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Katy E. Klymus

Catherine A. Richter

Nathan Thompson

Jo Ellen Hinck

Jess W. Jones

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Metabarcoding assays for the detection of freshwater mussels (Unionida) with environmental DNA

Katy E. Klymus[1] | Catherine A. Richter[1] | Nathan Thompson[1] | Jo Ellen Hinck[1] | Jess W. Jones[2]

[1]Columbia Environmental Research Center, U.S. Geological Survey, Columbia, MO, USA

[2]Department of Fish and Wildlife Conservation, U.S. Fish and Wildlife Service, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

Correspondence
Katy E. Klymus, Columbia Environmental Research Center, U.S. Geological Survey, Columbia, MO, USA.
Email: kklymus@usgs.gov

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Abstract

Freshwater mussels of the order Unionida are a widely distributed taxon that are important in maintaining freshwater ecosystems and are also highly imperiled throughout the world. Monitoring of mussel populations with environmental DNA (eDNA) is an attractive alternative to traditional methods because it is noninvasive and requires less labor and taxonomic knowledge from field personnel. We developed eDNA metabarcoding assays specific to freshwater mussels and tested them at six sites in the Clinch River, located in the southeastern United States. Our objective was to determine the utility of eDNA metabarcoding for future monitoring of mussel populations and restoration efforts in this watershed. Two metabarcoding assays that target the mitochondrial DNA regions of the cytochrome c oxidase subunit I (COI) and NADH dehydrogenase subunit (ND1) genes were developed and tested. Our assays appear to be order specific, amplifying members from the two families found in North America, Unionidae and Margaritiferidae, while not amplifying nontarget fish or other bivalve species. From the field collected samples, our assays together detected 19 species, eight of which are listed as federally endangered. The assays also detected 42%, 58%, and 54% of the species identified by recent quantitative visual mussel surveys at three sampling sites. Increased sampling effort by processing a greater water volume or number of samples will likely increase species detections. These eDNA metabarcoding assays may enable enhanced monitoring of freshwater mussel assemblages and subsequently inform conservation efforts.

KEYWORDS

biodiversity monitoring, bioinformatic processing, degenerate primers, high-throughput sequencing, species diversity

1 | INTRODUCTION

Freshwater mussels are bivalve mollusks belonging to the order Unionida (Williams et al., 2017). Mussels play major ecological roles in maintaining freshwater ecosystems worldwide through water filtration, nutrient cycling, habitat modification, and serving as food resources for other animals (Vaughn, 2018). However, they are one of the most imperiled taxa groups in the world (Lydeard

TABLE 1 Freshwater mussel and outgroup species used for primer design and evaluation of two metabarcoding assays

Family	Tribe	Genus	Species	Williams et al. (2017)	Species in the Clinch River	Primer Database		gDNA Tested	gDNA Amplified		Field eDNA Amplified	
						COI	ND1		COI	ND1	COI	ND1
Margaritiferidae	n/a	*Cumberlandia*	*monodonta*		X	X		X	X	X		
Margaritiferidae	n/a	*Cumberlandia*	*monodonta male*			X		X				
Unionidae	Amblemini	*Amblema*	*plicata*		X	X	X	X	X	X		
Unionidae	Amblemini	*Amblema*	*plicata male*			X						
Unionidae	Anodontini	*Alasmidonta*	*marginata*		X	X	X	X	X	X		X
Unionidae	Anodontini	*Alasmidonta*	*viridis*		X(NS)							
Unionidae	Anodontini	*Pegias*	*fabula*		X(NS)							
Unionidae	Anodontini	*Anodonta*	*californiensis*			X						
Unionidae	Anodontini	*Anodonta*	*nuttalliana*			X						
Unionidae	Anodontini	*Anodonta*	*oregonensis*			X						
Unionidae	Anodontini	*Lasmigona*	*costata*		X	X	X	X	X	X	X	X
Unionidae	Anodontini	*Lasmigona*	*holstonia*		X	X	X					
Unionidae	Anodontini	*Strophitus*	*undulatus*		X	X	X	X	X	X		
Unionidae	Lampsilini	*Actinonaias*	*ligamentina*		X	X	X	X	X	X	X	
Unionidae	Lampsilini	*Actinonaias*	*ligamentina male*			X						
Unionidae	Lampsilini	*Actinonaias*	*pectorosa*		X	X		X	X	X	X	X
Unionidae	Lampsilini	*Cyprogenia*	*stegaria*		X	X	X	X	X	X		
Unionidae	Lampsilini	*Dromus*	*dromas*		X	X	X					
Unionidae	Lampsilini	*Epioblasma*	*brevidens*		X	X	X	X	X	X	X	
Unionidae	Lampsilini	*Epioblasma*	*capsaeformis*		X	X	X	X	X	X	X	
Unionidae	Lampsilini	*Epioblasma*	*florentina aureola*	(*E. aureola*)	X		X*					
Unionidae	Lampsilini	*Epioblasma*	*haysiana*		X(EXT)							
Unionidae	Lampsilini	*Epioblasma*	*lenoir*		X(EXT)							
Unionidae	Lampsilini	*Epioblasma*	*stewardsonii*		X(EXT)							
Unionidae	Lampsilini	*Epioblasma*	*torsulosa gubernaculum*	(*E. gubernaculum*)	X(EXT)							
Unionidae	Lampsilini	*Epioblasma*	*torsulosa rangiana*	(*E. rangiana*)		X	X					
Unionidae	Lampsilini	*Epioblasma*	*triquetra*		X	X	X	X	X	X	X	
Unionidae	Lampsilini	*Epioblasma*	*florentina walkeri*	(*E. walkeri*)			X*					
Unionidae	Lampsilini	*Lampsilis*	*abrupta*		X	X						

(Continues)

TABLE 1 (Continued)

Family	Tribe	Genus	Species	Williams et al. (2017)	Species in the Clinch River	Primer Database		gDNA Tested	gDNA Amplified		Field eDNA Amplified	
						COI	ND1		COI	ND1	COI	ND1
Unionidae	Lampsilini	*Lampsilis*	*fasciola*		X	X	X				X	X
Unionidae	Lampsilini	*Lampsilis*	*ovata*		X	X	X	X	X			
Unionidae	Lampsilini	*Lampsilis*	*ovata male*			X						
Unionidae	Lampsilini	*Lampsilis*	*siliquoidea*					X	X	X		
Unionidae	Lampsilini	*Lemiox*	*rimosus*		X	X	X	X	X	X		
Unionidae	Lampsilini	*Lemiox*	*rimosus male*			X						
Unionidae	Lampsilini	*Leptodea*	*fragilis*		X(EP)	X	X					
Unionidae	Lampsilini	*Ligumia*	*recta*		X	X	X	X	X	X		
Unionidae	Lampsilini	*Ligumia*	*recta male*			X						
Unionidae	Lampsilini	*Medionidus*	*conradicus*		X	X	X	X	X	X	X	X
Unionidae	Lampsilini	*Potamilus*	*alatus*		X	X	X					
Unionidae	Lampsilini	*Ptychobranchus*	*fasciolaris*		X	X	X	X	X	X	X	X
Unionidae	Lampsilini	*Ptychobranchus*	*fasciolaris male*			X						
Unionidae	Lampsilini	*Ptychobranchus*	*subtentus*		X		X	X	X	X		X
Unionidae	Lampsilini	*Toxolasma*	*lividum*		X(EP)	X	X					
Unionidae	Lampsilini	*Truncilla*	*truncata*		X	X	X					
Unionidae	Lampsilini	*Villosa*	*fabalis*		X(EP)		X					
Unionidae	Lampsilini	*Villosa*	*iris*		X	X	X	X	X	X	X	X
Unionidae	Lampsilini	*Villosa*	*perpurpurea*	(*Venustaconcha trabalis*)	X(EP)		X					
Unionidae	Lampsilini	*Villosa*	*trabalis*	(*Venustaconcha trabalis*)	X		X					
Unionidae	Lampsilini	*Villosa*	*vanuxemensis*		X	X	X	X	X	X		
Unionidae	Pleurobemini	*Elliptio*	*crassidens*		X	X	X					
Unionidae	Pleurobemini	*Elliptio*	*dilatata*	(*Eurynia dilatata*)	X	X	X	X	X	X	X	X
Unionidae	Pleurobemini	*Fusconaia*	*barnesiana*	(*Pleuronaia barnesiana*)	X	X	X				X	X
Unionidae	Pleurobemini	*Fusconaia*	*cor*		X	X		X	X	X	X	
Unionidae	Pleurobemini	*Fusconaia*	*cuneolus*		X	X		X	X	X	X	X
Unionidae	Pleurobemini	*Fusconaia*	*subrotunda*		X	X	X	X	X	X		
Unionidae	Pleurobemini	*Hemistena*	*lata*		X	X	X				X	X

(Continues)

TABLE 1 (Continued)

Family	Tribe	Genus	Species	Williams et al. (2017)	Species in the Clinch River	Primer Database		gDNA Tested	gDNA Amplified		Field eDNA Amplified	
						COI	ND1		COI	ND1	COI	ND1
Unionidae	Pleurobemini	*Lexingtonia*	*dolabelloides*	(*Pleurnonaia dolabelloides*)	X	X	X	X	X	X		
Unionidae	Pleurobemini	*Plethobasus*	*cyphyus*		X	X	X	X	X	X		
Unionidae	Pleurobemini	*Pleurobema*	*cordatum*		X	X						
Unionidae	Pleurobemini	*Pleurobema*	*oviforme*		X	X						
Unionidae	Pleurobemini	*Pleurobema*	*rubrum*		X	X	X	X	X	X		
Unionidae	Pleurobemini	*Pleurobema*	*plenum*		X	X		X	X	X	X	
Unionidae	Pleurobemini	*Pleurobema*	*sintoxia*		X (EP)	X	X					
Unionidae	Pleurobemini	*Pleurobema*	*sintoxia male*			X						
Unionidae	Quadrulini	*Cyclonaias*	*tuberculata*		X		X	X	X	X	X	X
Unionidae	Quadrulini	*Quadrula*	*cylindrica*	(*Theliderma cylindrica*)	X		X					
Unionidae	Quadrulini	*Quadrula*	*intermedia*	(*Theliderma intermedia*)	X (EP)		X					
Unionidae	Quadrulini	*Quadrula*	*pustulosa*	(*Cyclonaias pustulosa*)	X	X	X	X	X	X		
Unionidae	Quadrulini	*Quadrula*	*sparsa*	(*Theliderma sparsa*)	X		X					
Bivalve outgroup species												
Cyrenidae	n/a	*Corbicula*	*fluminea*			X	X	X				
Mytilidae	n/a	*Mytilus*	*edulis*			X	X					
Dreissenidae	n/a	*Dreisena*	*polymorpha*			X						
Solemyidae	n/a	*Soleyma*	*velesiana*				X					
Trigoniidae	n/a	*Neotrigonia*	*margaritacea*				X					
Chordate outgroup species												
Percidae	n/a	*Sander*	*vitreus*			X						
Percidae	n/a	*Notropis*	*atherinoides*			X						
Cyprinidae	n/a	*Hypophthalmichthys*	*molitrix*			X		X				

et al., 2004; Strayer et al., 2004; Williams et al., 1993). North America has the greatest unionid mussel diversity worldwide (Graf & Cummings, 2007; Williams et al., 1993, 2017) with approximately 298 species; 72% of which are federally threatened, endangered, or of special concern (Williams et al., 1993). Population declines are often attributed to decreases in water quality, habitat loss caused by impoundment of rivers, pollution from agricultural, urbanized and industrial sources, and from ecological changes such as loss of host fishes and introduction of invasive species (Ricciardi et al., 1998; Williams et al., 1993). Mussel populations in North America are also experiencing unexplained mass mortality events (Haag, 2019; Waller & Cope, 2019).

Environmental DNA (eDNA) is DNA found in air, water, or soil, and detected without necessarily capturing the live target organism. The field of eDNA analysis has developed into a growing set of field and laboratory methods and has been applied to monitor a number of aquatic species both invasive and native (Belle et al., 2019).The two main strategies for species detection from eDNA are a targeted approach using PCR amplification from species-specific primers and a multispecies approach known as metabarcoding, which involves high-throughput sequencing of all the products of amplification from "universal" primers developed for a taxon of interest. Analysis of eDNA has been suggested as an additional tool for monitoring mussels, especially rare and endangered species (Cho et al., 2016; Gasparini et al., 2020; Prié et al., 2020). Monitoring of mussels with eDNA offers advantages of being less labor-intensive, less invasive for the organisms and their habitat, and potentially detecting cryptic individuals or life stages, or animals living in inaccessible habitats (Stoeckle et al., 2016). Metabarcoding for mussels offers further capabilities in detection of multiple species from the same set of samples, which is especially advantageous in the species-rich watersheds of the southeastern United States. The current rapid declines in freshwater mussel populations, combined with a lack of knowledge on the status of other populations, and the time-consuming nature of traditional visual mussel surveys demonstrates the need for new monitoring tools.

In 1998, a tanker truck over-turned on U.S. Route 460 in Tazewell County, Virginia spilling 1,350 gallons of Octocure-554 revised (a rubber accelerant used in various industries) into the Clinch River, killing an estimated 18,000 mussels of 16 species (Jones et al., 2001). Since then, the U.S. federal government through the U.S. Fish and Wildlife Services' Natural Resource and Damage Assessment and Restoration (NRDAR) program has implemented ongoing efforts to restore habitat and mussel populations. From 2010 through 2019, the NRDAR program in collaboration with the Virginia Department of Game and Inland Fisheries and Virginia Tech released 36,000 hatchery-reared mussels 1–3 years old (20–40 mm long) of 13 species at multiple sites in the Clinch River spill impact zone (Hyde & Jones, 2020). Subsequent traditional monitoring from 2015 to 2018 at restoration sites has shown survival and growth of all hatchery-reared species and thus the need for continued traditional monitoring as well as an opportunity to utilize eDNA monitoring to track restoration progress. Because the Clinch River has a high diversity of freshwater species with 46 extant mussel species, including 20 species listed as federally endangered (Jones et al., 2014) (Table 1), we attempted an eDNA metabarcoding approach.

Here, we describe the development of two metabarcoding primer sets that amplify the suite of Unionidae and Margaritiferidae species found in the Clinch River watershed. We verified the performance of these markers for amplifying genomic DNA from unionids in the laboratory and compared the eDNA metabarcoding survey results from water samples collected at multiple sites in the Clinch River with results from quantitative visual surveys at three sites conducted between 2016 and 2017 (Jones et al., 2018; Phipps et al., 2018). We expected to detect unionid mussels at all sampling sites using these two assays. We hypothesized that the two markers would identify similar but not necessarily the same suite of species, and we predicted that eDNA species detections would reflect the known assemblages at sites. Our results support the utility of eDNA metabarcoding as a complementary tool to traditional surveys for monitoring freshwater mussel populations.

2 | MATERIALS AND METHODS

2.1 | Primer development

We used sequences from two mitochondrial genomic regions for primer development: the cytochrome c oxidase subunit I (COI) gene region and NADH dehydrogenase subunit 1 gene region (ND1). These gene regions were chosen because: (a)—they have high levels of interspecies divergence, allowing for improved species level resolution compared to other mitochondrial loci; (b)—these are the two most abundant regions sequenced for this taxonomic group found in the National Center for Biotechnology Information's GenBank database (https://www.ncbi.nlm.nih.gov/genbank/). Sequences available for the known mussel species in the Clinch River system (Table 1) were downloaded from GenBank and added to our primer design database (Appendix S1). Ten of these species are believed to be extinct or extirpated, leaving 46 extant species in the system. Two of the extant species in the Clinch River did not have a representative sequence for either region (*Pegias fabula* and *Alasmidonta viridis*); however, there are representative sequences for the six extirpated species. Therefore, of the 56 species known to historically be in the Clinch system, we downloaded sequences for 50 of the species (Table 1).

Several bivalve mussel orders including Unionida are known for having doubly uniparental mitochondrial inheritance, in which males possess two distinct mitochondrial genomes, the female mitotype passed maternally and the male mitotype passed through the paternal lineage (Breton et al., 2007, 2011). This male mitotype is generally only found in the gametic tissue of males (Venetis et al., 2006). For the COI database, we included sequences of the male mitotype for eight Unionida species for which there were data in GenBank.

Sequences from five additional Unionida taxa not found in the Clinch system were included in the primer design databases as well.

These include *Anodonta californiensis*, *A. oregonesis*, *A. nuttalliana*, *Epioblasma rangiana*, and *E. walkeri* (Table 1). Thus, sequences from a total of 55 Unionida species plus male mitotype sequences from eight of these species were used in primer development. Finally, we included sequences from eight outgroup taxa, five non-Unionida bivalves and three fish species (Table 1). Accession number and number of sequences used in primer development can be found in Appendix S1 spreadsheets.

Sequences for each region were aligned in Geneious Prime 2019.1.3 (https://www.geneious.com) using MUSCLE (Edgar, 2004) and visually inspected. Within the Unionida order, classification and taxonomic refinement is ongoing with molecular data shedding new light onto phylogenetic relationships not discerned by earlier morphological assessments; therefore, we utilize the taxonomy and species names presented in Williams et al. (2017), which may differ from what is recorded in GenBank. We highlight these changes in Table 1. The species we utilized for primer development are reported in Table 1 as the name given to the sequence from the original GenBank submission with updated taxonomy in parentheses.

2.2 | Primer evaluation

We developed the first primer set based on the work of Cho et al. (2016) who created a number of species-specific primer sets for Canadian freshwater mussels using the COI gene region. Among the primers they developed was a pair (*Pfa*COI2) which amplified 28 of the 30 species they tested, including 17 species that are found in the Clinch River system. We visually evaluated the nucleotide variation between this primer set and our aligned sequences. We added degenerate sites to the *Pfa*COI2 primers where high amounts of nucleotide variation existed and labeled the new primer set as *Pfa*COI2_Degen. Three degenerate bases were added to the *Pfa*COI2_Degen_F, and two were added to the *Pfa*COI2_Degen_R primers (Table 2). For the second primer set, we focused on the alignment of sequences from the ND1 gene region and visually identified hypervariable regions flanked by conserved regions. We chose four forward and seven reverse primers as candidates to test. All primer pairs were then evaluated using IDT™'s OligoAnalyzer Tool for GC content, melting temperature and possible dimer formation.

The COI primer set and ND1 primer combinations were tested in the laboratory using genomic DNA collected from tissue and swab samples of both target and nontarget (*Corbicula* spp.) mollusks, as well as DNA extracted from silver carp (*Hypophthalmichthys molitrix*) fish tissue. As some of the Unionida species are endangered, we used noninvasive swabbing to collect DNA from species that we did not have tissue samples for. Swab samples were collected from mussels identified morphologically by trained technicians. DNA was extracted from tissue samples with the gMax mini genomic DNA kit (IBI Scientific). Swab samples were extracted using BuccalPrep Plus DNA Isolation Kit (Isohelix). For primer testing, we ran end-point PCR for both assays using extractions from 32 species, including two outgroup (nontarget) species. For *Cumberlandia monodonta*, we had two different extractions, one of the female mitotype and one of the male mitotype. Male mitotype DNA was extracted from released spermatozeugmata captured in the laboratory. Because the male mitotype extraction did not amplify with our assays nor with primers used to amplify male mitotype sequence of other freshwater mussels (Curole, 2004), we used primers designed in our laboratory that targeted a segment of the cytochrome *c* oxidase subunit II region in the male mitotype of *C. monodonta* to sequence verify the extraction (Appendix S2). PCR product was cleaned using the MinElute PCR Purification kit (Qiagen). Product was Sanger sequenced for product verification at the University of Missouri DNA Core laboratory and run on a 3730 × l 96-capillary DNA Analyzer (Applied Biosystems).

Primer sets *Pfa*COI2_Degen and ND1_Mini_F4R1_Degen were selected and used in subsequent testing with water samples. The COI assay amplifies a 238 base pair (bp) amplicon, and the ND1 assay amplifies a 227 bp amplicon. Primer sequences, amplicon lengths, and annealing temperatures are shown in Table 2.

2.3 | Water sampling and processing

Water samples were collected in the field on 19 September 2017 at six different known mussel beds along the Clinch River (Figure 1). A total distance of 216 river kilometers was covered with Indian Creek being the furthest upstream site and Kyles Ford being the furthest downstream site. A total of eight samples per site were collected at Indian Creek, Cleveland Island, Wallens Bend, and Kyles Ford. A total of 16 samples were collected at Bennett Island and Pendleton Island. All technicians collecting samples wore new, sterile disposable gloves for each set of samples. Water samples were taken using a three-meter long sampler constructed from PVC pipe that held four, 50-ml tubes.

TABLE 2 Primers used for each metabarcoding assay using the mitochondrial DNA gene regions cytochrome c oxidase subunit I (COI) and NADH dehydrogenase subunit 1 (ND1)

Primer name	Sequence 5′–3′	Anneal temp. (°C)	Amplicon size (bp)
*Pfa*COI2_Degen_F	ACACTCTTTCCCTACACGACGCTCTTCCGATCT**AGKCTTTTRATTCGDGCTGA**	50.4	238
*Pfa*COI2_Degen_R	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT**CCAGTHCCAACACCHCTCTC**		
ND1_Mini_F4_Degen	ACACTCTTTCCCTACACGACGCTCTTCCGATCT**CAAMTYCGAAARGGYCC**	51.0	227
ND1_Mini_R1_Degen	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT**GGCTCARCCTGYTATDARDGT**		

Note: For the first PCR round the assay specific (bold) section was used as a primer. For the second PCR round primers with the assay specific and Illumina® specific sequencing primer tail sections (underlined) were used. Annealing temperatures and amplicon size are included.

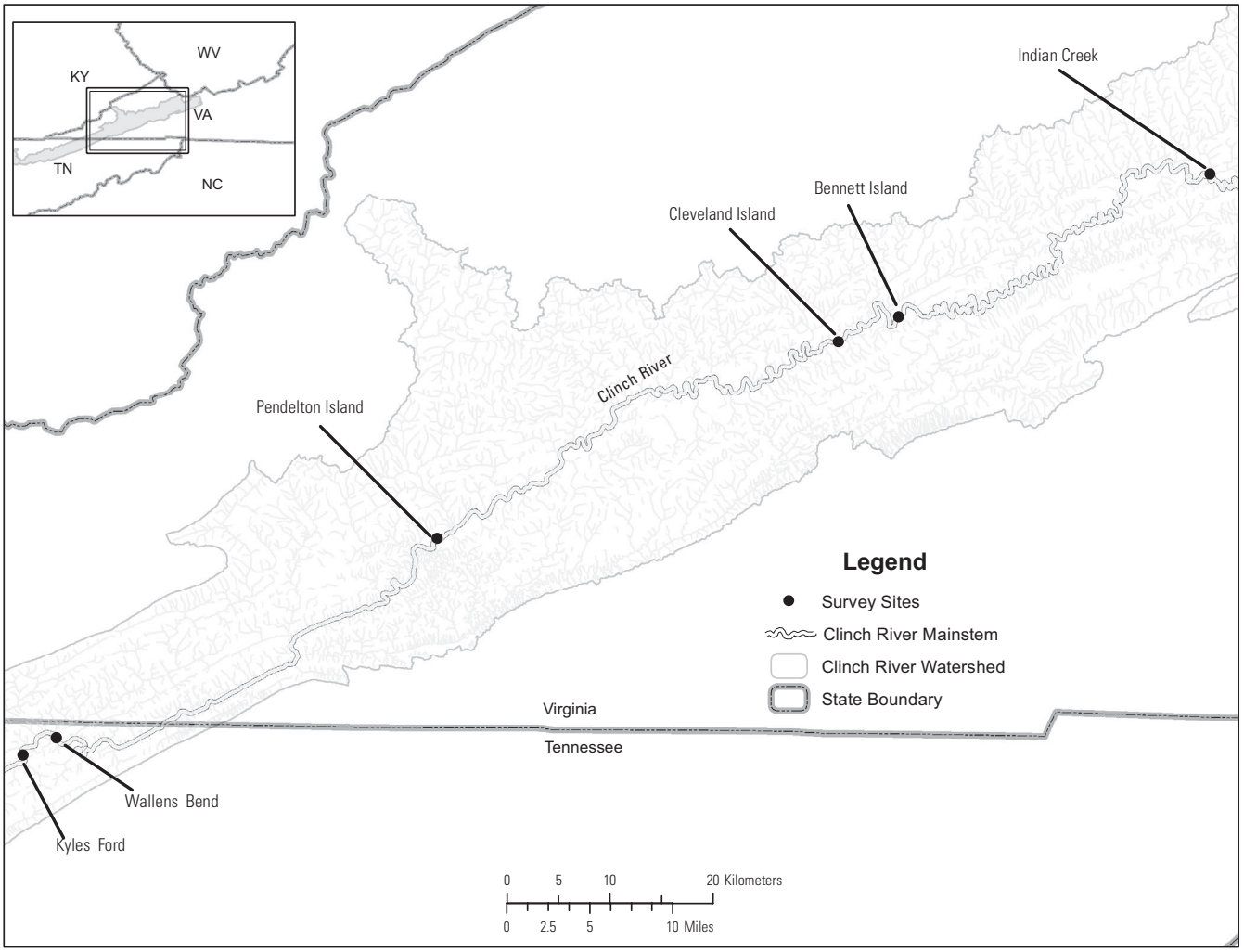

FIGURE 1 Map showing the six sites at which eDNA samples were collected in the Clinch River on September 19, 2017. The distance covers approximately 216 river kilometers (RKM). The confluence of Indian Creek with the Clinch is at 521 RKM, Bennett Island is at 447 RKM, Cleveland Island is at 436 RKM, Pendleton Island is at 364 RKM, Wallens Bend is at 309 RKM, and Kyles Ford is at 305 RKM. The direction of flow is from Indian Creek (upstream) to Kyles Ford (downstream)

Technicians waded into the water at approximately the middle of the stream and dipped the sampler into the river in the upstream direction to avoid potential contamination from waders that were not bleached between sites due to logistics. Sample tubes were dipped in the stream water, filled to 45 ml, capped, and immediately chilled in the dark on wet ice until they could be placed in a freezer. Field blanks were taken at three of the sites (Indian Creek, Pendleton Island, and Kyles Ford), which consisted of a 50-ml tube filled with distilled water at the field site. Samples were frozen and shipped overnight to the U.S. Geological Survey's Columbia Environmental Research Center (CERC), Columbia, Missouri, USA. The PVC water samplers were sprayed with a 10% bleach solution and dried in between sampling sites.

At the CERC genetics laboratory, frozen samples were thawed and concentrated via centrifugation. Water samples were centrifuged for 30 min at 5,100 RCF at 4°C. The water was decanted off, and the DNA pellet was suspended in 200 μl of the extraction kit's GST buffer (IBI Scientific) in 1.5-ml tubes. Samples were then stored at −20°C until DNA extraction.

For DNA extraction, samples were digested using proteinase K overnight in a shaking 60°C water bath and subsequently extracted using the gMax Mini Genomic DNA Kit (IBI Scientific) following the manufacturers specifications. Purified DNA from each 45 ml water sample was re-suspended in 50 μl of 10 mM Tris-HCL.

Prior to amplification with the metabarcoding primers and library preparation, all eDNA samples were tested for PCR inhibitors by running each sample with an internal positive control qPCR assay (Appendix S3). Samples were considered inhibited if the Cq value of the internal positive control (IPC) was equal to or greater than two cycles of the average PCR-negative control in which the only input DNA was that of the IPC.

2.4 | Sample library preparation and sequencing

We amplified samples with each marker independently, creating two sequencing sets, one for the COI marker and one for the ND1

marker. Sets included one or more positive control samples (four for the COI dataset and one for the ND1 dataset) to detect read carryover, as well as two PCR no-template reactions as our negative controls. Positive controls were genomic DNA from Unionida species that are not found in the Clinch River watershed (*Anodonta nuttalliana* for COI and *Lampsilis siliquoidea* for ND1). For the ND1 set, one of the negative controls was DNA from silver carp (*H. molitrix*). This sample was considered a negative control as no target template was introduced and we did not expect DNA from this nontarget species to amplify with the primers. Each set contained prepared libraries of the 55 field samples, three field blanks, laboratory positive controls, and negative controls. Thus, the COI set had a total of 64 samples and the ND1 set had 61 samples.

Each sample library was prepared for paired-end, high-throughput sequencing on the MiSeq platform (Illumina®) using a multiple PCR process modified from the two-step PCR described by Taberlet et al. (2018). In the first step, the target was amplified with assay specific primers (Table 2). A second round of PCR used the previous PCR cleaned product as template and primers that were tailed with 33- to 34-bp sequencing primer region on the 5′ end (Table 2). A reaction clean-up was also performed after the second round. For both markers, each sample was amplified using a 25 μl reaction volume, including 1X AmpliTaq Gold™ 360 Master Mix (Thermo Scientific), 600 nM of each primer, and 2 μl of template DNA. Conditions for the COI assay were as follows: 2 min initial denaturation at 94°C, followed by 35 cycles of 94°C for 15 s, 50.4°C for 30 s, and 72°C for 1 min. Conditions for the ND1 assay were as follows: 5 min initial denaturation at 95°C, followed by 35 cycles of 95°C for 30 s, 51°C for 30 s, and 72°C for 1 min. For the second PCR round using the Illumina sequencing region tailed primers, reaction conditions were the same for each primer pair, except using 300 nM of each COI tailed primer or 600 nM of each ND1 tailed primer and 2 ul of product. After each round of PCR, reactions were cleaned up using the MinElute PCR Purification kit (Qiagen). Size and quantity of product from each sample was measured using a QIAxcel® (Qiagen) instrument. Samples with quantifiable amounts of product were diluted to the same concentration. Product was then sent to the University of Missouri DNA Core laboratory for an additional round of PCR and sequencing.

The third and final PCR step added the paired-end indices (IDT™, Ultramer Oligos) as well as the P5 and P7 adaptor sequences, which enables the prepared product to bind onto the surface of the Illumina® MiSeq flowcell. The added indices allowed for the multiple libraries to be pooled together in a single MiSeq run. PCR was carried out in 50-μl reactions including 1X Phusion™ HF buffer, 200 nM of each indexed primer, 2 U of Finnzymes' Phusion™ High-Fidelity DNA polymerase (NEB®), and up to 28.5 μl of product from the previous PCR. Conditions consisted of a 3 min initial denaturation at 98°C, followed by 25 cycles of 98°C for 15 s, 55°C for 30 s, and 72°C for 30 s, with a final 7 min extension at 72°C. Samples were normalized to equal concentrations when possible (with the exception of samples that had little quantifiable product such as negative controls and field blanks). Then, equal volumes of libraries were pooled together into one set for each of the two markers.

The two sets were then run separately on an Illumina® MiSeq with 2 × 300 bp V3 chemistry. An additional 15% PhiX DNA spike-in control was added to improve library diversity and subsequent sequencing of reads.

2.5 | Bioinformatic processing

Sequence pairs (2 × 300) from the MiSeq runs were first joined using flash (Magoc & Salzberg, 2011). Primers at the ends of the successfully joined contigs were removed using cutadapt, and contigs were retained only if both primers were found (Martin, 2011). The usearch fastq filter program filtered those contigs whose expected number of errors was >0.5. All contigs were clipped from the 5′ end, and any contig shorter than 238 bp for the COI assay and 227 for the ND1 assay was rejected. The Qiime (Ver. 1.9.1) command split_libraries_fastq.py was used to format a fasta file of the cleaned, assembled, contigs (Caporaso et al., 2010). The outputs for all samples were concatenated into one file for clustering and chimera removal by the uparse method with a 97% threshold for clustering (Edgar, 2013). Taxonomy was assigned to a representative of each cluster of OTUs (operational taxonomic units) using BLASTn and the National Center for Biotechnology Information's nucleotide collection (nr/nt) database in May 2020 (NCBI, http://www.ncbi.nlm.nih.gov/) (Altschul et al., 1990; Camacho et al., 2009).

The search retained each OTU's top 100 BLAST hit sequences, scientific name, accession number, identity percentage, coverage percentage, and e- (expect) value. Only OTUs with >99% coverage were retained. We further assessed taxonomic identification manually to avoid sequence identification errors in GenBank (Axtner et al., 2019; Prié et al., 2020). Operational taxonomic units with BLAST hits having an identity (% similarity between query and subject sequence) ≥97% were considered identified to species level. We recorded which species were identified at this level as well as the first taxon identified below the 97% identity threshold. If a single OTU's BLAST search hit different species tied for best similarity, we retained the taxon identification belonging to the species found in the Clinch River, or we retained the multiple species identification for further evaluation. Finally, all OTUs with singleton reads were removed (Civade et al., 2016) (Appendix S4).

2.6 | Carryover calculations

Previous studies have found that reads from one sample can be found at low amounts in other samples through the processes of tag-jumping, index-hopping, sequencing carryover, or cross-contamination (Evans et al., 2017; Hanfling et al., 2016; Harper et al., 2019; Klymus et al., 2017; Schnell et al., 2015; Taberlet et al., 2018). In order to reduce the false positives from these processes, we included a positive control of either the Winged floater (*A. nuttalliana*) DNA

in our COI sequencing run or Fatmucket (*L. siliquoidea*) DNA in our ND1 sequencing run. Neither species is present in any of our field sites, thus any Winged floater or Fatmucket reads detected in the field samples is due to cross-contamination or sequencing carryover from these positive controls. We used the number of reads from the positive control species found in our field samples as a threshold. For each sample, we removed any OTU that had a read number less than or equal to the number of positive control reads identified in that sample Although similar to methods for handling carryover and cross-contamination in other studies our methods did differ from previously published work. For instance, our positive control samples used to assess carryover contained DNA extraction from one species and not DNA from multiple species or "mock communities" as used by Evans et al. (2017). Furthermore, our threshold determination method differs from that used Evans et al. (2017), which used the frequency of reads from field species that were found in their control libraries (mock communities, extraction blanks and no-template controls) as a minimum detection threshold.

2.7 | Data analysis

To create the final OTU table, sequences identified to the same taxon were collapsed into one OTU by summing the counts of all reads for that taxon. Species detection was recorded at a site if sequences for that species were found in any one of the site replicate samples. Reads from species in the negative controls remaining after the carryover threshold was applied were left as is and not removed. Species accumulation curves for each site were created using the replicate sample data and the Vegan 2.5-6 package in Rstudio (Oksanen et al., 2015; R Core Team, 2019). We also examined whether or not the number of COI and ND1 reads correlated with one another for the same sample at each site. Finally, we compared our eDNA results from three of the sites with visual survey data collected between 2016 and 2017 (Jones et al., 2018; Phipps et al., 2018).

3 | RESULTS

3.1 | Primer development

Our reference database of sequences used in primer design includes 55 mussel species across approximately 29 genera from the two North American families, Margaritiferidae and Unionidae (Table 1). Although not exhaustive, the reference species used to design primers ranged evenly across the known North American genera (Williams et al., 2017). The database includes sequences from 50 of the 56 Unionid mussel species known to occur historically in the Clinch River (Table 1). The COI database also included male mitotype sequences from eight of the species (Appendix S1). No male mitotype data for the ND1 region were found on GenBank. Based on these sequences, the primer pairs chosen had fewer base pair differences in the ingroup (Unionida mussels) compared to the outgroup species (fish and non-Unionida mussels) (Appendix S1). Within the Unionida species, differences of 3–5 bp per primer were noted between the female mitotype sequences and known male mitotype sequences included in the COI database. The only Clinch River species that are indistinguishable at these markers for which we have genetic sequence data are two pairs of species whose taxonomic classification has recently changed, *Villosa trabalis* and *V. perpurpurea* and *Pleurobema rubrum* and *P. sintoxia* (Appendix S5) (Lane et al., 2016; Williams et al., 2017). Other species not in our database may not be distinguishable from closely related species in this study, so future work should verify the ability of these markers to discriminate among a different suite of species.

3.2 | Primer evaluation

All genomic DNA extractions sequenced to the expected species. The extraction of male *C. monodonta* gametic tissue amplified with the in-house designed primers but we only obtained good quality sequence from the reverse read; nevertheless, its sequence was 100% identical to that of male *C. monodonta* mitotype. Of the 32 species for which we had genomic DNA extractions to test primers, including the two outgroup species, all 30 Unionida species amplified with both the COI assay (*Pfa*COI2_Degen primers) and the ND1 assay (ND1_Mini_F4R1_Degen primers). Neither assay amplified the two outgroup species (*C. fluminea* and *H. molitrix*) nor did they amplify male *C. monodonta* mitotype genomic DNA (Table 1).

3.3 | Water sampling, sequencing, and bioinformatic processing

Of the 64 field samples taken, we lost nine during shipping and processing. The remaining 55 samples plus three field blanks were processed, amplified and sequenced. Thus, the total number of samples sequenced per site were: Indian Creek (5), Bennett Island (14), Cleveland Island (5), Pendleton Island (16), Wallens Bend (7), and Kyles Ford (8). No PCR inhibition was detected in any eDNA sample using the qPCR assay.

For the COI assay run we recovered a total of 12,054,784 reads and retained approximately 55% after merging paired reads, trimming, quality checks and chimera removal. For the ND1 assay, we recovered 10,959,755 reads and retained approximately 49% after processing. See Appendix S6 for the number of reads retained throughout each quality control step. The sites or sample types with the highest number of median reads (Wallens Bend, Kyles Ford and the positive controls) also had the highest number of reads pass these initial filtering steps (Appendix S7).

Of the processed reads 91% of the COI reads and 90% of the ND1 reads were assigned to species level identification and only 4% and 7% of the processed reads from respective datasets led to a no Blast hit (Table 3). During taxonomy assignment, *Villosa taeniata* and *V. iris* were identified both with 100% identity to the same OTU in

the ND1 dataset. Given that *V. taeniata* is not in the region, we assigned the OTU to *V. iris*. In another case our COI dataset showed that *Lampsilis higginsii* and *Actinonaias ligamentina* both had 100% identity to the same OTU. Furthermore, sequences from these two species for the larger COI fragments available on GenBank had no fixed species differences between them. Because *L. higginsii* is not found in the Clinch River system, we designated these as *A. ligamentina*. These two species are distinguishable with the ND1 marker with four base pair differences. Appendix S4 provides the BLAST hits, percentage of identity, and taxonomic identification decision for OTUs in each dataset.

3.4 | Carryover calculations

Carryover from the positive control species made up approximately 9% of the COI field sample reads and 2% of the ND1 field sample reads. After applying this threshold and removal of singletons, 58% of the original merged COI reads and 83% of the original merged ND1 reads remained for the final analyses (Table 3). After the removal of reads from these steps, 36 OTUs were removed from the COI dataset and 0 OTUs were removed from the ND1 dataset. However even with the loss of OTUs from the COI dataset, all 17 species that the original OTU set collapsed into, remained in the final dataset, and thus, the threshold filtering did not remove any species from our final dataset (Table 3).

3.5 | Data analysis

From both datasets, sequences from 19 freshwater mussel species were detected from our Clinch River water samples (Figure 2). Eight of the 19 species identified are federally endangered (*Epioblasma brevidens*, *E. capsaeformis*, *E. triquetra*, *Fusconaia cor*, *F. cuneolus*, *Hemistena lata*, *Pleurobema plenum*, and *Ptychobranchus subtenus*). Seventeen species were identified with the COI primer set. The COI primer set did not identify *A. marginata* or *P. subtenus*. The ND1 primer pair identified 14 species while not detecting DNA from *A. ligamentina*, *E. brevidens*, *E. triquetra*, *F. cor*, or *P. plenum*. Finally, several OTUs identified equally as *E. rangiana* (*E. torulosa rangiana*) and *E. capsaeformis* in the COI dataset, indicating that our assay cannot distinguish between the two species. In fact, there are no base pair differences among *E. capsaeformis*, *E. walkeri*, *E. aureola*, and *E. rangiana* in the sequenced regions for either assay. These four species belong to a group of closely related mussels that typically have a <1% divergent at mitochondrial DNA (Jones & Neves, 2010; Jones et al., 2006). Because *E. rangiana* is not in the Clinch River, we identified these OTUs as *E. capsaeformis*. Additionally, one OTU met the

TABLE 3 Summary of the number of reads, operational taxonomic units (OTUs), and species produced for the COI and ND1 datasets

	COI	ND1
Sum of all raw reads	12,054,784	10,959,755
Sum of all reads post merging, trimming, and chimera removal	6,571,095	5,398,169
Sum of reads assigned to species level	5,957,097	4,869,161
Percentage of reads assigned to species level	91%	90%
Sum of reads assigned above species level (genus to family)	361,163	131,073
Percentage of reads assigned above species level (genus to family)	5%	2%
Sum of reads with no Blast hit	252,835	397,935
Percentage of reads with no Blast hits	4%	7%
Sum of reads after all filtering	3,810,192	4,488,164
Percentage of reads after all filtering	58%	83%
Sum of carryover reads in field samples	585,257	84,418
% of carryover reads in field samples	9%	2%
Number of OTUs	542	1,088
Number of OTUs after removal of no Blast hits	375	41
Number of OTUs at species level identification	158	27
Number of OTUs after carryover threshold filtering	122	27
Number of species	17	14

Note: The table shows the total number of raw reads and the number of reads after the initial bioinformatic processes of merging, trimming and chimera removal. Next, the table includes the number of reads assigned to species level, the number that resulted in no Blast hits and the number of reads produced after all filtering (only species level, removal of singletons, removal of the carryover threshold and removal of the positive control samples). The table also includes the total number and percentage of carryover reads found in field samples. Finally it shows the number of OTUs produced before and after filtering as well as the number of species the resulting OTUs collapsed into.

	COI – Percentage of Reads						ND1- Percentage of Reads					
	Indian Creek (5)	Bennett Island (14)	Cleveland Island (5)	Pendleton Island (16)	Wallens Bend (7)	Kyles Ford (8)	Indian Creek (5)	Bennett Island (14)	Cleveland Island (5)	Pendleton Island (16)	Wallens Bend (7)	Kyles Ford (8)
Actinonaias ligamentina				0.0941	7.6191	3.4348						
Actinonaias pecterosa	100.0000	0.0616	56.3297	1.2451	48.7767	74.0827	0.0876	51.1741	23.8576	0.5621	54.6858	68.6961
Alasmidonta marginata												0.0011
Cyclonaias tuberculata					0.3933	1.7724				0.0018	1.4778	0.0001
Epioblasma brevidens						5.4842						
Epioblasma capsaeformis				0.0528	0.2010	2.5128	0.0004			0.0018		1.2129
*Epioblasma OTU ?**					0.0034	0.0155						
Epioblasma triquetra		0.0003				1.3299						
Eurynia dilatata				0.0191	6.4847	3.5386	0.0105	0.0090	76.0898	0.0526	10.6724	2.2094
Fusconaia cor					0.0152	1.6235						
Fusconaia cuneolus				0.0294	4.7913	0.1103	0.0009	0.0035	0.0013	0.0241	7.6055	0.0002
Hemistena lata					3.8246		0.0013	0.0022		0.0170	8.8719	
Lampsilis fasciola					18.7808	2.1093	0.0135	1.8369	0.0306	0.0553	6.8119	15.1183
Lasmigona costata		10.9570					0.0004	4.2120		0.0054	3.2225	
Medionidus conradicus		0.0023	43.6703			0.2119	0.0009	26.1427		0.0054	0.0006	0.0005
Pleurobema plenum						0.0098						
Pleuronaia barnesiana		30.9767						0.6532				
Ptychobranchus fasciolaris					9.1099	2.6242	0.0028	0.0051		0.0562	2.5971	9.8679
Ptychobranchus subtenus							0.0007	0.0045	0.0013	0.0054	4.0484	2.8535
Villosa iris		58.0021		98.5595		1.1400	99.8811	15.9568	0.0193	99.2131	0.0060	0.0401
Number of Species	1	6	2	6	11	15	11	11	6	12	11	11
Total # Reads	5900	915545	1177	329541	1102988	1431197	459149	312324	300433	112079	1460724	1948970

FIGURE 2 A heatmap diagram (red = most abundant; dark green = least abundant per column) showing the percentage of reads assigned to each mussel species relative to the total reads at that site. Each sample was assayed with two metabarcoding primer sets that targeted either cytochrome c oxidase subunit I (COI) or NADH dehydrogenase subunit 1 (ND1) gene regions. The number in parentheses for each site indicates the total number of 45 ml water samples assayed. Bold are federally endangered species. The total number of species identified for each site is also included [Colour figure can be viewed at wileyonlinelibrary.com]

97% similarity threshold for identification to *E. rangiana/E. capsaeformis*; however, upon closer inspection the seven base pairs that differentiate it from known *E. rangiana/E. capsaeformis* sequences were identical to the sequence motif of *A. pectorosa*. These seven base pair differences were located in the middle portion of the sequence read surrounded by ends that match that of *E. rangiana/E. capsaeformis*. This OTU was only found in low abundance in one replicate sample of Kyles Ford (222 reads) and one replicate sample of Wallens Bend (37 reads) (Figure 2). This OTU that we labeled *Epioblasma OTU?* was not included as a species detection.

Over both datasets, *A. pectorosa* and *V. iris* make up the largest proportion of reads (Tables S1 and S2). Species richness is known to be highest at the downstream sites (Wallens Bend and Kyles Ford) relative to sites further upstream (Indian Creek), and the COI eDNA results followed this pattern (Jones et al., 2014) (Figures 2 and 3). This differs from the ND1 dataset where the number of species identified by eDNA is similar across all sites (Figures 2 and 3). The contamination reads in the blanks were generally low (range: 0–24,103 reads; median: 55 reads). Most blanks had *A. pectorosa* and *V. iris* reads as the highest contaminant (Tables S1 and S2).

We examined species identification across site replicates. Replicate sample data reflect the patterns we see in species richness when looking at sites as a whole. Within the COI data set, species richness appears to increase moving from upstream sites (Indian Creek) to downstream sites (Kyles Ford) whereas with the ND1 set, species richness is more evenly spread across sites (Figure 3). Within a site, we see large variation between replicates as well as across datasets (Figure 4). Looking across datasets, the COI dataset had 25 samples with zero amplification compared to the ND1 dataset which only had four samples that did not amplify. Across markers, the same replicate samples were not consistent in which species were amplified. For instance, replicate 4 from Indian Creek was mostly composed of *A. pectorosa* reads with the COI marker but when using the ND1 marker the major contributor

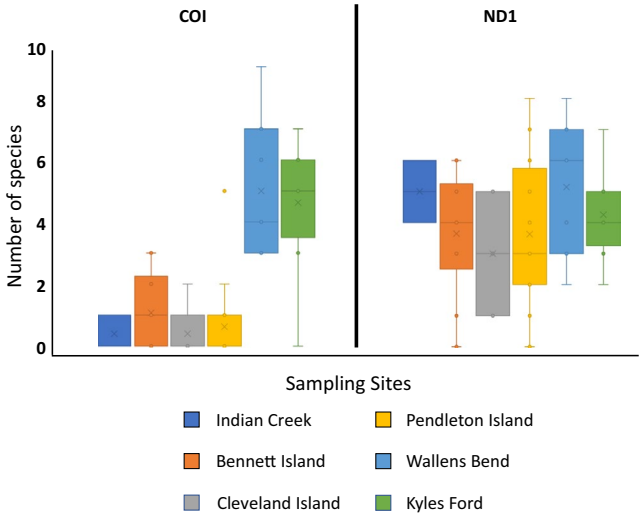

FIGURE 3 Box plots for each field site displaying the median (middle line) and mean number (x) of species identified among site sample replicates for each of the two datasets (COI and ND1). Whiskers represent the minimum and maximum number of species identified for each site among replicate samples. Single points represent outliers [Colour figure can be viewed at wileyonlinelibrary.com]

was *V. iris*. Similarly in replicate 6 of Wallens Bend, the majority of reads for the COI marker were identified from *A. pectorosa*, *H. lata*, and *L. fasciola*, whereas the majority of reads for the ND1 marker were identified from *A. pectorosa*.

Species accumulation curves for the COI dataset show that only the Wallens Bend samples begin to plateau. Curves from all other sites continued to increase, suggesting not enough sample replicates were taken at these sites to capture the species diversity (Figure S1a). For the ND1 dataset, Cleveland Island, Bennett Island, and Wallens Bend curves exhibited plateaus, whereas the other three sites did not show (Figure S1b). We did not observe strong correlations between the number of reads from each marker per site (Figure S2). The closest to the expected pattern was observed in our most species-rich sites, Wallens Bend and Kyles Ford. The pattern appears to be driven by *A. pectorosa*, which is one of the more abundant species at these two sites (Jones et al., 2014, 2018) with a high number of reads for both markers (Figure S3).

Finally, we compared the eDNA results with known quantitative visual survey data at three sites. Visual survey data from 2016–2017 detected more species than our eDNA samples (Jones et al., 2018; Phipps et al., 2018) (Figures 5 and S4). Across the three sites, the visual surveys identified 30 freshwater mussel species including 13 listed as federally endangered species. The eDNA surveys identified 19 species of which 8 are listed as federally endangered. Relative to the visual survey data at the three sites, eDNA samples identified 42% of the species at Cleveland Island, 58% at Pendleton Island, and 54% at Kyles Ford. The ND1 marker identified more species than COI at Cleveland Island and Pendleton Island; however, at Kyles Ford the COI marker outperformed the ND1 marker (Figure 5).

4 | DISCUSSION

We demonstrate two metabarcoding assays that amplify and sequence numerous North American mussel species from two Unionida families. Our assays were able to detect freshwater mussel DNA, including DNA from federally endangered species, using field collected samples. The assays appear to be Unionida specific as they did not amplify DNA from the nontarget taxa we tested nor did we observe off-target sequencing from our field samples. This decreases the chance of nontarget species DNA swamping out the signal from Unionida species and thereby increases the assays' sensitivity for detecting target species. Although designed for the Clinch River mussel assemblage, these markers amplify other North American species (e.g., *L. siliquoidea* and *A. nuttalliana*). We predict that they will also be effective for species outside of North America because they amplify an even more distantly related member from the sister family Margaritiferidae (*Cumberlandia monodonta*); however, further testing is needed to verify assay performance with other species.

Our field sampling demonstrates that although the two markers amplify the same species based on in vitro laboratory testing of DNA extractions, they differ in their ability to amplify the same species in the environment (in situ). Primer bias can lead to differential amplification from multi-target templates when target and primer sequences do not match perfectly. Interestingly, the species that each of the primer sets did not amplify in the field had either zero or one base pair difference in the primer sites (Appendix S1), suggesting that primer bias is unlikely to be the cause of the discrepancy. Despite detecting fewer species overall, the ND1 assay detected more taxa per site suggesting higher sensitivity compared to the COI assay. However, this may be because during library preparation, twice the amount of ND1 tailed primers was used relative to the same step for the COI assay. Regardless of the reason for the difference in specificity and sensitivity between the assays, the use of multiple markers for eDNA metabarcoding increased our overall species detection and species richness estimates, as has been found by others (Evans et al., 2017; Li et al., 2018; Thomsen et al., 2012).

Testing the assays at sites with well-studied mussel assemblages demonstrated their ability to detect a majority (mean of 53%) of the species detected by visual surveys. However, in our study, eDNA species detection relative to traditional survey detection is lower than what other studies have reported. For instance, Evans et al. (2017) found that metabarcoding detected all the fish species found using traditional sampling. Likewise, Prié et al. (2020) developed a 16S metabarcoding primer set for Eastern Palearctic unionid species, which detected greater than or equal to the number of species detected by traditional surveys. One reason for this difference may be the uneven sampling effort in the present study (i.e., number of replicate samples and total volume of water collected at each site). When assessing species detections, Prié et al. (2020) used samples that had up to 30 L of water filtered per site and Evans et al. (2017) used up to 31 samples that contained 250 ml each (a total of 7.75 L) to describe a 2.2-ha reservoir. Our sampling per site consisted of between 5 and

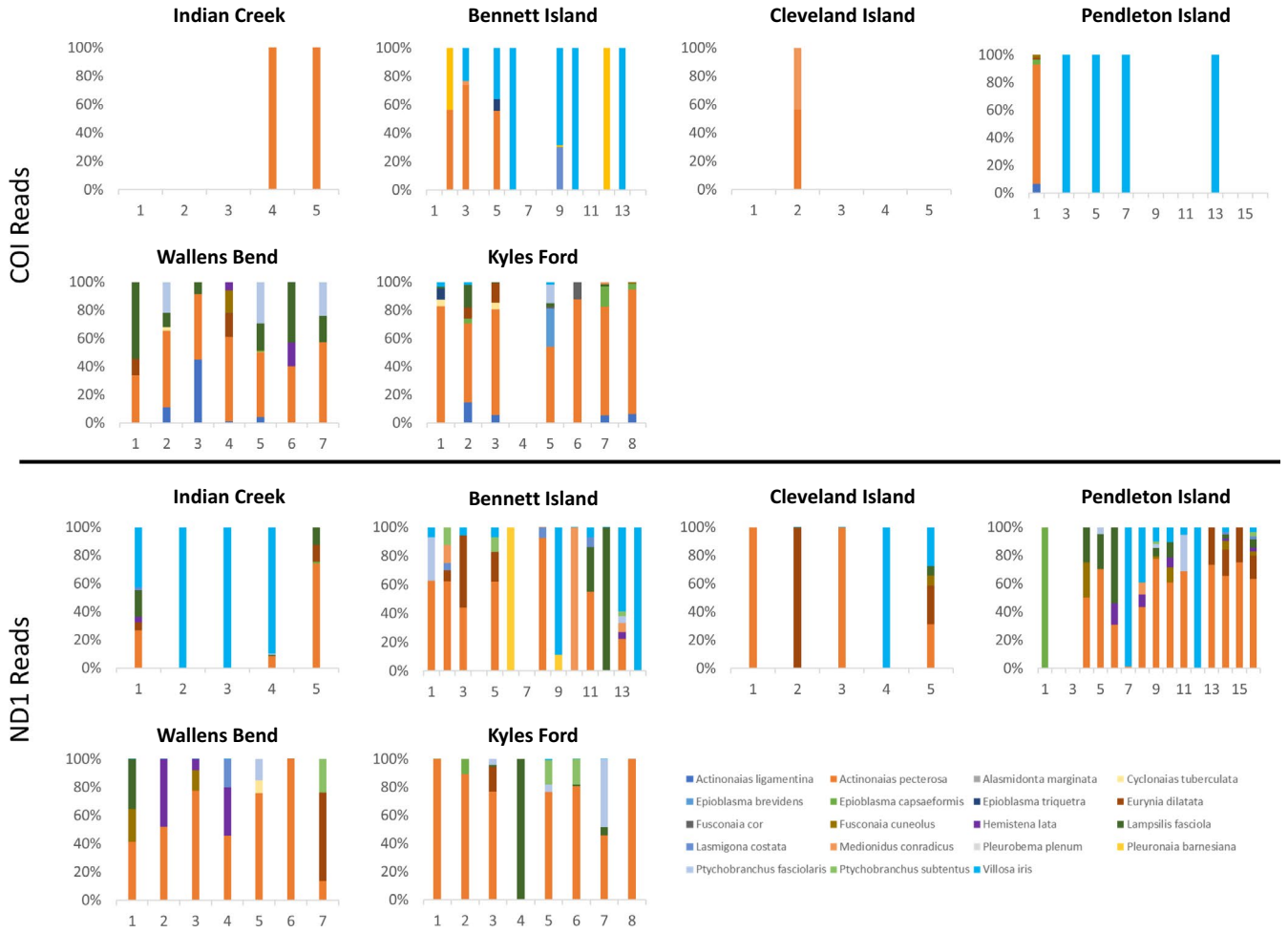

FIGURE 4 Plots of percentage species composition of each replicate sample per site for the COI (top) and ND1(bottom) datasets. Each color indicates a different species. The legend for both plots is included on the bottom panel [Colour figure can be viewed at wileyonlinelibrary.com]

16 replicate samples each containing 45 ml of water for a total volume per site of only 225 to 720 ml. Species accumulation curves also show that most sites were not adequately sampled. The presence of PCR inhibitors is a potential factor that can lower species detection (Gasparini et al., 2020; Jane et al., 2015; McKee et al., 2015); however, we found no evidence of inhibition in our samples in qPCR tests prior to sequencing. Given the lack of inhibition and the substantially smaller volume processed per site relative to other studies, sampling effort likely led to our lower species detections.

Species detection by eDNA can also be affected by species biomass and organism behavior (Spear et al., 2015; Wacker et al., 2019). Freshwater mussel species vary greatly in size, and mussel beds are made up of populations with various ages and size classes of individuals. Because species detection by eDNA surveys relies on the amount of shed DNA, species that are smaller or fewer in number can potentially be more difficult to detect (Wacker et al., 2019). For instance, *Medionidus conradicus* was one of the most abundant species found at Kyles Ford in visual surveys from 2016 to 2017; however, we recovered relatively few reads with our eDNA sampling in 2017. This species is smaller (30–40 mm long) in comparison to some of the other abundant taxa such as *A. ligamentina* (80–140 mm long) and *A. pectorosa* (80–150 mm long). Timing of freshwater mussel eDNA surveys will also affect results. For example, many mussel species at temperate latitudes in North America are near or at the substrate surface in the spring and early summer—typically from April through June—releasing glochidia and spawning, and then again in late summer and fall from September through early November. We would expect to detect more species during these times when mussels are actively filtering the water column and reproducing (Sansom & Sassoubre, 2017; Wacker et al., 2019).

Finally, the downstream movement of shed eDNA in riverine systems affects detection and is an area of active research with implications for eDNA sampling strategies in such environments. Transport distances from known populations can be relatively far. Deiner & Altermatt (2014) found DNA from a European freshwater mussel (*Unio tumidus*) 9 km downstream of the lake population. Sansom and Sassoubre (2017) detected eDNA of the fatmucket mussel (*L. siliquoidea*) 1 km downstream. However, other studies have detected freshwater mussels at much shorter distances, for instance Stoeckle et al. (2016) detected eDNA of *Margaritifera margaritifera* at 25 m downstream, but not beyond. The differences in results from these studies are likely a result of system specific dynamics.

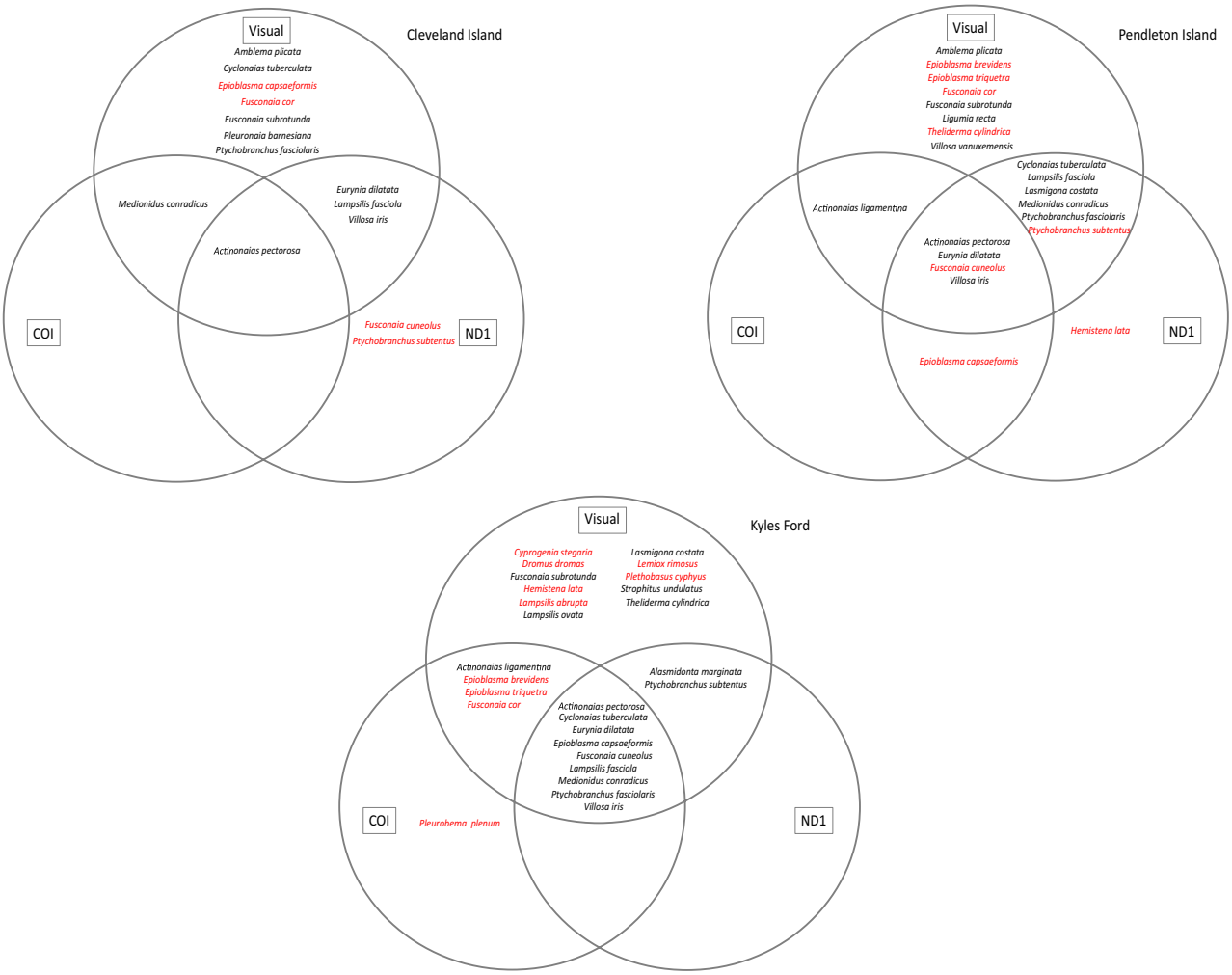

FIGURE 5 Venn diagrams representing overlap in species detection between visual survey data (Jones et al., 2018) and eDNA data (a) Cleveland Island (b) Pendleton Island and (c) Kyles Ford. Names in red are listed federally endangered species [Colour figure can be viewed at wileyonlinelibrary.com]

River hydraulics (i.e., size, discharge, mixing) (Pont et al., 2018), sedimentation (Shogren et al., 2017), and catchment networks (Deiner & Altermatt, 2014) all affect the movement of eDNA downstream. Li et al. (2018) and Pont et al. (2018) suggest that homogenization of eDNA signal occurs more rapidly in systems with larger discharge, carrying signal from upstream further down; whereas in smaller, lower discharge systems, transport distance is much shorter, and signal may be more reflective of local mussel assemblages. For larger rivers such as the Rhône with an average discharge of 2,154 m^3/s, Pont et al. (2018) suggest that sample sites to be 70 km apart. The Glatt River sampled by Deiner & Altermatt (2014) was reported to have an average discharge of 3.52 to 3.79 m^3/s and the authors suggest that sampling sites be between 5 and 10 km. Comparatively, the Clinch River is a medium size river with average discharge values from 59 m^3/s at the Tazwell gage to 52 m^3/s at the Looneys Gap gage (U.S. Geological Survey, 2020). Most of our site pairs are >10 km apart from one another with the exception of Wallens Bend and Kyles Ford which are only 4 km apart (Figure 1). The medium discharge values suggest our sampling could be indicative of local assemblages; however, other mussel beds are known between our sites and we cannot discount the possibility of an upstream signal in our samples. The mixing of eDNA in the water may also have impacted the high variation we observed among replicate samples at a site. Since we sampled directly at the mussel assemblage and not downstream, water was unlikely to be homogenously mixed. This, combined with the small volume of water collected per sample, likely led to the high variability observed among replicate samples.

Implementing an eDNA survey brings people from various disciplines together (Mosher et al., 2019). It is important for wildlife managers and other end-users of metabarcoding data to be aware of the influence that bioinformatic processing has on end results (Evans et al., 2017). Similarly working closely with trained biologists in the system of interest helps inform the molecular ecologist about unexpected results. In our study, the carryover thresholds applied to our datasets resulted in some species not being recovered at sites they are known to be (false negatives). For example, *L. fasciola*, a widely distributed species in the Clinch River that is found at all sites sampled by our study (Jones et al., 2014, 2018; Phipps et al., 2018). Our

ND1 analysis detected this species at all sites, but the COI assay only detected it at two sites based on the final data output. Upon further investigation the carryover threshold we applied using our positive control sample removed all reads of *L. fasciola* that were present in the raw COI dataset at all but two sites. Alternatively, such thresholds may not be strong enough and may result in false positives. In the ND1 dataset, *H. lata* is not known to be in Indian Creek or at Bennett Island, but six and seven reads were detected at those sites, respectively. This species is not located at these sites or upstream (Jones & Neves, 2004), suggesting that laboratory contamination or carryover led to detections in these samples. However, because no positive control reads were found in these samples, no reads were removed. It is unlikely that any cross-contamination or carryover threshold will result in an error free dataset. Stringent thresholds can remove true detections, and a lenient threshold can result in false positives. Increasing temporal sampling and number of replicate samples could increase confidence in rare detections and reduce this loss of true-positive detections for rare or low biomass species.

Finally, our 97% threshold value for species assignment or chimeric filtering may not be strict enough. The questionable *Epioblasma OTU?* that was identified may represent an unpublished sequence variant of *E. capsaeformis* or even an unsampled species. A close congener to *E. rangaiana/capsaeformis* is *E. gubernaculum*, a now extinct species which historically occurred in the river. No sequence information is known for this species; thus, it is possible that *Epioblasma OTU?* could be from a recently extinct species. It is also possible that this OTU is from an unsequenced male mitotype, as sampling occurred during the spawning period for several mussel species. However, upon comparison of the known male mitotype sequences to those of the female mitotype sequences (Appendix S5), male mitotypes differ 63% to 75% from female mitotype sequences, not 3%. Given this and the close resemblance of the middle section to *A. pectorosa* the most abundant species identified in our dataset, we suggest that this OTU may instead be a chimeric sequencing artifact that was not detected by our filtering.

In conclusion, our work adds to the growing literature which points to the suitability of eDNA analyses for monitoring mussel populations (Belle et al., 2019; Cho et al., 2016; Currier et al., 2018; Gasparini et al., 2020; Prié et al., 2020). The study verified two Unionida-specific metabarcoding assays suitable for freshwater mussel monitoring through eDNA sampling. Although visual surveys detected a greater number of species than our metabarcoding samples, increased sampling effort could increase the species diversity detected by these assays. Our assays did not appear to detect male mitotype sequence either in the laboratory or in the field, even though sampling took place during the spawning period for some species. Prié et al.'s (2020) 16S assay does detect some male mitotypes, and thus, a combination of these assays may be useful to understand reproductive timing in the field. Continued investigation into DNA transport dynamics in riverine systems will improve sampling strategy design, and ongoing refinement of bioinformatic processing will improve data interpretation. Despite the need for continued research to increase confidence of metabarcoding data, eDNA surveying has great potential for improving and aiding freshwater mussel conservation efforts.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

AUTHOR CONTRIBUTION

J.H., C.R., N.T., J.J., and K.K. conceived and designed the experiments; N.T, C.R., and J.J. collected samples, N.T. and K.K. performed the experiments; K.K. and C.R. analyzed the data; K.K. wrote the paper.

DISCLAIMER

Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

DATA AVAILABILITY STATEMENT

High-throughput sequence data can be found in NCBI's Sequence Read Archive under project PRJNA611800 and PRJNA612037 (https://www.ncbi.nlm.nih.gov/sra/). The data used in this study are available at: https://doi.org/10.5066/P9GO0A2R.

ORCID

Katy E. Klymus https://orcid.org/0000-0002-8843-6241

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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