



# Pindara revisited – evolution and generic limits in *Helvellaceae*

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## Key words

ascus croziers  
*Balsamia*  
*Barssia*  
*Helvella aestivalis*  
*Midotis*  
*Pezizomycetes*

**Abstract** The *Helvellaceae* encompasses taxa that produce some of the most elaborate apothecial forms, as well as hypogeous ascromata, in the class *Pezizomycetes* (*Ascomycota*). While the circumscription of the *Helvellaceae* is clarified, evolutionary relationships and generic limits within the family are debatable. A robust phylogeny of the *Helvellaceae*, using an increased number of molecular characters from the LSU rDNA, *RPB2* and *EF-1 $\alpha$*  gene regions (4299 bp) and a wide representative sampling, is presented here. *Helvella* s.lat. was shown to be polyphyletic, because *Helvella aestivalis* formed a distant monophyletic group with hypogeous species of *Balsamia* and *Barssia*. All other species of *Helvella* formed a large group with the enigmatic *Pindara* (*Helvella*) *terrestris* nested within it. The ear-shaped *Wynnella* constitutes an independent lineage and is recognised with the earlier name *Midotis*. The clade of the hypogeous *Balsamia* and *Barssia*, and *H. aestivalis* is coherent in the three-gene phylogeny, and considering the lack of phenotypic characters to distinguish *Barssia* from *Balsamia* we combine species of *Barssia*, along with *H. aestivalis*, in *Balsamia*. The closed/tuberiform, sparsoid *H. astieri* is shown to be a synonym of *H. lactea*; it is merely an incidental folded form of the saddle-shaped *H. lactea*. *Pindara* is a sister group to a restricted *Helvella*, i.e., excluding the *leucomelaena* lineage, on a notably long branch. We recognise *Pindara* as a separate genus and erect a new genus *Dissingia* for the *leucomelaena* lineage, viz. *H. confusa*, *H. crassitunicata*, *H. leucomelaena* and *H. oblongispora*. *Dissingia* is supported by asci that arise from simple septa; all other species of *Helvellaceae* have asci that arise from croziers, with one exception being the *alpina-corium* lineage of *Helvella* s.str. This suggests ascus development from croziers is the ancestral state for the *Helvellaceae* and that ascus development from simple septa has evolved at least twice in the family. Our phylogeny does not determine the evolutionary relationships within *Helvella* s.str., but it is most parsimonious to infer that the ancestor of the helvelloids produced sessile or shortly stipitate, cup-shaped apothecia. This shape has been maintained in some lineages of *Helvella* s.str. The type species of *Underwoodia*, *Underwoodia columnaris*, is a sister lineage to the rest of the *Helvellaceae*.

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## INTRODUCTION

The *Helvellaceae* contains species that produce some of the most elaborate apothecia in the class *Pezizomycetes*: sessile to stipitate, cup-shaped to expanded, saddle-shaped, ear-shaped or clavate, with the stipe when present terete, compressed, or ribbed/grooved. It includes also species that produce compressed hypogeous ascromata that are without active spore dispersal (ptychothecia and stereothecia). They occur on or in soil, or sometimes in connection with rotten wood or directly on wood. While several species/lineages have been suggested to be ectomycorrhizal, some connected to specific trees or shrubs (e.g., Palfner & Agerer 1998, Weidemann 1998, Murat et al. 2005, Tedersoo et al. 2006, Nguyen et al. 2013, Hwang et al. 2015), the lifestyle of many are yet to be settled.

Based on phylogenetic analyses of partial LSU and SSU rDNA the family *Helvellaceae* was emended to include the epigeous genera *Helvella*, *Wynnella* and *Underwoodia* and the hypogeous *Barssia* and *Balsamia* (O'Donnell et al. 1997). The epigeous taxa correspond to the tribe *Helvelleae* Dissing (1966) of *Helvellaceae*, including species with a well-differentiated

medullary and ectal excipulum, and broadly ellipsoid spores with one large guttule. Eckblad (1968) recognised a restricted *Helvellaceae* corresponding to this tribe. The broader family concept, including also the tribes *Gyromitreae* (*Gyromitra*, *Pseudorhizina*) and *Discineae* (*Discina*, *Neogyromitra* and *Rhizina*) (Dissing 1966, 1972), was maintained by most subsequent workers (e.g., Korf 1973a, Harmaja 1976, Abbott & Currah 1997). This was based mainly on the tetra-nucleate spores observed in these tribes, following Berthet (1964). Molecular data suggest, however, that tetra-nucleate spores is a plesiomorphic character rather than uniquely derived within *Helvellaceae*; the tribes *Gyromitreae* and *Discineae* form a sister group to *Morchellaceae* (with multi-nucleate spores), and *Helvellaceae* s.str. a sister group to *Tuberaceae* (with one to 18 nuclei per spore). This is the family concept accepted in this study.

While the circumscription of the *Helvellaceae* is clarified and supported by additional molecular phylogenetic studies (e.g., Hansen & Pfister 2006), the generic limits and relationships within the family are still poorly understood. *Wynnella* and *Pindara* have been variously treated as either part of the large genus *Helvella* or as separate monotypic genera. The taxonomic placement of *Pindara* has varied widely, from being originally described alongside cupulate helvellaceous taxa, i.e., *Acetabula* and *Macropodia* in 'Humariaceae' (Velenovský 1934, Svrček 1947), to suggested to belong to the inoperculate discomycetes because no operculum had been illustrated or noted (Eckblad 1968). It has also been considered to have a rather isolated position within *Pezizales*, because of the lack

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of carotenoid pigments, large fusoid multi-guttulate spores and cup-shaped to flattened apothecia 4–5 mm diam with a rather thick, 1–1.5 mm high, smooth white stipe (Svrček & Kubička 1968). For a period the asci were considered to be ‘suboperculate’ and *Pindara* was placed in the family *Sarcoscyphaceae* (Korf 1970, 1972, 1973a, Svrček 1976). The lack of carotenoids and the arrangement of the outer excipulum cells in rows perpendicular to the receptacle surface made Cabello (1988) suggest it is better accommodated in the *Helvellaceae*. Scanning electron microscopy has confirmed the asci of *Pindara* not to be thickened or with eccentric apices, i.e., subopercula, but with asci more like those found in the *Pezizineae* (e.g., *Caloscypha*; Harrington et al. 1999). A position in the *Helvellaceae* was substantiated by phylogenetic analyses of SSU rDNA sequences (Harrington et al. 1999, Landvik et al. 1999), and Landvik et al. (1999) ascribed it to their broad concept of *Helvella*.

*Wynnella silvicola* has been treated in a separate monotypic genus based on the narrowly ear-shaped apothecia, i.e., with a split to the base in one side, similar to species of *Otidea* and *Wynnea*. It has been considered to have a rather isolated position within *Helvellaceae*, placed in its own tribus *Wynneleae* (Le Gal 1947, Nannfeldt 1966); or maintained as closely related to *Helvella*, placed in tribus *Helvellae* (Dissing 1966, 1972; = *Helvellaceae* as currently recognised); or ascribed to the genus *Helvella* (Harmaja 1974, Häffner 1987, Abbott & Currah 1997, Franchi et al. 1999, Landvik et al. 1999). Within *Helvellaceae*, the tough consistency and reddish brown colours of the apothecia were emphasized as additional distinctive characters (e.g., Nannfeldt 1966, Dissing 1966, 1972, Eckblad 1968). The apothecial colours in *W. silvicola* have been suggested to correspond to those found in *Helvella aestivalis* (i.e., without a chemical analysis), differing markedly from all other known species of *Helvella* s.lat. (Dissing 1972, 1983, Dissing & Raitviir 1974). Also young spores of *H. aestivalis* have a reticulate pattern reminiscent of that found in spores of *Wynnella* (Dissing & Raitviir 1974). Nevertheless, emphasising the apothecial shape and anatomy, *H. aestivalis* was inferred even more closely related to species of *Helvella* sect. *Leucomelaenae* sensu Dissing (1966) and suggestive of a section of its own (Dissing & Raitviir 1974).

The structure of the excipulum in *Wynnella* has been described as similar or identical to *Helvella* (e.g., Dissing 1966, Eckblad 1968, Harmaja 1974), but noted to have a different ratio between the layers and possibly also differences in cell contents (Dissing 1972, Dissing & Raitviir 1974). Molecular phylogenies of SSU and/or LSU resolved *Wynnella* as a sister taxon to *Helvella* s.lat. (O’Donnell et al. 1997, Hansen & Pfister 2006, Læssøe & Hansen 2007, Landeros et al. 2015, Zhao et al. 2016a). Based on phylogenetic analyses of ITS and LSU rDNA sequences, an additional species of *Wynnella*, i.e., *W. subalpina*, has been proposed, i.e., a species which occurs in subalpine areas of western China (Zhao et al. 2016a).

Within the last six years species and subgroups of *Helvella* s.lat. have received renewed attention (Landeros et al. 2012, 2015, Nguyen et al. 2013, Zhao et al. 2015, 2016b, Skrede et al. 2017). These studies have focused on species delimitation including a limited number of molecular characters and/or morphological data. In this study we readdress the generic limits and relationships within *Helvellaceae* and focus primarily on the recognition of *Pindara*, *Wynnella* and the generic limits of *Helvella* s.lat., using an increased number of molecular characters, i.e., 4299 bp (excluding introns) from LSU, *RPB2* and *EF-1 $\alpha$* .

## MATERIALS AND METHODS

### Taxon sampling

Material of *Balsamia*, *H. aestivalis*, *Pindara*, *Wynnella* and a selection of *Helvella* species were collected and studied both fresh and dried under the light microscope, and are deposited in O, S and TUR. Additional collections in C, CUP, KUN, PRC, UC and UPS were studied. For molecular phylogenetic analyses we selected 32 collections from 21 species of *Helvella* representative for the seven sections erected by Dissing (1966), and the major lineages resolved in Skrede et al. (2017) (Table 1). The species concept of *Helvella* follows Skrede et al. (2017). We included 10 collections of *Pindara*, *Wynnella* and *H. aestivalis*: three of *P. terrestris* from two localities in Sweden and one in Finland; four of *Wynnella* from Canada, China, Germany and Switzerland; and three of *H. aestivalis* from Sweden and Norway. To cover additional genetic variation in *Helvella*, we retrieved sequences of *H. corbierei* (LSU, *RPB2*, *EF-1 $\alpha$* ) and *H. dryophila* (LSU) from GenBank. We likewise included LSU sequences from seven species of the hypogeous *Balsamia* and *Barssia* from GenBank to explore the limits of these genera to the rest of the *Helvellaceae*. For *B. cf. setchellii*, *RPB2* and *EF-1 $\alpha$*  sequences were also available and included. To mend the lack of *RPB2* and *EF-1 $\alpha$*  sequences for the GenBank retrieved *Balsamia*, we generated LSU, *RPB2* and *EF-1 $\alpha$*  sequences for *B. platyspora* (TUR206101). The isotype of the closed/tuberiform, sparassoid *H. astieri* was studied to explore whether this species belongs to *Helvella*. The type species of *Underwoodia*, *U. columnaris*, was included in the *Helvellaceae*, based on previous higher-level phylogenetic analyses of the LSU and SSU rDNA (e.g., O’Donnell et al. 1997, Hansen & Pfister 2006). *Underwoodia* has been shown to be polyphyletic, with *U. beatonii* and *U. singeri* being more closely related to the *Tuberaceae* (Bonito et al. 2013), and therefore these two species, along with *Gymnohydnotrya australiana* (the *Gymnohydnotrya* lineage) (Bonito et al. 2013), *Choiromyces venosus*, *Dingleya* sp., *Labyrinthomyces* sp., *Tuber borchii* and *T. melanosporum* (*Tuberaceae*) were included as sistergroup taxa and used for rooting purposes. This choice is based on higher-level phylogenetic analyses that has shown *Tuberaceae* to be the sister group of *Helvellaceae* (O’Donnell et al. 1997, Hansen & Pfister 2006, Hansen et al. 2008).

### Morphological techniques

Macroscopic descriptions are based on our own or other collectors’ field observations. Microscopic measurements and descriptions are based on dried material, unless otherwise stated. Structural features of the excipulum were studied using vertical, median sections made by hand. Hymenium elements were studied by teasing apart a piece of hymenium with a fine needle. Sections and pieces of apothecia were rehydrated in water for at least four hours. Measurements and descriptions were made on material mounted in water. When cells did not fully recover, 5 % or 10 % KOH was added after water. Melzer’s reagent (MLZ) and 10 % KOH were added to water mounts to observe reactions of exudates or other pigmentation. Congo red (CR) in ammonia was used to enhance the visibility of structures, especially the type of ascus bases. Microscopical observations were made with a Nikon 80i microscope using bright field and Nomarski Differential Interference Contrast (DIC). Microanatomical terminology follows Korf (1973a). Microscopic photos were taken with a Nikon Digital Sight DS-Fi1 camera. An exclamation point indicates that type specimen or other original material was examined by us.

Three different types of ascogenous hyphae have been found in the discomycetes, i.e., the acrorhynque, aporhynque and pleurorhynque types (Chadefaud 1943). Without studying the

**Table 1** Collections used in the molecular phylogenetic study, with voucher information and GenBank accession numbers. For type specimens (in **bold**) the original names are kept regardless of synonymy. Numbers in parentheses following species names indicate multiple collections of a single species. Sequences generated in this study are in **bold**.

Species	Collection no. (Herb.) or Herb./ Culture coll. no. <sup>1</sup>	Geographical origin, Year and Collector	hsp	LSU	GenBank accession no. <sup>2</sup>	EF-1 $\alpha$	RPB2
<i>Balsamia aestivalis</i> (1)	KH.10.117 (S)	Sweden, Lycksele Lappmark, 2010, K. Hansen, I. Olariaga & K. Gillen	-	<b>MK100249</b>	-	-	<b>MK113839</b>
<i>B. aestivalis</i> (2)	KH.10.133 (S)	Sweden, Lycksele Lappmark, 2010, K. Hansen, K. Gillen & I. Olariaga	-	<b>MK100250</b>	<b>MK113869</b>	-	<b>MK113840</b>
<i>B. aestivalis</i> (3)	O-253217	Norway, Oppland, 2009, T. Carlsen, I. Skrede & T. Schumacher	KY784200	<b>MK100251</b>	<b>MK113870</b>	-	-
<i>B. magnata</i>	JMT 13020 (OSC)	USA, CA, 1993, M. Castellano	-	U42683	-	-	-
<i>B. maroccana</i> (/Barssia)	AH44099; paratype	Morocco, 2014, J.L. Manjón, J. Álvarez-Jiménez & M.Á. Sanz	-	KM243654	-	-	-
<i>B. nigrens</i>	Trappe 19921 (OSC)	USA, CA, 1997, L. Criley	-	EU689425	-	-	-
<i>B. oregonensis</i> (/Barssia)	OSC 100014	USA	-	AY544652	-	-	-
<i>B. pleysporea</i>	TUR206101	Finland, Varsinais-Suomi, 2016, K. Ruokolainen with Lagotto Romagnolo	-	<b>MK100252</b>	<b>MK113871</b>	-	<b>MK113841</b>
<i>B. polysperma</i>	AH44225	Italy, 1999, A. Montecchi	-	KM243656	-	-	-
<i>B. cf. setchellii</i>	SRC868	USA, 2005, M.E. Smith	-	JQ925659	GU596459	-	JQ954469
<i>B. vulgaris</i>	AH44222	Italy, 2005, A. Montecchi	-	KM243651	-	-	-
<i>Choiromyces venosus</i>					Genome <sup>3</sup>		Genome <sup>3</sup>
<i>Dingleya</i> sp.	JT31575	Australia, 2006, J. Trappe	-	JQ925661	-	-	JQ954473
<i>Dissingia confusa</i> (1)	O-253269	Norway, Oppland, 2007, T. Schumacher & T. Vrålstad	-	<b>MK100253</b>	<b>MK113872</b>	-	<b>MK113842</b>
<i>D. confusa</i> (2)	O-253268	Norway, Buskerud, 2014, K. Sæbø	-	<b>MK100254</b>	<b>MK113873</b>	-	<b>MK113843</b>
<i>D. confusa</i> (3)	KH.12.75 (S)	Sweden, Jämtland, 2012, K. Hansen & X.H. Wang	-	<b>MK100255</b>	<b>MK113890</b>	-	<b>MK113844</b>
<i>D. crassitunicata</i>	O-253286	Canada, British Columbia, 1994, T. Schumacher	-	<b>MK100256</b>	<b>MK113874</b>	-	<b>MK113845</b>
<i>D. leucomelaena</i> (1)	KH.06.01 (FH)	USA, MA, 2006, G. Lewis-Gentry & K. Hansen	-	KC012682	KC109207	-	JX943751
<i>D. leucomelaena</i> (2)	DMS-9190862 (S)	Denmark, Møn, 2017, T. Læssøe	-	<b>MK100257</b>	<b>MK113835</b>	-	<b>MK113846</b>
<i>D. oblongispora</i>	O-166316	Norway, Oppland, 2004, T. E. Brandrud & E. Bendiksen	-	<b>MK100258</b>	<b>MK113836</b>	-	<b>MK113847</b>
<i>Gymnohydrotia australiana</i>	OSC 130601	Australia, 1996, S. Bobbin	-	JQ925663	JX022555	-	JQ954529 <sup>6</sup>
<i>Helvella acetabulum</i> (1)	C-F-81792	Denmark, Lolland, 2006, M. Sasa	-	<b>MK100259</b>	<b>MK113875</b>	-	<b>MK113848</b>
<i>H. acetabulum</i> (2)	KH.14.01 (S)	Sweden, Södermanland, 2014, S. Kyrk	-	<b>MK100260</b>	<b>MK113891</b>	-	<b>MK113849</b>
<i>H. alpina</i>	KH.12.69 (S)	Sweden, Åsele lappmark, 2012, K. Hansen & X.H. Wang	<b>MK179405</b>	<b>MK100261</b>	<b>MK113876</b>	-	<b>MK113850</b>
<i>H. astieri</i>	<b>Isotype, CUP 52755</b>	France, Var, 1972, J. Astier & J.-C. Donadini	<b>MK238676</b>	<b>MK129270</b>	-	-	-
<i>H. atra</i>	KH.10.97 (S)	Sweden, Lycksele Lappmark, 2010, K. Hansen, K. Gillen & I. Olariaga	<b>MK179402</b>	KC122771	<b>MK113877</b>	-	<b>MK113851</b>
<i>H. capucina</i>	O-253261	Norway, Oppland, Dovre, 2009, T. Carlsen, I. Skrede & T. Schumacher	-	<b>MK100262</b>	<b>MK113878</b>	-	<b>MK113852</b>
<i>H. camosa</i>	KH.10.277 (S)	Sweden, Gotland, 2010, K. Hansen, I. Olariaga & K. Gillen	-	<b>KY660042</b>	<b>KY660043</b>	-	<b>KY660044</b>
<i>H. corbieri</i> (1)	OSC 100019 (as <i>H. compressa</i> )	USA, OR, 2003, K. Hosaka	-	AY544655	DQ497604	-	DQ497613
<i>H. corbieri</i> (2)	PTR 763 (UC 1999259) (as <i>H. compressa</i> )	USA, CA, 2012, I. Singleton	<b>MK179406</b>	<b>MK100263</b>	<b>MK113879</b>	-	<b>MK113853</b>
<i>H. corium</i>	O-253279	Norway, Hordaland, 2014, T. Schumacher	-	<b>MK100264</b>	<b>MK113880</b>	-	<b>MK113854</b>
<i>H. costifera</i>	O-68514	Norway, Akershus, 2007, P.A. Bergersen	-	<b>MK100265</b>	<b>MK113881</b>	-	<b>MK113855</b>
<i>H. crispa</i>	KH.09.186 (S)	Sweden, Gotland, 2009, E.B. Jensen, K. Hansen & I. Olariaga	<b>MK179400</b>	<b>MK100266</b>	<b>MK113882</b>	-	<b>MK113856</b>
<i>H. dryophila</i>	MES218 (UC 1999238)	USA, CA, 2008, M.E. Smith	-	JQ925665	GU596456	-	JQ954477
<i>H. fallax</i>	KH.10.94 (S)	Sweden, Lycksele Lappmark, 2010, K. Hansen, I. Olariaga & K. Gillen	<b>MK179401</b>	<b>MK100267</b>	<b>MK113892</b>	-	<b>MK113857</b>
<i>H. lacunosa</i>	O-253320	Norway, Oppland, 2009, T. Carlsen, I. Skrede & T. Schumacher	-	<b>MK100268</b>	<b>MK113883</b>	-	<b>MK113858</b>
<i>H. macropus</i> (1)	O-292075	Norway, Akershus, 2009, C. Christiansen	-	<b>MK100269</b>	<b>MK113884</b>	-	<b>MK113859</b>
<i>H. macropus</i> (2)	KH.09.142 (S)	Norway, Nord-Trøndelag, 2009, K.M. Jenssen	<b>MK179399</b>	<b>MK100270</b>	<b>MK113885</b>	-	<b>MK113860</b>
<i>H. macropus</i> (3)	KH.12.05 (S)	Sweden, Lycksele Lappmark, 2012, K. Hansen & X.H. Wang	<b>MK179404</b>	<b>MK100271</b>	<b>MK113893</b>	-	<b>MK113861</b>
<i>H. rivularis</i>	KH.03.21 (FH)	Norway, Nordland, 2003, K. Hansen & C. Lange	<b>MK179398</b>	DQ191678	KC109208	-	JX943752
<i>H. solitaria</i>	O-253375	Norway, Oppland, 2009, T. Carlsen, I. Skrede & T. Schumacher	-	<b>MK100272</b>	<b>MK113837</b>	-	<b>MK113862</b>
<i>H. sublicia</i> (1)	KH.11.84 (S)	Sweden, Gotland, 2011, H. Knudsen & M. Sasa	<b>MK179403</b>	<b>MK100273</b>	<b>MK113894</b>	-	<b>MK113863</b>
<i>H. sublicia</i> (2)	KH.10.286 (S)	Sweden, Gotland, 2010, K. Hansen, K. Gillen & I. Olariaga	-	<b>MK100274</b>	<b>MK113886</b>	-	<b>MK113863</b>

Table 1 (cont.)

Species	Collection no. (Herb.) or Herb./ Culture coll. no. <sup>1</sup>	Geographical origin, Year and Collector	GenBank accession no. <sup>2</sup>			
			hsp	LSU	<i>EF-1<math>\alpha</math></i>	<i>RPB2</i>
<i>Labyrinthomyces</i> sp.	JT27750	Australia	-	JQ925670	-	JQ954480
<i>Midotis lingua</i> (1)	S. Huhtinen 82/98 (TUR 078781)	Canada, Quebec, 1982, S. Huhtinen	-	MK1100275	MK113887	-
<i>M. lingua</i> (2)	Sch 84.51 (C-F-57385)	Switzerland, Graubünden, 1984, H. Dissing	KY784396	MK1100276	MK113838	MK113864
<i>Pindara terrestris</i> (1)	KH.12.67 (S)	Sweden, Jämtland, 2012, X.H. Wang & K. Hansen	-	MK1100279	MK113889	MK113866
<i>P. terrestris</i> (2)	S-F327988 (dupl. UME)	Sweden, Västerbotten, 2011, N. & Z. Lipovac	-	MK1100280	MK113896	MK113867
<i>P. terrestris</i> (3)	T. Kekki 168 (TUR 196043)	Finland, Perä-Pohjanmaa, 2011, T. Kekki	-	MK1100281	MK113897	MK113868
<i>Tuber borchii</i>	Tbo3840 (University of Bologna Herb.)	Unknown geographic location	-	Genome <sup>4</sup> NESQ01000000	Genome <sup>4</sup> NESQ01000000	Genome <sup>4</sup> NESQ01000000
<i>T. melanosporum</i>	INRA-Clermont-Ferrand Tuber Collection strain Mel28	France, Bouche-du-Rhône, 1988, L. Rioussel	-	Genome <sup>5</sup> NZ_CABJ000000000	Genome <sup>5</sup> NZ_CABJ000000000	Genome <sup>5</sup> NZ_CABJ000000000
<i>Underwoodia beatonii</i>	JT28380	Australia	-	JQ925716	JX022613	JQ954528
<i>U. columnaris</i>	Kanouse1951	USA, MI	-	U42685	-	-
<i>U. cf. singeri</i>	MES161	Chile	-	JQ925718	JX022615	JQ954475 <sup>6</sup>
<i>Wynnella subalpina</i> (1)	Tribel, Microfungi exs. 83 (UPS F.-005952)	Germany, Bayern, 1993, A. Pilulukat	-	MK1100277	MK113895	-
<i>W. subalpina</i> (2)	KUN-HKAS 94928	China, Sichuan, 2015, S.H. Li	-	MK1100278	MK113888	MK113865

<sup>1</sup> Herbaria are cited according to acronyms in Index Herbariorum (<http://sweetgum.nybg.org/ih/>).<sup>2</sup> hsp: heat shock protein 90 (hsp); LSU: 28S large subunit of the rRNA gene; *EF-1 $\alpha$* : Translation elongation factor 1-alpha; *RPB2*: RNA polymerase II second largest subunit.<sup>3</sup> Murat et al. 2018a, <sup>4</sup> Murat et al. 2018a, <sup>5</sup> Martin et al. 2010.<sup>6</sup> It became apparent from our analyses that the *RPB2* sequences of *G. australiana* and *U. cf. singeri* in GenBank are switched, and therefore JQ954475 (as *G. australiana* in GenBank) is here used for *U. cf. singeri* and JQ954529 (as *U. cf. singeri* in GenBank) is used for *G. australiana*.

nuclear state in the ascogenous hyphae it may be difficult to distinguish the acrorhynque from the aporhynque type, and therefore we will here refer to the asci as arising either from croziers, i.e., the ascogenous hyphae being of the pleurorhynque type, or from simple septa, i.e., without the formation of croziers (ascogenous hyphae being aporhynque or acrorhynque).

### Molecular techniques

DNA was extracted from fresh apothecia stored directly in 1 % SDS extraction buffer (kept at -20 °C) or from dried apothecia, as outlined in Hansen et al. (1999) with the exceptions that fresh material was ground directly in an Eppendorf tube and dried material was not ground in liquid nitrogen but shaken in a cell disruptor (MINI-BEADBEATER™, BioSpec Products, Bartlesville, Oklahoma) for 20 s at 4600 rpm. Three gene regions were amplified: the 5' end of the nLSU rDNA, spanning domains D1 and D2 (c. 900 bp), part of the nuclear gene that encode the second largest subunit of RNA polymerase II (*RPB2*), 5–11 region (up to 2333 bp) (Liu et al. 1999, Hansen et al. 2005, 2013), and nearly the complete coding region of translation elongation factor 1-alpha (*EF-1 $\alpha$* ) (up to 1637 bp) (Rehner & Buckley 2005). The majority of the PCR amplifications were performed using Illustra™ Hot Start Mix RTG PCR beads (GE Healthcare, UK) according to the manufacturer's instructions and a smaller number using Takara EX® Taq DNA Polymerase (Takara, Dalian, China). The following PCR program was used to amplify the LSU: 5 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 60 °C and 90 s at 72 °C, and a final extension of 72 °C for 7 min. The annealing temperature was decreased to 55 °C when PCR failed or showed very weak bands. General PCR primers and programs for amplification of *RPB2* and *EF-1 $\alpha$*  followed Hansen et al. (2013). For problematic samples, where *RPB2* or *EF-1 $\alpha$*  did not successfully amplify, *Helvella* s.lat. specific internal primers were designed based on alignments of the readily obtained sequences. The new *RPB2* or *EF-1 $\alpha$*  primers and their position in the gene regions are provided in Table 2 and Fig. 1. For some material where PCR amplicons were weak and direct sequencing failed, PCR products were cloned using the Takara® pMD™18T cloning kit (Dalian, China) following the manufacturer's instruction. Colonies were screened for the presence of the desired products using primer pairs M13F and M13R. For each PCR product, one clone with the desired length of PCR product was sequenced. No heterozygous sites were observed in the sequences from direct sequencing and therefore it was considered unlikely that multiple copies of the gene would be present (and the risk of missing a different copy by sequencing only one clone would be very rare). A short region (up to 302 bp) of the heat shock protein 90 (hsp) was amplified for selected *Helvella* species using PCR primers and programs available in Skrede et al. (2017). PCR products (22  $\mu$ L) were either purified using 5.5  $\mu$ L mixture of FastAP Thermosensitive Alkaline Phosphatase and Exonuclease I (4 : 1) (Thermo Fisher Scientific Inc.), or using QIAquick Gel Extraction Kit (Qiagen™) when there were multiple bands, by following the manufacturers' instructions. The sequencing reactions were performed with BigDye™ Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems). The sequencing reactions were purified with DyeEX 96 Kit (Qiagen™) and run on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

### Sequence alignment and phylogenetic analyses

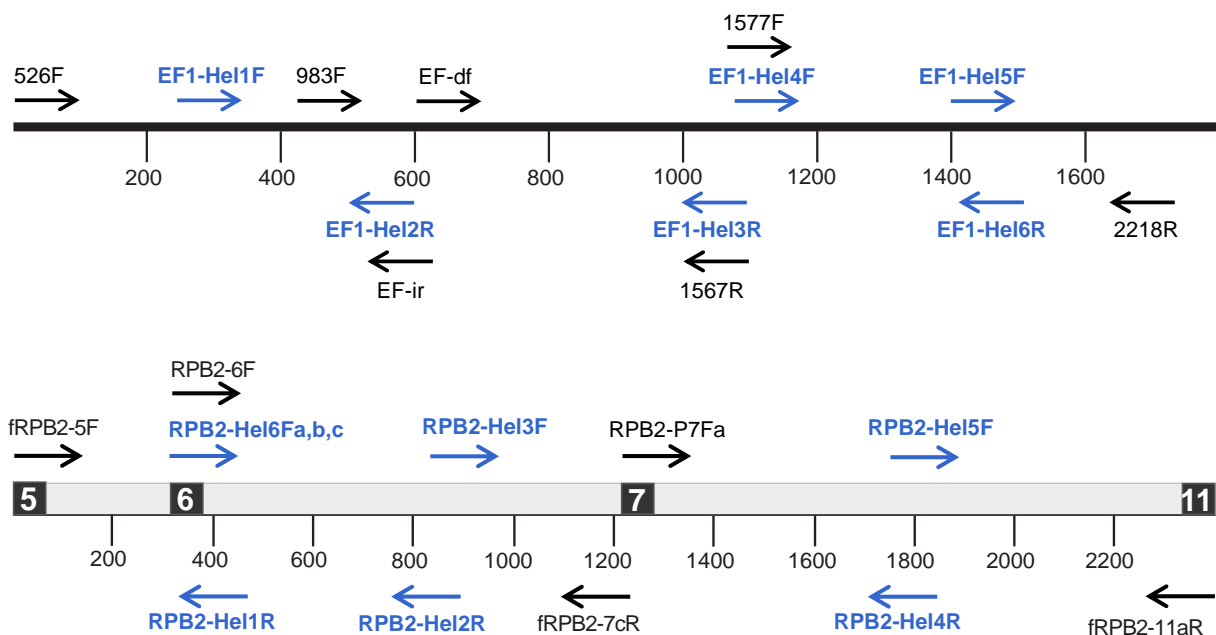
Sequences were edited and assembled with Sequencher v. 4.10.1 (Gene Codes Corp., Ann Arbor, MI) and are deposited in GenBank (Table 1). Sequences were aligned manually using Se-Al v. 2.0a11 (Rambaut 2002) and BioEdit v. 7.1.3.0 (Hall 1999). Separate alignments of the three individual gene regions (LSU, *RPB2* and *EF-1 $\alpha$* ) were prepared for phylogenetic analyses.

Incongruence among the three single genealogies was determined by comparing maximum likelihood bootstrap proportions (ML-BP) and Bayesian posterior probabilities (PP) for the same set of taxa. A conflict was assumed to be significant when two different relationships (one monophyletic and the other non-monophyletic) for the same set of taxa were both supported with ML-BP  $\geq 75\%$  and PP  $\geq 0.95$ . The introns in the protein-coding genes *RPB2* and *EF-1 $\alpha$*  were highly variable and could not be unambiguously aligned and were therefore excluded from the analyses. The combined alignment without introns (LSU: 1–890; *EF-1 $\alpha$* : 891–2170; *RPB2*: 2171–4299) is available from TreeBASE under S23492. All gene regions were analysed using the nucleotides. The two protein-coding genes were analysed with two partitions: i) first and second codon positions; and ii) third codon position. In the combined LSU-*EF-1 $\alpha$* -*RPB2* analyses, the LSU was specified as a distinct partition. Thus, the concatenated three-locus dataset was analysed with five partitions. Metropolis-coupled Markov chain Monte

Carlo (MCMCMC) methods as implemented in MrBayes v. 3.2.1 (Ronquist & Huelsenbeck 2003, Ronquist et al. 2012) and ML inference as implemented in RAxML v. 7.2.6 (Stamatakis 2006) were conducted to construct the single genealogies and the phylogeny from the three-locus concatenated dataset. *Tuber melanosporum* was specified as an outgroup for the analyses. The Bayesian analyses were run in parallel using model jumping (/mixed models) and gamma rates. All parameter values, except branch length and tree topologies, were unlinked and site-specific rates were allowed to vary across partitions. The analyses consisted of four parallel searches, each with four chains, run for 1 M generations, and initiated with random starting trees. The chains were sampled every 100 generations from the posterior distribution. Runs were inspected to make sure the average standard deviation of split frequencies went below 0.01 and effective sampling sizes were  $> 200$  in Tracer v. 7.1 (Rambaut et al. 2018). Posterior probabilities were calculated using the last 75 % of the trees sampled from the posterior

**Table 2** Newly designed internal primers of *RPB2* and *EF-1 $\alpha$*  in this study for PCR and sequencing, and unpublished *EF-1 $\alpha$*  primers (in **bold** by S. Rehner pers. comm.).

Locus	Primer	Sequences (5'–3')	Direction	Counterpart primer
<i>RPB2</i>	RPB2-Hel6Fa	TGGGGATTGTCTGCCCYGC	forward	fRPB2-7cR
	RPB2-Hel6Fb	TGGGGWTRGTYTYGCCBGC	forward	fRPB2-7cR
	RPB2-Hel6Fc	GYTYGCCBGCHGARACNCCVGA	forward	fRPB2-7cR
	RPB2-Hel1R	CCRATRGTGATRTAAGACATCA	reverse	fRPB2-5F
	RPB2-Hel2R	ACRATCATRACRCCTTCTC	reverse	RPB2-6F
	RPB2-Hel3F	AAGTTYGGTTGGGAAGGYTYGT	forward	fRPB2-7cR
	RPB2-Hel4R	AATTRTCTCCAATYTGDDGAA	reverse	RPB2-P7Fa
	RPB2-Hel5F	ACTAAYGCYGAGGGYTRAA	forward	fRPB2-11aR
	<i>EF-1<math>\alpha</math></i>	<b>983F</b>	<b>GCYCCYGGHCAYCGTGAYTTCAT</b>	forward
<b>EF-ir</b>		<b>GCRTGYTCNCGRGTGTGNCRCCTC</b>	reverse	526F
<b>1577F</b>		<b>CARGAYGT(C,G,T)TACAAGATYGGTGG</b>	forward	EF1-Hel6R, 2218R
EF1-Hel1F		CGTGGTATCACYATCGACATYGC	forward	1567R, EF1-Hel3R
EF1-Hel2R		CCVGCYTCGAACTCACCAGTC	reverse	526F
EF1-Hel3R		GRACCGTTCCAATACRCCTC	reverse	983F, EF1-Hel1F
EF1-Hel4F		CAAGATYGGYGGTATTGGAACGG	forward	EF1-Hel6R, 2218R
EF1-Hel5F		GCCCAGGKATTYTYATGAACC	forward	2218R
EF1-Hel6R		GCRAACTTGCAVGC AATRTG	reverse	EF1-Hel4F



**Fig. 1** *EF-1 $\alpha$*  and *RPB2* primers used in this study, with their positions shown by arrows on a schematic map of the gene region. Newly designed primers are in bold blue (see Table 2). Other primers are given in Hansen et al. (2013), or by S. Rehner (pers. comm.) for *EF-1 $\alpha$*  and for *RPB2* in Liu et al. (1999).

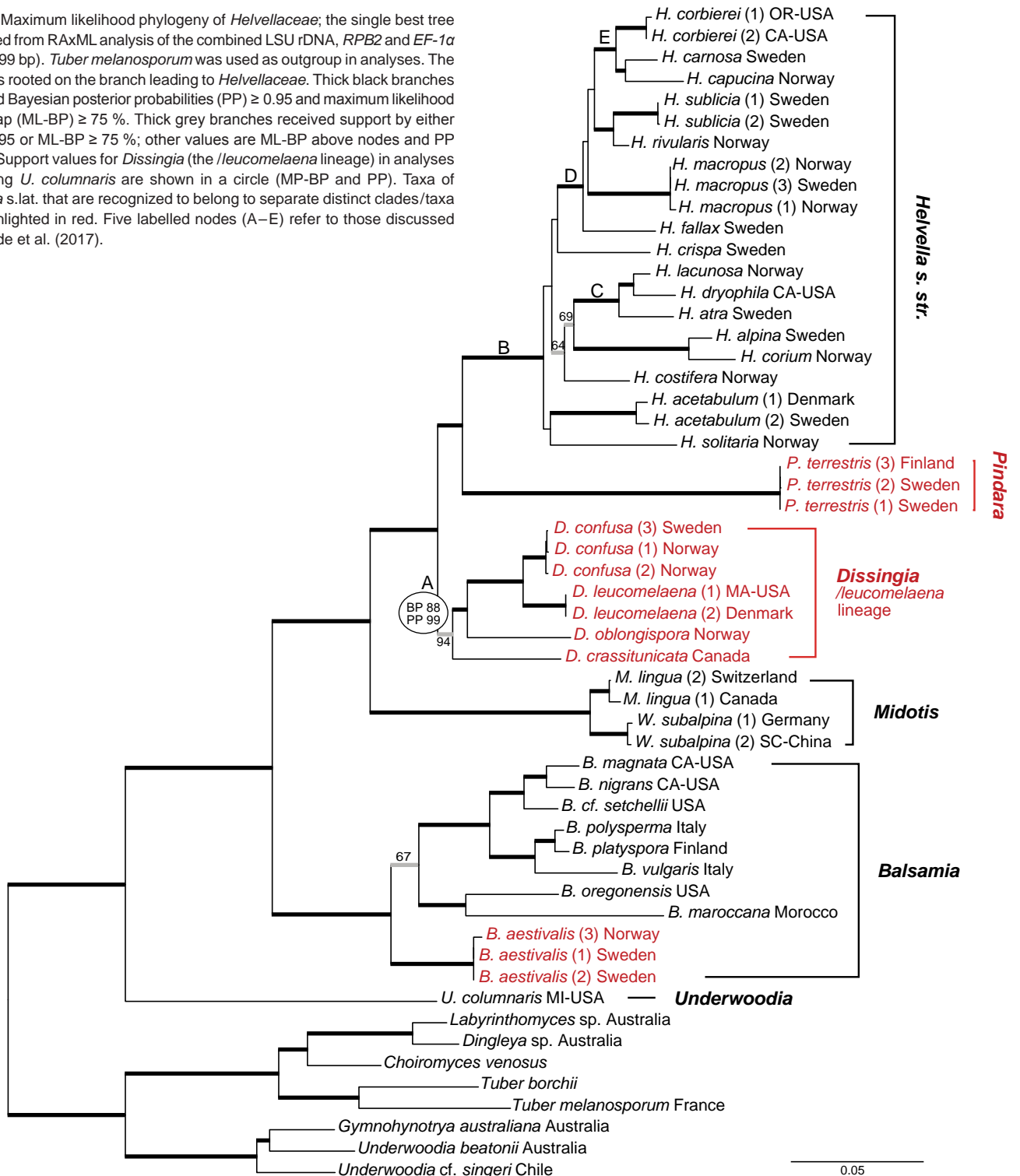
distribution. The incremental heating scheme for the analyses used the default settings in MrBayes, i.e., three heated chains and one cold chain. For the ML analyses a GTR-GAMMA model was assigned and all free model parameters estimated by the program. An ML bootstrap analysis (ML-BP) was performed, using 1 000 rapid bootstrapping replicates from random starting trees, followed by a subsequent ML search similarly using 1 000 replicates. An ML-BP  $\geq 75\%$  or PP  $\geq 95\%$  was considered as significant support for a node to be monophyletic. All datasets were in addition, analysed without *U. columnaris*, to test the effect of this taxon on the topology and support values, because it was only represented by a single gene region, the LSU rDNA.

**RESULTS**

**Congruence and data partitions, nucleotide sequences and introns**

We generated sequences from LSU, *RPB2* and *EF-1 $\alpha$*  to assess generic limits and relationships within *Helvellaceae*. A total of 144 sequences was obtained, with 96 new sequences reported here: 4272 bp from the protein-coding genes (31 sequences of *RPB2* and 34 sequences of *EF-1 $\alpha$* ) and 890 bp from 34 sequences of LSU from 35 collections (Table 1). The suggested DNA barcoding gene for *Helvella*, *hsp* (Skrede et al. 2017), was provided for 15 collections, to substantiate our

**Fig. 2** Maximum likelihood phylogeny of *Helvellaceae*; the single best tree produced from RAxML analysis of the combined LSU rDNA, *RPB2* and *EF-1 $\alpha$*  loci (4 299 bp). *Tuber melanosporum* was used as outgroup in analyses. The tree was rooted on the branch leading to *Helvellaceae*. Thick black branches received Bayesian posterior probabilities (PP)  $\geq 0.95$  and maximum likelihood bootstrap (ML-BP)  $\geq 75\%$ . Thick grey branches received support by either PP  $\geq 0.95$  or ML-BP  $\geq 75\%$ ; other values are ML-BP above nodes and PP below. Support values for *Dissingia* (the *leucomelaena* lineage) in analyses excluding *U. columnaris* are shown in a circle (MP-BP and PP). Taxa of *Helvella* s.lat. that are recognized to belong to separate distinct clades/taxa are highlighted in red. Five labelled nodes (A–E) refer to those discussed in Skrede et al. (2017).



identifications of species of *Helvella* s.lat. Three datasets were produced of LSU, *RPB2* and *EF-1 $\alpha$*  from 55 collections. Of the 55 collections included in the combined dataset, 11 collections lack *RPB2* and 10 collections lack *EF-1 $\alpha$* . In the combined dataset, sequences of all three markers were available for 75 % of the collections. For seven samples of *Balsamia*, *Barssia* and *U. columnaris* LSU sequences were retrieved from GenBank, and no *RPB2* and *EF-1 $\alpha$*  sequences were available for these. The three datasets were used to construct single genealogies that were inspected to ascertain similar topologies. After this, the three datasets were trimmed to include only 42 samples, i.e., those with all three loci, and their genealogies were similarly scrutinized for conflicts. No supported conflicts were detected between these genealogies derived from the LSU, *RPB2* and *EF-1 $\alpha$*  datasets.

### Combined three-gene phylogeny of the *Helvellaceae*

The three-gene phylogeny of the *Helvellaceae* is fully resolved and highly supported in all deeper branches as inferred by both Bayesian PP and ML-BP (Fig. 2). The genus *Helvella* is polyphyletic, because *H. aestivalis* forms a strongly supported monophyletic group with species of *Balsamia* and *Barssia* (PP/ML-BP 100 %). All other species of *Helvella* form a large group with *Pindara* (*Helvella*) *terrestris* nested within it, on notably the longest branch in the phylogeny. Confirming previous results *Midotis* (syn. *Wynnella*, see Taxonomy section below) is strongly supported as a sister group to *Helvella* s.lat. *Pindara* is strongly supported as a sister group to a restricted clade of *Helvella*, i.e., excluding the *lleucomelaena* lineage. The *lleucomelaena* lineage comprises *H. confusa*, *H. crassitunicata*, *H. lleucomelaena* and *H. oblongispora* (PP 94 %, ML-BP 76 %). *Pindara terrestris*, the *lleucomelaena* lineage, and *Midotis* are strongly supported as successive sister taxa to *Helvella* s.str. (all PP 100 %, ML-BP 95–100 %). *Midotis* comprises two strongly supported monophyletic groups that we recognize as species. Each of these is represented by two collections: *M. lingua* from Canada and Switzerland, and *W. subalpina* from China and Germany (Fig. 2). Based on our LSU sequence of the collection C-F-56847, *W. subalpina* also occurs in Switzerland; it is 100 % identical to the collections from China and Germany. The clade of *Balsamia*, *Barssia* and *H. aestivalis* forms a sister clade to the rest of the *Helvellaceae*, exempting *U. columnaris*. Both *Balsamia* and *Barssia* are supported as monophyletic, and resolved as sister groups although with low support in ML analysis (ML-BP 67 %). The type of *Underwoodia*, *U. columnaris*, represented only by an LSU sequence, is the earliest diverging lineage within *Helvellaceae*. Notably, the support values for the *lleucomelaena* lineage were raised when *U. columnaris* was excluded from the analysis (from PP 94 % to 99 %; ML-BP 83 % to 88 %), but otherwise the topology and support values were comparable/identical.

Several lineages were supported within *Helvella* s.str. corresponding to the lineages/clades discussed in Skrede et al. (2017). Four of these lineages, i.e., *H. corbierii-carnosa-capucina* (= clade E); *H. rivularis-sublicia*; *H. macropus*; and *H. fallax*, formed a highly supported clade (= clade D of Skrede et al. 2017) (Fig. 2, PP 100 %, ML-BP 81 %). A close relationship between two other lineages, *H. lacunosa-dryophila-atra* and *H. alpina-corium*, was also supported (PP 98 %, ML-BP 69 %). *Helvella costifera* formed a sister lineage to those (PP 98 %).

The hsp sequence of the closed/tuberiform, sparassoid *H. astieri* was 100 % identical to sequences of *H. lactea* (C-F-39379 (H374) and Fung. Exs. Suec. 1355 (H262) from Skrede et al. 2017).

## TAXONOMY

Based on the strong support provided by ML and Bayesian analyses of the combined LSU, *RPB2* and *EF-1 $\alpha$*  dataset and morphological characters, we propose the following generic delineation in *Helvellaceae*: a description of a new genus for the *lleucomelaena* lineage of *Helvella* s.lat., i.e., *Dissingia* gen. nov.; and combinations of *Barssia* and *H. aestivalis* in *Balsamia*. We recognise *Pindara* and *Midotis* (syn. *Wynnella*) as independent genera.

***Helvellaceae*** Fr., Syst. Mycol. 2: 1. 1822 ('*Elvellaceae*') (syn. *Balsamiaceae* E. Fisch.)

Included genera. *Balsamia*, *Dissingia*, *Helvella*, *Pindara*, *Midotis*, *Underwoodia* s.str.

The circumscription of the family follows Weber et al. (in O'Donnell et al. 1997).

***Balsamia*** Vittad., Monogr. Tuberc.: 30. 1831, emend. — Fig. 3

*Type species. Balsamia vulgaris* Vittad.

*Synonyms. Barssia* Gilkey, Mycologia 17: 253. 1925. — *Type species. Barssia oregonensis* Gilkey.

*Pseudobalsamia* E. Fisch., Ber. Deutsch. Bot. Ges. 25: 374. 1907. — *Type species. Pseudobalsamia setchellii* E. Fisch.

Ascoma epigeous cup-shaped, margin often split into lobes, sessile or with a short stipe (an apothecium), or semi-hypogeous to hypogeous closed and irregular globose to sub-globose, with a veined interior and a coarse peridium, sometimes with a depression that forms an irregular lateral or apical cavity (an infolded, compressed chambered to solid pycothecium). *Flesh/gleba* whitish to pale yellow or pale reddish. *Hymenium* exposed, dark reddish brown, often with a purplish tinge, or infolded irregular, pale, sometimes exposed in or open to the cavity of the ascoma. *Receptacle/peridial surface* brownish orange, dark reddish brown, to blackish brown, glabrous to very delicately pubescent, smooth to warty. *Stipe* inconspicuous, paler, with 1–4 blunt ribs or grooves. *Asci* 8-spored, operculate with forcible spore discharge, or without an opening with passive spore dispersal, cylindrical to clavate to ovoid, arising from croziers (for those studied carefully thus far for this feature), arranged in a palisade with paraphyses, or asci and paraphyses randomly distributed in the gleba. *Paraphyses* hyaline or apical cell with brownish to wine red guttules or homogenous content, ends at or extend above the asci. *Spores* uniseriate or clustered, ellipsoid, ovoid, hyaline, smooth with 1–3 large internal guttules, and sometimes with several smaller ones towards the poles. *Medullary excipulum/gleba* of *textura intricata*. *Outer excipulum/peridium* of angular to globose cells, outermost cells thin to thick-walled, with reddish brown walls, giving rise to the tomentum or warts.

Notes — We place *Barssia* in synonymy with *Balsamia* based on our molecular phylogenetic results (including the type species, Fig. 2) and the lack of morphological distinguishing features between the two genera (Læssøe & Hansen 2007, Crous et al. 2014). *Barssia* was described as a monotypic genus with emphasis on the ascomata having an apical depression with the outer excipulum/cortex complete, except where veins lined with hymenium open into the cavity (Gilkey 1925). This feature, however, is also present in some species of *Balsamia* (see e.g., Trappe 1979, Southworth et al. 2018). Gilkey (1925) did not compare *Barssia* and *Balsamia*, because she considered *Barssia oregonensis* to occur exclusively in North America. We transfer the epigeous *H. aestivalis* to *Balsamia* due to the ascertained close phylogenetic relationship between this spe-



**Fig. 3** *Balsamia*. a. *Balsamia aestivalis* apothecia; b. *Balsamia platyspora* ascomata; c–h. *Balsamia aestivalis*: c. ascospores; d–e. ascus base showing crozier (in CR); f. asci and paraphyses with reddish brown content; g. medullary excipulum of *textura intricata* (in KOH); h. outer excipulum of *textura angularis*, outermost cells elongated, forming short, hyphoid, fasciculate hairs with evenly brown content (in KOH); i. outermost hyphoid hairs (in water), end cell slightly club-shaped with a thin gelatinous or amorphous sheath (a, c–f: KH.10.133; b: TUR206101; g–i: KH.10.117). — Scale bars: c–f, i = 10  $\mu$ m, g–h = 20  $\mu$ m; a–c, f living material; d–e, g–i from dried material. — Photos: a, c–i K. Hansen; b S. Huhtinen.



cies and *Balsamia* spp., including the type species *B. vulgaris*. In our multi-gene phylogeny, species of *Balsamia* s.lat. form a coherent monophyletic group within *Helvellaceae*. Based on phylogenetic analyses of LSU rDNA sequences including additional sequences/species available from GenBank (not shown), the hypogeous species of *Balsamia* s.lat. represent four distinct lineages that are confined to either western North America or Europe. A fifth lineage is the epigeous *B. aestivalis* that has a true arctic-alpine-subalpine distribution. Supportive evidence for alpine *H. aestivalis* populations to be ectomycorrhizal has been provided by 100 % identical ITS sequences from an ectomycorrhizal rootlet of *Dryas octopetala* and *B. aestivalis* apothecial tissue (Weidemann 1998). Based on the Bayesian analyses of our combined dataset (Fig. 2) that suggests *B. aestivalis* is the earliest diverging lineage within *Balsamia* s.lat. (PP 95 %), we speculate that hypogeous species of *Balsamia* s.lat. originated from an ectomycorrhizal epigeous species with cup-shaped apothecia and a wide arctic-alpine-subalpine distribution, similar to *B. aestivalis*. Many hypogeous *Balsamia* s.lat. species are known from high elevations/mountain ranges in Europe, North Africa or North America. They are considered to be ectomycorrhizal based on tree association, i.e., inferred from the tree(s) growing beside the ascomata (e.g., Trappe et al. 2009). Only a limited number of molecular ectomycorrhizal community studies have documented *Balsamia* from root samples using ITS sequences (e.g., included in Southworth et al. 2018), but this is likely due to the methodology and sampling areas (the ITS region of *Balsamia* is long and difficult to amplify from rootlets, and the areas where the species occur has not been specifically targeted). *Balsamia alba* has been reported to form ectendomycorrhiza on *Pinus jeffreyi* with a thin or fragmented mantle, a well-developed Hartig net and intracellular colonization (Palfner & Agerer 1998).

Until recently, three to six species of *Balsamia* s.str. (incl. *Pseudobalsamia*) were recognised in western North America and two species in Europe (Gilkey 1954, Trappe 1979, Montecchi & Sarasini 2000). Based on phylogenetic analyses of the ITS rDNA and morphology, an additional eight new species were described from western North America (Southworth et al. 2018). For a long time, only one, or possibly two species were known in *Barssia* (Trappe 1979, Læssøe & Hansen 2007), but within the last four years three new species have been described in the genus. These new species were assigned to *Barssia* (and not *Balsamia*) because they formed a monophyletic group with the type species, *B. oregonensis*, in phylogenetic analyses of ITS and/or LSU sequences (Crous et al. 2014, Kaounas et al. 2015, Doğan et al. 2018). Two additional species have been assigned to *Barssia* based on morphological features, i.e., *B. peyronellii* (syn. *Stephensia peyronellii*) (Agnello & Kaounas 2017) and *B. yezomontana* (syn. *Phymatomyces yezomontanus*) (Trappe 1979). These are only known from the holotype specimens. We await further collections and study of these two species to better assess their identities.

***Balsamia aestivalis*** (R. Heim & L. Rémy) K. Hansen, Skrede & T. Schumacher, *comb. nov.* — MycoBank MB829142; Fig. 3a, c–i

*Basionym.* *Acetabula aestivalis* R. Heim & L. Rémy, Bull. Soc. Mycol. France 41: 460. 1925.

*Synonyms.* *Helvella aestivalis* (R. Heim & L. Rémy) Dissing & Raitv., Eesti NSV Tead. Akad. Toim., Biol. Ser. 23: 105. 1974. — *Lectotype.* R. Heim & L. Rémy, Bull. Soc. Mycol. France 41: pl. 29, f. 10–13. 1925. *Epitype.* FRANCE, Hautes-Alpes, Villard-Saint-Pancrace, under *Larix decidua*, 25 July 1997, G. Baiano & M. Filippa (LUG 8869). Types selected by Filippa & Baiano (2017).

?*Helvella pocillum* Harmaja, Karstenia 15: 30, 1976. — *Holotype.* SWEDEN, Torne Lappmark, Jukkasjärvi, Låktatjåkko, on bare soil, 17 Aug. 1946, L. Holm 472 (UPS)!

Selected illustrations — Heim & Remy (1925: f. 7–8, pl. 29: f. 10–13), Dissing & Raitviir (1974: f. 4), Schumacher & Mohn Jenssen (1992: 11), Baiano & Filippa (2000: 37, f. 12), Filippa & Baiano (2017: 119, 121).

Selected descriptions — Heim & Remy (1925: 460), Dissing & Raitviir (1974: 105), Dissing (1983: 176), Schumacher & Mohn Jenssen (1992: 11), Baiano & Filippa (2000: 35).

*Specimens examined and GenBank accessions for hsp sequences.* FRANCE, Savoie, Val Cenis, Col du Mont Cenis, 2100 m a.s.l., on very whitish calcareous soil, among *Dryas octopetala* and few *Salix reticulata*, 11 Aug. 2010, M. Filippa & M. Carbone (S-F335528) (MK408813). — ITALY, Trentino Alto Adige, Bolzano, Sesto Pusteria, Zsigmondy-Comici-Hütte, on the side of a trail at 2450 m a.s.l., on calcareous soil among dwarf *Salix* and *Dryas* sp., 8 Aug. 2000, E. Campo (S-F335530) (MK408814); Piemonte, Cuneo, Pontechianale, Fraz. Chianale, 1850 m a.s.l., on soil under *Larix decidua*, 21 June 2011, M. Carbone (S-F335531) (MK408815). — KYRGYZ REPUBLIC, Tien Shan, Kreet Moldotau, close to Karatal river, 29 July 1967, A. Raitviir (C-F-45329) (KY784401). — NORWAY, Oppland, Dovre, Grimsdalen, Tverrgjelet, in *Dryas* vegetation, 15 Aug. 2001, T. Schumacher (O-253218) (KY784312); *ibid.*, 15 Aug. 2007, T. Schumacher, TS 55.07 (O); *ibid.*, 8 Aug. 2009, T. Carlsen, I. Skrede & T. Schumacher, TS 23.09 (O-253217) (KY784200); Oppland, Lom, in Dryadion, 29 Aug 1957, F.-E. Eckblad (O-129530; as *H. pocillum*, det. Harmaja) (KY784456). — SWEDEN, Lycksele Lappmark, Vindelfjällen Nature Reserve, Brandsfjället, southern slope, 730–780 m a.s.l., calcareous rocks, among *Dryas octopetala* and *Salix reticulata* carpets, 21 Aug. 2010, K. Hansen, I. Olariaga & K. Gillen, KH.10.117 (S); Vindelfjällen Nature Reserve, Tångvattendalen, Rödingsfjället, southern slope, 690–750 m a.s.l., calcareous mountain, among *Dryas octopetala* and *Salix reticulata* carpets, 23 Aug. 2010, K. Hansen, I. Olariaga & K. Gillen, KH.10.133 (S); *ibid.*, on slope under stones, towards river coming down the mountain with *Dryas octopetala* and *Salix reticulata* carpets, KH.10.134 (S). — SWITZERLAND, Graubünden, S-Charl, on W side of the river a few m N of the bridge under Ravitschana on 'Blatt 259, Ofenpass', on soil, in area of *Larix* and *Picea* forest, 1749 m a.s.l., 5 Sept. 1979, H. Dissing Sch 79.132 (C-F-56913) (MK288024).

Notes — *Balsamia aestivalis* was originally described from South Eastern France, Briançon and MontGenevre, in Hautes-Alpes, at 1900 m a.s.l., under *Pinus* and *Larix* (Heim & Remy 1925). According to Dissing & Raitviir (1974) there is no authentic (type) material of *A. aestivalis* available (Heim in litt.). The original plate by Heim & Remy (1925: pl. 29, f. 10–13) was therefore recently selected as a lectotype (Filippa & Baiano 2017), supported by an epitype (LUG 8869) from a subalpine locality with *Larix decidua* close to the original locality in France. We apply here the name *B. aestivalis* to populations from both subalpine *Larix* or *Larix* and *Picea* forests (from Switzerland and France) and alpine *Dryas* carpets (from France, Italy, Norway, Sweden) based on low pair-wise nucleotide diversity in hsp sequences (0–2 bp) among the populations and only slight morphological differences. GenBank numbers for the newly obtained hsp sequences are listed with the 'Specimens examined and sequenced' above. Nevertheless, further studies using multiple genetic markers and a larger sampling of material are needed to determine if populations associated with *Dryas* represent a different species from those associated with *Larix* (*Picea/Pinus*). Apothecia produced in arctic-alpine areas with *Dryas* are generally smaller (0.5–2.5 cm diam and 0.5–2 cm high, vs 3–10 cm diam), and with darker/stronger pigment in the paraphyses apices and outermost excipulum cells, than those produced in subalpine areas with *Larix*. The morphological differences may be plastic and ascribed to the colder climate and stronger sun exposure. An hsp sequence obtained from the holotype of *H. pocillum* (GenBank MK288025) is 100 % identical to the hsp sequences from *B. aestivalis* occurring with *Dryas*, which ascertain that it is a member of *Balsamia*, and if not a synonym of *B. aestivalis* (as suggested with a question mark), it may prove to be an available name for the arctic-alpine populations. A collection from Norway (O-129530) published as *H. pocillum* by Harmaja (1977b), also represents *B. aestivalis* (GenBank KY784456; in Skrede et al. 2017). The ascus bases of *B. aestivalis* have been reported as both aporphynchous (Dissing

1983, Häffner 1987) and pleurorhynchous (Abbott & Currah 1997, Baiano & Filippa 2000, Filippa & Baiano 2017). We found the ascal bases in our material, and in the holotype of *H. pocillum*, to develop from croziers (Fig. 3d–e), i.e., pleurorhynchous.

Skrede et al. (2017) observed a surprisingly remote relationship between *H. aestivalis* and the rest of *Helvella* s.lat. and excluded it from the genus. The distinctiveness of *H. aestivalis* has been commented upon previously (Dissing & Raitviir 1974; see Introduction of the current paper). The apothecial margin that splits into large irregular teeth (/lobes), the characteristic reddish brown colours of the receptacle and the indistinct stipe are remarkable when compared to species of *Helvella* s.lat. The Swedish and Norwegian specimens collected and reported on here, showed similar dark brown hymenium with a red tinge and dark brown receptacle with copper-red tinges, reddish ochre to cream towards the short stipe and a toothed margin with broad lobes (Fig. 3a). The receptacle surface is without ribs, sub-pubescent or with delicate brown warts, towards the base and stipe smooth. The outer excipulum is noteworthy of an inner layer (56–70 µm broad) of irregularly arranged *textura angularis*, ending in an outer layer of angular to elongated clavate cells, with a reddish brown content, arranged perpendicular to the outer surface in rows of 2–4 cells with free ends (Fig. 3h–i). The layer of *textura angularis* in the outer excipulum was also observed in the Italian material from *Larix* forests. The paraphyses apices are clavate to capitate (5.5–8(–12) µm) with ± homogeneous, reddish brown content (Fig. 3f). The pigment does not dissolve in 10 % KOH, but a bright yellow pigment is exuded in MLZ.

*Balsamia aestivalis* has been reported from arctic Canada, Greenland and Svalbard and from subalpine and alpine zones of France, Italy, Norway, Sweden, Switzerland and Asia (Tien Shan) (see Dissing 1983, Baiano & Filippa 2000, and material examined in this study). The species typically occur in moist calcareous areas, often along streams (Dissing 1985, Baiano & Filippa 2000). In subalpine areas the species likely form ectomycorrhiza with *Larix* (*Picea/Pinus*) (inferred from the tree(s) growing beside ascomata), and in arctic-alpine areas with *Dryas* (Weidemann 1998; see above under *Balsamia*).

***Balsamia gunerii*** (H.H. Doğan et al.) K. Hansen & X.H. Wang, *comb. nov.* — MycoBank MB829143

*Basionym.* *Barssia gunerii* H.H. Doğan et al., Turkish J. Bot. 42: 637. 2018.

*Holotype.* TURKEY, Osmaniye Province, Kadirli, Uzunyazı plateau, Elmacık district (N37°42'170 E36°12'135), in humus soil under *Cedrus libani*, 1314 m a.s.l., 7 June 2016, Ş. Güneri (HHD17617 - Mushroom Application and Research Center of the Fungarium of Selçuk University, Konya, Turkey); isotype KONFUNGARIUM 5288).

Illustrations — Doğan et al. (2018: f. 2–4).  
Distribution — Turkey, 1300 m a.s.l.

Notes — *Barssia gunerii* has only been reported from the type locality, under *C. libani* that is native to a limited area in the mountains of Turkey, Syria and Lebanon. It is very closely related to *B. hellenica* and *B. maroccana* based on the ITS and LSU phylogenies given in Doğan et al. (2018) and the species boundaries need to be addressed with more loci and material from different localities. Spore size has been given as a feature to distinguish *B. gunerii* and *B. hellenica*, but spore measurements are overlapping.

***Balsamia hellenica*** (Kaounas et al.) K. Hansen & X.H. Wang, *comb. nov.* — MycoBank MB829144

*Basionym.* *Barssia hellenica* Kaounas et al., Ascomycete Org. 7: 213. 2015.

*Holotype.* GREECE, Parnitha Attica, under *Abies cephalonica*, 4 June 2015, V. Kaounas (MCVE 28663).

Illustrations — Kaounas et al. (2015: pl. 1–3, f. 1).  
Distribution — Mountain ranges in Greece, Turkey.

Notes — *Balsamia hellenica* was described from two localities in Greece, under *Abies cephalonica* that is native to the mountain ranges of Greece. It was described in the genus *Barssia* based on phylogenetic analyses of the LSU rDNA and ascoma with a conspicuous apical depression. This feature is not evident in the closely related *B. maroccana* (Kaounas et al. 2015). It has been reported from the Huzurlu high plateau (Gaziantep) in Turkey, under *Abies cilicica* subsp. *cilicica* in mixed forest, 1600 m a.s.l., based on morphological features (Uzun et al. 2018).

***Balsamia maroccana*** (G. Moreno et al.) K. Hansen & X.H. Wang, *comb. nov.* — MycoBank MB 829145

*Basionym.* *Barssia maroccana* G. Moreno et al., Persoonia 33: 263. 2014.

*Holotype.* MOROCCO, Azrou, province of Ifrane, *Cedrus atlantica* forest, 18 Nov. 2010, M.A. Sanz, J. Álvarez, P. Alvarado, J.L. Manjón (AH 39117).

Illustration — Crous et al. (2014: 262).  
Distribution — Morocco, Ifrane, 1760 m a.s.l.

Notes — This species was recently described from the Atlas Mountains of Morocco in *Cedrus atlantica* forests. So far it is known only from the type locality. The authors find only subtle (if any) morphological differences between *Balsamia* and *Barssia* (Crous et al. 2014), and *B. maroccana* lacks the principal feature of *Barssia* pointed out by Gilkey (1925), i.e., an apical depression in the ascoma covered by the peridium. *Balsamia maroccana* is morphologically very similar to *Balsamia polysperma*, differing only in the smaller ascomata, narrower spores and different ecology (Crous et al. 2014).

***Balsamia oregonensis*** (Gilkey) K. Hansen & X.H. Wang, *comb. nov.* — MycoBank MB829146

*Basionym.* *Barssia oregonensis* Gilkey, Mycologia 17: 254. 1925.

*Syntypes.* USA, Oregon, Benton County, Sulphur Springs Road, in earth one to three inches deep under leaf mould under *Rhamnus purshiana* ('tree of *Cascara sagrada*'), 12 Apr. 1925, H.P. Barss, 4833; *ibid.*, 26 Apr. 1925, 4834 (OAC Mycological herbarium).

Selected illustrations — Gilkey (1925: pl. 26, f. 5, 6).  
Selected descriptions — Gilkey (1925: 254; 1939: 26).  
Distribution — North America, Oregon and California, Poland, Tatra Mountains.

Notes — This species is well known from North America, but has also been reported from Poland, Tatra mountains, 950 m a.s.l. (Lawrynowicz & Skirgiello 1984). Originally it was collected under (the non-ectomycorrhizal) *Rhamnus purshiana*, but later reported as abundant with *Pseudotsuga menziesii* in north-western North America (Trappe et al. 2009). The single collection from Poland was found in calcareous soil (pH 6–6.5) in *Picea abies* forest (Lawrynowicz & Skirgiello 1984), and should be investigated using a molecular approach to ensure the species is present also in Europe.

***Dissingia*** K. Hansen, X.H. Wang & T. Schumach., *gen. nov.* — MycoBank MB829090; Fig. 4a–b, 5

*Etymology.* In honour of Dr Henry Dissing for his monumental work on *Helvella*.

*Type species.* *Dissingia leucomelaena* (Pers.) K. Hansen, X.H. Wang & T. Schumach.



**Fig. 4** Diversity of apothecial shapes and colours in *Dissingia* and *Helvella* s.str. a–e. Cup-shaped apothecia, subsessile to distinctly stipitate, ± blunt ribs gradually widening and branching toward the cup-attachment: a. *D. leucomelaena* (DMS-9190862); b. *D. confusa* (KH.12.75); c. *H. alpina* (KH.12.69); d. *H. macropus* (KH.09.142); e. *H. corium* (KH.09.25); f–g. cup-shaped apothecia with ribs extending onto the receptacle surface, but not reaching the margin, *H. acetabulum* (TL-12536, C); h–i. saddle shaped forms in *Helvella* s.str. with free margin in: h. *H. carnosa* (KH.10.277); or margin attached to the stipe: i. *H. atra* (KH.10.97). — All living material. — Photos: a, f–g T. Læssøe; b–e, h–i K. Hansen.

Included species. *D. confusa*, *D. crassitunicata*, *D. leucomelaena*, *D. oblongispora*.

*Apothecia* cup-shaped, mostly remaining concave when expanding, sessile, or with a ± distinct stipe; stipe short, broad or slender, with a few ± conspicuous, blunt ribs gradually widening and subdividing towards the cup attachment. *Hymenium* yellowish brown to greyish brown, to dark brownish black; receptacle surface even or with few to many blunt to angular ribs and grooves below, pubescent, upper part whitish to dark greyish brown, below concolorous or often gradually paler to almost whitish. *Asci* cylindrical, operculate, arising from simple septa, 8-spored. *Spores* ellipsoid, obtuse or attenuate, smooth. *Paraphyses* filiform, septate, with clavate or subcapitate tips.

**Notes** — The genus *Dissingia* conforms to sect. *Leucomelaenae* Dissing *sensu* N.S. Weber (1972) in being distinguished by asci that arise from simple septa. The species of *Dissingia* prefer calcareous soil, often in coniferous forests. The European species have been compared and distinguishing morphological and molecular characters presented (Harmaja 1979, Skrede et al. 2017).

***Dissingia confusa*** (Harmaja) K. Hansen & X.H. Wang, *comb. nov.* — MycoBank MB829091; Fig. 4b, 5e, g

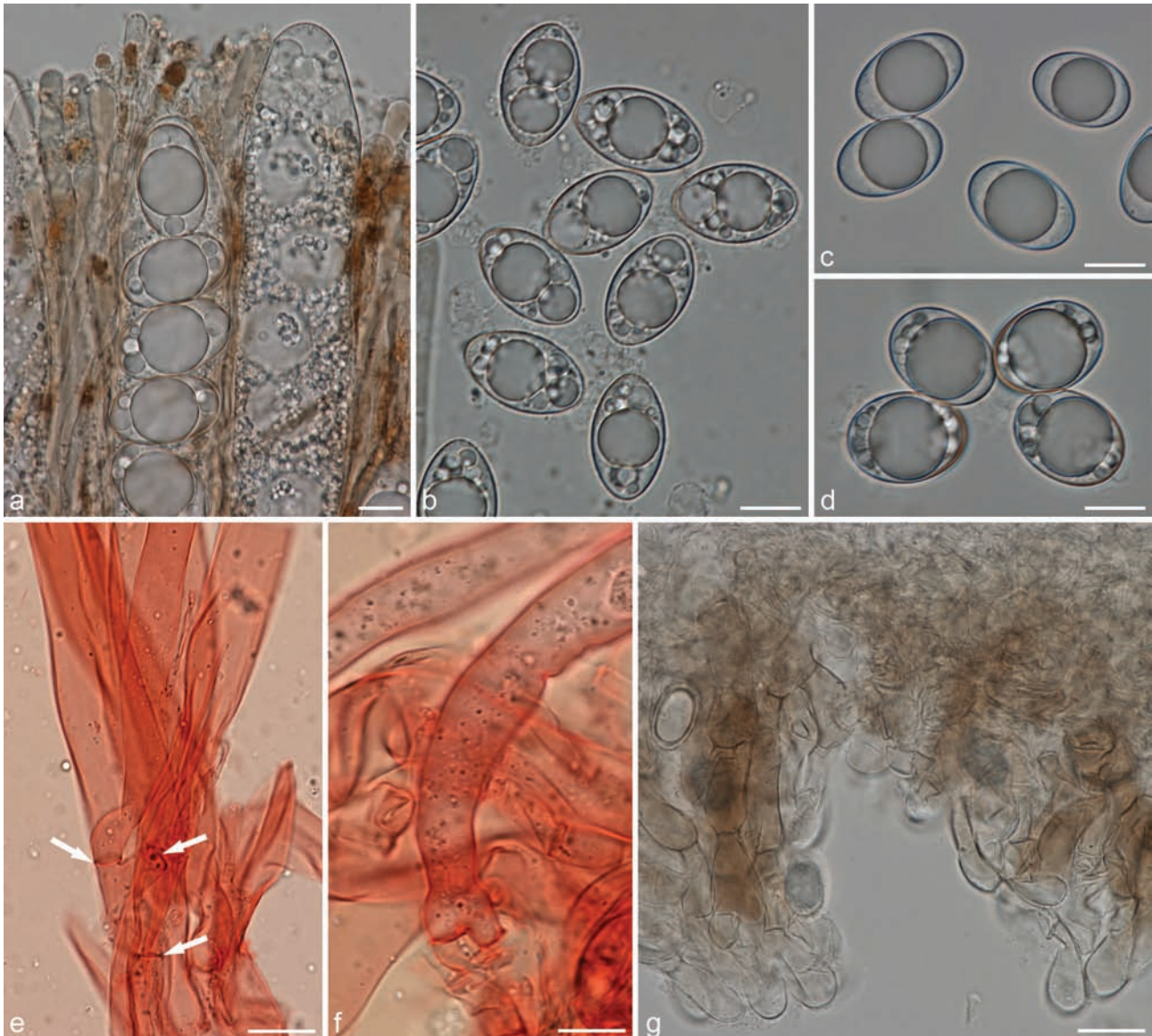
*Basionym.* *Helvella confusa* Harmaja, *Karstenia* 17: 43. 1977.

*Holotype.* DENMARK, Northern Jutland, Klitmøller, on calcareous soil, 15 May 1965, K. Toft & H. Dissing ('*H. solitaria*') (H). *Isotype* (C-F-70807 '*H. solitaria*').

Selected illustration — Dissing (1966: f. 10, as '*H. solitaria*').  
Selected descriptions — Harmaja (1977a: 43, 1979: 36), Dissing (1966: 42, as '*H. solitaria*').

**Distribution** — Europe (Norway, Sweden, Switzerland), probably Asia (India).

**Specimens examined.** NORWAY, Oppland, Dovre, Grimsdalen, Tollevshaugen, in pine forest reserve, 4 Aug. 2007, T. Schumacher & T. Vrålstad, TS 077.07 (O-253269); Buskerud, Gol, in moss along rivulet, 9 June 2014, K. Sæbø (O-253268). — SWEDEN, Jämtland, Korallgrottan Nature Reserve, trail to Korallgrottan, bank of the first river with bridge, upper to the bridge, on wet sandy soil, 487 m a.s.l., 31 Aug. 2012, K. Hansen & X.H. Wang, KH.12.73 (S); KH.12.75 (S); KH.12.76 (S).



**Fig. 5** *Dissingia* and *Helvella* s.str. a–d, g. Microscopic characters shared by the two genera; e–f. distinguishing ascus features. a. Asci and paraphyses, *H. capucina* (KH.10.135, S); b–d. spores: b. *H. macropus* (KH.10.192, S); c. *H. fibrosa* (KH.10.132, S); d. *H. levis* (KH.10.185, S); e. asci developing from simple septa (at arrows), *D. confusa* (KH.12.75); f. ascus base showing small hook from croziers, *H. carnososa* (KH.10.277); g. outer excipulum of elongated cells forming hyphoid, fasciculate hairs, *D. confusa* (KH.12.75). — Scale bars: a–f = 10 µm, g = 20 µm; a–d living material; e–g from dried material. — Photos: all K. Hansen.

***Dissingia crassitunicata*** (N.S. Weber) T. Schumach. & Skrede, *comb. nov.* — MycoBank MB829092

*Basionym.* *Helvella crassitunicata* N.S. Weber, *Beih. Nova Hedwigia* 51: 30. 1975.

*Holotype.* USA, Washington, Mount Rainier National Park, Narada Falls, 10 Aug. 1948, A.H. Smith 30052 (MICH-11561).

Selected illustration — Landeros et al. (2012: f. 24; type study).

Selected descriptions — Weber (1975: 30), Landeros et al. (2012: 47).

Distribution — North America (Canada, USA WA).

*Specimen examined.* CANADA, British Columbia, Whistler National Park, on soil in subalpine spruce forest, 13 Aug. 1994, T. Schumacher (O-253286).

***Dissingia leucomelaena*** (Pers.) K. Hansen & X.H. Wang, *comb. nov.* — MycoBank MB829093; Fig. 4a

*Basionym.* *Peziza leucomelas* Pers., *Mycol. Eur.* 1: 219. 1822.

*Synonym.* *Helvella leucomelaena* (Pers.) Nannf., in Lundell & Nannfeldt, *Fungi exs. Suec. Fasc.* 19–20: 21, no. 952. 1941. — *Lectotype.* NETHERLANDS, Herb. Persoon L 8945 – 6, selected by Abbott & Currah (1997). *Epitype:* SWEDEN, Gotland, Klintehamn, at the railway station, 7 May 1938, S. Lundell & E. Åberg (C, Fungi Exs. Suec. 952, 'Helvella leucomelas' (Pers.) Nannf.), selected by Skrede et al. (2017)!

Selected illustration — Dissing (1966: f. 8).

Selected description — Dissing (1966: 36).

Distribution — Cosmopolitan (Australia, Denmark, Sweden, USA MA, Chile).

Habitat/host — On calcareous soil, under conifers, usually *Pinus*.

*Specimens examined.* DENMARK, Møn, Busemark Mose, on sandy soil under *Pinus* in coniferous forest/plantation, 23 Apr. 2017, T. Læssøe, DMS-9190862 (S). — USA, Massachusetts, Bolton Lime Quarry and Kiln, Bolton, 7 May 2006, G. Lewis-Gentry & K. Hansen, KH.06.01 (FH).

***Dissingia oblongispora*** (Harmaja) T. Schumach. & Skrede, *comb. nov.* — MycoBank MB829094

*Basionym.* *Helvella oblongispora* Harmaja, *Karstenia* 18: 57. 1978.

*Holotype.* GERMANY, Bavaria, near Munich, on calcareous soil under conifers near the river Isar, 23 July 1969, A. Einhellinger (C)!

Selected illustration — Harmaja (1979: f. 7).

Selected description — Harmaja (1979: 36).

Distribution — Europe (Germany, Norway, Switzerland).

*Specimen examined.* NORWAY, Oppland, Lunner, Muttagrøvene naturminne, on calcareous gravel, 27 Aug. 2004, T. E. Brandrud & E. Bendiksen, TEB 278.04 (O-166316).

Notes — The macromorphology of *D. oblongispora* and *Helvella costifera* and *H. calycina* (of the *costifera* lineage in Skrede et al. 2017) is similar, but it is molecularly and anatomically easily distinguished from the two latter species. The holotype specimen is badly preserved and repeated efforts to retrieve DNA sequences failed.

***Helvella*** L., *Sp. Pl.* 2: 1180. 1753. ('*Elvela*') emend. — Fig. 4c–i, 5a–d, f

*Type species.* *Helvella crispa* Fr., *Syst. Mycol.* 2: 14. 1822.

Description in Skrede et al. (2017): 213 (under Morphology); restricted to species with asci arising from croziers, the ascogenous hyphae being of the pleurorhynque type, with the exception of *H. corium* and *H. alpina* of the *alpina-corium* lineage that have ascus bases with simple septa.

***Helvella lactea*** Boud., *Icon. Mycol.*, liste prélim.: 2. 1904

*Synonym.* *Helvella astieri* Korf & Donadini, *Rep. Tottori Mycol. Inst. (Japan)* 10: 397. 1973. — *Holotype.* FRANCE, Var, Sainte Baume, près de l'Hostellerie, on soil under *Quercus pubescens*, 17 Sept. 1972, J. Astier & J.-C. Donadini s.n. (PC); isotype (CUP 52755)!

Notes — *Helvella astieri* was described from a single collection of sparassoid ascomata (Korf 1973b). They are closed, tuberiform and sessile. The asci were still with operculum, although clearly not able to discharge their spores freely into the air. Our results suggest that *H. astieri* is simply an accidentally folded, sparassoid form of *H. lactea*. The hsp sequence of the isotype of *H. astieri* is 100 % identical to hsp sequences of *H. lactea* (obtained in Skrede et al. 2017). No LSU sequence is available from *H. lactea*. LSU sequences of *H. astieri* and *H. sublactea* differ in 5 bp (GenBank KT894833; cai663) and 6 bp (KT894834; Zhao1273) (from Wang et al. 2016). To our knowledge, only one additional collection has been reported as *H. astieri* (f. 6F in Læssøe & Hansen 2007), but that Danish collection represents a species of *Hydnotrya* based on LSU sequence comparisons.

***Midotis*** Fr., *Syst. Orb. Veg.* 1: 363. 1825: Fr., *Elench. Fung.* 2: 29. 1828. — Fig. 6

*Etymology.* Referring to king Midas in Greek mythology that was given the long ears of a donkey by Apollo.

*Type species.* *Midotis lingua* Fr. (only original species).

*Synonym.* *Wynnella* Boud., *Bull. Soc. Mycol. France* 1: 102. 1885. — *Type species.* *Wynnella silvicola* (Beck) Nannf.

Selected illustrations — Boudier (1909: n° 535, pl. 250, as '*Wynnella auricula*'), Svrček (1963: pl. 48), Van Vooren (2013: photos).

***Midotis lingua*** Fr., *Elench. Fung.* 2: 30. 1828: Fr., *Elench. Fung.* 2: 30. 1828 — Fig. 6a, c, e

*Neotype* designated here: SWITZERLAND, Graubünden, along the river E of Suras, Motta Nassa Donna, N46°43' E10°19', among mosses with *Tussilago*, *Saxifraga aizoides*, under *Pinus*, 31 Aug. 1984, H. Dissing Sch 84.51 (C-F-57385) ! MycoBank MBT384977

Selected description — Huhtinen (1985: 481).

*Specimens examined.* CANADA, Quebec, Poste-de-la-Baleine area, Manitoulin Islands, open dry heath with *Dryas* and *Vaccinium uliginosum*, 27 July 1982, S. Huhtinen 82/98 (TUR 078781). — SWITZERLAND, Graubünden, Las Paluda, on the ground under *Pinus*, 1750 m a.s.l., 29 Aug. 1984, H. Dissing (O-253387).

*Other material examined and referred to Wynnella subalpina.* CHINA, Sichuan, Jiuzhaigou, 15 Sept. 2015, S.H. Li, KUN-HKAS 94928. — GERMANY, Bayern, Oberbayern, Garmisch-Partenkirchen, Estergebirge, c. 3 km ESE of Farchant, northern slopes of the Wank Mtn, 1280 m a.s.l., on gravel, 30 June 1993, A. Pillukat (UPS F-005952, 84759. Tribel, Microfungi exs. 83). — KYRGYZ REPUBLIC, Tianshan Interior, Montes Naryntau, 22 July 1967, A. Raitviir (C-F-60841). — SWITZERLAND, Graubünden, Motta Jüda, Val Plavna, along path, 1600 m a.s.l., 30 Aug. 1979, E. Horak & H. Dissing, Sch 79.058 (C-F-56847).

Notes — We adopt the name *Midotis* for the genus commonly referred to as *Wynnella*. *Midotis lingua* was originally described as the only species of *Midotis* and it clearly represents an earlier name for *W. silvicola* or the recently described *W. subalpina*. Nannfeldt (1939) suggested this previously. Fries' recognition of *M. lingua* was based on material from Switzerland ('Vallisla'). Schleicher collected and sent the material to Fries who studied it, as indicated by the abbreviation 'v. s.' (*vidi siccam*, seen dried) (Fries 1828). No specimen of Schleicher's has been traceable, neither in the Fries herbarium (UPS, pers. comm. Å. Kruys) nor in the literature or exsiccate (checked in S and H; no exsiccate are available in UPS). The original description (Fries 1825,



**Fig. 6** *Midotis*. a, b. Apothecia; c. part of medullary excipulum of *textura intricata* and outer excipulum of pigmented long angular cells placed in a palisade, outermost cell club-shaped; d. close-up of outer excipulum cells; e. asci showing two-pronged remnants from croziers formation (in CR); f. close-up of the thick-walled interwoven hyphae of the medullary excipulum (in KOH) (a: *Midotis lingua* PRC 3971; b: *Wynnella subalpina* KUN-HKAS 94928; c, e: *Midotis lingua* S. Huhtinen 82/98; d, f: *Wynnella subalpina* UPS F-005952). — Scale bars: d–f = 10 µm; c = 20 µm; a, b living material; c–f from dried material. — Photos: a O. Koukol; b S.H. Li; c–f K. Hansen.

1828) emphasizes the distinctly ear-shaped apothecia, much elongated on the one side, with a coriaceous texture ('as a resupinate *O. leporina*'), up to 5 cm tall, with reddish brown colours. Unfortunately, *Midotis* has later been misinterpreted for some American tropical species, considered best typified by *Encoelia heteromera* (as *Peziza heteromera*, a species later referred to by Fries (1849) under *Midotis*) (Durand 1923) or by *Wynnea gigantea* (Clements & Shear 1931). These species are vastly different from *M. lingua*, a species occurring at high elevation or

in temperate regions. Notably Clements & Shear (1931) listed also *Wynnella* as a synonym of *Midotis*. We recognise *Midotis* as a distinct genus based on the narrowly elongate ear-shaped apothecia, the dark reddish brown to blackish brown hymenia and the concolorous, but slightly lighter, receptacle surface, with a contrasting light yellow to white base. Besides this, many have noted the tough ('horny') consistency of the apothecia of *Midotis* when dried (Kanouse 1949 as *O. auricula*, Nannfeldt 1966, Dissing 1972, Huhtinen 1985 as *H. silvicola*). Huhtinen



**Fig. 7** *Pindara terrestris*. a–c. apothecia; d. asci with bi-seriate spores; e. paraphyses apices with brownish to greyish brown content and surrounded by a greyish amorphous substance; f. hooked ascus base from crozier formation (in CR); g. subhymenium, medullary and outer excipulum; h. outer excipulum cells in rows forming pustule; i. fusoid spores, with single guttule formed by coalescence of multiple smaller guttules (a, d–e, g–i : S-F327988; b: KH.12.67, S; c, f: TUR196043). — Scale bars: f, h–i = 10  $\mu$ m; d–e, g = 20  $\mu$ m; a–c living material; d–i from dried material. — Photos: a N. & Z. Lipovac; b, d–g K. Hansen; c T. Kekki.

(1985) noted and illustrated thick-walled excipular hyphae in specimens from Canada (f. 10c; see also Fig. 6f this paper), compared to, e.g., those of *H. pezizoides* or *H. elastica*. We have observed the outer excipulum to be narrow (c. 50–70 µm) compared to a thick medullary excipulum composed of densely interwoven hyphae (c. 300 µm). The outermost cell of the outer excipulum is noticeably very long, club-shaped (35–50 × 11–15 µm at the widest part and 6–8 µm at septa), with the long axis perpendicular to the outer surface, with 2–3 additional shorter angular cells (10–16 µm long) in rows, forming a palisade (Fig. 6c–d, see also Huhtinen 1985: f. 10b–c; Eckblad 1968: f. 42). This structure may be responsible for the tough consistency. It should be noted that the illustration by Dissing (1966: f. 7) showing an outer excipulum composed of rows of 5–8 angular cells that are shorter and all of the same length, similar to those seen in *H. lacunosa* (in Dissing 1966: f. 6), does (mistakenly) not depict the material cited C-F-47570. We have restudied this material and the excipulum structure corresponds to the description and photos given here for *Midotis*. The outer excipulum cells and the paraphyses in *Midotis* are filled with yellowish to reddish brown granules in dried material. In MLZ the granules either dissolve or partially dissolve colouring the cells pale yellowish, or the pigment condenses and becomes refractive and yellowish. Molecular phylogenetic studies have placed *Midotis* (as *Wynnella*) as a sister lineage to *Helvella* (Hansen & Pfister 2006, Læssøe & Hansen 2007, Landeros et al. 2015, Zhao 2016a, Skrede et al. 2017). This is substantiated in the present study where *Midotis* forms a separate distinct sister group to the monophyletic group of *Helvella* s.str., *Pindara* and *Dissingia*. *Wynnella subalpina* was diagnosed with ‘a tea brown to blackish hymenium, a red, reddish brown to greyish yellow receptacle surface’ (Fig. 6b), as opposed to ‘a blood red to purple-brown hymenium, a medium red brown or pale red brown receptacle surface’ in *W. silvicola* (Zhao et al. 2016a). In addition, the spores of *W. subalpina* was said to be narrower than the spores in *W. silvicola*. For further comments see Discussion.

***Pindara*** Velen., Monogr. Discom. Bohemiae 1: 341. 1934 — Fig. 7

Type species. *Pindara terrestris* Velen. (only included species).

***Pindara terrestris*** Velen., Monogr. Discom. Bohemiae 1: 341, t. XXVI, f. 1. 1934.

Synonym. *Helvella terrestris* (Velen.) Landvik, Mycologia 91: 283. 1999. — Lectotype: CZECH REPUBLIC, Mnichovice, on sandy loamy soil along river, 8 Aug. 1927, Velenovský (PR 147368), selected by Svrček & Kubicka (1968). Other original material: 9 Aug. 1927 (PR 152821, 152822).

Selected illustrations — Van Vooren (2014: 43), Landvik et al. (1999: SEM of spores; f. 5).

Selected descriptions — Kristiansen (1984), Landvik et al. (1999: 283), our Notes below.

Specimens examined. FINLAND, Perä-Pohjanmaa, Rovaniemi, Marrasjärvi, grid 741987:342370, by a small creek, on moist sand with liverworts, 12 Aug. 2011, T. Kekki 168 (TUR 196043); Rovaniemi, Narkaus, Katiskonoja, grid 73557:4666, on a bank of a brook with mosses and tree roots, 8 Aug. 2018, T. Kekki 3148 (TUR). — NORWAY, Nord-Trøndelag, Verdal, Ramsås, along rivulet with *Trichophaea* sp., 28 Aug. 1983, S. Sivertsen 83-138 (C, duplicate in TRH). — SWEDEN, Västerbotten, Umeå, Tjälamark, in steep edge of a small tributary to the larger brook Kullabäcken c. 700 m north of Forslunda, N63°52' E20°11', on sandy soil among *Pellia*, 25 July 2011, N. & Z. Lipovac (S-F327988, dupl. UME); ibid., 4 Aug. 2011, N. & Z. Lipovac 1104 (UME); ibid., 15 Aug. 2012, N. & Z. Lipovac 1201 (S-F327988); Jämtland, Korallgrottan Nature Reserve, along trail to Korallgrottan, N64°53' E14°10', 469 m a.s.l., on sandy soil in side of rivulet (with *Trichophaea hybrida* KH.12.58) downstream toward the river Leipikälven, 28 Aug. 2012, K. Hansen & X.H. Wang, KH.12.67 (S).

Notes — This enigmatic species produces the smallest apothecia and longest spores (54–67 µm, Fig 7i) in *Helvellaceae*. The apothecia are purplish grey, stipitate, cupulate to flattened, with or without shallow grooves on the outer surface, cup 1–1.5 mm high and 3–6 mm broad, stipe 0.5–1.5 mm high (Fig. 7a–c). *Pindara terrestris* was for a long time known only from three localities in the Czech Republic (Velenovsky 1934, Svrček 1947, Svrček & Kubicka 1968), but later discovered in three localities in Norway (Kristiansen 1984, 1996, Landvik et al. 1999). It has also been reported from Sweden (Eriksson 2014), Croatia and Switzerland (Van Vooren 2014). Here we add one more locality from Sweden and two from Finland. We suggest the species has likely been overlooked, because of the small size and colour of the apothecia that easily blend into the substrate. The typical locality is moist/water soaked clayey soil in the edge of rivulets, often among liverworts. This is also a typical habitat for *Trichophaea hybrida* that has been reported along with *Pindara*.

Landvik et al. (1999) pointed out that the spores in *Pindara* (when dried) had a single large guttule, contrary to 4–6 guttules as given in the original description (Velenovsky 1934) and the description by Svrček (1947). In living material from Sweden and Finland, the spores were multi-guttulate with mostly 1 slightly larger guttule at each pole; in rehydrated material these guttules coalesce to one large guttule (Fig. 7i). In vital specimens the spores were also observed in two rows (bi-seriate) in the ascus. The asci of *P. terrestris* arise from croziers (Fig. 7f), and noteworthy, the paraphyses extend above the asci, have a brownish to greyish brown content, and apices surrounded by a greyish amorphous substance (Fig. 7e) with some yellow refractive, small guttules.

## DISCUSSION

The results presented here suggest two (or several) possible treatments of generic limits in the *Helvellaceae*: a very wide concept of *Helvella* including *P. terrestris* and *Midotis*, and even species of *Barssia* and *Balsamia*, or a more restricted concept of *Helvella* that reflects the three-gene phylogeny presented here (Fig. 2). Given the strong support provided by the molecular data and to reflect the morphological diversification observed among the major lineages, we have chosen to recognise the genera *Pindara* (monotypic), *Midotis* and *Balsamia*, and erect a separate new genus *Dissingia* for the *leucomelaena* lineage of *Helvella* s.lat. A restricted *Helvella* is with only one exception (the *alpina-corium* lineage) delimited by the type of ascus development, i.e., the asci arise from croziers. Asci in *P. terrestris*, *Midotis* spp. and *B. aestivalis* are also formed from croziers, while species in *Dissingia* are unique in having asci with simple septa at the bases. The asci in *Underwoodia columnaris* have been reported as pleurorhynchous (Abbot & Currah 1997). From this it is most parsimonious to infer that ascus development from croziers is the ancestral state for the *Helvellaceae* and that ascus development from simple septa has evolved at least twice within the family.

The type of ascus development was first noticed and used in *Helvella* by Weber (1972) to delimit one of several sections, sect. *Leucomelaenae* for *D. leucomelaena* (as *H. leucomelaena*), the only species (in Michigan) with aporhynchous asci; all other species showed pleurorhynchous asci. Later the North American *D. crassitunicata* (as *Helvella crassitunicata*) was described and added to the section (Weber 1975). Following this, the new European species *D. confusa* (as *H. confusa*) and *D. oblongispora* (as *H. oblongispora*), with aporhynchous asci, were placed in sect. *Leucomelaenae* (Harmaja 1977a, 1979). Häffner (1987) also recognized sect. *Leucomelaenae* with aporhynchous asci as the key character (including addi-



tionally *H. aestivalis*). Although Abbott & Currah (1997) did not use ascus development to delimit their subgenus *Leucomelaenae*, they placed all *Helvella* species with aporhynchous asci (*D. leucomelaena*, *D. crassitunicata* and *D. oblongispora*, all as *Helvella*) in this subgenus and noted that if these species were more closely related to each other, this feature would support the recognition of the section *Leucomelaenae* s.str. within their subgenus. This division of *Helvella* based on ascus development has been confirmed using phylogenetic analyses of LSU rDNA sequences (Landeros et al. 2015) and is substantiated with high support for all deeper branches in the *Helvellaceae* phylogeny by our multi-gene analyses (Fig. 2). The type of ascogenous hyphae has only to a limited degree been used as a feature in delimiting taxa at generic and higher levels within the Pezizomycetes (e.g., Berthet 1964, Kimbrough 1989) and is a character that should be studied further. All members of the suborder *Sarcoscyphineae*, the families *Caloscyphaceae* and *Discinaceae-Morchellaceae*, except for *Disciotis venosa*, have ascal bases with simple septa and for those species studied the ascogenous hyphae are of the aporhynque type (Berthet 1964). The suborder *Pyronemineae*, i.e., *Ascodesmidaceae*, *Pyronema* and *Coprotus*, was characterised as having the acrorhynque type of ascogenous hyphae (Kimbrough 1989), while most taxa in the suborder *Pezizineae* were considered to have the pleurorhynque or rarely aporhynque type. In one genus, *Pulvinula*, the presence or absence of croziers has been used to distinguish species (Pfister 1976).

We advocate reinstating *Pindara* as a distinct genus based on molecular and morphological characters. The large, narrowly fusiform spores with multiple guttules are unique within *Helvellaceae*. It has been suggested that the closest relatives of *Pindara* are to be found among the *Helvella* subgenera *Cupuliformae* or *Macropodes* sensu Abbott & Currah 1997 (Landvik et al. 1999, Van Vooren 2014) based on the stipitate-cupulate ascomata without external ribs and grooves (Fig. 7a–c), and the verruculose, fusoid ascospores (Fig. 7i), similar to characters observed in *H. macropus* (Fig. 4d, 5b). *Helvella rivularis* was also compared to *Pindara* because of its small ascoma size (3–9 mm). In combination with the study by Skrede et al. (2017), our three-gene phylogeny shows that *Pindara* constitute an independent lineage and is not closely related to the species of these subgenera (see placement of *H. macropus* (the only species in *Macropodes*); *H. rivularis* and *H. corium* (*Cupuliformae*) in Fig. 2). We also now know that contrary to *Pindara*, *H. rivularis* produces apothecia of a wide range of sizes (stipe 1.5–3.5 cm long, pileus 0.5–2.6 cm diam) (e.g., KH.03.21 (FH); see also Skrede et al. 2017).

The long branches leading to *Pindara* and *Midotis* suggest that these two taxa diverged a long time ago or the gene regions under study evolved at a higher rate in these two species than in closely related taxa. *Midotis* is morphologically unique within *Helvellaceae* in having narrowly hare ear-shaped apothecia in all developmental stages and differs also in microanatomy by its large, clavate-elongated outermost excipular cells (Fig. 6). The dark reddish colours of the apothecia are comparable to apothecial colours of *B. aestivalis* (Fig. 3a) (Dissing 1972, Dissing & Raitviir 1974 as *H. aestivalis*), colours that are not present in *Helvella* s.str.

In conclusion, our results support the view that gained support with Nannfeldt (1937), a scope intuitively given fifty years earlier by Quélet (1886) but with reasons very vaguely formulated. Nannfeldt (1937) clarified and exemplified that taxa of *Helvellaceae* with different apothecial shapes might be closely related: ‘... composite and simple, campanulate, mitrate and cupulate forms may be included in the same tribus’. This idea was accepted long ago (e.g., Dissing 1966, Weber 1972,

Häffner 1987, Abbott & Currah 1997) and species previously placed in five different genera, i.e., *Leptopodia*, *Cyathipodia*, *Acetabula*, *Macropodia* and *Helvella* sensu Boud. (Boudier 1885, 1907), and separated in two families, were merged in *Helvella*. Within *Helvella* s.lat. seven sections were described (Dissing 1966), although Dissing remarked ‘... no sharp lines can be drawn between ‘genera’ or even sections when considering macroscopic characters’. Since then several subdivisions have been proposed with slightly different boundaries (Weber 1972, Häffner 1987, Abbott & Currah 1997), still based mainly on stipe and apothecial shape and receptacle surface (smooth vs pubescent/hairy). For a summary of these subdivisions, see Landeros et al. (2015). Our multi-gene data do not determine the evolutionary relationships within *Helvella* s.str., but the early branching of *Dissingia* (/Sect. *Leucomelaenae*) suggests that the ancestor of *Helvella* s.lat. most likely produced sessile or shortly stipitate, cup-shaped apothecia – with an even receptacle surface or with blunt to angular ribs and grooves below (as in *Dissingia*, e.g., Fig. 4a, b). The cup-shape was maintained in some lineages of *Helvella* s.str., such as in the *alpinacorium* lineage that lack ribs on the receptacle surface (Fig. 4c, e); and the *acetabulum-solitaria* lineage (Fig. 4f–g) that have ribs, but the ribs do not extend to the margin. This is in agreement with previous molecular studies (Landeros et al. 2015, Skrede et al. 2017). It supports, at least partly, the idea illustrated by Dissing (1966: f. 1) that distinctly stipitate apothecial forms with solid terete and even stipes (e.g., *H. macropus*, *H. carnososa*, Fig. 4d, h) and forms with hollow chambered and ribbed stipes (e.g., *H. atra*, Fig. 4i), are derived from sessile forms or forms with short inflated hollow and ribbed stipes; and saddle-shaped or lobed apothecial forms (*H. carnososa*, *H. atra*, Fig. 4h–i) are likely derived from cup-shaped.

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