



## Morphology and phylogenic position of *Wynnella subalpina* sp. nov. (Helvellaceae) from western China

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### Abstract

The genus *Wynnella* has long been regarded as a monotypic genus, consisting only *W. auricula* (Schaeff) Boud. Morphological and phylogenetic studies were carried out on new collections of *Wynnella* from Tibet and Sichuan, in southwestern China. Morphology and phylogenies inferred from the internal transcribed spacer region of ribosomal DNA (ITS) and the D1–D3 region of 28S nrDNA (28S) sequences data clearly suggest these collections representing a new species in the genus. *Wynnella subalpina* is thus introduced and illustrated. The species is characterized by its rabbit ear-shaped apothecia, with a tea brown to blackish brown hymenium, a red, reddish brown to pale yellowish brown receptacle surface, and a costate stipe. Detailed macro- and micro-scopic descriptions, illustrations of microscopic characters and photographic plates are provided herein.

**Keywords:** new taxa, Pezizales, phylogeny, taxonomy

### Introduction

The genus *Wynnella* was introduced by Boud. (1885) to accommodate *Elvela auricula* Schaeff. *ss* Boud. (= *W. auricula* (Schaeff) Boud.). It is characterized by ear-shaped, two colored apothecia, ectal excipulum of *textura angularis*, medullary excipulum of *textura intricata*, subcylindrical, nonamyloid asci, and ellipsoide, uniguttulate ascospores (Nannfeldt 1931, Abbott and Currah 1997, Parslow and Spooner 2009). The systematic position of *Wynnella* in the family Helvellaceae was suggested with molecular phylogenetic studies (O'Donnell *et al.* 1997, Haltington *et al.* 1999, Landvik *et al.* 1999, Læssøe and Hansen 2007, Landeros *et al.* 2015). This genus has been regarded as monotypic with only *W. auricula* (Schaeff) Boud. (= *W. silvicola* (Beck) Nannf.), originally described from Europe, and then also reported in Asia and North America (Nannfeldt 1931, Harmaja 1974, Häffner 1987, Abbott and Currah 1997, Zhuang 2004, Kirk *et al.* 2008, Parslow and Spooner 2009).

During our study on the species diversity of Helvellaceae in China (Hwang *et al.* 2015, Zhao *et al.* 2015, 2016), it was noticed that four particular collections were frequently mislabeled as *Wynnella silvicola*. Our morphological observations and phylogenetic analyses based on ITS and 28S showed that these specimens is not *W. silvicola*, but represents a new species described herein as *W. subalpina*.

### Materials and Methods

#### Specimens and morphological studies

Specimens were collected in forests dominated by conifers, in Tibet and Sichuan Provinces, China and photographed *in situ*. Microscopic observations and photomicrographs were made as described in Zhao *et al.* (2015). The hemiamyloid reaction was tested in Melzer's reagent (Melzer's), J<sup>+</sup> for hemiamyloid reaction (solely red) and J<sup>-</sup> for negative

reaction. The examined specimens are deposited in the Herbarium of Cryptogams of Kunming Institute of Botany, Chinese Academy of Sciences (KUN-HKAS). Facesoffungi numbers and Index Fungorum numbers were obtained as detailed in Jayasiri *et al.* (2015) and Index Fungorum (2015).

**TABLE 1.** Specimens used in molecular phylogenetic studies and their GenBank accession numbers.

Fungal taxon	Specimen Voucher	ITS	28S	Reference
<i>Balsamia vulgaris</i>	AH 44222	KM243645	KM243651	Crous <i>et al.</i> 2014
<i>Balsamia vulgaris</i>	AH 44224	KM243647	KM243653	Crous <i>et al.</i> 2014
<i>Barssia hellenica</i>	MCVE 28663	KT350941	KT350940	Crous <i>et al.</i> 2014
<i>Barssia hellenica</i>	MCVE 28664	KT350942	KT350939	Crous <i>et al.</i> 2014
<i>Barssia maroccana</i>	AH 39117	KM243649	KM243655	Crous <i>et al.</i> 2014
<i>Barssia maroccana</i>	AH 44099	KM243648	KM243654	Crous <i>et al.</i> 2014
<i>Helvella compressa</i>	HKAS 45788	KU739801	KU739829	Zhao <i>et al.</i> 2016
<i>Helvella crispa</i>	HKAS 75434	JX462572	KR493479	Zhao <i>et al.</i> 2015
<i>Helvella dryophila</i>	UC 1860627	KC122828	KC122793	Nguyen <i>et al.</i> 2013
<i>Helvella dryophila</i>	UC 1999201	KC122831	KC122792	Nguyen <i>et al.</i> 2013
<i>Helvella dryophila</i>	UC 1999238*	KC122811	KC122772	Nguyen <i>et al.</i> 2013
<i>Helvella elastica</i>	HKAS83157	KU739799	KU739826	Zhao <i>et al.</i> 2016
<i>Helvella elastica</i>	HKAS83158	KU739800	KU739827	Zhao <i>et al.</i> 2016
<i>Helvella involuta</i>	HKAS 74281*	KT215608	KR493492	Zhao <i>et al.</i> 2015
<i>Helvella lacunosa</i>	HKAS 87877	KT894823	KT894830	Wang <i>et al.</i> 2016
<i>Helvella lacunosa</i>	HKAS 87878	KT894824	KT894831	Wang <i>et al.</i> 2016
<i>Helvella lacunosa</i>	KH.03.111	KC122808	KC122770	Nguyen <i>et al.</i> 2013
<i>Helvella lacunosa</i>	KH.10.97	KC122809	KC122771	Nguyen <i>et al.</i> 2013
<i>Helvella orienticrispa</i>	HKAS 74319*	JX462598	KR493503	Zhao <i>et al.</i> 2015
<i>Helvella pseudolacunosa</i>	HKAS 87594 *	KR493476	KT932629	Wang <i>et al.</i> 2016
<i>Helvella pseudolacunosa</i>	HMJAU 4533	KR493477	KT932630	Wang <i>et al.</i> 2016
<i>Helvella pseudoreflexa</i>	HKAS 74311*	JX462593	KR493500	Zhao <i>et al.</i> 2015
<i>Helvella rugosa</i>	HKAS 75442*	JX462575	KR493511	Zhao <i>et al.</i> 2015
<i>Helvella rugosa</i>	HKAS 87587	KR493478	KT932631	Zhao <i>et al.</i> 2015
<i>Helvella stevensii</i>	HKAS 83162	JX462567	KU739828	Zhao <i>et al.</i> 2015
<i>Helvella subglabra</i>	HKAS 78940	KU739802	KU167476	Zhao <i>et al.</i> 2016
<i>Helvella vespertina</i>	UC 1999193*	KC122846	KC122777	Nguyen <i>et al.</i> 2013
<i>Helvella vespertina</i>	UC 1999203	KC122856	KC122776	Nguyen <i>et al.</i> 2013
<i>Helvella vespertina</i>	UC 1999205	KC122857	KC122788	Nguyen <i>et al.</i> 2013
<i>Helvella zhongtiaoensis</i>	HKAS 74335	JX462578	KR493484	Zhao <i>et al.</i> 2015
<i>Helvella abella</i>	HKAS 83160	JX462568	KU739825	Zhao <i>et al.</i> 2016
<i>Tuber anniae</i>	JT 13209	HM485338	JQ925680	Crous <i>et al.</i> 2014
<i>Tuber anniae</i>	OSC 58992	NR119860	NG042661	O'Donnell <i>et al.</i> 1997
<i>Tuber bellisporum</i>	JT 6060	FJ89857	FJ809828	Crous <i>et al.</i> 2014
<i>Tuber bellisporum</i>	JT 7270	FJ89856	FJ809827	Crous <i>et al.</i> 2014

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TABLE 1. (Continued)

Fungal taxon	Specimen Voucher	ITS	28S	Reference
<i>Underwoodia beatonii</i>	JT 28375	JQ925654	JQ925715	Bonito <i>et al.</i> 2013
<i>Underwoodia beatonii</i>	JT 28380	JQ925655	JQ925716	Bonito <i>et al.</i> 2013
<i>Underwoodia cf. singeri</i>	MES 161	JQ925656	JQ925718	Bonito <i>et al.</i> 2013
<i>Wynnella silvicola</i>	NSW 6219	AF064596	U42682	O'Donnell <i>et al.</i> 1997
<i>Wynnella subalpina</i>	HKAS 45750*	<b>KX034101</b>	<b>KT581118</b>	This study
<i>Wynnella subalpina</i>	HKAS 78940	<b>KX034102</b>	<b>KT581119</b>	This study
<i>Wynnella subalpina</i>	HKAS 87730	<b>KX034103</b>	<b>KX034104</b>	This study

\* = Holotype

### Molecular procedures and phylogenetic analysis

Total DNA was extracted from silica-gel-dried materials using a CTAB procedure of Doyle (1987). 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. Amplification reactions were performed in an ABI 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA). The PCR program was 95 °C for 3 min; 94 °C for 50 s, 53 °C for 50 s, 72 °C for 80 s, 35 cycles; and 72 °C for 8 min. PCR products were purified with the Gel Extraction & PCR Purification Combo Kit (Spin-column, Biotek, Beijing, China), and then sequenced on an ABI-3730-XL sequence analyzer (Applied Biosystems, Foster City, CA, USA) using the same primers as those used in amplifications.

All ITS and 28S sequences of *Wynnella* in GenBank were retrieved, and combined with those generated in this study (Table 1). *Tuber anniae* W. Colgan & Trappe and *T. bellisporum* Bonito & Trappe were selected as outgroup taxa following O'Donnell *et al.* (1997), and representatives of other genera within Helvellaceae, viz. *Balsamia* Vittad., *Barssia* Gilkey, *Helvella* L. and *Underwoodia* Peck, were also included in the analyses.

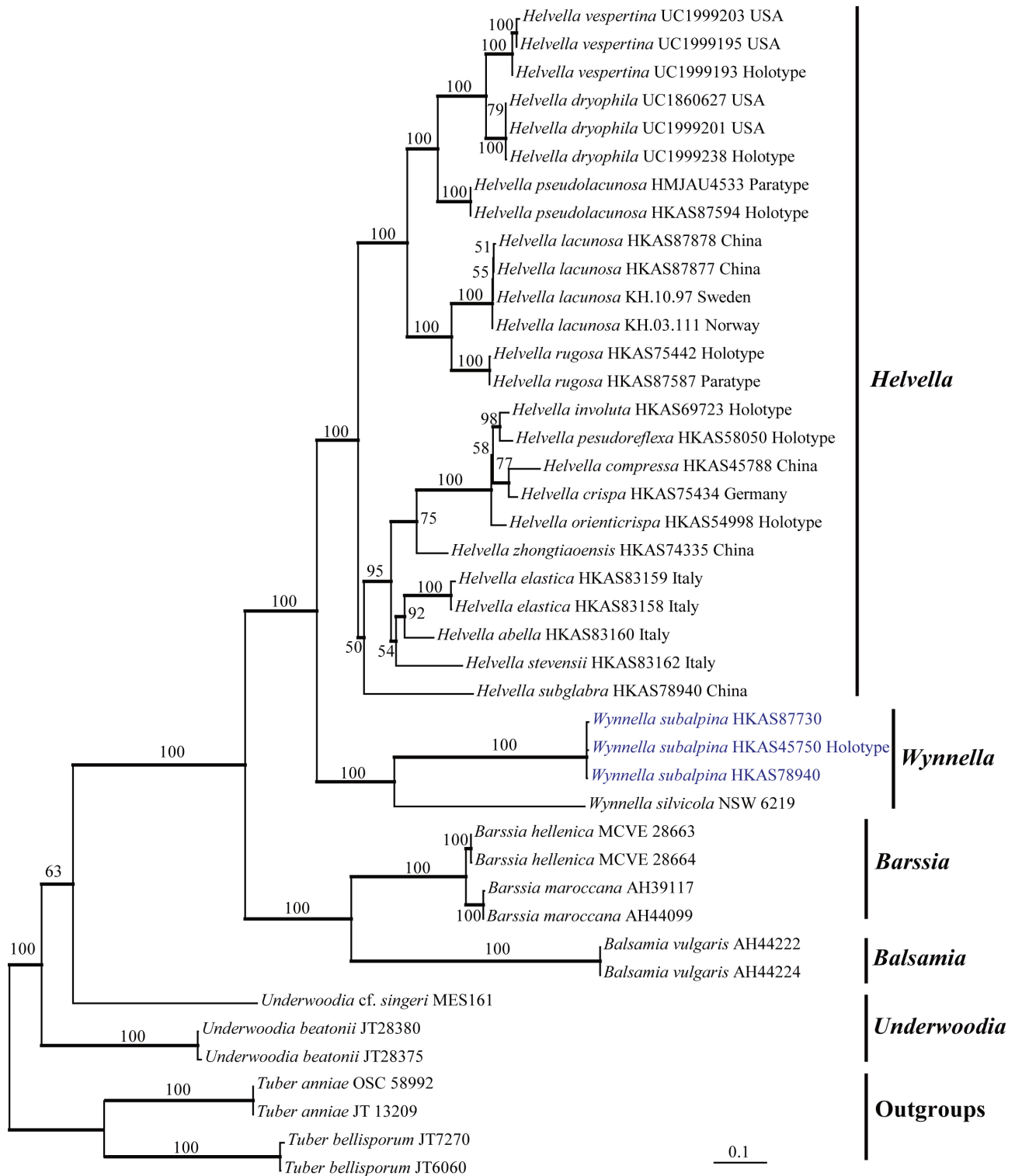
The ITS sequences and 28S sequences were aligned separately using MAFFT v6 (Katoh *et al.* 2005) and Bioedit v. 7.0.9 (Hall 1999) and resulted in two datasets. Sequence lengths of the ITS and 28S datasets are 1108 and 871 bp, respectively. To test for potential phylogenetic conflict between the two datasets, the partition homogeneity (PH) test was performed with 1,000 randomized replicates, using heuristic searches with the simple addition of sequences in PAUP\* 4.0b10 (Swofford 2002). The result showed that the phylogenetic signals within the two markers were not significantly in conflict ( $P < 0.5$ ). Consequently, the sequences of the two datasets were concatenated using Phyutility v2.2 (Smith and Dunn 2008) in order to carry out a combined analysis. Phylogenetic analyses of sequence data were carried out using RAxML v7.2.6 (Stamatakis 2006) for Maximum likelihood (ML) analyses and Mr Bayes v.3.0b4 (Ronquist and Huelsenbeck 2003) for Bayesian Inference (BI) analyses. Substitution models suitable for each partition in both datasets were determined with Akaike Information Criterion implemented in MrModeltest v2.3 (Nylander 2004). All parameters in ML analysis were kept at default, and statistical support values were obtained by nonparametric bootstrapping with 1000 replicates. BI analyses were performed with the Metropolis-coupled Markov chain Monte Carlo method under the GTR + T + I model. Analyses were run with four chains of 2,000,000 generations, and trees were sampled every 100<sup>th</sup> 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 resulted 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.

## Results

### Molecular phylogeny

The phylogenetic tree of most genera of Helvellaceae inferred from the ITS + 28S dataset using ML and BI analyses indicates the monophyly of *Wynnella*. The materials collected from southwestern China cluster in the *Wynnella* clade, but are distinct from the known species, *Wynnella silvicola* (Fig. 1). Therefore, a new species, is recognized to accommodate these collections.

Our analyses also indicated that *Wynnella* is a distinct genus of Helvellaceae, and is more closely allied to *Helvella* than *Balsamia*, *Barssia* and *Underwoodia* (Fig. 1), which is consistent with the suggestion of O'Donnell *et al.* (1997), Haltington *et al.* (1999), Landvik *et al.* (1999), Læssøe and Hansen (2007) and Landeros *et al.* (2015).



**FIGURE 1.** ML tree of ITS + 28S alignment (-ln L = -16562.411489). Only the topology generated from the ML analysis is shown. In the Bayesian analysis, PP > 0.95 are indicated with thick branch. New species are indicated with blue.

## Taxonomy

*Wynnella subalpina* Q. Zhao, Zhu L. Yang & K.D. Hyde, *sp. nov.* Fig. 2

Index Fungorum number: 551515, Facesoffungi number: FoF 01055



*Diagnosis*:—Rabbit-ear shaped apothecia, with a tea brown to blackish brown hymenium, a red, reddish brown to greyish yellow receptacle surface, and a costate stipe. Paraphyses filiform 3–4  $\mu\text{m}$  broad, and ascospores ellipsoid 19–23  $\times$  10.5–12  $\mu\text{m}$ .

*Etymology*:—*subalpina* is proposed for its occurrence in subalpine regions in western China.



**FIGURE 2.** *Wynnella subalpina* a. Typical mature specimens (a. HKAS 78940!, b. HKAS 45750!, HKAS 87730!); d. Receptacle surface of pileus; e-f Asci and paraphyses (HKAS 45750!); g-i Asci (HKAS 45750!); j = Ascospores. — Scale bars: a–c = 1 cm; c–i = 40  $\mu\text{m}$ ; i = 20  $\mu\text{m}$ .



*Apothecia* rabbit-ear shaped, 3–6 cm high, 1–4 cm broad, *hymenium* glabrous, tea brown to blackish brown, blackish brown when dry, margin rolled to hymenium when young, expanded at maturity; *receptacle surface* glabrous, red, reddish brown to greyish yellow, yellowish near the base. *Stipe* costate, glabrous, 0.5–1.5 × 0.5–2 cm, narrowing to base, light yellow to yellowish. *Medullary excipulum* 400–490 µm broad, of *textura intricata*, hyaline, composed of 2.5–5 µm broad hyphae, *J. Ectal excipulum* 100–150 µm broad, of *textura angularis*, pale yellow, lightly encrusted with pale brown crystals, end cells 30–60 × 10–20 µm, *J. Asci* arising from croziers, 8-spored, cylindrical to clavate, with apex rounded, 330–390 × 17–23 µm. *Paraphyses* filiform, 3–4 µm broad, brown, slightly short or exceeding the asci, hyaline in Melzer's reagent, light blue in cotton blue, enlarged apex 4–6 µm broad, *J. Ascospores* [60/2/2, in H<sub>2</sub>O] 19–23 × 10.5–12 µm [Q = 1.32–1.58, Q = 1.47 ± 0.06], ellipsoid, smooth-walled under the light microscope. *Odor* unique and strong when dried, but not detected when fresh.

*Habitat and distribution*:—Solitary to scattered on the ground or moss, under *Picea* spp. forest. Only known from high altitude localities in western China.

*Typification*:—CHINA. Tibet, Riwoqe County, on road (highway 317) from Riwoqe to Dingqing, 12 August 2004, Zhu L. Yang 4371 (holotype, HKAS 45750!).

*Additional material examined*:—CHINA. Tibet, Riwoqe County, on road (highway 317) from Riwoqe to Dingqing, 12 August 2004, Zhu L. Yang 4371 (isotype, FH 00301682!), Bomi County, on moss under *Picea* spp. forest, alt. 3600 m, 16 August 2014, Q. Zhao 2110 (HKAS 78940!); Sichuan Province, Jiuzhaigou County, on the ground, under *Picea* spp forest, alt. 3000 m, 20 June 2014, Q. Zhao 2050 (HKAS 87730!).

## Discussion

The debate over *Wynnella* and *Helvella* has lasted for several decades. Some mycologists treated *Helvella* in a broad sense, and took *Wynnella* as a later synonym (Harmaja 1974, Häffner 1987, Abbott and Currah 1997). Some thought *Wynnella* is an independent genus from *Helvella* (Zhuang 2004, Parslow and Spooner 2009, Landeros *et al.* 2015). Based on Bayesian and RAxML phylogenetic analyses (Fig. 1), we found that species in Helvellaceae are divided into four clades: the *Helvella* clade, the *Wynnella* clade, the *Balsamia/Barssia* clade and *Underwoodia* clade. This result is consistent with O'Donnell *et al.* (1997), Haltington *et al.* (1999), Landvik *et al.* (1999), Læssøe and Hansen (2007) and Landeros *et al.* (2015) with regard that *Wynnella* is an separate genus within Helvellaceae and is more closely allied to *Helvella* than to any other genera.

In China, *W. subalpina* has long been assigned to *W. silvicola* (= *W. auricula*), because of its rabbit ear-shaped apothecia, bi-colored fruiting bodies and similar ecological preference (Ying and Zang 1994, Mao 1998, Yuan and Sun 2007). However, several morphological features separate *W. subalpina* from the European *W. silvicola*. *Wynnella subalpina* has a tea brown to blackish brown hymenium, a red, reddish brown to greyish yellow receptacle surface, and ellipsoid ascospores (19–23 × 10.5–12 µm), while *W. silvicola* has a blood red to purple-brown hymenium, a medium red brown or pale red brown receptacle surface, and broadly ellipsoid ascospores (21.5–24 × 12.5–16.5 µm) (Abbott and Currah 1997, Parslow and Spooner 2009).

In the field, *Wynnella subalpina* is often confused by local residents with *Otidea* spp. However, *Otidea* spp. differs from *W. subalpina* by their filiform paraphyses curved or capitate at the apex, and elliptical, biguttulate ascospores (Kanouse 1949, Liu and Zhuang 2006).

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