O Romeo! Environmental DNA could prevent a tragedy for the elusive Chucky Madtom (*Noturus crypticus*)

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Cooperator Science Series # 169-2025

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This document (USGS IPDS #: IP-177206) was developed in conjunction with the U.S. Geological Survey, Tennessee Cooperative Fishery Research Unit in collaboration with the funding partner. The U.S. Fish and Wildlife Service provided funding for this project through the Tennessee Wildlife Resources Agency.

Recommended citation:

Paine, R.T.R., H. Swain-Menzel, A.E. Rosenberger, and A. Velasquez. 2025. O Romeo! Environmental DNA could prevent a tragedy for the elusive Chucky Madtom (*Noturus crypticus*). U.S. Department of Interior, Fish and Wildlife Service, Cooperator Science Series FWS/CSS-169-2025, Washington, D.C. https://doi.org/10.3996/css69379551

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ABSTRACT

Using environmental DNA (eDNA) surveillance methods, we report the first evidence of the persistence of the Chucky Madtom (*Noturus crypticus*) in Little Chucky Creek, Tennessee, which has been absent from conventional surveys since 2004, and in Dunn Creek, Tennessee, where it was last collected in 1940. This highlights the utility of eDNA for detecting cryptic, rare fish species that may persist at extremely low population densities when conventional surveys fail, as well as its effectiveness as a contemporary tool to guide targeted conventional sampling efforts; however, it is not intended to replace the 'in hand' detection of the species.

INTRODUCTION

Many freshwater fishes in the southeastern United States are threatened by human activities that have altered the landscape, such as water withdrawal or diversion, chemical runoff, sedimentation, deforestation, and introduction of nonnative species (Dudgeon et al. 2006; Jelks et al. 2008). Environmental policy frameworks can conserve natural resources and biodiversity through legislation such as the Clean Water Act, the National Environmental Policy Act, and the Endangered Species Act with their corresponding regulatory bodies. The research enterprise concurrently provides novel methods and conceptual frameworks to implement evidence-based conservation practices and streamline management (Nichols et al. 2017; Mace et al. 2018). Numerous species face extinction due to a variety of factors; however, the factors underlying population declines and range declines can remain unidentified, often due to insufficient knowledge regarding the fundamental biology and life history characteristics of numerous taxonomic groups (Cooke 2008; Sutherland et al. 2009; Lees et al. 2022). Tennessee's rivers and streams are home to a diverse array of fish species, including a disproportionately high number of threatened or endangered species (Etnier and Starnes 1993; Elkins et al. 2019). Additionally,

many of these species are poorly understood because they declined or disappeared before intensive investigations on their ecology could be initiated, such as Slender Chub (*Erimystax cahni*), Smoky Madtom (*Noturus baileyi*), and Chucky Madtom (*Noturus crypticus)* (Bauer et al. 1983; Etnier and Starnes 1993; Jenkins and Burkhead 1993). Some are even on the brink of extinction and may require prompt efforts to achieve successful conservation outcomes (Elkins et al. 2019; Black 2020; Paine et al. 2025).

Species presumed or suspected extinct but subsequently rediscovered after extended periods of non-detection are sometimes referred to as "Lazarus species" (Morrison et al. 2007). Rare and cryptic species can persist at low population densities without detection and may inhabit environments that present significant sampling challenges (Thompson 2004). As such, they represent critical conservation cases with limited information to guide intervention. In these instances, managers could waste resources by applying conservation measures to areas where the species is absent. Conversely, the most arguably irreversible error for a Lazarus species has been termed a "Romeo error," or its premature abandonment, contributing to or failing to prevent its demise (Collar 1998). Extensive sampling using high-detection methods and knowledge of the species' former range and habitat preferences could prevent a Romeo error by enhancing the discovery of Lazarus species (Thompson 2004; Fisher et al. 2011; Lopes et al. 2021; Oliveira-Carvalho et al. 2024). Such a method is available via the application of environmental DNA (eDNA), which can provide increased sensitivity for cryptic species that persist in low densities due to its capacity to use minimal genetic material shed into the environment to indicate species presence (Rees et al. 2014; Lopes et al. 2021). Rapid and extensive eDNA surveys provide a strategic approach for allocating resources for Lazarus species, guiding targeted, intensive

conventional sampling in areas with positive molecular detection (Valentini et al. 2016; Deiner et al. 2017).

The Chucky Madtom (*Noturus crypticus*) is a federally listed endangered species, historically known from two small streams, Little Chucky Creek and Dunn Creek, in eastern Tennessee, United States. A total of 14 individuals were collected from Little Chucky Creek, and none have been observed in over 20 years between 2004 and 2025 (Kuhajda et al. 2018). Researchers last captured a specimen from Dunn Creek in 1940, and the species is regarded as extirpated from this system (Burr et al. 2005). The last known living specimen was collected in 2004 from Little Chucky Creek, with no other individuals gathered despite extensive survey efforts (USFWS 2024). Ongoing presumed threats to this species include poor water quality, habitat deterioration, and invasive species (i.e., Virile Crayfish – *Faxonius virilis* and Kentucky River Crayfish – *Faxonius juvenilis*) (Kuhajda et al. 2018; Harris et al. 2020). Despite multiple survey efforts–with persistent threats in the watershed–its continued non-detection indicated the species was either extinct or "on the brink" of extinction (Kuhajda et al. 2018; Ceballos et al. 2020). Therefore, rapid, sensitive, and cost-effective survey efforts that could be applied extensively may be necessary to determine the current status of Chucky Madtom and guide conventional sampling efforts to areas where capturing a living individual is most likely. Our objective in this study was to use eDNA surveillance as a rapid and sensitive approach for detecting the Chucky Madtom throughout Little Chucky Creek and Dunn Creek.

METHODS

Study Area. -

Little Chucky Creek is a 32 river-km tributary stream to the Nolichucky River (USGS 2024). All of Little Chucky Creek has been designated as critical habitat for the Chucky

Madtom (Kuhajda et al. 2018). Many reaches contained habitat characteristics presumed suitable for the species (e.g., silt-free, flat gravel, cobble, and slab-rock boulders, shallow depths, gentle run-pool reaches) (Kuhajda et al. 2018). However, sediment input, presumably from agricultural runoff and cattle farming, undermines the presence of these habitat characteristics along most of the length of Little Chucky Creek (USFWS 2024).

Dunn Creek is located within the Little Pigeon River watershed, approximately 50 km southwest of Little Chucky Creek. Its headwaters originate in the Great Smoky Mountains National Park and terminate at the confluence with the East Fork Little Pigeon River. The upper reaches are characterized by forest and minimal rural development, while the downstream reaches are surrounded by farming and agriculture, albeit not to the extent of Little Chucky Creek (USGS 2023).

Quantitative PCR Assay Development. -

Initial candidate assay development followed methods described in Paine et al. (2025). We used the cytochrome oxidase b (cytb) gene to develop eDNA assays because of the number of sequences available for congeneric, sympatric, or possibly sympatric species. From the three cytb reference sequences available on GenBank (Accessions: DQ383657, DQ383658, DQ383659) for Chucky Madtom, we developed 10 candidate assays for *in silico* screening, from which two assays (NC100_CYTB and NC121_CYTB) were chosen for *in vitro* screening, which amplify a 100- and 121-base pair (bp) fragment, respectively. Assay specificity testing was conducted against the species list in Table 1, and includes species that are closely related and sympatric, e.g., Elegant Madtom (*Noturus elegans*), Mountain Madtom (*Noturus eleutherus*), Yellow Bullhead (*Ameiurus natalis*), and Flathead Catfish (*Pylodictus olivaris*).

All quantitative PCR (qPCR) amplification was conducted using the QuantStudio 3 qPCR thermocycler (Thermo Fisher Scientific, Waltham, MA). The two candidate assays were optimized independently *in vitro* against synthetic DNA, i.e., gBlocks ™ (IDT) –developed from the three reference sequences– and against genomic DNA extracted from fin clips of non-target species (Table 1). Chucky Madtom genomic DNA could not be used to optimize assays or as a positive control because no vouchers or tissues exist that have not been formalin-fixed; detailed information related to this issue is described in Paine et al. (2025). Optimization and specificity testing for both assays (singleplex) were conducted in 15-µL reactions with 2X TaqMan Environmental MasterMix 2.0 (7.5 µL), 10 µM of each primer (1.20 µL), 10 µM probe (0.3µL), 10X Internal Positive Control (IPC) Mastermix (0.75 µL) (Applied Biosystems™, TaqMan™) , 50X IPC DNA-template (0.15 µL) (Applied Biosystems™, TaqMan™), template DNA (1 µL), and water to volume. The optimized thermal profile for the NC121_CYTB assay was as follows: initial denature 95°C for 10 minutes, followed by 50 cycles of 95°C for 45 seconds, 63°C for 45 seconds, and 72°C for 45 seconds. The optimized thermal profile for the NC100_CYTB assay was the same as NC121_CYTB, with one difference (60°C annealing temperature). Because of the difference in annealing temperatures, assays could not be run in multiplex. Methods used to determine the limits of detection and quantification for each assay followed those described in Paine et al. (2025) (*sensu* Klymus et al. 2020). PCR inhibition was determined by calculating the IPC ΔCt between the standard curve dilutions and each sample (Turner et al. 2015). A sample was considered inhibited if the IPC ΔCt was delayed by >3 cycles (Turner et al. 2015; Sansom et al 2024).

Field Sampling. - Field sampling methodology followed Paine et al. (2025). Multiple sampling events (EVT) were conducted in Little Chucky Creek: one extensive, sampling event in Y2023 (i.e., Y23 – EVT I [September]) and two, intensive sampling events in Y2024 (i.e., Y24 – EVT I [May] and Y24 – EVT II [June]). Sites were systematically selected based on stream accessibility and included historical and new sites throughout Little Chucky Creek. Dunn Creek was sampled opportunistically for only one event in Y2024 (i.e., Y24 – EVT II [June]) at the locality where the 1940 specimen was collected (Figure 1).

Bottled water (1.5 L) was used for the field negative control at each site. At each site, 1.5-L samples for eDNA analysis were collected by filtering stream water through a 1.0-µm glass fiber filter at three different points perpendicular to flow (i.e., three independent replicates). Nitrile gloves were worn and switched throughout the sampling process and personnel and equipment were decontaminated with 20% bleach between sites and as needed. All samples were stored at -20°C in the field with a portable freezer and then transferred to -80°C until further processing (i.e., extractions and qPCR). All field equipment was thoroughly cleaned with 20% bleach for 10 minutes and thoroughly rinsed with deionized-water before sampling. Filter housings and associated sampling equipment were cleaned with 20% bleach for 10 minutes, washed in a laboratory dishwater, and autoclaved at 121°C for 1-hour before sampling. While in the field, all field equipment was washed with 20% bleach for 10 minutes (in the sun) and rinsed with bottled water before sampling in Dunn Creek to prevent possible transfer and contamination of eDNA from Little Chucky Creek.

DNA Extraction and Quantitative PCR. - DNA extraction methods followed Paine et al. (2025). All samples were extracted and purified with the Fecal Extraction Kit (IBI Scientific) using bead-beating homogenization during the lysis step. All samples were quantified using a

NanoDrop Spectrophotometer. Samples were further purified with the OneStep PCR Inhibitor Removal Kit (Zymo), if needed, after initial testing with qPCR.

All samples, including field blanks and extraction blanks, were amplified with the NC121_CYTB assay using the optimized chemical and thermal profiles mentioned above using 8 qPCR replicates. To quantify amplified DNA, a standard curve was generated from synthetic DNA in a 7-fold serial dilution (3,000,000 – 3 copies), with each dilution amplified in triplicate. PCR plate negative controls were run in triplicate. The DNA copy number was calculated using Paine et al. (2025). To confirm amplification of the target amplicon, qPCR reactions that yielded positive amplification were cleaned with the ExoSAP-it kit (ThermoFisher) for bi-directional Sanger sequencing.

RESULTS

Little Chucky Creek Survey

Of the three sampling events conducted in Little Chucky Creek (Y23-EVT I, Y24-EVT I, and Y24-EVT II), only two events were analyzed for the presence of Chucky Madtom eDNA (Y23-EVT I and Y24-EVT II). A total of six PCR reactions (from three independent replicates) yielded positive amplification (all from Y24 – EVT II) (Table 2). DNA concentrations in these reactions ranged from 0.946 to 2.139 copies/µL. The signals resulted from eDNA samples collected from three sites, upstream from where the last known individual was captured in Little Chucky Creek (Figure 1). Bi-directional Sanger sequencing of the six PCR reactions resulted in a 99-100% identity match to *Noturus crypticus* cytb reference sequences (Table 3). No amplification was observed in the Y23-EVT I samples (Table 2). The samples from Y24 – EVT I

are still being analyzed, but we report our current results here to expedite actionable science in the immediate future.

Standard performance fell within acceptable ranges across all runs for Y23-EVT I (Efficiency: 94.90-99.04%, $R^2 \geq 0.9985$ and slope: -3.3452 to -3.4504) and across all runs for Y24-EVT II (Efficiency: 94.60-100.69%, $R^2 \geq 0.9986$ and slope: -3.3054 to -3.4570). The maximum inhibition level for any samples collected and analyzed from Little Chucky Creek was 2.89 cycles. No amplification was observed in any extraction blank, field negative control, or PCR plate negative control.

Dunn Creek Survey

Five PCR reactions (from three independent replicates) yielded positive amplification in samples collected from Dunn Creek (Y24 – EVT II). DNA concentrations in these reactions ranged from 0.490 to 1.451 copies/µL. The signals resulted from eDNA samples collected below the confluence of Dunn Creek and Yellow Breeches Creek (DCCF) and within Dunn Creek (DNCK) (Table 2; Figure 1). No amplification was observed in samples collected from Yellow Breeches Creek (YBCK) (Table 2; Figure 1). Bi-directional Sanger sequencing of the five PCR reactions resulted in a >97% identity match to *Noturus crypticus* cytb reference sequences (n=4), with one qPCR reaction that did not return a sequence (Table 3).

Standard performance fell within acceptable ranges across all runs for Y24-EVT II (Efficiency: 97.88-99.12%, $R^2 \geq 0.9990$ and slope: -3.3433 to -3.3739). The maximum inhibition level for any samples collected and analyzed from Dunn Creek was 1.25 cycles. No amplification was observed in any extraction blank, field negative control, or PCR plate negative control.

DISCUSSION

Using eDNA methods, we provide the first evidence in over 20 years for the continued existence of the Chucky Madtom in Little Chucky Creek and the first evidence in over 80 years for its persistence in Dunn Creek. Environmental DNA is a sensitive tool for surveying locations where conventional surveys underperform. However, we emphasize that eDNA is best used in tandem with conventional methods, not as a replacement. In this instance, eDNA surveillance may help guide more intensive conventional survey efforts to locate living individuals for future propagation efforts.

Similar to conventional sampling, eDNA is not immune to sampling error or bias. For example, multiple assays are commonly used to detect rare species. While two assays were optimized and tested for specificity, only the NC121_CYTB assay was specific to the Chucky Madtom, while NC100_CYTB co-amplified DNA from the Pygmy Madtom (*N. stanauli*), a sister taxon. To our knowledge, while the Pygmy Madtom does occur in the Clinch River (>40 km north of Little Chucky Creek), it does not occur in either Little Chucky Creek or Dunn Creek (USFWS 2023). Still, given the cryptic nature of madtoms and their distributions, as evident in this study, we chose not to use the second assay to reduce the possibility of false positives. We emphasize the importance of testing eDNA assays against many individuals and species in the target sample region to develop the best *a priori* understanding of assay performance. When eDNA assays cannot be tested against all sympatric species, post-amplification methods (e.g., Sanger sequencing) are important to incorporate into the eDNA workflow to verify accurate target sequence amplification. Although we confidently identified eDNA signals from Chucky

Madtom, false positive detections can still result from the transport of eDNA (i.e., reporting that an individual is present at a site when eDNA originated from farther upstream), hence conventional sampling is important to confirm the location of a physical specimen (Burian et al. 2021).

Environmental DNA surveillance is also not exempt from false negative results. While we did rule out one source of false negatives (i.e., PCR inhibition that would prevent DNA from amplifying), many of our sampled sites were negative for detection. These should not be viewed as definitive negative detections because eDNA concentrations may fall below the limit of detection, which is to say that eDNA is present in a given reaction, but the concentration is so small that current instrumentation cannot measure a reliable signal (Klymus et al. 2020). Furthermore, an eDNA sample represents a single point in time, and a species can be detected at one point in time and not another. Thus, repeated sampling can increase or allow calculation of detection probability of a species within a given spatial extent (MacKenzie et al. 2002; Burian et al. 2021). For example, we did not detect Chucky Madtom eDNA in Little Chucky Creek in our first sampling event (Y23 – EVT I), but did detect eDNA in Little Chucky Creek during our third sampling event (Y24, EVT II) (Table 2). This may explain the failure to detect Chucky Madtom in Dunn Creek with conventional sampling, as limited surveillance has been conducted since its original capture in 1940 (Shute et al. 1997). Further, sampling in different seasons may increase detection probability (e.g., spawning vs non-spawning or summer vs fall), which may explain our detection/non-detection in Little Chucky Creek (Janosik and Johnston 2015; Paine et al. 2021).

Of major concern with this species are severe natural weather events. Our eDNA surveillance (and associated results) represent data collected before Hurricane Helene (Summer

2024). Excessive rainfall during this event greatly affected Little Chucky Creek, especially near its confluence with the Nolichucky River (Webb et al. 2024). While our eDNA signals were in upstream reaches, the entire Little Chucky Creek watershed experienced a 100-year precipitation event (Stevenson and Schumacher 2014, NOAA 2025) in association with Hurricane Helene, and Dunn Creek likely experienced similar conditions. The Chucky Madtom likely has low resilience to environmental disturbance, meaning it would be more difficult for this species to withstand and recover from this kind of stochastic and catastrophic event (Kuhajda et al. 2018). Intense, conventional surveillance of the area is needed to assess environmental damage and habitat alterations, and to assess whether living individuals are present and could be used for propagation efforts. If living individuals are found in either creek, it could mean bringing this species back from the brink with successful propagation efforts, as has been done with other species (e.g., *Noturus flavipinnis* - Yellowfin Madtom) (Rakes et al. 1999, Shute et al. 2005, Throneberry et al. 2009).

Our study highlights the importance of incorporating eDNA methods into surveillance efforts of fishes presumed extirpated or extinct. “Romeo errors” as a result of non-sensitive, conventional methods may lead agencies to believe that a species has disappeared, when in reality it may still exist (albeit in low densities) (e.g., Slender Chub [Paine et al. 2025] and Chucky Madtom [this manuscript]). Often, conventional sampling may be conducted in areas of familiarity (e.g., easy access, historical detection of target organism). The likelihood of detecting a target organism is decreased by only sampling a small reach or segment of the target organisms’ entire range. Environmental DNA surveillance allows for both extensive sampling (i.e., the target organisms’ entire range) and intensive sampling (i.e., focused sampling of a small reach or segment). This may give these “Lazarus species” or species “on the brink” a last chance

at detection by providing guidance to new areas previously unknown to researchers, whereby intensive, conventional efforts can be applied efficiently and systematically to increase capture of living individuals for future restoration goals.

Acknowledgements

The authors thank the Tennessee Wildlife Resources Agency with support from the U.S. Fish and Wildlife Service Section 6 program for support of this project. We are grateful to Joseph Calloway, who provided field assistance, enthusiasm, and knowledge to the project. We thank the Center for the Management, Utilization, and Protection of Water Resources at Tennessee Tech for graciously providing laboratory space to conduct analyses and administrative support. We thank Dr. Ben Keck and Jennifer Brummett from the University of Tennessee David A. Etnier Ichthyological Collection for providing tissue samples. We thank Conservation Fisheries, Inc. (CFI) for initial help with field site selection and site information. We also thank Mike Pinder and his team from the Virginia Department of Wildlife Resources for collecting tissues for another project that were used in this project. We thank Dr. Kit Wheeler and Dr. Katy E. Klymus for helpful comments and feedback on the initial drafts of the manuscript. Live vertebrates were not part of the research being reported.

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Table 1: List of species used for *in silico* and *in vitro* environmental DNA (eDNA) assay specificity testing. Chucky Madtom (*Noturus crypticus*) eDNA assays were tested against genomic DNA derived from all listed species, unless indicated otherwise. Species only used for *in silico* specificity testing are denoted with an asterisk (*). Collection locations indicate where individuals were collected from. Common and scientific names referenced from Page et al. 2023.

Family	Species	Common Name	Collection Location (River, Drainage)
Catostomidae	*Catostomus commersonii*	White Sucker	Little River, New River
	Hypentelium nigricans	Northern Hogsucker	Little River, New River
	*Moxostoma anisurum**	Silver Redhorse	
	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	
	*Moxostoma poecilurum**	Blacktail Redhorse	
	*Moxostoma robustum**	Robust Redhorse	
Centrarchidae	*Ambloplites rupestris**	Rock Bass	
	Lepomis auritus	Redbreast Sunfish	Clinch River, Clinch River
	Lepomis cyanellus	Green Sunfish	Peak Creek, New River
	Lepomis gibbosus	Pumpkinseed	Peak Creek, New River
	Lepomis gulosus	Warmouth	Harrel's Mill Pond, Chowan River
	*Lepomis humilis**	Orangespotted Sunfish	
	Lepomis macrochirus	Bluegill	Peak Creek, New River
	Lepomis megalotis	Longear Sunfish	Clinch River, Clinch River
	*Lepomis microlophus**	Redear Sunfish	Peak Creek, New River
	Micropterus dolomieu	Smallmouth Bass	Clinch River, Clinch River
	*Micropterus punctulatus**	Spotted Bass	
	Micropterus nigricans	Largemouth Bass	Peak Creek, New River
	*Pomoxis annularis**	White Crappie	
	Pomoxis nigromaculatus	Black Crappie	Peak Creek, New River
Cottidae	*Cottus carolinae**	Banded Sculpin	
Dorosomatidae	*Dorosoma cepedianum*	Gizzard Shad	Peak Creek, New River
Fundulidae	*Fundulus catenatus**	Northern Studfish	

	*Fundulus notatus**	Blackstripe Topminnow	
	*Fudulus olivaceus**	Blackspotted Topminnow	
Ictaluridae	*Ameiurus melas**	Black Bullhead	
	Ameiurus natalis	Yellow Bullhead	Clinch River, Clinch River
	*Ameiurus nebulosus**	Brown Bullhead	
	Ictalurus furcatus	Blue Catfish	Duck River, Duck River
	Ictalurus punctatus	Channel Catfish	South Fork Shenandoah River, South Fork Shenandoah River
	*Noturus albater**	Ozark Madtom	
	*Noturus baileyi**	Smoky Madtom	
	Noturus elegans	Elegant Madtom	Salt Lick Creek, Barren River; Trace Creek, Barren River
	Noturus eleutherus	Mountain Madtom	Clinch River, Clinch River; Harpeth River, Cumberland River; Little River, Tennessee River
	*Noturus exilis**	Slender Madtom	
	Noturus fasciatus	Saddled Madtom	Buffalo River, Tennessee River;
	*Noturus flavipinnis**	Yellowfin Madtom	
	Noturus flavus	Stonecat	Duck River, Duck River
	*Noturus funebris**	Black Madtom	
	Noturus gladiator	Piebald Madtom	Piney Creek, Hatchie River
	Noturus gyrinus	Tadpole Madtom	Mathis Creek, Hatchie River; North Reelfoot Creek, Mississippi River
	Noturus hildebrandi	Least Madtom	Piney Creek, Hatchie River; North Fork Forked Deer River, Mississippi River; Clarks Creek, Mississippi River
	Noturus insignis	Margined Madtom	Joes Creek, Pee Dee River
	Noturus miurus	Brindled Madtom	Tar Creek, Mississippi River; Spring Creek, Hatchie River
	Noturus nocturnus	Freckled Madtom	Big Cow Creek, Sabine River; Big Sandy River, Tennessee River
	Noturus phaeus	Brown Madtom	Indian Creek, Wolf River; Piney Creek, Hatchie River
	Noturus stanauli	Pygmy Madtom	Clinch River, Clinch River
	Pylodictis olivaris	Flathead Catfish	Kentucky Lake, Tennessee River
Leuciscidae	*Campostoma anomalum*	Central Stoneroller	Goose Creek, Roanoke River

Campostoma oligolepis	Largescale Stoneroller	South Fork Holston River, Holston River
Clinostomus funduloides	Rosyside Dace	Hungry Mother Creek, Holston River
Coccotis coccogenis	Warpaint Shiner	Big Moccasin Creek, Holston River
Cyprinella galactura	Whitetail Shiner	New River, New River
Cyprinella spiloptera	Spotfin Shiner	New River, New River
*Cyprinella whipplei**	Steelcolor Shiner	
Erimystax dissimilis	Streamline Chub	Clinch River, Clinch River
Erimystax insignis	Blotched Chub	Clinch River, Clinch River
Exoglossum maxillingua	Cutlips Minnow	Pigg River, Roanoke River
*Hybognathus nuchalis**	Mississippi Silvery Minnow	
Hybopsis amblops	Bigeye Chub	Clinch River, Clinch River
*Hybopsis amnis**	Pallid Shiner	
Hydrophlox rubricroceus	Saffron Shiner	Timbertree Branch, Holston River
Luxilus chrysocephalus	Striped Shiner	Timbertree Branch, Clinch River
Lythrurus ardens	Rosefin Shiner	Pigg River, Roanoke River
*Lythrurus fasciolaris**	Scarlet Shiner	
Lythrurus lirus	Mountain Shiner	Big Moccasin Creek, Holston River
*Miniellus boops**	Bigeye Shiner	
*Miniellus stramineus**	Sand Shiner	
Nocomis leptocephalus	Bluehead Chub	Pigg River, Roanoke River
Nocomis micropogon	River Chub	Clinch River, Clinch River
*Notropis ammophilus**	Orangefin Shiner	
*Notropis amoenus**	Comely Shiner	
Notropis micropteryx	Highland Shiner	Clinch River, Clinch River
Notropis photogenis	Silver Shiner	Clinch River, Clinch River
Notropis rubellus	Rosyface Shiner	New River, New River
Notropis telescopus	Telescope Shiner	Wolf Creek, New River
Paranotropis leuciodus	Tennessee Shiner	Clinch River, Clinch River
Paranotropis sp. cf. *spectrunculus*	Sawfin Shiner	Clinch River, Clinch River
Paranotropis volucellus	Mimic Shiner	Clinch River, Clinch River
Phenacobius uranops	Stargazing Minnow	Clinch River, Clinch River
Pimephales notatus	Bluntnose Minnow	Bluestone River, New River

	Pimephales promelas	Fathead Minnow	Hungry Mother Creek tributary, Holston River
	Rhinichthys atratulus	Eastern Blacknose Dace	Hungry Mother Creek tributary, Holston River
	Rhinichthys cataractae	Longnose Dace	Little River, New River
	Semotilus atromaculatus	Creek Chub	Mud Fork, New River
Percidae	*Allohistium cinereum*	Ashy Darter	Clinch River, Clinch River
	Etheostoma blennioides	Greenside Darter	Clinch River, Clinch River
	Etheostoma caeruleum	Rainbow Darter	Kimberling Creek, New River
	Etheostoma flabellare	Fantail Darter	Wrights Valley Creek, Clinch River
	*Etheostoma jessiae**	Blueside Darter	
	Etheostoma meadiae	Bluespar Darter	Clinch River, Clinch River
	Etheostoma simoterum	Snubnose Darter	Wrights Valley Creek, Clinch River
	Etheostoma zonale	Banded Darter	Clinch River, Clinch River
	Nothonotus camurus	Bluebreast Darter	Clinch River, Clinch River
	Nothonotus denoncourti	Golden Darter	Clinch River, Clinch River
	Nothonotus rufilineatus	Redline Darter	Clinch River, Clinch River
	Perca flavescens	Yellow Perce	Rock Island Creek, James River
	Percina aurantiaca	Tangerine Darter	Clinch River, Clinch River
	Percina burtoni	Blotchside Logperch	Clinch River, Clinch River
	Percina caprodes	Common Logperch	South Fork Holston River, S. Fork Holston River
	Percina evides	Gilt Darter	Clinch River, Clinch River
	*Percina sciera**	Dusky Darter	
	*Percina vigil**	Saddleback Darter	
Poeciliidae	*Gambusia affinis**	Western Mosquitofish	

Table 2: Results of cytochrome oxidase b environmental DNA (eDNA) surveillance for Chucky Madtom (*Noturus crypticus*) in Little Chucky Creek and Dunn Creek, Tennessee. Shown are detection results for each of the three sampling events that occurred during 2023 and 2024. Fractions represent the number of independent replicates that yielded amplification. Some samples have not been analyzed (NA) at the present time. Hyphens denote sites where samples were not collected. Note that the Site_ID label corresponds to Figure 1 and Table 3. LCCK – Little Chucky Creek; DNCK – Dunn Creek, DCCF – Dunn Creek confluence; YBCK – Yellow Breeches Creek; EVT = event.

	Little Chucky Creek			**Dunn Creek**
Site_ID	Y23 – EVT I (Sept. 2023)	Y24 – EVT I (May 2024)	Y24 -EVT II (June 2024)	Y24 – EVT II (June 2024)
DCCF	-	-	-	**1/3**
DNCK	-	-	-	**2/3**
YBCK	-	-	-	0/3
LCCK 1	0/3	NA	0/3	-
LCCK 2	0/3	NA	0/3	-
LCCK 3	0/3	NA	0/3	-
LCCK 4	0/3	NA	0/3	-
LCCK 5	0/3	NA	0/3	-
LCCK 6	0/3	NA	0/3	-
LCCK 7	0/3	NA	0/3	-
LCCK 8	-	NA	0/3	-
LCCK 9	-	NA	0/3	-
LCCK 10	-	NA	**2/3**	-
LCCK 11	0/3	NA	**2/3**	-
LCCK 12	-	-	-	-
LCCK 13	-	NA	**2/3**	-
LCCK 14	-	NA	0/3	-
LCCK 15	-	NA	0/3	-
LCCK 16	-	-	0/3	-
LCCK 17	0/3	NA	0/3	-
LCCK 18	-	NA	0/3	-
LCCK 19	0/3	NA	0/3	-
LCCK 20	-	NA	0/3	-
LCCK 21	-	NA	0/3	-
LCCK 22	-	NA	0/3	-
LCCK 23	-	NA	0/3	-
LCCK 24	-	NA	0/3	-
LCCK 25	-	NA	0/3	-
LCCK 26	0/3	NA	0/3	-

Table 3: Sanger sequencing results from all quantitative polymerase chain reaction (qPCR) reactions that yielded positive amplification of Chucky Madtom (*Noturus crypticus*) environmental DNA (eDNA) with the NC121_CYTB assay. Sequence identification was determined using the Basic Local Alignment Search Tool (BLAST) to compare to reference sequences from the genetic repository, Genbank. Note that all sequenced reactions are from samples collected in Y24 – EVENT II (June 2024). Sample IDs correspond to locations in Figure 1 and labels in Table 2 (LCCK – Little Chucky Creek; DNCK – Dunn Creek, DCCF – Dunn Creek confluence). Letters (A,B,C) at the end of Sample_IDs denote the specific independent replicate that yielded positive amplification. Some independent replicates (e.g., DNCK_C, DCCF_C) had multiple qPCR replicates that yielded positive amplification.

Sample_ID	**Location**	**Percent (%) Identity Match**
LCCK 10_B	Little Chucky Creek	99
LCCK 10_C	Little Chucky Creek	99
LCCK 11_B	Little Chucky Creek	100
LCCK 11_C	Little Chucky Creek	100
LCCK 13_A	Little Chucky Creek	100
LCCK 13_C	Little Chucky Creek	99
DCCF_C	Dunn Creek	99
DCCF_C	Dunn Creek	100
DNCK_B	Dunn Creek	99
DNCK_C	Dunn Creek	97
DNCK_C	Dunn Creek	No Sequence

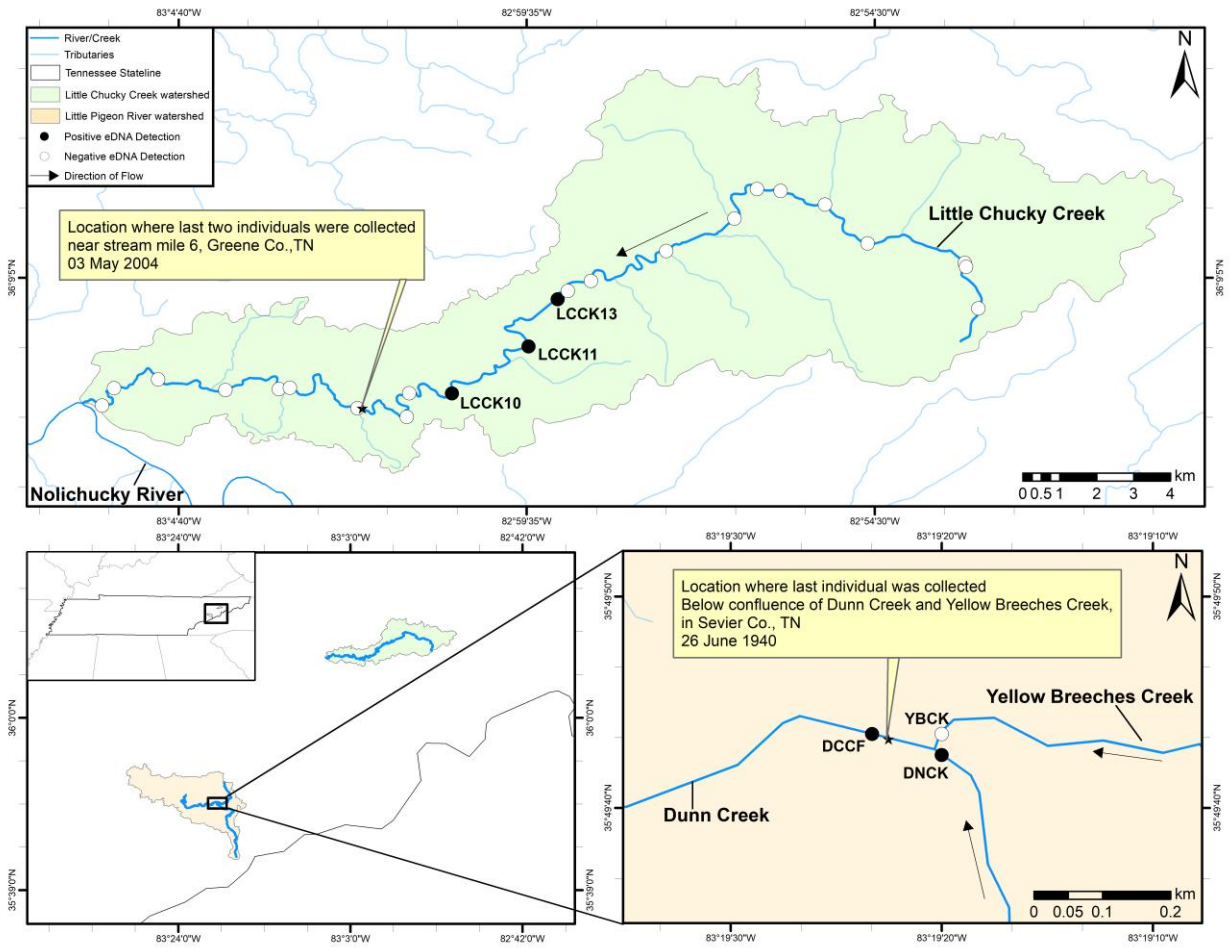

Figure 1. Overview of the sampling area for Chucky Madtom (*Noturus crypticus*) environmental DNA (eDNA) surveillance. (Bottom Left) State of Tennessee, United States with highlighted watersheds where sampling was conducted. (Top) Little Chucky Creek (LCCK) in its watershed, highlighted in green. (Bottom Right) Confluence (DCCF) of Dunn Creek (DNCK) and Yellow Breeches Creek (YBCK) in the Little Pigeon River watershed, highlighted in tan. Black stars denote locations where the last individual(s) were captured in both creeks. Map legend symbology is applicable to all three maps. Base map layers are from USA_States_Generalized (DHHS_Protect_Public) and USA Detailed Streams (esri_dm).