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Helvella sublactea sp. nov. (Helvellaceae) from southwestern China

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Abstract

A new species, *Helvella sublactea,* is discribed from southwestern China using morphological and molecular phylogenetic evidence. It is morphologically characterized by the combination of the following characters: saddle-shaped or irregular lobed pileus with milky white, greyish beige to taupe hymenium, margin fused with stipe, white receptacle surface glabrous and white stipe with deep longitudinal ribs. Molecular phylogenetic analyses, based on a combined ITS and 28S sequence data, showed that *H. sublactea* clustered with *H. lactea* but is clearly distinct from the latter. A description, figures, phylogenetic placement and comparison with similar taxa are presented.

Keywords: Pezizales, new taxa, phylogeny, morphology

Introduction

Helvella L. is one of generic names of mushrooms proposed by Linnaeus (1753), and has been widely studied by mycologists from all over the world (Dissing 1966, Weber 1972, Harmaja 1979, Korf 1985, Häffner 1987, Abbott and Currah 1988, 1997, Zhuang 2004, Nguyen *et al*. 2013). Species of *Helvella* are characterized by auriculoid, cupulate or irregularly lobed pileus that are generally distinguished by colours ranging from white to black and by presence of ribs on the stipe (Dissing 1966, Abbott and Currah 1997). To date, Index Fungorum lists 480 epithets at various levels in genus *Helvella*, including species, subspecies and varieties (http://www.indexfungorum.org/-Name/Names.asp, January 2016) and, of these, around 59 taxa were accepted (Kirk *et al*. 2008, Nguyen *et al*. 2013, Ariyawansa *et al*. 2015, Zhao *et al*. 2015).

This genus is widely distributed in China, and has been received much attention in China (Du *et al*. 1979, Liu and Cao 1988, Xu 2001, Zhuang 2004, Zhuang and Yang 2008, Ariyawansa *et al*. 2015, Hwang *et al*. 2015, Zhao *et al*. 2015). During a previous investigation of fungal resources of southwestern China (Zhao *et al*. 2015), we have found a taxon of this genus, saddle-shaped or irregular lobed pileus with milky white, greyish beige to taupe hymenium and white strongly ribbed stipe. A preliminary morphological comparison indicated that our collections are similar to *H. lactea* Boud. and *H. lacunosa* Afzel. : Fr in the Chinese literature (Liu and Cao 1988, Ying and Zang 1996, Mao 2000, Xu 2001, Yuan and Sun 2007). Further detailed morphological study indicated that our collections differ from *H. lactea* and *H. lacunosa* significantly (Dissing 1966, Abbott and Currah 1997, Nguyen *et al*. 2013). In this study, we used morphological and molecular evidence from combined the internal transcribed spacer region of ribosomal DNA (ITS) and the D1–D3 region of the 28S nuc rDNA sequence (28S) to assess the phylogenetic position of the fungus. The combination of morphological observations and molecular data showed that it is a new species of *Helvella*, and is described herein.

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Materials & methods

Specimens and morphological studies

Macroscopic and microscopic characters were described based on the fresh and dried materials collected in Yunnan, southwestern China. The specimens including type materials were deposited in the Herbarium of Cryptogams of Kunming Institute of Botany, Chinese Academy of Sciences (KUN-HKAS). Microscopic sections of the pileipellis and the surface of the stipe were made by free-hand with a razor blade under Olympus dp72 microscope. The dried specimens were cut and mounted in water to saturate the tissues for about 5 mins. Specimens were examined under a Nikon Microscope ECLIPSE 80i. Micrographs were taken under a Nikon 80i microscope using NIS-Elements D3.1 or F3.0 software. All microstructures were made from rehydrated materials. The notations "ascospores (n/m/p)" indicate that the measurements were made on "n" ascospores from "m" ascocarp of "p" collections. Dimensions of ascospores were presented in the notation (a–) b–c (–d), in which the range b–c represents a minimum of 90% of the measured values, and extreme values (a and d) were kept in parentheses. Q referred to the length / breadth ratio of ascospores. **Q** referred to the range Q of ascospores ± sample standard deviation.

DNA extraction and PCR amplification

Protocols for DNA extraction, PCR, and sequencing follow the procedures described in Zhao *et al*. (2015) and references therein. Universal primer pairs ITS5 / ITS4 (White *et al*. 1990) and LROR / LR5 (Vilgalys and Hester 1990) were used for the internal transcribed spacer region of ribosomal DNA (ITS), the D1–D3 region of 28S rDNA sequence (28S), respectively.

Sequence alignments and phylogenetic analyses

Sequences for each gene marker (Table 1) were carefully checked to exclude possible contamination, and were combined with sequences published in Nguyen *et al*. (2013) and Ariyawansa *et al*. (2015) were obtained from GenBank (Table 1). Each matrix was aligned using MAFFTv6.8 (Katoh *et al*. 2005) and manually optimized on BioEdit v7.0.9 (Hall 1999) or 4SALE v1.5 (Seibel *et al*. 2006).

TABLE 1. Fungal taxa analyzed and GenBank accession numbers for sequences used in this study.

Fungal taxon	Specimen Voucher	GenBank Accession		References
		ITS	nrLSU-rDNA	
Helvella dryophila	UC1999201	KC122831	KC122792	Nguyen *et al.* 2013
H. dryophila	UC1860627	KC122828	KC122793	Nguyen *et al.* 2013
H. dryophila	UC1999238 (holotye)	KC122811	KC122772	Nguyen *et al.* 2013
H. lacunosa	HC-PNNT-059	KC016124	—	Nguyen *et al.* 2013
H. lacunosa	HC-PNNT-235	KC016123	—	Nguyen *et al.* 2013
H. lacunosa	UC1999245	KC122820	—	Nguyen *et al.* 2013
H. lacunosa	MIN451143	KC122818	—	Nguyen *et al.* 2013
H. lacunosa	KH.10.97	KC122809	KC122771	Nguyen *et al.* 2013
H. lacunosa	KH.03.111	KC122808	KC122770	Nguyen *et al.* 2013
H. lacunosa	UC1999242	KC122823	—	Nguyen *et al.* 2013
H. lacunosa	UC1999241	KC122822	—	Nguyen *et al.* 2013
H. lacunosa	UC1999243	KC122824	—	Nguyen *et al.* 2013
H. lacunosa	UC1999199	KC122821	—	Nguyen *et al.* 2013
H. lacunosa	F1187326	KC122864	—	Nguyen *et al.* 2013
H. lacunosa	HKAS 87877	**KT894823**	**KT894830**	This study
H. lacunosa	HKAS 87878	**KT894824**	**KT894831**	This study

...Continued on next page

TABLE 1. (Continued)

Fungal taxon	Specimen Voucher	GenBank Accession		References
		ITS	nrLSU-rDNA	
H. cf. *lacunosa*	HC-PNNT-266	KC016116	—	Nguyen *et al.* 2013
H. cf. *lacunosa*	CB08303	KC016120	—	Nguyen *et al.* 2013
H. cf. *lacunosa*	HC-PNNT-149	KC016117	—	Nguyen *et al.* 2013
H. cf. *lacunosa*	CB08326	KC016115	—	Nguyen *et al.* 2013
H. cf. *lacunosa*	AR09690	KC016121	—	Nguyen *et al.* 2013
H. cf. *lacunosa*	CB08331	KC016122	—	Nguyen *et al.* 2013
H. cf. *lacunosa*	GO-2009-088	KC016119	—	Nguyen *et al.* 2013
H. cf. *lacunosa*	CB08367	KC016114	—	Nguyen *et al.* 2013
H. cf. *lacunosa*	GO-2009-279	KC016118	—	Nguyen *et al.* 2013
H. lactea	WCG1393	KC122826	—	Nguyen *et al.* 2013
H. pseudolacunosa	HMJAU4533	KR493477	KT932630	Ariyawansa *et al.* 2015
H. pseudolacunosa	HKAS87594 (holotype)	KR493476	KT932629	Ariyawansa *et al.* 2015
H. rugosa	HKAS87587	KR493478	KT932631	Ariyawansa *et al.* 2015
H. rugosa	HKAS75442 (holotype)	KR493475	KR493511	Ariyawansa *et al.* 2015
H. sublactea	HKAS90607	**KT894826**	**KT894833**	This study
H. sublactea	HKAS74226	**KT894827**	**KT894834**	This study
H. sublactea	HKAS69753 (holotype)	**KT894825**	**KT894832**	This study
H. sublactea	HKAS 69820	**KT894829**	—	This study
H. sulcata	F1142883	KC122825	—	Nguyen *et al.* 2013
H. aff. *sulcata*	UC1999239	KC122812	—	Nguyen *et al.* 2013
H. vespertina	UC1999203	KC122856	KC122776	Nguyen *et al.* 2013
H. vespertina	UC1999195	KC122855	KC122775	Nguyen *et al.* 2013
H. vespertina	UC1999193 (holotype)	KC122846	KC122777	Nguyen *et al.* 2013
H. zhongtiaoensis	HKAS74335	JX462578	KR493484	Zhao *et al.* 2015

Note: "—" shows no sequence in the GenBank database. GenBank accession numbers for sequences generated in this study are in boldface.

The phylogenetic analyses were based on two datasets: a single data set for 28S and a concatenated dataset of ITS and 28S. To investigate the potential conflict between ITS and 28S, the partition homogeneity or incongruence length difference test was performed with 1000 randomized replicates, using heuristic searches with simple addition of sequences in PAUP*4.0b10 (Swofford 2002). Since the result showed that the two different gene fragments were not in conflict ($P>0.5$), the two datasets were concatenated using Phyutility v.2.2 for further analysis (Smith and Dunn 2008).

For phylogenetic analyses based on the 28S, and the combined alignments, both Bayesian inference (BI) and maximum likelihood (ML) algorithms were employed by using MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) and RAxML v7.2.6 (Stamatakis 2006), respectively. Substitution models suitable for each partition in combined datasets were determined using Akaike Information Criterion implemented in MrModeltest v2.3 (Nylander 2004). All parameters in ML analysis were kept default, and statistical support values were obtained using nonparametric bootstrapping with 1000 replicates, and trees obtained prior to convergence were discarded before consensus tree. BI analyses were performed using the Metropolis-coupled Markov Chain Monte Carlo method under the GTR + I + G (ITS dataset), GTR + I (combined ITS + 28S dataset) model. Analyses were run with 4 chains of 1,000,000 generations, and trees were sampled every 100th generation. Bayesian posterior probabilities (BPP) values were obtained from the

50% majority-rule consensus tree, and branches with BPP > 95% were considered as significantly supported. The resulting trees were printed with FigTree v. 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) and the layout was done with Adobe Illustrator CS v. 6. *Helvella zhongtiaoensis* J.Z. Cao & B. Liu was chosen as outgroup taxon following Zhao *et al*. (2015).

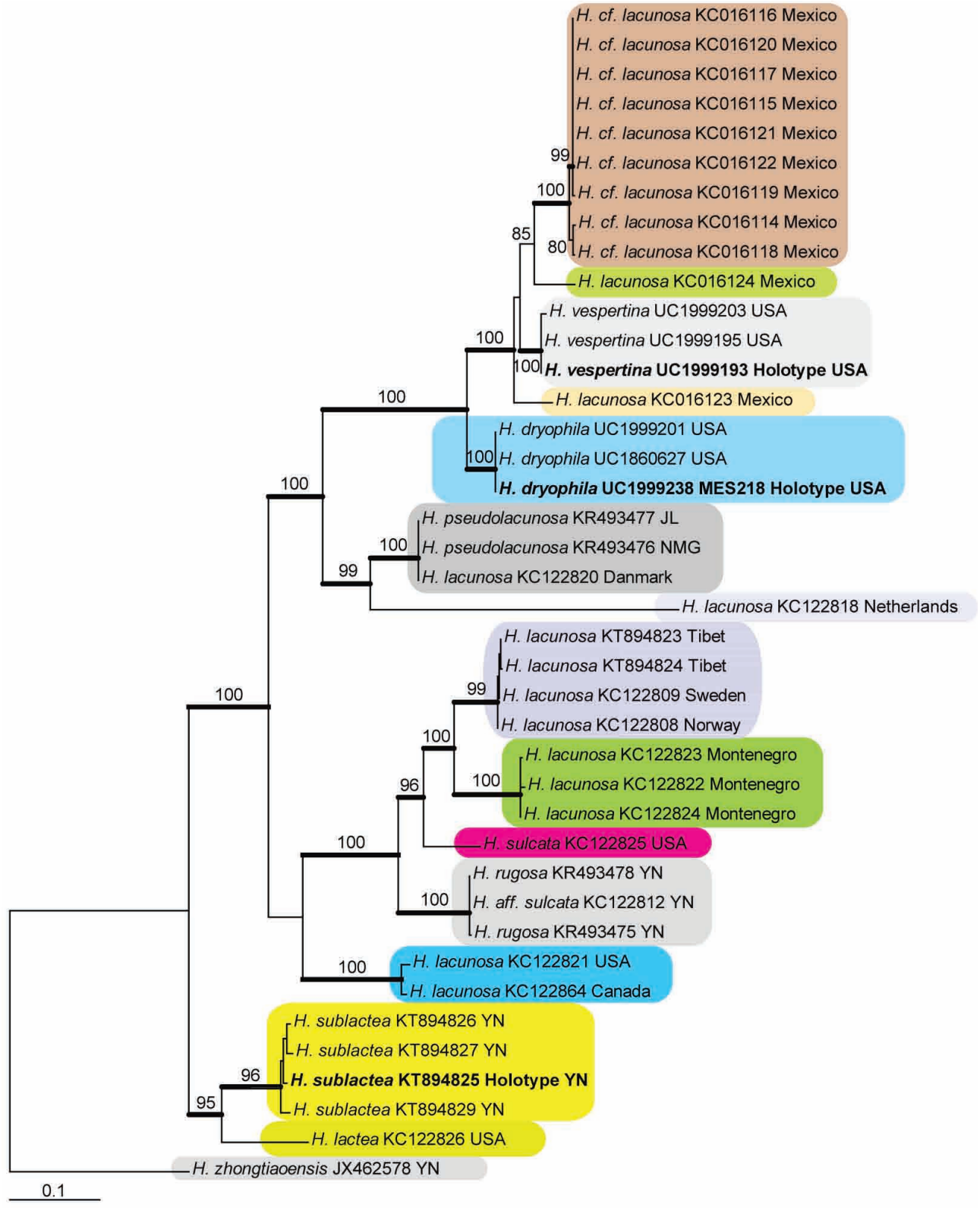

FIGURE 1. Phylogenetic tree inferred from most likelihood (ML) analysis and Bayesian inference (BI) using ITS data. Only the topology generated from the ML analysis is shown. Bayesian Posterior Probability > 0.95 is indicated with thick branch. Type sequences are in bold.

Results

Molecular data

Eleven sequences, six ITS and five 28S, were newly generated for this study (Table 1). For the ITS dataset, sequences representing most of the speces in the *Helvella* section *Lacunosae* were downloaded from GenBank (Nguyen *et al*. 2013, Ariyawansa *et al*. 2015). The final ITS dataset included 40 sequences (including 34 from GenBank; Table 1), representing 14 species, and the alignment contained 951 nucleotide sites (including gaps), of which 521 characters are constant, 118 parsimony-uninformative and 312 parsimony-informative. The combined ITS and 28S dataset consisted of 1,665 nucleotides (including gaps), with 1,268 characters are constant, 293 are parsimony-informative characters. In our phylogenetic analyses on both datasets using the ML and BI approaches, very similar estimates of tree topologies were produced (Figs. 1 and 2).

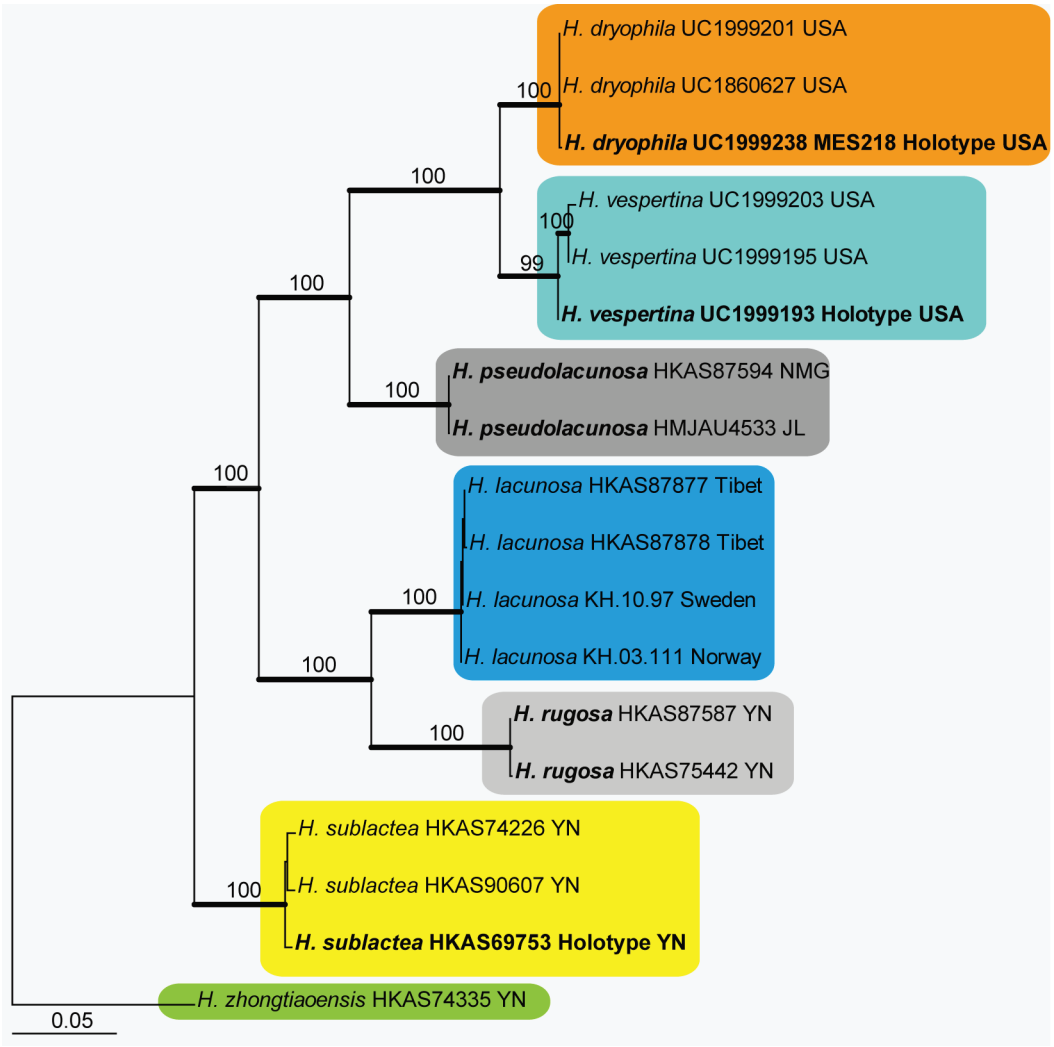

FIGURE 2. Phylogenetic tree generated from combined ITS and 28S dataset using ML method. Bootstrap values (> 50%) derived from ML analyses and posterior probabilities from Bayesian inference (> 0.95) are shown in the thick branches. Type sequences are in bold.

Bayesian and RAxML phylogenetic analyses showed that our collections clustered together and formed a well-supported clade with 96% bootstrap value and 1.00 posterior probability which are obviously distinct from related sequences available in the GenBank (Fig. 1). Phylogenetic analyses also showed that the new species grouped with *H. lactea* with 95% bootstrap value and 1.00 posterior probability as a sister group (Fig. 1).

Taxonomy

Helvella sublactea Q. Zhao, M. Wang & Y.C. Zhao, *sp. nov.* **Figure 3. a–j**
Mycobank: MB 815301

Etymology:—*sublactea* refers to the similarity of the eastern Asian material to *Helvella lactea*.

Pileus saddle-shaped or irregularly lobed, 1–2.5 cm high, 1–3.5 cm broad, margin fused with stipe; *hymenium* glabrous, milky white, greyish beige to taupe when fresh, beige when dried; *receptacle surface* glabrous, white when fresh, light grey when dried. *Stipe* 3–8 cm long, 0.3–1.5 cm broad, flaring and merging with pileus, white to creamy, becoming creamy when dried, finely pubescent, with deep longitudinal furrows, few anastomosis between ribs, basal mycelium white. *Medullary excipulum* 210–310 µm broad, of *textura intricata*, hyaline, 3–4 µm broad hyphae, blue in cotton blue. *Ectal excipulum* 55–70 µm broad, of *textura angularis*, hyaline, outermost cells 15–23 × 8–14 µm, clavate, evenly blue in cotton blue. *Stipitipellis* 77–110 µm, hyaline, terminal cells 14–26 × 8–16 µm, clavate, with a yellow refractive content in Melzer's reagent, blue in cotton blue. *Asci* 240–300 × 13–19 µm, pleurorhynchous base, 8-spored, uniseriate, subcylindrical to clavate. *Paraphyses* filiform, 3–4 µm broad, slightly exceeding the asci, with a yellow refractive content in Melzer's reagent, blue in cotton blue, apex apparently enlarged, 5–9 µm broad. *Ascospores* [100/3/3, in H_2O] (14–) 15–17(–18) × 10–12 µm [Q = 1.33–1.65, **Q** = 1.45 ± 0.07)] ellipsoid, smooth-wall under the light microscopy.

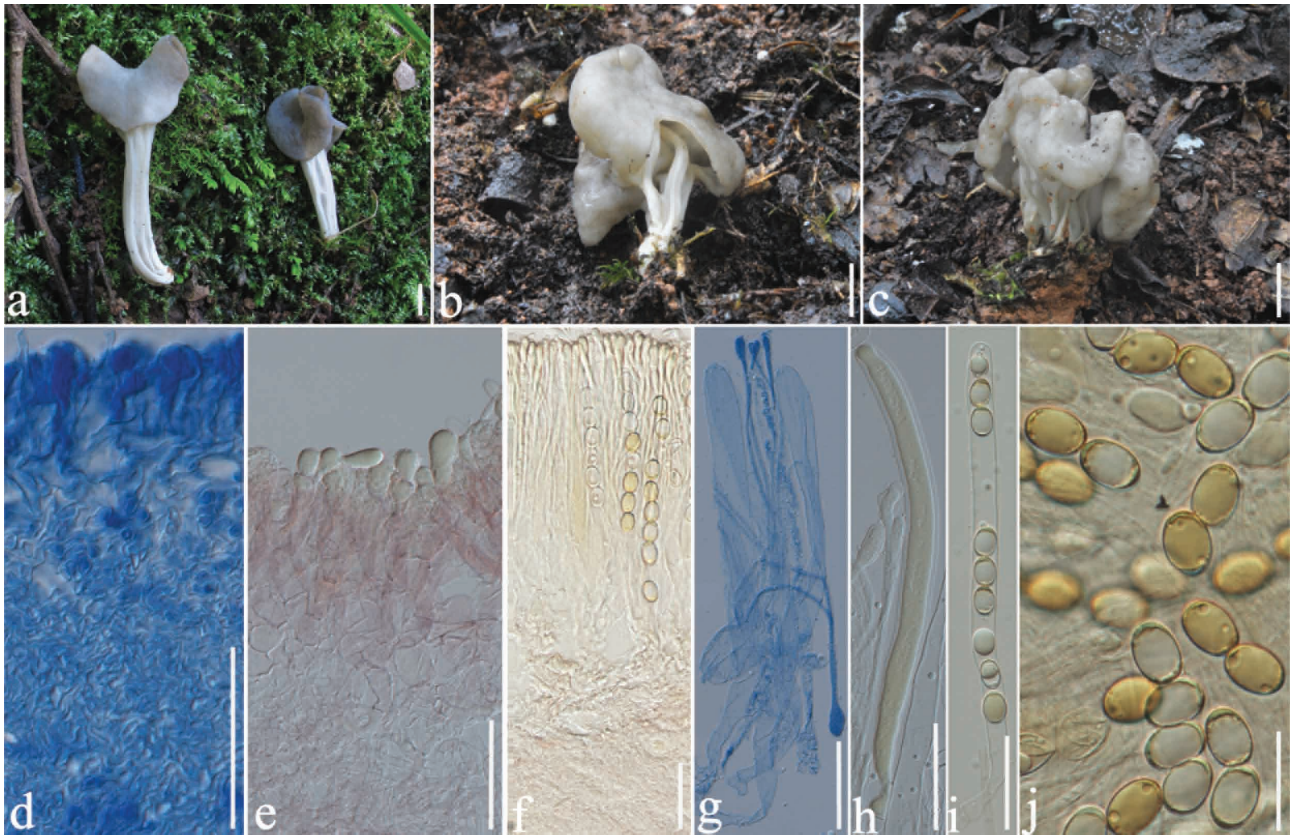

FIGURE 3. *Helvella sublactea* a–c. Typical mature specimens (a. HKAS90607, b–c. HKAS69753); d. ectal excipulum of pileus; e. stipitipellis; f–i. Asci and paraphyses; j. Ascospores. ─ Scale bars: a–c = 1 cm; d–j = 50 µm; j = 25 µm.

Habitat:—Solitary or gregarious on the ground under *Castanea* spp. or *Quercus* spp. forest.

Known distribution:—Only known from high altitude localities in southwestern China.

Type:—CHINA. Yunnan Province: Gucheng County, alt. 2500m, 22 Aug. 2010, *Qi Zhao1032* (holotype HKAS69753!).

Additional materials examined:—CHINA. Yunnan Province: Panlong County, Kunming Institute of Botany, 21 Aug. 2011, *Qin Cai 663* (HKAS90607!); Tengchong County, Puchuan, 11 Aug. 2011, *Qi Zhao1273* (HKAS74226!); Ducheng County, 30 Jul. 2011, *Li Ping Tang 1359* (HKAS68920!).

Discussion

Helvella sublactea is characterized by its saddle-shaped or irregular lobed pileus with milky white, greyish to taupe hymenium, margin fused with stipe, white receptacle surface glabrous, white stipe with deep longitudinal ribs, pleurorhynchous asci, apparently enlarged paraphyses apex and ellipsoid ascospores. Considering the combination of its irregularly lobed pileus, margin fused to stipe, greyish to taupe hymenium and strongly ribbed stipe, *H. sublactea* is placed in *Helvella* subgenus *Helvella* (Abbott and Currah 1997).

Phylogenetic analyses based on ITS revealed that *H. sublactea* has a close relationship with *H. lactea* (Fig. 1), which share connivent saddle-shaped, adnate or irregularly lobed pileus, whitish hymenium, white receptacle surface and white sulcate stipe. However, *H. sublactea* possessing milky white, greyish beige to taupe hymenium, and broader paraphyses apex, while the latter species has white hymenium that is never possessing greyish, and narrower paraphyses apex. In addition, *H. lactea* differs from *H. sublactea* in its broader ectal excipulum (70–85 µm broad) and medullary excipulum (230–460 µm broad) (Dissing 1966).

In the field, *H. sublactea* is often confused by local residents with the European species *H. lacunosa*. However, the ITS sequences clearly distinguish these two species as they fell into two distinct clades (Figs. 1 and 2). *Helvella lacunosa* differs from *H. sublactea* by its pileus and stipe pale greyish to greyish-brown to nearly black, inside of stipe generally has longitudinal chambers (Dissing 1966, Abbott and Currah 1997). *Helvella sublactea* is somewhat similar to *H. crispa* Scop. ex Fr., *H. involuta* Q. Zhao, Zhu L. Yang & K.D Hyde, *H. orienticripa* Q. Zhao, Zhu L. Yang & K.D Hyde, *H. pseudoreflexa* Q. Zhao, Zhu L. Yang & K.D Hyde and *H. zhongtiaoensis* Cao J.Z. & Liu in their whitish hymenium and white sulcate stipe. However, the latter five species have pubescent receptacle surface and inrolled free apothecial margin (Zhao *et al*. 2015).

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