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Source: Freshwater Mollusk Biology and Conservation, 27(1) : 16-26

Published By: Freshwater Mollusk Conservation Society

URL: https://doi.org/10.31931/fmbc-d-23-00003

Freshwater Mollusk Biology and Conservation 27:16–26, 2024

DOI:10.31931/fmbc-d-23-00003

REGULAR ARTICLE

SPECIES RICHNESS AND DISTRIBUTION OF SPHAERIIDAE SURVEYED WITH ENVIRONMENTAL DNA METABARCODING

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ABSTRACT

Freshwater bivalves of the family Sphaeriidae (fingernail, pea, and pill clams) are difficult to survey and identify due to their small size and overlapping morphological traits. Environmental DNA (eDNA) metabarcoding offers a cost-effective method for assessing species richness and distributional patterns at large scales. We evaluated sphaeriid species richness and distribution at 15 sites in the Maumee River, Ohio, USA, based on two eDNA metabarcoding assays (broad and targeted), and we compared our results with those from a traditional benthic macroinvertebrate survey. We detected seven molecular operational taxonomic units (MOTUs) in the Maumee River, including *Sphaerium transversum*, five MOTUs representing *Euglesa* spp., and one MOTU representing *Odhneripisidum* sp. *Sphaerium transversum* was widely distributed, occurring at 10 sites, but *Euglesa* and *Odhneripisidum* were restricted to one to four sites in the upper river. Distributional patterns were broadly similar between both metabarcoding assays and benthic surveys. However, eDNA metabarcoding provided species-level identifications, resulting in higher species richness. Environmental DNA sampling augments and enhances traditional benthic surveys, but greater eDNA sample replication is needed to improve detection, and additional sphaeriid reference sequences are needed to improve species-level identification.

KEY WORDS: environmental DNA, metabarcoding, fingernail clam, pea clam, pill clam, biodiversity assessment

INTRODUCTION

The freshwater bivalve family Sphaeriidae Deshayes, 1855 (fingernail, pea, and pill clams) occurs on every continent except Antarctica and currently contains 227 recognized species worldwide (Herrington 1962; Graf 2013; Lee 2019). Sphaeriids are present in virtually all freshwater habitats, including wetlands, lakes, and rivers. Although they often are the smallest freshwater bivalves (<25 mm shell length), they frequently are numerically dominant and ecologically important in nutrient cycling and energy transport (Burch 1975; Kuiper 1983; Lee 2019). Sphaeriidae contains two subfamilies. The Euperinae Heard, 1965, contains 33 species in 2 genera distributed throughout the Americas and the Afrotropics (Graf and Cummings 2023) and includes the invasive *Eupera cubensis* (Prime, 1865), which occurs in the Illinois River, USA, drainage near the Laurentian Great Lakes (Sneen et al. 2009). The Sphaeriinae Deshayes (1820) is widespread and species-rich. An estimated 35 species of Sphaeriinae occur in the Laurentian Great Lakes watersheds, with 24 reported from Lake Erie (NOAA and USEPA 2019; Trebitz et al. 2019).

Accurate morphological identifications of genera and species within Sphaeriidae are difficult due to plasticity of shell characters (Rassam et al. 2021). DNA sequences have been useful for resolving phylogenetic relationships and providing species diagnostics for this group (Lee and Ó'Foighil 2003; Schultheiß et al. 2008; Clewing et al. 2013). Recent phylogenetic studies indicate that Sphaeriinae includes five genera: *Afropisidium* Kuiper, 1962; *Euglesa* Jenyns, 1832; *Odhneripisidium* Kuiper, 1962; *Pisidium* Pfeiffer, 1821; and *Sphaerium* Scopoli, 1777. The genus *Musculium* Link, 1807, was subsumed under *Sphaerium* (Lee and Ó'Foighil 2003), while the genera *Afropisidium*, *Euglesa*, and *Odhneripisidium* formerly were contained in *Pisidium*. Additionally, DNA sequencing studies have identified cryptic

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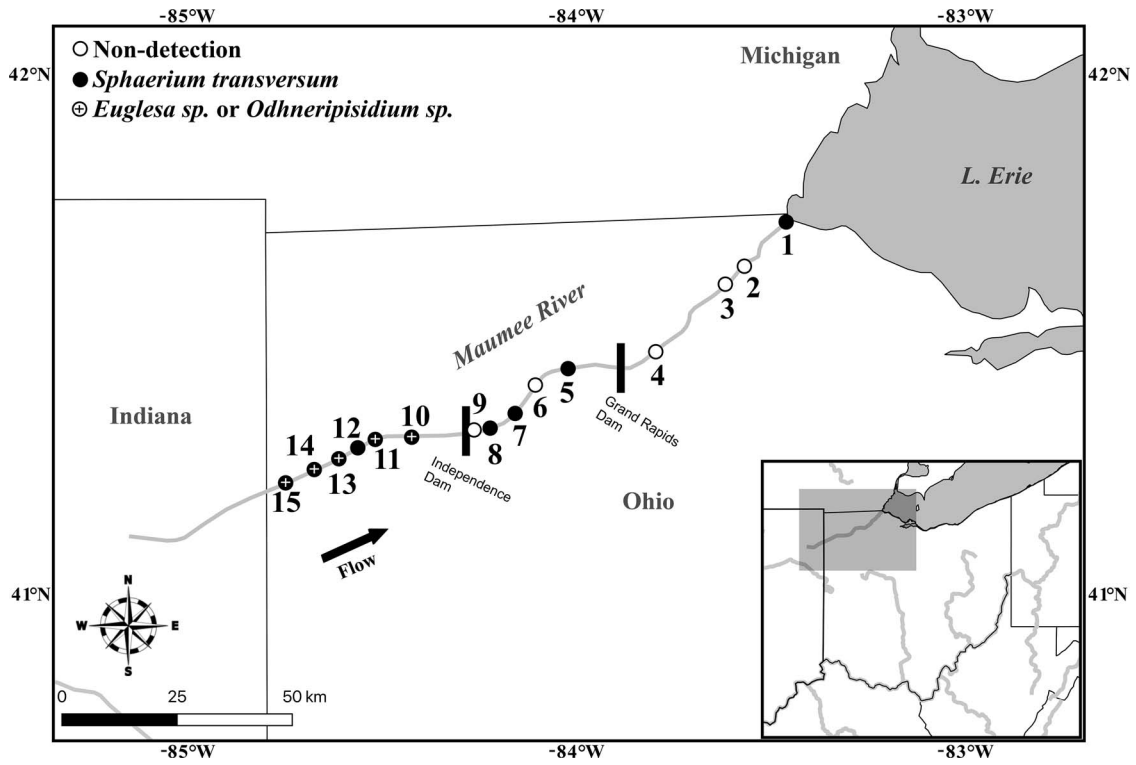

Figure 1. Map of the Maumee River showing sampling sites and eDNA detection of sphaeriid clams. Vertical black lines indication location of low head dams. Inset map shows location of the study area in Ohio.

sphaeriid species (Schultheiß et al. 2008; Clewing et al. 2013; Bößneck et al. 2016; Groh et al. 2020) while providing a better understanding of species distributions (Rassam et al. 2020).

Accurate identification methods and efficient survey approaches are needed to inform assessment of sphaeriid distribution and conservation status. For example, sphaeriid populations across the Great Lakes region have experienced large declines following dreissenid mussel invasions (Lauer and McComish 2001; Burlakova et al. 2018), and continued monitoring is needed for effective conservation. The analysis of environmental DNA (eDNA, genetic material released from urine, waste, mucus, or sloughed cells) provides an efficient method for surveying for a wide range of aquatic taxa (Beng and Corlett 2020; Deiner et al. 2021), including monitoring of invasive bivalves (Gingera et al. 2017; Cowart et al. 2018; Marshall and Stepien 2019; Marshall et al. 2021) and threatened freshwater mussels (Klymus et al. 2021; Marshall et al. 2022). In particular, eDNA may benefit diversity assessments of sphaeriids given the uncertainty surrounding their phylogenetic relationships and their high diversity in North American (Prié et al. 2021).

We compared the detection of sphaeriids using two types of eDNA metabarcoding assays (broad and targeted) versus a traditional benthic macroinvertebrate survey in the Maumee River, Ohio, USA. Benthic macroinvertebrate samples and eDNA samples were collected by the Ohio Environmental Protection Agency (OEPA) in 2012, and we reanalyzed the eDNA samples. We evaluated the ability of eDNA metabarcoding to (1) detect sphaeriids from locations where their presence was previously verified, and (2) characterize species-level diversity in the Maumee River. We discuss the potential of eDNA metabarcoding to facilitate accurate characterization of sphaeriid diversity.

METHODS

The Maumee River begins in Fort Wayne, Indiana, USA, at the confluence of the St. Marys and the St. Joseph rivers and flows 225 kilometers through northeastern Indiana and northwestern Ohio before discharging into Lake Erie (Fig. 1). The river drains 10,620 km^2, making it the largest watershed within the Great Lakes basin. The OEPA conducted a traditional benthic macroinvertebrate survey and collected eDNA samples from August 7 to August 28, 2012, at 15 sites in the Maumee River, Ohio, from river km 0.8 (near the river's mouth; 41.69, −83.47) to river km 158.4 (near the Indiana-Ohio border; 41.18, −84.73; OEPA 2014; Fig. 1). At each site, OEPA staff conducted a macroinvertebrate survey, which consisted of quantitative sampling by placing five modified Hester-Dendy samplers within the river for 6 wk and qualitative sampling with dip nets and hand sampling in different habitats (e.g., riffle, run, or pool) as outlined in OEPA (2008). All sphaeriids were identified to genera recognized at that time (*Sphaerium* or *Pisidium*); therefore, identifications of *Pisidium* may represent taxa from that genus or the now-recognized *Euglesa* or *Odhneripisidium*.

At each site, just prior to performing a traditional benthic macroinvertebrate survey, the OEPA collected a 1-L water sample 10 cm below the surface in a sterilized, bleach-washed Nalgene container, which was placed on ice in a sterile cooler and

transported to the Stepien laboratory at the University of Toledo, where it was frozen at −80°C until DNA was extracted in 2017. At three of the sites, eDNA was isolated and extracted by processing the water through a 0.2-µm PES filter with subsequent DNA extraction using a cetyl trimethyl ammonium bromide (CTAB) protocol (Klymus et al. 2017). At the remaining 12 sites, samples were processed by centrifuging and forming a pellet in 50 mL falcon tubes at 7,500 g for 30 min (Marshall and Stepien 2020). Genomic DNA from the pellets was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Germantown, MD, USA). All samples were processed with a Zymo Research One Step PCR Inhibitor Removal kit (Zymo Research, Irvine, CA, USA). A negative control of 250 ml deionized water was simultaneously extracted to test for possible laboratory contamination.

We examined sphaeriid occurrence in the Maumee River using archived eDNA samples that were previously extracted and processed for other taxonomic analysis. First, we used the results of Marshall and Stepien (2020), who used a broad-range mollusk metabarcoding assay (Mol16S; Klymus et al. 2017) as part of an assessment of overall macroinvertebrate communities. Second, we performed new analyses using a targeted sphaeriid-specific metabarcoding assay (Sph16S; Klymus et al. 2017). The Mol16S assay amplifies a 179–180 bp fragment of the 16S mitochondrial gene for sphaeriids and overlaps completely with the longer 259–260 bp fragment of the 16S gene amplified by Sph16S.

Amplification and library preparation for the Sph16S assay followed that of the Mol16S (Marshall and Stepien 2020) and is described briefly here. We included a short spacer region to increase library nucleotide diversity for enhanced cluster formation. We used a two-step PCR library preparation. The first PCR included 1× PCR buffer, 0.3 mM dNTPs, 0.5 µM of each primer, an additional 1.5 mM $MgCl_2$, 5 U AllTaq (Qiagen), 5 µl template DNA, and ddH_2O to total 50 µl. Conditions were 2 min initial denaturation at 95°C, followed by 40 cycles of 95°C for 5 s, 58°C for 15 s, and 72°C for 10 s, with no final extension. We processed first-step PCR products with a 0.7× HighPrep bead clean-up (MagBio Genomics, Gaithersburg, MD, USA, kit/AC60050), yielding the template for the second step. The second PCR incorporated Nextera paired-end indices (Illumina, San Diego, CA, USA, kit FC-121-1011), p5/p7 adaptor sequences, and eight base sample indices to distinguish among samples. This final reaction contained 1× PCR buffer, 0.2 mM dNTPs, 0.5 µM of each primer, 1.57 U NEB Hotstart Taq polymerase (New England Biolabs Inc., Ipswich, MA, USA), 2.5 µl from the previous PCR cleanup, and ddH_2O to total 25 µl. Conditions were 30 s initial denaturation at 95°C, followed by eight cycles at 95°C for 30 s, 55°C for 30 s, and 68°C for 1 min, with a final 2 min 68°C extension. We sized and quantified PCR products on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) prior to Illumina MiSeq sequencing conducted at the Ohio State University's Molecular and Cellular Imaging Center in Wooster, Ohio. Each PCR setup included the addition of a negative PCR control, which showed no amplification on gel electrophoresis.

Raw MiSeq data referencing the Mol16S assay used by Marshall and Stepien (2020) are available in the NCBI GenBank repository under BioProject PRJNA600479. We deposited raw MiSeq data for the Sph16S assay in the NCBI GenBank repository under BioProject PRJNA1024515.

We removed forward and reverse primer sequences from the demultiplexed reads using the Cutadapt plugin (Martin et al. 2011) in QIIME 2 (Bolyen et al. 2019). Next, we filtered and trimmed sequence reads using the denoising DADA2 plugin (Callahan et al. 2016) in QIIME 2 to truncate sequence reads based on the quality scores from the forward and reverse read files, estimate error rates, merge and dereplicate sequences into amplicon sequence variants (ASVs), and remove any erroneous or chimeric sequences. We clustered unique ASVs into molecular operational taxonomic units (MOTUs) using the QIIME 2 vsearch *de novo* with a 97% similarity threshold (Rognes et al. 2016).

We used the basic local alignment search tool (Camacho et al. 2009) and the National Center for Biotechnology Information (NCBI) GenBank nonredundant (nr) sequence database to identify MOTUs from sphaeriid taxa. We identified MOTUs to the species level if a sequence had >97% sequence match and to the genus level if it had >90% sequence match. We compared taxonomic classifications obtained from NCBI GenBank with species previously reported from the Great Lakes region (Appendix 1; NOAA and USEPA 2019). We used updated taxonomy for the subfamily Sphaeriinae following the MUSSEL Project database (Graf and Cummings 2023).

We constructed a phylogeny of the identified MOTUs and representative sphaeriid sequences from the NCBI GenBank based on a 259–260 bp region amplified with the Sph16S assay using the Maximum Likelihood method in the program Molecular Evolutionary Genetics Analysis (MEGA11; Tamura et al. 2021). We compared MOTUs produced by the Mol16s and Sph16S assays at each site and against the results of the OEPA benthic macroinvertebrate survey. We obtained sphaeriid occurrence records for the Maumee River and western Lake Erie from three online repositories (IdigBio 2023; GBIF 2023; UM Museum 2023).

RESULTS

All 15 samples were successfully amplified and sequenced using the Mol16S assay, but only 10 samples were successfully amplified and sequenced using Sph16S (Appendix 2). The Sph16S assay resulted in a total of 363,550 raw sequence reads (mean = 36,355.0 ± 1,717.5 standard error [SE]), with 51.70% (187,949 reads) passing through the filtering and merging bioinformatic processing. The Mol16S assay resulted in 1,420,366 raw sequence reads (mean = 94,691.1 ± 16,228.6), with 73.26% (1,040,617 reads) passing through the filtering and merging bioinformatic processing. Sphaeriid MOTU reads accounted for 100% of the final Sph16S dataset, but just 3.6% (± 1.6 SE, range = 0.0–18.2%) of the final Mol16S dataset (Appendix 2). The Sph16S assay resulted in a mean of 18,794.9 (± 1,058.2 SE) sphaeriid reads/sample, but the Mol16S assay resulted in a

Table 1. Taxonomic classification and percent identity for each sphaeriid molecular operational taxonomic unit (MOTU) detected in the Maumee River, Ohio, with the Sph16S and Mol16S metabarcoding assays.

MOTU	Taxonomic Classification	Sph16S	Mol16S
MOTU01	*Sphaerium transversum*	100.00	100.00
MOTU02	*Euglesa compressa*	100.00	100.00
MOTU03	*Euglesa casertana*	99.44	98.07
MOTU04	*Euglesa nitida*	99.44	99.23
MOTU05	*Euglesa fallax*	97.78	97.69
MOTU06	*Euglesa* sp.	96.11	96.54
MOTU07	*Odhneripisidium* sp.	93.33	94.64

mean of only 1,806.4 (± 774.6 SE) sphaeriid reads/sample (Appendix 2).

The Mol16S and the Sph16S datasets detected the same seven MOTUs in the Maumee River (Table 1). These were in three genera of Sphaeriinae: *Euglesa* (5 MOTUs), *Odhneripisidium* (1 MOTU), and *Sphaerium* (1 MOTU; Table 1). The single *Sphaerium* MOTU had 100% genetic match with *S. transversum* (Say, 1829) and was detected at all sites that amplified. Four of the five *Euglesa* MOTUs were identified to the species level as *E. compressa* (Prime, 1852; 100% match), *E. casertana* (Poli, 1791; 98.07–99.44% match), *E. nitida* (Jenyns, 1832; 99.23–99.44% match), and *E. fallax* (Sterki, 1896; 97.69–97.78% match) (Table 1 and Fig. 2; Appendix 3). The MOTU identified as *E. nitida* had high similarity (>97%) to four different species: *E. nitida*, *E. edlaueri* (Kuiper, 1960), *E. maaseni* (Kuiper, 1987), and *E. pseudosphaerium* (Favre, 1927), but only *E. nitida is* reported from North America. We were unable to identify one *Euglesa* MOTU to the species level. This MOTU clustered within a group of *E. fallax* sequences but had only a 96.11% match to any, falling below the 97% species-level threshold (Table 1 and Fig. 2; Appendix 3). We were unable to identify the single *Odhneripisidium* MOTU to the species level. This MOTU matched the Eurasian *O. annandalei* (Prashad, 1925) and the Asian *O. japonica* (Pilsbry and Hirase, 1908), but it had a less than 95% match, and neither of these species is reported from North America (Table 1 and Fig. 2; Appendix 3).

The Sph16S assay yielded positive detections at 10 sampling sites, and the Mol16S assay had positive detections at nine (Table 2 and Fig. 3). The two assays shared 22 of 26 detections (85% overlap), with each assay showing unique detections at two sampling sites. Numbers of read counts for each of the seven MOTUs were similar between the two assays ($R^2 = 0.913$, $P < 0.0001$; Table 2). For both assays, *S. transversum* made up a majority of the sequence reads (Mol16S: 74.7% ± 33.0 SD, Sph16S: 97.0% ± 5.2).

OEPA benthic macroinvertebrate surveys observed *Sphaerium* at ten of our study sites. Our eDNA assays detected *S. transversum* at eight of these ten sites, and at an additional two sites where OEPA did not report *Sphaerium* (Fig. 3). Benthic macroinvertebrate surveys observed species within the "*Pisidium*" group (sensu lato) from two sites, while our eDNA assays detected at least one *Euglesa* or *Odhneripisidium* MOTU at five sites, including one of those in agreement with visual observations (Fig. 3).

DISCUSSION

Our estimates of species distributions in the Maumee River from eDNA metabarcoding were broadly similar to those reported by the OEPA benthic macroinvertebrate surveys. As expected, eDNA metabarcoding improved species-level identifications, going beyond the "*Sphaerium*" or "*Pisidium*" designation. Taxonomic uncertainty associated with vague and overlapping morphological traits typically limits identification to the genus level, resulting in a loss of information about species distribution and status. We described five MOTUs to the species level (*E. compressa*, *E. casertana*, *E. fallax*, *E. nitida*, and *S. transversum*), with only two MOTUs being restricted to genus level identification (*Euglesa* sp. and *Odhneripisidium* sp.) due to lack of reference sequences. These two unidentified taxa illustrate limitations of existing DNA reference databases (Trebitz et al. 2015), as these sequences may belong to species that lack reference sequence data for 16S rDNA or belong to undescribed species. Cryptic species within the subfamily Sphaeriinae have been identified by combining molecular and morphological approaches (Guralnick 2005; Groh et al. 2020). The sequences reported here can be used to determine these identities in the future, as taxonomic advances are made and reference databases improve. The unknown *Euglesa* sp. group occurred within a cluster of *E. fallax* sequences, yet it fell below the 97% species level threshold. This may represent population genetic variation rather than separate species (Marshall and Stepien 2019), and further DNA sequence and morphological data would be needed to confirm.

Based on both eDNA and morphological surveys, *S. transversum* appears widespread throughout the Maumee River. A 2010 benthic survey near the mouth of the Maumee River (Ram et al. 2014) reported four sphaeriids based on morphological identifications including *S. transversum* (as *Musculium*), *E. compressa* (as *Pisidium*), and two taxa not found in our study: *S. securis* (Prime, 1852; as *Musculium*) and *S. simile* (Say, 1817). However, only *S. transversum* and *E. compressa* were confirmed with subsequent DNA analysis of collected specimens (supplementary data in Ram et al. 2014), matching our results. The three online repositories suggest that four species are the dominant sphaeriids within the lower reach of Maumee River and western Lake Erie, including three species we detected with eDNA (*E. casertana*, *E. compressa*, and *S. transversum*) and a fourth nondetected species, *S. striatinum* (Lamarck, 1818). Interestingly, these repositories indicate *S. striatinum* as the first or second most common species. While sphaeriid populations have declined across the Great Lakes region (Lauer and McComish 2001; Burlakova et al. 2018), it is unclear if our failure to detect *S. striatinum* is due to population declines or low eDNA sampling effort.

We did not detect sphaeriids at three sites where they were reported by OEPA benthic macroinvertebrate surveys. Five samples failed to amplify with the Sph16S assay, suggesting low concentration or absence of sphaeriid DNA. These same five samples

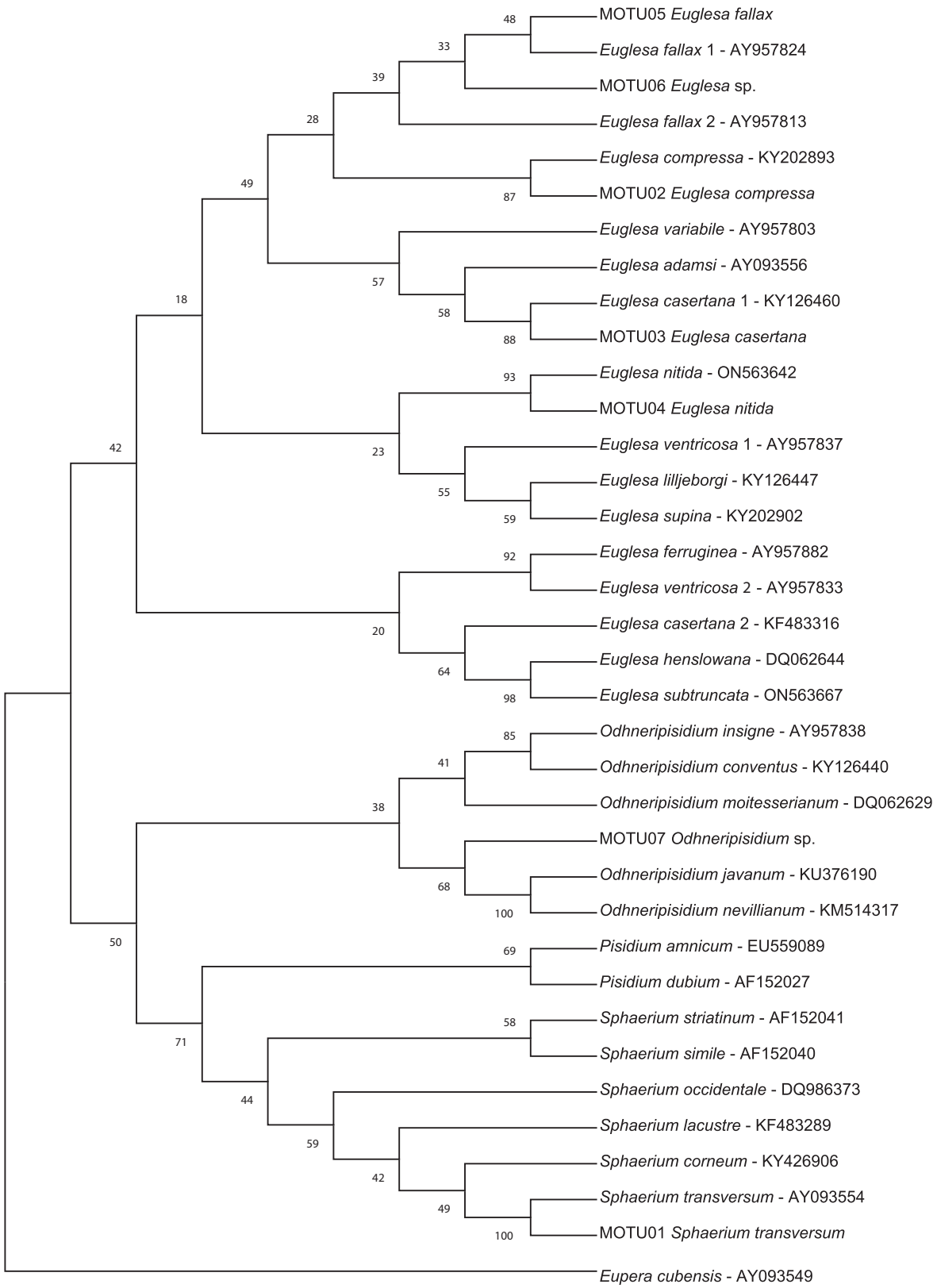

Figure 2. Phylogeny of the identified molecular operational taxonomic units (MOTUs) and representative Lake Erie sphaeriid sequences based on a 259–260 base pair region amplified with the Sph16S assay using the Maximum Likelihood method and Tamura-Nei model within the program Molecular Evolutionary Genetics Analysis (MEGA11). The bootstrap consensus tree is inferred from 500 replicates. Numbers at each node are the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test. Accession numbers represent sequences obtained from NCBI GenBank.

Table 2. Total read counts for each sphaeriid molecular operational taxonomic unit (MOTU) detected in the Maumee River, Ohio, with the Sph16S and Mol16S metabarcoding assays. Bold numbers indicate MOTU detection unique to one assay.

Sph16S	Species	Site 1	Site 5	Site 7	Site 8	Site 10	Site 11	Site 12	Site 13	Site 14	Site 15
MOTU01	*Sphaerium transversum*	21937	**19070**	18077	17854	24168	11853	21025	17146	15061	16336
MOTU02	*Euglesa compressa*	0	0	0	0	0	0	0	195	860	1940
MOTU03	*Euglesa casertana*	0	0	0	0	0	0	0	578	158	825
MOTU04	*Euglesa nitida*	0	0	0	0	0	0	0	28	175	255
MOTU05	*Euglesa fallax*	0	0	0	0	0	0	0	0	118	0
MOTU06	*Euglesa* sp.	0	0	0	0	0	0	0	0	74	0
MOTU07	*Odhneripisidium* sp.	0	0	0	0	136	**41**	0	39	0	0
	Total reads	21937	19070	18077	17854	24304	11894	21025	17986	16446	19356
	MOTU richness	1	1	1	1	2	2	1	5	6	4
Mol16S	Species	Site 1	Site 5	Site 7	Site 8	Site 10	Site 11	Site 12	Site 13	Site 14	Site 15
MOTU01	*Sphaerium transversum*	137	0	220	11	3206	45	1859	5593	6575	2960
MOTU02	*Euglesa compressa*	0	0	0	0	0	**57**	0	520	1147	1216
MOTU03	*Euglesa casertana*	0	0	0	0	0	0	0	1151	289	664
MOTU04	*Euglesa nitida*	0	0	0	0	0	0	0	138	296	214
MOTU05	*Euglesa fallax*	0	0	0	0	0	0	0	0	253	0
MOTU06	*Euglesa* sp.	0	0	0	0	0	0	0	**146**	210	0
MOTU07	*Odhneripisidium* sp.	0	0	0	0	136	0	0	53	0	0
	Total reads	137	0	220	11	3342	102	1859	7601	8770	5054
	MOTU richness	1	0	1	1	2	2	1	6	6	4

were successfully sequenced with the Mol16S assay, yet no sphaeriid sequences were detected. However, the OEPA survey did find sphaeriids at three of these five sites, suggesting that the single 1-L water sample was not always sufficient for collection of sphaeriid eDNA. Our study did not include replicate water sampling, and increasing the number of eDNA samples collected at each site likely would increase detection (Marshall et al. 2022). Along with increasing sample replication, sampling larger volumes of water can increase eDNA detection of bivalves (McKee et al. 2023). It also would be beneficial to sample water nearer the bottom, where sphaeriids occur.

The *Euglesa* and *Odhneripisidium* MOTUs appeared to be restricted to the upper reach of the Maumee River. The drainage area of the Maumee basin increases from 5,985 to 14,356 km^2 after the confluence of the Auglaize River near Independence Dam (our site 9; OEPA 2014). The resulting increase in river discharge may dilute eDNA, reducing the likelihood of sphaeriid detection (Curtis et al. 2021). Additionally, increases in discharge (cubic feet per second) typically result in greater eDNA transport distances (Jo and Yamanaka 2022), which, in turn, adds uncertainty to the determination of source location. Our investigation is limited due to the lack of sample replicates, and studies examining the spatial extent of eDNA recommend collecting several independent replicates throughout each reach (Bedwell and Goldberg 2020).

As expected, sphaeriid MOTUs accounted for a much greater number of read counts using the Sph16S assay compared to the Mol16S assay. Yet the two assays displayed large overlap in site-level sphaeriid MOTU detections. Despite the Mol16S assay amplifying a much broader range of taxa, when a MOTU had a low read count for Sph16S, it usually was likewise detected by the Mol16S assay. This suggests that the use of the family-specific Sph16S assay may not be warranted when interested in monitoring sphaeriids, as the Mol16S assay displayed similar sensitivity and can provide additional information on macroinvertebrate diversity (Marshall and Stepien 2020). On four of 26 occasions, a rarer sphaeriid MOTU was detected at a site with one assay but not the other. Considering the stochastic nature of PCR amplification, processing several PCR technical replicates could improve detection of rare sequences and may increase overlap between the assays (Shirazi et al. 2021).

Obtaining abundance estimates from eDNA metabarcoding datasets is challenging due to species-specific differences in eDNA shedding amounts and rates (e.g., differences in body size, life histories and spawning times, and metabolic activity), behavior, habitat differences, and PCR-based biases such as differential primer annealing and amplification (Ruppert et al. 2019). However, a metanalysis of eDNA metabarcoding studies suggested that sequence read counts often are correlated with species abundance or biomass (Keck et al. 2022). In our study, both assays indicated that *S. transversum* is the dominant species throughout the Maumee River, with *Euglesa* and *Odhneripisidium* being less abundant, based on their lower read counts. However, Klymus et al. (2017) reported lower read counts than expected for *Euglesa* (as *Pisidium*) based on known abundances

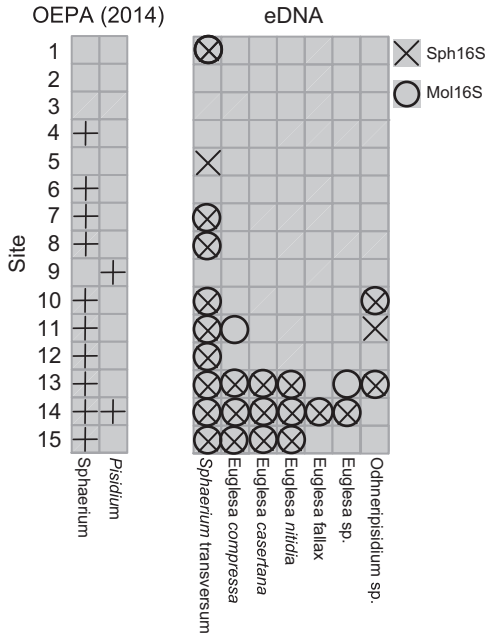

Figure 3. Comparison of sphaeriid clam detection using traditional benthic macroinvertebrate surveys (Ohio Environmental Protection Agency [OEPA]) and two eDNA metabarcoding assays (Sph16S and Mol16S) at 15 sites in the Maumee River, Ohio. Sphaeriids were identified by OEPA only to genus as *Sphaerium* or "*Pisidium*" (sensu lato).

in laboratory mesocosm trials. Because *Euglesa* and *Odhneripisidium* usually are smaller than *Sphaerium*, the former may shed less eDNA, influencing abundance estimates from eDNA sequence read counts.

Environmental DNA sampling is a valuable and cost-effective tool for large-scale, initial assessment of sphaeriid species richness and distributions (Prié et al. 2021). Additional eDNA studies, conducted in concert with traditional benthic surveys, would help to better understand possible sources of bias inherent in this approach. When unidentified MOTU sequences are found, traditional sampling can inform eDNA surveys by providing archived voucher specimens from which reference DNA sequences can be obtained.

ACKNOWLEDGMENTS

Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government. We thank members of the Stepien Genetics and Genomics Group (M. Snyder, D. Eddins, and S. Yerga-Woolwine) for laboratory assistance. Water samples from the Maumee River were provided by the Ohio EPA. Funding was provided through U.S. Environmental Protection Agency Grant Numbers GL-00E01289 and GL-00E01898 awarded to C.A.S.

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APPENDICES

Appendix 1. List of species in the family Sphaeriidae reported from the Laurentian Great Lakes region. "X" indicates occurrence in the watershed of each major lake. "16s sequences" is the number of reference sequences available for the mitochondrial 16S gene region on the NCBI GenBank database (https://www.ncbi.nlm.nih.gov, accessed September 16, 2023). Species occurrences are based on NOAA and USEPA (2019). Nomenclature follows Graf and Cummings (2023); former genera are given in parentheses.

Species	Superior	Michigan	Huron	Erie	Ontario	16S Sequences
Euglesa (*Pisidium*) *adamsi*	X	X	X	X	X	2
Euglesa (Pisidium) casertana	X	X	X	X	X	75
Euglesa (Pisidium) compressa	X	X	X	X	X	36
Euglesa (Pisidium) equilateralis	—	—	—	—	X	0
Euglesa (Pisidium) fallax	X	X	X	X	X	5
Euglesa (Pisidium) ferruginea	X	X	X	X	X	11
Euglesa (Pisidium) henslowana	X	X		X	X	17
Euglesa (Pisidium) lilljeborgi	X	X	X	X	X	7
Euglesa (Pisidium) milia	X	X	—	—	X	18
Euglesa (Pisidium) nitida	X	X	X	X	X	11
Euglesa (Pisidium) obtusale	—	X	X	—	X	0
Euglesa (Pisidium) rotundata	X	—	—	—	X	0
Euglesa (Pisidium) subtruncata	X	X	X	X	X	32
Euglesa (Pisidium) supina	—	—	—	X	X	18
Euglesa (Pisidium) variabile	X	X	X	X	X	7
Euglesa (Pisidium) ventricosa	X	X	X	X	X	5
Euglesa (Pisidium) walkeri	X	—	X	—	X	1
Ophneripisidium (Pisidium) conventus	X	X	X	X	X	1
Ophneripisidium (Pisidium) insigne	X	—	—	—	—	1
Ophneripisidium (Pisidium) moitessierianum	X	—	—	X	—	4
Pisidium amnicum	X	X	X	X	X	7
Pisidium cruciatum[a]	—	—	—	—	X	0
Pisidium dubium	X	X	X	—	X	1
Pisidium idahoense	X	X	X	X	X	0
Pisidium punctatum (= *P. simplex*)	X	X	X	X	X	0
Sphaerium corneum	X	X	X	X	X	50
Sphaerium (*Musculium*) *lacustre*	—	X	X	X	X	4
Sphaerium nitidum	X	X	X	—	X	0
Sphaerium occidentale	X	—	X	X	X	2
Sphaerium (*Musculium*) *partumeium*	X	X	—	—	X	1
Sphaerium rhomboideum	—	—	X	—	X	2
Sphaerium (*Musculium*) *securis*	X	X	X	X	X	1
Sphaerium simile	X	X	X	—	X	2
Sphaerium striatinum	X	X	X	X	X	5
Sphaerium (*Musculium*) *transversum*	—	X	—	X	X	1

[a] *Pisidium cruciatum* is considered present within Lake St. Clair between the Huron-Erie corridor (NOAA and USEPA 2019).

Appendix 2. Total number of raw sequencing reads per sample and the subsequent number of reads that passed the trimming and merging bioinformatic processing steps for samples collected at 15 sites in the Maumee River, Ohio, using the Sph16S or Mol16S metabarcoding assays.

Sph16S	Raw Reads	Primer Trimmed Reads	Merged Reads	Sphaeriid Reads	Percent Sphaeriid
Site 1	39,510	29,975	21,937	21,937	100
Site 2	—	—	—	—	—
Site 3	—	—	—	—	—
Site 4	—	—	—	—	—
Site 5	38,496	23,864	19,070	19,070	100
Site 6	—	—	—	—	—
Site 7	33,548	21,602	18,077	18,077	100
Site 8	29,896	20,009	17,854	17,854	100
Site 9	—	—	—	—	—
Site 10	34,603	30,131	24,304	24,304	100
Site 11	34,477	22,639	11,894	11,894	100
Site 12	34,643	27,163	21,025	21,025	100
Site 13	34,639	27,116	17,986	17,986	100
Site 14	33,867	23,867	16,446	16,446	100
Site 15	49,871	33,888	19,356	19,356	100
Total	363,550	260,254	187,949	187,949	—
Mean (SE)	36,355.0 (1,717.5)	26,025.4 (1,387.7)	18,794.9 (1,058.2)	18,794.9 (1,058.2)	100
Mol16S	Raw Reads	Primer Trimmed Reads	Merged Reads	Sphaeriid Reads	Percent Sphaeriid
Site 1	293,172	275,819	259,858	137	0.05
Site 2	172,378	163,021	100,799	0	0
Site 3	89,235	83,843	68,068	0	0
Site 4	68,288	63,819	46,101	0	0
Site 5	62,551	57,633	55,064	0	0
Site 6	55,179	50,705	41,726	0	0
Site 7	61,199	55,890	45,574	220	0.48
Site 8	59,527	55,048	36,558	11	0.03
Site 9	75,385	70,325	51,854	0	0
Site 10	95,639	89,361	66,198	3,342	5.05
Site 11	117,126	109,253	70,902	102	0.14
Site 12	83,855	77,426	54,214	1,859	3.43
Site 13	67,870	63,339	47,590	7,601	15.97
Site 14	60,973	56,981	48,156	8,770	18.21
Site 15	57,989	53,908	47,955	5,054	10.54
Total	1,420,366	1,326,371	1,040,617	27,096	—
Mean (SE)	94,691.1 (16,228.6)	88,424.7 (15,359.8)	69,374.5 (14,202.7)	1,806.4 (774.6)	3.6 (1.6)

Appendix 3. sequence data for the mol16s and sph16s assays for each of the seven sphaeriid MOTUS.

>MOTU01 *Sphaerium transversum* Sph16S
ACGTGGGAAAAACTGTCTCTTTTGTATATAAAGAAGTTTATTTTTGAGTGAAAAAGCTTAAATGTTTATAAAAGACGAGAAGACCCTATCGAACTTGAATTATTT
ATTTAAAAATTTAGATAAAGAAAGTTTAGTTGGGGAAACTTAAAGTAAAAAGTAACGCTTTATTTTTGTATCGGGATCCTATATTATAGAAAAATGAAAAAGT
TACCGTAGGGATAACAGCGCTTTCTTCTCTGAGAGGACTAATTAAAGAGTT

>MOTU01 *Sphaerium transversum* Mol16S
ATCGAACTTGAATTATTTATTTAAAAATTTAGATAAAGAAAGTTTAGTTGGGGAAACTTAAAGTAAAAAGTAACGCTTTATTTTTGTATCGGGATCCTATATTAT
AGAAAAATGAAAAAGTTACCGTAGGGATAACAGCGCTTTCTTCTCTGAGAGGACTAATTAAAGAGTTGGTTGCG

>MOTU02 *Euglesa compressa* Sph16S
ACGTGGGAAAAGCTGTCTCTTTTGTATAGAAAGAAGTTTATTTTTGAGTGAAAAAGCTTAAATATTTGTAAAAGACGAGAAGACCCTATCGAACTTGAATTGTG
TGTTTTAGTTTTGGAATACAGAAAGTTTAGTTGGGGAAACTTAAAGTTAAGAAAAACGCTTTTTTGTTATAAAATGATCCTGTATTATAGAAAAATGAAAAAG
TTACCGTAGGGATAACAGCGCTTTCTTCTCTGAGAGGACTAATCAAAGAGTT

>MOTU02 *Euglesa compressa* Mol16S
ATCGAACTTGAATTGTGTGTTTTAGTTTTGGAATACAGAAAGTTTAGTTGGGGAAACTTAAAGTTAAGAAAAACGCTTTTTTGTTATAAAATGATCCTGTATTAT
AGAAAAATGAAAAAGTTACCGTAGGGATAACAGCGCTTTCTTCTCTGAGAGGACTAATCAAAGAGTTGGTTGCG

>MOTU03 *Euglesa casertana* Sph16S
ACGTGGGAAAAACTGTCTCTTTTGTATATAAAGAAGTTTATTTTTGAGTGAAAAAGCTTAAATGTTTATAAAAGACGAGAAGACCCTATCGAACTTGAATTATGT
ATTGTAGATTTATAATGCAGAAAGTTTAGTTGGGGAAACTTAAAGTTAAGAAAAACGCTTTTTTGTTGTAAGATGATCCTGTATTATAGAAAAATGAAAAAGT
TACCGTAGGGATAACAGCGCTTTCTTCTCTGAGAGGACTAATCAAAGAGTT

>MOTU03 *Euglesa casertana* Mol16S
ATCGAACTTGAATTATGTATTGTAGATTTATAATGCAGAAAGTTTAGTTGGGGAAACTTAAAGTTAAGAAAAACGCTTTTTTGTTGTAAGATGATCCTGTATTAT
AGAAAAATGAAAAAGTTACCGTAGGGATAACAGCGCTTTCTTCTCTGAGAGGACTAATCAAAGAGTTGGTTGCG

>MOTU04 *Euglesa nitida* Sph16S
ACGTGGGAAAAGCTGTCTCTTTTATATAAAAAGAAGTTTATTTTTGAGTGAAAAAGCTTAGATGTTTATAAAAGACGAGAAGACCCTATCGAACTTGAATTATG
TGTTTAAGTTTTTAAGTACAAAAAGTTTAGTTGGGGAAACTTAAAGTTAAGAAAAACGTTTTTTTGTTATAAATTGATCCTGTATTATAGAAAAATGAAAAAG
TTACCGTAGGGATAACAGCGCTTTCTTCTCTGAGAGGACTAATTAAAGAGTT

>MOTU04 *Euglesa nitida* Mol16S
ATCGAACTTGAATTATGTGTTTAAGTTTTTAAGTACAAAAAGTTTAGTTGGGGAAACTTAAAGTTAAGAAAAACGTTTTTTTGTTATAAATTGATCCTGTATTAT
AGAAAAATGAAAAAGTTACCGTAGGGATAACAGCGCTTTCTTCTCTGAGAGGACTAATTAAAGAGTTGGTTGCG

>MOTU05 *Euglesa fallax* Sph16S
ACGTGGGAAAAGCTGTCTCTTTTATATAAAAAGAAGTTTATTTTTGAGTGAAAAAGCTTAGATGTTTATAAAAGAGAGAAGACCCTATCGAACTTGAATTATGT
GTTTTAGTTTTGGGGTACAGAAAGTTTAGTTGGGGAAACTTAAAGTTAAGAAAAACGCTTTTTTGTTGTAAAAATGATCCTATATTATGGAAAAATGAAAAA
GTTACCGTAGGGATAACAGCGCTTTCTTCTCTGAGAGGACTAATCAAAGAGTT

>MOTU05 *Euglesa fallax* Mol16S
ATCGAACTTGAATTATGTGTTTTAGTTTTGGGGTACAGAAAGTTTAGTTGGGGAAACTTAAAGTTAAGAAAAACGCTTTTTTGTTGTAAAAATGATCCTATATT
ATGGAAAAATGAAAAAGTTACCGTAGGGATAACAGCGCTTTCTTCTCTGAGAGGACTAATCAAAGAGTTGGTTGCG

>MOTU06 *Euglesa* sp. Sph16S
ACGTGGGAAAAGCTGTCTCTTTTATATAAAAAGAAGTTTATTTTTGAGTGAAAAAGCTTAGATGTTTGTAAAAGACGAGAAGACCCTATCGAACTTGAATTGT
GTGTCTTAGTTTTGGGGTACAGAAAGTTTAGTTGGGGAAACTTAAAGTTAAAAAAGAACGCTTTTTTTGTTTAAAAATGATCCTGTATTATAGAAAAATGAAA
AAGTTACCGTAGGGATAACAGCGCTTTCTTCTCTGAGAGGACTAATCAAAGAGTT

>MOTU06 *Euglesa* sp. Mol16S
ATCGAACTTGAATTGTGTGTCTTAGTTTTGGGGTACAGAAAGTTTAGTTGGGGAAACTTAAAGTTAAAAAGAACGCTTTTTTTGTTTAAAAATGATCCTGTATTA
TAGAAAAATGAAAAAGTTACCGTAGGGATAACAGCGCTTTCTTCTCTGAGAGGACTAATCAAAGAGTTGGTTGCG

>MOTU07 *Odhneripisidium* sp. Sph16S
ACGTGGGAAAAACTGTCTCTTTTGCATATGAAGAAGTTTATTTTTGAGTGAAAAAGCTTAGATTATTATAAAAGACGAGAAGACCCTATCGAACTTGAATTAGA
TGTTTGAGTTTTTGAATGTCAAAAGTTTAGTTGGGGAAACTTAAAGTAAAAAAGAACGCTTTATTTTTTTGTAATGATCCTGTAATACGGAAAAACGAAAAAG
TTACCGTAGGGATAACAGCGCTTTCTTCTCTGAGAGGACTAATTAAAGAGTT

>MOTU07 *Odhneripisidium* sp. Mol16S
ATCGAACTTGAATTAGATGTTTGAGTTTTTGAATGTCAAAAGTTTAGTTGGGGAAACTTAAAGTAAAAAAGAACGCTTTATTTTTTTGTAATGATCCTGTAATAC
GGAAAAACGAAAAAGTTACCGTAGGGATAACAGCGCTTTCTTCTCTGAGAGGACTAATTAAAGAGTTGGTTGCG