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Monitoring a minuscule madtom: Environmental DNA surveillance of the endangered pygmy madtom (*Noturus stanauli* Etnier & Jenkins 1980) in the Duck and Clinch rivers, Tennessee

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Abstract

Environmental DNA (eDNA) detection has been shown to be an effective biosurveillance tool for freshwater fishes, but further research is needed to apply eDNA detection tools to small and rare fishes in large rivers. We developed an eDNA surveillance assay and protocol for monitoring the presence of the pygmy madtom (*Noturus stanauli*), a federally protected freshwater fish endemic to the Clinch and Duck rivers in Tennessee (United States, North America). *Noturus stanauli* is a diminutive fish that is exceedingly rare throughout its range; it is currently known only from a 115-river-km section of the Duck River and a 5-river-km section of the Clinch River. The aim of this research was to develop an eDNA assay to detect the presence of *N. stanauli* in both the Duck and Clinch rivers. We used this newly developed eDNA protocol to assess detection as a function of water depth and to further delineate the distribution of *N. stanauli* in both the Duck and Clinch rivers. Field sampling was performed to delineate the extent of *N. stanauli*'s range in both rivers. Our results indicated that samples collected from three areas within the water column, as well as a sediment core samples, yielded equal detection rates. Our assay detected the presence of *N. stanauli* at a previously unknown site outside the current distribution in the Clinch River, located approximately 2.5-river-km downstream from the Tennessee-Virginia state line. We demonstrated that eDNA detection is a promising tool for delineating the distribution of *N. stanauli*; however, further research is needed to assess environmental and life history variables that influence eDNA detection probability of small fish in large rivers.

KEYWORDS

biosurveillance, conservation genetics, endangered species, environmental DNA, freshwater fish, madtom, mitochondrial DNA, quantitative PCR

1 | INTRODUCTION

Information gathered from biosurveillance efforts, such as the presence of species in a given area, population dynamics, and community structure helps resource managers discern current trends in biodiversity and allows for effective management and conservation plans to be developed and implemented (Leathwick et al., 2016; Primmer, 2006). The information obtained from biomonitoring, however, is only as reliable as

the tools and techniques that are used and require tests of data assurance. Traditional fish surveillance techniques, such as electrofishing and seining, can be difficult to standardize and can cause unintentional harm to both nontarget and target taxa in a system (Dwyer et al., 1993; Hayes et al., 1996; Reynolds, 1996; Snyder, 2003). Within the last decade, new molecular tools have been developed to survey rare and cryptic aquatic taxa, offering less-invasive approaches to monitoring fishes.

Environmental DNA (eDNA) monitoring utilizes the discarded biological components in an organism's habitat to determine the presence or absence of an individual in the area and has been especially useful for the surveillance of aquatic organisms. The water in aquatic systems contains genomic material derived from sloughed-off biological material (e.g., skin cells, slime coat, feces) of resident organisms. Species that otherwise can prove difficult to detect and capture using traditional methods can be identified in the local environment without any physical, visual, or auditory contact. Environmental DNA sampling has been shown to be more sensitive and reliable than traditional sampling techniques for rare or secretive animals (Janosik & Johnston, 2015; Pfleger et al., 2016) and for invasive species where early detection is vital to help prevent further spread (Díaz-Ferguson et al., 2014; Jerde et al., 2011). Environmental DNA is an easily optimized surveillance tool, as demonstrated by the vast number of studies that have successfully applied it to a wide range of taxa and systems. Each system and taxa pose different challenges for eDNA amplification and detection. Further research is needed on the effectiveness of eDNA surveillance for the detection of extremely rare species in large river systems.

Due to its diminutive size and reclusive behavior, *N. stanauli* has presented a difficult challenge for conventional surveillance efforts. *Noturus stanauli* is the smallest known member of the bullhead catfish family Ictaluridae (maximum total length 36–44 mm SL) and one of the smallest and rarest of fishes in North America; fewer than 50 specimens currently exist in collections (Bennett et al., 2009; Burr & Stoeckel, 1999; Etnier & Starnes, 1993; Figure 1). While much of its life history is still unknown, laboratory propagation has provided evidence that *N. stanauli* is ecologically similar to other members of the *Noturus* genus: nocturnal/crepuscular lifestyle, low annual fecundity, large embryo and hatchling sizes, cavity nesting, egg guarding, and benthic habitation (Burr & Stoeckel, 1999; Shute, 2001; Taylor, 1969; Wells & Mattingly, 2019). The limited distribution of *N. stanauli* is a leading factor contributing to its endangered status (USFWS, 2008). Historic records report the presence of *N. stanauli* from single locales in both the Clinch and Duck rivers, and several other sites of occurrence have been reported throughout the Clinch and Duck Rivers over the last few decades (Etnier & Jenkins, 1980; USFWS, 2008). Current information suggests that the Clinch River population is confined to a 5-km stretch, while the Duck River population appears to have a larger range across a 115-km area (USFWS, 2008). Although the Clinch and Duck river populations are separated by 1,055 river kilometers, no other occurrences are known from any other aquatic systems (USFWS, 2008).

Environmental DNA offers a less-invasive sampling approach for detecting the presence of *N. stanauli* than traditional survey techniques. Environmental DNA sampling eliminates capture- and handling-induced stress and mortality, while helping to obtain crucial distributional information needed for management agencies. However, eDNA surveillance has not been thoroughly tested in large river systems (≥ 6th order streams; Strahler, 1952) such as the Clinch and Duck rivers with a diminutive fish like *N. stanauli*. Here, we developed an eDNA field protocol for the detection of *N. stanauli*. Our specific objectives were to (a) develop and optimize an eDNA monitoring assay for *N. stanauli* using quantitative polymerase chain reaction (qPCR), (b) empirically test water sampling protocols for eDNA detection, and (c) further delineate the current distribution of *N. stanauli* in the Clinch and Duck river drainages. Results from this study provide insights into the design of eDNA assays for the detection of rare, diminutive fishes and advance disciplinary knowledge regarding the utility of molecular tools for biosurveillance in large riverine systems.

2 | MATERIALS AND METHODS

2.1 | Study area

The Clinch River originates in Virginia and runs southwesterly into eastern Tennessee. The Clinch River stretches 325 km

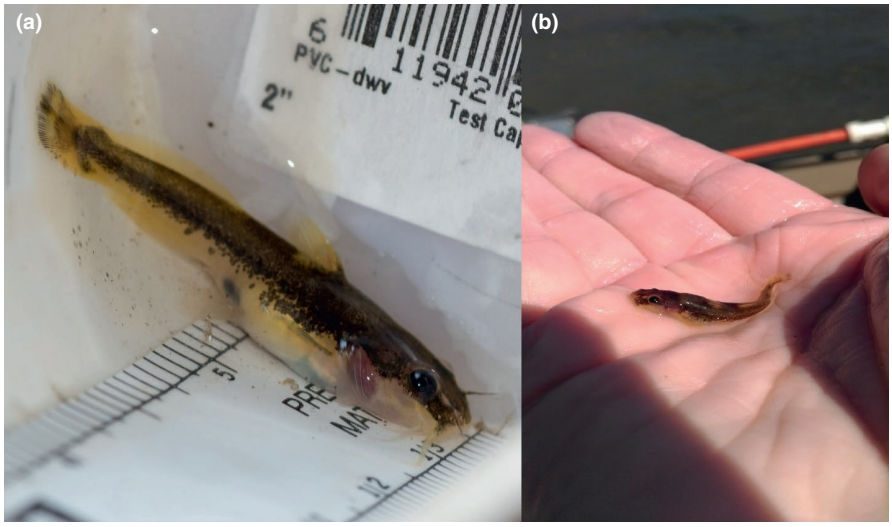


FIGURE 1 Pygmy Madtom, *Noturus stanauli*. (a) Gravid female (41 mm TL), collected in the Clinch River on 12 July 2017 (Wells, 2019). Photograph: Jennifer Caudle. (b) Unknown sex (34 mm TL), collected in the Duck River on 21 September 2016 (unpublished data, TVA). Photograph: Robert T. R. Paine

through Tennessee, spanning a number of different physiographic provinces, before ultimately draining into the Tennessee River (Figure 2b). Biodiversity within the Clinch River has been negatively impacted over the last few decades by anthropogenic influences (e.g., land-use practices, fly ash spills, and coal mining pollution) (USFWS, 2008).

The Duck River is the longest river entirely in Tennessee, originating in central Tennessee and spanning 457 km west-northwest as a tributary to the Tennessee River (Figure 2c). Hosting more than 150 fish species and 66 freshwater mussel species, the Duck River is not only the most biologically diverse river in North America, but one of the greatest freshwater biodiversity hotspots in a temperate region (Ahlstedt et al., 2017; Schilling & Williams, 2002). A majority of this diversity has been adversely affected by human activities including chemical run-off and sedimentation as a result of poor land-use practices or by the construction of dams (USFWS, 2008).

2.2 | Primer and probe design

2.2.1 | In silico

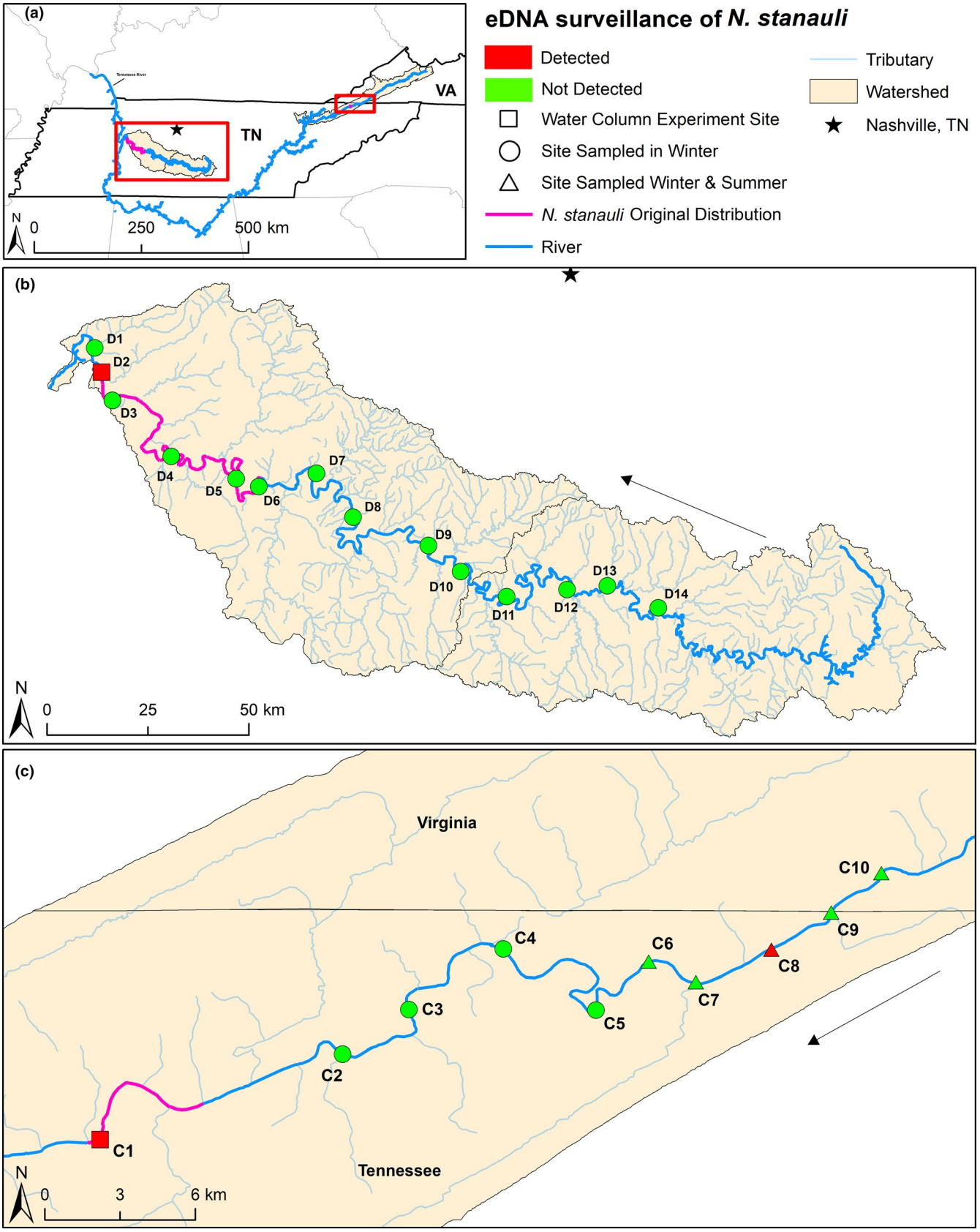
We designed a species-specific qPCR assay (e.g., primers and probe) to target a small fragment of the mitochondrial cytochrome b (*cyt b*) gene; this gene was targeted because of the successful amplification of mitochondrial loci in other eDNA studies and because of the availability of *cyt b* sequences for the genus *Noturus*. Eight sequences (1,036–1,137 bp), representing the *cyt b* region of *N. stanauli* from the Duck (n = 3; GQ153315-GQ153317) and Clinch rivers (n = 5; GQ153318-GQ153319, DQ383660-DQ383662), were downloaded from NCBI's genetic depository GenBank and were aligned using ClustalW (Thompson et al., 1994) in Bioedit Sequence Alignment Editor (Hall, 1999). A consensus sequence was generated from the eight *N. stanauli cyt b* sequences and imported into PrimerQuest (Integrated DNA Technologies) to identify primer and probe-binding sites. We used the default parameters in PrimerQuest with the exception of amplicon length, which was altered to search for amplicon lengths of 75–300 bp. A large portion of eDNA studies target DNA fragments ranging between 90 and 120 bp because shorter DNA fragments persist longer in the environment to allow species detection (Jo et al., 2017, 2019; Rees et al., 2014; Wei et al., 2018); however, longer fragments may be required to differentiate between closely related, sympatric species (Díaz-Ferguson & Moyer, 2014). Candidate oligos were retained based on fragment size and low homo- and heterodimerization potential (Bronnenhuber & Wilson, 2013).

Candidate oligos were compared with the GenBank nr database using the Basic Local Alignment Search Tool for nucleotide sequences (BLASTn) (Altschul et al., 1990) to assess similarity to nontarget sequences. Three criteria from the GenBank database were used to select candidate primers and probes for qPCR amplification including percent identity, e-value, and total score. Percent identity quantifies the similarity between queried sequences to other sequences in the database, where 100% is an identical match. The e-value score is the number of expected hits with a similar identity to the queried sequence that could be found just by chance, where a small value (e.g., $2e^{-06}$) indicates a better match. Lastly, the total score value is the sum of alignment scores of all segments from the same database sequence that match the queried sequence, where a higher value corresponds to a higher similarity to database sequences. Candidate oligos were selected based on the highest combination scores of the three criteria mentioned above. The resulting candidate oligos were imported into BioEdit and aligned to 109 *cyt b* sequences representing 18 ictalurid species from GenBank (Table 1; Table S1) for comparison to nontarget species. All 109 *cyt b* sequences come from individuals in North America. We retained primers with a minimum of two base pair mismatches and a probe with a minimum of one base pair mismatch to other Ictaluridae species (Wilcox et al., 2013).

2.2.2 | In vitro

Primer candidates were tested for amplification with *N. stanauli* genomic DNA extracted from 95% molecular-grade ethanol-preserved tissues from fin-clips or whole specimens of 11 individuals from the Clinch River. Tissues for other Ictaluridae species were collected from the Duck and Clinch rivers, via seining, electrofishing, or dip-netting. Primer candidates were all tested for cross-amplification of other ictalurid species (Table 1) using 1–3 individuals for each species (Bronnenhuber & Wilson, 2013). We did not test assay specificity against nonIctaluridae species, since our in silico results did not indicate any close-related species with high similarity to our oligos (see Results). All tissue-derived DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. Annealing temperature was optimized for candidate primers using a gradient thermocycler in 20-µl reactions containing 4 µl Flexi Buffer (5×), 2 µl $MgCl^{2+}$ (25 mM), 4 µl dNTPs (4 µM), 0.8 µl of each primer (10 µM), 0.2 µl *Taq* polymerase (5 U/µl), 4.2 µl of sterile PCR water, and 4 µl of template (15–50 ng/µl). Thermal cycling conditions were 95°C for 10 min, 35 cycles of 95°C for 30 s, 59–65°C for 60 s, 72°C for 60 s, and a final elongation of 72°C for 5 min. Results

FIGURE 2 (a) Overview of the study area in Tennessee (TN) and Virginia (VA), United States of America. The Duck and Clinch rivers are both tributaries of the Tennessee River. The study areas are highlighted with a red box. Maps of the Clinch River (b) and Duck River (c) showing locations of all sampled sites for water column experiment in summer 2017, and field sampling in winter 2017–2018 and summer 2018. Circles denote sites that were sampled only once during winter 2017–2018 season, while triangles indicate sites that were sampled twice, once during the winter 2017–2018 season and once during the summer 2018 season. Water column experiment sites were sampled during the winter 2017–2018 season field sampling. Black arrows denote the direction of flow for each river



were visualized in a 1.5% TBE agarose gel stained with GelRed under a UV Gel imager.

The candidate qPCR assay (i.e., primers and probe) was tested for specificity against *N. stanauli* and other sympatric ictalurid species, using the same tissue samples mentioned above, on a Roche

TABLE 1 List of sympatric ictalurid species with known occupancy in the Clinch and Duck rivers that were used for assay specificity optimization

Genera and species	Common Name	Location	
		Clinch	Duck
Noturus			
N. baileyi◊	Smoky Madtom		
N. eleutherus	Mountain Madtom	●	●
N. exilis	Slender Madtom		●
N. fasciatus	Saddled Madtom		●
N. flavipinnis	Yellowfin Madtom	●	
N. flavus	Stonecat		●
N. gyrinus	Tadpole Madtom		●
N. hildebrandi◊,*	Least Madtom		
N. miurus	Brindled Madtom		●
N. nocturnus	Freckled Madtom		●
Ictalurus			
I. furcatus	Blue Catfish	●	●
I. punctatus	Channel Catfish	●	●
Ameiurus			
A. melas	Black Bullhead	●	●
A. natalis	Yellow Bullhead	●	●
A. nebulosus	Brown Bullhead		●
Pylodictis			
P. olivaris	Flathead Catfish	●	●

Note: Reference sequences were downloaded from GenBank for in silico testing, and tissue samples were used for in vitro testing. Diamonds indicate species that do not have overlapping distribution with *N. stanauli*, but are closely related. Asterisks indicate that species has not been tested for specificity using the NS-193 assay. Black dots indicate if the species occurs in the Clinch or Duck River.

LightCycler 480 thermocycler, following methods similar to Díaz-Ferguson et al., 2014. Tissue-derived DNA from all species was standardized to a concentration of 20 ng/μl for assay specificity testing. All reactions were performed in 10-μl reaction volumes containing 5 μl of TaqMan® Environmental MasterMix (2×), 0.4 μl of each primer (10 μM), 0.2 μl ZEN double-quenched TaqMan® probe (10 μM), and 4 μl of template. All qPCRs were performed using the following optimized thermal profile: initial denaturation at 95°C for 10 min, followed by 50 cycles of 95°C for 30 s, 62°C for 60 s, and 72°C for 60 s.

A relative standard curve was developed to determine the limits of detection and limits of quantification by extracting and purifying DNA from *N. stanauli* tissue with an initial concentration of 28 ng/μl (Hunter et al., 2015). A 1:10 serial dilution was used to create a 6-fold serial dilution (2.8×10^{1} to 2.8×10^{-4} ng/μl. The limits of quantification were calculated as the average concentration of all replicates for the smallest serial dilution. All reactions for assay specificity and standard curve development were performed in triplicate. The reaction efficiency and R^2 for the standard curve and cycle threshold for unknown samples were calculated using the Second Derivative Maximum method within the Roche LightCycler 480 software. The assay (NS-193-F/R; Table 2) did not amplify in any of the nontarget species in silico or in vivo using either end-point (epPCR) or qPCR and was therefore determined to be specific to the amplification of *N. stanauli*; this assay was used for all subsequent eDNA sampling.

Three quality controls were used and repeated for each qPCR plate; these included two types of negative controls, a molecular-grade water substitution for DNA and a no template (no DNA or water) and positive control. The positive control, tissue-derived *N. stanauli* DNA of a known concentration equal to the median concentration of the standard curve, was used as an external standard on each qPCR plate to quantify amplified DNA in environmental samples and confirmed that our assay was amplifying correctly and efficiently during each run.

2.3 | Water column experimental design

A field study was performed in summer (July and August) 2017 to determine the optimal location within the water column for eDNA collection. We did not have permits to capture or harass this federally protected species and thus could not set up an experimental design where a known quantity of individuals was positioned in the river. We therefore conducted this field experiment under the premise that at least one individual *N. stanauli* was present at a chosen site. We chose to conduct our experiment at two sites (Frost Ford and Whirl Bar) that would have the greatest probability of occurrence based on past historic records and that we could easily access

TABLE 2 PCR and TaqMan qPCR primers/probe used to amplify a 253 bp region of the mitochondrial cytochrome oxidase b gene for *Noturus stanauli*

Oligo	Sequence (5′-3′)
NS-193-F (forward)	TTACTATGTCTTATTACACAAGTCCTAACA
NS-193-R (reverse)	GTAACAGTAGAATTACTCCGATG
NS-193-P (probe)	56FAM-TTGCATCTA/ZEN/CCTACATATTGGACGAGGCC-3IABkFQ
NS-620-R (reverse sequencing)	GGGTTATTGGAGCCTGTCTCA

Note: The probe used a double quencher molecule (ZEN & 3iABkFQ). The reverse sequencing oligo was used to confirm that our assay would amplify target fragments that contained a single nucleotide polymorphism in the reverse primer binding site.

(Figure 2b,c). Frost Ford has produced the highest catch rate of *N. stanauli* throughout its known distribution in the Clinch River (unpublished data, Tennessee Valley Authority [TVA] and Conservation Fisheries, Inc. [CFI]; Wells, 2019). Whirl Bar is the historic paratype locality that has not been sampled in more than 30 years. However, upstream sites have yielded specimens in the past three years (R.T.R.P., personal observations; unpublished data, TVA). We collected water samples at two sites: (a) Frost Ford (C1) in the Clinch River (3 August 2017) and (b) Whirl Bar near Rex May Lane (D2) in the Duck River (26 July 2017). We targeted four vertical strata: three in the water column at surface (SURF), middle (MID), and epibenthic (EPIB) locations, plus a sediment sample (CORE) (Figure 3).

Water sample collection protocols followed the procedure described by Mahon et al. (2010). Our study area targeted four points at each site in both rivers, where one 4-L water sample was collected at each of the vertical strata (for both sites, $n = 12$; 4 per strata), in addition to sediment core samples ($n = 4$). All water samples were collected in 4-L Nalgene bottles that were sterilized with a 20% bleach solution, thoroughly rinsed with tap water, and then autoclaved before sampling. Sediment samples were collected in sterilized, 50-ml conical tubes. Water samples were obtained at a river depth of approximately 100 cm at each point. Water samples for SURF, MID, and EPIB strata were collected, in order, at approximately 1–10 cm, 40–50 cm, and 90–100 cm, respectively. Bottles were held in front of the researcher collecting the water, and each sample bottle was submerged and positioned at the appropriate stratum with the lid securely attached. The lid was removed to allow water to enter to the bottle and resecured while still positioned at the targeted stratum.

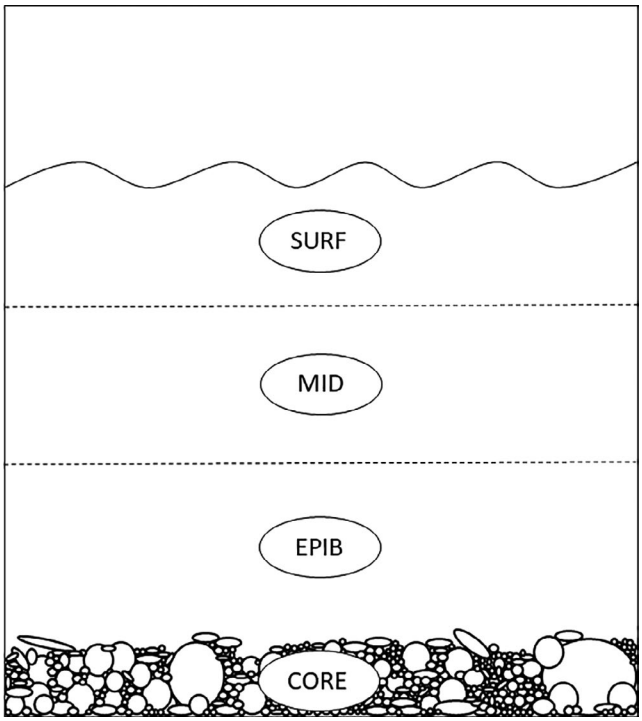


FIGURE 3 Longitudinal cross section schematic for the water column experiment part of the study showing the layout of the river strata: surface (SURF), middle (MID), and epibenthic (EPIB), where water samples were collected. A sediment sample was collected from the benthic substrate (CORE)

We chose to collect sediment core samples because madtoms are known to live in the interstitial cavities of the epibenthic substrate (Gibbs et al., 2014; Simonson & Neves, 1992). A sediment core sample was taken at each point by driving a PVC pipe (2.5 cm diameter) 30.5 cm into the sediment. The end of the pipe sticking out of the water was then capped creating a vacuum to pull up the core. The sediment at both sites can be characterized as a mix of sand, small pebbles (1–10 mm) and small-medium gravel (2.5–15 cm width) in areas of flowing water, and fine sediment with small pebbles (1–10 mm) in areas of slackwater (Bovee, 1982). We targeted the portion of the substrate that was likely to come into contact with the animal itself, or at least contained eDNA that represented a contemporary signal. The top portion of the core (7.5 cm) was discarded to help remove eDNA that might have originated from the water that came in contact with the core. The bottom portion of the core (7.5 cm) was removed because any eDNA recovered from this part of the core likely represents eDNA from an individual that is no longer in the area or alive. The remaining portion of the core was placed in a 50-mL conical tube. The PVC pipe was sterilized with 20% bleach and thoroughly rinse with deionized water between each sediment core sampling. Negative control bottles containing deionized or ultra-pure water were collected at each point to assess no cross-contamination occurred between points within each river. The lid for each negative control bottle was opened and exposed to the air for 10 s while standing in the stream. The lid was resecured and the negative control bottle was submerged approximately 10 cm. All bottles and tubes were dried of excess water using disposable paper towels, sterilized by wiping with a disposable paper towel soaked with 20% bleach, wiped down with a disposable paper towel soaked in deionized water, sealed with parafilm, and transported back to the laboratory on ice for filtration. Any waste (paper towels, gloves, and excess parafilm) was placed in a zip-lock bag for disposal and kept separated from samples and sampling supplies. All filters were previously aliquoted in a sterilized PCR hood for each field negative control and sample before sampling occurred to reduce potential contamination of filters, including individual filters for each negative control. All water samples were vacuum-filtered onto 1.0-μm glass fiber filters (Whatman®) within 24 hr and stored in 50-mL conical tubes at −20°C until DNA extraction. Field negative controls were filtered before each set of water samples to reduce potential contamination. All sediment samples were also stored at −20°C until DNA extraction. All filtration equipment was decontaminated with a 20% bleach solution for 10 min and thoroughly rinsed with deionized water after processing the water from each sample.

2.4 | Field sampling and water filtration protocol

All field sampling followed the protocols similar to Mahon et al. (2010). Surface water samples (depth range: 5–15 cm) were

collected at 14 sites in the Duck River (n = 14) from 21 to 28 November 2017 (winter 2017), 10 sites in the Clinch River (n = 10) from 26 to 31 January 2018 (winter 2018), and 5 sites in the Clinch River (n = 5) on 14 July 2018 (summer 2018). Water samples collected in the Clinch River (summer 2018) were from the same sites in the Clinch River (winter 2018) sampling. All bottles were sterilized with a 20% bleach solution for 10 min, thoroughly rinsed with tap water, and then autoclaved before sampling. In-stream conditions in both rivers varied among sites, for example, depth and discharge, and therefore, water samples were collected as close to the thalweg (lowest point in the river) as wadable conditions permitted (Pilliod et al., 2013; Erickson et al., 2016). A total of 16 L (four 4-L subsamples) of water was collected in Nalgene bottles at each site along a 100-m reach, along with one 4-L negative control in a downstream to upstream direction. Negative control bottles containing deionized water were treated as sample bottles to assess no cross-contamination occurred between sites in the field and were always collected first at the most downstream end of the reach for each site. The lid for the negative control was removed for 10 s while standing in the water, securely reattached, and the bottle was submerged as described above in the *Water Column Experiment Design* section. All bottles were dried of excess water, sterilized, sealed with parafilm, and transported back to the laboratory on ice for filtration as mentioned above in the water column experimental design section.

All 16-L samples from each site were vacuum-filtered onto 1.0-μm glass fiber filters (Whatman®) within 24 hr and stored in 50-mL conical tubes at −20°C until DNA extraction. All filtration equipment was decontaminated with a 20% bleach solution for 10 min and thoroughly rinsed with deionized water after processing the water from each site.

2.5 | eDNA extraction and qPCR amplification

2.5.1 | Water samples

Due to clogging from riverine debris, water samples from each site required multiple filters to process (Table S2,S3). To collect a representative eDNA sample from each site, we extracted eDNA from one-quarter of each filter from each sample and pooled the resulting extracts. For example, if a site required 12 filters to process the water from that site, then we performed 12 extractions and then pooled all 12 extracts (Table S2). The QIAamp Powerfecal DNA Kit (Qiagen®) was used to extract and purify eDNA from filters per the manufacturer's protocol, with two exceptions: (a) a FastPrep tissue homogenizer (Thermo Savant) was used at 6 m/s for 60 s for the lysis step and (b) DNA was eluted in 20 μl (instead of the recommended 100 μl) of the provided buffer solution. The full, eluted 20 μl, was placed back in the spin column and eluted a second time to increase eDNA yield. An extraction blank was performed during each round of eDNA extractions to assess potential cross-contamination. The extraction blank was performed by conducting the extraction process with all kit materials and chemicals, but no filter is placed in the spin column to assess extraction reagent contamination (Dougherty et al., 2016). The pooled, purified eDNA from each site resulted in different volumes for each sample. We accounted and standardized for this difference in volume, that is, different dilutions of eDNA, by diluting all samples with the QIAamp Powerfecal DNA kit elution buffer to an average of 15 ng/μl (range: 14.8–15.2 ng/μl) before amplification with qPCR. All samples were quantified using a Nanodrop Spectrophotometer.

2.5.2 | Sediment samples

The conical tubes containing our sediment samples were placed on a bench-top vortexer, and the sediment samples were homogenized by vortexing for 30 s. Individual spatulas were used to place 0.5 ml of sediment material from each conical tube into an extraction tube. All spatulas were sterilized with 20% bleach, rinsed with tap water, and autoclaved before use. We performed eDNA extractions from sediment samples using the same QIAamp Powerfecal kit and protocol mentioned above. To provide more comparable results between water and sediment samples, we extracted an equal number of subsamples from each sediment core sample equal to the average number of filters for water samples at each point in each river (Table S3). For example, if the average number of filters used to filter water samples at Point 1 in the Clinch River was 4, then we extracted 4 subsamples from the sediment core from Point 1.

All samples were tested for proper PCR inhibition removal before any PCR analyses were performed. An aliquot (18 μl) from each pooled sample from both rivers was spiked with 2 μl (1 ng/μl) of *N. stanauli* DNA, for a total of 20 μl. The concentration of our DNA used to spike the aliquots was quantified with the Qubit Fluorometer 2.0 (Thermo Fisher Scientific) using the High Sensitivity, dsDNA BR Assay kit (Qubit™), and diluted with the AE buffer provide in the Qiagen DNeasy Blood and Tissue Extraction kit. We performed epPCR and qPCR amplification, in triplicate, on all samples using the chemistry and thermal profiles mentioned above. The final DNA template concentration in epPCR and qPCR for the samples spiked with 2 μl (1 ng/μl) of *N. stanauli* DNA was 0.02 ng/μl. All replicates for all samples yielded positive amplification for epPCR (i.e., target fragment with equal brightness in gel among all replicates for all samples) and qPCR (i.e., all replicates for all samples were within 1 cycle threshold of each other and cross the set C_t threshold) indicating that all samples have been properly purified during DNA extraction.

Presence of *N. stanauli* eDNA was assessed using qPCR with the same chemistry and thermal profile as detailed in the in vitro section. All samples, including all field negative controls, extraction blanks, and PCR negative controls, were independently amplified two times with eight technical PCR replicates each (Ficetola et al., 2015; Jerde et al., 2011; Mahon et al., 2013). Results for epPCR were visualized on a 1.5% TBE agarose gel stained with GelRed, while standard curve analysis and cycle threshold values were used to determine

positive detection in qPCRs. A sample was determined to be positive for detection if ≥ 2 technical replicates for both independent rounds of PCR yielded positive amplification (sensu Ficetola et al., 2015). If one or both independent rounds exhibited positive amplification in ≤ 1 technical replicate, then a third independent round of PCR was conducted.

We validated all positive reactions with Sanger sequencing to confirm that the correct target fragment was amplified (Barnes & Turner, 2016; Jerde et al., 2011; Mahon et al., 2010). Reactions with a positive signal were cleaned using exonuclease I and shrimp alkaline phosphatase (New England Biolabs) for bidirectional Sanger sequencing on an ABI 3730-automated sequencer (MCLab). All resulting sequences were aligned and ends trimmed using Sequencher version 5.2 (Gene Codes Corp.). We verified the identity of our positive eDNA sequences by comparing them to published reference sequences in the GenBank *nr* database using the BLASTn (Altschul et al., 1990) and to our *Noturus* spp. *cyt b* reference alignment to visually confirm similarity to *N. stanauli*.

2.6 | Statistical analyses

To assess statistical significance for eDNA detection probability between molecular methods and sampled strata, our water column experiment dataset was formatted to a 2 × 2 × 104 contingency table for statistical analyses. We used three nominal variables: (a) "METHOD," which includes epPCR and qPCR, (b) "DETECTION," which includes detection (i.e., samples that met our 2 technical replicate minimum criteria) and nondetection (i.e., samples that did not meet our 2 technical replicate minimum criteria), and (c) "STRATUM," which includes our four strata in the water column and sediment (i.e., SURF, MID, EPIB, and CORE). A Woolf test was performed to test for the homogeneity of the data and is appropriate for 2 × 2 × k contingency tables with small replication (Mehta et al., 1985; Paul & Donner, 1992). The Cochran–Mantel–Haenszel test is a type of chi-square test that is optimal for datasets with a small replication size and was performed to assess whether the data exhibited independence (Zhang & Boos, 1997). Lastly, a Fisher's exact test was performed on the water column samples to test for statistical differences in detection probability among the different vertical strata (SURF, MID, and EPIB) and core samples within each river, as well as differences between detection rates using epPCR and qPCR. All statistical analyses were conducted with $\alpha = 0.05$ in program R version 3.5.1 (R Development Core Team, 2017).

3 | RESULTS

3.1 | Primer and probe design and assay specificity

From eight aligned sequences of *N. stanauli*, *cyt b*-specific primers were developed that amplify a 253 bp length fragment. The forward primer (NS-253-F) showed high specificity to *N. stanauli* in silico (percent identity = 100%, e-value = $2e^{-06}$, and total score = 60) with a minimum of three mismatches between *N. stanauli* and all other ictalurid species. The reverse primer (NS-253-R) had a comparable specificity (percent identity = 100%, e-value = 0.010, and total score = 46.1) with a minimum of two mismatches between *N. stanauli* and other ictalurid species in silico, except for *N. fasciatus* which was an exact match. The probe-binding region (NS-253-P) also had high similarity (percent identity = 100%, e-value = $7e^{-06}$, total score = 58) with at least one mismatch between *N. stanauli* and other ictalurid species.

The BLASTn alignment of our primer and probe oligos to sequences in the GenBank database did indicate similarity to other fishes (e.g., *Eigenmannia macrops*, *Atractosteus tristoechus*, and Characiformes) and reptiles (e.g., *Iguana delicatissima*, *Scelarcis perspicillata*). All fishes that showed similarity have never been reported to occur in any Tennessee river system, nor have any of the exotic reptiles been found in Tennessee. Additionally, these fish and reptile species are unlikely to occur in Tennessee due to suboptimal environmental conditions conducive to survival and thus pose no risk of cross-amplification with our qPCR assay.

The candidate reverse primer was designed over a polymorphic site (A/G) that is present in some of the Clinch River individuals ($n = 3$) (MT622529–MT622539) (Figure S1). Individuals in our study from the Duck River do not contain the single nucleotide polymorphism (SNP). To assess whether our assay could detect both alleles, we performed qPCR amplification using the same chemistry and thermal profile mentioned above, on 11 individuals from the Clinch River. Bidirectional sequencing was performed using our candidate forward primer and another reverse primer (NS-620-R) that flanks our candidate reverse primer. For the 11 *N. stanauli* individuals that we used, ($n = 3$) contained the polymorphism; however, all 11 individuals produced efficient amplification via epPCR and qPCR. Additionally, Sanger sequencing confirmed both alleles were detected in samples from the water column and field sampling parts of our experiment.

3.2 | qPCR limits of detection

The target DNA concentration and cycle threshold (C_T) values for all replicates of all serial dilutions were correlated ($r^2 = 0.997$; efficiency = 99%; slope = −3.45; intercept = 29.152). Based on our serial dilutions, the lower limit of eDNA detection for *N. stanauli* was 2.8×10^{-4} ng/μl with a C_T value of 40.7–42.3 required for the minimum DNA detection. The limit of quantification was calculated as 3.83×10^{-4} ng/μl. Amplification that results late in the reaction (≥ 45) can be indicative of a false-positive signal; thus, our standard curve dilutions and reported positive signals fall within acceptable qPCR parameters (Burns & Valdivia, 2008; Caraguel et al., 2011; Laramie et al., 2015). Additionally, any positive signal that falls outside of the limits of quantification were not considered as reliable detection.

3.3 | Water column experiment

3.3.1 | Duck River

Noturus stanauli eDNA was detected with only epPCR in the Duck River for our water column experiment. A single positive replicate was observed among the majority of all strata from all four points (Table 3); however, only one of the CORE samples yielded positive detection for *N. stanauli* eDNA using the criteria of ≥ 2 technical replicate amplifications for positive detection. A total of 13 reactions containing our target fragment were bidirectionally sequenced, and all 13 reactions produced sequence reads with a ≥ 99% match to *N. stanauli* reference sequences in GenBank. Additionally, among all water strata samples, we observed a nontarget fragment (75 bp). We randomly selected 10 reactions that contained this nontarget fragment and also bidirectionally sequenced these reactions. All reactions containing the nontarget fragment produced small length (<75 bp) and poor-quality sequences and were likely the result of occasional primer dimerization. Furthermore, while no amplification was observed in the Duck River water strata samples with qPCR, our positive control did exhibit efficient amplification, which indicated there was no inhibition or reaction-efficiency problems associated with the assay.

3.3.2 | Clinch River

Noturus stanauli eDNA was detected in the Clinch Rivert with both epPCR and qPCR, in all water column strata samples, with the exception of one MID, one EPIB, and two CORE samples (Table 3). A total of 28 epPCRs containing our target fragment were bidirectionally sequenced, and all 28 reactions produced sequence reads with a ≥ 99% match to *N. stanauli* reference sequences in GenBank. Like the Duck River samples, a nontarget fragment (75 bp) was observed in several reactions across all water strata samples. We randomly selected 28 reactions with the nontarget fragment for sequencing, and all reactions produced small length (<75 bp) and poor-quality sequences, as was seen in the Duck River samples.

TABLE 3 Water column detection results for end-point PCR (epPCR) and quantitative PCR (qPCR) analysis

	C1 (Clinch River)								D2 (Duck River)							
	epPCR				qPCR				epPCR				qPCR			
	R1	R2	R3	D	R1	R2	R3	D	R1	R2	R3	D	R1	R2	R3	D
Point 1																
SURF	4	4		+	3	7		+	1	3	1	−	0	0		−
MID	5	5		+	6	7		+	1	2	1	−	0	0		−
EPIB	2	3		+	3	4		+	0	0		−	0	0		−
CORE	4	4		+	3	5		+	0	0		−	0	0		−
Point 2																
SURF	6	7		+	8	7		+	0	1	0	−	0	0		−
MID	4	3		+	3	5		+	1	1	1	−	0	0		−
EPIB	4	4		+	5	5		+	0	1	0	−	0	0		−
CORE	8	8		+	7	7		+	0	0		−	0	0		−
Point 3																
SURF	5	4		+	2	3		+	0	2	1	−	0	0		−
MID	8	6		+	6	6		+	0	3	0	−	0	0		−
EPIB	1	3	1	−	2	3		+	0	3	0	−	0	0		−
CORE	2	3		+	0	4	1	−	2	2		+	0	0		−
Point 4																
SURF	5	4		+	3	5		+	1	0	0	−	0	0		−
MID	2	4		+	1	3	1	−	1	0	0	−	0	0		−
EPIB	3	3		+	4	2		+	0	1	0	−	0	0		−
CORE	1	3	1	−	2	2		+	1	0	0	−	0	0		−

Note: Water samples (4-L) were collected from four strata (surface (SURF), middle (MID), epibenthic (EPIB), and sediment core (CORE)) at four different points (Point 1, 2, 3, and 4) at site C1 (Clinch River) and site D2 (Duck River). Results are reported as the number of eight total technical replicates that yielded positive amplification during each amplification round (R1, R2, R3) and the decision (D) as to if the sample was classified as positive detection (+) or negative detection (−). A third round (R3) of amplification was only necessary for the decision if <2 technical replicates failed to amplify our target fragment in either R1 or R2. Number for negative controls are not shown since all negative controls yielded no amplification, indicating no cross-contamination occurred.

For the Clinch River water strata samples, a total of 31 qPCRs that contained positive amplification for *N. stanauli* eDNA were selected for sequencing. The majority (27 of 31) of the reactions produced sequence reads that had a ≥ 99% match to *N. stanauli* reference sequences in GenBank. The remaining four reactions either contained poor-quality raw sequences or did not match any reference sequence in GenBank. The amplification curves for these reactions demonstrated efficient amplification similar to reactions that did produce a sequence read and GenBank match, but due to a limited amount of PCR product we were unable to have these four samples resequenced to confirm. All negative controls, that is, field, extraction, and PCR, failed to amplify for both rivers, indicating no cross-contamination occurred during the experiment.

3.4 | Statistical results

A Woolf test and a Cochran–Mantel–Haenszel test confirmed our data exhibited both homogeneity and independence, respectively (Woolf Test: $\chi^2 = 0$, $p = 1$; Cochran–Mantel–Haenszel test: $M^2 = 3$, $p = .3916$). A Fisher's exact test was used to test for differences between epPCR and qPCR at each stratum within each river and indicated no significant differences ($p = 1.00$).

3.5 | Field sampling

In the winter 2017–2018, we did not detect *N. stanauli* eDNA at any of the 10 sites in the Clinch River or any of the 14 sites in the Duck

TABLE 4 Field sampling data for *Noturus stanauli* eDNA surveillance in the Clinch and Duck Rivers

Site	Observed distribution	Season	Volume (L)	eDNA detected	No. Positive qPCRs		Sanger sequence	% Identity
					1st Round	2nd Round		
C1	Y	W	16	N	0/8	0/8		
C2	N	W	16	N	0/8	0/8		
C3	N	W	16	N	0/8	0/8		
C4	N	W	16	N	0/8	0/8		
C5	N	W	16	N	0/8	0/8		
C8	N	W	16	N	0/8	0/8		
	N	S	16	N	0/8	0/8		
C7	N	W	16	N	0/8	0/8		
	N	S	16	N	0/8	0/8		
C6	N	W	16	N	0/8	0/8		
	N	S	16	Y	3/8	5/8	6	99%–100%
C9	N	W	16	N	0/8	0/8		
	N	S	16	N	0/8	0/8		
C10	N	W	16	N	0/8	0/8		
	N	S	16	N	0/8	0/8		
D1	N	W	16	N	0/8	0/8		
D2	Y	W	16	N	0/8	0/8		
D3	Y	W	16	N	0/8	0/8		
D4	Y	W	16	N	0/8	0/8		
D5	Y	W	16	N	0/8	0/8		
D6	Y	W	16	N	0/8	0/8		
D7	N	W	16	N	0/8	0/8		
D8	N	W	16	N	0/8	0/8		
D9	N	W	16	N	0/8	0/8		
D10	N	W	16	N	0/8	0/8		
D11	N	W	16	N	0/8	0/8		
D12	N	W	16	N	0/8	0/8		
D13	N	W	16	N	0/8	0/8		
D14	N	W	16	N	0/8	0/8		

Note: Site abbreviations correspond to sites in Figure 2 and are listed in a downstream to upstream orientation for each river. The observed distribution column denotes if a sampled site was in the known distribution of *N. stanauli* (Y = Yes, N = No). The season column indicates the time of year when samples were collected (W = Winter, S = Summer). Sanger sequencing was performed on three replicates from each qPCR round.

River, with either epPCR or qPCR (Table 4). However, resampling of the Clinch River in summer 2018 yielded positive detection at a new site outside the historic range, C8 (Figure 2c; Table 4) located approximately 2.5 km downstream from the Tennessee-Virginia state line.

We confirmed the eDNA signal detected at site C8 with epPCR where 8 of 8 technical replicates yielded positive amplification of our target fragment and all negative controls were clean indicating no cross-contamination. Our qPCR assay produced amplification products in multiple replicates in both independent rounds (Table 4). Three randomly selected reactions from both qPCR rounds ($n = 6$) were bidirectionally sequenced. All six reaction generated sequences that resulted in a 99%–100% identity match to *N. stanauli* in the GenBank database (Table 4).

4 | DISCUSSION

Noturus stanauli presents a challenging species for monitoring given its small size and rarity in two large river systems. In this study, a species-specific assay was developed and tested for detection of this rare and diminutive fish throughout two rivers. Despite the challenging dynamics of this system, our assay was able to detect a signal from *N. stanauli* at the type locality in the Clinch River (Figure 2c, Table 3), at a paratype locality in the Duck River (Figure 2b, Table 3), and at a new site outside of its historic distribution in the Clinch River (Figure 2c, Table 4). Although the primary focus of our study was to provide delineation of *N. stanauli*, we were also able to test and provide insight to mechanistic underpinnings of eDNA detection of small, cryptic fish in large rivers through the context of life history traits and environmental variability.

While many eDNA studies have focused their sampling efforts on collecting surface water samples, few studies have adapted their sampling methods to fit the life history of the target species (Eichmiller et al., 2014; Moyer et al., 2014). We initially hypothesized that water collections at the epibenthic strata within the water column would be more effective at detection of *N. stanauli* based on the benthic life history of this species. One study using three fish species that exhibit different spatial distributions in the water column (e.g., pelagic, benthopelagic, and benthic) found that sampling location had no significant effect on DNA concentration or detection probability (Hinlo et al., 2017). Our findings match those in Hinlo et al. (2017) and indicate that eDNA may be more homogenized in a riverine (lotic) system compared with lentic systems, like those in Moyer et al., 2014. We also detected an eDNA signal in sediment samples from the Duck and Clinch Rivers, similar to Turner et al. (2015), indicating that eDNA concentration may be greater in sediment compared with the water column overhead. Genomic molecules bound to sediment particles have demonstrated reduced rates of degradation; thus while these signals can represent true positives from the target organism, the spatiotemporal inferences drawn from sediment samples may be limited (Cai et al., 2006).

Our field sampling efforts suggested a broader distribution of *N. stanauli* in the Clinch River than previously known based on traditional sampling methods (Figure 2c). Based on streamside observation, site C8 does not display typical habitat characteristics reported from other sites with known *N. stanauli* occurrences (e.g., Etnier & Jenkins, 1980). The section of the river where this positive detection occurred appears to contain mostly low-gradient, run-pool habitat with relatively slow local water velocities. The substrate is characterized as having a large amount of fine sediment with medium gravel to small cobble (2.5–15.0-cm width) (Bovee, 1982), sparsely dispersed throughout. Traditional surveillance is needed to confirm the origin of the detected eDNA because the signal could have originated from further upstream and from feces deposited by an animal that consumed *N. stanauli* (Jane et al., 2015; Merkes et al., 2014). The probability of a live individual is high in this stretch of the river, however, according to recent MaxEnt habitat modeling (Wells, 2019). Incorporation of eDNA detection probability models, as well as production, transportation, and decay models, would improve our ability to estimate the movement of eDNA in large rivers and help to refine eDNA surveillance techniques, especially for small, cryptic fish.

It is known that environmental variability and biotic factors can influence eDNA detection, although the exact relationship can differ from system to system. Detection probability was greater in both rivers in summer than in winter months. Warmer temperatures increase the metabolic activity of fishes leading to an increased production of biological matter that is shed into the water, and thereby increase eDNA production in the water (Gillooly et al., 2001; Takahara et al., 2012). Furthermore, historic and new records indicate that *N. stanauli* has a prolonged summertime spawning period, ranging from late May to early September (USFWS, 2008; Wells & Mattingly, 2019). Our greater detection rate in summer in the Clinch River is likely a result of congregating and nesting individuals associated with reproductive activity, which would lead to a greater abundance of individuals, increased eDNA concentration, and greater detection probability in a local area (Janosik & Johnston, 2015; Takahara et al., 2012; Wells, 2019). Reproductive and population-level information of *N. stanauli* is limited, but this species appears to be a nest-guarder like other *Noturus* species and many more individuals have been collected in the Clinch River than in the Duck River (Shute, 2001; USFWS, 2008; Wells & Mattingly, 2019). With limited breeding habitat, several individuals could be forced into occupying a localized area, like site C1 (Figure 2c), for spawning, which would increase eDNA concentration in the localized area.

We were unable to collect samples in a framework that would allow us to develop modeling for detection probability or occurrence estimates. A species' detection probability with eDNA is directly related to the abundance of individuals and the distance from the individuals at the point where water is collected (Boothroyd et al., 2016; Pilliod et al., 2013). Our study was conducted with the intention of delimitation, not occupancy estimation, hence collecting (and pooling) a large amount of water (16-L) at each field site. Even with traditional sampling methods, estimating abundance for *N. stanauli* at a site is difficult at best; thus, we cannot accurately quantify the abundance of individuals at a given site. As suggested by Jerde

et al. (2011), repeated sampling trips to the same sites may be a better option to develop eDNA occurrence estimates for *N. stanauli* and further deduce environmental variables that affect detection probability and help reduce false-negative results.

In our study, false-negative results were likely to occur from three factors: (a) the two-replicate minimum criterion, (b) the length of the target amplicon, and (c) our limit of detection. First, several of the water column samples yielded positive amplification in <2 replicates for one or more independent rounds of PCR amplification (Table 3). These reactions likely represent amplified *N. stanauli* eDNA (as confirmed by Sanger sequencing); however, our two-replicate minimum rule meant that the samples were still classified as negative. While contamination of samples would also correctly sequence, all of our negative controls failed to amplify our target fragment indicating that contamination is not likely in this case. Due to the random dispersion of DNA in the system, it is possible that only a few target DNA molecules were collected in the sample, which would then limit the number of target DNA molecules in each PCR (see Furlan et al., 2016). Some studies justify designation of positive samples based on one technical replicate in the cases of rare species (Carim et al., 2019; Janosik & Johnston, 2015). We justified using a two-replicate minimum because detection probability and occurrence estimates for *N. stanauli* were unknown due to limited information, but this criterion may have been too conservative in our study (Ficetola et al., 2015). Second, shorter fragments are known to persist in the environment for longer periods of time (Jo et al., 2017). It is possible we did not detect our target fragment at some sites where it was truly present because there is theoretically a smaller abundance of longer fragments present. In instances where multiple closely related species occupy the same habitat, like *Noturus*, a longer fragment is required to differentiate between all sympatric species. The use of multiple markers can be more beneficial for true-positive detection of the target species when longer fragments are used for surveillance of a single species (Farrington et al., 2015). Lastly, our detection of *N. stanauli* could be limited by the lower limit of our standard curve. The lowest concentration on our standard curve was 2.8×10^{-4} ng/μl (or ~100,00 DNA copy number), compared with contemporary eDNA studies that can detect lower orders of magnitude of eDNA concentrations (1.0×10^{-8} or 1 DNA copy number) (Carim et al., 2019). It is plausible that we did not detect an eDNA signal that might have been present at a lower concentration; however, we only report results that fall within the parameters of our relative standard curve which provides validation for the signals that we did detect. Absolute standard curves developed with synthetic DNA, for example, gBlocks, can be used to accurately quantify the lower limits of detection and lower limits of quantification compared with our relative standard curve where the specific copy number is unknown (Carim et al., 2019; Klymus et al., 2019).

While qPCR is cited as being the more sensitive compared with epPCR (Jerde et al., 2013; Turner et al., 2014), we unexpectedly detected *N. stanauli* eDNA in the Duck River with epPCR and not with qPCR. One explanation for these results could be that the primer oligos are not species specific and may co-amplify DNA from an unknown and related *Noturus* species, while the addition of the probe in the qPCR amplification makes this assay species specific. Phylogenetic relationships between species in *Noturus* are still unresolved and indicate more undescribed species may be present than are currently described and recognized (Bennett et al., 2009; Hardman, 2004; Near & Hardman, 2006). At the time our primers were designed and field tested, *N. fasciatus* was a newly described species that occurs in the Duck River with *N. stanauli*. It was not until after the submission of this manuscript that sequences were made available through GenBank for *N. fasciatus* and we discovered that the reverse primer was an exact match. However, all positive reactions were sequenced and identified as *N. stanauli* with the GenBank database, indicating that our eDNA signals are true and our results are sound. This issue denotes the need for further life history and morphometric studies that can help resolve phylogenetic relationships between species in this cryptic genus to increase effective assay design and the reliability of eDNA surveillance. Contamination is also another plausible explanation for positive eDNA amplification; however, all of our field, extraction, and PCR-negative controls were negative for amplification of our target fragment.

Our study highlights the potential application of eDNA surveillance to the detection of rare and cryptic fishes in large rivers and identifies environmental variables that may influence eDNA detection in these systems. *Noturus stanauli* is a unique fish among the fauna in Tennessee river systems, being the smallest ictalurid species in the world and habitating two of the state's largest rivers. Biosurveillance of *N. stanauli* is inherently challenging for both traditional and molecular methods. While there can be unforeseen environmental variables that should be considered in experimental design, eDNA monitoring offers a more efficient sampling protocol compared with traditional aquatic sampling methods for *N. stanauli*. In particular, the ability to collect surface samples reduces and potentially eliminates the need to enter the water, reducing contamination risk and harm to animals and associated habitat. In order to fully incorporate eDNA surveillance into routine management and conservation plans, both the biological and environmental factors that directly contribute to eDNA ecology (i.e., origin, transport, persistence, and fate) must be well understood. Future eDNA surveys in the Clinch River are of special interest to state agencies due to the undefined distribution of *N. stanauli*. More surveillance is needed to confirm the potential presence in the Virginia reaches of the Clinch River, which would require new or revised conservation strategies for both Tennessee and Virginia if found.

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CONFLICT OF INTEREST

None declared.

AUTHORS CONTRIBUTIONS

This study was conducted as part of a dissertation project (RTRP) in the School of Environmental Studies at Tennessee Technological University, Cookeville, TN. HTM and CRH developed the initial study concept, and RTRP conducted field collection, data analysis, and data interpretation. All three authors contributed to study design and manuscript synthesis.

DATA AVAILABILITY STATEMENT

All raw sequences generated from *N. stanauli* tissues for this study have been archived in NCBI's GenBank repository database. All raw data will be provided upon request.

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REFERENCES

Ahlstedt, S. A., Powell, J. R., Butler, R. S., Fagg, M. T., Hubbs, D. W., Novak, S. F., Palmer, S. R., & Johnson, P. D. (2017). Historical and current examination of freshwater mussels (Bivalvia: Margaritiferidae, Unionidae) in the Duck River basin Tennessee. *Malacological Review*, *45*, 1–163.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Barnes, M. A., & Turner, C. R. (2016). The ecology of environmental DNA and implications for conservation genetics. *Conservation Genetics*, *17*, 1–17. https://doi.org/10.1007/s10592-015-0775-4

Bennett, M. G., Heath Howell, J., Kuhajda, B. R., & Wood, R. M. (2009). Mitochondrial DNA divergence in the critically imperiled pygmy madtom, *Noturus stanauli* (Siluriformes: Ictaluridae). *Journal of Fish Biology*, *75*(9), 2363–2372.

Boothroyd, M., Mandrak, N. E., Fox, M., & Wilson, C. C. (2016). Environmental DNA (eDNA) detection and habitat occupancy of threatened spotted gar (*Lepisosteus oculatus*). *Aquatic Conservation*, *26*, 1107–1119. https://doi.org/10.1002/aqc.2617

Bovee, K. D. (1982). *A guide to stream habitat analysis using the instream flow incremental methodology*. US Fish and Wildlife Service Biological Services Program Report FWS/ OBS-82/26. US Fish and Wildlife Service, Fort Collins, CO. 248 pp.

Bronnenhuber, J. E., & Wilson, C. C. (2013). Combining species-specific COI primers with environmental DNA analysis for targeted detection of rare freshwater species. *Conservation Genetics Resources*, *5*, 971–975. https://doi.org/10.1007/s12686-013-9946-0

Burns, M., & Valdivia, H. (2008). Modelling the limit of detection in real-time quantitative PCR. *European Food Research and Technology*, *226*(6), 1513–1524. https://doi.org/10.1007/s00217-007-0683-z

Burr, B. M., & Stoeckel, J. N. (1999). *The natural history of madtoms (genus* Noturus*), North America's diminutive catfishes*. In Catfish 2000: Proceedings of the International Ictalurid Symposium. American Fisheries Society, Symposium (Vol. *24*, pp. 51–101).

Cai, P., Huang, Q., & Zhang, X. (2006). Interactions of DNA with clay minerals and soil colloidal particles and protection against degradation by DNase. *Environmental Science and Technology*, *40*, 2971–2976. https://doi.org/10.1021/es0522985

Caraguel, C. G., Stryhn, H., Gagné, N., Dohoo, I. R., & Hammell, K. L. (2011). Selection of a cutoff value for real-time polymerase chain reaction results to fit a diagnostic purpose: Analytical and epidemiologic approaches. *Journal of Veterinary Diagnostic Investigation*, *23*(1), 2–15. https://doi.org/10.1177/104063871102300102

Carim, K. J., Dysthe, J. C., McLellan, H., Young, M. K., McKelvey, K. S., & Schwartz, M. K. (2019). Using environmental DNA sampling to monitor the invasion of nonnative *Esox lucius* (northern pike) in the Columbia River basin, USA. *Environmental DNA*, *1*, 215–226.

Díaz-Ferguson, E., Herod, J., Galvez, J., & Moyer, G. (2014). Development of molecular markers for eDNA detection of the invasive African jewelfish (*Hemichormis letourneuxi*): A new tool for monitoirng aquatic invasive species in National Wildife Refuges. *Management of Biological Invasions*, *5*(2), 121–131.

Díaz-Ferguson, E., & Moyer, G. (2014). History, applications, methodological issues and perspectives for the use environmental DNA (eDNA) in marine and freshwater environments. *Revista De Biologia Tropical*, *62*(4), 1273–1284. https://doi.org/10.15517/rbt.v62i4.13231

Dougherty, M. M., Larson, E. R., Renshaw, M. A., Gantz, C. A., Egan, S. P., Erickson, D. M., & Lodge, D. M. (2016). Environmental DNA (eDNA) detects the invasive rusty crayfish *Orconectes rusticus* at low abundances. *Journal of Applied Ecology*, *53*, 722–732.

Dwyer, W. P., Fredenberg, W., & Erdahl, D. A. (1993). Influence of electroshock and mechanical shock on survival of trout eggs. *North American Journal of Fisheries Management*, *13*(4), 839–843. https://doi.org/10.1577/1548-8675(1993)013<0839:IOEAMS>2.3.CO;2

Eichmiller, J. J., Bajer, P. G., & Sorensen, P. W. (2014). The relationship between the distribution of common carp and their environmental DNA in a small lake. *PLoS One*, *9*(11), e112611.

Erickson, R. A., Rees, C. B., Coulter, A. A., Merkes, C. M., McCalla, S. G., Touzinsky, K. F., Walleser, L., Goforth, R. R., & Amberg, J. J. (2016). Detecting the movement and spawning activity of bigheaded carps with environmental DNA. *Molecular Ecology Resources*, *16*, 957–965.

Etnier, D. A., & Jenkins, R. E. (1980). *Noturus stanauli*: A new madtom catfish (Ictaluridae) from the Clinch and Duck Rivers, Tennessee. *Bulletin of the Alabama Museum of Natural History*, *5*, 17–22.

Etnier, D. A., & Starnes, W. C. (1993). *The fishes of Tennessee*. University of Tennessee Press.

Farrington, H., Edwards, C. E., Guan, X., Carr, M. R., Baerwaldt, K., & Lance, R. F. (2015). Mitochondrial genome sequencing and development of genetic markers for the detection of DNA of invasive bighead and silver carp *(Hypophthalmichthys nobilis and H. molitrix)* in environmental water samples from the United State. *PLoS One*, *10*(2): e0117803.

Ficetola, G. F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., Gielly, L., Lopes, C. M., Boyer, F., Pompanon, F., Rayé, G., & Taberlet, P. (2015). Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. *Molecular Ecology Resources*, *15*(3), 543–556. https://doi.org/10.1111/1755-0998.12338

Furlan, E. M., Gleeson, D., Hardy, C. M., & Duncan, R. P. (2016). A framework for estimating the sensitivity of eDNA surveys. *Molecular Ecology Resources*, *16*(3), 641–654.

Gibbs, W. K., Miller, J. E., Throneberry, J. K., Cook, S. B., & Kulp, M. A. (2014). Summer habitat use and partitioning by two reintroduced rare madtom species. *Journal of Freshwater Ecology*, *29*(2), 243–258. https://doi.org/10.1080/02705060.2014.881308

Gillooly, J. F., Brown, J. H., West, G. B., Savage, V. M., & Charnov, E. L. (2001). Effects of size and temperature on metabolic rate. *Science*, *293*(5538), 2248–2251. https://doi.org/10.1126/science.1061967

Hall, T. A. (1999). *BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT*. In Nucleic Acids Symposium Series (Vol. *41*, No. 41, pp. 95–98). [London]: Information Retrieval Ltd., c1979–c2000.

Hardman, M. (2004). The phylogenetic relationship among *Noturus* catfishes (Siluriformes: Ictaluridae) as inferred from mitochondrial gene cytochrome *b* and nuclear recombination activating gene 2. *Molecular Phylogenetics and Evolution*, *30*, 395–408.

Hayes, D. B., Ferreri, C. P., & Taylor, W. W. (1996). Active fish capture methods. In B. R. Murphy, & D. W. Willis (Eds.), *Fisheries techniques*, 2nd ed. (pp. 193–220). American Fisheries Society.

Hinlo, R., Furlan, E., Suitor, L., & Gleeson, D. (2017). Environmental DNA monitoring and management of invasive fish: Comparison of eDNA and fyke netting. *Management of Biological Invasions*, *8*(1), 89–100. https://doi.org/10.3391/mbi.2017.8.1.09

Hunter, M. E., Oyler-McCance, S. J., Dorazio, R. M., Fike, J. A., Smith, B. J., Hunter, C. T., Reed, R. N., & Hart, K. M. (2015). Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive Burmese pythons. *PLoS One*, *10*(4), e0121655. https://doi.org/10.1371/journal.pone.0121655

Jane, S. F., Wilcox, T. M., McKelvey, K. S., Young, M. K., Schwartz, M. K., Lowe, W. H., Letcher, B. H., & Whiteley, A. R. (2015). Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Molecular Ecology Resources*, *15*, 216–227. https://doi.org/10.1111/1755-0998.12285

Janosik, A. M., & Johnston, C. E. (2015). Environmental DNA as an effective tool for detection of imperiled fishes. *Environmental Biology of Fishes*, *98*(8), 1889–1893. https://doi.org/10.1007/s10641-015-0405-5

Jerde, C. L., Chadderton, W. L., Mahon, A. R., Renshaw, M. A., Corush, J., Budny, M. L., Mysorekar, S., & Lodge, D. M. (2013). Detection of Asian carp DNA as part of a Great Lakes basin-wide surveillance program. *Canadian Journal of Fisheries and Aquatic Science*, *70*, 522–526. https://doi.org/10.1139/cjfas-2012-0478

Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters*, *4*(2), 150–157. https://doi.org/10.1111/j.1755-263X.2010.00158.x

Jo, T., Arimoto, M., Murakami, H., Masuda, R., & Minamoto, T. (2019). Particle size distribution of environmental DNA from the nuclei of marine fish. *Environmental Science and Technology*, *53*(16), 9947–9956. https://doi.org/10.1021/acs.est.9b02833

Jo, T., Murakami, H., Masuda, R., Sakata, M. K., Yamamoto, S., & Minamoto, T. (2017). Rapid degradation of longer DNA fragments enables the improved estimation of distribution and biomass using environmental DNA. *Molecular Ecology Resources*, *17*(6), e25–e33.

Klymus, K. E., Merkes, C. M., Allison, M. J., Goldberg, C. S., Helbing, C. C., Hunter, M. E., Jackson, C. A., Lance, R. F., Mangan, A. M., Monroe, E. M., Piaggio, A. J., Stokydyk, J. P., Wilson, C. C., & Richter, C. A. (2019). Reporting the limits of detection and quantification for environmental DNA assays. *Environmental DNA*, *2*(3), 271–282. https://doi.org/10.1002/edn3.29

Laramie, M. B., Pilliod, D. S., & Goldberg, C. S. (2015). Characterizing the distribution of an endangered salmonid using environmental DNA analysis. *Biological Conservation*, *183*, 29–37. https://doi.org/10.1016/j.biocon.2014.11.025

Leathwick, J. R., Collier, K. J., Hicks, B. J., Ling, N., Stichbury, G. L., & de Winton, M. (2016). Predictions of establishment risk highlight biosurveillance priorities for invasive fish in New Zealand lakes. *Freshwater Biology*, *61*(9), 1522–1535. https://doi.org/10.1111/fwb.12792

Mahon, A. R., Jerde, C. L., Galaska, M., Bergner, J. L., Chadderton, W. L., Lodge, D. M., Hunter, M. E., & Nico, L. G. (2013). Validation of eDNA surveillance sensisitive for detection fo Asian carps in controlled and field experiments. *PLoS One*, *8*(3), e58316.

Mahon, A. R., Rohly, A., Budny, M., Elgin, E., Jerde, C., Chadderton, W., & Lodge, D. M. (2010). *Environmental DNA monitoring and surveillance: Standard operation procedures*. Report to the United States Army Corps of Engineers, Environmental Laboratories, Cooperative Environmental Studies Unit, Vicksburg, Mississippi.

Mehta, C. R., Patel, N. R., & Gray, R. (1985). Computing an exact confidence interval for the common odds ratio in several 2 × 2 contingency tables. *Journal of the American Statistical Association*, *80*(392), 969–973.

Merkes, C. M., McCalla, S. G., Jensen, N. R., Gaikowski, M. P., & Amberg, J. J. (2014). Persistence of DNA in carcasses, slime, and avian feces may affect interpretations of environmental DNA data. *PLoS One*, *9*(11), e113346.

Moyer, G. R., Díaz-Ferguson, E., Hill, J. E., & Shea, C. (2014). Assessing environmental DNA detection in controlled lentic systems. *PLoS One*, *9*(7), e103767. https://doi.org/10.1371/journal.pone.0103767

Near, T. J., & Hardman, M. (2006). Phylogenetic relationships of *Noturus stanauli* and *N. crypticus* (Siluriformes: Ictaluridae), two imperiled freshwater fish species from the southeastern United States. *Copeia*, *2006*(3), 378–383.

Paul, S. R., & Donner, A. (1992). Small sample performance of tests of homogeneity of odds ratios in K 2× 2 tables. *Statistics in Medicine*, *11*(2), 159–165. https://doi.org/10.1002/sim.4780110203

Pfleger, M. O., Rider, S. J., Johnston, C. E., & Janosik, A. M. (2016). Saving the doomed: Using eDNA to aid in detection of rare sturgeon for conservation (Acipenseridae). *Global Ecology and Conservation*, *8*, 99–107. https://doi.org/10.1016/j.gecco.2016.08.008

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2013). Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. *Canadian Journal of Fisheries and Aquatic Science*, *70*, 1123–1130. https://doi.org/10.1139/cjfas-2013-0047

Primmer, C. (2006). Genetic characterization of populations and its use in conservation decision-making in fish. In J. Ruane, & A. Sonnino (Eds.), *The role of biotechnology in exploring and protecting agricultural genetic resources* (pp. 97–104). FAO.

R Development Core Team. (2017). *R: A language and environment for statistical computing*. R Foundation for Statistical Computing: Vienna, Austria. ISBM: 3–900051-07-0. http://www.R-project.org/

Rees, H. C., Maddison, B. C., Middleditch, D. J., Patmore, J. R. M., & Gough, K. C. (2014). The detection of aquatic animal species using environmental DNA – A review of eDNA as a survey tool in ecology. *Journal of Applied Ecology*, *51*, 1450–1459.

Reynolds, J. B. (1996). Electrofishing. In B. R. Murphy, & D. W. Willis (Eds.), *Fisheries techniques*, 2nd ed. (pp. 221–253). American Fisheries Society.

Schilling, E. M., & Williams, J. D. (2002). Freshwater mussels (Bivalvia: Margaritiferidae and Unionidae) of the lower Duck River in middle Tennessee: A historic and recent review. *Southeastern Naturalist*, *1*(4), 403–414.

Shute, J. R. (2001). Spawning the rare pygmy madtom, *Noturus stanauli*. *American Currents*, *27*(1), 19–21.

Simonson, T. D., & Neves, R. J. (1992). Habitat suitability and reproductive traits of the orangefin madtom, *Noturus gilberti* (Pisces: Ictaluridae). *The American Midland Naturalist*, *127*(1), 115–124. https://doi.org/10.2307/2426327

Snyder, D. E. (2003). Invited overview: Conclusions from a review of electrofishing and its harmful effects on fish. *Reviews in Fish Biology and Fisheries*, *13*(4), 445–453. https://doi.org/10.1007/s11160-004-1095-9

Strahler, A. N. (1952). Hypsometric (area-altitude) analysis of erosional topology. *Geological Society of America Bulletin*, *63*(11), 1117–1142.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., & Kawabata, Z. I. (2012). Estimation of fish biomass using environmental DNA. *PLoS One*, *7*(4), e35868. https://doi.org/10.1371/journal.pone.0035868

Taylor, W. R. (1969). A revision of the catfish genus *Noturus* Rafinesque with an analysis of higher groups in the Ictaluridae. *Bulletin of the United States National Museum*, (282), 1–315. https://doi.org/10.5479/si.03629236.282.1

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, *22*(22), 4673–4680. https://doi.org/10.1093/nar/22.22.4673

Turner, C. R., Miller, D. J., Coyne, K. J., & Corush, J. (2014). Improved methods for capture, extraction, and quantitative assay of environmental DNA from Asian bigheaded carp (*Hypophthalmichthys* spp.). *PLoS One*, *9*(12), e114329.

Turner, C. R., Uy, K. L., & Everhart, R. C. (2015). Fish environmental DNA is more concentrated in aquatic sediments than surface water. *Biological Conservation*, *183*, 93–102. https://doi.org/10.1016/j.biocon.2014.11.017

USFWS (United States Fish and Wildlife Service). (2008). *5-year review, Pygmy Madtom (*Noturus stanauli*)*. Federal Register 58 FR 25758.

Wei, N., Nakajima, F., & Tobino, T. (2018). A microcosm study of surface sediment environmental DNA: decay observation, abundance estimation, and fragment length comparison. *Environmental Science & Technology*, *52*(21), 12428–12435. https://doi.org/10.1021/acs.est.8b04956

Wells, W. G. (2019). *Aspects of life history, species-habitat associations, species-community associations, and distribution of the pygmy madtom*, Noturus stanauli (Doctoral dissertation, Tennessee Technological University).

Wells, W. G., & Mattingly, H. T. (2019). Preliminary analysis of age-class structure and longevity for the endangered Pygmy Madtom. *Copeia*, *107*(3), 447–450. https://doi.org/10.1643/CI-19-186

Wilcox, T. M., McKelvey, K. S., Young, M. K., Jane, S. F., Lowe, W. H., Whiteley, A. R., & Schwartz, M. K. (2013). Robust detection of rare species using environmental DNA: The importance of primer specificity. *PLoS One*, *8*(3), e59520.

Zhang, J., & Boos, D. D. (1997). Mantel-Haenszel test statistics for correlated binary data. *Biometrics*, *53*, 1185–1198. https://doi.org/10.2307/2533489

SUPPORTING INFORMATION

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

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