Surveying the biodiversity of the early diverging fungal phylum Cryptomycota using a targeted PCR approach

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TABLE OF CONTENTS

LIST OF FIGURES

Figure

LIST OF TABLES

Table

Abstract

Surveying the biodiversity of the early diverging fungal phylum Cryptomycota using a targeted PCR approach

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Cryptomycota (Greek: crypto-, hidden; + Greek myco, fungus) is a recently discovered, massively diverse, and widespread lineage of fungi, known primarily from environmental sequences. Only one formally described and successfully cultured genus, *Rozella*, exists to date. My study describes the biodiversity and ecological distribution of Cryptomycota among a variety of environmental samples, including freshwater, marine, soil, and *Daphnia*. Several studies on Cryptomycota diversity to date have detected the groups among unidentified eukaryote sequences from environmental molecular surveys. However, my study is unique for creating and using primers for the 18S rRNA gene designed to specifically target Cryptomycota sequences from the environment. This study expands the boundary of current Cryptomycota sequence diversity by detecting 44 unique OTUs distributed in 8 clades. I found the greatest diversity of Cryptomycota sequences from freshwater samples. The primers developed in this study can serve to the Cyptomycota diversity in future Cryptomycota molecular diversity studies.

CHAPTER I

Introduction and summary of work

1.1 Introduction

Humans are currently in a race against time to document the planet's fleeting biodiversity. The biodiversity of organisms is important to understand from an ecological perspective because ecosystem functioning is affected by the entire diversity of relationships among the organisms that inhabit it. Estimates of biodiversity have increased astoundingly with the advancements in molecular sampling methods, and the increased computational feasibility for analyzing sequence data of many taxa (Zwickl & Hillis, 2002). However, many overlooked areas still remain, including a group of early diverging fungi, recently named Cryptomycota or Rozellomycota (Jones et al. 2011a). Fungi in particular, are integral in a diversity of ecosystem functional roles, but a huge majority pose challenges for understanding because of our inability to culture and observe them (James & Berbee, 2012; Jones et al. 2011a; Rosling et al. 2011).

Cryptomycota's consideration as true fungi is contended because while Cryptomycota has fungal characteristics, it is distinct from other fungi by not having chitin in its cell wall during the trophic stage, the major growing stage, in its lifecycle (Held 1981). Chitin is considered an important wall constituent in fungi, allowing their characteristic osmotrophic feeding habit (Jones et al. 2011b). Even in other basal fungi, such as Chytridiomycota and Blastocladiomycota, species can be saprotrophs or epibiotic or endobiotic parasites, but present

evidence indicates that their nutrition is always osmotrophic (Stajich et al. 2009). The observed lack of chitin in *Rozella*'s trophic stage suggests a different feeding strategy, possibly the ancestral retention of the protistian ability to phagocytose host cytoplasm (Powell, 1984; Lara et al. 2010). Despite feeding habits unconventional to fungi, chitin has been observed in the inner layer of resting spores, and immature resting spores, in some species of *Rozella*, as indicated with calcofluor white stain and the presence of a fungal-specific chitin synthase gene (James & Berbee, 2012).

Rozella and Cryptomycota are likely closely related to other groups known to be protist-like obligate endoparasites, with reduced morphologies and genomes, the microsporidia. *Rozella* and microsporidia both lack a cell wall when invading host cells, so the plasma membrane of the parasite makes direct contact with the cytoplasm of the host cell (James et al. 2006b). Another group of endoparasites appears to be related to Cryptomycota, the aphelids, a group of endoparasitic algal parasites that are also suspected of phagocytosing host cytoplasm (Karpov et al. 2013).

Species of *Rozella* also ecologically and morphologically resemble some members of the phylum Chytridiomycota. Both require water for a uniflagellate zoospore stage (Lara et al. 2010). However, knowledge of life stages of Cryptomycota is still very incomplete. Only three morphologies have been observed in freshwater environments: uniflagellate zoospores, non-flagellated cells attached to other eukaryotic microorganisms (e.g. *Rozella* parasites of,

Blastocladiomycota, Oomycota, and heterotophic stramenopiles (Gleason et al. 2012)), and non-flagellate cysts (Jones et al. 2011a).

Ecological studies of *Rozella* revealed several species to be obligately biotrophic primary and hyper endoparasites, and they can only be grown in a dual culture with their hosts (Held 1981). *Rozella coleochaetis* is one species in Cryptomycota reported to be a primary parasite of the green alga *Coleochaete* (Sparrow 1965). Studies on parasitism in freshwater lakes often cite primary consumer zoosporic fungal parasites, especially chytrids, infecting phytoplankton primary producers, and they likely have some degree of regulation on producer populations (Kagami et al. 2007; Held 1981; Powell 1984; van Donk & Bruning 1995). *Rozella* and other parasitic fungi, especially on phytoplankton, usually have species- or genus-host level specificity, and the infection of one algal species can favor the development of other algal species through competitive release, which affects community composition, like that of cyanobacteria (Canter 1972). Several *Rozella* species are known to be secondary consumers or hyperparasites and likely regulate the size of populations of primary consumer parasitic zoosporic fungi in lakes (Gleason et al. 2012). The freshwater diatom *Asterionella formosa*, is often parasitized by the chytrid, *Zygorhizidium affluens*, which is often hyperparasited by *Rozella parva* (Reynolds 1984). Cryptomycota hyperparasitism of other fungal parasites of planktonic algae adds to trophic chain length and ecosystem complexity. Establishing these ecological roles of *Rozella* and their hosts is important to understand the complexities of food web functioning, stability. This information

could be useful for natural control systems for cyanobacteria (Canter 1972), and monitoring other ecosystem functions such as rates and efficiency of nutrient transfer (Gleason et al. 2012).

1.2 Summary of work

To learn more about the ecological roles and relationships of Cryptomycota, sequences from multiple environments were compared using phylogenetic and habitat data. I hypothesized that comparing 18S rRNA Cryptomycota gene sequence data from a variety of habitat types will yield distinct clades of Cryptomycota associated with particular habitats.

The diversity of Cryptomycota was investigated from 5 habitats: Florida and Michigan freshwater sediment, Florida marine water sediment, Michigan soil, and Michigan *Daphnia* spp. Within habitats, an effort was made to vary sampling sites conditions, such as forest type in soil samples, and depth of collection in sediment samples.

After collecting samples, DNA was extracted. A major novelty in this project was the development of Cryptomycota-targeting primers (developed using alignment of 18S rRNA sequences and not discussed further in this paper). Trials of various primer combinations and PCR conditions were attempted, with a primary objective of retrieving the largest diversity of Cryptomycotan sequences within these samples. We anticipated a phylogenetic analysis of these recovered sequences to be informative to the specificity of certain primers for certain clades of Cryptomycota.

CHAPTER II

Biodiversity and phylogeny of Cryptomycota

2.1 Introduction

Thus far, Cryptomycota have consistently branched with fungi in all phylogenies constructed, and are parsimoniously considered belonging to fungi because of the presence of a gene for chitin synthase II in their genomes (James et al. 2006 a, b, Lara et al. 2010, Jones et al. 2011b, James & Berbee 2012). A lack of observed chitinous cell walls during the trophic stage is not enough evidence to discern whether they are the most basal fungi lineage, because this characteristic is derived in other fungi. More accurate phylogenies for Cryptomycota within fungi will require additional taxon sampling (Zwickl and Hillis, 2002).

Molecular surveys have recovered Cryptomycota sequences from a variety of environments including lakes, rivers, a drinking water treatment system, aquifers, soil, and oceans (Jones et al. 2011b). Current sequencing data indicates Cryptomycota has extensive biodiversity, potentially larger than the rest of the known fungi (Jones et al. 2011b), however, due to limited sampling, the scope of that evolutionary and ecological diversity is still very narrow.

With the use of multiple Cryptomycota-targeting primers, we hoped to recover a wide range of Cryptomycota sequences from a variety of habitats. The distribution of these Cryptomycota sequences may serve as a basis to better understand the community compositions and dynamics of these organisms

within these sampled ecosystems. With a limited amount of sequence data, additional samples will greatly contribute to the extent of known Cryptomycotan diversity and the distribution of diversity among different ecosystems, especially with the use of this study's newly developed Cryptomycota-targeting primers.

2.2 Materials and Methods

Sampling

Samples were collected from Michigan and Florida in fall of 2012. Samples were collected in sterile 15 ml centrifuge tubes 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, varied in depth of collection (0.05-10m) 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 5 Florida freshwater sediment samples from University of Florida Natural Area Teaching Laboratory (NATL) and ponds on campus and 4 Florida Marine sediment samples from Honeymoon Island State Park were collected. These Florida samples varied in depths (0.05-1.0 m) and were all collected with sterilized turkey basters. *Daphnia* spp. samples were harvested from 11 lakes in Waterloo State Recreation Area and Pinckney State Recreation Area, using a plankton net. For each location, 100 *Daphnia* were separated from other organisms with a glass micropipette. A total of 12 Michigan soil samples were collected from the O horizon in Nichol's Arboretum, Pinckney State Recreation Area, Ella Mae Power Park, Rotary Park, and Manistee National Forest.

DNA extraction, PCR, and sequencing

DNA was extracted from sediment and soil samples using a MoBio PowerSoil DNA isolation kit. Freshwater and marine samples were first centrifuged at 4000 rpm for 6 minutes. 250mg of sediment or soil was used in the extraction protocol.

DNA was extracted from *Daphnia* after isolating and grinding with a pestle. 250 μl of 2X CTAB extraction buffer was added and the mixture was incubated at 65° C for 60 min, and vortexed once during incubation. An equal volume of chloroform-isoamyl alcohol (24:1) was added after incubation and the tube was shaken to an emulsion and periodically shaken for several minutes. Tubes were centrifuged at 13000 rpm for 12 min. The upper aqueous layer was removed with a micropipette and put in a new tube. A 2/3 volume cold isopropyl alcohol was added to the aqueous phase and mixed. This solution was stored in -20 °C over night to allow the DNA to precipitate. The next day the tube was centrifuged at 13000 rpm for 7 min and the supernatant poured out twice. 1 mL of ethanol was added, the tube was shaken gently, and the ethanol was poured out. Tubes were then dried in an Eppendorf Vacufuge for 10 min. Finally, DNA was resuspended in 50 μl of water and stored at -20 °C. DNA concentration for each sample was visualized with electrophoresis, and diluted ≈1-5 ng/μl.

Primers were created to target 18S rRNA gene region and modeled to selectively target Cryptomycota based on current sequence data of Cryptomycota (Figure 1). This study tested 18 primer combinations from 14 primers (8 forward, 6 reverse).

Primer	**Sequence 5'-3'**	**Specificity**
AU4v2	GCCTCACTAAGCCATTC	Cryptomycota
AU2	TTTCGATGGTAGGATAGDGG	Fungi
SR1R	TACCTGGTTGATYCTGCCAGT	Eukaryotes
SR6.1	TGTTACGACTTTTASTTCCTCT	Eukaryotes
CRYPTO2-1F	CAGTAGTCATATGCTTGTCC	Cryptomycota
CRYPTO2-2F	CACAGGGAGGTAGTGACAG	Cryptomycota
ROZELLA-1F	CGCAAATTACCCAATGG	*Rozella*
ROZELLA-1R	TTTCTCATAAGGTGCCRATGA	*Rozella*
CRYPTO1A-1F	TTGGATAACTGAGGTAATTCTT	Cryptomycota
CRYPTO1A-1R	GAATTTCACCTCTAACGTYTC	Cryptomycota
CRYPTO1B-1F	GACCTTGTGTCGACGACGTA	Cryptomycota
CRYPTO1B-1R	CCTCTAGCTTCGGAATACGA	Cryptomycota
CRYPTO2A-1F	AAAAACCAATGCCTTCGG	Cryptomycota
CRYPTO2A-1R	TGTCAATCCTTCCCATGTCC	Cryptomycota

Table 1: **Primers sequences** including the level of their specificity. Refer to Figure 1 for their relative locations on the 18S rRNA gene.

These primers varied in their specificity for Cryptomycota, and some primers combinations included one general eukaryote primer. The 5 best combinations were predominately used in sequencing. The primers and conditions, selected for amplifying the

sequences used in the phylogeny include: CRYPTO2-1F/AU4v2; CRYPTO2-2F/AU4v2; ROZELLA-1F/AU4v2; CRYPTO1B-1F/CRYPTO1B-1R; CRYPTO2-1F/CRYPTO2A-1R.

PCR products were sequenced by the University of Michigan DNA Sequencing Core. Samples that produced unclear, overlapping chromatograms (presumably because of amplifying more than one target sequence) were cloned to separate the multiple sequences.

Cloning

Cloning was performed on 8 of the samples that produced dirty chromatograms (2 soil, 2 *Daphnia*, 2 FL freshwater, 1 MI freshwater sediment, and 1marine sediment) using TOPO® TA Cloning Kit. The *E. coli* cells were plated on LB plates with Ampicillin and X-gal. *E.coli* cells that took the fungal plasmid were screened for by direct PCR of bacterial colonies using M13F and M13R primers at 55 C annealing. A total of 18 cloning PCR products were sequenced.

Sequence analysis, alignment, and phylogenetic analysis

Sequence chromatograms were analyzed, cleaned, and assembled using Sequencher 4.1. Samples producing noisy chromatograms were cloned and reanalyzed. Cleaned sequences were compared to sequences in GenBank using BLASTn (Table 2). A phylogeny was constructed of the sequences from this study by 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 manually constructed in MACCLADE 4.08 (Maddison & Maddison 2000), and the phylogeny was estimated with PHYMLv2.4.4 (Guindon & Gascuel 2003). The best fitting model of substitution (Tim3 + G) for analysis was selected using the Akaike Information Criterion in JMODELTEST 0.1.1 (Posada 2008).

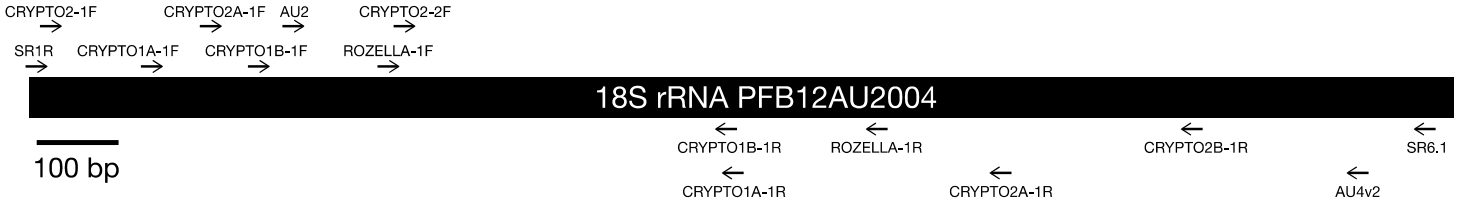

Figure 1: **Primer map** the location and amplification directions of the primer sequences on the 18S rRNA gene

2.3 Results

DNA was extracted from 47 samples collected for this study (15 Michigan freshwater, 5 Florida freshwater, 4 marine, 11 *Daphnia*, and 12 soil), 37 samples were tried in PCR amplifications, and 35 had DNA amplified by one of the top 5 Cryptomycota-targeting primer combinations (Table 3). Some proportion of this amplified DNA were not Cryptomycota based on DNA sequencing results, although the vast majority of clean, uncloned sequences were (Table 2). The greatest proportion of usable and informational sequences came from cloning.

The 11 sequenced clone colonies from primer combination CRYPTO2-2F/AU4v2 consistently produced cleanest, most easily assembled sequences, with only one non-Cryptomycotan (301.1_34_clone), probably a chytrid.

The 4 sequenced clone colonies from primer combination CRYPTO1-2F/AU4v2 forward and reverse sequences were not assembled 2/4 of the time (presumable due to the length of the fragments), but yielded no non-Cryptomycotan sequences.

The 3 sequenced clone colonies from primer combination CRYPTO2-1F/CRYPTO2A-1R were all from *Daphnia* and yielded exclusively non-Cryptomycotan sequences, *Skistodiaptomus* sp. (copepod) and *Arachnula* sp. (vampyrellid, data not shown).

A total of 13 clades of Cryptomycota were observed with bootstrap supports greater than 50%, denoted with a bold internode (Figure 2). The color designations were generalized for the location and habitat information provided

with the environmental sequences in GenBank amongst 5 major categories: freshwater, marine, soil, animal waste, wastewater, and anoxic environments.

Of the 13 Cryptomycota clades, 5 clades contained representatives of one habitat exclusively, 6 clades contained 2 habitat types, and 2 clades contained representatives from 3 habitat types. Of the 44 OTUs in this study, 23 were distributed among 8 of the 13 Cryptomycota clades.

A fair diversity of clades were targeted by the 5 primer pairs, covering 8 of the 13 Cryptomycota clades. The primer combination CRYPTO2-2F/AU4v2 targeted members in clades 4, 6 and 7. CRYPTO2-1F/AU4v2 targeted clades 3 and 8. ROZELLA-1F/AU4v2 targeted clades 1 and 10. CRYPTO2-1F/CRYPTO2A-1R targeted a member in clade 3. CRYPTO1B-1F/CRYPTO1B-1R was able to target clade 13.

The 5 primer combinations that amplified the sequences for constructing this phylogeny frequently amplified multiple sequences per sample, and occasionally targeted non-Cryptomycotan sequences to varying degrees.

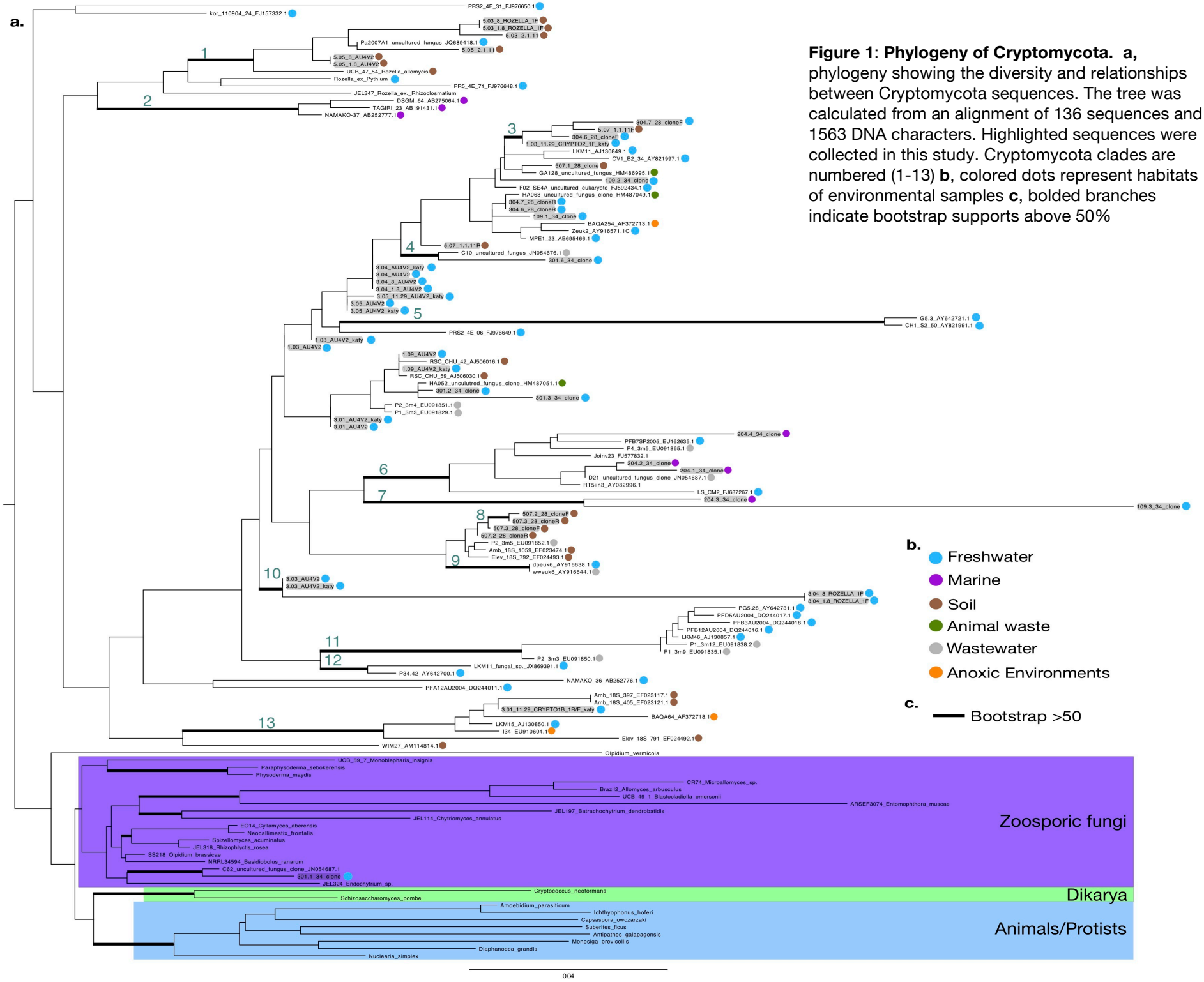

Figure 1: **Phylogeny of Cryptomycota. a,** phylogeny showing the diversity and relationships between Cryptomycota sequences. The tree was calculated from an alignment of 136 sequences and 1563 DNA characters. Highlighted sequences were collected in this study. Cryptomycota clades are numbered (1-13) **b**, colored dots represent habitats of environmental samples **c**, bolded branches indicate bootstrap supports above 50%

Sample name	F-Primer	R-Primer	Habitat	BLASTn Results: Excluding Uncultured/Environmental Sample Sequences	Query Coverage	Max ID	Including Uncultured/Environmental Sample Sequences	Query Coverage	Max ID
>3.04_1.8_ROZELLA-1F	ROZELLA-1F	AU4v2	FWFL	Rozella sp. JEL347 isolate	98%	90%	Rozella sp. JEL347 isolate	98%	90%
>3.04_1.8_AU4V2	ROZELLA-1F	AU4v2	FWFL	Fungal sp. LKM11	100%	92%	Uncultured eukaryote gene	100%	97%
>5.03_1.8_ROZELLA-1F	ROZELLA-1F	AU4v2	SOIL	Rozella allomycis	100%	91%	Uncultured fungus clone Pa2007A1	100%	94%
>5.05_1.8_AU4V2	ROZELLA-1F	AU4v2	SOIL	Rozella allomycis	100%	94%	Uncultured fungus clone Pa2007A1	100%	96%
>5.03_8_ROZELLA-1F	ROZELLA-1F	AU4v2	SOIL	Rozella allomycis	100%	91%	Uncultured fungus clone Pa2007A1	100%	94%
>5.05_8_AU4V2	ROZELLA-1F	AU4v2	SOIL	Rozella allomycis	100%	94%	Uncultured fungus clone Pa2007A1	100%	96%
>3.04_8_ROZELLA-1F	ROZELLA-1F	AU4v2	FWFL	Rozella sp. JEL347	98%	90%	Rozella sp. JEL347	98%	90%
>3.04_8_AU4V2	ROZELLA-1F	AU4v2	FWFL	Fungal sp. LKM11	100%	92%	Uncultured eukaryote gene	100%	97%
>1.03_AU4V2	CRYPTO2-2F	AU4v2	FWMI	Fungal sp. LKM11	99%	94%	Uncultured fungus clone HA052	100%	97%
>1.09_AU4V2	CRYPTO2-2F	AU4v2	FWMI	Fungal sp. LKM11	100%	94%	Uncultured fungus clone HA052	100%	98%
>3.01_AU4V2	CRYPTO2-2F	AU4v2	FWFL	Fungal sp. LKM11	100%	93%	Uncultured fungus clone HA052	100%	97%
>3.03_AU4V2	CRYPTO2-2F	AU4v2	FWFL	Fungal sp. LKM11	100%	92%	Uncultured fungus clone HA052	100%	95%
>3.04_AU4V2	CRYPTO2-2F	AU4v2	FWFL	Fungal sp. LKM11	100%	94%	Uncultured eukaryote clone F02_SE4A	100%	98%
>3.05_AU4V2	CRYPTO2-2F	AU4v2	FWFL	Fungal sp. LKM11	100%	94%	Uncultured fungus clone C10	100%	96%
>1.03_11.29_CRYPTO2-1F_katy	CRYPTO2-1F	CRYPTO2A-1R	FWMI	Hyaloraphidium curvatum strain	98%	87%	Uncultured eukaryote gene	99%	98%
>3.05_11.29_AU4V2_katy	CRYPTO2-2F	AU4v2	FWFL	Fungal sp. LKM11	95%	94%	Uncultured fungus clone	100%	97%
>3.01_11.29_CRYPTO1B-1R/F_katy	CRYPTO1B-1F	CRYPTO1B-1R	FWFL	Endochytrium ramosum isolate JEL 402	99%	98%	Uncultured alveolate clone G40	99%	99%
>1.03_AU4V2_katy	CRYPTO2-2F	AU4v2	FWMI	Fungal sp. LKM11	100%	94%	Uncultured fungus clone HA052	100%	97%
>1.09_AU4V2_katy	CRYPTO2-2F	AU4v2	FWMI	Fungal sp. LKM11	97%	93%	Uncultured fungus clone HA052	100%	98%
>3.01_AU4V2_katy	CRYPTO2-2F	AU4v2	FWFL	Fungal sp. LKM11	100%	93%	Uncultured fungus clone HA052	100%	97%
>3.03_AU4V2_katy	CRYPTO2-2F	AU4v2	FWFL	Fungal sp. LKM11	100%	93%	Uncultured fungus clone HA052	100%	96%
>3.04_AU4V2_katy	CRYPTO2-2F	AU4v2	FWFL	Fungal sp. LKM11	100%	94%	Uncultured eukaryote gene	100%	98%
>3.05_AU4V2_katy	CRYPTO2-2F	AU4v2	FWFL	Fungal sp. LKM11	93%	93%	Uncultured fungus clone	100%	96%
>5.07_1.1.11F	CRYPTO2-2F	AU4v2	SOIL	Powellomycetaceae sp.	99%	94%	Uncultured fungus clone	99%	98%
>5.07_1.1.11R	CRYPTO2-2F	AU4v2	SOIL	Rozella sp. JEL347 isolate	99%	91%	Uncultured eukaryote gene	100%	97%
>5.03_2.1.11	ROZELLA-1F	AU4v2	SOIL	Rozella allomycis	99%	92%	Uncultured fungus clone Pa2007A1	100%	95%
>5.05_2.1.11	ROZELLA-1F	AU4v2	SOIL	Rozella allomycis	99%	98%	Rozella allomycis	99%	98%
>301.6_34_clone	CRYPTO2-2F	AU4v2	FWFL	Catenomyces sp. JEL342	95%	89%	Uncultured fungus clone C10	95%	96%
>204.1_34_clone	CRYPTO2-2F	AU4v2	MFL	Candida sp. BG01-7-26-005A-2-1	95%	87%	Uncultured fungus clone D21	95%	94%
>507.1_28_clone	CRYPTO2-1F	AU4v2	SOIL	Hyaloraphidium curvatum strain SAG 235-1	96%	88%	Uncultured eukaryote	95%	98%
>507.2_28_cloneF	CRYPTO2-1F	AU4v2	SOIL	Fungal sp. LKM11	96%	93%	Eimeriidae environmental sample clone	97%	99%
>507.2_28_cloneR	CRYPTO2-1F	AU4v2	SOIL	Rhizophydium elyensis isolate	95%	85%	Eimeriidae environmental sample clone	95%	96%
>507.3_28_cloneF	CRYPTO2-1F	AU4v2	SOIL	Rhizophydium elyensis isolate AFTOL-ID 693LKM11	96%	85%	Eimeriidae environmental sample clone	96%	95%
>507.3_28_cloneR	CRYPTO2-1F	AU4v2	SOIL	Fungal sp. LKM11	95%	93%	Eimeriidae environmental sample clone Amb_18S_883	95%	99%
>304.7_28_cloneR	CRYPTO2-1F	AU4v2	FWFL	Fungal sp. LKM11	95%	95%	Uncultured fungus clone HA068	95%	99%
>109.1_34_clone	CRYPTO2-2F	AU4v2	FWMI	Endogone aggregata voucher OSC:130580	94%	89%	Uncultured fungus clone HA068	94%	99%
>109.2_34_clone	CRYPTO2-2F	AU4v2	FWMI	Hyaloraphidium curvatum strain SAG 235-1	95%	88%	Uncultured fungus clone GA128	94%	95%
>109.3_34_clone	CRYPTO2-2F	AU4v2	FWMI	Cercozoa sp. TGS3 gene	95%	97%	Uncultured eukaryote clone Amb_18S_1350	95%	98%
>204.2_34_clone	CRYPTO2-2F	AU4v2	MFL	Gaertneriomyces semiglobifer strain BK91-10	94%	88%	Uncultured fungus clone D21	94%	97%
>204.3_34_clone	CRYPTO2-2F	AU4v2	MFL	Cercomonas sp. HFCC 901	95%	92%	Soil flagellate AND6	95%	92%
>204.4_34_clone	CRYPTO2-2F	AU4v2	MFL	Rhizophlyctis rosea strain JEL 318	94%	87%	Uncultured eukaryote clone Joinv23	95%	93%
>301.1_34_clone*	CRYPTO2-2F	AU4v2	FWFL	Catenomyces sp. JEL342	94%	94%	Uncultured fungus clone C62	94%	95%
>301.2_34_clone	CRYPTO2-2F	AU4v2	FWFL	Blastocladiales sp.	96%	88%	Uncultured fungus clone HA052	96%	99%
>301.3_34_clone	CRYPTO2-2F	AU4v2	FWFL	Triparticalcar arcticum	95%	88%	Uncultured fungus clone HA052	95%	96%

Table 2: **BLASTn results by sample**. All sequences that nested within Cryptomycota. Query coverage is the percent of the query sequence from GenBank that overlaps the subject sequence. Max Identity is the percent similarity between the query and subject sequences over the length of the coverage area. The BLASTn hit with the highest query coverage and max identity scores (generally uncultured or environmental sequences), were added to the phylogeny. FWFL= freshwater Florida; FWMI= freshwater Michigan; MFL= marine Florida. *Determined non-Cryptomycota

Sample #	Location	Primer: CRYPTO2-1F/ AU4v2 Annealing temp ° C: 61° C	Primer: CRYPTO2-2F/ AU4v2 Annealing temp ° C: 58.6° C	Primer: ROZELLA-1F/ AU4v2 Annealing temp ° C: 55° C	Primer: CRYPTO1B-1F/ CRYPTO1B-1R Annealing temp ° C: 60° C	Primer: CRYPTO2-1F/ CRYPTO2A-1R Annealing temp ° C: 55° C
1.01	UM Herbarium pond	x	x		x	x
1.02	Cedar Lake		x			
1.03	Crooked Lake	xCLONED	**x**	x	o	**x**
1.04	Mill Lake		o	o		
1.05	Pickerel Lake	x	x	o	x	x
1.06	Pickerel Lake		X	x		
1.07	Bruin Lake		O	o		
1.08	Gosling Lake		O			
1.09	Gosling Lake		**xCLONED**			
1.1	Halfmoon Lake		X			
1.11	Crooked Lake		X			
1.12	South Lake		X			
1.13	North Lake		X			
1.14	Sullivan Lake		X			
1.15	Walsh Lake		X			
2.01	Honeymoon Island W	X	X		x	o
2.02	Honeymoon Island W					
2.03	Honeymoon Island E	X	o	o	o	o
2.04	Honeymoon Island marsh		**xCLONED**			
3.01	Gainesville pond	x	**xCLONED**/xCLONED	o	**x**	x
3.02	UF campus pond	x	o	xCLONED		
3.03	UF campus pond	x	**x**	x	x	x
3.04	UF campus stream	**xCLONED**	**x**	xCLONED		
3.05	UF NATL pond	x	**x**	x	x	x
4.01	Cedar Lake	o	x		x	xCLONED
4.02	Mill Lake		o			x
4.03	Walsh Lake	x	o	o	x	x
4.04	South Lake		o			xCLONED
4.05	Pickerel Lake		o			x
4.06	Sullivan Lake		o			xCLONED
4.07	Crooked Lake		o			x
4.08	Appleton Lake		o			x
4.09	Little Appleton Lake		o			xCLONED
4.1	Whitmore Lake		O			
4.11	Woodland Lake		O			
5.01	Crooked Lake	x	xCLONED		x	o
5.02	Nichol's Arboretum		X			
5.03	Nichol's Arboretum	x	X	xCLONED	x	o
5.04	Ella Mae Power Park	o	X	o		
5.05	Rotary Park	o	X	**x**		
5.06	Ella Mae Power Park	x	X	o		
5.07	9 Mile/Meadowbrook	**xCLONED**	**X**	o		
5.08	Ella Mae Power Park		X			
5.09	9 Mile/Meadowbrook		X			
5.1	Rotary Park		X			
5.2	Manistee Nat Forest					
5.21	Manistee Nat Forest					
	(-) control *Mixia osmmundae*	o	o	o	x	x
	(+) control *Rozella allomycis*	**x**	**x**	**x**	**x**	**x**

Table 3: **Primer success by sample.** The 5 primer combinations, their optimal annealing temperatures, and samples attempted in this study. x= observable bands after PCR; o= attempted, with no PCR amplification; xCLONED= PCR product was cloned; **confirmed Cryptomycota DNA**/confirmed non-Cryptomycota DNA. Samples: 1.01-1.15, Michigan freshwater; 2.01-2.04, Florida marine; 3.01-3.05, Florida freshwater; 4.01-4.11. Daphnia; 5.01-5.21. Soil.

2.4 Discussion

Clades with 50% and higher bootstrap support usually included environmental reference sequences from GenBank, with the exception of clades 3, 7, 8, and 10, which were formed with sequences from this study only. The top GenBank matches for the samples in this study, discovered using BLASTn (Table 2), were also added to the phylogeny. Considering our inclusion of GenBank's similar Cryptomycota environmental samples, these data support that these clades 3, 7, 8, and 10 are newly documented.

Cryptomycota appear to be extremely diverse among freshwater habitats, and only one of the 13 clades lacks a freshwater representative, with 55 total freshwater sequences. This apparent diversity may partially be a product of the study's bias towards freshwater samples. In all, 14/35 samples with amplified DNA were from Florida or Michigan freshwater. Cryptomycota from marine environments, in which they have been scarcely documented (Jones et al. 2011b), span a diverse 3 clades with 7 sequence representatives (4 from this study). Cryptomycota in soil was limited to only 4 clades, despite having 21 sequence representatives (9 from this study).

Little target overlap was observed between primers, and the ones selected for this study appear to have a fairly wide range for targeting Cryptomycota. Clades varied greatly in their sequence quantities and habitat proportions. Some clades, such as 2, 5 and 8 were highly specific and contained representatives from a single habitat type.

No clean sequences of Cryptomycota were found in any samples of *Daphnia* in this study. This may be a result of only cloning *Daphnia* samples using one unsuccessful primer pair, CRYPTO2-1F/CYPTO2A-1R, amplifying sequences similar to *Skistodiaptomus* sp*.* and *Arachnula* sp*.* (data not shown). This pair did successfully in amplify Cryptomycota DNA from a Michigan freshwater ecosystem. However, the fact that CRYPTO2-1F/CRYPTO2A-1R also amplified the positive control, *Mixia osmundae* (dikaryotic fungus*),* and other non-target sequences indicates the pair is not very Cryptomycota specific. Further efforts to amplify Cryptomycota in these *Daphnia* samples may include the use of different, or more specific primer pairs, such as CRYPTO1B-1F/CRYPTO1B-1R.

Future studies could test additional potential primer combinations in untested samples, especially from underrepresented habitats on the phylogeny. Two of the preliminarily tested primer combinations, CRYPTO2A-1F/AU4v2 and ROZELLA-1F/ROZELLA-1R, yielded promising results by selectively amplifying the positive control, *R. allomycis,* and not the negative control, *Mixia osmundae*. These pairs were only tested on several representative samples, and the DNA was not sequenced.

Cloning contributed a significant number of high-quality sequences to this study. A more thorough analysis of already cloned samples and additional cloning in more samples should be conducted. Of the 8 cloned environmental samples, 7 yielded a diversity of Cryptomycota sequences. Sufficient confidence that all sequences from a multi-sequence environmental sample are

represented requires 6+ *E. coli* colonies per sample to be selected for amplification.

The extensive range in biodiversity of the sequences recovered with our targeting primers further supports that Cryptomycota is a highly diverse lineage, especially in freshwater habitats. The fact that this massive diversity of organisms is only now being documented suggests they may have very integrated ecosystem relationships, such as the parasitic habits observed in species of the genus *Rozella*. Our emerging knowledge of the evolutionary relationships and habitat preferences between clades of Cryptomycota will help us explain their ecological roles and understand greater intricacies in ecosystem functioning.

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