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The *Helvella corium* species aggregate in Nordic countries – phylogeny and species delimitation

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Abstract: Mycologists have always been curious about the elaborate morphotypes and shapes of species of the genus *Helvella*. The small, black, cupulate *Helvella* specimens have mostly been assigned to *Helvella corium,* a broadly defined morpho-species. Recent phylogenetic analyses, however, have revealed an aggregate of species hidden under this name. We performed a multispecies coalescent analysis to re-assess species limits and evolutionary relationships of the *Helvella corium* species aggregate in the Nordic countries. To achieve this, we used morphology and phylogenetic evidence from five loci – heat shock protein 90 (*hsp*), translation elongation factor 1-alpha (*tef*), RNA polymerase II (*rpb2*), and the 5.8S and large subunit (LSU) of the nuclear ribosomal DNA. All specimens under the name *Helvella corium* in the larger university fungaria of Norway, Sweden and Denmark were examined and barcoded, using partial *hsp* and/or *rpb2* as the preferential secondary barcodes in *Helvella*. Additional fresh specimens were collected in three years (2015–2018) to obtain *in vivo* morphological data to aid in species discrimination. The *H. corium* species aggregate consists of seven phylogenetically distinct species, nested in three divergent lineages, *i.e. H. corium, H. alpina* and *H. pseudoalpina sp. nov.* in the /alpina-corium lineage, *H. alpestris, H. macrosperma* and *H. nannfeldtii* in the /alpestris-nannfeldtii lineage, and *H. alpicola* as a weakly supported sister to the /alpestris-nannfeldtii lineage. Among the seven species, the ribosomal loci expressed substantial variation in evolutionary rates, suggesting care in the use of these regions alone in delimitation of *Helvella* species. Altogether, 469 out of 496 available fungarium specimens were successfully barcoded.

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INTRODUCTION

Helvella is a species-rich genus of ascomycetous fungi (*Pezizales, Helvellaceae*) probably consisting of more than 100 species worldwide. Recently, the broad concept of *Helvella* was restricted, *i.e.* to exclude some para- and polyphyletic groups of species now referred to the segregate genera *Balsamia, Dissingia, Pindara* and *Wynnella* (Hansen *et al.* 2019). Originally, *Helvella* included only stipitate-capitate (*i.e.* non-cupulate) taxa, but cupulate species, which are today included in *Helvella*, were assigned to *Peziza* and/or *Acetabula* (*Pezizaceae*) (Fries 1822, Fuckel 1870). Quélet (1886), and later Nannfeldt (1937) and Dissing (Dissing 1966a, b), introduced a broad generic concept that includes both stipitate-capitate (saddle-shaped) and stipitate-cupulate taxa in *Helvella* (*sensu lato*). They considered apothecial gross morphology too variable and phenotypically plastic to be useful in genus level delineation. Instead, microanatomy of excipular tissues was introduced as more useful for this purpose. Species with similar microanatomy, the capitate as well as the cupulate, were thus included in *Helvella*. Still, apothecial gross morphology was retained as an important characteristic in delineation of subgroups (sections) within the genus (Boudier 1907, Dissing 1964, 1966b).

Helvella corium, first described as *Peziza corium* by Weberbauer (1873), is a medium-sized, stipitate-cupulate black species. It has seen many revisions over time. It was referred to as *Cyathipodia* and *Leptopodia* by Boudier (1907, 1910) until it was accommodated in *Helvella* (Massée 1895). Over the years, a few additional species have been introduced as closely related to *H. corium*. *Helvella alpestris* was described from alpine areas of France (Boudier & Fischer 1894, Boudier 1895), *H. arctica* (Nannfeldt 1937) from Abisko in Northern Sweden and *H. arctica* var. *macrosperma* (Favre 1955) from the Swiss Alps. The latter variety was later given specific rank by Fellner & Landa (1991), as has been followed by subsequent authors (Van Vooren 2014, 2015, Skrede *et al.* 2017). Contrary, in his monumental work on *Helvella*, Dissing (1966b) included *H. alpestris*, *H. arctica* and *H. arctica* var. *macrosperma* as part of his broad concept of *H. corium.*

Traditionally, fungal species have been recognised based on morphological characters, ecology and/or mating behavioural traits. Since many morphological traits in fungi show a high degree of phenotypic plasticity (West-Eberhard 1989), it has often been challenging for taxonomists to interpret and understand patterns of fungal relationships. Today, with the availability of molecular data, the concept of genealogical species recognition [*i.e.* recognition of species based on congruent gene trees across multiple unlinked loci (Taylor *et al.* 2000, Dettman *et al.* 2003)] has completely out-performed traditional morphological species concepts in this group of organisms.

Molecular markers used to infer phylogenies and erect phylogenetic species in fungi are diverse, including both RNA-coding, non-coding and protein coding loci. Several studies

have pointed out that single-copy, protein-coding markers are preferred and may perform better than multi-copy ribosomal genes in delimiting fungal species (Raja *et al.* 2011, Stielow *et al.* 2015, Hansen & Olariaga 2015). For species delineation the commonly used protein-coding loci include β-tubulin (*tub2*), the translation elongation factor 1-α (*tef*) and the DNA directed RNA polymerase II core subunits (*rpb1* and *rpb2*). The latter locus has proven useful in resolution of deep as well as shallow clades of ascomycetes (Spatafora *et al.* 2006, Hofstetter *et al.* 2007, Schoch *et al.* 2009). Recently, the minichromosome maintenance complex component 7 (*mcm7*) and the ribosome biogenesis protein *tsr1* were added to the list of valuable markers for resolving high- as well as low level taxonomic units in *Ascomycota* (Aguileta *et al.* 2008, Raja *et al.* 2011). In *Helvella* a portion of the heat shock protein 90 (*hsp*), in combination with *rpb2*, were shown to have strong discriminating power at the species level (Skrede *et al.* 2017). *Hsp* was also shown to be successfully amplified from fungaria specimens. This is probably due to the short sequence length (*i.e*. 272 bp). It was introduced as a most useful secondary barcode marker in *Helvella* (Skrede *et al.* 2017).

In the study by Skrede *et al.* (2017), species limits, phylogeny and taxonomy within *Helvella* were assessed, using a multilocus genealogical approach. They found that the morphospecies concept of *H. corium* in fact represented a pseudo-cryptic species aggregate that comprised five phylogenetic species nested in two divergent evolutionary lineages: the /alpina-corium lineage with *H. corium* and *H. alpina,* and the /alpestris-nannfeldtii lineage with *H. alpestris, H*. *macrosperma* and *H. nannfeldtii.* It was suggested as well that a sixth species, *i.e*. *H. alpicola,* might belong to this species aggregate, but its phylogenetic placement within the genus was unresolved (Skrede *et al.* 2017). The phylogenetic species of this species aggregate are morphologically very similar and therefore difficult to distinguish in the field. They also occupy overlapping habitats of the alpine zone, often in close proximity to *Salix* spp. (Weidemann 1998).

It is against this background that we aimed to get a better resolution of the species-level relationships within the *H. corium* species aggregate, using a broader set of genetic markers. In addition, we wanted to re-evaluate morphological characters used in species discrimination.

MATERIALS AND METHODS

The study is based on a set of newly collected specimens of the *Helvella corium* aggregate in the years 2015 to 2017 and samples deposited as *H. corium* and associated species in the Nordic University fungaria of O, TRH, BG, TROM, S, UPS, GB, UME, and C (in total 496 specimens). All specimens were identified by molecular barcoding (Tables 1, S1).

DNA extraction and PCR

A small (sesame seed sized) piece of the stipe of each fungarium specimen was put in CTAB Lysis buffer (BioChemica, Panreac AppliChem) for DNA extraction. For the specimens that were newly collected for this study, fresh pieces of ascomata were put directly in CTAB buffer before drying the whole ascocarp at 30 °C for 24 h.

Prior to DNA extraction, one tungsten bead was added to each sample, *i.e*. specimens in CTAB Lysis buffer (AppliChem Panreac). Samples were first frozen, then incubated at 65 °C on a heating block for 30 min, and subsequently vortexed thoroughly. Samples were frozen a second time, and allowed to thaw at room temperature before DNA extraction. DNA was extracted using the E.Z.N.A.®HP Fungal DNA Kit (Omega Biotek D3195), following a slightly modified version of the manufacturer's protocol for dried samples, *i.e.* the optional step of adding 10 µL 2-mercaptoethanol was undertaken, and the column was equilibrated with NaOH. The DNA was eluted in 50 µL elution buffer to increase DNA concentration.

For barcoding purposes, efforts were made to PCR amplify a partial segment of the heat shock protein 90 (*hsp*) for all sampled specimens. In addition, a subset of 80 specimens, of which 21 represented outgroup taxa, were selected to infer multilocus phylogenies (Table 1). Within the species aggregate an effort was made to include broad taxon sampling. For some species, this proved difficult due to lack of collections (*i.e. H. alpicola, H. macrosperma*). Five loci were targeted: a 272 bp region of the protein-coding gene *hsp*, a 356 bp region of the protein-coding gene *rpb2*, a 571 bp region of the protein-coding gene *tef*, a 743 bp region of the large ribosomal subunit LSU (including the D1 and D2 regions), and the whole internal transcribed spacer region (ITS1, 5.8S, ITS2). Only the highly conserved 160 bp region of the 5.8S ribosomal RNA region was used in the phylogenetic analyses since the complete ITS region is difficult to align across the whole species aggregate. The primers used are shown in Table 2.

For all PCR reactions, PuReTaq Ready-To-Go PCR Beads (GEhealthcare, Waukesha, WI) were used in 25 µL reactions. The following PCR protocols were used to amplify the five loci: 4 min at 95 °C, 40 (50 for LSU) cycles of 25 s (30 s for *hsp* and LSU) at 95 °C, 30 s at 53 °C (58 °C for *hsp*, 52°C for LSU) and 60 s at 72 °C, followed by a 10 min extension at 72 °C and an indefinite hold at 10 °C. Amplified PCR products were visualized with electrophoresis on 1 % agarose gels. For PCR reactions that yielded product, 5 µL PCR product was purified with 0.2 µL ExoSAP-IT (GEhealthcare) and 1.8 µL H_2O. Samples were then run on a thermocycler at 37 °C for 15 min, followed by 80 °C for 15 min. Cleaned PCR product was diluted with 45 µL water per sample. 5 µL PCR product and 5 µL primer were added to clean tubes and labelled before sequencing. Sanger sequencing was performed by GATC Biotech (Constance, Germany).

Phylogenetic inference and species delimitation

Sequence assembly and editing was done using Geneious v. 9.1.6 (http://www.geneious.com, Kearse *et al.* 2012). All sequences were manually inspected and edited. Preliminary alignments were made using MAFFT v. 7.309 (Katoh & Standley 2013) within Geneious v. 9.1.6, under default parameter settings. All alignments were inspected and manually adjusted when necessary.

Substitution models for each locus were determined based on the AICc model selection criterion (small-sample-size corrected version of Akaike information criterion) as implemented in PartitionFinder v. 1.1.1 (Lanfear *et al.* 2017). Search was set to "greedy" and branch lengths set to "linked".

In recent years, new methods and software for delimiting species based on multilocus data have become available [*e.g*. BPP (Yang & Rannala 2010), Structurama (Huelsenbeck *et al.* 2011), PTP (Zhang *et al.* 2013), and DISSECT (Jones *et al.* 2015)]. Based on DISSECT, STACEY (Jones 2017) was introduced, as a package implemented in BEAST2 (Bouckaert *et al.* 2014).

Table 1. *Helvella* specimens included in the phylogenetic analyses of this study. Specimens included in the final analysis are marked with an asterisk. All specimens are marked with sample ID and fungaria ID. Type specimens are written in bold. GenBank accession numbers are added for all obtained sequences. X indicates sequence was not long enough for publishing in GenBank.

Species	Sample and fungarium ID	Locality	Collection year	GenBank accession number				
				hsp	*rpb2*	*tef*	LSU	ITS
H. corium	H2184, O-255756*	Svalbard. Longyearbyen	2017	MN692370	MN692314	MN658196	MN655873	MN656185
	H547, O-255757*	France. Savoie	1992	MN692363	MN692309	MN658197	MN655870	MN656165
	H436, O-253281	Svalbard. Spitsbergen. Kongsfjorden	1988	KY784528	KY772771	–	KY773163	–
	H950, TROM-F-610059	Norway. Nordland. Saltdal. Junkerdalen	2016	MN692364	MN692312	–	MN655880	–
	H956, O-255753	Norway. Hedmark. Folldal. Einunndalen	2016	MN692365	MN692313	–	MN655875	–
	H957, O-255754	Norway. Sør-Trøndelag. Oppdal. Vinstradalen	2016	MN692366	MN692310	–	MN655871	–
	H1958, TROM-F-610014	Norway. Troms. Salangen	2017	MN692369	–	–	MN655876	MN656172
	H248, O-253277	Norway. Hordaland. Ulvik. Finse	1996	KY784366	KY772614	–	KY773075	–
	H1088, C-F-86904	Greenland. Thule Airbase	1994	MN692367	–	–	MN655872	–
	H1089, C-F-63828	Greenland. Mestervig. Ochenpas	1968	MN692368	–	–	MN655874	–
	H294, C-F-16568	Russia. North Ural Mountaint	1990	KY784407	KY772654	–	KY773100	–
H. alpina	H2106, O-255751*	Norway. Oppland. Dovre. Grimdalen	2017	MN692362	MN692307	MN689291	MN655865	MN656181
	H1124, C-F-55730*	Greenland	1987	MN692361	MN692306	MN658193	MN655863	MN656171
	H223, **O-253228***	France. Savoie	1992	KY784343	KY772593	MN658192	KY773054	–
	H336, O-253227	Canada. British Colombia. Whistler	1994	KY784439	KY772690	–	KY773116	–
	H1159, C-F-54601	Norway. Nordland. Rana. Virvassdalen	1979	MN692359	X	–	MN655864	–
	H540, C-F-34420	Russia. Khatanga airport	NA	MN692358	MN692305	–	MN655862	–
	H1095, C-F-50287	Greenland. Sdr. Strømfjord	1982	MN692360	–	–	–	–
H. pseudoalpina	H498, O-255748*	Svalbard. Longyearbyen	2015	MN692354	MN692302	MN689292	MN655869	MN656164
	H1965, TROM-F-610048*	Norway. Troms. Balsjord	2017	MN692355	MN692303	MN658194	MN655866	MN656173
	H1966, TROM-F-610049*	Norway. Troms. Balsjord	2017	MN692356	MN692304	MN658195	MN655868	–
	H2278, TRH-F-20631	Norway. Nordland, Saltdal, Junkerdalen. Bibeldalen	1988	MN692357	MN692308	–	MN655867	–
H. alpicola	H231, O-253226*	Switzerland. Graubunden	1984	KY784349	KY772598	MN689298	KY773061	–
	H952, TROM-F-610061*	Norway. Nordland. Saltdal. Junkerdalen	2016	MN692344	MN692296	MN658206	MN655847	–
	H953, TROM-F-610062*	Norway. Nordland. Saltdal. Junkerdalen	2016	MN692345	MN692297	MN658207	MN655848	–

Table 1. (Continued).

Species	Sample and fungarium ID	Locality	Collection year	GenBank accession number				
				hsp	*rpb2*	*tef*	LSU	ITS
	H1439, C-F-53856	Norway. Nordland. Rana	1972	MN692346	–	–	–	–
	H1534, C-F-103009	Norway. Nordland. Rana	1971	X	X	–	–	–
H. nannfeldtii	H1972, TROM-F-610034*	Norway. Troms. Kåfjord	2017	MN692330	MN692291	MN658199	MN655861	MN656174
	H954, O-255744*	Norway. Hedmark. Folldal	2016	MN692328	MN692290	MN689293	MN655860	MN656167
	H2104, O-255745*	Norway. Oppland. Dovre. Grimsdalen	2017	MN692327	MN692293	MN658200	MN655859	–
	H027, **O-253338***	Norway. Oppland. Dovre. Grimsdalen	2009	KY784203	KY772447	KY772836	KY772919	X
	H1994, TROM-F-610036*	Norway. Troms. Målselv	2017	MN692326	MN692292	MN689294	MN655856	–
	H212, O-253332*	France. Savoie. Val d'Isere	1992	MN692323	KY772585	KY772888	KY773044	–
	H216, O-253333*	France. Savoie. Bon Valle	1992	KY784337	KY772589	KY772891	KY773048	–
	H545, O-255740	Austria. Tirol. Obergurgl	–	MN692324	MN692288	–	MN655858	–
	H564, O-255741	France. Savoie. Val d'Isere	1992	MN692325	MN692289	–	MN655857	–
	H1105, C-F-86947	Greenland. Narssarssuaq.	1991	MN692329	–	–	–	–
Helvalla sp	H1995, O-255738*	Norway. Troms. Målselv	2017	MN692339	MN692283	MN689299	MN655845	MN656178
	H1996, O-255739*	Norway. Troms. Målselv	2017	MN692340	MN692284	MN689300	MN655846	MN656179
H. alpestris	H460, ex DAOM574891*	Canada. Nuvanut	2014	KY784542	KY772789	MN658202	MN655837	MN656163
	H548, O-255736*	France. Savoie. Val d'Isere	1992	MN692332	MN692276	MN658203	MN655835	MN656166
	H2111, O-255737*	Norway. Oppland. Dovre. Grimsdalen	2017	MN692341	MN692280	MN658205	MN655834	MN656183
	H865, S-F-122351*	Norway. Oppland. Dovre. Grimsdalen	1985	MN692334	MN692277	MN658204	MN655838	–
	H1098, C-F-50667	Greenland. Ella Island. St. Elvdal E of the house.	1982	MN692342	MN692279	–	MN655839	–
	H719, UPS-F-145393	Sweden. Torne Lappmark. Jukkasjärvi	1946	MN692333	X	–	MN655836	–
	H916, TROM-F-11410	Norway. Nordland. Fauske. Blåmannsisen	1967	MN692335	MN692278	–	–	–
	H928, TROM-F-11403	Norway. Troms. Tromsø. Tromsø museum	1980	MN692336	MN692311	–	MN655833	–
	H1103, C-F-86999	Greenland. Std. Strømfjord, 4km E of airport	1988	MN692337	–	–	–	–
	H1115, C-F-86617	Greenland. Jameson Land. Valley W of Nathorts fjeld	1989	MN692338	–	–	MN655834	–

Table 1. (Continued).

Species	Sample and fungarium ID	Locality	Collection year	GenBank accession number				
				hsp	*rpb2*	*tef*	LSU	ITS
	H1190, C-F-45351	Norway. Nordland. Fauske. Blåmannsisen	1967	MN692343	–	–	–	–
H. macrosperma	H2146, O-F-285169*	Norway. Oppland. Lom	2007	MN692322	MN692286	MN658201	MN655844	MN656184
	H2100, TROM-F-610004*	Norway. Troms. Balsfjord	2017	MN692321	MN692285	MN658198	MN655843	MN656180
	H947, TROM-F-610056*	Norway. Nordland. Junkerdalen	2016	MN692318	MN692281	MN689295	MN655841	–
	H029, O-253328*	Norway. Oppland. Dovre. Grimsdalen	2007	KY784205	KY772449	KY772838	KY772921	MN656159
	H047, O-253329*	Norway. Oppland. Dovre. Grimsdalen	2009	KY784217	KY772462	KY772850	KY772936	–
	H1982, TROM-F-610001*	Norway. Troms. Målselv	2017	MN692319	MN692282	MN689297	MN655842	MN656176
	H050, O-253330*	Norway. Oppland. Dovre. Grimsdalen	2009	MN692316	KY772464	KY772852	KY772938	–
	H053, O-253331*	Norway. Oppland. Dovre. Grimsdalen	2009	MN692317	KY772466	MN689296	KY772939	–
	H1997, TROM-F-610017	Norway. Troms. Balsfjord. Lakselvbukt	2017	MN692320	MN692287	–	MN655840	–
H. arctoalpina	H033, O-253237*	Norway. Oppland. Dovre. Grimsdalen	2009	KY784207	KY772453	KY772841	KY772924	–
H. acetabulum	H225, O-253212*	Norway. Oppland. Dovre. Grimsdalen	1984	KY784344	KY772594	KY772894	KY773055	MN656162
H. subilica	H148, O-70080*	Norway. Akershus. Asker	1994	KY784281	KY772531	KY772880	KY772997	MN656160
H. rivularis	H1978, O-255764*	Norway. Troms. Balsfjord	2017	MN692371	MN692295	MN689306	MN655850	MN656175
H. fallax	H018, O-253351*	Norway. Oppland. Dovre. Grimsdalen	2009	KY784195	KY772439	KY772830	KY772913	–
H. pezizoides	H061, O-253366*	Sweden. Halmstad	2009	KY784225	KY772471	KY772854	KY772945	–
H. scyphoides	**H140**, **O-65348***	Norway. Hedmark. Åmot	2002	KY784273	KY772523	KY772879	KY772989	–
Helvalla sp2	H1983, O-255763*	Norway. Troms. Målselv	2017	MN692351	MN692294	MN689305	MN655849	MN656177
H. macropus	H238, O-291425*	Norway. Rogaland	2009	KY784356	KY772605	KY772896	KY773067	–
H. fibrosa	H240, O-291352*	Norway. Sør-Trøndelag	2008	KY784358	KY772607	KY772898	KY773069	–
H. lacunosa	H1041, O-255761*	Norway. Nordland. Saltdal. Junkerdalen	2016	MN692347	MN692299	MN689302	MN655855	MN656169
H. atra	H1055, O-255762*	Norway. Hedmark. Kvikne	2016	MN692348	MN692300	MN689304	MN655852	MN656170
H. philonotis	H2110, O-255760*	Norway. Oppland. Dovre. Grimsdalen	2017	MN692353	MN692301	MN689303	MN655853	MN656182
H. calycina	H022, O-253255*	Norway. Oppland. Dovre. Grimsdalen.	2009	KY784198	KY772442	KY772833	KY772915	MN656158
H. costifera	H247, O-253283*	Norway. Oppland. Vågå	1998	KY784365	KY772613	KY772900	KY773074	–
H. crispa	H235, O-360158	Norway. Nordland. Andøy	2005	KY784353	KY772602	–	KY773065	–
H. hyperborea	H1309, C-F-55004	Norway. Nordland. Rana	1981	MN692349	X	MN689301	–	–

Table 1. (Continued).

Species	Sample and fungarium ID	Locality	Collection year	GenBank accession number				
				hsp	*rpb2*	*tef*	LSU	ITS
H. hypocrateriformis	**H275, C-F-57126**	Switzerland. Graubünden	1982	KY784390	KY772638	–	–	–
H. pulla	H149, O-069282	Norway. Møre og Romsdal. Nesset	2008	KY784282	KY772532	–	KY772998	MN656161
H. bicolor	H1033, O-255759	Norway. Nordland. Saltdal. Junkerdalen	2016	MN692352	MN692298	–	MN655851	MN656168
H. capucina	H1051, O-255758	Norway. Hedmark. Kvikne. Innerdalsvatnet	2016	MN692350	MN692315	–	MN655854	–

Table 2. PCR and sequencing primers used to amplify the *Helvella corium* species aggregate and relevant outgroup taxa in the study.

Locus[1]	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
hsp	H_hspf [4]: CRGGCATCCGGGTGACGTAAT	H_hspr[4]: AGGGKGTTGTCGACTCCGAGG
rpb2	H_rpb2r2[4]: TCCACAATCTGCATCCCGATTC	H_rpb2f[4]: CCAGACATGGACAGAAGGTTGAG
tef	EF595F[3]: CGTGACTTCATCAAGAACATG	EF1160R[3]: CCGATCTTGTAGACGTCCTG
LSU	H_LSUf1[2]: AGCGGAGGAAAGAAACCAAC	H_LSUr2[2]: TCCCAACAGCTATGCTCCTAC
ITS	ITS5[5]: GGAAGTAAAAGTCGTAACAAGG	ITS4[5]: TCCTCCGCTTATTGATATGC
ITS2	ITS3[5]: GCATCGATGAAGAACGCAGC	ITS4[5]: TCCTCCGCTTATTGATATGC

[1]LSU: 28S large subunit ribosomal RNA, domains D1/D2; *rpb2*: RNA polymerase II, second largest subunit; *hsp*: heat shock protein 90; *tef*: translation elongation factor 1-alpha; ITS: The internal transcribed spacer region (ITS1, 5.8S and ITS2).

[2]From Landeros *et al*. (2015), modified in Skrede *et al*. (2017).

[3]From (Kauserud & Schumacher 2001).

[4]From Skrede *et al*. (2017).

[5]From White *et al.* (1990).

This analytical tool is based on multispecies coalescent theory (Rannala & Yang 2003, Degnan & Rosenberg 2009, Yang & Rannala 2010), and utilizes multilocus data and Bayesian inference to estimates gene trees, the species tree and species delimitations simultaneously. In this study, molecular species delimitation was performed using STACEY. All possible species combinations were assessed, treating each individual as a hypothetical species (Heled & Drummond 2010, Jones *et al.* 2015). In the resulting maximum clade credibility tree, each cluster represents a putative species under the multispecies coalescent model.

Input xml files were prepared in BEAUti v. 2.4.7 (Bouckaert *et al.* 2014) and the corresponding substitution models were set according to results from PartitionFinder. Bayesian posterior probabilities of different species scenarios were estimated using a strict clock model for all loci. The following priors were adjusted: PopPriorScale was given a lognormal distribution with M = -7 and S = 2, and the CollapseWeight a beta distribution with alpha = 1 and beta = 1. The collapse height was set to 0.00001. All other priors were accepted as the defaults in BEAUti. The analyses were run for 115 M generations and sampled at every 5 000th. The output file was inspected in Tracer v. 1.6.0 (Rambaut *et al.* 2018) to ensure convergence of the MCMC chains (ESS values > 200). Output trees were processed in TreeAnnotator v. 2.4.7 (supplied with the BEAST package), where burnin was set to 10 %, and a maximum clade credibility tree was produced. The tree was displayed with FigTree v. 1.4.3 (tree.bio.ed.ac.uk/software/figtree/). The final visualization was done using iTOL (Letunic & Bork 2016) and edited in Adobe Photoshop.

Cluster analyses were performed using SpeciesDelimitationAnalyser (Jones *et al.* 2015), where burn-in was set to 10 %, collapseheight to 0.001 and simcutoff to 0.95. Visualization was done in RStudio (RStudio Team 2016) using PlotSimMatrix [script provided in the supplementary information for DISSECT (Jones *et al.* 2015)].

In addition, maximum likelihood (ML) analyses were performed using RAxML v. 7.2.8 (Stamatakis 2006, 2014) as implemented in Geneious. For these analyses, Rapid Bootstrapping and search for best-scoring ML tree algorithms were applied. Bootstrap analyses were performed with 1 000 pseudo-replicates.

Morphological examinations

Macromorphological traits of cup and stipe as well as ecological traits were noted on site during fieldwork. Microanatomical examinations and morphological descriptions were done after molecular identification of specimens. Detailed microscopical examination of selected specimens was done on dried material (number of specimens examined in parentheses): *Helvella corium* (4), *H. alpina* (3), *H. pseudoalpina* (3), *H. alpestris* (4), *H. macrosperma* (4), *H. nannfeldtii* (4), and *H. alpicola* (3). Manually cut sections and squash preparations of rehydrated apothecia were studied and observed in distilled water and lactophenol cotton blue (LCB). For each specimen, 20 ascospores were measured, and measurements of other character states were measured in 10 examples. The terminology of microanatomical terms follows Korf (1973).

RESULTS

Sequence amplification

Out of 469 barcoded specimens, 281 were assigned to *Helvella corium s. str*., 12 specimens to *H. alpina*, nine specimens to *H. pseudoalpina sp. nov*., nine specimens to *H. alpicola*, 69 specimens to *H. nannfeldtii*, 37 specimens to *H. alpestris*, and five specimens to *H. macrosperma*. Based on barcode sequences obtained, 47 specimens were referred to a wide range of species outside the *H. corium* species aggregate (*e.g*. *H. arctoalpina, H. solitaria, H. rivularis* and *H. philonotis*).

Amplicons were not produced for all individuals originally targeted for multilocus phylogenies; from 80 initial individuals, 80 *hsp*, 71 *rpb2*, 48 *tef*, 73 LSU and 29 5.8S sequences were obtained (Table 1). The *tef* and ITS regions proved especially difficult to amplify from fungarium specimens. The protein-coding genes *MCM7* and *TSR1* were targeted in initial studies, but the available universal primers failed to amplify these regions in the *Helvella corium* species aggregate.

Phylogenetic inference and species delimitation

Sequences for four loci (*hsp*, *rpb2*, *tef* and LSU) were obtained from 47 out of 80 specimens. The multispecies coalescent analysis using STACEY was thus based on 47 accessions (32 individuals of the *Helvella corium* species aggregate plus 15 individuals representing outgroup taxa). Twenty-nine 5.8S sequences were included *a posteriori* to the data set. The run converged with ESS values > 200 for all parameters. The resulting maximum clade credibility tree (based on 21 600 sampled trees, 2 400 trees were discarded as burn-in) is shown in Fig. 1. Bayesian posterior probabilities (BBP) are shown above branches. For simplicity, maximum likelihood RAxML bootstrap values (MLB) were also

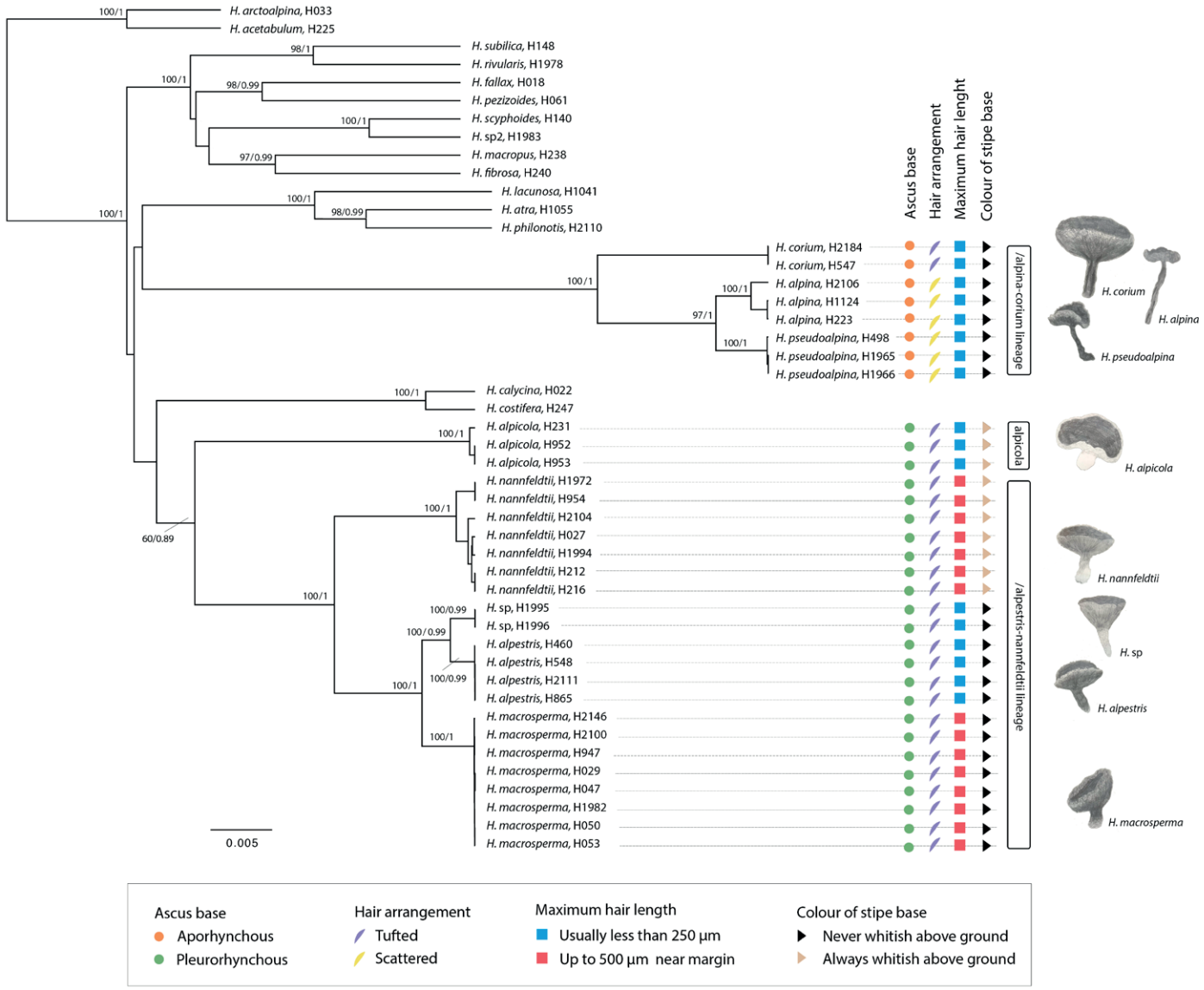

Fig. 1. Maximum clade credibility tree of the *Helvella corium* species aggregate, along with outgroup taxa, resulting from the multilocus coalescent analyses in STACEY (Beast2). The analysis is based on partial sequences of the heat shock protein 90 (*hsp*), the nuclear ribosomal large subunit (LSU, including D1/D2 domains), the second largest subunit of RNA polymerase II (*rpb2*), the translation elongation factor 1-α (*tef*) and the complete 5.8S ribosomal RNA. RAxML maximum likelihood bootstrap values (added manually) > 60 % and Bayesian posterior probability (BPP) > 0.8 are shown above nodes. Selected morphological character states are mapped on the tree, as explained in detail in the results section. The scale bar reflects the number of substitutions per site. Drawings: S.B. Løken.

added to the tree, since the topologies resulting from the two multilocus analyses were congruent.

The multispecies coalescent analysis supported seven distinct clades of the *Helvella corium* species aggregate, distributed in three evolutionary lineages: (1) the /alpina-corium lineage (1.0 BBP, 100 % MLB) with *H. corium*, *H. alpina* and *H. pseudoalpina* (2) the /alpestris-nannfeldtii lineage (1.0 BBP, 100 % MLB) with *H. alpestris, H. macrosperma and H. nannfeldtii;* and (3) *H. alpicola* (Fig. 1). *Helvella pseudoalpina* was strongly supported as sister to *H. alpina* (1.0 BPP, 97 % MLB) and *H. corium* as sister to this clade (1.0 BPP, 100 % MLB). *Helvella alpestris* and *H. macrosperma* were supported as sister species (1.0 BPP, 100 % MLB) and *H. nannfeldtii* as sister to this subclade (1.0 BPP, 100 % MLB). Two specimens (denoted "*H.* sp") were separated as a distinct clade from *H. alpestris* (0.99 BBP, 100 % MLB), yet with a short branch length. These two individuals had *rpb2* sequences similar to *H. macrosperma* and *hsp* sequences similar to *H. alpestris*. *Helvella alpicola* was recovered as sister to the /alpestris-nannfeldtii lineage with moderate support (0.89 BBP, 60 % MLB). The average distance between sister species varied from 0.005 to 0.015 substitutions per site. According to the inferred phylogeny, the *H. corium* species aggregate represents a paraphyletic group, although with low support for basal nodes (Fig. 1). A run based on the full dataset (*i.e.* 80 individuals, with missing data) produced the same topology and delimitation of species.

For the seven supported clades, Bayesian inference with STACEY and ML analyses showed a high degree of congruence. Resolution was poor outside the identified clades, and the majority of the basal nodes had very low posterior probabilities (as well as bootstrap support). The placement of the lineages within the genus *Helvella* was therefore difficult. STACEY showed topological congruence among all gene trees, but gene trees produced by the ML analysis showed incongruence regarding the basal nodes as well as the placement of the two specimens denoted *"H.* sp". In the *hsp* gene tree these specimens were nested within *H. alpestris* and in the *rpb2* gene tree within H. *macrosperma*. For the other two markers, maximum likelihood analyses placed them as sister to *H. alpestris*.

The cluster analysis performed with SpeciesDelimitation Analyser produced a similarity matrix separated into seven distinct clusters with strong support within (red colour) and low support in-between (white colour) (Fig. 2). The individuals within the clusters had zero posterior probability of belonging to a different cluster, as indicated by the white colour that separates the clusters. The in-between singletons represent *Helvella* outgroup taxa outside the species aggregate under study. Some clusters exhibited considerable intraspecific genetic structure. The specimens denoted "*H.* sp." displays some degree of separation from *H. alpestris,* yet not significant enough for STACEY to delimit them as a separate species. *Helvella nannfeldtii* exhibited extensive intraspecific variation, but the STACEY analysis still accepted all included individuals as belonging to the same species.

Performance of molecular markers

The three protein-coding loci (*hsp*, *rpb2* and *tef*) provided good phylogenetic signal and showed low levels of intraspecific genetic variation. The LSU marker also clearly separated six of the seven species, but displayed slightly more intraspecific genetic variation compared to the protein-coding loci. The ITS region was found to be highly variable, and an objective alignment of this region across the two divergent lineages of the *H. corium* aggregate was not achievable. The conserved part of this region, *i.e.* the 5.8S gene, however, provided valuable phylogenetic signal and was therefore included as a fifth locus in the phylogenetic analyses. We obtained complete sequences from the ITS region (ITS1, 5.8S and ITS2) from six of the species, while for the seventh species, *i.e. H. alpicola*, we only succeeded in obtaining ITS2 and parts of 5.8S. The pairwise similarity identity across the ITS1 and ITS2 regions of the *H. corium* species aggregate is presented in Table 3.

Little or no intraspecific variation was found in LSU and the ITS region of *H. corium, H. alpina, H. pseudoalpina, H. alpicola, H. alpestris* and *H. macrosperma*. This contrasted the high levels of intraspecific genetic variation observed in the ribosomal gene clusters (LSU and ITS) of *H. nannfeldtii*. Two distinct genetic groups of LSU (H1972 and H954 *vs*. the rest of individuals of *H. nannfeldtii* included in the phylogeny) showed a considerable number of substitutions between them. Some within-group variation was also observed. From two to 43 LSU substitutions separated the specimens of *H. nannfeldtii* included in this dataset. The two LSU groupings also largely corresponded to genetic groups observed in the ITS region of this species. For comparison, 25 LSU substitutions separated specimens of *H. corium* from specimens of *H. alpina.* Seven LSU substitutions separated specimens of *H. macrosperma* from specimens of *H. alpestris*.

Morphology and taxonomy

Due to the large degree of morphological similarity of species recognized as parts of the *H. corium* aggregate, a conclusive identification of the pertinent species in the field proved challenging. Still, after more careful examinations, we observed that morphological characters largely corresponded to the seven

Table 3. Pairwise percent identity in the ITS-region (ITS1 and ITS2) between species of the *Helvella corium* species aggregate. Individuals of *H. nannfeldtii* included in table represent one of the genetic groupings within the species. NA indicates no obtained sequence.

	H. corium	***H. alpina***	***H. pseudoalpina***	***H. alpicola***	***H. nannfeldtii***	***H. alpestris***	***H. macrosperma***
H. corium	100/100	86/81–84	85/80–82	NA/67	57–60/61	61–63/68–71	59–62/65–71
H. alpina	86/81–84	96–100/ 96–100	91–92/92–93	NA/61–64	59–60/56–60	62–63/68	60–62/65–69
H. pseudoalpina	85/80–82	91–92/92–93	100/100	NA/63	58/58–61	60/68–71	60/67–71
H. alpicola	NA/67	NA/61–64	NA/63	NA/100	NA/76	NA/83–85	NA/78–84
H. nannfeldtii	57–60/ 61	59–60/56–60	58/58–61	NA/76	100/100	81–82/83	80–81/81–85
H. alpestris	61–63/68–71	62–63/67–69	60/68–71	NA/83–85	81–82/82–84	100/100	94/97–98
H. macrosperma	59–62/65–71	60–62/65–69	60/67–71	NA/78–84	80–81/81–85	94/97–98	100/100

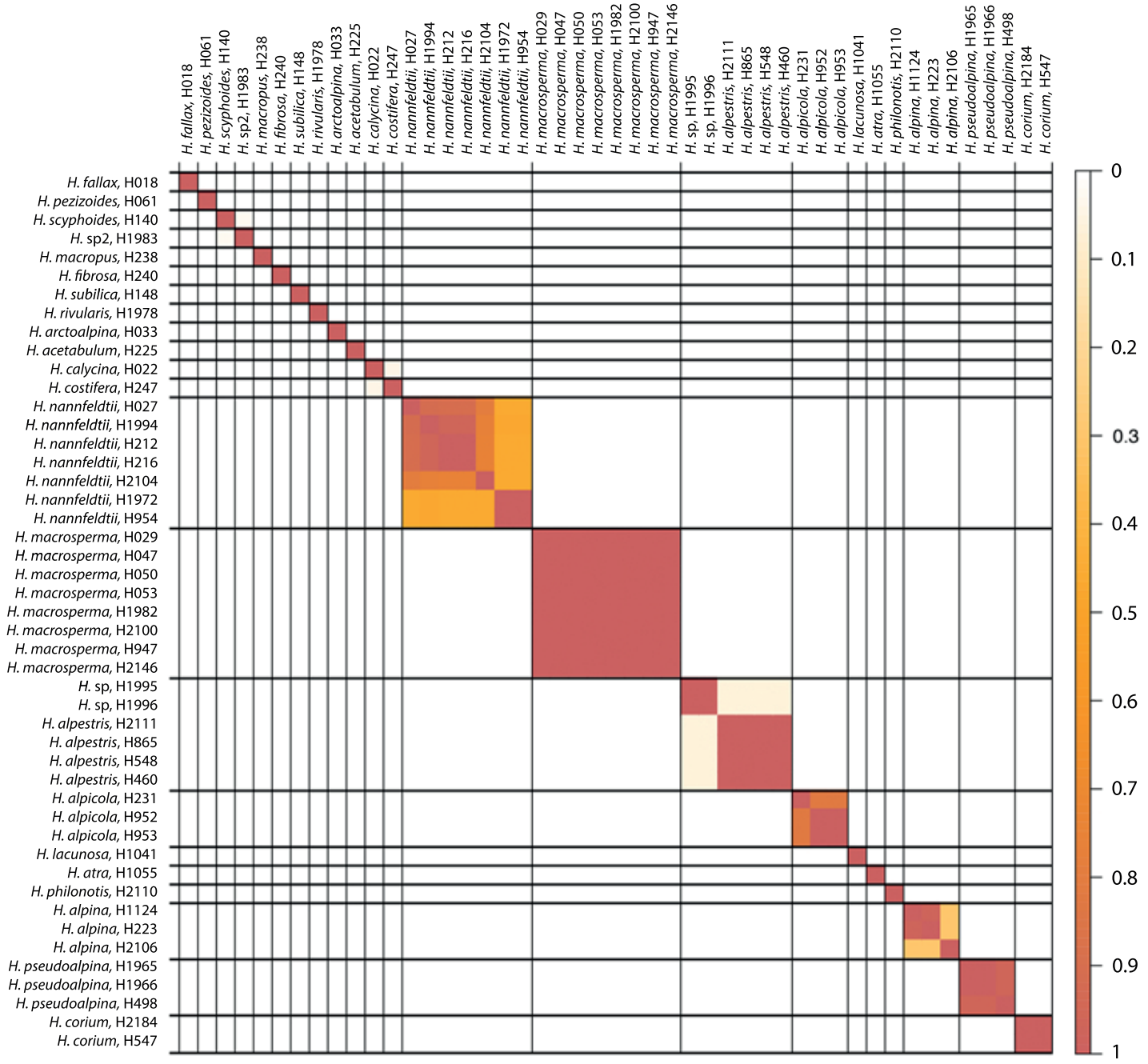

Fig. 2. Similarity matrix showing Bayesian posterior probabilities (BPP) for pairs of individuals from the *Helvella corium* species aggregate and outgroup taxa belonging to the same cluster from the STACEY analysis. Red means 1.0 BPP, white means 0.0 BBP. For this analysis, collapseheight was set to 0.001 and simcutoff to 0.95.

supported clades obtained from the STACEY analysis (Fig. 1). The seven clusters are therefore acknowledged here as "good" species. In *H. nannfeldtii*, the genetic subgrouping observed in STACEY was not observed in distribution of morphological characters. The two specimens annotated as *Helvella* sp. showed some degree of macro-morphological differentiation, but the sampled material was insufficient for recognition as a new species at this point.

A synoptic key, as well as emended taxonomic descriptions of the inferred phylogenetic species of the *Helvella corium* species aggregate, is presented below.

1. ..... *H. corium*
2. ..... *H. alpina*
3. ..... *H. pseudoalpina*
4. ..... *H. alpicola*
5. ..... *H. nannfeldtii*
6. ..... *H. alpestris*
7. ..... *H. macrosperma*

Apothecium

a. Receptacle and hymenium blackish to black all over **1, 2, 3, 5, 6, 7**
b. Receptacle brownish, hymenium blackish **4**
c. Cup up to 3.5 cm diam **1**
d. Cup always less than 3.5 cm diam **2, 3, 4, 5, 6, 7**
e. Receptacle surface little to moderately pubescent, with scattered, short-celled, hyphoid hairs **2, 3**
f. Receptacle surface moderately pubescent, with tufts of hyphoid hairs usually less than 250 µm in length **1, 4, 6**
g. Receptacle surface heavily pubescent, with tufts of hyphoid hairs reaching 250–500 µm in length towards the margin **5, 7**

Stipe

a. Slender, more than two times longer than apothecium width **2, 3**
b. Thick, equal to or less than apothecium width **1, 4, 5, 6, 7**
c. Brown-blackish above, whitish below **4, 5**
d. Black above, whitish only below ground **1, 6, 2, 3, 7**
e. Terete (cylindrical) without groove **4, 5, 6, 7**
f. +/- Grooved **1, 2, 3**

Ascus type

a. Aporhynchous **1, 2, 3**
b. Pleurorhynchous **4, 5, 6, 7**

Ascospores

a. Predominantly exceeding 20 µm in length **1, 4, 6**
b. Predominantly less than 20 µm in length **2, 3, 5, 7**
c. With one large internal guttule, sometimes so large that the spore appears empty **1, 3, 4, 5, 6, 7**
d. With one large internal guttule and several small droplets towards the poles **2**

Description of species

Helvella corium (O. Weberb.) Massee, *Brit. Fung. Fl*. **4**: 463. 1895. Fig. 3A–C, E.
Synonym: *Helvella arctica* Nannf., *Svensk Bot. Tidskr*. **31**: 60. 1937.
Detailed list of synonyms in Skrede *et al*. (2017).

Apothecium ± regularly stipitate-cupulate, black all over, cup up to 3.5 cm wide. *Stipe* uniformly black, above ground portions black, predominantly with longitudinal grooves that do not continue onto receptacle. *Receptacle* surface densely pubescent with brown-walled hyphoid hairs forming fascicles 150–360 µm long, fascicles gradually increasing in length towards margin where they form distinct triangular tufts; in specimens from arctic-alpine areas often with white crystals, individual hair cells up to 20 µm broad. *Medullary excipulum* of densely interwoven *textura intricata*, hyphae 3–7 µm broad, septate. *Outer excipulum* of *textura angularis*, innermost cells irregular in shape and size, walls pale brown, outermost cells cylindrical or club-shaped, with subhyaline or brownish walls, individual cells 10–25 × 8–30 µm, arranged in tufts of fascicled hyphae. *Asci* aporhynchous, 210–340 × 11.3–18.8 µm. *Ascospores* ellipsoid, with one large internal guttule, 16.3–22.5 × 10–13.8 µm. *Paraphyses* septate, cells with brownish walls along the whole length, gradually increasing in pigmentation towards the tips, 2.5 µm broad below, gradually enlarged to 3.8–7.0 µm at the subcapitate tips.

Included specimens for macro- and microanatomical examinations: **France**, Savoie, *T. Schumacher* [H547] (O-255757). **Sweden**, Torne Lappmark, Jukkasjärvi, E of Abiskojok, Aug. to Sep. 1928, *J.A. Nannfeldt* [H292] (C-F-92111-Fung. Exs. Suec. 369 **isotype** of *H. arctica* Nannf.). **Norway**, Troms, Salangen, 8 Aug. 2017, *S.B. Løken & T. Schumacher* [H1958] (TROM-F-610014); Finnmark, Alta, Talvik, 12 Aug. 2017, *S.B. Løken & T. Schumacher* [H1970] (TROM-F-610015); Troms, Balsfjord, Lakselvbukt, 20 Aug. 2017, *S.B. Løken & T. Schumacher* [H1998, H1999, H2101] (TROM-F-610004, TROM-F-610017, TROM-F-610040); Nordland, Saltdal, Junkerdalen, Aug. 2016, *S.B. Løken & T. Schumacher* [H950] (TROM-F-610059); Hedmark, Folldal, Einunndalen, Aug. 2016, *S.B. Løken & T. Schumacher* [H955] (O-255766); Sør-Trøndelag, Oppdal, Vinstradalen, Aug. 2016, *S.B. Løken & T. Schumacher* [H957] (O-255754). **Svalbard**, Longyeardalen, Longyearbyen, 20 Jul. 2017, *S.B. Løken & B.A. Granbo* [H2184] (O-255756).

Notes: *Helvella corium* forms a well-supported lineage with *H. alpina* and *H. pseudoalpina.* Eleven *hsp*, 10 *rpb2*, 18 *tef*, 25 LSU, zero 5.8S and 96–99 ITS substitutions separate *H. corium* and *H. alpina.* Ten *hsp*, nine *rpb2*, 16 *tef*, 26 LSU, zero 5.8S and 111–112 ITS substitutions separate *H. corium* and *H. pseudoalpina*. *Helvella corium* is by far the most common species of the *H. corium* species aggregate and is also the only species that thrives in temperate as well as in the boreal and arctic-alpine biomes. The holotype of *H. corium* [**Poland**, Georgenberg Landeck, May 1870, *Weberbauer* (WRSL)] has not been examined. Due to its old age it is presumably unsuitable for molecular identification. We base our concept of *Helvella corium* on the **epitype** designated by Skrede *et al.* (2017) [**Denmark**, Mid Zealand, Kirke Hvalsø, Brødlesgård, 2 Jul. 1984, *U. Søchting* (C-F-71638)].

Fig. 3. Photos of fresh (A–D, G) and dried (E–F) apothecia of the /alpina-corium lineage and *H. alpicola* of the *Helvella corium* species aggregate. **A.** *H. corium.* **B.** *H. corium* (H2184). **C.** *H. corium* (H1958). **D.** *H. alpina* (H336). **E.** *H. corium* (H1998). **F.** *H. pseudoalpina* (H2278). **G.** *H. alpicola* (H552). Scale bars = 1 cm. Photos: B–E: S.B. Løken; A, G: T. Schumacher.

Helvella alpina Skrede *et al*., *Persoonia* **39**: 19. 2017. Fig. 3D.

Apothecium regularly stipitate-cupulate, black all over, occasionally with white crystals at margin, cup 0.8–2.0 cm wide. *Stipe* slender, solid to hollow, above ground portions black, occasionally with a few longitudinal grooves, 0.2–0.3 cm thick, 1.0–3.5 cm long. *Medullary excipulum* of *textura intricata*, hyphae 2–5 µm wide, hyaline. *Outer excipulum* of *textura angularis*, cells 10–25 µm diam, intermixed with subhyaline to brown-walled hyphae, turning out perpendicularly to receptacle surface. *Receptacle* surface subpubescent, with scattered, brown-walled hyphoid hairs, not tufted, individual hairs 60–200 (occasionally up to 350) µm long, composed of ovoid to subglobose cells, up to 20 µm broad. *Asci* aporhynchous, 230–340 × 12.5–15 µm. *Ascospores* ellipsoid, with one large internal guttule and several smaller ones towards the poles, 16.3–21.3 × 10–13.8 µm. *Paraphyses* 2.0–2.8 µm broad below, septate, cells with brownish walls along the whole length, gradually enlarged to 4.0–6.5 µm at the subcapitate tips.

Included specimens for macro- and microanatomical examinations: **Canada**, British Columbia, Whistler National Park, 13 Aug. 1994, *T. Schumacher* [H336] (O-253227). **France**, Savoie, Plan des Evettes, 26 Aug. 1992, *T. Schumacher* [H223] (O-253226 holotype). **Russia**, Khatanga Air Port, *H.F. Gøtzsche* [H540] (C-F-34420). **Sweden**, Torne Lappmark, Jukkasjärvi, 1945, *G. Degelius* [H711] (UPS-F-145392). **Norway**, Nordland, Ballangen, Langvatn, 8 Aug. 1970, *O. Skifte* [H921] (TROM-F-41055); Oppland, Dovre, Grimsdalen, 23 Aug. 2017 [H2106] (O-255751); Troms, Tromsø, *ca*. 2 km W of Breivikeidet, 29 Aug. 1954, *F.E. Eckblad* [H2162] (O-F-174772).

Notes: *Helvella alpina* is sister species to *H. pseudoalpina* from which it diverges in one substitution in *hsp*, one substitution

in *rpb2*, six substitutions in *tef*, 10 substitutions in LSU, zero substitutions in 5.8S and 52–61 substitutions in ITS.

Helvella pseudoalpina S.B. Løken, Skrede & T. Schumach. ***sp. nov.*** MycoBank MB833241. Fig. 3F.

Etymology: From Greek "false" and Latin "occurring in mountainous regions", referring to morphological resemblance to *H. alpina*.

Typus: **Norway**, Nordland, Saltdal, Junkerdalen, Bibeldalen, 28 Aug. 1988, *L. Ryvarden* [H2278] (**holotype** TRH-F-20631).

Apothecium regularly stipitate-cupulate, black all over, cup 0.8–1.5 cm wide. *Stipe* slender, solid, above ground portions black, occasionally with a few longitudinal grooves, 0.2–0.3 cm thick, 1.0–3.5 cm long. *Receptacle* surface subpubescent, with scattered, brown-walled hyphoid hairs, 90-250 µm long. *Medullary excipulum* of *textura intricata*, hyphae septate, hyaline to subhyaline, generally 3–6 µm broad, intermixed with swollen cells up to 15 µm broad. *Outer excipulum* of *textura angularis*, cells 20–45 µm diam, brown-walled, intermixed with subhyaline to brown-walled hyphae, cells in rows turning out perpendicularly to receptacle surface, individual cells 12–30 × 7–25 µm, outermost cells subcapitate to capitate in shape., *Asci* aporhynchous, 250–330 × 12.5–17.5 µm. *Ascospores* ellipsoid, with one large internal guttule, 15-21.3 x 10-13.8 µm. *Paraphyses* 2.5 µm broad below, septate, walls light brownish along the whole length, gradually enlarged to 5.0–8.8 µm at the subcapitate tips.

Included specimens for macro- and microanatomical examinations: **Greenland**, Qeqertarsuaq (Godhavn), 11 Aug. 1977, *P.M. Petersen* [H349] (C-F-63820). **Norway**, Troms, Tromsø, Tromsdalen, 27 Aug. 1961, *O. Skifte* [H941, H942, H943] (TROM-F-11412, TROM-F-11405, TROM-F-11404); Troms, Balsfjord, Mestervik, 9 Aug. 2017, *S.B. Løken & T. Schumacher* [H1965, H1966, H1967] (TROM-F-610048, TROM-F-610049, TROM-F-610050); Nordland, Saltdal, Junkerdalen, Bibeldalen, 28 Aug. 1988, *L. Ryvarden* [H2278] (TRH-F-20631 **holotype**). **Svalbard**, Longyeardalen, Longyearbyen, Aug. 2015, *S. Svantesson* [H498] (O-255748).

Notes: *Helvella pseudoalpina* is sister species to *H. alpina* from which it diverges in one substitution in *hsp*, one substitution in *rpb2*, six substitutions in *tef*, 10 substitutions in LSU, zero substitutions in 5.8S and 52 to 61 substitutions in ITS.

Helvella alpicola Skrede *et al*., *Persoonia* **39**: 19. 2017. Fig. 3G.

Apothecium regularly stipitate-cupulate, cup 0.5–1.5 cm wide, hymenium greyish black, receptacle dark greyish. *Stipe* terete, greyish to whitish below, 0.2–0.3 cm broad, 0.5–1.8 cm high, with 2–3 shallow grooves at base. *Receptacle* surface densely pubescent, covered with multiseptate, subhyaline, hyphoid hairs forming fascicles near margin, fascicles 60–350 µm long, individual hair cells 10–40 × 7.5–25 µm, with conspicuous brown pigments at septa. *Medullary excipulum* of loose *textura intricata*, hyphae 2–5 µm broad, hyaline. *Outer excipulum* of *textura angularis*, cells 10–27 µm diam. *Asci* pleurorhynchous, 250–400 × 12.5–18 µm. *Ascospores* ellipsoid, with one large internal guttule, 18–22.5 × 11–15 µm. *Paraphyses* 2.5–3.8 µm below, walls light brownish along the whole length, septate, gradually enlarged to 5–10 µm at the clavate tips.

Included specimens for macro- and microanatomical examination: **Switzerland**, Graubünden, Inn at Resgia, 26 Aug. 1984, *H. Dissing* [H231] (O-253226). **Norway**, Nordland, Saltdal, Junkerdalsura. 27 Aug. 1988, *A.E. Torkelsen* [H175] (O-185924 **holotype**); Nordland, Saltdal, Junkerdalsura, 11 Aug. 2016, *S.B. Løken & T. Schumacher* [H552, H553, H554, H952, H953] (TROM-F-610052, TROM-F-610053, TROM-F-610054, TROM-F-610061, TROM-F-610062).

Notes: *Helvella alpicola* is nested in a divergent sister lineage to the /alpestris-nannfeldtii lineage (Fig. 1). It diverges from *H. nannfeldtii* in four substitution in *hsp*, 11 substitutions in *rpb2*, 12 substitutions in *tef* and 52 substitutions in LSU.

Helvella nannfeldtii Skrede *et al*., *Persoonia* **39**: 33. 2017. Fig. 4A–C.

Apothecium stipitate-cupulate, 1–3 cm wide, hymenium black, receptacle dark brown to black, rarely with white crystals at margin. *Stipe* short, terete, solid, greyish black above, whitish below in above ground portions, 0.2–0.3 cm wide, 0.5–2 cm long. *Receptacle* surface pubescent, covered with subhyaline to light brown-walled hyphoid hairs forming hyphal fascicles, individual hairs 190–500 µm long, irregularly shaped and often much constricted at septa, toward the margin increasing in length and forming distinct triangular tufts. *Medullary excipulum* of loosely interwoven *textura intricata*, hyphae 3–5 µm broad. *Outermost excipulum* of *textura angularis*, thick-walled, cell walls dark brown, 15–30 µm diam, intermixed with broad short-segmented brownish hyphae forming a *textura intricata*. *Asci* pleurorhynchous, 250–340 × 13.75–20 µm. *Ascospores* ellipsoid, with one large internal guttule, 15–22.5 × 10–15 µm. *Paraphyses* 2.5–3.8 µm broad below, septate, walls dark brown along upper two thirds, gradually increasing in pigmentation towards tips, gradually enlarged to 5–8.8 µm at the subcapitate tips.

Included specimens for macro- and microanatomical examinations: **France**, Savoie, Val d'Isere, Gorges du Mal, 31 Aug. 1992, *T. Schumacher* [H212] (O-253332); Savoie, Bon Valle, Sur Arc, 2 Sep. 1992, *T. Schumacher* [H216] (O-253333). **Norway**, Oppland, Dovre, Grimsdalen, Veslegrimsa, 8 Aug. 2009, *T. Carlsen, T. Schumacher & I. Skrede* [H027] (O-253338 **holotype**); Hedmark, Folldal, Einunndalen, Aug. 2016, *S.B. Løken & T. Schumacher* [H954] (O-255744); Troms, Kåfjord, Guolasjärvi, 13 Aug. 2017, *S.B. Løken & T. Schumacher* [H1971, H1972] (TROM-F-610033, TROM-F-610034); Troms, Målselv, Frøkentindelva, 16 Aug. 2017, *S.B. Løken & T. Schumacher* [H1984, H1985] (TROM-F-610031, TROM-F-610032); Troms, Målselv, Iselvdalen, 19 Aug. 2017, *S.B. Løken & T. Schumacher* [H1991, H1992, H1993, H1994] (TROM-F-610020, TROM-F-610002, TROM-F-610005, TROM-F-610036); Oppland, Dovre, Grimsdalen. 23 Aug. 2017, *S.B. Løken & T. Schumacher* [H2103, H2104] (O-255765, O-255745).

Notes: *Helvella nannfeldtii* forms a well-supported lineage with *H. alpestris* and *H. macrosperma*. Four *hsp*, two *rpb2*, seven *tef*, 47 LSU, two 5.8S and 138–139 ITS substitutions separate *H. nannfeldtii* and *H. alpestris.* Three *hsp*, one *rpb2*, five *tef*, 43 LSU, 3 5.8S and 139 ITS substitutions separate *H. nannfeldtii* and *H. macrosperma*.

Helvella alpestris Boud., *Bull. Soc. Bot. France* **41**: CCXL. 1894. Fig. 4D–F.

Apothecium stipitate-cupulate to almost plane, black all over, predominantly with white crystals at margin, cup 0.5–1.8 cm

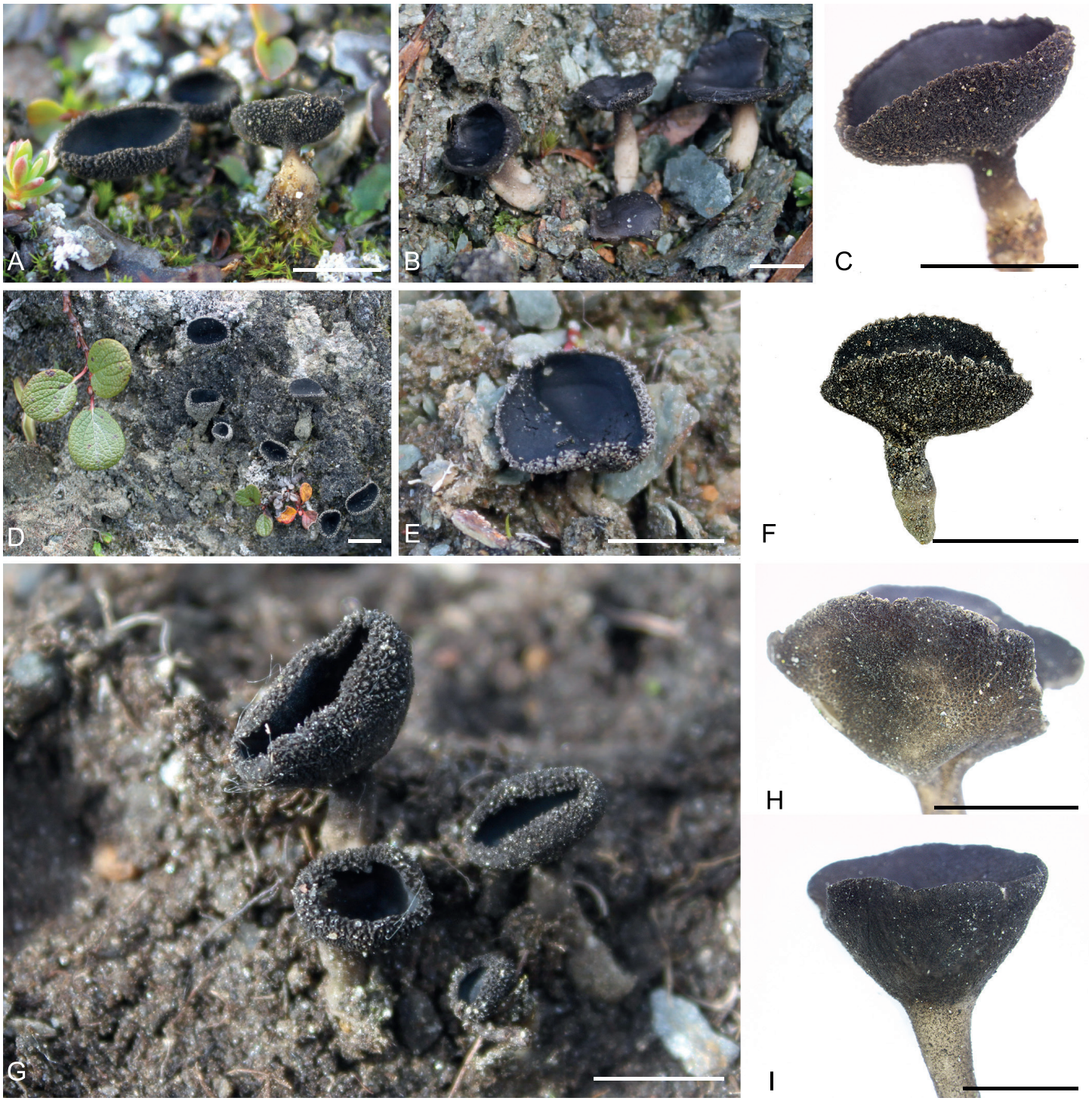

Fig. 4. Photos of fresh apothecia from the /alpestris-nannfeldtii lineage of the *Helvella corium* species aggregate. **A.** *Helvella nannfeldtii* (H1971). **B.** *H. nannfeldtii* (H2104). **C.** *H. nannfeldtii* (H1971). **D.** *H. alpestris* (H2111). **E.** *H. alpestris* (H2102). **F.** *H. alpestris* (H2111). **G.** *H. macrosperma* (H1982). **H.** *H. macrosperma* (H2100). **I.** *Helvella* sp. (H1996). Scale bars = 1 cm. Photos: S.B. Løken.

wide. *Stipe* terete, solid, above ground portions black, 0.2–0.4 cm broad, 0.4–1.5 cm high. *Medullary excipulum* of *textura intricata*, hyphae 2–5 µm wide. *Outer excipulum* of brown-walled globose to angular cells, 10–20 µm diam. *Receptacle* surface densely pubescent, covered with dark brown-walled hyphoid hairs forming fascicles, hairs 50–380 µm long, gradually increasing in length towards the margin where they form distinct triangular tufts, individual hair cells 10–40 × 7.5–17.5 µm. *Asci* pleurorhynchous, 230–350 × 12–17.5 µm. *Ascospores* ellipsoid, with one large internal guttule, 16–22.5 × 10–13.8 µm. *Paraphyses* septate, 2.5–3.75 µm broad below, walls brownish along the whole length, gradually increasing in pigmentation towards apex, enlarged to 4.5–8.8 µm at the gnarled, clavate tips.

Included specimens for macro- and microanatomical examinations: **Canada**, Nunavut, Kitikmeot Region, above Bloody Falls, 19 Jul. 2014, *J.M. Saarela, P.C. Sokoloff & R.D. Bull* [H460] (O ex DAOM-574891). **France**, Savoie, Val'dIsere, *T. Schumacher* [H548] (O-255736). **Norway**, Troms, Tromsø, Tromsø museum, 10 Jul. 1980, *S. Sivertsen & O. Skifte* [H928] (TROM-F-11403); Oppland, Dovre, Grimsdalen, Jegerhøi, 23 Aug. 2017, *S.B. Løken & T. Schumacher* [H2111] (O-255737); Finnmark, Porsanger, Lakselv, 16 Jul. 1961, *F.E. Eckblad* [H2154] (O-F-174783).

Notes: *Helvella alpestris* is sister species to *H. macrosperma* from which it diverges in one substitution in *hsp*, one substitution in *rpb2*, four substitutions in *tef*, seven substitutions in LSU, one substitution in 5.8S and 42–43 substitutions in ITS. Specimens are found on bare silt or slate grounds, usually in close proximity to *Salix reticulata*. One of the genotypes for 5.8S and ITS2 (*i.e.* S1) from root tips of *S. reticulata*, obtained in the work by Weidemann (1998), proved identical to the 5.8S/ITS2 genotypes of *H. alpestris* provided in the present study.

Helvella macrosperma (J. Favre) R. Fellner & Landa, *Česka Mykol*. **45**: 35. 1991. Fig. 4G–H.

Apothecium regularly stipitate-cupulate, black all over, rarely with white crystals at the margin, 0.5–2 cm wide. *Stipe* short, terete, solid, above ground portions black, 0.4–1.2 cm high, 0.2–0.4 cm broad. *Receptacle* and stipe surface densely pubescent, covered with dark, brown-walled hyphoid hairs, forming fascicles, hairs 180–500 µm long, gradually increasing in length towards margin where they form distinct triangular tufts; individual hair cells 10–40 × 10–20 µm, constricted at septa and with conspicuous incrusted pigments on the interior of the cell walls. *Medullary excipulum* of loosely interwoven *textura intricata*, hyphae 3–5 µm broad, short-celled; outer excipulum of brown-walled globose to angular cells, 10–20 µm diam, turning out perpendicularly to the receptacle surface. *Asci* pleurorhynchous, 240–330 × 10–20 µm. *Ascospores* broadly ellipsoid, with one large internal guttule, 17.5–22.5 × 11.3–13.8 µm. *Paraphyses* septate, 2.5–3.75 µm broad below, walls brownish along the whole length, gradually increasing in pigmentation towards tip, enlarged to 5–7.5 µm at the gnarled, clavate tips.

Included specimens for macro- and microanatomical examinations: **Norway**, Nordland, Saltdal, Junkerdalen, Aug. 2016, *S.B. Løken & T. Schumacher* [H947] (TROM-F-610056); Troms, Målselv, Håkåfjellet, 16 Aug. 2017, *S.B. Løken & T. Schumacher* [H1982] (TROM-F-610001); Troms, Balsfjord, Lakselvbukt, 20 Aug. 2017, *S.B. Løken & T. Schumacher* [H1997, H2100] (TROM-F-610017, TROM-F-610004); Oppland, Lom, Høyrokampen at Bøvertun, 31 Aug. 2007, *A.K. Wollan* [H2146] (O-285169); Oppland, Dovre, Grimsdalen, Veslegrimsa, 2007, *Master Field Course* [H029] (O-253328).

Notes: *Helvella macrosperma* is sister species to *H. alpestris* from which it diverges in one substitution in *hsp*, one substitution in *rpb2*, four substitutions in *tef*, seven substitutions in LSU, one substitution in 5.8S and 42–43 substitutions in ITS.

DISCUSSION

Phylogenies based on a few, phylogenetically highly informative loci are still preferred when delimiting species and inferring relationships among species of fungi. Indeed, the larger amount of sequence data produced by next generation sequencing techniques is not necessarily associated with better phylogenetic signal and resolution (Lemmon & Lemmon 2013). Multilocus phylogenies of phylogenomic data often include extensive non-phylogenetic signal and may produce conflicting, yet highly supported, phylogenetic trees (Philippe *et al.* 2011). Thus, the choice and number of genetic markers necessary to infer reliable evolutionary histories of species is still an important matter of debate in phylogenetics (Rokas *et al.* 2003, Aguileta *et al.* 2008, Balasundaram *et al.* 2015, Stielow *et al.* 2015), and should be evaluated individually for each group under study. The present study confirmed the high informativeness of selected single-copy protein-coding loci, in combination with ribosomal markers, in delimiting species. Altogether, five markers (*hsp, rpb2*, *tef*, LSU and 5.8S) were sufficient to get a stable support for species recognition as well as shallow clades in our phylogeny. Not unexpectedly, this selection of markers was not sufficient to infer a stable topology of the deeper branches of the tree (Fig. 1). The inclusion of highly conserved loci, e.g. SSU, *mcm7* and/or *tsr1*, may have improved the support for basal nodes. We initially attempted to include *mcm7* and *tsr1*, which had been shown to reveal higher-level relationships in *Helvella* and other ascomycete genera (Raja *et al.* 2011, Zhao *et al.* 2015, Mark 2016), however, PCR-amplification using general primers for these regions failed in our study. It was concluded that the design of new, specific *Helvella* primers for these loci are needed if these markers are to be used in future phylogenetic studies of this genus. The ITS region also proved difficult to amplify with universal ITS primers, as also demonstrated in previous studies (Landvik *et al.* 1999, Skrede *et al.* 2017). Nevertheless, we succeeded in obtaining complete ITS sequences for six out of seven species of the *H. corium* species aggregate. In general, the ITS region displayed extremely high levels of interspecific variability across the *Helvella* species under study, making it impossible to construct objective alignments across the /alpestris-nannfeldtii and /alpina-corium lineages jointly. ITS is thus considered unsuitable as a phylogenetic marker across these lineages, as also demonstrated for the genetically divergent *Helvella* genus as a whole (Landvik *et al.* 1999, Skrede *et al.* 2017).

Nevertheless, the ribosomal loci, *i.e.* LSU and ITS, successfully delimited six out of seven species in the *Helvella corium* species aggregate. The seventh species, *i.e. H. nannfeldtii*, showed remarkably high levels of intraspecific divergence in these ribosomal regions. The high level of intraspecific variability in *H. nannfeldtii* observed in Fig. 2 can be explained by its variability in the LSU region, as no other locus displayed comparable levels of intraspecific divergence. Conflicting gene trees, as observed in *H. nannfeldtii,* may result from intraspecific recombination, which implies that the two genetic clusters observed are not reproductively isolated (Taylor *et al.* 2000). Based on the observed inconsistency, these two divergent clusters are not considered to represent two cryptic species. This is also demonstrated by the output from the STACEY analysis (Fig. 2), where all individuals of *H. nannfeldtii* were regarded as belonging to the same species.

The LSU and ITS nrDNA regions are present in numerous copies in fungal genomes. These copies form gene families that are expected to be subject to concerted evolution (Arnheim *et al.* 1980, Strausbaugh 2001), where homogenization of paralogous genes is achieved through homologous recombination. However, exceptions do occur, resulting in heterogeneous copies within a genome and apparent heterozygosity (Selosse *et al.* 2016). Substantial intragenomic heterozygosity of ribosomal DNA is thought to be infrequent (Thiéry *et al.* 2016), but is still observed in the fungal kingdom (Stensrud *et al.* 2007, Nilsson *et al.* 2008, Harder *et al.* 2013, Lindner *et al.* 2013). Thus, if the primers used in this study amplified paralogs of LSU and ITS in *H. nannfeldtii,* we might have been comparing variable non-homologous sequences.

As the same level of intraspecific variation is not observed in any other species of the *H. corium* species aggregate, we conclude that the ribosomal DNA of *H. nannfeldtii* is evolving in a different manner and at a different rate compared to even its closest relatives.

The STACEY analysis inferred seven well-supported clusters in the present species aggregate (Figs 1, 2). Though, when a limited number of individuals and loci are used, STACEY may over-estimate the number of clusters (Toprak *et al.* 2016, Sukumaran & Knowles 2017). However, as morphological examinations supported the seven clusters, we feel confident they represent well-defined species. The STACEY analysis is based on the multispecies coalescent model and assumes random mating and that no gene flow occurs after speciation (Leaché *et al.* 2014, Xu & Yang 2016). While restricted gene flow between sister species will not severely effect species tree topology (Heled & Drummond 2010, Toprak *et al.* 2016), gene flow between non-sisters will lead to incongruence between gene trees and species trees (Taylor *et al.* 2000, Leaché & Fujita 2010). Speciation is seldom instantaneous in nature, thus violations to the implied evolutionary model might have occurred in our dataset. In this study, the Bayesian inference shows no topological incongruence across loci or between gene trees (except for within *H. nannfeldtii*) and species trees, but the ML analyses raises uncertainty regarding the placement of the possibly new but unnamed species of *Helvella* sp. It is not fully understood how well STACEY handles the inclusion of hybrid individuals (Wagner *et al.* 2017). Still, if *Helvella* sp. represents hybrid individuals, *i.e.* as a result of hybridisation between the sister species *H. alpestris* and *H. macrosperma*, it will not have severe effect on the overall tree topology.

In this study, using a molecular genealogical approach as basis for classification, allowed us to get a better understanding of the taxonomical value of morphological characters in the *Helvella corium* species aggregate. Although previous authors have recognized the morphological variation in *H. corium s. lat.* (Boudier 1907, Nannfeldt 1937, Favre 1955, Dissing 1964, 1966a, b), an accurate delimitation of species within this aggregate has not existed before molecular data were introduced (Skrede *et al.* 2017). In Dissing's (1966) comprehensive review of *Helvella* in Europe, the new section Macropodes was proposed to include all *Helvella* species with cupulate apothecia and a pubescent to villose receptacle surface, a solid stipe with or without furrows and ribs, and ribs not extending from the stipe onto the receptacle. The section Macropodes then included *Helvella corium*, *H. macropus, H. villosa* (= *H. fibrosa*) *H. cupuliformis* (= *H. hypocrateriformis*) and *H. queletii* (= *H. solitaria*). As already shown by Skrede *et al.* (2017), and further supported by this study, these macro-morphological characters are useless in recognizing an infrageneric phylogenetic classification. Indeed, the combination of stipitate-cupulate and pubescent *Helvella* species is found scattered across many lineages in the genus.

Our study of the /alpina-corium and /alpestris-nannfeldtii evolutionary lineages of *Helvella* further elaborates on the morphological differentiation between species. We identified several informative characters that are useful in discriminating among lineages and species. The ascus development, whether aporhynchous or pleurorhynchous (Chadefaud 1943, Berthet 1964), is considered valuable for defining lineages and sections of *Helvella* (Weber 1972, Landeros *et al.* 2015), and even genera of the family *Helvellaceae* (Hansen *et al.* 2019). Aporhynchous asci seem important to delineate the /alpina-corium lineage (Häffner 1987), while pleurorhynchous asci characterise the /alpestris-nannfeldtii lineage (Van Vooren 2014, 2015, Skrede *et al.* 2017). Indeed, aporhynchous asci represent a synapomorphy for the /alpina-corium lineage (Fig. 1). Skrede *et al.* (2017) also acknowledged the diagnostic value of arrangement of hyphoid hairs on the receptacle surface, also confirmed in this study: hairs are scattered in *H. alpina* and *H. pseudoalpina*, and primarily tufted (in fascicles) in *H. corium, H. nannfeldtii, H. alpestris, H macrosperma* and *H. alpicola.* Additional taxonomically informative morphological characters in this species aggregate include: (1) colour of stipe base (white in above ground portions in *H. nannfeldtii* and *H. alpicola*); (2) hair length (hairs approaching 500 µm near the margin in *H. nannfeldtii* and *H. macrosperma*); (3) ascospore size (predominantly more than 20 µm in length in *H. corium, H. alpicola* and *H. alpestris* vs. less than 20 µm in *H. alpina, H. pseudoalpina, H. nannfeldtii* and *H. macrosperma*); (4) ascospore morphology (one large guttule in *H. corium, H. pseudoalpina, H. alpicola, H. alpestris, H. macrosperma, and H. nannfeldtii* vs. one large guttule and several smaller ones at the poles in *H. alpina*); and (5) shape of paraphysis tips (clavate in the /alpestris-nannfeldtii lineage *vs*. subcapitate in the /alpina-corium lineage).

In describing *Helvella arctica,* Nannfeldt (1937) paid a great deal of attention to the external, white crystals observed on the marginal receptacle hairs in this species. This trait played a major role in distinguishing it against *H. corium.* Later, Dissing (1966) concluded that this trait represented a mere adaptation to alpine environments, and synonymized *H. arctica* with *H. corium.* This latter disposition also had molecular support in the work by Skrede *et al.* (2017), who corroborated the synonymy of the two taxa. In fact, in the present study it was found that white crystalline deposits on receptacle hairs are frequently observed under alpine conditions of all species of the *Helvella corium* species aggregate, however, being most prominent in *H. alpestris* and *H. corium*.

Evidently, considerable genetic differentiation has occurred independently of morphological differentiation among species that belong to the *Helvella corium* species aggregate, resulting in the species sharing many morphological traits. This phenotypical similarity may possibly represent morphological stasis. It has been argued, generally speaking, that morphological stasis may result from strong stabilizing selection for adaptation to harsh environments (Nevo 2001, Lumbsch & Leavitt 2011). In fact, many cryptic and pseudo-cryptic plant species are found in harsh alpine and Arctic regions (Grundt *et al.* 2006, Skrede *et al.* 2008, Brochmann & Brysting 2010), and morphological similarity in non-sister species may be due to strong adaptive value of certain morphological traits (Bickford *et al.* 2007). This may also apply to the genetic divergent, but pseudo-cryptic nature of the *H. corium* species aggregate. In addition, it should be noted that the species under study show a high degree of niche conservatism in favouring calcareous soils and growth in close proximity to *Dryas octopetala* and/or *Salix* spp. These morphologically similar, yet genetically divergent species appear in sympatry in alpine regions of the northern hemisphere. Thus, we hope that the species delimitations made in this study will provide a valuable basis for future investigations of the specific ecological roles of such species in the natural systems they may occupy.

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Table S1. Specimens of the *Helvella corium* species aggregate in this study. Specimen ID as well as geographical origin (with coordinate data) is shown for each specimen. "NA" means non annotated data. Type specimens are written in bold.