



Taxonomy and systematics

## Advances in the phylogeny of *Helvella* (Fungi: Ascomycota), inferred from nuclear ribosomal LSU sequences and morphological data

*Avances en la filogenia de Helvella (Fungi: Ascomycota), inferida de secuencias nucleares ribosomales de la subunidad grande y datos morfológicos*

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Received 6 February 2015; accepted 1 June 2015

Available online 10 November 2015

### Abstract

Phylogenetic relationships in the genus *Helvella* were investigated using both LSU rDNA sequences and morphological features. The latter were coded and included in the analyses, resulting in better-supported clades. *Helvella* is monophyletic and *Wynnella* is its sister genus. Aporhynchous vs. pleurorhynchous asci are the synapomorphies for the 2 subgenera, *Leucomelaenae* and *Helvella*, which were confirmed as natural groups. Other recovered supported clades were section *Leucomelaenae* in the subgenus *Leucomelaenae*, and sections *Elasticae*, *Helvella*, and *Lacunosae* within the subgenus *Helvella*. Generic description, comments on taxonomically informative features, species complexes, and species that need typification are presented.

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**Keywords:** Type specimens; Generic delimitation; Ecology; Distribution

### Resumen

Se investigaron las relaciones filogenéticas en el género *Helvella*, utilizando secuencias de la subunidad grande del ADN ribosomal y características morfológicas; estas últimas se codificaron para incluirlas en el análisis, lo que resultó en clados con mejor soporte. *Helvella* es un grupo monofilético y *Wynnella* es su género hermano. Las ascas con base simple vs. las ascas con gancho en la base son sinapomorfias que ayudan en el reconocimiento de 2 subgéneros, *Leucomelaenae* y *Helvella*, que se confirman como grupos naturales. Otros clados con soporte son la sección *Leucomelaenae* en el subgénero *Leucomelaenae* y las secciones *Elasticae*, *Helvella* y *Lacunosae* dentro del subgénero *Helvella*. Se presentan la descripción genérica, comentarios de las características taxonómicamente importantes, complejos de especies y especies en las que es necesario nombrar tipos.

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**Palabras clave:** Especímenes tipo; Delimitación genérica; Ecología; Distribución

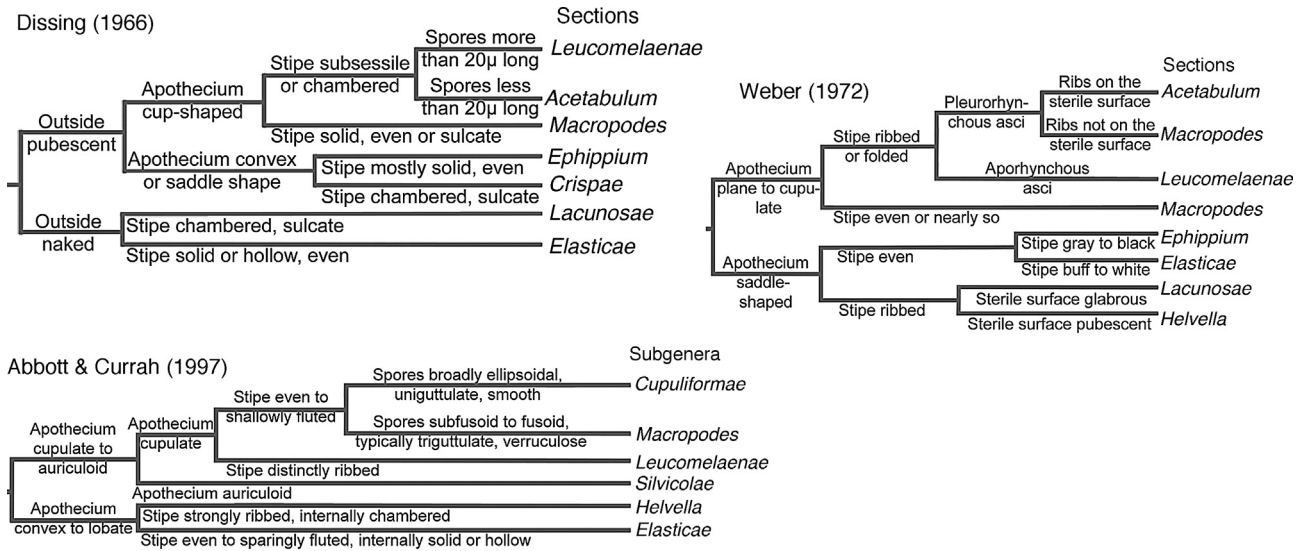
### Introduction

The genus *Helvella* L. (Linnaeus, 1753) contains approximately 52 species (Kirk, Cannon, & David, 2008). Its limits were established by Nannfeldt (1937) based on excipular tissue

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Peer Review under the responsibility of Universidad Nacional Autónoma de México.

Figure 1. Traditional infrageneric classifications of *Helvella*.

and ascospore characteristics. He described the excipulum as having 2 layers, one with intricate interwoven hyphae and the other with almost isodiametrical cells. Later, Korf (1952) named these types of tissues *textura intricata*, which constitutes the medullary excipulum, and *textura angularis* to *prismatica* for tissues in the ectal excipulum. Also, Nannfeldt (1937) described the ascospores of *Helvella* as having a large central guttula, sometimes with small apical guttulae, and tetra-nucleate (with 2 nuclei at each end).

Phylogenetic studies indicate that the family Helvellaceae is a monophyletic group (Hansen & Pfister, 2006; Læssøe & Hansen, 2007; O'Donnell, Cigelnik, Weber, & Trappe, 1997) consisting of 5 genera: *Balsamia* Vittad., *Barssia* Gilkey, *Helvella*, *Underwoodia* Peck, and *Wynnella* Boud. In all these studies, few sequences of *Helvella* were included, and the phylogenetic position of *Wynnella silvicola* (Beck) Nannf., which had been regarded as *H. silvicola* (Beck) Harmaja by some authors (Abbott & Currah, 1997; Häffner, 1987; Harmaja, 1974), was not established. In fact, Parslow and Spooner (2009) mentioned that the actual taxonomic position of *Wynnella* would be found only after a thorough study of *Helvella* spp., combining molecular and morphological characters. The genus *Wynnella* is monotypic, macromorphologically characterized by a reddish brown ear-shaped apothecium, which distinguishes it from *Helvella*, with a cup-shaped to saddle shaped apothecium. Microscopically, both genera do not differ in any essential character (Dissing, 1966; Eckblad, 1968).

Several infrageneric classifications have been proposed based on morphological characters (Fig. 1). Dissing (1966) split the genus in 7 sections; Weber (1972) recognized Dissing's 7 sections, some having different limits, and added ascus development (aporhynchous vs. pleurorhynchous) as a diagnostic character. The most recent infrageneric classification of *Helvella* was proposed by Abbott and Currah (1997), accepting 6 subgenera, based on macro- and micromorphological features.

Because the sister group of *Helvella* and its infrageneric relationships are not known, this study aims to (1) investigate the

position of *Wynnella*, and (2) improve our understanding of the phylogenetic relationships within *Helvella* using morphological data and DNA sequences of the nuclear ribosomal large subunit (nrLSU) region. For the first aim, all genera of Helvellaceae were included, as well as other genera of Discomycetes. For the second aim, representative taxa of all known sections were examined (including type specimens) from 12 countries in America, Africa, Asia, Europe, and Oceania.

## Materials and methods

Eighteen type specimens and 91 additional ones from 16 herbaria: DAOM, ENCB, FCME, H, IBUG, K, MICH, NY, O, OSC, OULU, PRM, S, TAAM, UPS, and WTU, were studied. Herbaria abbreviations follow Thiers (2012). The material was studied according to the methodology described by Landeros, Iturriaga, and Guzmán-Dávalos (2012), that is to cut sections of the apothecia and stipes directly from the dry ascoma with a razor blade. Tissue sections were placed first in 96% ethyl alcohol and subsequently in water, Melzer's reagent, or cotton blue-lactophenol (Largent, Johnson, & Watling, 1977). The terminology used here is that described by Landeros et al. (2012), except for the use of the dextrinoid term with Melzer's reagent, here abbreviated as Dx+, instead of hemiamyloid (rr+), which was incorrectly used by Landeros et al. (2012) because that term applies to the use of lugol, not Melzer's reagent (Baral, 1987).

### DNA extraction, amplification and sequencing

The proteinase K protocol (Aljanabi & Martínez, 1997) was used. The DNA pellet was diluted in 100–500  $\mu$ l of TE (Tris 10 mM pH 7.4, EDTA 0.2 mM), kept at room temperature overnight, and stored at  $-20^{\circ}\text{C}$  until the DNA amplification step.

The polymerase chain reaction (PCR) was performed to amplify the D1 and D2 domains of the nrLSU. A Taq DNA polymerase Recombinant Kit (Invitrogen) was used. Primer pairs

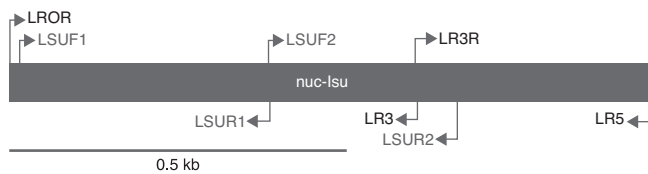


Figure 2. Location of the primers used in this work. Primers designed specifically for *Helvella* are shaded gray.

LROR-LR3 (White, Bruns, Lee, & Taylor, 1990) were used to amplify the first 600 bp. Because the DNA of 25 ascomata appeared to be deteriorated, mainly in the type specimens, we designed 4 additional *Helvella*-specific internal primers using the Lasergene Primer Select Ver. 7.1.0 (44) (DNASar, Inc.) software. These primers amplify segments of approximately 300 bp and were designated as: LSUF1 (AGCGGAGGAAAGAAACCAACAG), LSUR1 (CTCTACTTGTGCGCTATCGGTCT), LSUF2 (AGACCGATAGCGCACAAGTAGAG), and LSUR2 (TCCCAACAGCTATGCTCCTACTC) (Fig. 2).

DNA amplification was performed in an Eppendorf Mastercycler Personal 5332 thermocycler using the protocol of White et al. (1990) with some modifications. The amplification program included a denaturation period of 5 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 52–54 °C for 90 s, and extension at 72 °C for 105 s. A final extension cycle of 72 °C for 10 min ended the process and the reaction was held at 4 °C. GFX PCR DNA columns and Gel Band Purification Kits (GE Healthcare) were used to purify PCR products, following the manufacturer's instructions.

The sequencing reaction was carried out with BigDye Terminator v3.1 (Applied Biosystems, Wellesley, MA). Sequencing products were purified using an AutoSeq G-50 Dye Terminator Removal Kit (GE Healthcare), following the instructions of the manufacturer. Sequences were visualized using an ABI-Prism 310 Genetic Analyzer (Applied Biosystems).

### Phylogenetic analysis

**Taxon sampling.** Sequences of the nrLSU DNA were generated from 42 specimens (including 11 type specimens) of *Helvella*, representing 26 species, plus one sequence of *Wynnella silvicola* (Table 1). Furthermore, 42 additional sequences of *Helvella* were obtained from GenBank, plus another of *W. silvicola*, and 15 of the outgroup. The 92 sequences assembled for this study come from specimens of 12 countries.

**DNA alignment.** Sequences were edited using Chromas Pro Ver. 1.41 (Technelysium Pty, Ltd, Tewantin, Qld, Australia). Every sequence was then subjected to a BLAST search in GenBank and ambiguous sequences were removed. DNA alignments were checked by eye and manually corrected when necessary using MacClade 4.0 (Maddison & Maddison, 2000).

**Morphological data.** A matrix of 15 morphological and chemical characters were used. Appendix I shows character coding and Appendix II the morphological and chemical matrix.

**Phylogenetic inference.** The first phylogenetic analysis was done to determine the position of the genus *Wynnella* and the second

to establish the phylogeny within *Helvella*. In the first analysis, a matrix comprising species of Helvellaceae, Tuberaceae, Rhiziniaceae, and Discinaceae with 101 LSU sequences and 487 bp was constructed; 459 bp remained when ambiguous regions were excluded. To determine the phylogeny of *Helvella*, 68 specimens producing a matrix of 499 bp and 15 morphological and chemical data was used. Phylogenetic analyses were conducted using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference. MP was executed in PAUP\* 4.0b10 (Altivec) (Swofford, 2002), with the following parameters: gaps as missing characters, heuristic searches with changes among character states having equal weights, 1,000 replicates, tree-bisection-reconnection (TBR) as the branch swapping algorithm, branches collapsed if maximum branch length was zero, and molecular data as the “DNA” option and morphological and chemical data as “mixed”. MP bootstrap support (BS-MP) was obtained from 1,000 replicates (Felsenstein, 1985). RAxML 7.2.6 (Stamatakis, 2006), implemented in raxml-GUI 0.93 (Silvestro & Michalak, 2010), was used for the ML search, consisting of 100 replicates to find the best ML tree using the GTRGAMMA model for molecular data and the GTR model for morphological and chemical data; bootstrap support (BS-ML) was calculated with 1,000 replicates using the same models. Bayesian analyses were conducted in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The best substitution model, GTR+G+I, was determined by Modeltest 3.7 (Posada & Crandall, 1998). The parameters in the Bayesian search were set as GTR model, rates = invgamma, ngen = 100,000,000, nruns = 4, samplefreq = 100, and the first 50% of the samples were discarded. For morphological and chemical data, the program MrBayes was run with nst = 1 (basic model) and rates = gamma.

### Results

The first analysis (Fig. 3) shows *Wynnella silvicola* as the sister group of *Helvella* (BML: 97, BMP: 88, PP: 100), i.e., our analysis supports the proposal of Dissing (1966), who considered it as a separate genus. Thus, *W. silvicola* was used as the outgroup in the second analysis.

The ML phylogram yielded 2 major clades (Fig. 4): (1) *Helvella* subgen. *Leucomelaena* (BML: 55, BMP: 75, PP: –) formed by *Acetabula calyx* Sacc. (= *H. leucomelaena*), *H. leucomelaena* (Pers.) Nannf., and *H. oblongispora* Harmaja, and (2) *Helvella* subgen. *Helvella* (BML: 85, BMP: 73, PP: 99) formed by the remaining *Helvella* species. Within subgen. *Helvella*, 3 monophyletic groups were supported: (1) sect. *Lacunosae* (BML: 60, BMP: 67, PP: 100) [*H. alpestris* Boud. sensu Häffner (1987), *H. dryophila* Vellinga & N.H. Nguyen, *H. fusca* Gillet, *H. lacunosa* Afzel., *H. aff. sulcata*, and *H. vespertina* N.H. Nguyen & Vellinga], (2) sect. *Helvella* (BML: 80, BMP: 92, PP: 100) [*H. crispa* (Scop.) Fr. and *H. maculata* N.S. Weber], and (3) sect. *Elasticae* (BML: 60, BMP: 59, PP: 96) [*H. albella* Qué., *H. compressa* (Snyder) N.S. Weber, *H. connivens* Dissing & M. Lange, *H. elastica* Bull., and *H. stevensii* Peck]. Unfortunately, several species affinities could not be elucidated.

Table 1  
Sequenced specimens of *Helvella* and outgroup.

Species	Infrageneric position	Collection data, date (Herbarium)	Country of origin	GenBank (accession no.)
<i>Acetabula calyx</i>	D, W, A & C – <i>Leucomelaenae</i>	P.A. Saccardo, without date [Isotype, K (M): 15990]	Italy	JX993051
<i>Helvella acetabulum</i>	D, W – <i>Acetabulum</i> ; A & C – <i>Leucomelaenae</i>	M.A. Hernández 164, 3 November 1994 (IBUG) Bellis Kullman, 21 May 2001 (TAA179637, TAAM) M.G. Wood, 28 May 2010 (UC 1999256)	Mexico Estonia USA	JX993048 <sup>a,b</sup> AJ972414 <sup>c</sup> KC122805
<i>H. albella</i>	D – <i>Ephippium</i> ; W, A & C – <i>Elasticae</i>	A. Jakobson et al., 14 September 2001 (TAA175705, TAAM)	Estonia	<sup>a</sup> AJ972411
<i>H. alpestris</i>	D, W – <i>Acetabulum</i> ; A & C – <i>Leucomelaenae</i>	P. Marstad 128-93, 22 July 1993 (O) Anders & Wollan 34, 31 August 2007 (O)	Norway Norway	JX993049 JX993050
<i>H. atra</i>	D, W – <i>Ephippium</i> ; A & C – <i>Elasticae</i>	R. Healy RH1213, 28 July 2010 (UC 1999253) TAA179690 (TAAM)	USA Estonia	<sup>c</sup> KC122802 <sup>d</sup> AJ972413
<i>H. compressa</i>	D – <i>Ephippium</i> ; W, A & C – <i>Elasticae</i>	D.E. Stuntz 12143, 30 October 1960 (WTU)	USA	JX993052
<i>H. aff. compressa</i>	D – <i>Ephippium</i> ; W, A & C – <i>Elasticae</i>	–	USA	<sup>e</sup> AY544655
<i>H. connivens</i>	D – <i>Ephippium</i> ; W, A & C – <i>Elasticae</i>	A.H. Smith 25872, 21 July 1947 (Holotype, MICH)	USA	JX993053
<i>H. costifera</i>	D, W – <i>Acetabulum</i> ; A & C – <i>Leucomelaenae</i>	T. Ulvinen, 4 August 1994 (OULU) U. Nummela-Salo & P. Salo 5318, 7 August 1998 (H) I. Kytövuori 92-352, 16 August 1992 (H)	Finland Finland Norway	JX993054 JX993055 JX993056
<i>H. aff. costifera</i>	D, W – <i>Acetabulum</i> ; A & C – <i>Leucomelaenae</i>	M. Medina & I. García 1104, 3 August 1975 (ENCB) F. Landeros 3358, 27 September 2009 (IBUG) F. Tapia 2301, 22 August 2004 (IBUG)	Mexico Mexico Mexico	JX993057 JX993058 JX993059
<i>H. crispa</i>	D <sup>f</sup> , W, A & C – <i>Helvella</i>	L. Guzmán-Dávalos 10453, 24 August 2008 (IBUG) F. Landeros 3355, 26 September 2009 (IBUG) DSH97–050 (FH)	Mexico Mexico USA	JX993060 JX993061 <sup>a</sup> AY789399
<i>H. cupuliformis</i>	D, W – <i>Macropodes</i> ; A & C – <i>Cupuliformae</i>	T. Eriksson & R. Morander, 21 July 1948 (Paratype, UPS)	Sweden	JX993062
<i>H. dryophila</i>	D, W – <i>Lacunosae</i> ; A & C – <i>Helvella</i>	N.S. Weber 6373, 10 November 1990 (OSC) MES215 M.E. Smith MES218, 6 April 2008 (UC 1999238) N.H. Nguyen, 7 January 2012 (UC 1999201) L. Grubisha, 12 January 2002 (UC 1860627) J.M. Trappe, 12 January 2002 (UC 1860642) E.C. Vellinga, 15 January 2012 (UC 1999226)	USA USA USA USA USA USA	<sup>a</sup> U42681 <sup>g</sup> JQ925665 <sup>c</sup> KC122772 <sup>c</sup> KC122792 <sup>c</sup> KC122793 <sup>c</sup> KC122794 <sup>c</sup> KC122795
<i>H. elastica</i>	D, W, A & C – <i>Elasticae</i>	L. Guzmán-Dávalos 7554, 11 September 1998 (IBUG)	Mexico	JX993063
<i>H. ephippium</i>	D, W – <i>Ephippium</i> ; A & C – <i>Elasticae</i>	–		<sup>h</sup> JN048874
<i>H. aff. ephippium</i>	D, W – <i>Ephippium</i> ; A & C – <i>Elasticae</i>	O. Rodríguez 1022, 19 August 1994 (IBUG)	Mexico	JX993064
<i>H. fusca</i>	W – <i>Lacunosae</i> ; D <sup>e</sup> , A & C – <i>Helvella</i>	G. Bresadola, 21 May 1898 (Epitype, S)	Italy	JX993065
<i>H. griseoalba</i>	D, W – <i>Acetabulum</i> ; A & C – <i>Leucomelaenae</i>	N.J. Smith 982, 10 June 1968 (Holotype, MICH)	USA	JX993066
<i>H. lacunosa</i>	D, W – <i>Lacunosae</i> ; A & C – <i>Helvella</i>	K.H.03.111, 27 August 2003 (FH) K. Hansen, K. Gillen & I. Olariaga, 20 August 2010 (S)	Norway Sweden	<sup>c</sup> KC122770 <sup>c</sup> KC122771
<i>H. aff. lacunosa</i>	D, W – <i>Lacunosae</i> ; A & C – <i>Helvella</i>	MES286 L. Guzmán-Dávalos 9002, 18 October 2003 (IBUG) A. Castro-Castro, 7 October 2010 (IBUG) F. Padilla, 20 August 1985 (IBUG)	USA Mexico Mexico México	<sup>g</sup> JQ925666 JX993067 JX993068 KC019115



Table 1 (Continued)

Species	Infrageneric position	Collection data, date (Herbarium)	Country of origin	GenBank (accession no.)
<i>H. leucomelaena</i>	D, W, A & C – <i>Leucomelaenae</i>	P. Billekens & J. Legarde, 22 May 1982 (H)	Netherlands	JX993069
		F.D. Calonge 15990, 11 May 1980 [K, ex herb. MA-Fungi 15990]	Spain	JX993070
		Beauglehole & Fuhrer (Beaton 333), 23 July 1966 (K)	Australia	JX993075
		K. Hansen & G. Lewis-Gentry, K.H.06.01 (FH) D.J. Klein, 16-Mar-2012 (UC 1999257)	USA	<sup>i</sup> KC012682
<i>H. aff. leucomelaena</i>	D, W, A & C – <i>Leucomelaenae</i>	E.C. Vellinga, 18 March 2012 (UC 1999258)	USA	<sup>c</sup> KC122798
<i>H. macropus</i>	D, W, A & C – <i>Macropodes</i>	MES198	USA	<sup>g</sup> JQ925667
		O. Rodríguez 3409, 24 August 2008 (IBUG)	Mexico	JX993071
		F. Landeros 3396a, 9 October 2010 (IBUG)	Mexico	JX993072
		F. Landeros 3396c, 9 October 2010 (IBUG) M.E. Smith MES198	Mexico	JX993073
<i>H. maculata</i>	D <sup>e</sup> , W, A & C – <i>Helvella</i>	N.J. Smith 2124, 5 October 1968 (Holotype, MICH)	USA	JX993074
		E.C. Vellinga, 28 January 2012 (UC 1999255)	USA	<sup>c</sup> KC122797
		N.H. Nguyen, 10 December 2007 (UC 1999251)	USA	<sup>c</sup> KC122800
<i>H. oblongispora</i>	D, W, A & C – <i>Leucomelaenae</i>	M. Korhonen 5421, 22 August 1983 (H)	Sweden	JX993076
<i>H. pallidula</i>	D, W – <i>Macropodes</i> ; A & C – <i>Cupuliformae</i>	U. Söderholm 2916, 17 June 1999 (H)	Austria	JX993077
<i>H. robusta</i>	D, W – <i>Acetabulum</i> ; A & C – <i>Leucomelaenae</i>	N.J. Smith 397, 24 July 1967 (Holotype, MICH)	USA	JX993078
<i>H. solitaria</i>	D, W – <i>Acetabulum</i> ; A & C – <i>Leucomelaenae</i>	R.M. Danielson 459, 30 August 1972 (Holotype, DAOM)	Canada	JX993079
<i>H. stevensii</i>	D – <i>Ephippium</i> ; W, A & C – <i>Elasticae</i>	–		<sup>j</sup> AM397273
		Herrera de Duero 1294, 31 March 2001 (MA Fungi 54822)	Spain	KP860988
		A. González, J.C. Campos et al., G.P. 1395, 7 April 2001 (MA-Fungi 73836)	Spain	KP860989
<i>H. subglabra</i>	D – <i>Ephippium</i> ; A & C – <i>Elasticae</i>	Stevens, 23 June 1905 (NY)	USA	JX993080
<i>H. aff. sulcata</i>	D, W – <i>Lacunosae</i> ; A & C – <i>Helvella</i>	N.J. Smith 2145, 13 October 1968 (Holotype, MICH)	USA	JX993081
<i>H. ulvinenii</i>	D, W – <i>Acetabulum</i> ; A & C – <i>Leucomelaenae</i>	C.T. Rogerson, 29 July 1962 (NY)	USA Mexico	JX993082
		De Ávila 13B, without date (ENCB)	Mexico	JX993083
		F. Landeros 1848, 7 August 2004 (IBUG)		JX993084
<i>H. vespertina</i>	D, W – <i>Lacunosae</i> ; A & C – <i>Helvella</i>	H. Harmaja, 25 August 1979 (Holotype, H)	Finland	JX993085
<i>Peziza subclavipes</i>	D, W, A & C – <i>Macropodes</i> <i>Wynnella</i> A & C – Subgenus <i>Silvicolae</i>	E.C. Vellinga, 27 December 2011 (UC 1999195)	USA	<sup>c</sup> KC122775
		S. Branco, 7 January 2012 (UC 1999203)	USA	<sup>c</sup> KC122776
		BAMS, 11 October 2011 (UC 1999193)	USA	<sup>c</sup> KC122777
		BAMS, 11 October 2011 (UC 1999194)	USA	<sup>c</sup> KC122778
		N.H. Nguyen, 19 November 2011 (UC 1999206)	USA	<sup>c</sup> KC122780
		M. Brown, 29 October 2011 (UC 1861236)	USA	<sup>c</sup> KC122781
		E.C. Vellinga, 23 October 2011 (UC 1999197)	USA	<sup>c</sup> KC122782
		N.H. Nguyen, 19 November 2011 (UC 1999207)	USA	<sup>c</sup> KC122783
		N.H. Nguyen, 20 November 2011 (UC 1999212)	USA	<sup>c</sup> KC122784
		A. Ward, 22 November 2011 (UC 1999214)	USA	<sup>c</sup> KC122785
		N.H. Nguyen, 28 January 2012 (UC 1999215)	USA	<sup>c</sup> KC122786
		BAMS, 7 January 2012 (UC 1999202)	USA	<sup>c</sup> KC122787
		N.H. Nguyen, 19 November 2011 (UC 1999205)	USA	<sup>c</sup> KC122788
		N.H. Nguyen, 20 November 2011 (UC 1999213)	USA	<sup>c</sup> KC122789
		N.H. Nguyen, 19 November 2011 (UC 1999209)	USA	<sup>c</sup> KC122790
		N.H. Nguyen, 20 November 2011 (UC 1999210)	USA	<sup>c</sup> KC122791
<i>Wynnella silvicola</i>	D, W – Genus <i>Wynnella</i> A & C – Subgenus <i>Silvicolae</i>	N.A.F. Ellis, 27 September 1882 (Lectotype, NY)	USA	JX993086
		N.S. Weber 6219 (OSC)	USA	<sup>k</sup> U42682

Table 1 (Continued)

Species	Infrageneric position	Collection data, date (Herbarium)	Country of origin	GenBank (accession no.)
<i>Wynnella silvicola</i>	Outgroup	N.S.Weber 6219 (OSC)	USA	JX993087
<i>Balsamia magnata</i>				U42683
<i>Balsamia nigrens</i>				EU669425
<i>Barssia oregonensis</i>				U42684
<i>Barssia oregonensis</i>				NG027601
<i>Barssia cf. oregonensis</i>				AY544652
<i>Choiromyces alveolatus</i>				AF435826
<i>Discina macrospora</i>				U42678
<i>Gyromitra melaleucoides</i>				U42680
<i>Labyrinthomyces varius</i>				U42689
<i>Rhizina undulata</i>				Q220410
<i>Tuber melanosporum</i>				AF435821
<i>Tuber rufum</i>				DQ191676
<i>Underwoodia beatonii</i>				JQ925715
<i>Underwoodia beatonii</i>				JQ925716
<i>Underwoodia columnaris</i>	U42685			

Symbology: D – Dissing (1966), W – Weber (1972), A & C – Abbott and Currah (1997).

<sup>a</sup> From Tedersoo et al. (2006), included in the morphological study.

<sup>b</sup> As “*H. leucomelaena*” in Tedersoo et al. (2006).

<sup>c</sup> From Nguyen et al. (2013), not studied morphologically.

<sup>d</sup> From Tedersoo et al. (2006), not studied morphologically.

<sup>e</sup> From AFTOL (unpublished), not studied morphologically.

<sup>f</sup> As *Helvella* Sect. *Crispa*. In the case of the species not included by D, W, and A & C, their location is in accordance with the morphologic characteristics of the section.

<sup>g</sup> From Bonito et al. (2013), not studied morphologically.

<sup>h</sup> From Alvarado et al. (2011), not studied morphologically.

<sup>i</sup> From Hansen et al. (2013), not studied morphologically.

<sup>j</sup> Kellner et al. (unpublished), not studied morphologically.

<sup>k</sup> O’Donnell et al. (1997) included in the morphological study.

### Updated generic description

**Helvella** (as *Elvela*) L., *Sp. pl. 2: 1180 (1753)*

*Type species: Helvella mitra* (as *Elvela*) L., *Sp. pl., Edn 2 2: 1180 (1753)*

≡ *Helvella crispa* (Scop.) Fr., (*Lundae*) 2(1): 14 (1822)

Ascomata epigeous, stipitate, occasionally sessile, solitary to gregarious. Apothecia cupulate, discoid, convex, saddle-shaped, bi-, tri-, or irregularly lobed, but never auricularoid nor completely fused with the stipe; hymenium white, cream, buff, brown, gray, red brown, dark brown, or black, occasionally mottled (shades of grayish brown); apothecial sterile surface smooth or ribbed, glabrous, subpubescent, or pubescent, same color as hymenium or lighter. Stipe terete, even, with clefts, costate, or lacunose, white, cream, buff, brown, gray, or black, glabrous, subpubescent, or pubescent, internally solid, hollow, or chambered; base with white mycelium. Asci cylindrical, hyaline, operculate, base pleurorhynchous or aporhynchous, non-amyloid (J-), 8-spored. Ascospores ellipsoid to broadly ellipsoid, subglobose, subfusoid to fusoid, frequently with a central guttule or rarely triguttulate, hyaline, smooth or verrucose, tetranucleate at maturity. Paraphyses straight, clavate, septate, simple or branched, normally thin-walled, rarely thick-walled or with a thick-walled cap or collar at the apex, hyaline, with light brown or dark brown pigments in the wall, cytoplasm, and/or encrusted on the wall, these pigments visible or not with

cotton blue. Apothecial tissues divided in 2 layers: medullary and ectal excipulum; stipe tissues also separated in an outer and inner layer. Medullary excipulum and stipe inner layer of *textura intricata*, hyaline or light brown, some species with dextrinoid reaction (Dx+) with Melzer’s reagent. Ectal excipulum and stipe outer layer of *textura angularis*, hyaline, with light or dark brown pigments in the wall, cytoplasm, and/or encrusted on the wall, pigments visible or not with cotton blue, some species Dx+. Hairs may be present on the apothecial sterile surface and on the stipe surface, with the same microstructure in all species that present them, grouping into hyphal clusters or fascicles, hyaline, light brown, or dark brown, pigments present in the wall, cytoplasm, and/or encrusted on the wall, pigments visible or not with cotton blue, some species Dx+. Basal mycelial hyphae septate, hyaline.

### Habit, habitat, and distribution

Solitary or in groups, some scattered, in soil of conifer and angiosperm forests. Common in temperate and arctic habitats, almost exclusively distributed in the Northern Hemisphere; only *Helvella elastica*, *H. fibrosa* (Wallr.) Korf, *H. lacunosa* (*sensu lato*), *H. papuensis* Dissing, and *H. sp.* (aff. *H. pezizoides*) have been reported from a tropical region, in Papua New Guinea (Dissing, 1979). Some species have been cited from Argentina and Australasia (Gamundi, 2010; Rifai, 1968).

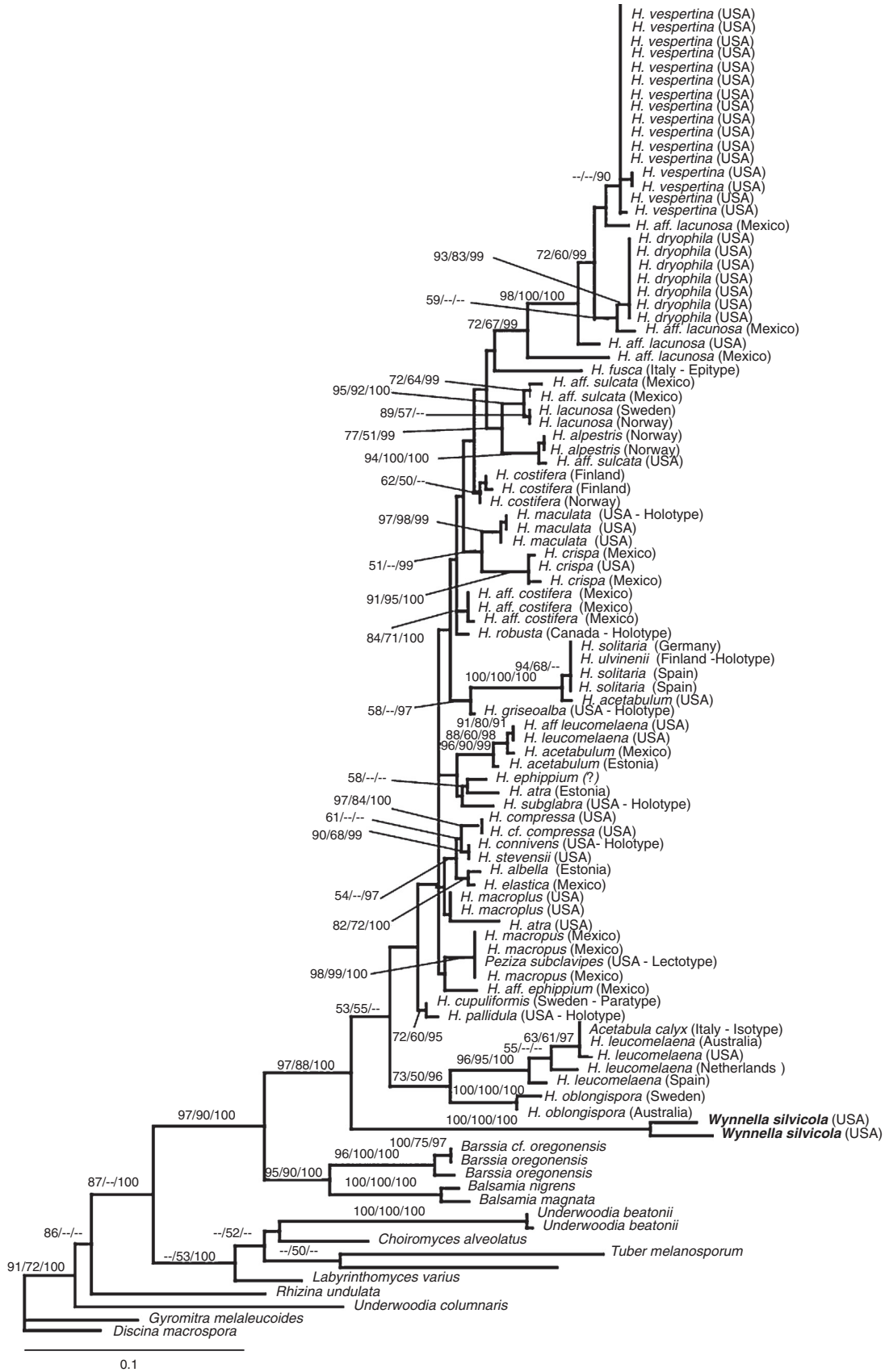


Figure 3. Phylogenetic position of *Wynnella* based on maximum parsimony analysis of large nuclear subunit rDNA sequences. Maximum likelihood bootstrap support (BML) and parsimony bootstrap support (BMP) >50%, and Bayesian posterior probability (PP) values >95% are given at the internodes (BML/BMP/PP).

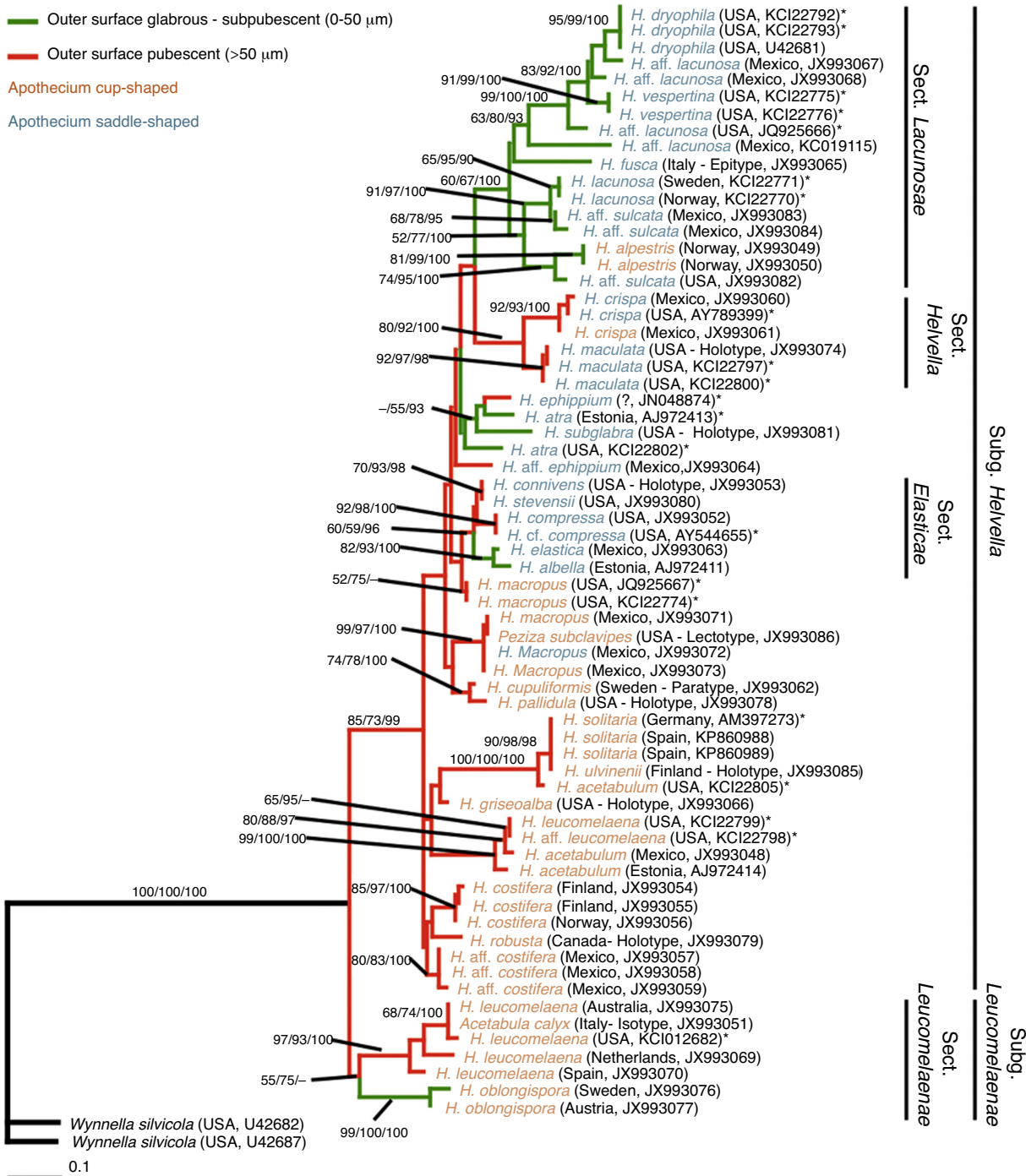


Figure 4. Phylogeny of *Helvella* generated from maximum likelihood analysis of large nuclear subunit rDNA sequences and morphological data. Maximum likelihood bootstrap support (BML), parsimony bootstrap support (BMP) >50%, and Bayesian posterior probability (PP) values >95% are given at the internodes (BML/BMP/PP). \*Specimens not studied morphologically.

## Discussion

### The misunderstood concept of *Helvella*

Since the last century, Nannfeldt (1937) argued that *Acetabula* (Fr.) Fuckel [represented here by *Helvella acetabulum* (L.) Quél.], *Cyathipodia* Boud. (represented here by *H. cupuliformis* Dissing & Nannf.), *Leptopodia* Boud. (represented here by *H. albella*, *H. alpestris*, *H. atra* J. König, *H. elastica*,

*H. ephippium* Lév., and *H. stevensii*), *Macropodia* Fuckel or *Macroscyphus* Nees ex Gray [represented here by *H. macropus* (Pers.) P. Karst., *Peziza subclavipes* W. Phillips & Ellis], and *Paxina* Kuntze (represented here by *A. calyx*, *H. acetabulum*, *H. compressa*, *H. costifera* Nannf., and *H. leucomelaena* in this paper) were untenable. They were described based on the shape of the apothecia and stipes, e.g., *Macropodia* with cup-shaped apothecia and *Leptopodia* with lobed apothecia. However, it is common to find ascomata with young cup-shaped apothecia that



later become lobed (as in *H. crispa* and *H. macropus*). None of these genera can be separated from *Helvella* because they are: (1) microscopically similar, and (2) do not form supported monophyletic clades.

Some authors did not follow the Nannfeldt's concept of *Helvella*. Dennis (1981) and Breitenbach and Kränzlin (1984) continued using the genera *Cyathipodia*, *Leptopodia*, *Macropodia* or *Macroscyphus*, and *Paxina*, as well as *Helvella*. In contrast, Eckblad (1968) placed *Underwoodia columnaris* Peck and *U. fuegiana* (Speg.) Gamundi under *Helvella*, and this placement was followed by Harmaja (1974), who synonymized *U. beatonii* Rifai as *H. beatonii* (Rifai) Harmaja. However, Abbott and Currah (1997), Ainsworth, Sparrow, and Sussman (1973), Dissing (1966, 1972), Gamundi (2010), Korf (1972), and Rifai (1968), considered *Underwoodia* as an independent genus. Phylogenetic studies have corroborated the last point of view (Hansen & Pfister, 2006; Læssøe & Hansen, 2007; O'Donnell, Cigelnik, Weber, & Trappe, 1997, and this work). Although *Underwoodia* has a medullary excipulum of *textura intricata* and ectal excipulum of *textura angularis* as do all species of *Helvella*, and this character was used by Eckblad (1968) to consider *Underwoodia* as a synonym of *Helvella*, the apothecium of *Underwoodia* is fully adhered to the stipe, unlike any apothecium seen in *Helvella*. Also, ascospore ornamentation is very evident inside the asci (not seen in *Helvella* ascospores), and some species have hooked paraphyses (*U. beatonii* and *U. columnaris*), while all the species of *Helvella* have straight paraphyses.

The taxonomic position of *Wynnella* has been controversial. On the basis of microscopic features, Harmaja (1974) transferred *W. silvicola* to *H. silvicola*. Abbott and Currah (1988), following Harmaja, erected *Helvella* sect. *Silvicolae* to place this species. Subsequently, they raised its rank to subgenus (Abbott & Currah, 1997). However, Ainsworth et al. (1973), Dissing (1966, 1972), Dissing, Eckblad, and Lange (2000), Eckblad (1968), Korf (1972), and Parslow and Spooner (2009) recognized *Wynnella* as an independent genus from *Helvella*. Phylogenetic studies of Helvellaceae have suggested a sister group relationship between *Helvella* and *Wynnella* (Hansen & Pfister, 2006; Harrington, Pfister, Potter, & Donoghue, 1999; Læssøe & Hansen, 2007; Landvik, Kristiansen, & Schumacher, 1999; O'Donnell et al., 1997), which was also found in this study. Like *Underwoodia* and *Helvella*, *Wynnella* has a medullary excipulum of *textura intricata* and ectal excipulum of *textura angularis*, but its apothecia are auriculoid (similar to a rabbit's ear), different to any apothecia seen in *Helvella*. A complete circumscription of *Helvella* based mainly on Nannfeldt (1937), Dissing (1966, 1972), and Rifai (1968), and following the phylogenetic results previously obtained and the ones generated in this study, is presented above in Results.

#### Comments about diversity and distribution of the species of *Helvella*

Excluding synonyms and species transferred to other genera, 36 species have been recognized for Europe (Calonge & Arroyo, 1990; Dissing, 1966; Dissing et al., 2000; Häffner,

1987; Van Vooren, 2010). In North America, 32 species have been registered (Abbott & Currah, 1997; Nguyen, Landeros, Garibay-Orijel, Hansen, & Vellinga, 2013; Weber, 1972), 20 for Asia (Kaushal, 1991; Zhuang, 1995), 3 for South America (Dissing, 1966; Gamundi, 2010; Wright & Albertó, 2006), 3 for Africa (Dissing, 1966), and 2 species for Australia (Rifai, 1968). *Helvella leucomelaena* is the most widely cited, recorded from every continent. Similarly, *H. lacunosa* and *H. solitaria* P. Karst. are located on 4 continents. However, it has recently been revealed that *H. lacunosa* is a complex of species, where the American specimens do not correspond to its European counterpart (Nguyen et al., 2013). This probably occurs with other species with a broad distribution such as *H. leucomelaena* and *H. costifera* as already seen from our data (Fig. 4), but we need more detailed studies to confirm it.

On the other hand, *H. alpestris*, *H. dovrensis* T. Schumach., *H. fusca*, *H. latispora* Boud., *H. oblongispora*, *H. paraphysitorquata* I. Arroyo & Calonge, and *H. ulvinenii* Harmaja have been reported only from Europe (Calonge & Arroyo, 1990; Dissing, 1966; Schumacher, 1992). *Helvella compressa*, *H. crassitunicata* N.S. Weber, *H. griseoalba* N.S. Weber, *H. maculata*, and *H. robusta* S.P. Abbott are exclusively known from America (Abbott & Currah, 1997; Weber, 1972, 1975). *Helvella papuensis* is only recorded from a tropical region of Papua New Guinea (Dissing, 1979) and *H. aestivalis* (R. Heim & L. Rémy) Dissing & Raitv., *H. dovrensis*, and *H. verruculosa* (Berk. & M.A. Curtis) Harmaja have an arctic and alpine distribution (Abbott & Currah, 1997). It is necessary to examine *Helvella* species that have been described from Asia to determine their status.

#### Infrageneric classification of *Helvella*

The analysis combining DNA sequences, morphological, and chemical data generated trees with better-supported clades, which demonstrates the importance of “total evidence”, where all relevant data are considered in phylogenetic analysis (Eernisse & Kluge, 1993).

Our results do not completely support any previous infrageneric *Helvella* classification. However, there is enough current evidence to divide *Helvella* in 2 subgenera based on ascus development that is also supported by phylogenetic data (Fig. 4). *Helvella* subgen. *Leucomelaenae* is monotypic with sect. *Leucomelaenae* Dissing sensu Weber (1972) and includes species with aporhynchous asci. On the other hand, *Helvella* subgen. *Helvella* comprises species with pleurorhynchous asci. The present study failed to determine several species affinities at section level, as for *H. acetabulum*, *H. costifera*, *H. griseoalba*, *H. robusta*, and *H. ulvinenii* within *Helvella* sect. *Acetabulum* Dissing sensu Weber (1972). Although *H. atra*, *H. ephippium*, and *H. subglabra* N.S. Weber, representing *Helvella* sect. *Ephippium* Dissing sensu Weber (1972), formed a clade, this was unsupported statistically. Furthermore, the phylogenetic results did not support the association of the species of *Helvella* sect. *Macropodes* Dissing sensu Weber (1972), which includes *H. cupuliformis* and *H. macropus*. It is necessary to use other DNA regions to resolve their affinity.

Most of the monophyletic clades are defined by more than one morphological feature. The exception is the clade representing *Helvella* sect. *Leucomelaenae*, which is characterized only by the presence of aporhynchous asci. Figure 4 shows that the main features used by Abbott and Currah (1997), Dissing (1966), and Weber (1972) to define infrageneric taxa (pubescence and apothecium shape) are present throughout the phylogram, and only in combination can they be used as diagnostic characters of the clades.

#### *Helvella* subgen. *Helvella*

≠ subgen. *Helvella*, in Abbott and Currah (1997)

Asci pleurorhynchous, i.e., ascus base forked, because a crozier is formed at the base.

*Type species:* *Helvella crispa* (Scop.) Fr.

Abbott and Currah (1997) circumscribed subgen. *Helvella* for species with lobed apothecia and a costate or lacunose stipe; instead, we consider this subgenus to include all the species with pleurorhynchous asci. Phylogenetically supported sections in this subgenus are *Elasticae*, *Helvella*, and *Lacunosa*.

#### *Helvella* subgen. *Leucomelaenae* S.P. Abbott emend. Landeros & Guzm.-Dáv.

Asci aporhynchous, i.e., ascus base simple, because no crozier is formed at the base.

*Type species:* *Helvella leucomelaena* (Pers.) Nannf.

It is characterized by its aporhynchous asci. In contrast, Abbott and Currah (1997) delineated the subgenus for species with a cup-shaped apothecium and a costate stipe, without considered how the ascus was formed. We recognize sect. *Leucomelaenae* Dissing sensu N.S. Weber (1972).

#### Taxonomically informative features

Comments on all the characters that have been used in *Helvella* are presented, mentioning if they have any taxonomical value.

**Apothecia.** Weber (1972) and Abbott and Currah (1997) considered the shape of the apothecia as one of the most important features for recognition of sections or subgenera within the genus. *Helvella acetabulum*, *H. costifera*, *H. cupuliformis*, *H. ulvinenii*, and *H. verruculosa* have cup-shaped apothecia throughout their entire development. In contrast, *H. albella*, *H. compressa*, *H. elastica*, and *H. lacunosa* exhibit lobed apothecia. However, some species like *H. crispa*, *H. ephippium*, and *H. macropus* have cupulate apothecia when young and lobed apothecia when mature, and sometimes even cupulate apothecia may have mature ascospores. Another relevant taxonomic character is the way in which the apothecium margin is attached to the stipe, a feature almost exclusively found in sect. *Lacunosa* and in *H. robusta* in sect. *Acetabulum*. Therefore, the classification of *Helvella* based on apothecia shape and attachment is

problematic. In the light of the phylogenetic results (Fig. 4), it is desirable to re-evaluate these characters more carefully in each species by studying various collections and assessing the degree of variation.

**Stipe.** Its shape is a diagnostic character in the genus. Weber (1972) described the stipe as even, costate, lacunose, and sulcate. The first 3 are easy to distinguish, but sulcate is hard to differentiate from costate. For this reason, we consider sulcate and costate as synonyms, meaning that the stipe has only longitudinal ribs. Sometimes species with a smooth stipe can develop folds or clefts mainly in old ascomata [e.g., *Helvella atra*, *H. corium* (O. Weberb.) Masee, *H. pezizoides* Afzel., *H. stevensii*], but they never look like true ribs as in *H. acetabulum*, *H. costifera*, and *H. sulcata* Afzel. On the other hand, *H. costifera*, *H. palustris* Peck, and *H. sulcata* have a costate stipe with one or 2 transverse ribs, but it never looks like a lacunose stipe, which has both longitudinal and transversal ribs, giving the stipe the appearance of having deep holes, e.g., in *H. lacunosa*. Because we have seen specimens of *H. acetabulum* and *H. crispa* with either costate or lacunose stipes, we believe it is important to know the degree of variation among and within species. Therefore, we consider stipe shape (even, costate, and lacunose) as an important taxonomic character.

**Hymenium and stipe color.** Weber (1972) regarded hymenium and stipe color as valuable features at the infrageneric classification level, while Abbott and Currah (1997) did not use these as main characters for their sections. We agree with Weber (1972) that hymenium and stipe colors are useful, but only for species distinction and in combination with other characters. For instance, *Helvella* sect. *Elasticae* Dissing sensu Weber (1972) is defined by its tan to gray-brown hymenial surface and buff to nearly white stipe, besides the lobed apothecium and even stipe. Hymenium color is also helpful for identifying species. For example, fresh specimens of *H. griseoalba* have a hymenium with grayish tones, which separates them from fresh specimens of *H. costifera*, which have brownish tones.

**Ribs.** Rib structure is a useful character to distinguish some species. In *Helvella acetabulum*, sharp ribs contrast with the blunt ribs of *H. costifera* and *H. griseoalba*. Another feature to take into consideration is the distance from the ribs to the margin of the apothecial sterile surface. We recognize 2 categories in this character: (1) ribs missing or reaching a quarter or less in the lower part of sterile surface (e.g., *H. leucomelaena*, *H. solitaria*, and *H. sulcata*), and (2) ribs reaching half or to the edge of the apothecium, i.e., in the same ascoma of *H. acetabulum* or *H. costifera* ribs may arrive halfway, some to three quarters, and others to the edge of the apothecial sterile surface. Although ribs can be simple, forked, or anastomosing, this feature is uninformative because some species have simple ribs when immature and forked when mature, or in the same stage ribs may be both simple and forked.

**Asci.** Weber (1972) considered ascus development (aporhynchous vs. pleurorhynchous) as a useful character. Indeed, she restricted *Helvella* sect. *Leucomelaenae* to include only species with aporhynchous asci. In this work, we found that *Helvella*

sect. *Leucomelaena* Dissing *sensu* Weber (1972) is monophyletic (Fig. 4), with aporhynchous asci its synapomorphy.

**Ascospores.** Ascospores have little taxonomic value, except in a few species of *Helvella*. *Helvella crassitunicata* and *H. pocillum* Harmaja have large ascospores (Landeros et al., 2012). Likewise, *H. macropus* and *H. terrestris* (Velen.) Landvik develop fusoid to subfusoid ascospores (Abbott & Currah, 1997; Landvik et al., 1999). Abbott and Currah (1997) established ascospore ornamentation as an important taxonomic feature. Unfortunately, ornamentation is variable among ascospores of the same species. The verrucose appearance is due to remnants of the secondary wall of the ascospores, which might adhere to the primary wall (Schumacher, pers. com. in Landeros et al., 2012). It is common in many ascospores of the same specimen that such remnants do not adhere, and therefore, ascospores remain smooth. For this reason, Eckblad (1968) described the ascospores of *Helvella* as having “false ornamentation”.

**Paraphyses.** Their shape is useful at the generic level in Helvellaceae, but not for infrageneric classification or to separate species. In *Helvella*, all species have septate, simple or branched, and straight paraphyses, in at least one stage of their development. Vite-Garín, Villaruel-Ordaz, and Cifuentes (2006) described species with non-septate (e.g., *H. acetabulum*, *H. corium*, *H. cupuliformis*, and *H. elastica*) and/or unbranched paraphyses (e.g., *H. acetabulum*, *H. atra*, *H. corium*, *H. costifera*, and *H. crispa*). We studied some of the specimens Vite-Garín et al. (2006) mentioned and found that paraphyses are always septate and either branched or unbranched in the same specimen.

**Hyphal fascicles.** Microscopically, hairs on the apothecial surface are formed by hyphal fascicles. In the present work, following Landeros et al. (2012), we use the terms: (1) glabrous, when such hyphal fascicles are absent; (2) subpubescent, when hyphal fascicles are shorter than 50  $\mu\text{m}$ ; and (3) pubescent, when hyphal fascicles are longer than 50  $\mu\text{m}$ . Dissing (1966) considered hairs on the apothecial sterile surface as an important criterion in his infrageneric classification. In Figure 4, all species of *Helvella* sect. *Lacunosae* are glabrous-subpubescent, while all species of sections *Helvella* and *Macropodes* (*H. cupuliformis* and *H. macropus*) are pubescent. However, in *Helvella* sect. *Elasticae* *sensu* Dissing (1966) there are species with glabrous, subpubescent, and pubescent ascromata. In this case, this feature is useful to recognize species, such as *H. albella* and *H. elastica*, which have a glabrous to subpubescent surface. Therefore, this is an important feature for recognizing species, but it must be used in conjunction with other characteristics to separate sections.

**Pigments.** For Landeros et al. (2012), the presence of pigment in the ascroma has taxonomic value at species level. When present, pigments have to be evaluated from 3 points of view: (1) color, which may vary from dark to light brown; (2) structure location, pigments can be found in paraphyses, ectal excipulum cells, stipe outer layer cells, and apothecial and stipe hyphal fascicles, and (3) cellular location, in the wall, encrusted on the wall, and/or in the cytoplasm. We also recommend using cotton blue

to evaluate whether the pigments are visible or not with this stain. Encrusted pigment on the wall (mainly in the paraphyses) is variable between ascromata of the same species; if present, it is always visible with cotton blue.

**Dextrinoid reaction (Dx+).** A red reaction in Melzer's reagent, assessed according to Leonard (2006), is a meaningful character from taxonomic and phylogenetic perspectives. All species of *Helvella* sect. *Elasticae* present at least one Dx+ tissue, but this feature is not exclusive to this section; e.g., *H. acetabulum*, *H. robusta*, and *H. subglabra* also have at least one type of Dx+ tissue, and the reaction can be used to recognize these species. Because the reaction is especially evident in stipe tissues, it is important to look in the entire ascroma for it and to describe the tissue where it occurs.

**Cell width in the ectal excipulum and stipe outer layer, and breadth of both layers.** We noticed that cell width in the ectal excipulum and outer stipe layer of each ascroma is the same, independent of the tissue origin of the section. The thickness of the ectal excipulum may vary slightly; it is thinner toward the edge and wider in the center of the apothecium, while in the stipe the outer layer is uniform. *Helvella oblongispora* is unique in these layers being very wide, both composed of very large cells, almost the same width as in the medullary excipulum. Therefore, this feature is only important for recognizing *H. oblongispora*.

**Ecology.** Hobbie, Weber, and Trappe (2001) mentioned as uncertain the mycorrhizal status of *Helvella*; however, Tedersoo, Hansen, Perry, and Kj oller (2006) considered all the species as forming ectomycorrhizae, hence studies are needed to confirm the status of the genus. Some taxa are associated with specific trees, e.g., *H. fusca* (Dissing, 1966; Landeros et al., 2012) and *H. leucopus* var. *populina* I. Arroyo & Calonge (in Calonge, 2000) always grow near or under *Populus* L. Recently, on the basis of molecular data, 2 cryptic species of the *H. lacunosa* complex from western North America were found (Nguyen et al., 2013); *H. dryophila* is associated with *Quercus* L. and *H. vespertina* with conifers. Furthermore, *Helvella* species can be restricted to a vegetation type or ecosystem, e.g., *H. papuensis* associates with tree species of *Castanopsis* (D. Don) Spach and *Lithocarpus* Blume in subtropical rain forests (Abbott & Currah, 1997; Dissing, 1979) and *H. aestivalis* and *H. verruculosa* in the tundra of arctic and alpine regions (Abbott & Currah, 1997).

Phenology is also cited as an important feature; *H. ephippium* fruits in July and August in USA (Weber, 1972) and *H. leucopus* Pers. from March to May in Europe (Dissing, 1966). The fruiting period can also be longer; *H. elastica*, *H. lacunosa*, and *H. macropus* fruit from June to November (Dissing, 1966; Weber, 1972). Some species fruit at different times in different countries; *H. leucomelaena* fruits from May to July in Sweden, from January to May in France, from April to June in British Columbia and Washington, and from May to September in Alberta (Abbott & Currah, 1997; Dissing, 1966). There are species that can be distinguished from other similar species on the basis of phenology; *H. albella* has an autumnal fruiting pattern, whereas *H. compressa* fruits in the spring. In



addition to the information of vegetation and phenology, soil type was proposed by [Dissing \(1966\)](#) as an important feature to recognize some species. For example, he described *H. corium*, *H. leucomelaena*, *H. queletii* Bres., and *H. solitaria* growing in calcareous soil.

#### *Species complex and misunderstood species*

*Helvella alpestris*. This species is cited in [Index Fungorum \(2015\)](#) as a synonym of *H. corium* (O. Weberb.) Masee, but we treat it as an independent species, as [Mycobank \(2015\)](#). [Boudier \(1895\)](#) described *H. alpestris* with ascospores  $22\text{--}25 \times 14\text{--}15 \mu\text{m}$ , stipe “sulcatum” (ribbed) and “. . . *extus sub lente breviter velutinum, non squamoso-hirtum*” (under lens surface slightly velutinous, non squamous-hirsute). [Dissing \(1966\)](#) considered *H. alpestris* as a synonym of *H. corium*, with ascospores  $18\text{--}20\text{--}22 \times 11\text{--}12.1\text{--}13 \mu\text{m}$ , stipe with grooves near the base, and surface pubescent to villose, which, according to Boudier’s protologue corresponds to an incorrect interpretation of *H. alpestris*. Later, [Häffner \(1987\)](#) separated both species, describing *H. corium* with ascospores  $(15.3\text{--}) 17.3\text{--}21.3$  ( $\text{--}22$ )  $\times (8.5\text{--}) 9\text{--}12.7 \mu\text{m}$ , stipe even, and pubescent apothecial sterile surface. For Häffner, *H. alpestris* has ascospores  $(15\text{--}) 17\text{--}20.5$  ( $\text{--}22.1$ )  $\times 10.2\text{--}13.3 \mu\text{m}$ , stipe with well-defined ribs, and glabrous to subpubescent apothecial sterile surface. Except for the ascospore size, the interpretation of Häffner matches Boudier’s description of *H. alpestris*. The specimens that we studied and sequenced have ascospores similar to those cited by Häffner; hence, in this paper we follow this interpretation. However, it is necessary to sequence material of *H. corium* to confirm if they are different.

*Helvella atra*. [Landeros et al. \(2012\)](#) mentioned several features to distinguish this species from *H. subglabra*. Furthermore, in the tree of [Figure 4](#), the sequences of 2 specimens of *H. atra* from the USA are not grouped together, indicating that another species could be split. Therefore, it is necessary to include specimens from other parts of the world in the analyses, mainly from Europe, as *H. atra* was described from Iceland, to elucidate its status and typify it because there is no type material.

*Helvella costifera*. This species was described by [Nannfeldt \(in Lundell & Nannfeldt, 1953\)](#) from Sweden. Later, [Weber \(1972\)](#) described *H. griseoalba* and [Harmaja \(1978\)](#) *H. hyperborea* Harmaja. [Landeros et al. \(2012\)](#), with a study of the type specimens and additional material, proposed that *H. hyperborea* is a synonym of *H. costifera*, but *H. griseoalba* is an independent species. We sequenced European specimens from Finland and Norway and found that they grouped in one supported clade ([Fig. 4](#)), except the holotype of *H. griseoalba*, which agrees with [Landeros et al. \(2012\)](#). In fact, *H. griseoalba* is closer to the *H. solitaria* clade than to any member of *H. costifera* group. On the other hand, there is a species complex, formed by European specimens that correspond to *H. costifera* and Mexican specimens belonging to a different undescribed species ([Fig. 4](#)).

*Helvella ephippium*. This species has a small apothecium, less than 1.5 cm diam. Only *H. pocillum*, *H. rivularis* [Dissing](#)

& [Sivertsen](#), and *H. terrestris* can be as small, but all these species have a cup-shaped, well-defined apothecium, while *H. ephippium* has an involute, irregularly lobed to saddle-shaped apothecium. [Léveillé \(1841\)](#) did not mention the size of the ascospores, he only wrote “rondes et transparentes”. [Masee \(1895\)](#) was the first to describe them,  $16\text{--}18 \times 10 \mu\text{m}$ . More recently, [Dissing \(1966\)](#) measured ascospores  $19\text{--}20.4\text{--}21.5 \times 11\text{--}11.6\text{--}12 \mu\text{m}$ , while [Weber \(1972\)](#) described them as  $16.5\text{--}19 \times 10.5\text{--}12 \mu\text{m}$  and [Abbott and Currah \(1997\)](#) as  $17\text{--}21 \times 10.1\text{--}13 \mu\text{m}$ . We included 2 sequences in our analyses, one as *H. ephippium* from Europe (unfortunately with no more information on locality and collector) ([Alvarado et al., 2011](#)), and another as *H. aff. ephippium* from Mexico, which we proved to be unrelated ([Fig. 4](#)). On the basis of this phylogenetic result and the differences in ascospore size found by different authors, we think it is a complex. Thus, it is necessary to first lectotypify *H. ephippium* with the figure of [Léveillé \(1841\)](#) and then epitypify it with a specimen to clarify this group of species.

*Helvella fibrosa* [= *H. villosa* (Hedw. ex Kuntze) [Dissing & Nannf.\]. Not shown in the phylogenetic tree. \[Korf \\(2008\\)\]\(#\) concluded that \*Peziza fibrosa\* is the correct name for this species because “\*H. villosa\* \(Hedw. ex Kuntze\) \[Dissing and Nannf.\]\(#\)” is a later homonym of a name proposed by \[Schaeffer \\(1774\\)\]\(#\) for a different species. Also, \[Korf \\(2008\\)\]\(#\) designated Hedwig’s figure as the lectotype of \*H. fibrosa\* and specimen of \[Lundell, Nannfeldt, and Holm \\(Fungi exsiccata Suecici praesertim Upsaliensis # 3262, 1985\\)\]\(#\) as the epitype. On the other hand, \[Weber \\(1972\\)\]\(#\) proposed \*H. pallidula\* N.S. Weber as a new taxon based on the absence of pigmentation in the ascoma tissues. We agree with \[Häffner \\(1987\\)\]\(#\) and \[Abbott and Currah \\(1997\\)\]\(#\), in considering \*H. pallidula\* as a synonym of \*H. fibrosa\* because when we studied the holotype of the former we observed light brown pigments in the paraphyses, ectal excipulum, and outer layer of stipe, and yellowish pigments in the apothecial and hyphal fascicles of the stipe, as in \*H. fibrosa\*. It is necessary to include sequences of specimens from several countries in different continents in the phylogenetic analyses to establish if all specimens cited as \*H. fibrosa\* correspond to the same species.](#)

*Helvella lacunosa* and *H. sulcata*. These 2 species were described by [Afzelius \(1783\)](#). Since the last century, their delimitation has been controversial, being considered as synonyms ([Abbott & Currah, 1997; Dissing, 1966; Häffner, 1987](#)) and as separate taxa ([Landeros & Guzmán-Dávalos, 2013; Weber, 1972](#)). Recently, 2 species of the *H. lacunosa* complex in the western USA were segregated and described ([Nguyen et al., 2013](#)) because their DNA sequences do not correspond to the European material of this species. A careful study is needed to determine the number of species included in this complex and to elucidate the status of some species such as *H. sulcata*.

*Helvella leucomelaena*. We sequenced 4 specimens of this species ([Table 1](#)), 3 from Europe (one of them labeled as the isotype of *Acetabula calyx*) and one from Australia, and all were grouped in a supported clade ([Fig. 4](#)). *Acetabula calyx* is cited in the [Index Fungorum \(2015\)](#) as a synonym



of *H. solitaria*; however, morphological data and LSU rDNA sequence of the isotype, indicate that it must be a synonym of *H. leucomelaena*. Of 4 sequences labeled as *H. leucomelaena* in GenBank (AJ972414, KC012682, KC122799, KC122798), only one (KC012682 from US) grouped with our sequences of this species (Fig. 4). We checked the source specimen [Bellis Kullman, 21 May 2001 (TAA179637, TAAM)] for sequence AJ972414 and found pleurorhynchous asci, so it corresponds to *H. acetabulum*; in fact, AJ972414 groups with *H. acetabulum* from Mexico and Estonia. We did not study the US specimens from which sequences KC122798 and KC122799 were obtained; thus, we do not know whether they have aporhynchous or pleurorhynchous asci, but we suspect they correspond to *H. acetabulum* in their phylogenetic position (Fig. 4). Harmaja (1977, 1978, 1979) proposed *H. confusa* Harmaja, *H. oblongispora*, and *H. pedunculata* Harmaja, which are very similar to *H. leucomelaena*. After morphologically studying the holotype of *H. confusa* (unpublished data) and specimens labeled as *H. pedunculata* by Harmaja (unpublished data), we have concluded that we do not have evidence to consider these 2 species as independent taxa. On the other hand, we sequenced 2 specimens labeled as *H. oblongispora*, one of them determined by Harmaja, and they were grouped in another clade, thus corroborating the observations of Abbott and Currah (1997), who considered *H. confusa* and *H. pedunculata* as synonyms of *H. leucomelaena*, but *H. oblongispora* as an independent taxon.

*Helvella macropus*. It can be identified by its fusoid to subfusoid ascospores within the asci. *Helvella terrestris* also has fusoid to subfusoid ascospores, but larger, 50–65 × 12–15 μm (Landvik et al., 1999) instead of 20–26 × 9–12 μm in *H. macropus* (Dissing, 1966). Peck (1902) proposed *H. macropus* var. *brevis* Peck, based on specimens with smaller ascospores, with darker hymenia, and very short stipes. Later, Harmaja (1974) elevated it as *H. brevis* (Peck) Harmaja, considering the ascospore size and ecological features (Weber, 1972), as did Abbott and Currah later (1988). However, Weber (1972) argued that the variation between the 2 taxa merely represents different responses to environmental conditions. In fact, later Abbott and Currah (1997) reconsidered their position and placed *H. brevis* in synonymy with *H. macropus*. Figure 4 shows that specimens of *H. macropus* from USA and Mexico do not form a clade; thus, future studies need to include European sequences of *H. macropus* to determinate its relation with the American specimens.

*Helvella stevensii*. This species was considered a synonym of *H. latispora* by Häffner (1987) and Abbott and Currah (1997), but we agree with Dissing (1966) in considering these species as independent taxa (see discussion by Landeros & Guzmán-Dávalos, 2013). On another hand, we studied authentic material of *H. stevensii* and the holotype of *H. connivens*, but we did not find any difference. In fact, their DNA sequences are identical; therefore, we agree with Weber (1972) and Abbott and Currah (1997) that *H. connivens* is the same as *H. stevensii*.

DNA sequences of *H. latispora* specimens from Europe need to be analyzed to determine the relation of this species to *H. stevensii* in section *Elasticae*.

## Acknowledgments

To the curators of the herbaria DAOM, ENCB, FCME, FH, H, IBUG, K, MA, MICH, NY, O, OSC, OULU, PRM, S, TAAM, UPS, WTU, and XAL for the loan of types and other specimens. The first author thanks the Universidad Autónoma de Querétaro for its support and Conacyt for a scholarship grant for his doctoral studies. To Greg Bonito (Duke University, USA) for providing literature on *Helvella* and Richard Korf (Cornell University, USA) for his nomenclatural advice and correcting a first draft of the manuscript. Funds were obtained from Universidad de Guadalajara (projects 72640, 88682, 108721, PIFI 2008–2009).

## Appendix I. Codification of morphological and chemical characters

1. Apothecium shape when mature:	(0) auriculoid, (1) cup-shaped, (2) lobed
2. Apothecium margin:	(0) free, (1) attached to the stipe
3. Apothecium sterile surface:	(0) glabrous to subpubescent, (1) pubescent
4. Ribs on the apothecium sterile surface:	(0) missing or reaching only up to ¼ of the surface, (1) reaching from ¼ to the apothecium edge
5. Stipe shape:	(0) ribbed, (1) even
6. Type of ribs on the stipe:	(0) only longitudinal ribs, (1) with longitudinal and transversal ribs (forming cavities, lacunose)
7. Edge of the ribs on the stipe:	(0) sharp, (1) blunt
8. Stipe surface:	(0) glabrous to subpubescent, (1) pubescent
9. Stipe color:	(0) dark tones, (1) light tones
10. Ascus type:	(0) pleurorhynchous, (1) aporhynchous
11. Reaction to Melzer's solution of apothecium medullary excipulum:	(0) negative, (1) dextrinoid
12. Reaction to Melzer's solution of apothecium ectal excipulum:	(0) negative, (1) dextrinoid
13. Reaction to Melzer's solution of stipe inner layer:	(0) negative, (1) dextrinoid
14. Reaction to Melzer's solution of stipe outer layer:	(0) negative, (1) dextrinoid
15. Ascospores shape:	(0) ellipsoid, (1) fusoid to subfusoid



	1	2	3	4	5	6	7	8	9	1	1	1	1	1	1
										0	1	2	3	4	5
<i>H. aff. sulcata</i> JX993083	2	1	0	1	0	0	1	0	1	0	1	0	0	0	0
<i>H. aff. sulcata</i> JX993084	2	1	0	1	0	0	1	0	1	0	0	0	0	0	0
<i>H. ulvinenii</i> JX993085	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0
<i>H. vespertina</i> KC122776	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>H. vespertina</i> KC122775	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
<i>Peziza subclavipes</i> JX993086	1	0	1	0	1	?	?	1	1	0	0	0	0	0	1

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