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Surveying the biodiversity of the Cryptomycota using a targeted PCR approach

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ABSTRACT

Cryptomycota is a newly discovered phylum of early-diverging fungi that is estimated to comprise a massive amount of unstudied biodiversity. Known primarily from environmental DNA sequences, the group contains few formally described and successfully cultured genera, all known as obligate endoparasites. Numerous studies have detected Cryptomycota as DNA sequences in aquatic, terrestrial, and animal associated habitats, though no study to date has specifically targeted the group by PCR. In this study, we developed Cryptomycota-specific and preferential primers for the 18S rRNA gene in order to facilitate studies on the biodiversity and ecology of the group. The diversity of sequences and habitat association of Cryptomycota was surveyed across three environments: freshwater sediment, marine sediment, and soil. The new primers were able to recover a large proportion of Cryptomycota sequences: of 56 sequences cloned or directly sequenced, 44 were unique OTUs and (39/44) 88.6 % were phylogenetically affiliated with Cryptomycota. This study expands the boundary of current Cryptomycota sequence diversity; unique Cryptomycota OTUs were spread across six of 12 supported Cryptomycota sub clades. Freshwater sediments and soil were consistently amplified with the Cryptomycota-preferential primers. Our results suggest that Cryptomycota are ubiquitous and phylogenetically diverse components of essentially all terrestrial and aquatic ecosystems, whose ecological functions should be better explored.

Introduction

Among the many groups containing overlooked biodiversity on Earth, fungi are projected to comprise a large proportion. Currently, the number of described fungal species is approximately 100 000 (Kirk et al., 2008), but recent biodiversity estimates based on DNA sequence analysis suggest as many as 5.1 million or more species of fungi may exist (Blackwell, 2011; Taylor et al., 2014). Fungi have a diversity of ecological roles and, therefore, cataloging their true biodiversity will be essential for understanding ecosystem functioning. Many undescribed fungi are likely to be microscopic and among the early-diverging branches, because these are enriched for obligately biotrophic species, or species with complex growth requirements such as the anaerobic rumen chytrids and the pathogens of invertebrates (Gleason et al., 2010). The difficulty of culturing these obligate parasites greatly limits our ability to research and observe them relative to other fungi. Only with recent molecular advances have we been able to target the missing diversity with techniques such as DNA

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sequencing and *in situ* DNA hybridization (Jobard et al., 2010; Jones et al., 2011b; Rosling et al., 2011; Nagahama et al., 2011; Monchy et al., 2011; Glass et al., 2013).

One of the groups of uncultured fungi that avoided circumscription until the widespread use of DNA methods for community studies is the recently described phylum known variously as Cryptomycota (Jones et al., 2011a), Rozellomycota (Corsaro et al., 2014), or Rozellida (Lara et al., 2010). We use Cryptomycota throughout for consistency. Cryptomycota were first recognized as a clade uniting diverse environmental sequences and the parasitic zoosporic fungus *Rozella* (Lara et al., 2010; Jones et al., 2011b). Cryptomycota became an evolutionary curiosity because of their placement on the earliest diverging branch within the true fungi (Jones et al., 2011b; James et al., 2013), and their intermediate features between true fungi and protists. For example, *Rozella* is known for having a trophic phase without a chitinous cell wall but makes a resting sporangium with a chitinous wall (James and Berbee, 2012). These traits have led some authors to place Cryptomycota outside of the fungi, either in the polyphyletic phylum Choanozoa (Cavalier-Smith, 2013) or in a new superphylum Ophisthosporidia (Karpov et al., 2014b).

Two other groups of parasites appear to be related to the Cryptomycota, the aphelids (Karpov et al., 2012), and the microsporidia (James et al., 2013). Aphelids comprise three described genera known as zoosporic endoparasites of phytoplankton (Karpov et al., 2014a). Microsporidia are better known and are endoparasites of a variety of animals and protozoa and have evolved a number of defining traits such as a specialized infection apparatus, known as a polar tube, and loss of a mitochondrial genome (Keeling and Fast, 2002). The phylogenetic placement of the Aphelida is still under consideration, and depending on the analysis, the Aphelida and Cryptomycota may be a clade together with Microsporidia or Aphelida may diverge before on a separate branch (Karpov et al., 2012; Letcher et al., 2013; Corsaro et al., 2014). Uniting all species in this clade of endoparasites at the base of the fungal tree is the absence of a cell wall in the tropic phase, so that the plasma membrane of the parasite makes direct contact with the cytoplasm of the host cell (Karpov et al., 2014a,b). Both aphelids and *Rozella* are suspected of phagocytosing host cytoplasm (Karpov et al., 2012; Powell, 1984).

Current sequencing data indicate Cryptomycota has extensive phylogenetic diversity, potentially larger than the rest of the known fungi (Jones et al., 2011b). However, they are largely unknown in their morphology, because nearly all of the diversity is from environmental DNA. The aquatic and marine habitats in which Cryptomycota appear to thrive suggest a requirement for water, consistent with an uniflagellate zoospore stage (Lara et al., 2010). Using probes specific to a sequence type recovered from a pond in Exeter, U.K., Jones et al. (2011b) observed three morphologies: uniflagellate zoospores, non-flagellated cells attached to other eukaryotic microorganisms, such as diatoms, and non-flagellated cysts lacking a chitinous cell wall. Cryptomycota life cycles, therefore, remain largely unknown and must be extrapolated from basic biology of named members cultured with their hosts. Species of *Rozella* are known as primary parasites of water molds (Oomycota), zoosporic fungi (Blastocladiomycota, Chytridiomycota), and reportedly the green alga, *Coleochaete* (Held, 1981). These parasitic interactions appear to be genus or species-specific based on experimental inoculations (Held, 1974; Karling, 1942). *Rozella allomycis* is probably the most well known species in Cryptomycota, and is a primary parasite of the water mold, *Allomyces* spp. (Blastocladiomycota).

More recently, two new parasites, intermediate in morphology between microsporidia and *Rozella* have been described. *Paramicrosporidium* is an endonuclear parasite of amoebae (Corsaro et al., 2014), and *Mitosporidium daphniae* is a gut parasite of *Daphnia* (Haag et al., 2014). Both species have morphologies intermediate between microsporidia and *Rozella*, such as the absence of a flagellum, reduced or absent mitochondria, and small (1 μm) chitinous spores with a polar tube. *Mitosporidium* was considered the earliest diverging microsporidian, while *Paramicrosporidium* was classified as a member of Rozellomycota (Cryptomycota). The two new genera group among the molecular sequences previously considered Cryptomycota, though the two are not actually a clade. These observations lead to the hypothesis that many of the members of Cryptomycota have a microsporidian-like morphology, characterized by endoparasitic growth without a cell wall, absence of a flagellum, and reproduction with small chitinous spores.

Despite the uncertain biology of the group, it is clear that the group is widely distributed, as molecular surveys have recovered sequences from numerous environments including lakes, rivers, drinking water treatment systems, aquifers, soil, and oceans (Jones et al., 2011b). These fungi are quite common in aquatic and marine environments where their abundance may range from 0.02 to 30 % of all eukaryotic sequences, with an average of 3.5 % across 13 studies (Livermore and Mattes, 2013). While direct ecosystem effects from parasites of producers are more readily observed, *Rozella* and similar hyperparasites may have a considerable regulatory role on population sizes of zoosporic fungi that are parasites of primary consumers in lakes (Gleason et al., 2012, 2014). The hyperparasite *R. parva*, is a primary parasite of the chytrid *Zygorhizidium affluens*, which in turn parasitizes the freshwater diatom *Asterionella formosa* (Reynolds, 1984). Hyperparasitic interactions on parasites of primary consumers may consequentially affect producer populations in a trophic cascade. Parasites are generally underrepresented in food webs, but as primary parasites the role of Cryptomycota and Aphelida on ecological processes should also be evaluated. The infection of one algal species can favor the development of other algal species through competitive release, which influences community composition and regulates producer populations (Kagami et al., 2007; Held, 1981; Powell, 1984; van Donk and Bruning, 1995).

Cryptomycota diversity has been categorized on the basis of habitat origin (Jones et al., 2011b; Livermore and Mattes, 2013), but our ability to identify ecological patterns that could explain their function in the environment is impeded by either insufficient sampling of the tree or short next-generation DNA sequences. Better understanding of the ecology and morphology has been difficult for the group and, therefore, while it is clear Cryptomycota are pervasive, frequently encountered in a

variety of habitats, and likely parasitic, it is mostly uncertain which hosts they may have or if they may actually contain saprotrophic or bacterivorous taxa. As a primary step towards characterizing Cryptomycotan diversity, we developed primers that show a broad preference for the clade, and demonstrate how they can be utilized in a variety of habitats to refine ecological hypotheses of their function.

The principle aim of this study was to evaluate the suitability of PCR primers to recover the range of diversity in Cryptomycota sequences from a variety of habitats and to detect patterns in the distribution of diversity. Our Cryptomycota-preferential primers were tested on DNA from freshwater sediment, marine sediment and soil samples. The diversity and distribution of the recovered Cryptomycota sequences may assist future studies on community composition and eventually interactions within these ecosystems.

Materials and methods

Sampling

Samples were collected from Michigan and Florida in the fall of 2012. Samples were collected in sterile 15 ml centrifuge tubes, transported to the lab on ice, and stored at −80 °C until DNA extraction. A total of 15 Michigan freshwater sediment samples from Waterloo State Recreation Area and Pinckney State Recreation Area, varying in depth below the water surface (0.05–10 m), were collected; shallower sediments were collected with a sterilized turkey baster, and deeper sediments were collected with an Ekmen Bottom Grab sampler. A total of six Florida freshwater sediment samples from University of Florida Natural Area Teaching Laboratory (NATL),

Table 1 – List of samples utilized and DNA amplification results with the most successful primer combinations

Location	Sample ID	CRYPTO2-1F/ AU4v2	CRYPTO2-2F/ AU4v2	ROZELLA-1F/ AU4v2	ROZELLA-1F/ ROZELLA-1R
Freshwater sediment (Michigan)					
UM herbarium pond, Washtenaw Co.	101	○	○	●	○
Cedar Lake, Washtenaw Co.	102	–	○	–	–
Crooked Lake#1, Washtenaw Co.	103	○	●	○	–
Mill Lake, Washtenaw Co.	104	–	–	–	–
Pickerel Lake (~15 m), Washtenaw Co.	105	–	○	–	–
Pickerel Lake (~0.05 m), Washtenaw Co.	106	○	○	○	–
Bruin Lake, Washtenaw Co.	107	–	–	–	–
Gosling Lake (~5 m), Livingston Co.	108	–	–	–	–
Gosling Lake (~0.05 m), Livingston Co.	109	○	●+⊗c	●	–
Halfmoon Lake, Washtenaw Co.	110	○	○	●	●
Crooked Lake#2, Washtenaw Co.	111	○	○	–	–
South Lake, Washtenaw Co.	112	–	○	–	–
North Lake, Washtenaw Co.	113	○	○	–	–
Sullivan Lake, Washtenaw Co.	114	○	○	–	–
Walsh Lake, Washtenaw Co.	115	○	○	–	–
Marine sediment					
Honeymoon Island west (gulfside), Pinellas Co.	201	○	○	–	–
Honeymoon Island east (intercoastal), Pinellas Co.	203	○	–	–	–
Freshwater sediment (Florida)					
Honeymoon Island pond, Pinellas Co.	3 204	○	●c	–	–
Residential pond, Alachua Co.	301	○	●+⊗c	–	–
UF campus, Shands Cancer Hospital pond, Alachua Co.	302	○	–	○	●
UF campus, Hume Pond, Alachua Co.	303	–	●	○	○
UF campus, creek, Alachua Co.	304	●c	●	●	●
UF campus, NATL pond, Alachua Co.	305	○	●	○	–
Soil					
Waterloo State Rec., Crooked Lake, Washtenaw Co.	501	○	○	–	–
Nichols Arboretum (mesic), Washtenaw Co.	502	○	○	–	–
Nichols Arboretum (hydric), Washtenaw Co.	503	–	○	●	–
Ella Mae Power Park (mesic), Oakland Co.	504	–	○	–	–
Rotary Park (hydric), Oakland Co.	505	–	○	●	–
Ella Mae Power Park (mesic/disturbed), Oakland Co.	506	○	○	–	–
9 Mile/Meadowbrook forest (mesic), Oakland Co.	507	●c	●	–	–
Ella Mae Power Park (hydric), Oakland Co.	508	○	○	–	–
9 Mile/Meadowbrook forest, Oakland Co.	509	○	○	–	–
Rotary Park, Oakland Co.	510	–	○	–	–

● = sequenced and confirmed Cryptomycota; ⊗ = sequenced and confirmed non-Cryptomycota; ○ = suspected Cryptomycota; – = no amplification in sample; ●c = clone library. Confirmed Cryptomycota were determined by direct sequencing or cloned sequence analysis. A large proportion of the unsequenced DNA amplifications are suspected also to be Cryptomycota due to the high proportion (89.3 %) of Cryptomycota to non-Cryptomycota sequenced.

ponds near campus, and a marsh on Honeymoon Island State Park were collected. Two Florida marine sediment samples were also collected in Honeymoon Island State Park. Florida sediment samples varied in depths (0.05–1.0 m) and were all collected with a sterilized turkey baster. A total of 10 Michigan soil samples were collected from Nichol's Arboretum, Pinckney State Recreation Area, Ella Mae Power Park, and Rotary Park from O_a horizons (Table 1).

DNA methods

DNA was extracted from sediment and soil samples using a MoBio PowerSoil DNA isolation kit. Freshwater and marine sediment samples were first centrifuged at 1 500 g for 6 min. Sediment or soil weighing 250 mg was used in the extraction protocol. The DNA was resuspended in 50 μl of water and stored at −20 °C. The DNA concentration for each sample was checked by electrophoresis and diluted to 1–5 ng μl^{-1}.

Primers were created to target the 18S rRNA gene region of Cryptomycota based on current sequence data of Cryptomycota (Fig 1). This study tested 18 primer combinations from 14 primers (eight forward, six reverse) varying in specificity for Cryptomycota. Table 2 shows the primers involved in combinations that were verified to amplify Cryptomycota DNA. PCR was conducted with Ex Taq DNA Polymerase (Takara) using 2 mM $MgCl_2$ in 12.5 μl reactions with 5 μl of diluted DNA. PCR conditions were as follows: 94 °C for 3 min; 35 cycles of 94 °C for 1 min, the primers' optimum annealing temperature (ranging between 55 and 61 °C) for 30 s, and 72 °C for 1 min; 72 °C for 7 min. PCR products were purified using ExoSAP-IT (Promega) and sequenced by the University of Michigan DNA Sequencing Core. Samples that produced unclear, overlapping chromatograms were cloned.

Cloning libraries were obtained for five of the samples that produced noisy chromatograms (one soil, three FL freshwater, and one MI freshwater sediment) using the TOPO® TA Cloning Kit (Invitrogen). *Escherichia coli* (TOP10) transformants were analyzed by direct PCR of bacterial colonies using M13F and M13R primers. The colony PCR conditions were as follows: 94 °C for 7 min; 35 cycles of 94 °C for 1 min, 55 °C for 30 s, and 72 °C for 1 min; 72 °C for 7 min. Cloning libraries contained sequences from 5 to 8 sub-cloned PCR products per sample.

Sequence analysis

Sequence chromatograms were analyzed, trimmed, and assembled using Sequencher 4.1 (GeneCodes). Edited sequences were compared to sequences in GenBank using BLASTn (Table 3). Sequences were eliminated as being from the same OTU if they differed by ≤3 bp or if they had max identity scores above 99 %. Two putative chimeras were removed after their identification using chimera-slayer in mothur v.1.32.1 (Schloss et al., 2009) using a reference set of 203 Cryptomycotan and other fungal 18S sequences.

A phylogeny was constructed using the sequences from this study in comparison to those of Jones et al. (2011b) with the inclusion of additional related environmental sequences from GenBank. The alignment of 18S rRNA sequences was constructed using MAFFT v7 (Katoh et al., 2002) with the parameters E–INS–i. Following removal of the ambiguously aligned regions using Gblocks 0.91b (Castresana, 2000) with parameters for a less stringent selection (allow smaller final blocks, allow gap positions within the final blocks and allow less strict flanking positions). The phylogeny was estimated with RAxML v 7.2.8 (Stamatakis, 2006) using the GTR + G model with support estimated by 1 000 bootstrap pseudoreplicates.

Sequences recovered in this study were deposited in GenBank (Accession numbers KP096134–KP096173).

Table 2 – 18S rRNA primer sequences and their intended targets

Primer	Sequence 5′-3′	Preferential target
AU4v2	GCCTCACTAAGCCATTC	Cryptomycota
CRYPTO2-1F	CAGTAGTCATATGCTTGTCC	Cryptomycota
CRYPTO2-2F	CACAGGGAGGTAGTGACAG	Cryptomycota
ROZELLA-1F	CGCAAATTACCCAATGG	*Rozella*
ROZELLA-1R	TTTCTCATAAGGTGCCRATGA	*Rozella*
CRYPTO1B-1F	GACCTTGTGTCGACGACGTA	Cryptomycota
CRYPTO1B-1R	CCTCTAGCTTCGGAATACGA	Cryptomycota
CRYPTO2A-1R	TGTCAATCCTTCCCATGTCC	Cryptomycota

Results and discussion

We designed and tested 18 primer pair combinations to selectively amplify Cryptomycota across a variety of habitats using alignments of sequences classified as Cryptomycota (Jones et al., 2011b) with other fungi and eukaryote outgroups. Primers designated "CRYPTO" were designed to broadly target the clade, while primers labeled "ROZELLA" were designed to target a more restricted subset of sequences related to *Rozella* (Table 2). Primer AU4v2 is a modification of the fungal specific primer AU4 originally reported by Vandenkoornhuyse et al. (2002). Of the primers tested, six combinations of primers

Fig 1 – Primer map showing the location and orientation of primer sequences on the 18S rRNA gene.

Table 3 – BLASTn results by sample

OTU	Additional recoveries	p1	p2	p3	p4	p5	p6	Top BLASTn including environmental sequences	GenBank ACCN	Query coverage	Max ID
CES304-1		●						Uncultured fungus clone HA068	HM487049	60	99
CES304-2		●						Uncultured fungus clone HA068	HM487049	60	99
CES304-3		●						Uncultured fungus clone C10 18S	JN054676	89	99
CES304-4		●						Uncultured fungus clone C10 18S	JN054676	88	99
CES304-5	1	●						Uncultured eukaryote gene for 18S	AB695466	100	99
CES304-6		●						Uncultured eukaryote gene	AB695466	95	98
CES304-7		●						Uncultured eukaryote gene for 18S	AB695466	100	97
CES507-8	1	●						Uncultured eukaryote gene for 18S	AB695466	99	98
CES507-9		●						Eimeriidae environmental sample clone	EF023661	96	99
CES507-10		●						Eimeriidae environmental sample clone	EF024493	96	94
CES507-11		●						Eimeriidae environmental sample clone	EF024493	100	97
CES507-12		●						Uncultured eukaryote gene for 18S	AB695512	99	93
CES507-13		●						Eimeriidae environmental sample clone	EF024493	100	95
CES301-14	2		●					Uncultured fungus clone HA052 18S	HM487051	99	97
CES109-15			●					Uncultured fungus clone HA068	HM487049	99	99
109_p2_14			●					Uncultured eukaryote clone Amb	EF023895	99	98
CES109-17	1		●					Uncultured fungus clone HA052 18S	HM487051	100	97
CES109-18			●					Uncultured fungus clone HA011	HM487053	100	99
CES109-20			●					Uncultured rhizosphere zygomycete	AJ506030	100	98
301.1_34			●					Uncultured fungus clone C62	JN054682	94	95
CES301-21			●					Uncultured fungus clone C10	JN054676	100	96
301_p2_18	1		●					Eimeriidae environmental sample clone	EF023410	100	89
CES301-22			●					Uncultured fungus clone HA052	HM487051	99	99
CES301-23			●					Uncultured fungus clone HA052	HM487051	99	96
301_p2_31			●					Chytridiomycota clone PA2009A3	HQ191313	100	96
CES303-24	1		●					Uncultured fungus clone HA052 18S	HM487051	100	95
CES304-25	1		●					Uncultured eukaryote clone F02_SE4A	FJ592434	100	97
CES305-26			●					Uncultured fungus clone C10	JN054676	100	96
CES3204-27			●					Uncultured fungus clone D21	JN054687	100	94
CES3204-28			●					Uncultured fungus clone D21	JN054687	94	97
3204_p2_16			●					Soil flagellate AND6 18S	AY965860	99	92
CES3204-29			●					Uncultured eukaryote clone Joinv23	FJ577832	100	93
CES3204-30			●					Uncultured soil fungus clone 564	GU568155	100	97
CES507-31			●					Uncultured eukaryote gene	AB695466	78	97
CES305-32	1		●	●				Uncultured fungus clone C10	JN054676	100	97
CES304-33	1			●				Uncultured eukaryote gene	AB695466	100	97
101_p3_11				●				Uncultured fungus clone Pa2007A1	JQ689418	99	96
CES304-34	1			●				*Rozella* sp. *JEL347 isolate*	AY601707	98	90
CES503-35	1			●				Uncultured fungus clone Pa2007A1	JQ689418	100	95
CES505-36				●				Uncultured fungus clone Pa2007A1	JQ689418	100	96
CES505-37				●				*Rozella allomycis 18S*	AY635838	99	98
CES302-38					●			Uncultured fungus clone Pa2007A1	JQ689418	99	95
CES103-39						●		Uncultured eukaryote clone A0Esp_2	KC911733	100	98
CES301-40							●	Uncultured alveolate clone G40 18S	EU910606	100	99

The 44 unique OTUs recovered with Cryptomycota-preferential primers from 12 samples via cloning (29) and direct sequencing (16). The sequence names not in bold type are confirmed Cryptomycota and included in the phylogeny (except for 101_p3_11). The five other sequences are considered non-Cryptomycota. OTUs were considered identical if sequences varied ≤3 bp, or had max identity scores above 99 %. These sequences recovered repeatedly are indicated in the "Additional Recoveries" column. Sequencing results were compared to GenBank including environmental sequences. Sample ID habitat key: 1- Michigan freshwater sediment, 2- Marine sediment, 3- Florida freshwater sediment, 5- Michigan soil. Primer combination key: p1- CRYPTO2-1F + AU4v2; p2- CRYPTO2-2F + AU4v2; p3- ROZELLA-1F + AU4v2; p4- ROZELLA-1F + ROZELLA-1R; p5- CRYPTO2-1F + CRYPTO2A-1R; p6- CRYPTO1B-1F + CRYPTO1B-1R. Optimum primer annealing temperatures: p1- 61 °C, p2- 58.6 °C, p3- 55 °C, p4- 55 °C, p5- 60 °C, p6- 60 °C.

were confirmed to successfully amplify Cryptomycota. A high percentage (89.3 %) of these recovered sequences were phylogenetically affiliated with Cryptomycota (Table 3). Two of the most versatile primer pairs (CRYPTO2-1F + AU4v2 and CRYPTO2-2F + AU4v2) are considered to be "Cryptomycota-preferential" as they also amplify the 18S rRNA fragment from control sequences, including the basidiomycetes *Mixia osmundae* and *Wolfiporia cocos* (Table 4). Primer pairs ROZELLA-1F + AU4v2 and ROZELLA-1F + ROZELLA-1R appear to be specific to Cryptomycota because they did not amplify control templates from other fungal phyla. On the other hand, ROZELLA-1F + AU4v2 and ROZELLA-1F + ROZELLA-1R only produced amplification products for 11/33 and 5/33 environmental DNA samples respectively, whereas CRYPTO2-1F + AU4v2 and CRYPTO2-2F + AU4v2 produced products for 22/33 and 28/33 (Table 1).

Using direct sequencing or subcloning of PCR products, we investigated the phylogenetic origin of the amplified products. Cloned samples contributed the majority of clean sequences, while direct sequencing usually resulted in overlapping chromatograms, indicating multiple templates per amplicon. The primers selected appear fairly specific to Cryptomycota with 50/56 of the total recovered sequences, and 39/44 unique OTUs grouping with *Rozella* and other Cryptomycota from GenBank (Fig 2). The five unique OTUs not grouping with Cryptomycota were excluded from the phylogeny and were most closely related to Chytridiomycota, Cercozoa, and Arthropoda (Table 3).

A quarter of the sequences (11/44) were recovered multiple times. Sequences were considered identical OTUs if they had max identity scores above 99 % or $\leq$3 bp differences. When duplicate sequences were recovered, they were usually from the same habitat and sample. However, the OTU named CES301-14 was recovered three times with sequencing, twice from a Florida freshwater sample (301) and once from a Michigan freshwater sample (103). Recovering identical sequences from regions so different geographically suggests there are cosmopolitan species of Cryptomycota.

Overall, recovering duplicate sequences was uncommon, even across the multiple considered studies of Cryptomycota (Fig 2), and we expect sampling our sites more thoroughly would reveal much more sequence diversity. All of our DNA extractions came from 250 mg of sediment or soil per sample, which is likely not truly representative of habitat diversity. A soil fungal diversity study (Taylor et al., 2010) found more than 200 fungal OTUs in an equivalent mass of soil in Alaska with only 14 % sequence overlap in a sample taken 1 m away. Sequence repeats within these small samples suggests there may not be hundreds of Cryptomycotan OTUs in less than 1 g of soil. On the other hand, because direct sequencing rarely resulted in clean DNA sequences, we believe that most samples contained multiple cryptomycete sequences amplifiable by the primers. Most samples (30/33) had amplifications from some primer combination and the more thorough analysis of five samples conducted via cloning yielded a high proportion of Cryptomycota (24/29). Therefore, we believe that many of the positives in Table 1, represent successful amplification of Cryptomycota, and label them as "suspected Cryptomycota" despite the absence of clean sequences to verify them.

By using samples from diverse habitat types, we aimed to reveal phylogenetic patterns to see whether certain clades are over-represented in various habitats. Previous studies have found Cryptomycota are highly diverse in freshwater habitats, yet have identified clades that were dominated by marine or aquifer sequences (Livermore and Mattes, 2013). We found freshwater sediment and soil representatives both widely distributed across the Cryptomycota phylogeny. Two clades (V and X) appeared to contain a high proportion of sequences from soil across studies, while three other clades (I, III, and VIII) had a high proportion of freshwater and waste water sequences (Fig 2; clades were assigned to groups with bootstrap support over 70 %).

The phylogenetic diversity of Cryptomycota was high within habitat types and within samples. For example, sample 507, Michigan soil from a mesic, deciduous forest contained sequence diversity that spanned 4/12 Cryptomycota clades (Fig 2). Considering that the diversification of the Cryptomycota may be as ancient as the rest of the fungi, given its phylogenetic placement as the sister-group to remaining fungi, the larger clades identified might be considered subphyla or classes, e.g., clades I, III, and V. By comparison to Fungi, subphyla tend to show high levels of ecological diversity, but also trends. For example, the Pucciniomycotina comprise saprobes, entomopathogens, and mycoparasites, but is primarily known for containing the plant pathogenic rusts (Aime et al., 2006). Other large fungal taxa, such as Dothideomycetes and Pezizomycotina could be similarly characterized as having exceedingly large ecological breadth, but some specialization. These clade-level differences can be detected when comparing across very different habitat types.

Table 4 – Ability of primer combinations to amplify non-Cryptomycota DNA from cultures. Primers appear to, nonetheless, preferentially amplify Cryptomycota in mixed DNA samples

	CRYPTO2-1F/AU4v2	CRYPTO2-2F/AU4v2	ROZELLA-1F/AU4v2	ROZELLA-1F/ROZELLA-1R
(+) control				
Rozella allomycis	x	x	x	x
(−) controls				
Mixia osmundae	x	x	–	–
Rhizopus sp.	–	–	–	–
Wolfiporia cocos	–	x	–	–
Archaeorhizomyces finlayi	x	x	–	–
Batrachochytrium dendrobatidis	x	–	–	–

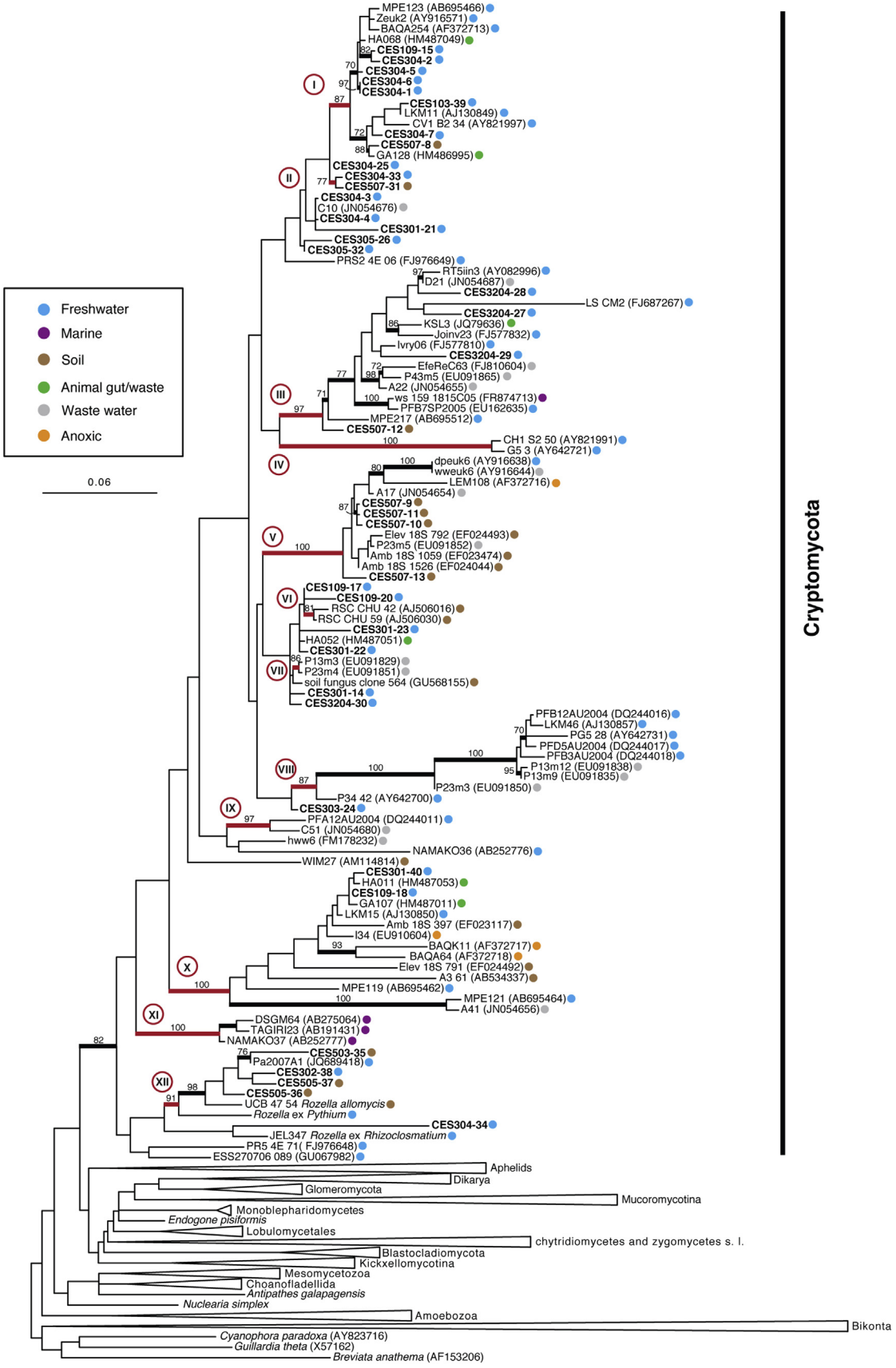

Fig 2 – 18S rDNA-based maximum likelihood (ML) phylogeny showing the diversity of Cryptomycota sequences and habitat from the environmental sampling in this study (bolded names) and GenBank sequences. The tree was constructed using 1 349 characters. Branches in bold type indicate ML bootstrap values ≥70 %. Twelve Cryptomycota clades (I–XII) with strong bootstrap support were identified. See Suppl. Fig 1 for the tree with the relationships among non-Cryptomycota sequences displayed.

For example, Alaska soils (Taylor et al., 2014) and coral reefs (Amend et al., 2012) are drastically different habitats whose fungal communities share many of the same taxonomic classes. However, the two communities differ dramatically when looking at the abundance of these classes. Leotiomycetes are much more common in soils than corals and *vice versa* for Eurotiomycetes, due to the propensity for the former to associate with plants and the latter with animals. By analogy, when sampling the numerous clades of Cryptomycota across habitats, we might expect to find almost all of them in a particular habitat type, but depending on the habitat, certain clades will be expected to predominate.

The proportion of unique Cryptomycota sequences relative to total Cryptomycota sequences recovered was highest in Michigan freshwater (6/7), and Michigan soil (10/12), and lowest in Florida freshwater (23/30). However, this finding is probably related to quality of sample site and depth of analysis. For example, sample 304 was a stream on University of Florida's campus, and although 10 unique Cryptomycota OTUs were found, it was from 14 successful Cryptomycota sequence recoveries (cloning and direct), using three primer combinations. This particular site is probably lower in overall sequence diversity, but the unequal sampling effort makes this conclusion tentative. The soil sample 507, from a beech maple forest in Oakland County, Michigan contained seven unique Cryptomycota OTUs from eight total Cryptomycota sequences using two primer combinations, by comparison. To fairly estimate which habitat types are most phylogenetically diverse for Cryptomycota more sequences need to be analyzed from samples and from multiple habitat types. Future studies include utilizing the most broad primer pair CRYPTO2-2F and AU4v2 to survey a diversity of habitats using next-generation sequencing methods.

Cryptomycota is a phylogenetically diverse but ecologically mysterious group of early-diverging, fungi or fungal-like eukaryotes. Their pervasiveness in soil and aquatic environments suggest they probably influence ecosystems significantly, through control of primary producer, consumer, and saprobe populations as parasites. The Cryptomycota-preferential primers developed and tested in this study will assist future efforts exploring this overlooked group and the planet's hidden fungal diversity in new environments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2014.11.004.

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