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Detection of an Enigmatic Plethodontid Salamander Using Environmental DNA

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The isolation and identification of environmental DNA (eDNA) offers a non-invasive and efficient method for the detection of rare and secretive aquatic wildlife, and it is being widely integrated into inventory and monitoring efforts. The Patch-Nosed Salamander (*Urspelerpes brucei*) is a tiny, recently discovered species of plethodontid salamander known only from headwater streams in a small region of Georgia and South Carolina. Here, we present results of a quantitative PCR-based eDNA assay capable of detecting *Urspelerpes* in more than 75% of 33 samples from five confirmed streams. We deployed the method at 31 additional streams and located three previously undocumented populations of *Urspelerpes*. We compare the results of our eDNA assay with our attempt to use aquatic leaf litterbags for the rapid detection of *Urspelerpes* and demonstrate the relative efficacy of the eDNA assay. We suggest that eDNA offers great potential for use in detecting other aquatic and semi-aquatic plethodontid salamanders.

THE indirect detection of vertebrates through the isolation and identification of DNA shed into the environment (i.e., environmental DNA or eDNA) is a promising new technique that is being rapidly integrated into wildlife inventory and monitoring programs (Bohmann et al., 2014). For example, eDNA detection methods have been used for the detection of invasive species (Ficetola et al., 2008; Jerde et al., 2011) and species of conservation concern (Olson et al., 2012; Spear et al., 2015). In particular, eDNA assays hold great potential for the detection of organisms that are difficult to locate with traditional methods, such as those with secretive behavior.

The utility of environmental DNA assays has now been demonstrated in a variety of taxa, including amphibians (Ficetola et al., 2008; Dejean et al., 2011; Goldberg et al., 2011; Thomsen et al., 2011; Olson et al., 2012; Pilliod et al., 2013, 2014; Spear et al., 2015). Most studies using eDNA to detect amphibians have been conducted in lentic systems, but a smaller subset (Goldberg et al., 2011; Olson et al., 2012; Pilliod et al., 2013; Spear et al., 2015) have taken place in lotic systems, where the dynamics of flowing water can cause important differences in the distribution and persistence of eDNA (Pilliod et al., 2014).

The Patch-Nosed Salamander (*Urspelerpes brucei*; hereafter referred to only as *Urspelerpes*) is a recently discovered species of plethodontid salamander (Camp et al., 2009). *Urspelerpes* is always found in association with small, first- or second-order headwater streams; larvae are aquatic, and adults are associated with cover and leaf litter on the margins of the streambed. *Urspelerpes* reaches a maximum size of approximately 26 mm snout–vent length (SVL). At the beginning of our study, *Urspelerpes* was known from just ten headwater streams in an approximately 7 km^2 region of northeastern Georgia and western South Carolina (Camp et al., 2012). Due to the extremely limited extent of its known distribution, *Urspelerpes* is of high conservation concern, and a better understanding of its full distribution remains a critical need.

Here we present the results of two attempts to design and implement a standardized method to locate previously undocumented populations of *Urspelerpes*. First, we used aquatic leaf litterbags to sample *Urspelerpes* and estimate detection probability at three streams of confirmed presence of *Urspelerpes*. Second, we designed a species-specific qPCR-based assay to amplify and detect eDNA from *Urspelerpes*. We tested the eDNA assay both *in silico* and against DNA extracted from *Urspelerpes* and 16 other potentially sympatric plethodontid salamanders to ensure specificity, then estimated detection probability by sampling at five streams of confirmed presence of *Urspelerpes*. We then deployed this assay at streams with potential, but unconfirmed, *Urspelerpes* in an attempt to locate previously undocumented populations.

MATERIALS AND METHODS

Leaf litterbag surveys.—We initially attempted to develop a standardized method for the detection of *Urspelerpes* using leaf litterbags (Pauley and Little, 1998). We constructed leaf litterbags measuring approximately 25 cm × 40 cm from 1 cm^2 plastic mesh. We placed 25 leaf litterbags at 3 m intervals in each of three streams of confirmed presence of *Urspelerpes* (Stream 1, Stream 2, and Stream 3) and checked the litterbags for salamanders after 24 and 48 hours. We conducted these surveys once per month from April to October 2010, for a total of 14 visits to each stream.

Quantitative PCR assay design.—We used Biosearch Technologies's online software (https://www.biosearchtech.com/bhqprobes; Biosearch Technologies, Inc.) and published mitochondrial sequence data (cytochrome *b*; GenBank FJ917634) of *Urspelerpes* to design primers and a fluorescent

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Table 1. Species-specific primers and probe used for the qPCR assay.

Oligo	Name	Sequence
Forward primer	Urspelerpes_Cytb_1_F	5′–CGATACCGCCTCAGCCTTT–3′
Reverse primer	Urspelerpes_Cytb_1_R	5′–CTCCGTTAGCGTGGGTGTT–3′
Probe	Urspelerpes_Cytb_1_Pr	5′–FAM-TTCAGTAGCCCACATCTGTCGTGA-BHQ-1–3′

probe for the amplification of *Urspelerpes* DNA (Table 1). To examine assay specificity *in silico*, we used Primer-BLAST (http://ncbi.nlm.nih.gov/tools/primer-blast/) to look for sequence similarities in non-target taxa. BLAST results showed no sequences with <6 total mismatches to the forward and reverse primers. The closest non-target match to our full assay (including the primer pair and probe) was from *Gyrinophilus porphyriticus* (GenBank EU336389.1), which had nine mismatches (85.5% similarity). We tested assay specificity by creating serial dilutions of DNA extracted from tissues of target (*Urspelerpes brucei*) and non-target species (*Desmognathus aeneus, D. conanti, D. folkertsi, D. marmoratus, D. monticola, D. ocoee, D. quadramaculatus, Eurycea aquatica, E. cirrigera, E. guttolineata, E. quadridigitata, E. wilderae, Gyrinophilus porphyriticus, Hemidactylium scutatum, Pseudotriton montanus,* and *P. ruber*).

Sample collection.—In March 2013 and September 2013, we collected water samples from five streams of confirmed presence of *Urspelerpes* (hereafter called "confirmed streams") and again from one of those streams (Stream 5) in April 2014. Between March 2013 and April 2014, we collected water samples from 31 headwater streams in northeastern Georgia and northwestern South Carolina where *Urspelerpes* had not previously been documented (hereafter called "unconfirmed streams"). Due to the limited distribution of *Urspelerpes* and its potential sensitivity to disturbance, we follow Camp et al. (2009) and omit any additional information about the location of these streams.

At each stream, we collected three 1 L water samples and one 1 L field negative control (store-bought distilled water), similar to the protocol developed by Pilliod et al. (2013). Samples were collected in sterile, single-use plastic water bottles and stored in a cooler until filtration. All samples were filtered within 12 hours of collection with either a hand vacuum pump or an electrical vacuum pump through 0.45 μM cellulose nitrate filter paper (Whatman International, LTD or Thermo Fisher Scientific). Filters were stored in 95% ethanol at –20°C until DNA extraction.

DNA extraction and purification.—For tests of assay specificity, DNA was extracted from tail-tips using a Qiagen DNeasy Tissue and Blood Kit (Qiagen, Inc.). For eDNA samples, we extracted DNA from one-half of each filter using a Qiagen DNeasy Tissue and Blood Kit (Qiagen, Inc.) with the additional use of a QIAshredder Kit (Qiagen, Inc.) after the lysis step (Goldberg et al., 2011). If qPCR reactions of eDNA samples from confirmed streams appeared inhibited, we treated the extracted DNA with a OneStep PCR Inhibitor Removal Kit (IRK; Zymo Research) to remove potential PCR inhibitors and re-ran the samples. All eDNA samples from streams that had not previously been surveyed were preemptively treated with the IRK. All eDNA extractions were carried out in a "clean room" dedicated to low-copy DNA extractions and PCR/qPCR preparations at the University of Georgia's Department of Environmental Health Science.

Quantitative PCR.—All eDNA samples, DNA samples, and negative controls were run in triplicate, with standards (0.057, 0.0057, and 0.00057 ng/μL) created from a serial dilution of DNA extracted from tissue of *Urspelerpes* (original concentration = 57 ng/μL) run in triplicate with every plate. All qPCR reactions included the addition of an internal positive control (IPC) to assess potential PCR inhibition or assay failure and a no template control to assess potential contamination.

Quantitative PCR reactions (15 μL) were set up using 3.75 μL sample eDNA/DNA and 11.25 μL of a master mix containing the following per-reaction components: 7.5 μL Quantitect Multiplex PCR Mix (Qiagen, Inc.), 0.06 μL each 100 μM primer (0.4 μM final concentration), 0.03 μL 100 μM probe (0.2 μM final concentration), 1.6 μL TaqMan Exogenous Internal Positive Control 10X Exo IPC Mix (Applied Biosystems), 0.33 μL of TaqMan Exogenous Internal Positive Control 50X Exo IPC DNA (Applied Biosystems), 1.67 μL RNase-free water. All qPCR preparations were carried out in the aforementioned "clean room." Quantitative PCR reactions were run in 96-well optical qPCR plates on an Applied Biosystems StepOnePlus™ (Life Technologies Corp.) using the follow thermocycling protocol: 95°C for 15 minutes, 50 cycles of 95°C for 60 seconds and 60°C for 60 seconds.

To test qPCR assay specificity, DNA extracted from tissues of each of 16 sympatric or potentially sympatric plethodontid salamander species were pooled by species, diluted to 0.001X from stock concentrations ranging from 20 to 150 ng/μL, and run in triplicate alongside the standards created from a serial dilution of DNA extracted from tissue of *Urspelerpes*. The 0.001X dilution was chosen to approximate the highest typical concentrations found in extracted eDNA (Spear et al., 2015).

Data analysis.—We used the StepOne™ software v 2.3 (Life Technologies) to set a manual amplification threshold near the beginning of exponential amplification in the no template control. We considered wells showing exponential amplification of target DNA surpassing this threshold to be positive. We considered wells showing no exponential amplification or delayed amplification ($C_t \geq$ three more than the C_t of the no template control) to be inhibited. We considered *Urspelerpes* eDNA to be present in a sample if at least one (1/3) qPCR replicate tested positive, and we considered *Urspelerpes* to be present in a stream if at least one (1/3) sample tested positive. However, we considered the potential outcome of just one positive qPCR replicate in a single sample to be weak evidence of presence. Multiple positive qPCR replicates and/or multiple positive samples from a stream, in the absence of amplification in the negative control, provide the strongest evidence of presence.

Schmidt et al. (2013) divided the per-sample probability of detecting a target species with an eDNA assay into two components: availability (i.e., the probability of capturing eDNA in a sample from a site of confirmed presence) and detection probability (i.e., the probability of detecting eDNA with a PCR assay from a sample that contains eDNA of the

Table 2. Results from confirmed streams, showing all three sampling periods. The Detection column indicates whether *Urspelerpes* eDNA was detected in at least one sample. The + Samples column indicates shows how many water samples showed amplification in at least one qPCR reaction. The + qPCRs column indicates how many qPCR replicates showed amplification of *Urspelerpes* eDNA.

Stream	Known?	Detection	March 2013 + Samples	March 2013 + qPCRs	September 2013 + Samples	September 2013 + qPCRs	April 2014 + Samples	April 2014 + qPCRs
1	+	+	3/3	4/9	2/3	4/9	–	–
2	+	+	2/3	3/9	3/3	8/9	–	–
3	+	+	2/3	4/9	2/3	3/9	–	–
4	+	+	2/3	5/9	2/3	4/9	–	–
5	+	+	2/3	4/9	3/3	7/9	3/3	6/9

target species). We acknowledge the presence of imperfect detection in both components of our assay; however, for the sake of this study, we were interested solely in the overall per-sample detection probability (p_{eDNA}), which we estimated directly as the proportion of samples from five confirmed streams in which *Urspelerpes* eDNA was detected, assuming detection probabilities to be equal among streams. To compare our eDNA assay with previous attempts to survey for *Urspelerpes* with leaf litterbags, we also estimated the overall per-visit detection probability for the latter method. We estimated overall per-visit detection probability (p_{leaf}) as the proportion of visits to a confirmed site (Streams 1–3) in which *Urspelerpes* was detected, where one visit consisted of checking all 25 leaf litterbags in a stream. For both methods, we calculated and plotted the cumulative probability of detection (p^*) after n samples or visits, respectively, using the function dbinom in R version 3.1.1 (R Core Team, 2014).

RESULTS

Leaf litterbag survey results.—We detected *Urspelerpes* on just two occasions in our leaf litterbag surveys: one individual found in Stream 1 in April 2010 and one individual found in Stream 2 in September 2010. Thus, we estimated the overall per-visit detection probability of the leaf litterbag assay to be 0.048 (i.e., two detections in 42 visits).

Assay specificity and efficacy.—DNA extracted from tissues of *Urspelerpes* showed consistent amplification, and no amplification was observed for any of the 15 non-target species tested. None of our negative controls or no template controls showed amplification at any point of the study.

We detected *Urspelerpes* eDNA in at least two samples during every visit to each confirmed stream, with amplification occurring in a total of 26/33 samples and 49/99 qPCR replicates (Table 2). Thus, we estimated the overall per-sample detection probability (p_{eDNA}) to be 0.788. We plotted the cumulative detection probabilities for both leaf litterbag surveys and eDNA surveys in Figure 1.

Environmental DNA sampling results.—We detected *Urspelerpes* eDNA in three new streams (Stream A, Stream B, and Stream H) out of 31 streams surveyed. At these three streams, we detected amplification in at least two samples and in a total of 8/9 samples and 12/27 qPCR replicates (Table 3). We have since revisited all of these three streams and have found larvae and/or adults of *Urspelerpes* in two of them (Streams B and H).

DISCUSSION

Overall, our results demonstrate the high specificity and efficacy of our qPCR-based eDNA assay for the detection of *Urspelerpes*. The lack of amplification of DNA extracted from other plethodontid salamanders shows that the assay is species-specific, and the lack of amplification in any field negative controls or no template controls suggests that contamination was not a significant factor in our study. Detection probabilities of *Urspelerpes* were much higher with eDNA than with leaf litterbag surveys (0.788/sample vs. 0.048/visit), with a 95% cumulative detection probability reached after just two eDNA samples (each with three qPCR replicates), compared to 62 visits using leaf litterbag surveys. Although leaf litterbags have been effective in detecting *Urspelerpes* when left to soak for a longer time (i.e., 1–2 months; Camp, pers. obs.), low detection probabilities without a high investment of time and labor deemed this method impractical for our purposes.

One particular benefit of our study system is the short length and discrete nature of the streams surveyed. In many larger lotic systems, determining the actual occupied area from a positive eDNA result is at least partially dependent upon the linear movement of eDNA through the system. Because the streams we sampled for *Urspelerpes* exist for only some hundreds of meters, our inference of occupancy is very geographically restricted. Future efforts should continue to focus on understanding temporal and spatial variability in detection probabilities with eDNA assays, especially in lotic

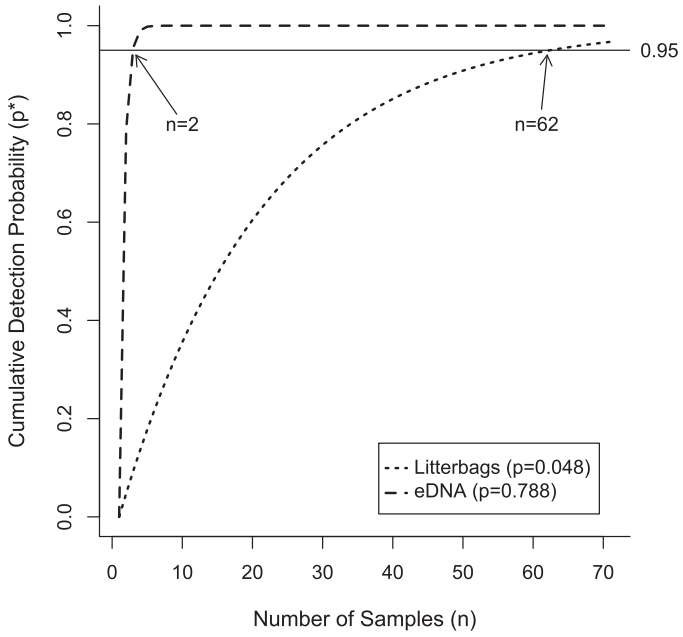

Fig. 1. Cumulative detection probabilities for both the leaf litterbag survey and eDNA assay. The 95% cumulative detection probability threshold is shown with a solid horizontal line, and arrows point to where each method reaches this threshold.

Table 3. Results from unconfirmed streams. The Detection column indicates whether *Urspelerpes* eDNA was detected in at least one sample. The + Samples column indicates shows how many water samples showed amplification in at least one qPCR reaction. The + qPCRs column indicates how many qPCR replicates showed amplification of *Urspelerpes* eDNA.

Stream	Known?	Detection	+ Samples	+ qPCRs
A	–	+	3/3	6/9
B	–	+	2/3	3/9
C	–	–	0/9	0/9
D	–	–	0/9	0/9
E	–	–	0/9	0/9
F	–	–	0/9	0/9
G	–	–	0/9	0/9
H	–	+	3/3	3/9
I	–	–	0/9	0/9
J	–	–	0/9	0/9
K	–	–	0/9	0/9
L	–	–	0/9	0/9
M	–	–	0/9	0/9
N	–	–	0/9	0/9
O	–	–	0/9	0/9
P	–	–	0/9	0/9
Q	–	–	0/9	0/9
R	–	–	0/9	0/9
S	–	–	0/9	0/9
T	–	–	0/9	0/9
U	–	–	0/9	0/9
V	–	–	0/9	0/9
W	–	–	0/9	0/9
X	–	–	0/9	0/9
Y	–	–	0/9	0/9
Z	–	–	0/9	0/9
AA	–	–	0/9	0/9
BB	–	–	0/9	0/9
CC	–	–	0/9	0/9
DD	–	–	0/9	0/9
EE	–	–	0/9	0/9

systems. For example, several studies (Pilliod et al., 2013, 2014; Jane et al., 2014; Moyer et al., 2014) have explicitly tested how physical, chemical, and biological stream processes affect eDNA detection probabilities in streams. Understanding how these factors influence detection is critical for the incorporation of eDNA assays into inventory and monitoring programs.

Quantitative PCR-based eDNA assays can be non-invasive and effective methods of detecting amphibians in aquatic systems. Leaf litterbag surveys are less invasive than other common survey methods (e.g., dipnetting surveys), but they still disturb aquatic habitats. However, the collection of eDNA requires very little disturbance to aquatic habitats, which is a particularly important consideration for inventory and monitoring efforts of rare and range-restricted species like *Urspelerpes*. Relative to the cost of labor necessary to check leaf litterbags a sufficient number of times to achieve a 95% detection probability, eDNA assays are also relatively inexpensive. For example, each sample in this study cost approximately $20, including $4.50 for collection and filtration, $4.50 for DNA extraction, and $11 for qPCR reagents, but excluding labor. Our results demonstrate the efficacy of a qPCR-based eDNA assay for the detection of *Urspelerpes* and suggest the potential utility of this technique for the detection of other aquatic and semi-aquatic plethodontid salamanders with similar life histories. Additionally, because plethodontid salamanders exhibit a wide variety of life and natural histories, they provide an interesting system for exploring the utility of eDNA assays as tools for ecological studies.

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LITERATURE CITED

Bohmann, K., A. Evans, M. T. P. Gilbert, G. R. Carvalho, S. Creer, M. Knapp, D. W. Yu, and M. de Bruyn. 2014. Environmental DNA for wildlife biology and biodiversity monitoring. Trends in Ecology & Evolution 29:358–367.

Camp, C. D., T. Lamb, and J. R. Milanovich. 2012. *Urspelerpes* Camp, Peterman, Milanovich, Lamb, Maerz, and Wake. Catalogue of American Amphibians and Reptiles 885:1–3.

Camp, C. D., W. E. Peterman, J. R. Milanovich, T. Lamb, J. C. Maerz, and D. B. Wake. 2009. A new genus and species of lungless salamander (family Plethodontidae) from the Appalachian highlands of the south-eastern United States. Journal of Zoology 279:86–94.

Dejean, T., A. Valentini, A. Duparc, S. Pellier-Cuit, F. Pompanon, P. Taberlet, and C. Miaud. 2011. Persistence of environmental DNA in freshwater ecosystems. PLOS ONE 6:e23398.

Ficetola, G. F., C. Miaud, F. Pompanon, and P. Taberlet. 2008. Species detection using environmental DNA from water samples. Biology Letters 4:423–425.

Goldberg, C. S., D. S. Pilliod, R. S. Arkle, and L. P. Waits. 2011. Molecular detection of vertebrates in stream water: a demonstration using Rocky Mountain tailed frogs and Idaho giant salamanders. PLOS ONE 6:e22746.

Jane, S. F., T. M. Wilcox, K. S. McKelvey, M. K. Young, M. K. Schwartz, W. H. Lowe, B. H. Letcher, and A. R. Whiteley. 2014. Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. Molecular Ecology Resources 15: 216–227.

Jerde, C. L., A. R. Mahon, W. L. Chadderton, and D. M. Lodge. 2011. "Sight-unseen" detection of rare aquatic species using environmental DNA. Conservation Letters 4: 150–157.

Moyer, G. R., E. Díaz-Ferguson, J. E. Hill, and C. Shea. 2014. Assessing environmental DNA detection in controlled lentic systems. PLOS ONE 9:e103767.

Olson, Z. H., J. T. Briggler, and R. N. Williams. 2012. An eDNA approach to detect eastern hellbenders (*Cryptobranchus a. alleganiensis*) using samples of water. Wildlife Research 39:629–636.

Pauley, T. K., and M. Little. 1998. A new technique to monitor larval and juvenile salamanders in stream habitats. Banisteria 12:32–36.

Pilliod, D. S., C. S. Goldberg, R. S. Arkle, and L. P. Waits. 2013. Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. Canadian Journal of Fisheries and Aquatic Sciences 70:1123–1130.

Pilliod, D. S., C. S. Goldberg, R. S. Arkle, and L. P. Waits. 2014. Factors influencing detection of eDNA from a stream-dwelling amphibian. Molecular Ecology Resources 14:109–116.

R Core Team. 2014. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/

Schmidt, B. R., M. Kéry, S. Ursenbacher, O. J. Hyman, and J. P. Collins. 2013. Site occupancy models in the analysis of environmental DNA presence/absence surveys: a case study of an emerging amphibian pathogen. Methods in Ecology and Evolution 4:646–653.

Spear, S. F., J. D. Groves, L. A. Williams, and L. P. Waits. 2015. Using environmental DNA methods to improve detectability in a hellbender (*Cryptobranchus alleganiensis*) monitoring program. Biological Conservation 183:38–45.

Thomsen, P. F., J. Kielgast, and L. L. Iversen, C. Wiuf, M. Rasmussen, M. T. P. Gilbert, L. Orlando, and E. Willerslev. 2011. Monitoring endangered freshwater biodiversity using environmental DNA. Molecular Ecology 21:2565–2573.