Environmental DNA (eDNA) surveillance of the federally threatened Slender Chub (*Erimystax cahni*) in the Clinch River and Powell River

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Cooperator Science Series # 157-2024

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This document (USGS IPDS #: IP-156546) was developed in conjunction with the funding partner, Tennessee Tech University and the Tennessee Cooperative Fishery Research Unit (U.S. Geological Survey, Tennessee Wildlife Resources Agency, Tennessee Tech University, U.S. Fish and Wildlife Service and Wildlife Management Institute cooperating). Funding for this project was provided by the U.S. Fish and Wildlife Service: Cooperative Agreement G21AC10868-01.

Recommended citation:

Paine, R.T.R., Rogers, M.W., and Rosenberger, A.E. 2024. Environmental DNA (eDNA) surveillance of the federally threatened Slender Chub (*Erimystax cahni*) in the Clinch River and Powell River. U.S. Department of Interior, Fish and Wildlife Service, Cooperator Science Series FWS/CSS-157-2024, Washington, D.C. https://doi.org/10.3996/css55463605

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Final Report: July 25, 2024

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Photos from left to right and top to bottom: *Erimystax insignis* (N. Burkhead); *Erimystax dissimilis* (D. A. Neely); and *Erimystax cahni* (N. Burkhead)

This document was developed in conjunction with the U.S. Geological Survey and the Tennessee Cooperative Fishery Research Unit in collaboration with the funding partner. Funding for this project was provided by the U.S. Fish and Wildlife Service: Cooperative Agreement G21AC10868-01.

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ABSTRACT

The slender chub (*Erimystax cahni*) is a federally threatened fish native to and geographically restricted to eastern North America. More specifically, the Powell, Clinch, and lower Holston Rivers in Tennessee were historical collection areas. Habitat degradation from multiple sources, including surface mining, agriculture, dams, and urbanization, is associated with the decline of this species - an obligate inhabitant of gravel shoals in large rivers. As of 1964, only 15 voucher specimens were known and no living specimens were documented for decades. A federal recovery plan and Species Status Assessment were developed to determine if slender chub still exists and could be restored. Given the lack of recent observations using conventional sampling to search for its presence, we used environmental DNA sampling to determine their potential presence. Our specific objectives were to (1) develop a quantitative polymerase chain reaction (qPCR) assay aimed at species-specific detection and (2) sample historically known areas of collection. We sampled 43 sites in the Clinch and Powell Rivers. For the first time in almost two decades, we provide evidence for the continued existence of a putatively extinct species. We detected evidence of *E. cahni* in both the Clinch and Powell Rivers, but only at a few sites. We cannot confirm the presence of *E. cahni*, and positive eDNA matches could be attributed to amplification from a hybrid. Our results do indicate slender chub DNA perseveres in the Clinch and Powell Rivers and can inform resource agencies of localities to pursue on-the-ground searches for the slender chub with conventional methods (e.g., snorkeling) and potential restoration and recovery sites.

INTRODUCTION

The state of Tennessee hosts the largest number of freshwater fish species (>300) in the United States (Etnier and Starnes 1993). Many of these species have reduced native ranges due to habitat modifications, and some are federally listed as threatened or endangered. Thus, a greater understanding of their biology would benefit successful conservation and recovery strategies (Elkins et al. 2019). The slender chub (*Erimystax cahni*) is a federally threatened fish native to the Clinch, Powell, and lower Holston Rivers in Tennessee (USFWS 2008). Human-induced habitat alterations, including surface mining, agriculture, creation of reservoirs by dams, and urbanization, are associated with the decline of this species and degradation of associated habitat quality and biodiversity (USFWS 2008). Although reintroduction and establishment of other rare species (e.g., yellowfin madtom [*Noturus flavipinnis*] and freshwater mussels) indicate improvements in habitat quality during recent decades, no slender chub have been captured in almost 20 years (USFWS 2008). Cryptic and isolated populations of slender chub are presumed to still exist in the Clinch and Powell Rivers (USFWS 2008) due to the capture of suggested hybrids and putative sightings in the last eight years (USFWS 2008; Petty et al. 2014, 2016). More specialized and targeted monitoring would be helpful to assess the presence of *E. cahni*.

Accurate detection of rare fishes can require specialized biological monitoring due to several factors, such as cryptic behavior, preferred habitat, or rarity (MacKenzie and Royle 2005; Jerde et al. 2011; Pilliod et al. 2014; Wedderburn 2018). Monitoring for the presence of fishes that might be presumed extirpated or extinct adds another level of difficulty for biological surveys (Pilliod et al. 2014; Pfleger et al. 2016; Brys et al. 2020; Mason et al. 2020; Janosik et al. 2021). Capturing slender chub with conventional methods, such as seining, has been described as difficult and ineffective (USFWS 2008). Low catchability may be attributed to the fact that *E. cahni* is a fast-moving, benthic fish, and the species may exhibit natural population fluctuations that reduce detection probability during periods of lower abundance (Etnier 1994; USFWS 2008). Given the unknown presence of *E. cahni* in its home range and capture difficulty, previous research has suggested that molecular surveillance tools could offer an alternative option for detection and monitoring (Petty et al. 2016).

Molecular surveillance has become more commonplace in the last decade for rare and invasive species due to its efficiency and accuracy compared to conventional surveillance methods. Specifically, environmental DNA (eDNA) uses the discarded biological material in water (e.g., skin cells, slime coat, feces) to discern the potential presence of a target species in a system with minimal disturbance to the environment (Jerde et al. 2011). Environmental DNA surveillance is easily optimized for single-species detection or entire community surveillance. Single-species detection is achieved by designing species-specific oligos (i.e., primers and probes), typically for quantitative polymerase chain reaction (qPCR) analyses. Quantitative PCR can provide a more rapid and sensitive detection tool than conventional techniques due, in part, to the ability to detect low copy numbers of DNA (Bustin et al. 2009; Takahara et al. 2013).

Our objective was to survey the Clinch and Powell Rivers using molecular surveillance with qPCR to provide a rapid monitoring effort to determine the presence of *E. cahni* throughout its historically reported distribution.

METHODS

Study Area

The Clinch and Powell Rivers have headwaters in Virginia and run southwesterly into eastern Tennessee. The Powell River (3^{rd}-order) spans more than 300 km and drains approximately 2,471 km^2 in both Virginia and Tennessee before reaching its confluence with the Clinch River in Norris Reservoir. The Clinch River (4^{th}-order) spans more than 480 km before reaching its confluence with the Tennessee River and crosses multiple physiographic provinces (order and length information obtained from U.S. Geological Survey National Hydrography Dataset, following Jones et al. 2022). Both rivers are home to multiple endemic fish and mussel species, many of which are listed as threatened or endangered (USFWS 2008).

qPCR Assay Development

No living or molecular-grade preserved specimens of *E. cahni* exist to generate new sequences. We therefore used all available mitochondrial reference sequences from GenBank (n=3; Table 1). Reference sequences are representative of the cytochrome oxidase I (COI) and cytochrome

oxidase b (cytb) genes. We used the three reference sequences in the PrimerQuest™ Tool (IDTDNA) with default settings to develop candidate primer and probe oligos. Candidate assays were tested against synthetic DNA, i.e., gBlocks™ (IDTDNA), developed from the three references sequences mentioned above. All qPCR reactions were conducted in 15-µL volumes with 2X TaqMan Environmental MasterMix (7.5 µL), 10 µM of each primer (1.20 µL), 10 µM probe (0.38 µL), 10X Internal Positive Control (IPC) Mastermix (0.75 µL), 50X IPC DNA-template (0.15 µL), target DNA (2 µL), and water to volume. The IPC mastermix and DNA-template are components used to determine if PCR inhibition has occurred during a run. We first conducted a gradient PCR to optimize annealing temperatures. The optimized thermal profile used for all qPCR reactions included an initial denature step of 95°C for 10 minutes, followed by 50 cycles of 95°C at 15 seconds, 62°C at 30 seconds, and 72°C at 30 seconds. All plates were run with a 7-fold concentration standard curve (3,000,000 – 3 copies) derived from a synthetic DNA gBlock of the targeted region, as well as negative controls (i.e., PCR-grade water substituted for template DNA). Copy number was calculated using the formula:

$$DNA\ Copy\ Number = \frac{(A)(6.022 \times 10^{23})}{(B)(1 \times 10^{9})(660)}$$

where *A* is the amount (ng) of starting DNA and *C* is the basepair (bp) count of the target fragment. All samples, standard curve concentrations, and PCR negative controls were amplified in triplicate.

Limits of Detection and Quantification

We followed the methods published in Klymus et al. (2020) to determine the limits of detection (LOD) and limits of quantification (LOQ). We used a set of serial dilutions with 16 replicates per dilution to determine the effective LOD and LOQ for both the COI and cytb assays. Concentrations used to determine LOD and LOQ ranged from 5,000,000 – 0.3 copies/µL using the optimized chemistry and thermal profile mentioned above. We used the provided script (qPCR.-LoD-calculator.R) in R-studio (R v4.2.2) to conduct these analyses (R-Core Team 2022; R-Studio Team 2020). The LOD was calculated where 95% of the replicates of the lowest copy number standard dilution amplified. The LOQ was determined by the lowest copy number dilution with <35% coefficient of variation (CV). Detection of *E. cahni* eDNA was determined positive if one qPCR replicate yielded amplification before the 40 cycles (Westhoff et al. 2022).

Assay Specificity

Reference sequences for 111 species (Table 2) for the COI and cytb genes were downloaded from Genbank and aligned to *E. cahni* reference sequences. Reference sequences represented species that were either sympatric or likely sympatric with *E. cahni* and encompassed a wide phylogenetic range of families and genera. We curated sequences from a variety of locations to account for a range of genetic variation that may exist because no genomic tissue was available for *E. cahni* (Table 2). All of the primer and probe candidate oligos were aligned and compared to the respective gene region to visually assess species specificity *in silico*. Additionally, voucher specimens and tissues were collected from 33 species, including two sister taxa, streamline chub (*Erimystax dissimilis*) [n=13] and blotched chub (*Erimystax insignis*) [n=8]. Three individuals from all taxa, except for the two sister taxa, were used for assay specificity testing. Fish species were caught and identified via electrofishing and seining by Virginia Department of Wildlife Resources (VADWR) personnel under VADWR protocols and permitting requirements. Voucher specimens were stored in 100% ethanol, and fin-clips were stored in RNALater. All vouchers and specimens were stored at -20°C. Tissues from all individuals were extracted using the gMAX Mini Genomic DNA Kit (IBI Scientific) per the manufacturers protocol. Both the COI and cytb assay were tested using the optimized chemistry and thermal profile mentioned above.

Field Sampling

The Clinch and Powell Rivers were sampled during three different events (survey periods) over the summer 2022 (Event I: June 6-9, Event II: June 27-30, and Event III: July 19-22). A total of 43 sites were sampled in the Clinch River (n=23) and Powell River (n=20) during each of the three sampling events (Figure 1). All samples were collected by vacuum filtration (Citizen Science eDNA sampler [Smith-Root™]) using 1.0-µm pore size (47 mm), glass fiber filters secured in polypropylene filter housings and attached to each filter housing was a 15-cm silicon tube that allowed for precision targeting in the water column. All water samples were collected at a depth between 5 and 8 cm.

We sampled sites that were previously surveyed by Conservation Fisheries Inc. (Knoxville, Tennessee) and new sites that were opportunistically chosen based on accessibility to the rivers

(Figure 1). We targeted areas where reaches would include a wide variety of habitat but centered around riffle areas that fit the description of the preferred habitat for *E. cahni* (Etnier and Starnes 1993). Each site comprised a 50-m reach, where three, 1-L biological samples were collected in a downstream to upstream direction and always collected in front of the researcher to prevent sample contamination. A negative control was collected before sampling at each site by filtering 1-L of distilled water. Filtered samples were placed in 5-mL conical tubes and placed on ice while in the field and stored at -20°C within 12 hours in a portable freezer. All filters were transported back to the laboratory and placed in -80°C until DNA extractions were performed.

All containers, ice chest, and other equipment were decontaminated with 20% bleach (30 seconds) and thoroughly washed with deionized water before sampling. Equipment was decontaminated in the field using the same protocol (e.g., waders or sampling equipment) whenever it came into contact with river water. All filtration housings, silicone tubes, and tweezers used to filter and process samples in the field were sterilized with 20% bleach, washed with deionized water, and autoclaved at 121°C for 60 minutes before use. All filtration equipment, storage tubes, and gloves were kept separate from all other equipment to reduce contamination risk. Nitrile gloves were worn and switched between each sample at each site. All personnel that entered the water wore waders that were decontaminated with 20% bleach and rinsed with deionized water after each site.

DNA Extraction

Samples were extracted using the Fecal Extraction Kit (IBI Scientific™) following the manufacturer provided protocol except for the digestion and elution steps. One-quarter of each filter was cut into several small pieces and placed inside the provided 2-mL bead-beating tube. All tubes were homogenized at 6.0 m/s for 1 minute. Our change to the elution step was to perform a double elution with a smaller volume. We eluted the first time with 25 µL and conducted a second elution step with 25 µL. The elutent from both steps was pooled together for a total of 50 µL. DNA extractions were performed in a room where no PCR occurs to prevent possible cross-contamination. The immediate working area was sterilized with 20% bleach, and all tweezers and scissors used to process filters were heat sterilized after each use at >800°C for 30 seconds.

qPCR Analyses and Sequencing

All samples were amplified using the selected EC_COI122 assay with the optimized chemistry and thermal profile outlined above. The PCR product from any samples that yielded amplification of the target fragment was cleaned with the Small DNA Fragment Extraction Kit (IBI Scientific™) and sequenced via bi-directional Sanger sequencing to confirm accurate amplification of the target fragment. Furthermore, any sample that yielded amplification with the EC_COI122 was further tested with the EC_CYTB122 assay.

RESULTS

Assay Development and Quality Controls

Our initial attempts to design multiple assays yielded four assays for the COI region and two assays for the cytb region. After aligning all six assays to our reference sequences, we selected one assay from each gene for further testing, EC_COI122 and EC_CYTB122 (Table 1) based on *in silico* species specificity. All 111 species in the alignment had at least 1 basepair (bp) difference in both the forward and reverse primer regions, with most species having 2 bp differences in both primer regions. Additionally, all species had at least 1 bp difference in the 3' end of each primer region. Furthermore, all species had ≥ 4 bp differences in the probe binding region. Both assays exhibited specificity to the target species, with no amplification observed for any of the 33 species tested. Although we conducted 50 cycles of amplification, both assays showed efficient amplification for the 3-copy standard below the 40-cycle mark.

Limits of Detection and Quantification

Our analyses indicated for the COI assay that the LOD was 1.49 copies, while the LOQ was 88 copies. The PCR efficiency for all LOD/LOQ runs ranged from 93.89–100.27%, indicating reliable estimates of target detection and quantification. Typically, genomic DNA (gDNA) is used as a positive control for qPCR analyses, but, given the possible extinction of this species and lack of non-formalized tissue samples, no gDNA was available for use. However, all standards that used synthetic DNA amplified robustly. Additionally, IPCs amplified as expected, indicating no inhibition. All negative controls showed no indication of amplification (i.e., no cross-contamination).

Field Sampling and Detection

A total of 43 sites were sampled from the Clinch River (n=23) and the Powell River (n=20) during three different sampling events during summer 2022. We detected eDNA signals with the EC_COI122 assay at three sites, site 6 and 8 in the Powell River and site 20 in the Clinch River (Figure 1, Table 3). The PCR efficiency for all runs was within normal ranges (94.71–101.91%; R^2: 0.991–1.0).

The amplified DNA concentration from all three sites was low (~1 copy/μL) and falls below the LOQ; thus we sequenced each sample three times in case of sequencing failure due to low concentration. The generated sequences from each sample were aligned and trimmed to our reference sequences, and we visually confirmed that our generated sequences matched the reference sequences to ensure that each base pair between the two sequences matched. The consensus sequence from each generated pair of sequences was compared to the Genbank genetic repository using the Basic Local Alignment Search Tool (BLAST), and all nine sequences exhibited 100% match to known reference sequences of *Erimystax cahni*. Samples from all three sites were amplified with the EC_CYTB122 assay; however, no amplification of the target fragment was observed. All negative controls (i.e., field, extraction blanks, and PCR) exhibited no amplification, indicating no cross-contamination occurred.

Given the low number of eDNA detection events, no robust occupancy modelling could be developed, nor could a definitive detection probability be calculated. In lieu of this, we report raw detection probabilities and raw occupancy. Out of the total attempted detection events, the raw occupancy was 6% (3 positive detections out of 43 sites). At sites where eDNA was detected, the raw detection probability was 11% (3 positive detections in 27 samples in sites where the species was detected).

DISCUSSION

Assay Development

We found no indication of cross-amplification, and the assays we developed performed robustly in a biologically diverse assemblage. Voucher specimens of rare and extinct species are exceedingly valuable and rare, and thus destruction or even partial destruction is often limited or

not allowed. Furthermore, as is the case with many fish vouchers, including *E. cahni*, the only voucher specimens available are formalin fixed. Formalin fixation limits the extraction of useable genomic DNA, although some methods were successful. However, previous research (Shedlock et al. 1997; Chakraborty et al. 2006; Hykin et al. 2015; Jo et al. 2017) indicates that the older a fixed sample becomes, the probability of extracting useable genomic DNA diminishes (Ferrer et al. 2007; Do and Dobrovic 2015; Hedegaard et al. 2014). The ability to develop eDNA assays with minimal genetic material and reference sequences is promising for future endeavors with eDNA surveillance of rare and (possibly) extirpated or extinct species.

The detection and quantification of eDNA for rare and extinct species can be very difficult given the already low population abundance. Although the estimated abundance of *E. cahni* is unknown, lack of detection or capture in the last 20 years indicates it is likely very low. Given the low population abundance and size of the two rivers we sampled, basic detection of this species with eDNA likely requires a high number of samples and sampling events (as for this study). The use of multiple assays may be more successful for robust detection of rare, aquatic species. Although we did design and use two eDNA assays, only one was successful at detecting *E. cahni* eDNA. The target fragment length can also influence detection; however, both of our assays were the same size (122 bp), thus low abundance (*sensu* low DNA concentration in the sample) is likely the overriding factor contributing to low eDNA detectability of this species.

Field Sampling and Detection

For the first time in almost two decades, we provide evidence for the continued existence of a putatively extinct species in the Clinch and Powell Rivers. Detection in the Powell River was at two sites in the Tennessee reaches. The two sites are approximately 35.85 river-km apart. Both sites exhibit similar habitat (i.e., long riffle/run reaches dominated by cobble and bedrock). Detection in the Clinch River was in the Virginia reach of the Clinch River near the confluence of Copper Creek. This site occurs near a bend in the river characterized by several riffles dominated by a mix of gravel, cobble, boulders, and bedrock. Microhabitat characteristics at sampling sites were not a quantitative component of the project; however, future projects could incorporate that as an objective to inform future sampling. None of the three sites where slender

chub were previously detected with conventional methods revealed detection of the species using eDNA.

Our evidence indicates that this species or some hybrid form of *E. cahni* still exists in the Clinch and Powell Rivers. Environmental DNA uses mitochondrial DNA that is maternally inherited. Thus, we cannot preclude that our detections could have originated from a hybridized individual. In past years, hybrid individuals have been reported (USFWS 2008), although no individuals have been confirmed as such (USFWS 2008; Petty et al. 2014, 2016). Extensive conventional sampling over a wide distributional range is not feasible; however, more targeted intensive conventional sampling could be conducted at these three sites and the surrounding area to confirm the presence of living individuals in-hand.

We cannot confirm that specific spatial location of the source of the eDNA signals we detected at these sites. In the Powell River, site 7 is located 2.18 river-km upstream from site 6, and site 9 is located 5.31 river-km upstream from site 8. Similarly, in the Clinch River, site 21 is 3.42 river-km upstream from site 20. Given the proximity of upstream sites with negative detection, we can estimate that eDNA transport may be limited to a few kilometers. The mechanistic underpinnings controlling the transport and degradation of eDNA are complex; however, previous research in the Clinch River indicates that for rare, benthic species, eDNA transport is minimal (<1 km) (Paine et al. 2020). For riverine fishes, other research has also shown that eDNA transport is likely < 300 m, depending on flow and substrate interactions (Jane et al. 2015; Shogren et al. 2017).

POTENTIAL FUTURE DIRECTIONS

Environmental DNA surveillance has been demonstrated to be a rapid, sensitive tool for surveillance of rare aquatic species, and now, putatively extinct species. For rare fishes that have a low detection probability with conventional surveillance techniques, eDNA surveys can help guide agencies to target specific areas with more intense field efforts. In the case of *E. cahni*, agencies and managers could conduct more intensive sampling in the 40 river-km range between sites 6 and 8 in the Powell River if the goal is to corroborate species' presence. The Powell River is smaller than the Clinch River, and thus makes conventional sampling much easier, especially

during base-flow conditions. This may provide the best possibility of capturing live individuals for propagation and conservation efforts. However, attempts could be made to capture individuals in the Clinch River near the site of our detection as well, to not preclude possible genetic variation that may exist between the two disjunct populations.

Recent developments in metabarcoding-eDNA methodology imparts metabarcoding surveillance with levels of sensitivity comparable to those of qPCR surveillance techniques, with the added benefit of multispecies detection (Lanzén et al. 2017; Sato et al. 2017; Alberdi et al. 2018; Harper et al. 2018; Muha et al. 2021). Metabarcoding allows for a more comprehensive picture of community composition in each area, providing not only species presence or absence information, but valuable ecological insight related to possible species relationships and abiotic associations. The ability to extract useable genomic DNA from *E. cahni* vouchers would allow for the development of reference sequences that could be used for metabarcoding surveillance. Although we were successful at developing protocols to extract usable genomic DNA from formalin-fixed vouchers of *E. dissimilis* and *E. insignis*, we plan to work towards the same goal for *E. cahni*. Previous experiments with formalin-fixed *E. cahni* tissues have yielded mixed success, likely resulting from the age (1970s) of the vouchers specimens and the (unknown) original protocol used to fix the specimens. However, even without a reference sequence, tandem conventional and molecular sampling in the indicated reaches of the Clinch and Powell Rivers could prove more robust methods for making sympatric associations with metabarcoding data if a living *E. cahni* individual is captured.

ACKNOWLEDGEMENTS

The authors thank the U.S. Fish and Wildlife Service for funding this project (Cooperative Agreement G21AC10868-01). The Center for the Management, Utilization and Protection of Water Resources at Tennessee Tech is thanked for graciously providing laboratory space to conduct analyses and administrative support. Conservation Fisheries, Inc., and the Virginia Department of Wildlife Resources provided fish tissue samples following state protocols and permitting requirements. The authors thank Dr. Ben Keck and Jennifer Brummett for loaning us *E. cahni* voucher specimens. We owe a huge thank you to Eduardo Toala-Hidalgo for field sampling assistance and Jessee Griffith for laboratory assistance. We thank Dr. Aubree Hill, Dr. Shannon

Brewer, Dr. Katy Klymus, and Janet Carter for helpful comments and feedback on the initial drafts of the manuscript. Lastly, we dedicate this manuscript to Sandy Garrison, whose kindness, patience, and dedication made it possible to write and obtain the grant that supported this project. While she is no longer with us in body, she will always be with us in our hearts and research.

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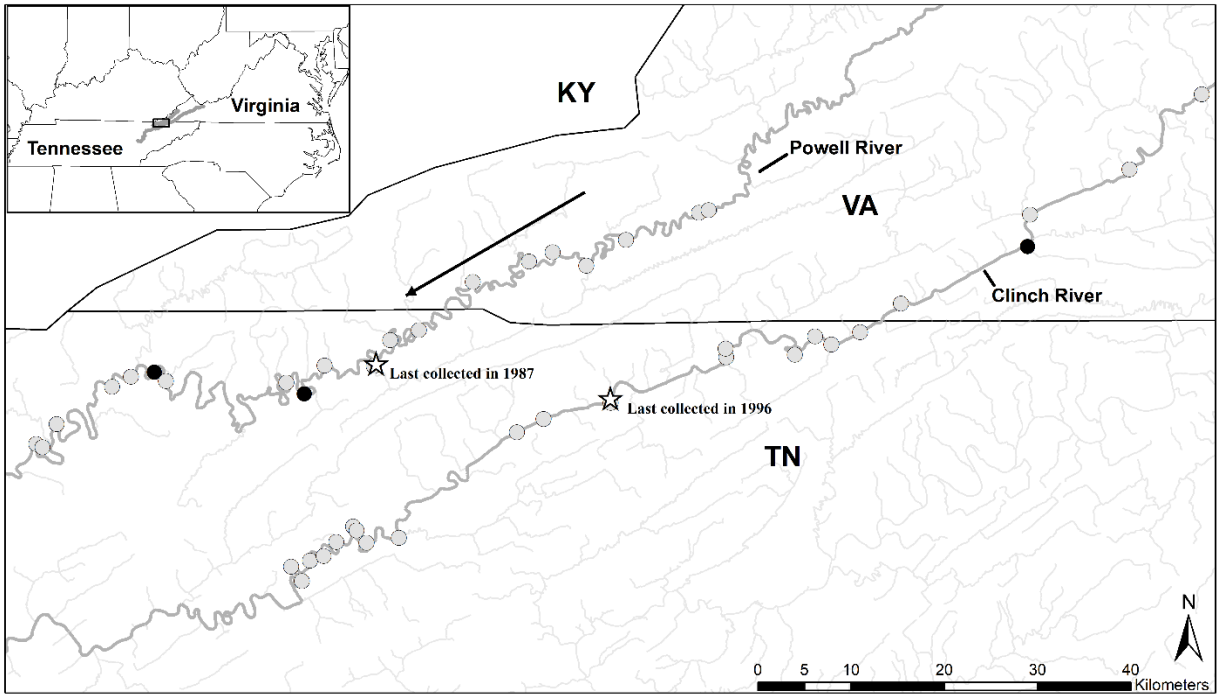

Figure 1: Sampling locations in the Clinch and Powell Rivers for the summer 2022 slender chub (*Erimystax cahni)* environmental DNA survey. Non-detection (gray) and Detection (black) of *E. cahni* eDNA are represented with closed circles (Detection, Sites 6 and 8 Powell River; Site 20 Clinch River). Hollow stars denote the last location in each river where living *E. cahni* individuals were captured. Sites are numerically ordered from downstream to upstream for both rivers (Table 3, this document).

Table 1: Quantitative polymerase chain reaction (qPCR) assays developed from reference sequences for slender chub (*Erimystax cahni*) environmental DNA (eDNA) surveillance. Accession numbers for the reference sequences used for assay development are also provided.

Oligo	Sequence (5' – 3')	Accession Seq
EC_COI122_FWD	CAGCCATCTCTCAGTACCAGA	HQ557200.1, JN025336.1
EC_COI122_REV	CTATGCTTCTTACTGACCGAAAC	HQ557200.1, JN025336.1
EC_COI122_PROBE	AGTCCTTCTTCTTCTGTCCCTACCTGT	HQ557200.1, JN025336.1
EC_CYTB122_FWD	CGTAATATACACGCTAATGGCGC	AY486010.1
EC_ CYTB 122_REV	AAATATCGGAGTGGTCCTGC	AY486010.1
EC_ CYTB 122_PROBE	ATTTACATGCACATTGCCCGAGGC	AY486010.1

Table 2: List of species used for assay specificity testing. All species listed have reference sequences that were aligned to those of *Erimystax cahni* and environmental DNA (eDNA) assay candidate oligos. Asterisks (*) denote species that were used for quantitative polymerase chain reaction (qPCR) specificity testing *in vitro*. Collection locations are provided for tissues and vouchers used for *in vitro* specificity testing.

Species	Common Name	Collection Location (River, Drainage)
*Campostoma anomalum**	central stoneroller	Goose Creek, Roanoke River
*Campostoma oligolepis**	largescale stoneroller	South Fork Holston, Holston River
Carassius auratus	goldfish	
Carassius carassius	Crucian carp	
Carassius cuvieri	Japanese crucian carp	
Carassius gibelio	Prussian carp	
Carpiodes cyprinus	quillback	
*Catostomus commersonii**	white sucker	Little River, New River
Chrosomus erythrogaster	southern redbelly dace	
Chrosomus saylori	laurel dace	
Clinostomus elongatus	redside dace	
*Clinostomus funduloides**	rosyside dace	Hungry Mother Creek, Holston River
*Cyprinella galactura**	whitetail shiner	New River, New River
*Cyprinella spiloptera**	spotfin shiner	New River, New River
Cyprinella venusta	blacktail shiner	
Cyprinella whipplei	steelcolor shiner	
Cyprinus carpio	common carp	
Erimonax monachus	spotfin chub	
*Erimystax dissimilis**	streamline chub	Clinch River, Clinch River
*Erimystax insignis**	blotched chub	Clinch River, Clinch River
Etheostoma aquali	coppercheek darter	
Etheostoma bison	buffalo darter	
Etheostoma blennius	blenny darter	
Etheostoma blennioides	greenside darter	
Etheostoma caeruleum	rainbow darter	
Etheostoma camurum	bluebreast darter	
Etheostoma chlorobranchium	greenfin darter	
Etheostoma chlorosomum	bluntnose darter	
Etheostoma cinereum	ashy darter	
Etheostoma crossopterum	fringed darter	
Etheostoma denoncourti	golden darter	
Etheostoma flabellare	fantail darter	
Etheostoma flavum	saffron darter	
Etheostoma gracile	slough darter	
Etheostoma histrio	harlequin darter	
Etheostoma jessiae	blueside darter	
Etheostoma kennicotti	stripetail darter	
Etheostoma lawrencei	headwater darter	
Etheostoma luteovinctum	redband darter	
Etheostoma meadiae	bluespar darter	
Etheostoma nigripinne	blackfin darter	

Etheostoma nigrum	johnny darter	
Etheostoma oophylax	guardian darter	
Etheostoma parvipinne	goldstripe darter	
Etheostoma percnurum	duskytail darter	
Etheostoma pseudovulatum	egg-mimic darter	
Etheostoma punctulatum	stippled darter	
Etheostoma rufilineatum	redline darter	
Etheostoma simoterum	snubnose darter	
Etheostoma spectabile	orangethroat darter	
Etheostoma stigmaeum	speckled darter	
Etheostoma striatulum	striated darter	
Etheostoma tennesseense	Tennessee darter	
Etheostoma tippecanoe	Tippecanoe darter	
Etheostoma variatum	variegate darter	
Etheostoma zonale	banded darter	
Etheostoma zonistium	bandfin darter	
Exoglossum laurae	tonguetied minnow	
*Exoglossum maxillingua**	cutlips minnow	Pigg River, Roanoke River
Hybognathus nuchalis	Mississippi silvery minnow	
*Hybopsis amblops**	bigeye chub	Clinch River, Clinch River
Hybopsis dorsalis	bigmouth shiner	
*Hypentelium nigricans**	northern hogsucker	Little River, New River
*Luxilus chrysocephalus**	striped shiner	Timbertree Branch, Clinch River
*Luxilus coccogenis**	warpaint shiner	Big Mocassin Creek, Holston River
*Lythrurus ardens**	rosefin shiner	Pigg River, Roanoke River
Lythrurus fasciolaris	scarlet shiner	
Lythrurus fumeus	ribbon shiner	
*Lythrurus lirus**	mountain shiner	Big Mocassin Creek, Holston River
Lythrurus umbratilis	redfin shiner	
Macrhybopsis gelida	sturgeon chub	
Macrhybopsis meeki	sicklefin chub	
Macrhybopsis storeriana	silver chub	
*Moxostoma breviceps**	smallmouth redhorse	Clinch River, Clinch River
*Moxostoma carinatum**	river redhorse	Clinch River, Clinch River
*Moxostoma cervinum**	blacktip jumprock	Pigg River, Roanoke River
*Moxostoma duquesnei**	black redhorse	Clinch River, Clinch River
*Moxostoma erythrurum**	golden redhorse	Clinch River, Clinch River
Moxostoma macrolepidotum	shorthead redhorse	
Nocomis effusus	redtail chub	
*Nocomis leptocephalus**	bluehead chub	Pigg River, Roanoke River
*Nocomis micropogon**	river chub	Clinch River, Clinch River
Notemigonus crysoleucus	golden shiner	
Notropis ariommus	popeye shiner	
Notropis atherinoides	emerald shiner	
Notropis blennius	river shiner	
Notropis boops	bigeye shiner	
Notropis buccatus	silverjaw minnow	
Notropis buchanani	ghost shiner	

Notropis chrosomus	rainbow shiner	
*Notropis leuciodus**	Tennessee shiner	Clinch River, Clinch River
Notropis micropteryx	highland shiner	
Notropis photogenis	silver shiner	
*Notropis rubellus**	rosyface shiner	New River, New River
*Notropis rubricroceus**	saffron shiner	Timbertree Branch, Holston River
Notropis rupestris	bedrock shiner	
Notropis shumardi	silverband shiner	
*Notropis sp cf spectrunculus**	sawfin shiner	Clinch River, Clinch River
Notropis spectrunculus	mirror shiner	
Notropis stramineus	sand shiner	
*Notropis telescopus**	telescope shiner	Wolf Creek, New River
*Notropis volucellus**	mimic shiner	Clinch River, Clinch River
Notropis wickliffi	channel shiner	
Opsopoeodus emiliae	pugnose minnow	
*Pimephales notatus**	bluntnose minnow	Bluestone River, New River
*Pimephales promelas**	fathead minnow	Hungry Mother Creek tributary, Holston River
Pimephales vigilax	bullhead minnow	
*Rhinichthys atratulus**	eastern blacknose dace	Hungry Mother Creek tributary, Holston River
*Rhinichthys cataractae**	longnose dace	Little River, New River
Rhinichthys obtusus	western blacknose dace	
*Semotilus atromaculatus**	creek chub	Mud Fork, New River

Table 3: Results of cytochrome oxidase I (COI) environmental DNA (eDNA) surveillance for *Erimystax cahni* in the Clinch and Powell Rivers. Shown are detection results for each of the three sampling events that occurred during summer 2022. Fractions represent the number of technical replicates where amplification was observed. Sample names that yielded amplification are provided in parentheses.

SITE	CLINCH			POWELL		
	Event I	Event II	Event III	Event I	Event II	Event III
1	0/3	0/3	0/3	0/3	0/3	0/3
2	0/3	0/3	0/3	0/3	0/3	0/3
3	0/3	0/3	0/3	0/3	0/3	0/3
4	0/3	0/3	0/3	0/3	0/3	0/3
5	0/3	0/3	0/3	0/3	0/3	0/3
6	0/3	0/3	0/3	1/3 (P6BI)	0/3	0/3
7	0/3	0/3	0/3	0/3	0/3	0/3
8	0/3	0/3	0/3	1/3 (P8BI)	0/3	0/3
9	0/3	0/3	0/3	0/3	0/3	0/3
10	0/3	0/3	0/3	0/3	0/3	0/3
11	0/3	0/3	0/3	0/3	0/3	0/3
12	0/3	0/3	0/3	0/3	0/3	0/3
13	0/3	0/3	0/3	0/3	0/3	0/3
14	0/3	0/3	0/3	0/3	0/3	0/3
15	0/3	0/3	0/3	0/3	0/3	0/3
16	0/3	0/3	0/3	0/3	0/3	0/3
17	0/3	0/3	0/3	0/3	0/3	0/3
18	0/3	0/3	0/3	0/3	0/3	0/3
19	0/3	0/3	0/3	0/3	0/3	0/3
20	0/3	0/3	1/3 (C20BIII)	0/3	0/3	0/3
21	0/3	0/3	0/3	-	-	-
22	0/3	0/3	0/3	-	-	-
23	0/3	0/3	0/3	-	-	-

Note: All samples that yielded positive amplification were Sanger sequenced and compared to the Genbank genetic repository using the Basic Local Alignment Search Tool (BLAST), and resulted in 100% identity match to *E. cahni* reference sequences.

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