amplification of the LSU region was obtained from the *H. splendidissima* 2 specimen. The PCR product was 589–675 bp (ITS) and 1064–1085 bp (LSU). The ITS data matrix comprises a total of 42 sequences (including 36 from GenBank). This dataset is 1070 base pairs long and contains 572 (53.5%) variable sites. Of these, 399 (44.0%) are parsimony informative. The LSU data matrix comprises a total of 46 sequences (including 41 from GenBank). This dataset is 1451 base pairs long and contains 394

(27.2%) variable sites. Of these, 255 (17.6%) are parsimony informative. The combined dataset comprises a total of 48 taxa (including 42 from GenBank) and is 2521 base pairs long. The topologies of the combined ITS and LSU Bayesian, maximum likelihood and maximum parsimony trees are congruent, and all the three analyses have reported high support values for most branches (FIG. 1). *Haasiella* clearly belongs to the Hygrophoroid clade. The genera that were recognized in the Hygrophoroid clade by Matheny et al. (2006), Binder et al. (2010), Ovrebo et al. (2011) and Seitzman et al. (2011) also belong to this clade in our analyses, with high bootstrap support, with the exception of the genus *Cuphophyllum* (Donk) Bon that falls outside the clade (FIG. 1). *Haasiella* sequences form a distinct clade (BPP 0.82) together with *Hygrophorus* Fr. and *Chrysomphalina* Cléménçon sequences. *Haasiella* clusters as sister to *Hygrophorus* (BPP 1 and MLB 65%), and *Chrysomphalina*

is basal to this clade. The four *H. splendidissima* collections and the one *H. venustissima* collection cluster together into a unique, strongly supported clade (BPP 1, MLB 100% and MPB 100%). The five *Haasiella* ITS sequences and the four *Haasiella* LSU sequences show a pairwise percent identity of 99.3 and 99.9, respectively. The *Haasiella/Hygrophorus/Chrysomphalina* clade is sister to the clade consisting of *Gliophorus/Humidicutis* and *Hygrophorus auratocephalus* (Ellis) Murrill.

DISCUSSION

Generic placement, Haasiella/Chrysomphalina connections and phylogenetic position.—Published as *Agaricus (Clitocybe) venustissimus* Fr. (Fries 1861) the taxon later was considered a *Clitocybe* (Karsten 1879), an *Omphalia* (Quélet 1886), a *Hygrophoropsis* (Haas 1958), a *Gerronema* (Singer 1961, 1964; Haas 1965), a “*Chrysomphalina*” (Haas 1962, invalid genus) and also an *Omphalina* s.l. by Lange (1981, 1992) and Ludwig (2001). However Kotlaba and Pouzar (1966) had established the genus *Haasiella* to accommodate *A. venustissimus* and the new species *H. splendidissima*. Singer (1975, 1986) recognized *Haasiella* but only as a section of his heterogeneous genus *Gerronema*, while Kühner (1980), Cléménçon (1982), Moser (1983), Kost (1986), Kuyper (1986, 1995), Norvell et al. (1994), Romagnesi (1995), Bon (1997), Horak (2005) and Elborne (2008) accepted it as a distinct genus.

Haas (1962) considered *Agaricus venustissimus* and *Agaricus chrysophyllus* Fr. to be very closely related and congeneric, due to the colored spore print, and invalidly established the genus “*Chrysomphalina* Haas” (typified by *C. chrysophylla*). These generic affinities among the two taxa were highlighted again by Raithelhuber (1973) with the new combination *Haasiella chrysophylla* (Fr.) Raithelh. Cléménçon (1982) validated the genus *Chrysomphalina* including the type, *C. chrysophylla* (Fr.) Cléménçon, and *Gerronema strobodes* (Berk. & Mont.) Singer but excluding *Haasiella* as a related but distinct genus.

Haasiella shares with *Chrysomphalina* a lignicolous habitat, a yellow-pinkish spore print, a thickening hymenium (production of basidia of different lengths on short subhymenial elements (Kühner 1928; Cléménçon 1982, 2004; Kost 1986), a bidirectional hymenial trama, elongated basidia and the presence of carotenoid pigments (Arpin 1966; Fiasson and Bouchez 1968; Arpin and Fiasson 1971; Gill and Steglich 1987), even though in *Haasiella* there is a majority of γ -carotenoids while in *Chrysomphalina* they are outnumbered by β -carotenoids (Fiasson and Bouchez 1968, Kühner 1980). *Haasiella* differs mainly in having thick-walled metachromatic spores and a

slightly gelatinized pileipellis (Kost 1986, Norvell et al. 1994). The two species of *Haasiella* so far recognized are said by some authors to be strictly terrestrial (e.g. Kost 1986, Norvell et al. 1994), but many collections were found on stumps or wood debris (Kotlaba and Pouzar 1966; Baiano and Forno 1983; Døssing 1980; Romagnesi 1995; Vila and Caballero 2007; Roux 2006; Dam 2007; Elborne 2008; Eyssartier and Roux 2011; Consiglio et al. 2012). *Haasiella* species are probably saprotrophic on wood and woody debris, according to these data and our observations.

In the pre-molecular era the *Haasiella/Chrysomphalina* genus pair was regarded as belonging to either the Tricholomataceae R. Heim ex Pouzar (Kühner 1980; Moser 1983; Singer 1986; Kuyper 1986, 1995; Bon 1997; Horak 2005) or, due to the presence of carotenoids and elongated basidia, to the Cantharellaceae J. Schröt. (Fiasson and Bouchez 1968; Norvell et al. 1994; Romagnesi 1995, 1996), a family well circumscribed also on the basis of these pigments (Fiasson 1968; Fiasson et al. 1970; Mui et al. 1998). Romagnesi (1996) even established the tribus Chrysomphalineae in the Cantharellaceae to accommodate the gilled agarics with carotenoids (*Haasiella*, *Chrysomphalina* and *Phyllotopsis* E.-J. Gilbert & Donk ex Singer; see Arpin 1966; Fiasson and Bouchez 1968; Fiasson 1969).

Recent DNA studies (Lutzoni 1997; Moncalvo et al. 2000, 2002; Redhead 2002a; Matheny 2006; Binder et al. 2010; Ovrebo et al. 2011) have highlighted that *Chrysomphalina chrysophylla* is closely related to the Hygrophoraceae Lotsy of the Hygrophoroid clade. Depending on taxon sampling, genetic loci studied and phylogenetic analysis methods used, *Chrysomphalina* has been resolved as sister to *Hygrocybe* (Fr.) P. Kumm. (Redhead et al. 2002a), sister to *Hygrophorus* (Moncalvo 2000, 2002, Binder et al. 2010), allied to other hygrophoroid genera (Lutzoni 1997), allied to *Hygrocybe* and *Chromosera* Redhead, Ammirati & Norvell (Matheny et al. 2006) and as sister to *Gliophorus laetus* (Ovrebo et al. 2011).

Based on the above molecular results, Knudsen and Vesterholt (2008), in *Funga Nordica*, classified *Chrysomphalina* in the Hygrophoraceae and *Haasiella* also was placed in the same family due to its morphological and pigment affinities with *Chrysomphalina*.

Our data support that *Haasiella* is a genus distinct from *Chrysomphalina* and that it belongs to the Hygrophoraceae where it is sister to *Hygrophorus* (FIG. 1). *Hygrophorus* shares with *Haasiella* the distant lamellae and elongated basidia but differs in the absence of carotenoid pigments (Gill and Steglich 1987; Lübken et al. 2006; Teichert et al. 2008), the non-metachromatic spores, the bilateral hymenophoral trama and the strictly terrestrial basidiomes (Singer 1986, Bon 1990).

Sporal metachromacy in *Haasiella* is a feature that is not shared by any other member of the Hygrophoroid clade. The character also is sparingly present in other agaricoid, gilled genera, being so far known only in the Leucocoprineae Singer (*Macrolepiota* Singer, *Leucogaricus* Locq. ex Singer, *Leucocoprinus* Pat., *Chlorophyllum* Masee [Singer 1986]) in the Agaricoid clade (Matheny et al. 2006) and for *Tricholoma cookeanum* Bertault & Malençon (Bon 1991) in the Tricholomatoïd clade (Matheny et al. 2006).

Gliophorus Herink and *Humidicutis* (Singer) Singer (+ *Hygrophorus auratocephalus*) are sister to the clade formed by *Chrysomphalina*, *Haasiella* and *Hygrophorus* (Fig. 1).

Interspecific relationships.—*Haasiella venustissima* is a rarely collected and infrequently illustrated species (Haas 1965; Nothnagel 1974; Døssing 1980; Baiano and Forno 1983; Kelderman 1996; Moser and Jülich 1997; Chiaffi and Surault 1996b; Gyosheva 2000; Vila and Caballero 2007; Dam 2007; Consiglio et al. 2012), and it has been included in the red data lists of rare and threatened macromycetes of several European countries (e.g. Senn-Irlet et al. 2007; Laurent-Dargent 2009; Karasch and Hahn 2009). It is well characterized by having mono- and bisporic basidia and lacking clamp connections. According to the karyological studies by Lamoure (in Kühner 1980), Kost (1986) and Chiaffi and Surault (1996a), the spores, young basidia and subhymenial hyphae are mononucleate, a feature indicating an apomictic way of life. Such a life cycle is not uncommon in Agaricales (Whitehouse 1949; Lamoure 1957, 1960; Kühner 1980; Kües et al. 2011) and known for species in *Mycena* (Kühner 1938, Lamoure 1955), *Omphalina* s.l. (Lamouré 1968, 1969), *Coprinus* s.l. (Lange 1952), *Agaricus* (Volkova et al. 2003, Kamzolkina et al. 2006), *Favolaschia* (Vizzini et al. 2009) and *Hygrocybe* (Boertmann 2010).

Haasiella splendidissima differs in having tetrasporic basidia and clamp connections and in being putatively heterothallic (Kotlaba and Pouzar 1966; Horak 1968; Cléménçon 1982). It originally was described from Slovakia (Kotlaba and Pouzar 1966) and then collected again only five times, from Spain (Vila and Caballero 2007), France (Roux pers comm), the Republic of Moldova (Roux 2006), Austria (Moser and Jülich 1997) and also from Switzerland (Roffler and Ardüser 2010). Since the original description some authors have recognized *H. splendidissima* as a distinct species (Singer 1975, 1986; Cléménçon 1982; Moser 1983; Kuyper 1995; Chiaffi and Surault 1996a; Bon 1997; Horak 2005; Elborne 2008), others have regarded it as a taxon subordinate to *H. venustissima* (Ludwig 2001 as *Omphalina venustissima* var. *splendidissima* [Kotl. & Pouzar] E. Ludw.; Roux 2008 as *H.*

venustissima var. *splendidissima* [Kotl. & Pouzar] P. Roux); still others only as an informal tetrasporic variant of *H. venustissima* deserving no formal taxonomic rank (Romagnesi 1995, Vila and Caballero 2007). The latter taxonomic decision was based possibly on the fact that Vila and Caballero (2007) collected the specimens of *H. splendidissima* at the same site where they had found specimens of *H. venustissima*.

Our phylogenetic analysis highlights that all the *Haasiella* sequences (eight of *H. splendidissima* and two of *H. venustissima*) are almost identical. The five ITS sequences display a pairwise percent identity value of 99.3. According to this value and accepting an intraspecific ITS variability lower than 3% (Nilsson et al. 2008), *H. splendidissima* and *H. venustissima* could be considered conspecific, *H. splendidissima* being only a tetrasporic strain (population) of *H. venustissima* with a heterothallic life cycle. A similar situation was demonstrated for *Agaricus bisporus* (J.E. Lange) Imbach, where three different types of life cycles are present in cross-fertile populations (Kamzolkina et al. 2006).

The bisporic and non-clamped populations of *H. venustissima* seem to be relatively more common and widespread than the tetrasporic ones due to their apomictic (homothallic) way of life. Murtagh et al. (2000) suggested that these apomictic mechanisms might be an adaptation to circumstances in which opportunities for outbreeding arise infrequently with the consequent removal of the time-consuming necessity of encountering a suitable mate.

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