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Solving the ecological puzzle of mycorrhizal associations using data from annotated collections and environmental samples – an example of saddle fungi

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Summary

The relation between ecological and genetic divergence of *Helvella* species (saddle fungi) has been perplexing. While a few species have been clearly demonstrated to be ectomycorrhizal fungi, ecological roles of many other species have been controversial, alternately considered as either saprotrophic or mycorrhizal. We applied SATé to build an inclusive deoxyribonucleic acid sequence alignment for the internal transcribed spacers (ITS) of annotated *Helvella* species and related environmental sequences. Phylogenetic informativeness of ITS and its regions were assessed using PhyDesign. Mycorrhizal lineages present a diversity of ecology, host type and geographic distribution. In two *Helvella* clades, no *Helvella* ITS sequences were recovered from root tips. Inclusion of environmental sequences in the ITS phylogeny from these sequences has the potential to link these data and reveal *Helvella* ecology. This study can serve as a model for revealing the diversity of relationships between unculturable fungi and their potential plant hosts. How non-mycorrhizal life styles within *Helvella* evolved will require expanded metagenomic investigation of soil and other environmental samples along with study of *Helvella* genomes.

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Introduction

Ascomycetes account for about half of all fungal species, including between 1.5 to 7.1 million species according to a number of studies (Bass and Richards, 2011). Within the Ascomycetes, the Pezizomycotina contains several families that produce large ascocarps, including the well-known morels and truffles. Some of these fungi are found to be associated with particular plants, and mushroom collectors often rely on these associations for successful foraging. While fungi–plant associations are considered to be widely distributed and enhance functioning of the plant–soil interface, little is known for most of these potential mycorrhizal ascomycetes (Tedersoo *et al.*, 2009a, 2014). One morphologically diverse group of Ascomycetes featuring identifiable fruiting bodies is *Helvella*, the saddle fungi (Helvellaceae). Species of *Helvella* are characterized by auriculoid, cupulate or irregularly lobed apothecia that are generally distinguished by colours ranging from white to black and by presence of ribs on the stipe (Abbott and Currah, 1997). Among the 52 species that are recognized within the genus *Helvella* (Kirk *et al.*, 2008), some common *Helvella* species names likely refer to multiple cryptic species associated with divergent biogeographic lineages. For instance, recently a Western North American clade of *Helvella* was identified by analysis of molecular sequence data, and a new species was identified within the *Helvella lacunosa* complex based on extensive sampling of morphological and biogeographic representatives from North America and Europe (Nguyen *et al.*, 2013). While *Helvella* species are regarded as common macrofungi in most temperate forests, and have been widely reported from Europe, North America, Asia and Australia, very little is known about the trophic status of these fungi (Abbott and Currah, 1997).

One challenge to inferring phylogenies that would accurately differentiate subgenera within *Helvella* is that the ribosomal deoxyribonucleic acid (rDNA) internal transcribed spacer (ITS), including ITS1, 5.8S rDNA and ITS2

sequences, exhibits a high variation in rates across sites. The consequent uncertainties regarding the phylogeny of *Helvella* obstruct attempts to understand the potentially dynamic evolution of *Helvella* life history. *Helvella elastica, H. macropus* and *H. atra* have been reported as saprotrophic species (Anderson and Ickis, 1921; Revett, 2002; Munguia *et al*., 2003; Iršėnaitė and Kutorga, 2006). Other studies have indicated that several *Helvella* species have ectomycorrhizal associations (Maia *et al*., 1996; Weidemann, 1998; Rinaldi *et al*., 2008; Tedersoo and Smith, 2013). Indeed, diagnostic isotopic approaches supported ectomycorrhizal life history of *Helvella*, albeit with a low statistical confidence (Hobbie *et al*., 2001). These approaches suggested that *Helvella* and the closely related genera *Barssia, Dingleya* and *Labryinthomyces* were mycorrhizal, yet the five isolates of *Helvella* exhibited diverse levels of enrichment or depletion of $\delta^{13}C$ and $\delta^{15}N$, for which the uncertainties were too large for conclusive interpretation (Hobbie *et al*., 2001). An alternative approach to investigating the life history of *Helvella* is to perform analysis of DNA markers for unculturable microbes in environmental samples alongside sequences from annotated specimens, potentially providing insight into the systematic placement of environmental DNAs, as well as facilitating the proper identification of environmental ITS sequences (Brock *et al*., 2009). In a study of potential pezizalean ectomycorrhiza, a phylogeny based on the rDNA large subunit revealed four unknown ectomycorrhizal sequences nested firmly within *Helvella* (Tedersoo *et al*., 2006), supplying the first direct evidence from root tips that implies an ectomycorrhizal lifestyle for several *Helvella* species.

Despite the challenges that ensue when using ITS for phylogenetic inference, the ITS region has advantages that encourage further studies: practically, it is easy to amplify, and theoretically, it combines resolution at multiple scales: the rapidly evolving ITS1, the conserved 5.8S and the moderately rapid ITS2 regions (Toju *et al*., 2012; Nilsson *et al*., 2014). Phylogenetic informativeness can be quantitatively estimated and has been profiled for many genes used in fungal phylogenetic research (Schoch *et al*., 2012; Townsend *et al*., 2012). While the usefulness of the diverse ITS regions has been qualitatively evaluated many times for resolving phylogenies at diverse levels (Hillis and Dixon, 1991; Gardes and Bruns, 1993; Scorzetti *et al*., 2002; Nilsson *et al*., 2006; 2008; Porter and Golding, 2011), it has never been quantitatively evaluated (Hershkovitz and Lewis, 1996; Wang *et al*., 2011; Nilsson *et al*., 2014). Comparison of sequences of genetic markers from root tip mycelia to annotated species has been demonstrated to be an efficient approach for identifying mycorrhizal associations in some fungal species (Nilsson *et al*., 2006; Uehling *et al*., 2012). Including ITS sequences from environmental soil and root tip samples was found to be very helpful for revealing the diversity, distribution and ecology of another terrestrial ascomycetes group, Geoglossaceae, for which the recently developed alignment program SATé (Liu *et al*., 2009) was used to produce alignments of highly variable ITS sequences from distantly related species (Wang *et al*., 2011).

In this study, we resolved the phylogeny of the *Helvella* using ITS sequence data, characterizing the phylogenetic informativeness of the ITS1, ITS2 and 5.8S rDNA regions over time. Using SATé, we aligned unidentified *Helvella* ITS sequences from recent studies assessing fungal diversity in soil and plant root samples, sequences of annotated herbarium collections and sequences of undescribed species of *Helvella* from China, then inferred the phylogenetic relationships between these diverse accessions. Applying the resulting phylogeny, our aims are: (i) to assess the diversity of mycorrhizal relationships between *Helvella* species and plants, (ii) to investigate possible saprotrophic lineages within *Helvella* and (iii) to examine the role that ecology might play in the evolution of *Helvella*.

Materials and methods

Data mining

ITS sequences of Helvellaceae, including the 5.8S rRNA, were downloaded from the International Nucleotide Sequence Database (INSD: GenBank, the European Molecular Biology Laboratory (EMBL) and DataBank of Japan (DDBJ); Fig. 1; Table S1 and S2). *Gyromitra* and *Morchella* were also included as outgroups (O'Donnell *et al*., 1997). We sequenced 30 ITS sequences (Table S1) extracted from Chinese and European collections using primer pair ITS1F (Gardes and Bruns, 1993) and ITS4 (White *et al*., 1990). All annotated ITS sequence data from *Helvella* were used to conduct BLAST searches (Altschul *et al*., 1997) against environmental sequences in the INSD, and additional searches for sequences similar to *Helvella* sequences were also performed with the genus search function of *emerencia* (Ryberg *et al*., 2009). Pairwise alignments of the ITS were generated and compared for sequence similarity using the function NEEDLEALL in the EMBOSS suite (Rice *et al*., 2000), with a gap penalty of 10.0, and an extend penalty of 0.5.

Phylogenetic inference

Nucleotide sequences were aligned with SATé (Liu *et al*., 2009). SATé alignments were performed in six runs of 50 iterations under default settings. The best-scored alignment was manually adjusted with MacClade 4.0 (Maddison and Maddison, 2000). Phylogenies were inferred by analysis by MrBayes 3.1 (Huelsenbeck and

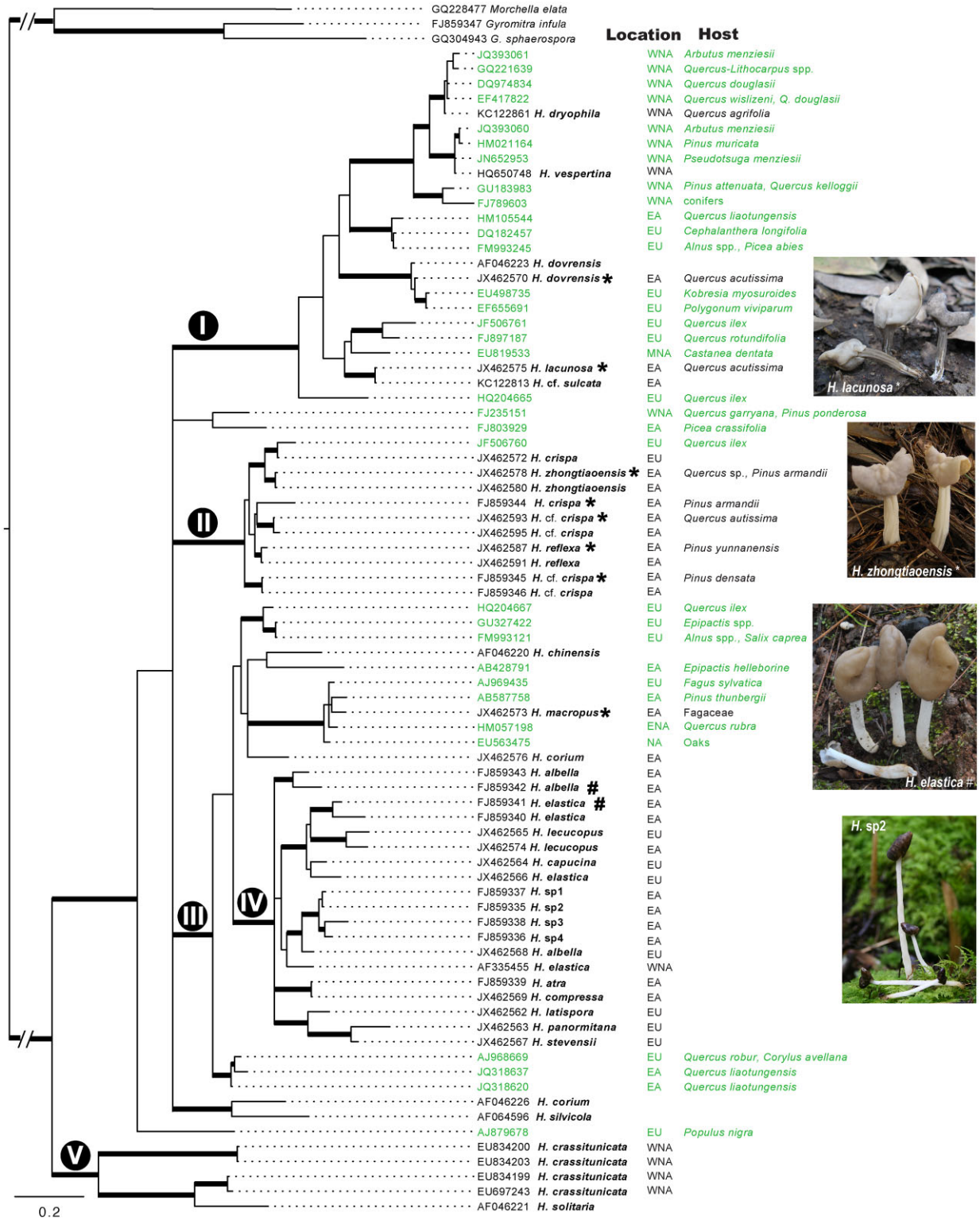

Fig. 1. Internal transcribed spacers phylogeny of *Helvella* and related environmental samples of uncultured ectomycorrhiza (highlighted in green). Bayesian posterior probabilities (BPP) > 95% are indicated as bold internodes. Annotated collections with a mycorrhizal association identified in root samples collected from the same location are marked with *. Similar ITS sequences were not recovered from environmental samples collected from the same location for the two *Helvella* species marked with a pound (#) symbol. Abbreviations for locations: East Asia (EA), Europe (EU), Eastern North America (ENA), Middle North America (MNA), Western North America (WNA) and South America (SA). Insert: habitat picture of *H. lacunosa* (HKAS75442) from Clade I, *H. zhongtiaoensis* (HKAS74335) from Clade II, *H. elastica* (HKAS55000) and an unidentified *Helvella* species (HKAS55224) from Clade IV.

Ronquist, 2001) via Metropolis-coupled Markov chain Monte Carlo specifying a GTR + Γ + I model. Four chains of 2 000 000 generations were computed. Trees were sampled every 100th generation. After 100 000 generations, likelihood approached a stable value. Trees obtained prior to convergence were discarded, and those obtained following convergence were used to generate a 50% majority-rule consensus tree.

Phylogenetic informativeness profiling

Phylogenetic informativeness (PI) of ITS1, ITS2 and 5.8S rDNA for resolving *Helvella* phylogeny was estimated using PHYDESIGN (Townsend, 2007; Lopez-Giraldez and Townsend, 2011; Townsend *et al*., 2012). The input tree was the consensus Bayesian tree generated in the above Bayesian analysis. It was scaled relative to the most basal divergence (set to age 1.0) rather than being scaled in a known unit of time. Heavily gapped regions and indels were removed from the best-scored SATÉ alignment, then the alignment was provided as input to perform rate estimation at every site by HYPHY (Pond *et al*., 2005), as implemented in PHYDESIGN (Lopez-Giraldez and Townsend, 2011) with the time-reversible model.

Helvella mycorrhizae morphology and verification

Potential mycorrhizae formed between *Helvella* species and root tips were investigated at the location where ascocarps were collected. A core of soil (about $5 \times 5 \times 5$ cm^3) beneath the ascocarps was screened for root tips, and up to 20 root tips were sampled at each location. Excavated roots were placed in polypropylene bags, transported back to the laboratory within 1.5 h, stored at 4 C, then processed within 2 days. Root samples were examined microscopically, surface-sterilized with 99% ethanol and 3.125% NaOCl, then washed with sterile water prior to DNA extraction. Fungal specific ITS sequences were amplified using primers ITS1F and ITS4. Cloning of polymerase chain reaction products was accomplished with pGEM-T Easy Vector system II kits and JM109 competent cells from Promega (Madison, Wisconsin). Selected colonies were sequenced on an ABI3730 DNA analyser and ABI BIGDYE 3.1 terminator cycle sequencing kit (Shanghai, China). Deoxyribonucleic acid samples of *Helvella* ascocarps were used as positive controls for root samples collected from same location: 10 to 15 colonies were randomly selected for each root tip sample. For root samples that showed no *Helvella*-affiliated ITS sequences, an additional 15 colonies were sequenced. Identical ITS sequences derived from root samples and from annotated *Helvella* specimens were deemed indicative of a specific mycorrhizal association (Fig. S1; Table S3).

Results and discussion

Phylogeny of Helvella and affiliated environmental sequences

The final dataset contained 81 ITS sequences, including 33 obtained from root tips in 24 published studies of environmental fungal diversity (Fig. 1; Table S2). The alignment included 1643 aligned positions (TreeBase http://purl.org/phylo/treebase/phylows/study/TB2:S15730). Analysis of pairwise similarity among all *Helvella* ITS sequences by NEEDLEALL (Rice *et al*., 2000) revealed an average score of 45.53 (45.5% pairwise similarity), with a maximum score of 97.9 within unidentified *Helvella* species (FJ859335 and FJ859337) and a minimum score of 8.9 between *H. lacunosa* (AJ544211) and a short environmental sequence (EF655691). The ITS phylogeny based on the best-scored SATÉ alignment was well resolved for most *Helvella* species (Fig. 1). Several major clades received strong support [Bayesian posterior probabilities (BPP) > 95%]. Clade I included *H. dryophila, H. vespertina, H. dovrensis*, a subclade of *H. lacunosa* and *H*. cf. *sulcata* collected from China, as well as many uncultured ectomycorrhizal species. Several subclades were also strongly supported (BPP > 95%) within Clade I, including a subclade composed of *H. dovrensis* and two environmental ITS sequences isolated from ectomycorrhizal roots of mountain-dwelling herbaceous plants, and a subclade of nine environmental ITS sequences from roots of oaks, conifers, bearberry and madrone from western North America, plus *H. dryophila* and *H. vespertina*. Clade II included *H. crispa, H. reflexa* and four Chinese collections morphologically similar to European *H. crispa*, and a single sequence isolated from oak roots in France. Clade III comprised *H. chinensis*, and *H. macropus* collected from China, three well-supported groups of root samples and Clade IV, and *H. macropus* was positioned in one of the root samples groups. Clade IV contained only herbarium collections of diverse species, including four collections of *H. elastica* from Italy, Canada (AF335455) and China. The basal Clade V contained only annotated collections *H. crassitunica* and *H. solitaria*; however, *H. crassitunica* was non-monophyletic. A recent study of *Helvella* intensively sampled in western North America with a focus on species morphologically similar to *H. lacunosa* (Nguyen *et al*., 2013), and the ITS phylogeny presented here share the same topology, suggesting that a rich diversity of root associations has evolved in Clade I. In addition to the well-resolved phylogeny for known *Helvella* species, our results strongly support the placement of species represented by environmental ITS in Clades I, II and III with *H. dovrensis*, *H. dryophila*, *H. vespertina, H. lacunosa*, *H. chinensis* and *H. macropus*. Interestingly,

the uncultured species in the environmental samples in these clades were all associated with plant root tips (Fig. 1; Table S2).

Phylogenetic informativeness of ITS regions across the evolutionary history of Helvella

After removal of heavily gapped regions, the PhyDesign input alignment included 221 base pairs (bp) of ITS1, 152 bp of 5.8S rDNA and 257 bp of ITS2. Both ITS1 and ITS2 exhibited peaks of informativeness at recent times (Fig. 2). While informativeness of the ITS1 region dropped abruptly in the epoch of rapid radiation observed for *Helvella* species in Clades I, II and III, predicted probability of resolution of these nodes based on rates of evolution of sites used remained highest for ITS1 despite its potential to be subject to noise. Internal transcribed spacers 2 was consistently informative for inferring relationships above the species level. Conservation of 5.8S rDNA, with just 18 informative sites out of just 40 variable sites, compromised its utility for recent inference, but likely increased resolution for deeper nodes. Furthermore, the lack of noise in 5.8S presented little to no potential for misleading inference. Analysis of the whole SATé alignment, which included 72% gapped ITS1 and 57% gapped ITS2, also demonstrated high informativeness of ITS1 and ITS2 for recent events even after scaling down the informativeness inferred by the percentage of gapped columns (data not shown). Because the phylogenetic informativeness of ITS1 reaches peak informativeness in relatively recent history and drops precipitously at deeper divergence times, its utility is likely to be compromised for deeper inferences in this time scale by phylogenetic noise (molecular convergence or parallelism). Despite the dynamic differences in recent phylogenetic informativeness of ITS1 and ITS2, both regions were anticipated to provide very good to strong resolving power for internodes of interest across the *Helvella* phylogeny, with ITS1 featuring a 1–5% higher probability of correct signal for more the recent internodes of Clades II and IV (Fig. 2). Presenting less noise and nearly equivalent signal at deeper nodes, ITS2 almost surpassed ITS1 in probability of providing resolution for the deepest node analysed (node V). ITS1 or ITS2 alone were each predicted by this means to provide sufficient resolution for these five nodes. Accordingly, the concatenated sequence of ITS1, ITS2 and the 5.8S rDNA region was predicted to and provided sufficient power in our Bayesian analysis to resolve branches I to V with high posterior probability (> 99%). While the inferred probability of correct signal estimated for genes or different regions is very useful in relative assessment – choosing proper gene markers for certain epochs, it nonetheless only serves as an indirect predictor of level of support for certain branches or nodes.

Environmental investigation of Helvella-affiliated genes for selected species

From root samples collected at the location where ascocarps of *Helvella* species were observed, we verified mycorrhizae using ITS sequences for eight species morphologically identified as *H. dovrensis*, *H. lacunosa*, *H. zhongtiaoensis*, *H. reflexa*, *H.* cf. *crispa* and *H. macropus*. *Helvella* ITS sequences were dominant in these samples and were perfect matches to ITS sequences from ascocarps recovered at the same location for all eight samples, which were distributed throughout Clade I, II and III in the ITS phylogeny, adjoining environmental sequences of *Helvella* reported from plant roots (Fig. 1). Morphologically, these mycorrhizae possessed pale-coloured mantle with extended extra-radical hyphae in collections of *Helvella reflexa – Pinus yunnanensis*, and *Helvella dovrensis – Quercus acutissima* (Fig. S1). Multiple fungal ITS sequences were also amplified using fungal specific ITS primers (ITS1 and ITS4) from root tips sampled from locations where two common *Helvella* species, *H. albella* and *H. elastica*, in clade IV were collected. However, no *Helvella* ITS sequences identified in ascocarps were successfully recovered from these root tips.

Diversity of Helvella mycorrhizal associations

Host types and geographic distribution provide further support for the inferred subgenus groupings within the ectomycorrhizal clade I, which was examined with much more inclusive sampling in Nguyen and colleagues (2013). As in the phylogeny based on collections, root symbionts of *Helvella* collected from the US west coast grouped in several sub-clades in clade I. The host plants are mostly oaks or members of the Pinaceae (Nguyen *et al*., 2013). Samples from Europe, Asia and the US west coast shared clades (Fig. 1), with host type consisting mainly of deciduous trees in Fagales and Salicaceae, except three sequences isolated from conifers on the west coast of the United States (Smith *et al*., 2007; Morris *et al*., 2008a; Mühlmann *et al*., 2008; Palmer *et al*., 2008; Tedersoo *et al*., 2009b; Branco and Ree, 2010; Gladish *et al*., 2010; Wolfe *et al*., 2010; Richard *et al*., 2011; Southworth *et al*., 2011; Kranabetter *et al*., 2012; Kennedy *et al*., 2012; Peay *et al*., 2011; Wang *et al*., 2012). In the Northern Hemisphere, temperate forests typically are abundantly populated with trees classified in the Fagaceae and Pinaceae. Species in these plant families exhibit associations with diverse phylogenetic clades of ectomycorrhizal fungi, including root-endophytic ascomycetes (Toju *et al*., 2013a,b; Yamamoto *et al*., 2014). GenBank EU498735 was obtained in the Austrian Alps from the sedge *Kobresia myosuroides*, an

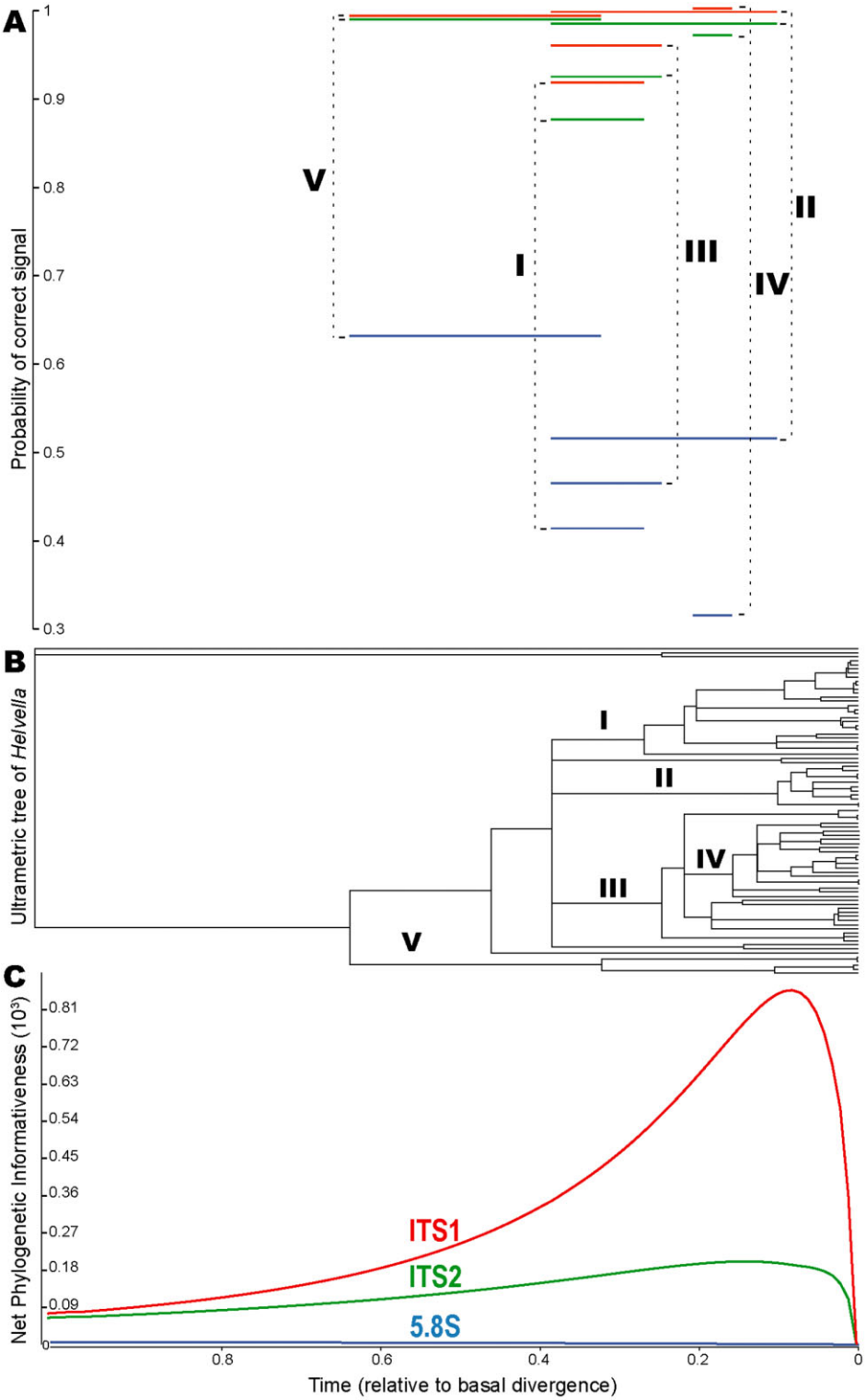

Fig. 2. Usefulness of ITS sequences in resolving *Helvella* phylogeny. A. For each branch I–V in the diagrammatic phylogeny below, three horizontal bars indicate probability of correct signal (scale at left) for different ITS regions, ITS1 (red), ITS2 (green) and 5.8S rDNA (blue). B. Ultrametric phylogeny of *Helvella* with the major clades I–V labeled above branches. C. Phylogenetic informativeness profiles for ITS1 (red), ITS2 (green) and 5.8S rDNA (blue). Areas of the informativeness curve where informativeness declines with time can contribute significant noise to inferred phylogenies. The x-axis is in arbitrary units of time scaled relative to the basal divergence, which was assigned to be 1.0 in the past. The y-axis is calculated net phylogenetic informativeness ($\times 10^3$).

herbaceous plant with a circumboreal distribution primarily inhabiting arctic and alpine environments (Mühlmann and Peintner, 2008). Similarly, EF655691 was collected from the herbaceous *Polygonum viviparum* from arctic-alpine habitats. Other ectomycorrhizal species identified from this plant all belong to the basidiomycetes (Mühlmann and Peintner, 2008; Mühlmann *et al*., 2008). The ITS phylogeny suggested that *H. dovrensis* shares a clade with the two *Helvella* sequences associated with alpine herbaceous plants. Before we observed *H. dovrensis* associated with *Quercus acutissima* at high altitudes (> 2000 m) in Southwest China, *H. dovrensis* had only been found in eroded *Dryas* (Rosaceae) vegetation in alpine regions in Europe, including the Norwegian mountains and the Alps (Schumacher, 1992). Diverse host types, including conifers, *Castanopsis*, *Fagus*, *Populus* plants and several species of *Quercus*, are also observed in other mycorrhizal lineages in *Helvella* in Clade II and III (Murat *et al*., 2005; Kjøller, 2006; Tedersoo *et al*., 2006; 2009b; Morris *et al*., 2008b; Karpati, 2010; Obase *et al*., 2011; Richard *et al*., 2011; Zhang *et al*., 2013). Although only one environmental ITS sequence was positioned in clade II, field investigations confirmed associations with roots of *Quercus*, *Pinus* or *Castanopsis* species, for five *Helvella* isolates in clade II (Fig. 1; Table S3). It is interesting to see *Helvella* sequences DQ182457, GU327422 and AB428791 found in root samples of land orchids *Epipactis* or *Cephalanthera* species (Ogrua-Tsujita and Yukawa, 2008; Malinova *et al*., 2012). Apparently, these associations evolved independently in three clades. All orchids are mycoheterotrophic at some point in their life cycle, and orchid seed obtains its carbon from fungal symbionts, which typically are basidomyceteous fungi (McCormick *et al*., 2012). While 'atypical' plant–fungal associations, such as ectomycorrhizal fungi on possibly arbuscular mycorrhizal plants, are actually not rare in forest ecosystems (Toju *et al*., 2014), interconnections between ectomycorrhiza and orchid mycorrhiza have been rarely observed (van der Heijden *et al*., 2015). Orchid plants independently evolved mycorrhizal associations by recruiting new endophytic lineages, many of which also have a saprotrophic, free-living stage (Selosse *et al*., 2009; van der Heijden *et al*., 2015).

Non-ectomycorrhizal lineages within Helvella

For many species within clade IV, including *H. elastica* and *H. atra*, which are commonly found in temperate forests (Anderson and Ickis, 1921; Revett, 2002; Munguia *et al*., 2003; Iršėnaitė and Kutorga, 2006), we found no evidence supporting a mycorrhizal association between these fungi and plants. Another common saddle fungus widely reported in Europe, North America and Asia, *H. lacunosa* is described from Sweden and taxonomically problematic (Nguyen *et al*., 2013) and its ecological status is ambiguous. Researchers used different approaches to assess ecology of *H. lacunosa*, and conclusions of research studies have been conflicting on the saprobic and ectomyccorhizal lifestyles of this broadly defined morphological species (Anderson and Ickis, 1921; Iršėnaitė and Kutorga, 2006; Barroetaveña *et al*., 2007; Smith *et al*., 2007). Proper inference of ecology for these species requires a more inclusive sampling.

Sampling sequence markers from environmental samples should provide more accurate and direct evidence of ecological roles for fungi. However, assessing a saprotrophic lifestyle in many *Helvella* species will require expanded metagenomic investigation on plant materials and soil samples and study genomes of these fungi. The lack of sequences from root tips appearing in clade IV and V might suggest that no mycorrhizal association is formed by *Helvella* species in these two clades. If so, it is conceivable that the presence of these *Helvella* species has not yet been sufficiently sampled from the soil or other substrates they inhabit. On the other hand, recent environmental studies have intensively sampled soil and other environmental materials from regions where *Helvella* species have been found (e.g. O'Brien *et al*., 2005; Buée *et al*., 2009; Jumpponen and Jones, 2009). Thus, an alternative explanation is that species in this clade might have evolved associations with plants other than those intensively sampled in clade I; for example, with *Dryas* and *Salix* species, as demonstrated in Weidemann (1998). Although *Dryas* and *Salix* species are common elements in temperate forests and alpine regions, root symbionts of these plants, which are of low perceived economic or ecological significance, have been poorly studied. By observation of changes in ectomycorrhizal community structure over 2 or more months, *Helvella* species have been identified as late colonizers (Kjøller, 2006), which suggests that time of sampling can be critical in detecting *Helvella* associations with plant roots. Examining root samples from *Helvella* locations in this study provided direct evidence that *Helvella* species in clades I, II and III form mycorrhizal associations with various plants. However, we did not observe mycorrhizal roots from *Helvella* species in clades IV and V.

Conclusion

Increased understanding of taxonomy and additional environmental sampling are critical for a better characterization of the overall diversity and specific ecologies of fungi. Our study has demonstrated the usefulness of aligning sequences from root tips alongside annotated specimens of *Helvella* for the assessment of associations between plants and this group of fungi. Consistent with previous

studies, our observations suggest that mycorrhizal lineages are distributed across the *Helvella* phylogeny. In two *Helvella* clades, no *Helvella* ITS sequences were recovered from root tips. Molecular data from more exhaustively sampled environmental elements are critical to understand ecological roles of *Helvella* species in these two clades. Our integrative phylogenetic analysis demonstrates the utility of applying environmental sequence data to reveal the ecology of unculturable fungi and the diversity of relationships between fungi and their potential plant hosts.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1. *Helvella mycorrhizae* identified from root samples. (A) *Helvella reflexa* from *Pinus yunnanensis*, (B) *H. crispa* from *P. armandii*. (C) *Helvella* cf. *crispa* from *P. densata*, (D) *H. zhongtiaoensis* from *Quercus acutissima*, (E) *H.* cf. *lacunosa* from *Q. acutissima*, (F) *H.* cf. *crispa* from *Q. acutissima*, (G) *H. zhongtiaoensis* from *Q. franchetii*, (H) *H. dovrensis* from *Q. acutissima* and (I) *H. macropu*s from *Castanopsis delavayi*.

Table S1. Herbarium collections studied from China and Europe.

Table S2. Additional ITS sequences investigated in this study.

Table S3. Environmental investigation observed identical ITS sequences between plant root tips and *Helvella* collections from the same locations.