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## Conservation of biotrophy in Hygrophoraceae inferred from combined stable isotope and phylogenetic analyses

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**Abstract:** The nutritional modes of genera in Hygrophoraceae (Basidiomycota: Agaricales), apart from the ectomycorrhizal *Hygrophorus* and lichen-forming taxa, are uncertain. New  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values were obtained from 15 taxa under Hygrophoraceae collected in central Massachusetts and combined with isotopic datasets from five prior studies including a further 12 species using a data standardization method to allow cross-site comparison. Based on these data, we inferred the probable nutritional modes for species of *Hygrophorus*, *Hygrocybe*, *Humidicutis*, *Cuphophyllus* and *Gliophorus*. A phylogeny of Hygrophoraceae was constructed by maximum likelihood analysis of nuclear ribosomal 28S and 5.8S sequences and standardized  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values were used for parsimony optimization on this phylogeny. Our results supported a mode of biotrophy in *Hygrocybe*, *Humidicutis*, *Cuphophyllus* and *Gliophorus* quantitatively unlike that in more than 450 other fungal taxa sampled in the present and prior studies. Parsimony optimization of stable isotope data suggests moderate conservation of nutritional strategies in Hygrophoraceae and a single switch to a predominantly ectomycorrhizal life strategy in the lineage leading to *Hygrophorus*. We conclude that Hygrophoraceae of previously unknown nutritional status are unlikely to be saprotrophs and are probably in symbiosis with bryophytes or other understory plants.

**Key words:** *Cuphophyllus*, ecology, *Gliophorus*, *Humidicutis*, *Hygrocybe*, *Hygrophorus*, nutritional status

### INTRODUCTION

Hygrophoraceae is a widely distributed and conspicuous group in Agaricales. The monophyly of the family has yet to be established (Moncalvo et al. 2002), but it includes several genera of mushroom-forming species, such as *Hygrophorus* Fr., *Hygrocybe* (Fr.) P. Kumm., *Gliophorus* Herink, *Cuphophyllus* (Donk) Bon (approx. syn. *Camarophyllus* [Fr.] P. Kumm.) and *Humidicutis* Singer. Lichenizing forms, such as *Lichenomphalia* (Moncalvo et al. 2002), a symbiont with green algae, also are present in the group. Recent molecular evidence also supports the inclusion in Hygrophoraceae of *Dictyonema* C. Agardh ex Kunth, a lichenizing partner of cyanobacteria. Lichenizing basidiomycetes are few, and finding a concentration of those engaged in this form of symbiosis within a single family (Lawrey et al. 2009) raises questions about the nutritional status of the non-lichenizing Hygrophoraceae to which they are related. For the most part we lack evidence supporting the classification of genera in Hygrophoraceae as either ectomycorrhizal (ECM) or saprotrophic. Conflicting taxonomic schemes (Singer 1957, Hessler and Smith 1963, Arnolds 1986, Bougher and Young 1997) also have made it difficult to address the issue of nutritional status.

The sole non-lichenizing genus in Hygrophoraceae for which the nutritional strategy has been demonstrated is *Hygrophorus* (Fr.), several species of which have been shown to be ECM by direct examination of the morphology of mycorrhizal root tips (Agerer 2006) as well as molecular analysis of ectomycorrhizae (Peter et al. 2001, Douglas et al. 2005). Tedersoo et al. (2010) consider *Hygrophorus* a likely but not proven ECM genus, and so we have included it as a taxon of unknown status in our analyses rather than including it among known ECM taxa. The status of other genera within Hygrophoraceae is uncertain. *Cuphophyllus* is considered possibly ECM by Bougher (1994) and Agerer (2006), but no experimental evidence or direct observations support this status. The case for *Humidicutis*, species of which were formerly in *Hygrophorus*, is similar. Bougher (1994) suggested that *Humidicutis* might be ECM, but only the correlation with the presence of some trees provides evidence (Nantel and Neumann 1992, Bougher 1994). The taxonomy of *Humidicutis* remains problematic; the type species is *H. marginata* (Peck) Singer, but the closely related *Hygrophorus auratoce-*

*phalus* (Ellis) Murrill has not yet been transferred into *Humidicutis*. *Hygrocybe* has been considered to be probably saprotrophic (Griffith et al. 2002), but Bougher (1994) includes the genus among possibly ectomycorrhizal fungi along with Australian species of *Gliophorus*. Species of *Gliophorus*, such as *G. laetus*, were treated until recently as a section in *Hygrocybe*. Recent phylogenetic analysis has suggested that *Gliophorus* is more closely related to *Humidicutis* (Matheny et al. 2006). Ecological study of vascular plant and *Hygrocybe* spp. richness has found little correlation between the two (Öster 2008), but field observations from Arnolds (1981) and Griffith et al. (2002) as well as our own observations suggest that *Hygrocybe* as well as *Gliophorus*, *Humidicutis* and *Camarophyllus* most often occurs with bryophytes. Bryophytes are adapted for maximizing their uptake of nitrogenous nutrients and are thought to interact with cyanobacteria and algae (Turetsky 2003).

Stable isotope analysis has proven to be a useful method to elucidate the ecological roles of basidiocarp-producing fungi as either saprotrophs or partners in ECM symbiosis with vascular plants (Griffith 2004). Due to isotopic fractionation during the exchange of carbohydrates and nutrients, ECM fungi are consistently depleted in the proportion of  $^{13}\text{C}$  (Högberg et al. 1999) and enriched in  $^{15}\text{N}$  relative to the saprotrophic taxa with which they occur in the same patch of habitat (Gebauer and Taylor 1999, Hobbie et al. 1999). With few exceptions, such as species that may engage in proteolysis or use refractory nitrogen compounds from mineralized soil horizons (Gebauer and Taylor 1999), stable isotope analysis is helpful in clarifying the status of fungi of uncertain nutritional status. The ascomycete *Leotia lubrica* is an excellent example of this; its ecological role has been supported as mycorrhizal by examining its isotopic signature (Zeller et al. 2007). However the proportions of  $^{13}\text{C}$  and  $^{15}\text{N}$  in fungi are influenced by a number of site-specific environmental factors (Taylor et al. 1997, Gebauer and Taylor 1999, Henn and Chapela 2001, Griffith et al. 2002, Griffith 2004, Trudell et al. 2004, Hart et al. 2006, Zeller et al. 2007, Mayor et al. 2008), which include soil chemistry, humidity and available sunlight. Sampling from a single locality is necessary to avoid error resulting from variation in environmental factors across sites, and a means of standardizing data is required to compare isotopic data among studies (Mayor et al. 2008).

A number of studies have measured the stable isotope ratios across Basidiomycota to investigate their roles in the flow of nutrients in various habitats (Taylor et al. 1997; Hobbie et al. 1999, 2001; Högberg et al. 1999; Gebauer and Taylor 1999; Trudell et al.

2004; Hart et al. 2006; Zeller et al. 2007; Mayor et al. 2008). Few studies have focused on comparative analyses of multiple species within clades to determine nutritional mode diversity therein. One such investigation found a high degree of nutritional strategy homogeneity among genera *Gliophorus*, *Hygrocybe*, *Cuphophyllus* and *Camarophyllus* (Hygrophoraceae) in low-nutrient grassland in Wales (Griffith et al. 2002). In that study investigators demonstrated that the species sampled had unusual  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values, markedly different from saprotrophs sampled from the same habitat but similar to clavarioid fungi. While broader studies have incidentally sampled several *Hygrophorus* spp. (TABLE I), Griffith et al. (2002) has been the only one to focus on other genera under Hygrophoraceae.

Attempts to culture Hygrophoraceae axenically have been unsuccessful (Griffith et al. 2002), and it has been suggested that this resistance to culturing plus extreme stable isotope values and frequent association with bryophytes could indicate an unusual nutritional strategy (Tedersoo et al. 2010). In the present study we investigated whether stable isotope analysis can clarify the nutritional strategies of taxa within Hygrophoraceae and whether these strategies are conserved within and among these taxa. We generated new stable isotope data from collections of diverse Hygrophoraceae fruiting bodies in a single locality to avoid confounding environmental factors. We then collected data from prior investigations that recorded  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values from at least one Hygrophoraceae species along with values both for characterized ECM and saprotrophic fungi. We standardized data from all studies with a transformation scheme to isolate differences due to fungal biology from those due to environmental variations. This approach let us compare isotopic profiles regardless of the geographical origin of the exemplars from which they were generated. Finally, we performed phylogenetic analysis of diverse Hygrophoraceae and estimated the distribution of the standardized isotope characters with parsimony optimization. This approach let us predict the distribution of probable nutritional modes across Hygrophoraceae.

#### MATERIALS AND METHODS

*Specimen collection and preservation.*—Fruiting bodies were collected in the Tom Swamp section of Harvard Forest (Petersham, Massachusetts) Apr–Oct 2009. Specimens were identified morphologically to genus (Barron 1999, Hesler and Smith 1963, Phillips 1991, Robert et al. 2005, Wilson 2006) and preserved by drying with air circulation at 32 C or lower. Specimens collected for this study have been deposited in the Clark University herbarium (<http://www.clarku.edu/faculty/dhibbett/clarkfungaldb/>).

TABLE I. Hygrophoraceae exemplars from all sites with the GenBank accession numbers for the ITS sequences used for phylogenetic analysis

Taxon	GenBank accession number (ITS)	Site, study	$\sigma$ s from site $\delta^{13}\text{C}$		$\sigma$ s from site $\delta^{15}\text{N}$	
			SAP	ECM	SAP	ECM
<i>Camarophyllus borealis</i> <sup>a</sup>	HM020684	West Brookfield, MA, present	NA	NA	NA	NA
<i>Cuphophyllus lacmus</i>	HM020690	Tom Swamp, present	-3.26	-4.54	7.07	2.40
<i>Cuphophyllus</i> sp.	HM020683	Tom Swamp, present	-3.22	-4.45	9.65	3.99
<i>Gliophorus laetus</i>	HM020692	Tom Swamp, present	-2.60	-3.04	3.68	0.32
<i>Gliophorus</i> sp.	HM020676	Tom Swamp, present	-2.44	-2.67	5.36	1.35
<i>Hygrophorus auratocephalus</i> <sup>b</sup>	GU256223 et al.	Tom Swamp, present	-2.32	-2.40	6.16	1.85
<i>Hygrocybe</i> "toe-head"	None	Guyana, Mayor et al. 2008	-2.90	-2.79	2.92	0.95
<i>Hygrocybe</i> cf. <i>cantharellus</i>	HM020689	Tom Swamp, present	-3.16	-4.32	4.45	0.79
<i>Hygrocybe miniata forma longipes</i>	HM020677	Tom Swamp, present	-2.66	-3.18	4.27	0.68
<i>Hygrocybe punicea</i> <sup>a</sup>	HM020682	Tom Swamp, present	NA	NA	NA	NA
<i>Hygrocybe</i> sp.	HM020686	Tom Swamp, present	-2.74	-3.37	6.36	1.97
<i>Hygrocybe</i> sp.	HM020687	Tom Swamp, present	-3.91	-6.02	4.84	1.03
<i>Hygrocybe</i> sp.	HM020688	Tom Swamp, present	-3.52	-5.13	4.29	0.70
<i>Hygrophorus agathosmus</i>	AY586660	Stadsskogen, Taylor et al. 2003	-3.95	-1.95	1.46	0.16
<i>Hygrophorus bakerensis</i>	AF042623	Deer Park, Trudell et al. 2004	-1.55	-0.12	2.83	-0.65
<i>Hygrophorus camarophyllus</i>	None	Stadsskogen, Taylor et al. 2003	-1.19	0.90	0.46	-0.82
<i>Hygrophorus camarophyllus</i>	None	Deer Park, Trudell et al. 2004	-1.55	-0.12	3.19	-0.47
<i>Hygrophorus camarophyllus</i>	None	Snowbowl, Hart et al. 2006	-1.33	0.47	-1.00	-1.57
<i>Hygrophorus chrysodon</i>	AY586661, DQ071733	Deer Park, Trudell et al. 2004	-1.69	-0.37	1.38	-1.38
<i>Hygrophorus eburneus</i>	AF430279	Deer Park, Trudell et al. 2004	-1.69	-0.37	2.59	-0.77
<i>Hygrophorus flavodiscus</i>	GU289651	Tom Swamp, present	-0.72	1.23	3.95	0.49
<i>Hygrophorus fuliginosus</i>	HM020693	Tom Swamp, present	-1.82	-1.26	5.64	1.53
<i>Hygrophorus lindtneri</i>	None	Breuil, Zeller et al. 2007	-6.56	-2.53	-2.34	-2.17
<i>Hygrophorus olivaceoalbus</i>	AY586662	Stadsskogen, Taylor et al. 2003	-2.30	-0.24	-0.98	-2.25
<i>Hygrophorus olivaceoalbus</i>	AY586662	Hoh, Trudell et al. 2004	-1.63	0.45	3.45	-0.53
<i>Hygrophorus purpurascens</i>	None	Deer Park, Trudell et al. 2004	-1.12	0.64	5.29	0.59

Specimens without accession numbers were not used in phylogenetic reconstruction.

<sup>a</sup>Not be used in plots of isotopic values (no exemplar of the taxon was collected from Site 4 or Site 5 at Tom Swamp) but were included in phylogenetic analysis.

<sup>b</sup>Isotopic values are a mean from multiple specimens collected at the same site.

All Hygrophoraceae specimens used for isotopic analysis were collected from two boggy sites designated Site 4 (42°30.791'N, 72°12.783'W, 242 m) and Site 5 (42°30.778'N, 72°12.837'W, 256 m). Both sites contained profuse bryophytes. Trees at Site 5 are predominantly *Tsuga canadensis* and *Pinus strobus* mixed with a few hardwoods, such as *Acer* spp. and *Fagus* spp. Site 4 is an open area covered mainly with *Sphagnum* and containing *Larix laricina*, *Acer rubrum* and *Vaccinium* spp. A slow-moving brook drains from Site 5 into Site 4. Sporocarps from Hygrophoraceae taxa also were collected from other local sites both within and outside Tom Swamp for use in molecular analysis (TABLE I).

*Stable isotope analysis.*—Fungal samples were analyzed for  $\delta^{13}\text{C}$ ,  $\delta^{15}\text{N}$ , % C, and % N by continuous flow with a Costech ECS4010 elemental analyzer (Costech Analytical Technologies Inc, Valencia, California) coupled with a DELTA<sub>plus</sub> XP isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany) at the University of New Hampshire Stable

Isotope Laboratory. All carbon and nitrogen isotope data are reported in  $\delta$  notation according to this equation:  $\delta\text{X} = [(\text{R}_{\text{sample}}/\text{R}_{\text{standard}}) - 1] * 1000$  where X is  $^{13}\text{C}$  or  $^{15}\text{N}$  and R is the ratio  $^{13}\text{C}/^{12}\text{C}$  or  $^{15}\text{N}/^{14}\text{N}$ . All  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values were normalized on VPDB ( $\delta^{13}\text{C}$ ) and AIR ( $\delta^{15}\text{N}$ ) reference scales with the following internationally calibrated standards and values: IAEA CH6 (-10.45‰), CH7 (-32.15‰), N1 (0.4‰) and N2 (20.3‰). Laboratory working standards included NIST 1515 (apple leaves), NIST 1575a (pine needles) and tuna muscle, as well as *Boletus* quality control.

*Molecular analysis.*—DNA was isolated from dried fruiting bodies. For smaller specimens approximately 5 mg were used with the EZNA Forensic DNA Kit (Omega Bio-Tek, Norcross, Georgia). For larger specimens approximately 20 mg were ground in liquid nitrogen and extracted in 600  $\mu\text{L}$  3% SDS buffer (0.15M NaCl, 50 mM Tris, 50 mM EDTA) at 65 C for 1 h, purified with phenol chloroform and chloroform and precipitated with 10  $\mu\text{L}$  3M sodium acetate in isopropyl alcohol. The resulting pellets were washed with

TABLE II. Mean and standard deviation values for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  for saprotrophic and ECM exemplars from each site

Site	$\delta^{13}\text{C}$ , SAP		$\delta^{15}\text{N}$ , SAP		$\delta^{13}\text{C}$ , ECM		$\delta^{15}\text{N}$ , ECM	
	(‰)	$\sigma$	(‰)	$\sigma$	(‰)	$\sigma$	(‰)	$\sigma$
Breuil-Chenue, France	-22.53	0.93	-3.25	1.43	-26.24	0.93	3.13	4.48
Deer Park Rd, Washington, USA	-23.33	1.40	-1.20	1.66	-25.41	0.79	5.65	3.31
Hoh River, Washington, USA	-22.90	1.23	-2.38	1.47	-25.23	0.73	4.70	3.78
Snowbowl, Arizona, USA	-22.03	1.18	1.94	2.35	-24.10	1.07	5.23	3.58
Stadsskogen, Sweden	-23.08	1.27	1.55	3.33	-25.71	1.23	5.87	3.37
Tom Swamp, Massachusetts, USA	-23.91	1.44	-2.34	2.01	-25.73	0.63	4.00	3.27
Upper Potaro River, Guyana	-24.87	1.32	1.59	2.20	-25.95	0.98	5.64	2.48

70% ethanol, dried and resuspended in 100  $\mu\text{L}$  sterile  $\text{dH}_2\text{O}$ . DNA was further diluted up to 500-fold for use as a PCR template.

Two sequences were amplified from the nuc rDNA of each specimen. For the sequence containing ITS1, ITS2 and the 5.8S ribosomal subunit, primers ITS-1F and ITS4 (White et al. 1990) were employed. For the 28S ribosomal subunit primers LR0R and LR7 were used (<http://www.biology.duke.edu/fungi/mycolab/primers.htm>). PCR for both regions proceeded by initial denaturing at 95 C for 2 min and 35 cycles of denaturing at 94 C for 45 s, annealing at 50 C for 1:10 and extension at 72 C for 2 min. Each PCR reaction mixture contained 14.5  $\mu\text{L}$  sterile  $\text{dH}_2\text{O}$ , 2.5  $\mu\text{L}$  10 $\times$  PCR buffer, 0.5  $\mu\text{L}$  dNTP, 1.25  $\mu\text{L}$  of each primer in the pair and 0.25  $\mu\text{L}$  Paq5000 DNA polymerase (Agilent Technologies, Santa Clara, California). Five microliters purified DNA was added to each reaction mixture.

PCR products were checked by electrophoresis on a 1% agarose gel, viewed with ethidium bromide in UV light, purified by ethanol precipitation and sequenced with either the two ITS primers or the nested primers LR0R, LR3R, LR5 and LR7 for the 28S subunit using the ABI Prism BigDye-terminator cycle sequencing kit (Applied Biosystems, Foster City, California). Sequencing reaction products were purified by ethanol precipitation, resuspended in 15  $\mu\text{L}$  formamide and analyzed with an ABI 3130 Genetic Analyzer. The resulting sequences were edited with Sequencher 3.1 (GeneCodes Corp., Ann Arbor, Michigan).

*Phylogenetic analysis.*—Existing ITS and 28S sequences were located in GenBank with a combination of BLAST queries with newly generated sequences as queries and text-based searches for Hygrophoraceae.

New and existing 28S sequences were aligned initially on the MAFFT Web server 6 (<http://mafft.cbrc.jp/alignment/server/index.html>, Katoh et al. 2002) with the E-INS-I algorithm. Further refinement was performed with Mesquite 2.72 (Maddison and Maddison 2009). The same procedure was followed for aligning the 5.8S sequences from the ITS regions of the 50 taxa. Sequences from the two regions were concatenated for maximum likelihood analysis on the RAxML BlackBox server (<http://phylobench.vital-it.ch/raxml-bb/index.php>, Stamatakis et al. 2008) with *Typhula phacorrhiza* as an outgroup (Matheny et al. 2006). Bootstrap values were determined from 1000 replicates. The resulting tree was viewed in Mesquite. Alignments and trees were deposited in TreeBase (accession number S10612).

*Transformation, coding and analysis of isotopic data.*—Isotopic data from the present study were combined with those from previous studies that included at least one species of Hygrophoraceae (Mayor et al. 2008). Because  $^{13}\text{C}$  and  $^{15}\text{N}$  levels in the same taxon can be influenced by numerous environmental factors, such as elevation, humidity and soil chemistry, a standardization approach was employed. First, mean  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  for the known saprotrophic and ectomycorrhizal taxa, excluding Hygrophoraceae and taxa of uncertain nutritional status, from each individual site in each published study (TABLE II). Next, a standard deviation for each isotopic proportion was determined for each nutritional strategy at each site. Hygrophoraceae species collected from a site were treated as being of unknown nutritional type and their  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  data transformed into a number of standard deviations from the means for each isotope in collective saprotrophs and ECM taxa from the same site (TABLE I). Thus for each Hygrophoraceae species four transformed values were obtained ( $\delta^{13}\text{C}$  standard deviations from the saprotrophic mean,  $\delta^{13}\text{C}$  standard deviations from the ECM mean,  $\delta^{15}\text{N}$  standard deviations from the saprotrophic mean and  $\delta^{15}\text{N}$  standard deviations from the ECM mean). When two or more exemplars of a species were collected from a given site, a mean was taken to arrive at a single value. The absolute value of the number of standard deviations was used as a measure of distance to estimate the nutritional character state for each taxon across all studies (TABLE III).  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  were treated as independent characters.

TABLE III. Character coding scheme for standard deviations of stable isotope values used in parsimony optimization

$\sigma$ s from SAP mean (absolute value)	$\sigma$ s from ECM mean (absolute value)	State
< 2	> 2	Probably saprotrophic
< 2	< 2	Either saprotrophic or ECM (equivocal)
> 2	< 2	Probably ECM
> 2	> 2	Neither probably saprotrophic nor probably ECM

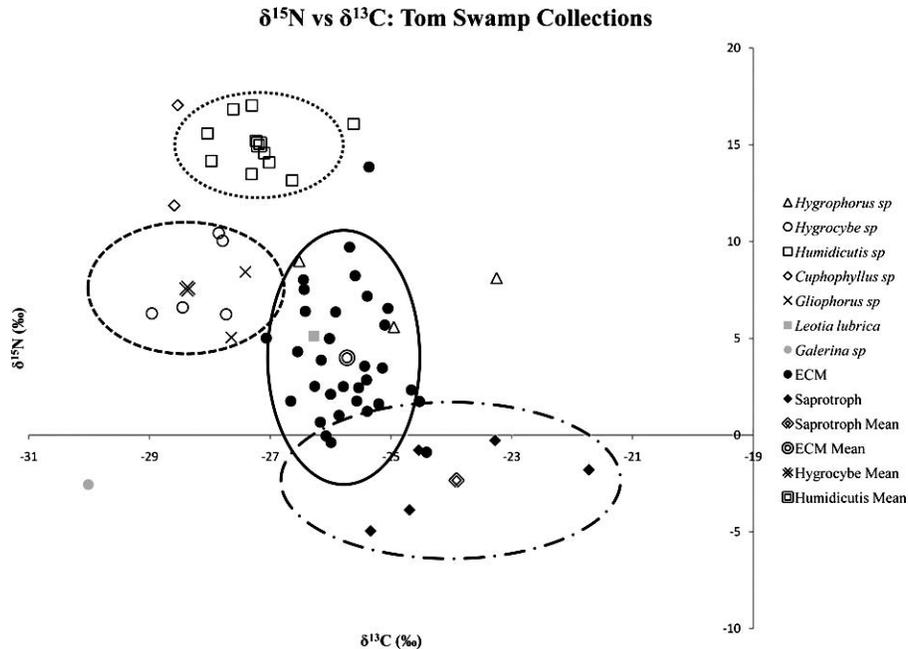


FIG. 1. Plot of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  absolute values for specimens collected from Tom Swamp. Ovals delineate two standard deviations from the mean for each of four groups (*Humidicutis*, *Hygrocybe*, ECM and saprotrophic fungi). The double-lined point at the center of each oval is the combined mean for both isotopes. The outlying *Hygrophorus* (upper right) is an exemplar of *H. flavodiscus* collected from a location in Tom Swamp other than sites 4 and 5.

Four character states were created for each stable isotope based on whether the distance value was more or less than two standard deviations from each of the means (TABLE III). For example a specimen with a  $\delta^{13}\text{C}$  value less than two standard deviations of the site saprotrophic mean at which it was collected was coded as “probably saprotrophic”. The same coding scheme also was applied to the specimen’s  $\delta^{15}\text{N}$  value. OTUs for which nLSU and 5.8S sequences but no isotopic data were available (*Lichenomphalia umbellifera*, *Dictyonema minus* and *Typhula phacorrhiza*) were left as ambiguous, even in cases where the nutritional mode previously had been established.

The resulting states were mapped onto the molecular phylogenetic tree in Mesquite with unordered parsimony as the optimality criterion, and ancestral state reconstruction was performed with the TRACE module for each of the two characters individually. The absolute values from the Tom Swamp and the transformed  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values for all taxa across all reviewed studies also were plotted in Excel 2007 to view the differences between Hygrophoraceae and other taxa.

## RESULTS

*Tom Swamp specimens.*—Twenty-three Hygrophoraceae sporocarps from sites 4 and 5 were collected and used in isotopic analysis (two *Cuphophyllus*, six *Hygrocybe*, 10 *Humidicutis*, two *Gliophorus* and three *Hygrophorus*). Thirty-two sporocarps from character-

ized ECM taxa and five from characterized saprotrophic taxa also were analyzed (SUPPLEMENTAL TABLE I). One specimen of *Hygrocybe punicea* was collected from a different site in the Tom Swamp tract, and one *Camarophyllus borealis* fruiting body was collected from outside Tom Swamp (TABLE I). These two specimens were used only for phylogenetic analysis.

The mean  $\delta^{13}\text{C}$  values for all *Hygrocybe* and *Humidicutis* spp. demonstrate substantial depletion versus both non-Hygrophoraceae saprotrophic ( $-23.91\text{‰}$ ) and ECM ( $-25.73\text{‰}$ ) species (TABLE I). When the values for all specimens are plotted (FIG. 1) clustering of individual values within two standard deviations of the mean for each of the four groups (known ECM fungi, known saprotrophic fungi, *Hygrocybe* spp. and *Humidicutis* spp.) is seen. The outlying *Hygrophorus flavodiscus* in the upper right quadrant of the figure was collected at a site fewer than 100 yards from Site 5, demonstrating the variation in stable isotope readings, which can occur due to differing conditions. Minimal overlap occurs between groups; each of the four appears to have a distinct combined stable isotope signature recognizable as differing from the other three. The  $\delta^{13}\text{C}$  values of the *Hygrocybe*/*Gliophorus* cluster show consistently more depletion than either those of ECM or saprotrophic taxa, but the  $\delta^{15}\text{N}$  are comparable to those of known ECM fungi. *Humidicutis* demon-

strates both more depleted  $^{13}\text{C}$  and greater  $^{15}\text{N}$  enrichment compared to both saprotrophic and ECM fungi. The two *Cuphophyllus* exemplars do not fall into either of the two other Hygrophoraceae clusters with *Cuphophyllus lacmus* having isotopic values slightly closer to the *Hygrocybe/Gliophorus* group and *Cuphophyllus* sp. closer to *Humidicutis* but both falling beyond two standard deviations for both heavy isotopes. Both *Cuphophyllus* spp. are more depleted for  $^{13}\text{C}$  and more enriched for  $^{15}\text{N}$  than either ECM or saprotrophic fungi.

A single exemplar of a *Galerina* sp. morphologically similar to *Galerina paludosa* (Redhead 1981), collected from a *Sphagnum* hummock at Site 4, also had depleted  $^{13}\text{C}$  compared to both ECM and saprotrophic fungi, and the value for this isotope is similar to that for *Hygrocybe* exemplars. Fruiting bodies of the ascomycete *Leotia lubrica* had stable isotope values consistent with known ECM taxa.

**Isotopic data across studies.**—Transformation and comparison of stable isotope data across seven studies (Tom Swamp, present study; Stadsskogen, Sweden, Taylor et al. 2003; Deer Park Road, Washington, USA, Trudell et al. 2004; Hoh River, Washington, USA, Trudell et al. 2004; Snowbowl, Arizona, USA, Hart et al. 2006; Upper Potaro River, Guyana, Mayor et al. 2008; Breuil-Chenue, France, Zeller et al. 2007) demonstrated a remarkable consistency in the transformed distances for all taxa despite widely differing environmental conditions (FIG. 2). When all data are plotted in a single graph a large majority of taxa of the same nutritional type across studies fall within two standard deviations of the mean for that type, whether ECM or saprotroph, allowing for consistency in comparing data from taxa under Hygrophoraceae. Transformed data points for all but two exemplars of *Hygrophorus* are within two standard deviations of the mean for known ECM species (FIG. 3). In contrast, all exemplars of *Hygrocybe*, *Humidicutis*, *Gliophorus* and *Cuphophyllus* are more than two standard deviations from the mean for ECM species. When Hygrophoraceae are compared to the means for saprotrophic taxa across studies only two *Hygrophorus* specimens fall within two standard deviations of the mean (FIG. 2). The other Hygrophoraceae sampled from Tom Swamp are thus outside where they would likely fall if they were typical of either known ECM or saprotrophic fungi with which they occur in the same patches of habitat. *Hygrophorus*, which is likely to be ECM, has  $\delta^{13}\text{C}$  values within two standard deviations of the mean for saprotrophs in all but two cases, but its  $\delta^{15}\text{N}$  values show more enrichment for that isotope and are in keeping with ECM fungi from the same sites.

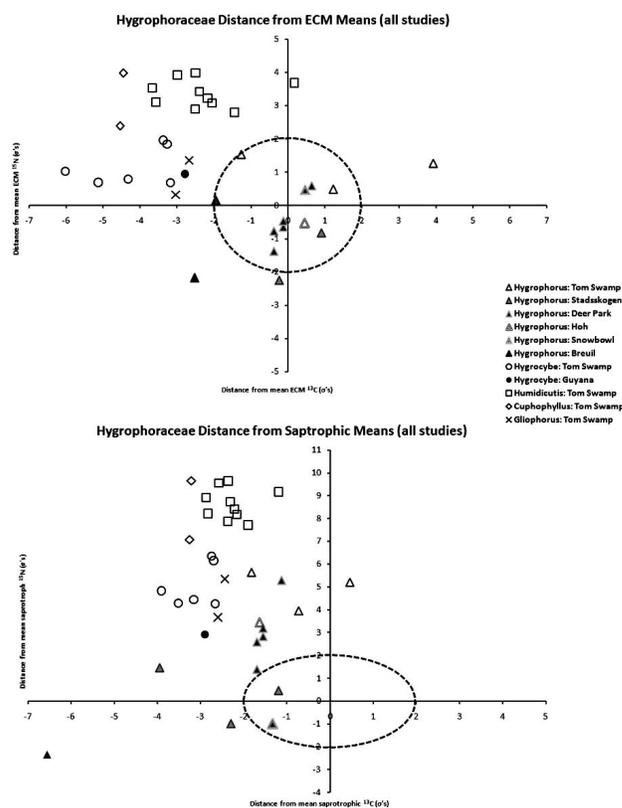


FIG. 2. Plots of all stable isotope data points for Hygrophoraceae from all studies. The dashed oval centered at the origin of each plot delineates two standard deviations from the mean for all known ECM (top) and saprotrophic (bottom) specimens in each of the studies. Data from most *Hygrophorus* exemplars cluster with ECM but not saprotrophic fungi. Other Hygrophoraceae genera do not cluster with ECM or saprotrophic fungi, indicating a different nutritional strategy. Data points for non-Hygrophoraceae fungi are not shown.

**Molecular phylogeny.**—The initial alignment of the nLSU region contained 50 taxa and 1414 characters. Further refinement resulted in an alignment of 1355 characters when unalignable regions had been removed. Alignment of the 5.8S region contained 50 taxa and 161 characters.

Maximum likelihood analysis of combined nLSU + 5.8S produced a tree (FIG. 3) with moderate to weak bootstrap support at the deepest nodes, which are also short branches. The *Cuphophyllus* clade (clade 1) is resolved as the sister of all other Hygrophoraceae sampled (BS 82%), although monophyly of the remaining Hygrophoraceae is not strongly supported. Clade 2, *Hygrophorus* + *Humidicutis* + *Gliophorus* + *Lichenomphalia* + *Dictyonema*, reconstructs the lichenizing Hygrophoraceae (Lawrey et al. 2009) (clade L) as the sister of the other three genera, again with weak bootstrap support. A monophyletic *Humidicutis* clade

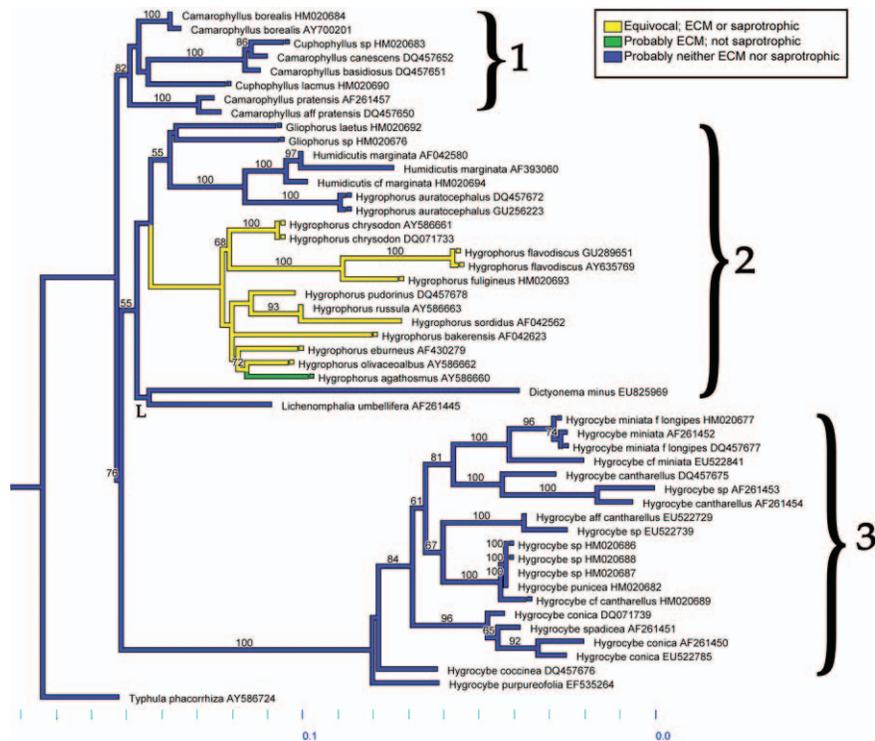


FIG. 3. Parsimony optimization of the  $\delta^{13}\text{C}$  character states mapped onto the nLSU + 5.8S phylogram. Clades are indicated by the numbered brackets; clade 1 is composed of *Cuphophyllum* spp., clade 2 of *Gliophorus* + *Humidicutis* + *Hygrophorus* + lichenizing *Hygrophoraceae* (indicated by an L), and clade 3 of *Hygrocybe* spp. Bootstrap support > 50% is above branches. Length = two steps.

was strongly supported (BS 100%). *Gliophorus* shares a more recent common ancestor with *Humidicutis* than it does with *Hygrophorus*. Each of the genera appears here as monophyletic. Clade 3, composed entirely of *Hygrocybe* spp., is at the end of a significantly longer and better supported branch (BS 100%) than that leading to the *Hygrophorus* et al. clade.

*Parsimony optimization of stable isotope data.*—Mapping of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  character states onto the molecular phylogeny suggests that the isotopic profiles are conserved among closely related taxa except for isolated changes in *Hygrophorus*. For  $\delta^{13}\text{C}$  (FIG. 3) all *Hygrophorus* spp. possessed the same state (equivocal), except *H. agathosmus* (ECM-like). All other taxa for which data were available were more than two standard deviations from both ECM and saprotrophic fungi sampled from the same site. For  $\delta^{15}\text{N}$  (FIG. 4) *Hygrocybe* is entirely ECM-like as are *Gliophorus* and *Hygrophorus* spp. other than *H. chrysodon* and *H. olivaceoalbus*, both of which are equivocal (gray branches). *Cuphophyllum* and *Humidicutis* are uniformly more than two standard deviations from the means of both the known ECM and saprotrophic taxa with which they occur at a given site.

## DISCUSSION

Most prior stable isotope analyses incidentally have sampled only a few *Hygrophorus* spp. and a single unidentified *Hygrocybe* referenced as “toe-head” (Mayor et al. 2008). The exception to this was a survey of taxa in Welsh grasslands that included several *Hygrocybe*, *Camarophyllum* and *Gliophorus* exemplars (Griffith et al. 2002). Our study is the first to sample diverse species across *Hygrophoraceae* from a single woodland locality, including exemplars of *Hygrophorus* and *Humidicutis* along with co-occurring saprotrophic and ECM taxa. Griffith et al. (2002) compared  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values in the *Hygrophoraceae* sampled to saprotrophic fungi from the same site but included only co-occurring clavarioid putatively ECM taxa, relying on data from Kohzu et al. (2000) and Hobbie et al. (2001) for comparisons to other ECM basidiomycetes. Nevertheless our results largely agreed with the pattern of stable isotopes noted in Griffith et al. (2002). Our study found for the first time that *Humidicutis* spp. also have  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values unlike previously characterized ECM and saprotrophic taxa. When non-*Hygrophorus* *Hygrophoraceae* were compared to ECM basidiomycetes, the  $\delta^{13}\text{C}$  values in non-*Hygrophorus* *Hygrophoraceae* were lower than in

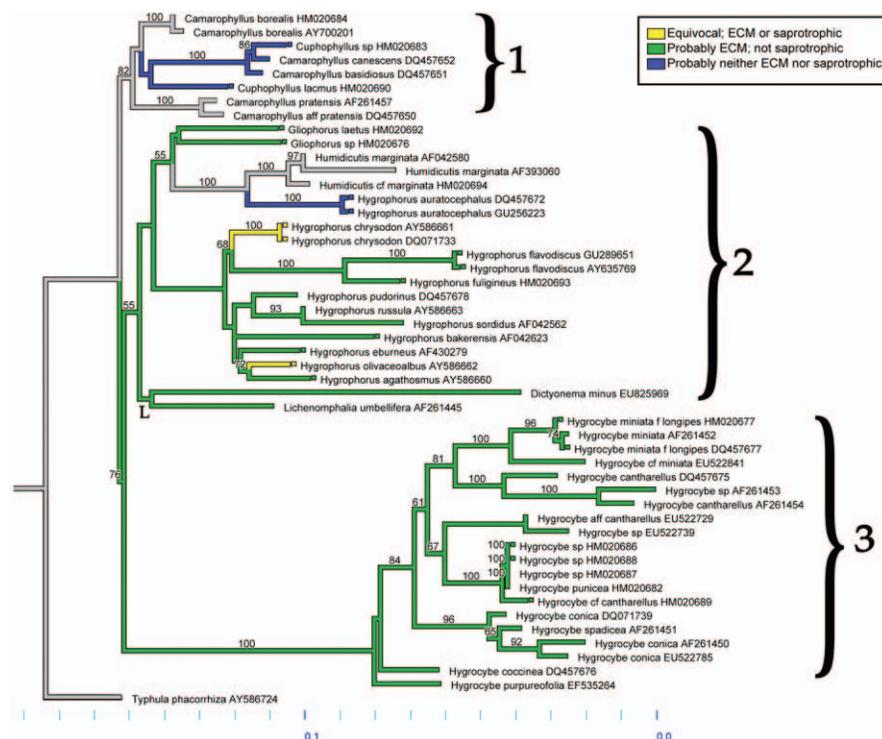


FIG. 4. Parsimony optimization of the  $\delta^{15}\text{N}$  character states mapped onto the nLSU + 5.8S phylogram. Clades are indicated by the numbered brackets; clade 1 is composed of *Cuphophyllus* spp., clade 2 of *Gliophorus* + *Humidicutis* + *Hygrophorus* + lichenizing Hygrophoraceae (indicated by an L) and clade 3 of *Hygrocybe* spp. Bootstrap support > 50% is above branches. Gray branches are those for which no state has been inferred. Length = four steps.

characterized ECM taxa.  $\delta^{15}\text{N}$  values are similar to other ECM fungi only in *Hygrocybe* spp. from forest habitat. While nothing in our study explains the source of this difference between our study and Griffith's European grassland *Hygrocybe*, in light of the known sensitivity of *Hygrocybe* to soil nitrogen content, differences between forests and grasslands in soil chemistry, such as pH and availability of ammonium versus that of nitrate, should be considered potential influences (Keizer 1993, Griffith et al. 2002). Variations in  $\delta^{15}\text{N}$  values in conspecific fungi also have been linked to temperature and humidity (Henn and Chapela 2001, Griffith et al. 2002, Mayor et al. 2008). Our own finding of significant variation in the isotopic profile of *Hygrophorus flavodiscus* collected from nearby sites with differing soil conditions (FIG. 1) demonstrated the importance of collecting specimens from the same site when analyzing stable isotope content in Hygrophoraceae.

A number of factors related to fungal biochemistry and morphology have been proposed to influence  $\delta^{15}\text{N}$  in fungi other than their nutritional status (Gleixner et al. 1993, Taylor et al. 1997, Gebauer and Taylor 1999, Griffith et al. 2002, Hobbie and Agerer 2010). Nothing in our investigation of fruiting bodies collected from Tom Swamp supports these mechanisms as playing a

significant part in observed values from that site. With a simple transformation method we were able to compare isotopic data across several diverse sites that incorporate stable isotope data from nearly 500 exemplars of Basidiomycota diversity (SUPPLEMENTAL TABLE I). While some outliers do occur (SUPPLEMENTAL FIG. 1), they are few, suggesting that factors such as carbon isotope fractionation between substrates and fruiting bodies (Gleixner et al. 1993), mycelial exploration types (Hobbie and Agerer 2010) and use of nitrogen from different soil profiles (Taylor et al. 1997), do not cause sufficient variability to affect our analysis when our data normalization technique is used. For example *Hygrophorus olivaceoalbus* was sampled in two of the studies we reviewed (Taylor et al. 2003, Trudell et al. 2004) and appears isotopically typical in our analysis (FIG. 2). DNA sequences from *H. olivaceoalbus* have been recovered from mineral eluvial soil profiles, as have various *Lactarius* and *Russula* sequences (Landeweert et al. 2003). Among the hundreds of exemplars of ECM fungi across the studies we reviewed, only two exemplars had  $\delta^{15}\text{N}$  values similar to those of *Humidicutis* or *Cuphophyllus* (SUPPLEMENTAL FIG. 1).

Analysis of isotopic data from Tom Swamp (FIG. 1) delineates three moderately conserved profiles in

Hygrophoraceae. *Hygrophorus* spp. are ECM-like for both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ . *Hygrocybe* and *Gliophorus* are more depleted in  $^{13}\text{C}$  than known ECM taxa but similar in  $^{15}\text{N}$ . *Humidicutis* is more depleted in  $^{13}\text{C}$  and much more enriched in  $^{15}\text{N}$  than ECM basidiomycetes. No Hygrophoraceae from Tom Swamp have combined isotopic profiles resembling saprotrophic fungi from the site. The same result holds when isotopic data from multiple sites are transformed and plotted (FIG. 2). Two *Cuphophyllus* exemplars have  $\delta^{13}\text{C}$  values similar to those in both *Hygrocybe* and *Humidicutis* but slightly different  $\delta^{15}\text{N}$  values that prevent their inclusion in either of the two delineated nutritional modes seen in those genera.

Our phylogenetic reconstruction of Hygrophoraceae (FIG. 3) agrees with the topology of that section of the phylogeny in Matheny et al. (2006) except for the weakly supported placement of lichenizing taxa as the sister clade of *Hygrophorus* + *Humidicutis* + *Gliophorus* in the present study. Our phylogeny also agrees with the maximum likelihood reconstruction in Lawrey et al. (2009), which supported *Dictyonema* as a genus in Hygrophoraceae. Deep nodes were not well supported by bootstrap analysis, but *Cuphophyllus*, *Humidicutis* (including *Hygrophorus auratocephalus*) and *Hygrocybe* all are resolved as well supported clades. *Gliophorus* and *Hygrophorus* were not well supported but are in agreement with current taxonomy. Our results are consistent with much more intensively sampled phylogenies of Hygrophoraceae (Lodge et al. unpubl.).

Parsimony optimization mapping of the  $\delta^{13}\text{C}$  character onto the Hygrophoraceae molecular phylogeny (FIG. 3) suggests that the extreme depletion of  $^{13}\text{C}$  in clades other than *Hygrophorus* are widespread and conserved in the family. While *Hygrophorus* exhibits a character state like that of both saprotrophic and ECM taxa outside Hygrophoraceae, *Cuphophyllus*, *Hygrocybe*, *Humidicutis* and *Gliophorus* all have transformed values more than two standard deviations from the mean for all other saprotrophs and ECM taxa samples across all reviewed studies. Within *Hygrophorus* only *H. agathosmus* (Taylor et al. 2003) is inferred as probably ECM and not saprotrophic. The most parsimonious explanation for this scenario is that there has been a single switch from the presently uncharacterized “probably neither ECM nor saprotrophic” nutritional mode in all other Hygrophoraceae to the more ECM-like state in *Hygrophorus*. The switch is inferred to have occurred along the branch separating *Hygrophorus* from the other taxa examined. No members of Hygrophoraceae are inferred to be probably saprotrophic and probably not ECM in this analysis.

Mapping of the  $\delta^{15}\text{N}$  character with parsimony optimization (FIG. 4) presents a more complex series

of shifts. Extreme  $^{15}\text{N}$  enrichment, greater than that in other ECM fungi, is seen in both the *Cuphophyllus* and *Humidicutis* clades. The character in *Hygrocybe* as well as *Hygrophorus* is consistent with known ECM taxa. Two additional shifts to a less ECM-like, equivocal state are noted within *Hygrophorus*. Both *H. chrysodon* (Trudell et al. 2004) and *H. olivaceoalbus* (Taylor et al. 2003, Trudell et al. 2004) appeared in the present reconstruction to have independently switched to a somewhat different nutritional strategy than other Hygrophoraceae sampled. Once again none of the Hygrophoraceae in any of the studies were inferred to be exclusively saprotrophic and only two OTUs are even equivocally so. Both equivocal OTUs are in *Hygrophorus*, which contains species characterized as ECM by both morphological study (Agerer 2006) and analyses of ITS sequences from root tips (Peter et al. 2001, Douglas et al. 2005).

Based on our analyses incorporating both  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  values *Hygrocybe* should not be considered a saprotrophic clade. The case is the same for the other clades recovered in this study. The only species we have reconstructed even as equivocally saprotrophic for both nitrogen and carbon acquisition are *Hygrophorus chrysodon* and *Hygrophorus olivaceoalbus*, and because we have no information regarding the condition of the fruiting bodies sampled or the conditions at the precise locations from which they were collected we must allow that unknown factors may have affected the isotopic data for these taxa. One of our collections of *Hygrophorus flavodiscus* from Tom Swamp and one of *Hygrophorus lindtneri* from France (Zeller et al. 2007) each varied by several standard deviations from means for ECM taxa at their respective sites, demonstrating the variability of isotopic data due to changes in the exemplar's immediate environment (FIG. 2). Both yielded low  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values unlike all other Hygrophoraceae and in fact unlike any other fungus among the ~ 500 samples taken in all studies reviewed (SUPPLEMENTAL TABLE I).

The result that many Hygrophoraceae fell more than two standard deviations from the means for one or both stable isotope values in characterized ECM and saprotrophic taxa suggests that the nutritional strategy of these Hygrophoraceae differs from those characterized fungi. We can only speculate presently as to what the specific strategy might be. Hygrophoraceae exhibiting the unusual profiles have been described in the literature as bryophilous in European grassland setting (Arnolds 1981, Griffith et al. 2002), and they also occurred with bryophytes in the sites from which we recovered them in a North American woodland. These observations suggest that Hygrophoraceae are biotrophic on either bryophytes or with another bryophyte-associated organism. The

only other basidiomycete in our study exhibiting the degree of  $^{13}\text{C}$  depletion found in non-*Hygrophorus* Hygrophoraceae was *Galerina* growing on *Sphagnum* that was morphologically similar to *Galerina paludosa*, demonstrated as being biotrophic on *Sphagnum* (Redhead 1981). Our phylogenetic analysis reconstructed lichenizing Hygrophoraceae (Lawrey et al. 2009) as sister taxa of *Hygrophorus* and *Humidicutis* (FIG. 3). It thus is possible that some non-lichenizing Hygrophoraceae derive a portion of their carbon from algae (as does *Lichenomphalia*) or cyanobacteria (as does *Dictyonema*) (Lawrey et al. 2009) as either mutualists, parasites or necrotrophs. On the other hand, if non-lichenizing Hygrophoraceae other than *Hygrophorus* are indeed saprotrophic, it is conceivable that the differences we see in these genera could result from their exploitation of algae as a carbon source as some other saprotrophic basidiomycetes are capable of doing (Hutchison and Barron 1997, Hobbie and Boyce 2010).

Our findings were consistent with a model in which Hygrophoraceae outside genus *Hygrophorus* are exchanging nutrients with a partner. The same mechanisms that result in the depletion of  $^{13}\text{C}$  and enrichment of  $^{15}\text{N}$  in ECM fungi relative to saprotrophs cause even greater respective  $^{13}\text{C}$  depletion and  $^{15}\text{N}$  enrichment in members of Hygrophoraceae in which  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values were more than two standard deviations from the means for both saprotrophs and confirmed ECM taxa. Hygrophoraceae in which the “neither saprotrophic nor ECM” character state has been noted are found in nitrogen-poor habitats. It is possible that in such habitats Hygrophoraceae are forced to sequester most of their nitrogen in  $^{15}\text{N}$ -depleted chitin during growth, leaving a residual,  $^{15}\text{N}$ -enriched protein pool (Taylor et al. 1997) to serve as the nitrogen source for fruiting body formation.

These nitrogen and carbon isotope patterns suggest that *Cuphophyllus*, *Humidicutis*, *Hygrocybe* and *Gliophorus* are not saprotrophic, but are biotrophic with an as yet unknown partner.  $\delta^{13}\text{C}$  values are particularly suggestive of an association with bryophytes, algae or understory vascular plants. Further investigation of possible interactions among *Hygrocybe*, *Gliophorus*, *Cuphophyllus* and *Humidicutis* and other organisms in their environment will be necessary to clarify the source of the evidence we have uncovered against these genera being exclusively saprotrophic.

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