Testate amoebae (Arcellinida, Amoebozoa) community diversity in New England bogs and fens assessed through lineage-specific amplicon sequencing

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ABSTRACT

Testate amoebae (order Arcellinida) are abundant in freshwater ecosystems, including low pH bogs and fens. Within these environments, Arcellinida are considered top predators in microbial food webs and their tests are useful bioindicators of paleoclimatic changes and anthropogenic pollutants. Accurate species identifications and characterizations of diversity are important for studies of paleoclimate, microbial ecology, and environmental change; however, morphological species definitions mask cryptic diversity, which is a common phenomenon among microbial eukaryotes. Lineage-specific primers recently designed to target Arcellinida for amplicon sequencing successfully captured a poorly-described yet diverse fraction of the microbial eukaryotic community. Here, we leveraged the application of these newly-designed primers to survey the diversity of Arcellinida in four low-pH New England bogs and fens, investigating variation among bogs (2018) and then across seasons and habitats within two bogs (2019). Three OTUs represented 66% of Arcellinida reads obtained across all habitats surveyed. 103 additional OTUs were present in lower abundance with some OTUs detected in only one sampling location, suggesting habitat specificity. By establishing a baseline for Arcellinida diversity, we provide a foundation to monitor key taxa in habitats that are predicted to change with increasing anthropogenic pressure and rapid climate change.

INTRODUCTION

Testate amoebae (Arcellinida, Amoebozoa) are unicellular eukaryotes that thrive in freshwater habitats, including lakes, soils, and low-pH bogs and fens (Mitchell et al., 2008; Swindles et al., 2016). Within aquatic microbial food webs, Arcellinida consume bacteria, fungi, algae, and small metazoa (Creevy et al., 2018; Foissner, 1987; Geisen et al., 2015; Gilbert et al., 2000; Yeates and Foissner, 1995). In addition, some species have established symbioses with photosynthetic algae (Gomaa et al., 2014; Weiner et al., 2022). When the amoebae perish, their tests are preserved in peats and sediments, creating a fossil record that has been incorporated into studies of paleoclimate and early eukaryotic evolution (Kosakyan et al., 2016; Lahr et al., 2015, 2019; Mitchell et al., 2008; Porfirio-Sousa et al., 2017; Porter, 2016; Porter et al., 2003). Arcellinida are also sensitive to environmental perturbations and have been used as indicator taxa to evaluate habitat quality (Nasser et al., 2020; Soler-Zamora et al., 2021; Whittle et al., 2019).

The size, shape, and composition of the amoeba test has historically been used to define species within the Arcellinida (Jassey et al., 2011; Kosakyan et al., 2016; Meisterfeld, 2002; Mieczan and Tarkowska-Kukuryk, 2017; Steele et al., 2018). These morphological classifications serve as the foundation for bioindicator and fossil record studies. However, recent studies using various molecular markers examined the genetic diversity of closely-related species of *Nebela* (Heger et al., 2011; Kosakyan et al., 2013, 2012; Lara et al., 2008; Singer et al., 2015), *Difflugia* (Gomaa et al., 2015, 2012; Macumber et al., 2020), and *Hyalosphenia* (Oliverio et al., 2015, 2014) and demonstrated the presence of cryptic lineages (Heger et al., 2013; Kosakyan et al., 2012; Oliverio et al., 2014; Singer et al., 2019). Efforts to resolve the diversity and geographic distribution of Arcellinida are on-going; such information is critical to understand

small-scale distribution patterns, investigate the molecular mechanisms that underlie niche environmental adaptations, and detect changes in response to environmental disturbances.

Morphological and genetic studies of Arcellinida biogeography have highlighted limited distribution patterns correlated with environmental factors (Heger et al., 2013; Singer et al., 2019; Smith et al., 2008). For example, the taxa *Lesquereusia*, *Planocarina carinata*, and larger *Difflugia* were observed in moist environments with higher pH optima, while *Hyalosphenia subflava* and *Nebela tincta* exhibit opposite habitat preferences (Booth, 2001). Several other studies of biogeography have identified relationships between Arcellinida community composition and soil moisture content (Heal, 1964; Mitchell et al., 2008; Warner et al., 2007), pH, and macronutrients (Mitchell et al., 2008; Tolonen et al., 1992). Genetic analyses of variable regions in the SSU-rDNA and the mitochondrial cytochrome oxidase gene subunit 1 (COI) detected multiple genotypes within the species *Hyalosphenia papilio* and noted their restricted distribution patterns that were not detected when assessing the distribution of the morphospecies (Heger et al., 2013; Oliverio et al., 2014; Singer et al., 2019). This underscores the need for molecular tools in studies of Arcellinida biodiversity and biogeography. Ruggiero et al. (2020) addressed this need by developing Arcellinida-specific primers that target the hypervariable V6 and V7 regions of the SSU-rDNA. These primers were used to investigate the spatial variability of Arcellinida communities along a transect from the forest to the water's edge in a low-pH bog (Hawley Bog, Hawley, MA, USA), and detected habitat preferences within some lineages (Ruggiero et al., 2020).

In this study, we expand on the work of Ruggiero et al. (2020) by deploying the Arcellinida-specific primers to characterize the testate amoebae communities across four low-pH bogs and fens in New England sampled across multiple locations in 2018 and then intensively

within two locations in the spring and fall of 2019. Though we precluded from replicating sampling in 2020, we examine both geographic and seasonal diversity in Arcellinida across these samples, establishing a baseline for future molecular studies in similar habitats.

METHODS

Sampling Approach

Arcellinida community diversity was assessed in samples collected from low-pH bogs and fens across New England, USA, in 2018 and 2019 (Figure S1 and Table S1). Sampling sites included Hawley Bog (Hawley, MA), Harvard Forest (Petersham, MA), Big Heath Bog (Acadia National Park, ME), and Orono Bog (Orono, ME). In the spring and summer of 2018, we aimed to capture variation at broad geographic scales (Table 1), sampling three bogs – Big Health (Maine), Harvard Forest (Massachusetts) and Hawley Bog (Massachusetts). In 2019, we sampled across habitats within two bogs (Hawley Bog and Orono Bog (Maine), visiting each location only twice. Regrettably, replication in 2020 was precluded by the pandemic.

The focal bogs vary in abiotic and biotic features. Hawley Bog, Orono Bog and Big Heath Bog are characterized as low-nutrient bogs with floating *Sphagnum* mats (Davis and Anderson, 2001; Kearsley, 1999). Harvard Forest, on the other hand, represents a nutrient-rich fen with a low-pH pond (Swan and Gill, 1970). Collectively, these sites represent ecologically and geographically distinct ecosystems to explore testate amoebae diversity. To sample the Arcellinida community, moss was removed at each sampling site and stored in a container for transport back to the laboratory. At the time of sampling, environmental parameters including pH, overall moisture level (wet vs. dry), and vegetation density were recorded (Table S1).

Sampling Frequency

From April to October 2018, Hawley Bog and Harvard Forest were sampled monthly excluding June. The two sampling sites in Maine (Big Heath Bog and Orono Bog) were sampled in May

and September 2018. At Hawley Bog, two transects spanning approximately 30 m were sampled at ~6-10 m intervals from the forest edge across the bog to open water. Harvard Forest was sampled at three locations: along a roadside and at two sites on the shore of Harvard Pond. Acadia Bog and Orono Bog were sampled from sites along boardwalks that traversed the open bog (Figure S1; Table S1).

For 2019, sampling of Hawley Bog occurred twice in June and once in September. In June, higher resolution transects sampled 15 sites, ~5-10 m apart, from the forest edge to open water. In September, transects across various environments in Hawley Bog were collected: two from the open bog, two from the forest edge, and one from the sedges along the open water. These transects were sampled at 4 m intervals. Several individual samples were collected from transition zones, described as qualitative shifts in vegetation from the forest edge to the open bog and from the open bog to sedges. Orono Bog was sampled in May and October 2019. Samples were collected from 12 sites along the boardwalk, spanning the forest to the open bog.

Sample Preparation

Arcellinida were isolated for microscopy by placing five moss strands in a 50 mL conical tube with 30 mL of in-situ water corresponding to each field site. In-situ water was filtered through a 2 μm Isopore filter (MilliporeSigma, MA, USA) and autoclaved to minimize microbial growth . Tubes were gently inverted to dislodge Arcellinida, then the water was poured through a 300 μm sieve into a petri dish to remove the moss and larger particles. All samples were prepared in duplicate except those collected in September and October 2018.

For molecular analyses, 7.5 g of moss (wet weight) was weighed from each sample within one day of collection and placed in a 50 mL conical tube. The moss was washed using the

same technique described for the microscopy samples. Water collected in the petri dish was then returned to the 50 mL conical tube and placed in a centrifuge for 1 minute at 1,800 rcf. The supernatant was removed, then 1 mL RLT buffer (Qiagen, USA) was added to each sample and mixed gently by pipetting. Samples were divided evenly into four 1.5 mL centrifuge tubes and stored at -80°C for DNA and RNA extraction (two replicates each). One replicate for each extraction method was chosen at random for amplification and sequencing, with the other remained stored at -80°C in the event of failure in downstream analyses.

DNA and RNA Extraction, Amplification, and Sequencing

DNA was extracted using the ZR Soil Microbe DNA Miniprep™ extraction kit (Zymo Research, CA, USA). RNA was extracted from samples using the Qiagen RNeasy Mini Kit (Qiagen, USA). DNA contamination was removed from RNA extractions using the TURBO DNA-free™ Kit (Invitrogen, USA), then RNA was reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis System (Thermofisher, USA) following the standard manufacturer's protocol. All extracts were stored at -20°C. Selected samples (n=201, Table S2) were amplified using the Arcellinida-specific primers, ARC SSU 2088 and ARC SSU 2435, which target the hypervariable V6 and V7 regions of the SSU- rDNA (approximately 210 base pairs; Ruggiero et al., 2020). Cycling conditions were identical to those described in Ruggiero et al., 2020. All PCRs were performed in triplicate to minimize PCR bias (Jung et al., 2012; Lahr and Katz, 2009), which can lead to the formation of chimeras and the overrepresentation of biodiversity. Gel electrophoresis was performed to confirm sample amplification and PCR products were pooled. A second PCR was performed to add sequence library adapters to the PCR product (described in Ruggiero et al., 2020); to avoid overamplification at this step, we dilute

PCR products either 1:10 or 1:50 for weak and bright bands respectively, and then sent the products to University of Rhode Island Genomics and Sequencing Center for paired-end sequencing on the Illumina MiSeq 2 X 150 platform.

Cell Counts

Testate amoebae were identified and enumerated from live samples (n=329) using an inverted light microscope. Each sample prepared in a Petri dish was examined for 20 minutes to ensure consistent survey effort across samples as our goal was to compare abundant taxa across samples, and not characterize every species in each sample. We counted only morphologically-distinct Arcellinida genera *Heleopera* sp., *Nebela* sp., *Arcella sp*., *Centropyxis* sp., and *Difflugia* sp., as well as the morphospecies *Hyalosphenia elegans* and *Hyalosphenia papilio*. All counts were completed within a week of sampling and some moss samples were prepared and counted in duplicate, with the results averaged in downstream analyses (Table S4).

Data Analysis

Sequences were trimmed to remove adaptors by the University of Rhode Island Genomics and Sequencing Center and quality-checked using the methods described in Sisson et al. (2018) and documented in GitHub (https://github.com/jeandavidgrattepanche). In brief, operational taxonomic units (OTUs) were identified using the software program SWARM (v2), with a parameter distance of 1 to account for SNPs that could represent experimental error (Mahe et al., 2015). The NCBI comparative basic local alignment search tool (BLAST) was used to identify and remove non-Arcellinida OTUs. Sequences that did not have E-value ratios higher than 10^{-15} were removed. For taxonomic assignment, a phylogenetic tree was built with the identified Arcellinida OTUs, other Amoebozoa taxa, and outgroups downloaded from GenBank (Table

S3). A second tree was constructed that contained only Arcellinida taxa (Figure 1). Arcellinida, Amoebozoa and outgroup sequences were aligned using the L-INS-i algorithm in MAFFT (Katoh and Standley, 2013) and a constrained tree was produced using RAxML v 8.0 using the substitution model GTRGAMMA (Stamatakis, 2014). To compare Arcellinida communities across samples, OTUs were rarified to 5,000 reads per sample (a number chosen after visual inspection of quality of all sections) and samples containing less than 5,000 reads were excluded from further analyses. This approach allows us to compare the relative abundance of OTUs across samples. The software package ggplot2 (v3.4.0; Wickham, 2014) available in R (v 4.2.2; R Core Team, 2022) was used to construct bar plots demonstrating the relative abundances of different Arcellinida taxa in the samples. An annotated tree was produced with iToL V5 (Letunic and Bork, 2021).

RESULTS

With the goal of furthering our understanding of Arcellinida community diversity, we assessed both DNA and RNA amplicon libraries and compared the resulting ~210 bp OTUs to existing sequences on GenBank. We sampled in two distinct efforts: across three sites at various intervals (determined based on accessibility from our lab) in 2018 and then within sites in two seasons in 2019 (Table 1). We were unable to repeat within site sampling in 2020 given the global pandemic, and so we focus here on broad biodiversity patterns.

Sequencing Arcellinida communities across four low-pH New England bogs and fens yielded 1,155,000 reads (after curation, see methods) from 231 samples (Table S1) that clustered into 106 Arcellinida OTUs (Table S2). These OTUs are presented in a phylogenetic tree (Figure 1) that includes 56 additional sequences from GenBank representing the diverse Arcellinida

taxonomic groups available at the time of the study (2019-2021; Table S4). We assigned preliminary species designation using NCBI BLAST (Table S2) and then updated these taxonomic assignments based on the position of the OTUs on the phylogenetic tree constructed with high-quality references (Table S2, Fig 1). As with all short-read studies, the aim here is to estimate community patterns and not to reconstruct phylogeny and we have used names associated with GenBank sequences; readers looking for more up-to-date phylogenetic estimates of Arcellinida might start with Lara et al. (2008), Gomaa et al. (2012), Kosakyan et al. (2012), and González-Miguéns et al. (2022).

Four OTUs were highly abundant in all samples, accounting for 67% of all Arcellinida reads and appearing in over 88% of all samples (n=231, Tables S1 and S2). We identified these sequences as *Physochila griseola* (OTU2), *Arcella cf.* (OTU1), and *Hyalosphenia papilio* (OTU4 and OTU5, Table S2, Figure 1). The abundance of *Arcella* and *Hyalosphenia papilio* in these bogs and fens is well-supported by morphological observations (Fig 2c,d), but we did not identify the *P. griseola* morphospecies in our surveys; this taxon was likely categorized as *Nebela*-like by the team. The majority of less abundant OTUs were also detected in samples from all four sites; however, some OTUs (e.g. 32, *Nebela cf*; 445, *Hyalosphenia papilio*; 389, *Nebela cf.*) were present in samples from only one or two study sites (Figure 1, Table S2).

We compared Arcellinida lineages from RNA-derived amplicon libraries across sites and months to examine how geographic distance and environmental factors influence the composition of the active community (Figures 2 and 3). Monthly samples from Hawley Bog and Harvard Forest in 2018 showed some differences in community composition (Figure 2). Arcellidae sequences dominant across all months sampled in Harvard Forest, with the greatest genus-level diversity occurring in early May (Figure 2a, b). We also find that *P. griseola* OTUs

were more abundant at Hawley Bog in the warmer months (May-September), but *H. papilio* and *A. vulgaris* reads contributed to a larger fraction of the communities in May and *H. papilio* and *Nebela cf.* sequences were more abundant in October (Figure 2a).

We enumerated tests for five genera (*Nebela, Arcella, Heleopera, Centropyxis,* and *Difflugia*) and two morphospecies, *H. papilio* and *H. elegans* in samples collected across sites in 2018. A comparison of the morphological communities across season and locations in 2018 is shown in Figure 2c,d. The Arcellinida communities were similar across the months sampled in Hawley Bog, with samples dominated by the morphospecies *H. papilio* and *H. elegans*. *Heleopera*-like cells were more abundant in the spring and fall, although these cells were not abundant in the OTU libraries. Between sites, communities sampled from Harvard Forest contained many more *Arcella* cells than Hawley Bog (Figure 2c,d).

Sampling in 2019 focused on variation within two bogs – Hawley Bog and Orono Bog (Table 1) – across two sampling periods. Here, transects from the forest edge to the sedges near open water in Hawley Bog and comparisons between the forest and open bog in Orono show simple patterns across the ecological gradients; however, there are some differences in the relative abundances of taxa in these communities (Figure 3), including many Hawley Bog reads in the forest and forest-to-open bog transition zone belonging to taxa that did not closely match any published Arcellinida reference sequences (see 'other' Figure 3, Table S2).

DISCUSSION

Arcellinida communities characterized by exclusively using microscopy overlook cryptic diversity that may be specific to particular environmental regimes and/or geographical regions. To address prevailing questions about testate amoebae biogeography and ecology, we deployed

high-throughput sequencing primers to target the hypervariable V6 and V7 regions of the SSU-rDNA of Arcellinida following Ruggiero et al. (2020). Here, we used these primers to conduct molecular surveys across four low-pH bogs and fens in New England to characterize diversity across sites (2018) and then within only two bogs (2019; Table 1).

A total of 106 Arcellinida OTUs were identified using a combination of NCBI BLAST searches and phylogenetic comparisons (Table S2). The three most abundant OTUs were identified as the species *Physochila griseola*, *Arcella vulgaris*, and *Hyalosphenia papilio* (Figure 1). We hypothesize that the ubiquity and abundance of these three taxa represent clonal populations that may be best adapted to the current conditions of the bogs and fens, and that this indicates high dispersal potential across the region. In other protists, like ciliates and diatoms, restricted gene flow between individuals may act as a mechanism for speciation (De Decker et al., 2018; Moerman et al., 2022) and can highlight environmental or geographical barriers between populations. Given the limited information of a single marker gene, and especially the short sequences characterized here, it is also possible that sub-populations exist within Arcellinida morphospecies across New England; however, this hypothesis requires further investigation with multigene assessments of variation (e.g. SNPs, species tree reconciliation). Within our marker gene dataset, some less abundant Arcellinida OTUs were specific to one or two habitats (Figure 1), suggesting New England bogs and fens are reservoirs of diversity, and these strains may become dominant clonal populations under different environmental conditions.

Building on the observations of Ruggiero et al. (2020) regarding the restricted distribution of some Arcellinida taxa, the molecular surveys conducted in this study aimed to explore the temporal and spatial variability of Arcellinida communities. In Hawley Bog and Harvard Forest samples from 2018, the relative abundances of the three most abundant genera in

the amplicon libraries, *Physochila*, *Hyalosphenia*, and *Arcella*, did not show clear seasonal community shifts (Figure 2). One trend we did observe is that *P. griseola* reads were more abundant in the summer months in Hawley Bog and May samples from Harvard Forest (Figure 2a,b). Warner et al. (2007) found trends in the prevalence of *P. griseola* when sampling a small *Sphagnum* peatland in Southern Ontario, Canada, correlated with higher water table measurements in May. *H. papilio* and *Arcella*-like sequences, on the other hand, were more abundant during the summer and early fall months (Figure 2a,b). In northern Poland, mixotrophic species, like *H. papilio*, were more abundant in samples collected in August characterized by warmer temperatures and higher light levels (Marcisz et al., 2014). More broadly, data from our transects revealed the taxonomic composition of the Arcellinida communities were heterogeneous, though we see some evidence of transition from *P. griseola* and *Arcella*-like from the forest to the sedges in Hawley Bog (Fig. 2). In addition, we observed differences in the abundances of *Arcella*-like, *H. papilio*, and novel lineages between the forest and open bog in Orono from 2019 (Figure 3). Though these differences in the relative abundances suggest the environmental gradients may be drivers in the distribution of taxa, further assessment of such trends will required sampling across additional years, something we were unable to do given constraints of global pandemic in 2020.

The Arcellinida communities characterized morphologically by quantifying the abundances of five distinct genera and two morphospecies (Figure 2c, d) showed distinct patterns between sites sampled in 2018. Harvard Forest Arcellinida communities differed from Hawley Bog (Figure 2c) and were dominated by *Arcella* morphotypes (Figure 2d). These observations are consistent with the amplicon libraries (Figure 2b), where *Arcella*-like sequences represented up to 95% of the community in some Harvard Forest samples (Table S2). Samples collected

from Hawley Bog were abundant in *H. papilio* and *H. elegans* (Figure 2c). No *P. griseola* were identified morphologically in these samples despite abundant signatures in the amplicon libraries. *P. griseola* recycles the scales of other testate amoebae to build its test (Armynot du Châtelet et al., 2015); therefore, it is possible this species was misidentified. Conversely, *Heleopera* cells were enumerated in Hawley Bog and Harvard Forest, but reads were absent in most amplicon libraries from the same moss samples, suggesting either difficulties extracting DNA from this genus or primer mismatches that prevented amplification. Ruggiero et al. (2020) noted similar challenges recovering *Heleopera* DNA signatures with the Arcellinida-specific primers, highlighting the continued need to collect both morphological and molecular data when surveying Arcellinida diversity.

Few studies have assessed the seasonal composition of Arcellinida communities, and most have relied on morphological classifications to describe this diversity (Arrieira et al., 2015; Davidova et al., 2008; Lamentowicz et al., 2013), likely missing cryptic species that can only be identified by molecular signatures (Heger et al., 2013, Oliverio et al., 2014, Ruggiero et al., 2020). In sensitive habitats, like bogs and fens, it is critical to assess the diversity of indicator species, like the Arcellinida, to develop baselines for future change. Here, we showcase molecular methods that characterize Arcellinida communities across New England, focusing on the taxonomic identity of amplicon sequences and setting the stage for future studies that combine fluorescent microscopy and 'omics scale data.

All communities contained some novel and/or taxonomically ambiguous OTUs, highlighting the poorly characterized fraction of Arcellinida diversity on GenBank (Fig. 1-3). It is likely that these unplaced reads represent some of the over 1000 described morphospecies for which there are no molecular references (Meisterfeld, 2002). In future work, the design of

fluorescence *in situ* hybridization probes targeting sequences of novel clades will connect morphology to molecular signatures.

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DATA AVAILABILITY

All sequences are available on GenBank as indicated in Table S1. The multi-sequence alignment and original tree files used to assign taxonomy have been made available through FigShare (https://figshare.com/account/home#/projects/159104).

COMPETING INTERESTS

The authors declare no competing interests related to the submission of this article.

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FIGURE LEGENDS

Figure 1. An iToL cladogram depicting the phylogenetic position of the 106 OTUs detected in this study relative to 52 Arcellinida reference sequences available through GenBank. Sequences per sample were rarified to 5,000 reads and the total number of reads per OTU were summarized in the innermost ring. Shading in the outer rings corresponds to observation frequencies for OTUs at each site across all samples.

Figure 2. Both molecular (a, b) and morphological (c, d) show the prevalence of the genus *Hyalosphenia* at the nutrient-rich Hawley Bog (a, c) while Arcellidae dominate in the nutrient-rich fen at Harvard Forest (b, d). Other taxa – Nebelidae, *Heleopera, Physochila* – show more complex patterns across time and space. Relative read abundances for Hawley Bog (a) and Harvard Forest (b) highlight differences in the most prevalent genera by site. Microscopy counts for Hawley Bog (c) and Harvard Forest (d) indicate a decline in live cells in the fall. When comparing approaches, *Physochila griseola* was detected using molecular methods, but was not identified *via* microscopy. Conversely, *Heleopera* was absent from most sequencing libraries despite being observed in microscopy samples.

Figure 3. The OTU relative abundances of Arcellinida communities assessed from 2019 samples show considerable variation both within and between Hawley Bog (a) and Orono Bog (b). Transitional zones captured gradients from forest (F) to open bog (OB), or from open bog to sedges (S).

Figure S1. A map of sampling sites across New England.

Figure

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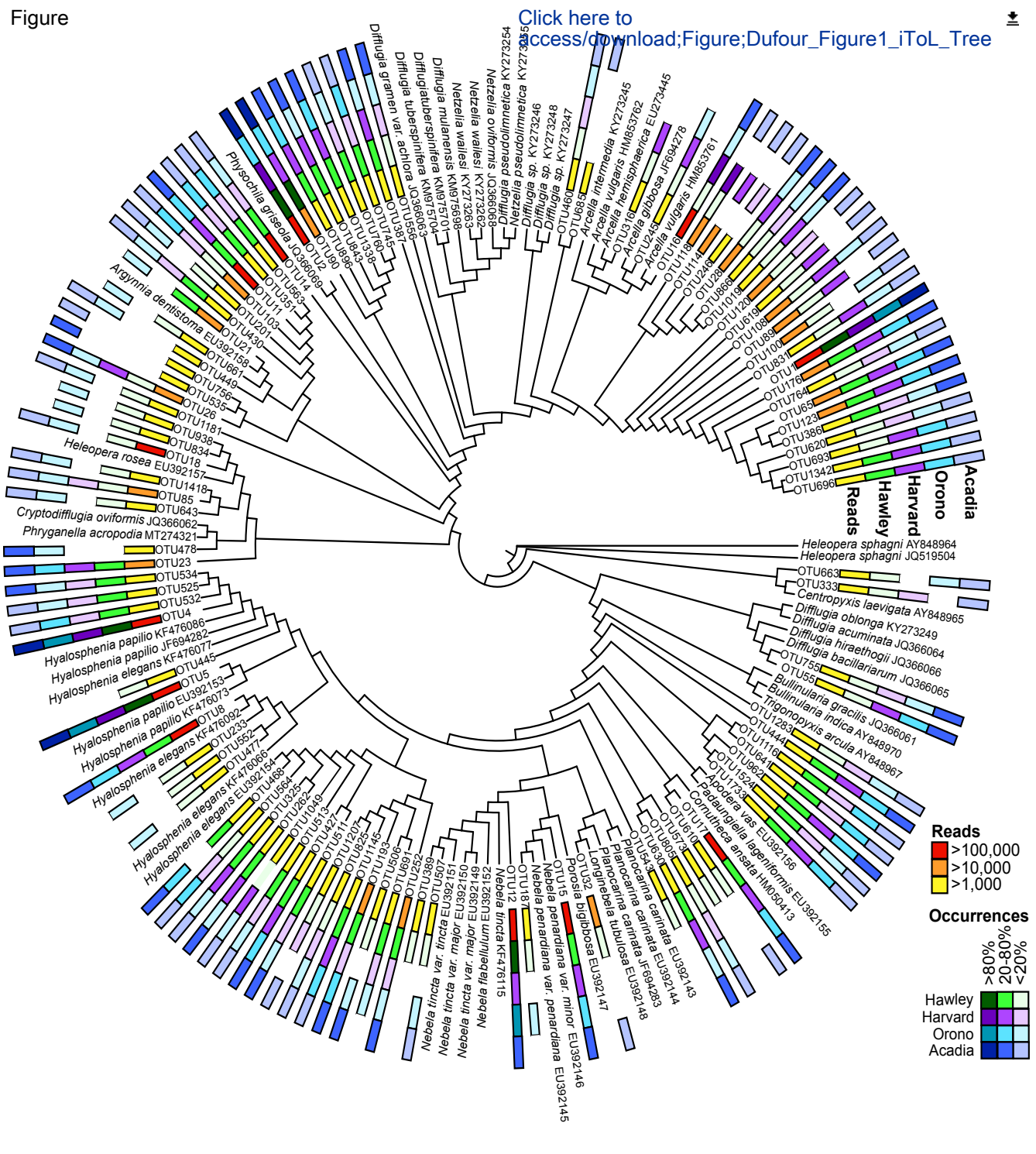


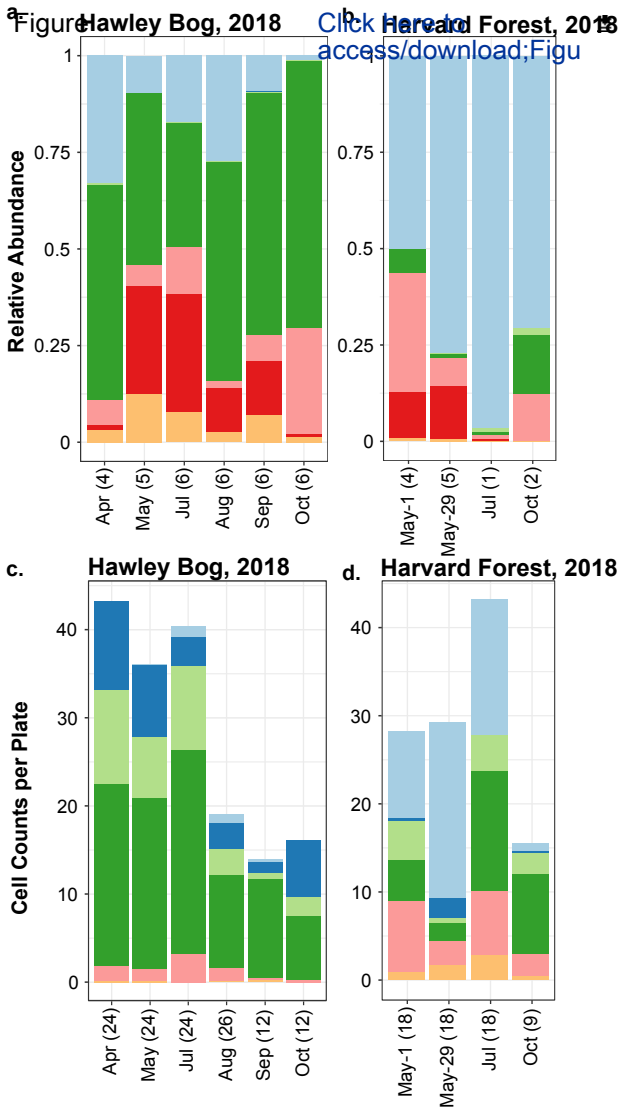
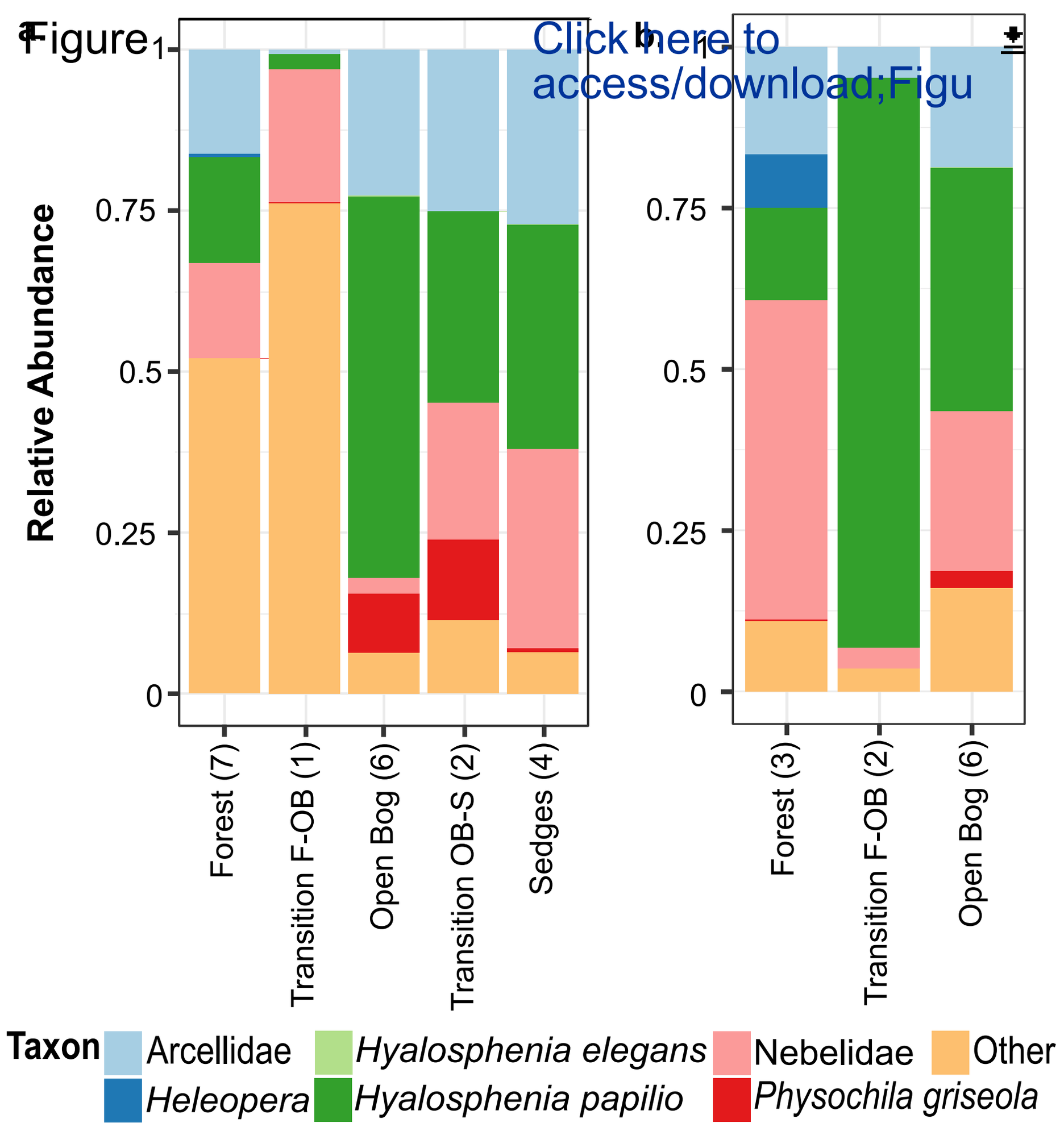


Figure 1



Figure

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