Surveillance of a federally protected freshwater fish using loop-mediated isothermal amplification (LAMP) and eDNA

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Abstract

Environmental DNA (eDNA) has increasingly been used in the surveillance of imperiled aquatic species. Despite recent efforts in drawing genetic material from the environment, there are still pitfalls surrounding this field. We created a novel protocol which implements loop-mediated isothermal amplification (LAMP) to detect target DNA. Our methods are applied here in the surveillance of *Etheostoma trisella*, the Trispot Darter, a freshwater fish that recently received protection under the U.S. Endangered Species Act. Water samples (n = 256) were collected at sites in Alabama and Georgia to determine whether *E. trisella* still occupies historic sites and whether it inhabits previously unknown areas. We found evidence of *E. trisella* presence in 69 water samples while 187 were negative. Our LAMP protocol is capable of amplifying low quantities of DNA in the water, and is a robust technique for freshwater species surveillance. Verification of positive results from eDNA experiments is essential to confirm reaction reliability. Application of methods such as ours are necessary for recognizing species under threat that require conservation.

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Running Head: Surveillance of protected fish

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ABSTRACT

Environmental DNA (eDNA) has increasingly been used in the surveillance of imperiled aquatic species. Despite recent efforts in drawing genetic material from the environment, there are still pitfalls surrounding this field. We created a novel protocol which implements loop-mediated isothermal amplification (LAMP) to detect target DNA. Our methods are applied here in the surveillance of *Etheostoma trisella* , the Trispot Darter, a freshwater fish that recently received protection under the U.S. Endangered Species Act. Water

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Keywords

Etheostoma trisella

environmental DNA

loop-mediated isothermal amplification

freshwater

1 INTRODUCTION

The conservation of imperiled species requires an understanding of current population stability and distribution. However, the surveillance of aquatic species via the employment of traditional methods such as netting and electrofishing are labor intensive, expensive, and disruptive to the species of conservation concern (Jerde et al., 2011). Many species that require monitoring are rare, and, in fact, the probability of detecting aquatic taxa using traditional methods is low when species density falls below a specific threshold (Dejean et al., 2012). Furthermore, due to permitting restrictions, it is difficult to rapidly assess populations of species protected by law. Some studies have found that sampling methods such as electrofishing do not accurately determine the abundance of targeted taxa despite their invasive nature (Wilcox et al., 2016). Several eDNA studies report that published methods did not capture the true diversity or abundance of taxa known from traditional sampling (Cilleros et al., 2019; Drinkwater et al., 2019). As such, a noninvasive, supplemental method is required in order to monitor and provide protection to as many imperiled species as possible.

Environmental DNA (eDNA) is the genetic material found in environmental samples such as water, sediment, and air. Representing a conglomeration of genetic material sourced to different organisms, eDNA molecules originate from extracellular DNA (i.e., natural cell death followed by cell lysis), cellular DNA (e.g., epithelial cells, mucus, and feces) or microbial organisms (Díaz-Ferguson & Moyer, 2014; Nagler et al., 2018; Pietramellara et al., 2009; Taberlet et al., 2012). DNA from environmental sources was first utilized in the 1980's for the extraction of microbial DNA from sediment and later for bacterial biomass assessment (Ogram et al., 1987; Paul et al., 1996). Environmental DNA is now used in aquatic systems to detect invasive species, monitor populations of threatened taxa, and trace fecal contaminants (Díaz-Ferguson & Moyer, 2014; Goldberg et al., 2011; Jerde et al., 2011; Layton et al., 2006).

The detection of target eDNA using traditional polymerase chain reaction (PCR) and quantitative PCR (qPCR) at natural sites has shown limited applicability in both freshwater and marine habitats (Foote et al., 2012; Grey et al., 2018). In an effort to increase the utility of eDNA, we have developed an eDNA detection method that applies loop-mediated isothermal amplification (LAMP). Previous authors have prospected the utility of LAMP for animal eDNA detection, but this study is the first to demonstrate feasibility for freshwater fishes (Lee, 2017; Rees et al., 2014). LAMP amplifies target sequences with high specificity by initially binding four different primers to six complementary regions of the target DNA; annealing of two primers to four target regions maintains specificity as amplification progresses (Notomi et al., 2000). This method uses a *Bst* DNA polymerase under isothermal conditions to produce multiple stem-loop DNA structures (Notomi et al., 2000). Furthermore, LAMP has the capability of amplifying small quantities of target DNA with detection possible with as few as six copies in solution during experimentation (Notomi et al., 2000).

While the use of genetic material isolated from nonliving sources for the detection of targeted taxa is not novel, our custom molecular methods are. We present here the application of our new method to the surveillance of *Etheostoma trisella* (Percidae) populations.*E. trisella* , the Trispot Darter, is a small, freshwater fish endemic

to the Coosa River system of northwestern Georgia, southeastern Tennessee, and northeastern Alabama (Page & Burr, 2011). The currently understood range in Georgia and Tennessee includes the Conasauga River system upstream of the confluence with the Coosawattee River including Mill Creek and below Carter's Lake, and the Oostanaula River between Arumuchee Creek and Calhoun (Boschung & Mayden, 2004; *Rare Species Status Maps* , 2020). In Alabama, *E. trisella* has recently been recovered in Little Canoe Creek and Ballplay Creek (*FishMap.Org* , 2018; O'Neil et al., 2009). *Etheostoma trisella* occupies a specialized niche and migrates between two seasonally interconnected habitats (Ryon, 1986). During the non-breeding season (mid-April to mid-October), *E. trisella* populates peripheral zones characterized by the presence of gravel, vegetation, and a slow moving current. Seasonal rainfall allows the darter to migrate upstream to the breeding grounds where they spawn from January to March. This habitat consists of streams in seepage waters of pastures and floodplains containing high amounts of vegetation. Given that*E. trisella* has a severely limited distribution and is highly impacted by anthropogenic activity, the darter was listed as "Threatened" under the Endangered Species Act (ESA) in 2019 (*Endangered and Threatened Wildlife and Plants; Threatened Species Status for Trispot Darter* , 2018). Therefore, surveillance of *E. trisella* populations is essential in understanding how to best implement conservation efforts.

2 MATERIALS AND METHODS

2.1 Sample Collection

We collected environmental water samples (n = 256) during 2019 (January-March) at 136 sites encompassing 17 watersheds in Georgia and Alabama, USA (Figure 1). Collection sites were chosen to collectively satisfy the following criteria: 1) localities of documented *E. trisella* captures, 2) localities proximal to documented *E. trisella* captures, and 3) localities where *E. trisella* have not been documented. For each collection, two sealed 0.5L plastic water bottles were emptied of the manufacturer's contents and filled with environmental water. Negative field controls were implemented as suggested by Goldberg et al. (2016).

2.2 eDNA Extraction

We captured eDNA from environmental water samples by filtering through Whatman Microfiber Filters (GE Healthcare, Chicago, IL, USA) housed in Nalgene Analytical Test Filter Funnels (Thermo Fisher Scientific, Waltham, MA, USA) using the Geopump Peristaltic Pump (Geotech Environmental Equipment, Denver, CO, USA). Duplicate water samples were combined during filtration. Filters containing residual particles were cut into halves; one half was stored in 70% ethanol at 4 °C and the other half used immediately in DNA extraction. DNA extraction was performed using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and the QIAshredder (Qiagen, Hilden, Germany). DNA quality and quantity were determined using standard gel electrophoresis on a 1.5% agarose gel and a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Purified eDNA was stored at 4 °C. Filtration of water and extraction of eDNA were performed in a lab environment cleaned with a 10% bleach solution.

2.3 eDNA Detection

We detected *E. trisella* presence by implementing a custom LAMP protocol. LAMP primers were designed in PrimerExplorer (v. 5) within the mitochondrial genome with specificity for the *E. trisella*cytochrome *b* (cyt *b*) gene (Table 1) and optimized in a temperature titration (*LAMP Primer Designing Software:PrimerExplorer* , 2015; Sandel et al., 2020). Primer specificity was verified using Primer-BLAST; our external primers alone yielded*E. trisella* as the only expected target (Ye et al., 2012). LAMP was performed with the following specifications suggested by the manufacturer: 12.5µL LavaLAMP DNA Master Mix (2X; Lucigen, Middleton, WI, USA), 2.5µL target-specific primer assay (Table 1), 1.0µL green fluorescent dye (Lucigen, Middleton, WI, USA), bovine serum albumin (0.4 mg/mL; Thermo Fisher Scientific, Waltham, MA, USA), 7.5µL Invitrogen UltraPure DNase/RNase-Free Distilled Water (Thermo Fisher Scientific, Waltham, MA, USA), and 1.0µL template DNA for a total reaction volume of 25.0µL. Reactions were performed on a Stratagene Mx3000P (Agilent Technologies, Santa Clara, CA, USA) under the following conditions: 4 min 90°C preheat, 30 min 74°C temperature hold, standard dissociation curve, and 5 min 95°C enzyme deactivation. All reactions were performed in triplicate including positive and negative controls. To ensure that our primers amplify different

genetic populations of *E. trisella* , we performed our LAMP protocol on a subset of fish representing three genetically distinct populations (unpublished data). Although our primers were designed to target *E. trisella* only, we determined that two species of darter could potentially be cross-amplified. To account for this possibility, we screened DNA samples from *E. jordani* and *E. rupestre* , genetically similar species of darter that may be detectable in our water samples.

Results were visualized using standard gel electrophoresis on a 1.5% agarose gel, amplification plots and dissociation curves on the Stratagene Mx3000P, and quantitative electrophoresis on a QIAxcel Advanced System (Qiagen, Hilden, Germany). We performed quantitative electrophoresis using the QIAxcel DNA High Resolution Kit (Qiagen, Hilden, Germany) combined with a 15bp/600bp QX Alignment Marker (Qiagen, Hilden, Germany) and 25-500bp QX DNA Size Marker (5ng/µL) under the manufacturer's suggested 0H800 method. A positive status was assigned to experimental samples if they produced DNA segments comparable in size to at least three of the five segments characterizing the positive control. In addition, samples were only deemed positive upon reaching a positive result in a second replicate (Mahon et al., 2013). A subset of positive products was purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced with a SimpleSeq Kit (Eurofins Genomics, Louisville, KY, USA) to verify template amplification. We used NCBI BLAST to determine the origin of each DNA fragment (Altschul et al., 1990). Statistical analyses were performed using the R Programming Language including Fisher's Exact Tests and covariance to determine which factors may influence detection, if any (R Core Team, 2018).

3 RESULTS

After screening environmental water samples, 187 samples were found to be negative for *E. trisella* presence and 69 positive (Figure 1). At sites in Alabama, 24.5% of water samples screened positive (n = 151) while 30.5% of samples in Georgia were positive (n = 105). Water samples from different states were obtained by different groups of collectors, and we show that detection of *E. trisella* was not influenced by collector ($p = 0.3911$). However, the date of water collection did influence detection ($p < 0.05$). *Post hoc*pairwise analyses indicate that detections were lowest during the beginning of our collection period (January) and increased in February through March ($p < 0.05$). The sampling scheme (i.e., north/south or east/west) did not confound this temporal trend (Cov = -0.11921).

Our primers amplified all genetic lineages of *E. trisella* tested. With several mismatched sites in our primers, amplification of DNA belonging to other *Etheostoma* species was not a source of false positives as neither *E. jordani* nor *E. rupestre* amplified using our *E. trisella* primer set. Sequence data confirmed amplification of *E. trisella* DNA in a positive control and two environmental water samples (Table 2). The positive control DNA sample collected from *E. trisella* tissue matched across its entirety to an *E. trisella* voucher specimen. The two eDNA fragments matched*E. trisella* DNA across > 95.0% of nucleotides with over 98.0% sequence coverage. One environmental sample however, did not match to *E. trisella* and the source of the eDNA molecule(s) sequenced could not be resolved. The sequence showed 91.49% nucleotide identity to the bacterium *Rahnella aquatilis* , but at only 16.0% coverage. In thirteen instances, environmental negative controls screened positive. Attempts to extract positive bands from an agarose gel for sequencing were inconclusive and did not provide any usable data. The number of environmental negative controls that screened positive did not show an association with collector ($p = 0.3834$).

4 DISCUSSION

Using noninvasive methods for the surveillance of imperiled species is an attractive alternative to traditional methods, and we have presented our contribution to this innovation. We screened 256 water samples from Alabama and Georgia for *E. trisella* eDNA and were able to detect their presence using our LAMP protocol. A comparable study was completed that assessed *E. trisella* presence using eDNA and PCR in some of the same Alabama streams which showed fewer positive detections (1.3%) than we reported here (24.5%) (Johnston & Janosik, 2019).

In verifying that our field and laboratory protocols were sufficient to detect *E. trisella* eDNA, we tested several positive controls known to contain *E. trisella* DNA. Because our primer design was broad enough to

amplify DNA in positive control fish from different genetic populations, we infer that our methods should be capable of detecting fish from any of our localities. Including the proportion of negative environmental controls, we were also able to rule out bias in detection from the investigator ($p = 0.3834$). From these data we conclude that all samples were handled in a similar fashion through the point of collection, transport, and processing.

The protocol that we designed includes several checks to determine if false positives are present. Our pipeline required negative and positive controls as well as cleaning lab equipment with bleach solution as described above. We also sequenced positive samples to verify the DNA source. We detected *E. trisella* presence in 34.2% of the environmental negative controls implemented ($n = 38$). We were unable to isolate and sequence DNA fragments from these samples, but hypothesize that amplification in negative controls may result from contamination or amplification of eDNA from other sources. The incidence of false positives in LAMP studies has been previously documented in disease prevalence studies including that of malaria and thalassemia (Kollenda et al., 2018; Wang et al., 2020). The addition of quenched fluorescent primers to LAMP has been suggested to reduce the number of false positives(Hardinge & Murray, 2019). Our reusable, bleached filter funnels are a potential source of contamination, and closed filters are an alternative that may reduce risks (Spens et al., 2017). Although our primers were designed with specificity for *E. trisella* , the LAMP protocol may cross-amplify DNA of other unintended targets. We determined that our primers matched *E. trisella* along the entirety of the cyt *b* gene and mismatched *E. jordani* and*E. rupestre* at multiple nucleotides (18 and 26 sites respectively). We have shown that our primers show high specificity for the intended target, and are not a likely source of false positives. We postulate that environmental negative control water samples taken at Shoal Creek on the Cahaba River screened positive due to contamination or cross-amplification of a target not yet documented in genetic databases. Inspection of the eDNA sequence that matched poorly to*Rahnella aquatilis* showed no sites that were likely for binding of our primers. We consider this sequence unresolved and not explicitly identified as *Rahnella aquatilis* .

We stress that caution should be used when interpreting negative results. Thus, the lack of detection at sites does not necessarily indicate that *E. trisella* are not present at those localities, but that our methods did not detect their presence. When utilizing eDNA, false negatives may arise because template DNA is not amplified using molecular methods despite presence of the target organism (Darling & Mahon, 2011). Failure to amplify template DNA may occur because of DNA degradation, low quality or quantity DNA, low primer specificity, PCR inhibitors, and failed reactions (Klymus et al., 2015; Nathan et al., 2015). In addition, replicates are required to accurately determine diversity and detect rare species (Fonseca, 2018).

We report that detection probabilities varied significantly with the date of collection, with the number of detections increasing at the end of our collection period (i.e., expected end of spawning period). The life history of *E. trisella* may play a particularly strong role in why detections varied over time. *E. trisella* move between main and spawning channels. Although we did sample from potential spawning grounds throughout the expected spawning season, it is possible that our collections did not coincide with the fish's movement. Perhaps, *E. trisella* were not yet present at sites sampled early in the spawning season. Furthermore, DNA molecules may not be distributed homogenously in water bodies due to the number of organisms upstream, river flow rate, and the degree of water column mixing (Jerde et al., 2011). Duplicate samples from the same localities over time would be useful in clarifying this postulation. We plan to collect additional water samples as well as conduct confirmatory surveys to capture live *E. trisella* specimens coinciding with sites described here.

We have presented here our novel approach to noninvasive surveillance of freshwater species. Our protocol has provided evidence that loop-mediated isothermal amplification is a robust method for detecting low quantities of eDNA in the water and can be modified for application in other systems. We acknowledge that results of eDNA surveillance efforts should be interpreted with caution and require subsequent sequencing to confirm detection of the intended target.

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DATA ACCESSIBILITY STATEMENT

All sequences are available on Genbank under the following accession numbers: MT490609, MT490610, and MT490611.

AUTHOR CONTRIBUTIONS

The research project was designed by MWS with contributions from authors; water samples were collected by AP; laboratory work and analyses were designed and conducted by KMF and MWS; and the paper was written by KMF with contributions from all of the authors.

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