An improved assay for eDNA detection of four-toed salamanders (*Hemidactylium scutatum*): Significance of mitochondrial genome region in primer development.

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Introduction

Environmental DNA (eDNA) utilizes DNA that is released from organisms into the environment to detect their presence and provides an effective, non-invasive method to determine organism presence or absence, and perhaps abundance, in an efficient manner[1,2,3]. We developed species-specific oligos to detect *Hemidactylium scutatum* (four-toed salamander).

Figure 1. *Hemidactylium scutatum* (four-toed salamander). Photo courtesy of Todd Pierson.

The plethodontid *Hemidactylium scutatum* is a small, lungless, fossorial salamander. Of the eight plethodontids, it has the largest geographic span, ranging from Nova Scotia to Florida in the east and Minnesota, Oklahoma, and Louisiana in the west[4].

H. scutatum is primarily terrestrial; however, adult females communally nest in sphagnum moss on the banks of vernal pools and the species has a brief larval stage within vernal pools[5]. During these times, individuals are likely to shed their DNA into the vernal pools; this provides a window of opportunity to detect this species via eDNA surveys[5].

A previously published eDNA assay designed based on a New York-collected *H. scutatum* specimen was successful in detecting New York but not Kentucky-collected *H. scutatum* specimens[5].

The wide range of *H. scutatum* provides a unique opportunity to examine the significance of phylogeography on the effectiveness of eDNA assays. In this case, the two assays target different regions of the mitochondrial genome; the New York assay targets the intergenic spacer region (IGS) while the Kentucky assay targets cytochrome b (cytb), regions known to display different rates of evolution[6].

The objectives of this study were to:

A) Develop and validate a qPCR protocol to detect Kentucky *H. scutatum* specimens using eDNA.
B) Field validate this assay during the *H. scutatum* breeding season in known breeding sites.
C) Compare the efficacy of this assay (targeting cytb) with the efficacy of the previously developed New York assays (targeting IGS region) in both New York and Kentucky-collected *H. scutatum* specimens.

Methods

Sequencing

H. scutatum DNA was acquired from a specimen collected in Robinson Forest (Breathitt and Knott Counties, KY) and published primers were used to amplify a 600 BP region of cytochrome b (cytb). PCR products were bidirectionally sequenced via Sanger using either the F or R primer; all sequencing was conducted by ACGT, Inc. (ACGTinc.com).

Methods

Primer Design

Potential assays (forward primer, reverse primer, and probe) were designed based on this cytochrome b sequence using PrimerQuest® software and aligned them with potential sympatric sequences. Final oligos were selected based on mismatches with sympatric species.

Table 1. Quantitative PCR assay developed for *H. scutatum*. The assay amplifies a 98 BP region of cytochrome b.

Oligo	Tm (°C)	Length (BP)	Sequence (5'-3')
Forward primer	62	22	CACAAATTGTCACCGGCTTATT
Reverse primer	62	22	GTAGTTTACGTCTCGGCAGATG
Probe	67	67	ACAGCAGACACTTCCTCAGCATTT

In Silico testing

Primers were tested for specificity against the 21 sympatric or potentially sympatric salamander species. Mismatches were quantified via alignment in MEGA X. Since prediction of primer specificity based solely on the number and position of mismatches can be misleading, in addition a machine learning program (eDNAssay[7]) was used to predict probabilities of amplification.

In Vitro testing

End-point PCR reactions with forward and reverse primers (no probe) were used to assess primer specificity. Tissue DNA from sympatric salamander species was extracted using a DNeasy® Blood and Tissue Kit (Quaigen), according to protocol. End-point reactions were run with target and six closely related sympatric species (40 cycles).

In Situ testing

Water samples were collected from potential four-toed habitats within Kentucky. One liter samples were collected in new, sterile containers.

Water eDNA Extraction

Environmental DNA extraction was performed using a modified version of an established protocol[8]. The extraction was conducted using a DNeasy® Blood & Tissue Kit (Qiagen).

eDNA quantification

Extracted DNA was quantified using a StepOnePlus™ Real-Time PCR system. Standard curves were generated using synthetic DNA (gBlock™, IDT™) to both enable data reporting in copy number and assess lowest observed limits of detection and quantification[7].

Inhibitor removal

Each sample was treated with a OneStep Inhibitor Removal Kit (Zymo Research) to remove any potential PCR inhibition.

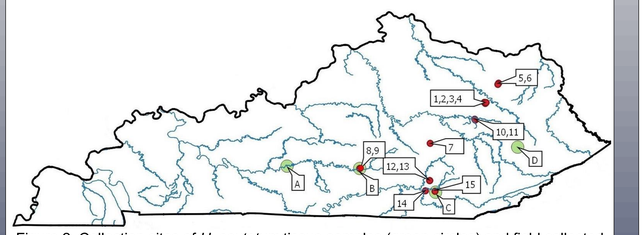

Figure 2. Collection sites of *H. scutatum* tissue samples (green circles) and field collected water samples (red circles) in Kentucky, U.S.A. Numbers (1-15) refer to eDNA water sample field collection sites (Table 3), letters (A-D) refer to *H. scutatum* tissue samples.

In Vitro testing - NY and multiple KY specimens

Both the primers developed in this study (KY) and the primers developed in the NY study were tested in vitro against four KY collected *H. scutatum* specimens and one NY collected specimen. Reactions were conducted at an AT of 60°C for 40 cycles.

Results

Specificity testing

Probe, F primer, and R primer showed a minimum total of 9 mismatches with all sympatric species tested. Amplification probability predicted by the machine learning program for the target *H. scutatum* sequence was 0.851 (Table 2). Probabilities for non-target sympatric species ranged from 0.131 to 0.286 and were all well below the recommended threshold of 0.55[7].

Table 2. Alignment results of 21 sympatric or potentially sympatric species with a *H. scutatum* sequence. All sympatric species sequences were obtained from GenBank.

Sympatric species	Amp. prob.	FP mismatch	RP mismatch	P mismatch	Seq. accession #	*In vitr*
Hemidactylium scutatum	0.851	0	0	0	XXXXXX	-
Pseudotriton ruber	0.233	6	4	4	OQ376719	Y
Pseudotriton montanus	0.188	7	4	6	MW319716.1	N
Gyrinophilus porphyriticus	0.222	7	2	5	MZ507696.1	Y
Eurycea cirrigera	0.194	5	4	6	MZ485475.1	Y
Eurycea lucifuga	0.214	5	3	4	KT873718.1	Y
Eurycea longicauda	0.219	5	3	4	AY528403.1	N
Eurycea bislineata	0.178	6	3	7	AY528402	N
Desmognathus fuscus	0.181	5	7	6	MZ485476.1	Y
Desmognathus monticola	0.203	4	6	2	MZ418126.1	N
Desmognathus ochrophaeus	0.163	6	4	4	EU314289	N
Desmognathus welteri	0.250	5	6	4	EU314293	N
Desmognathus conanti	0.242	4	7	4	EU314275.1	N
Plethodon glutinosus	0.204	5	2	5	MN723529.1	N
Plethodon dorsalis	0.235	4	5	5	GQ464404	N
Plethodon richmondi	0.286	4	4	8	AY378072	N
Ambystoma barbouri	0.231	5	3	2	OL456142.1	N
Ambystoma opacum	0.131	4	4	3	KT780868.1	N
Ambystoma jeffersonianum	0.250	6	0	3	KT780869.1	Y
Ambystoma maculatum	0.185	4	4	3	MZ485477.1	N
Ambystoma tigrinum	0.206	5	5	3	MZ962317.1	N
Notophthalmus viridescens	0.221	4	3	5	AY691731	Y

Primer testing

End-point PCR reactions run using our (KY) primers (designed from the cytb region) and template DNA from all KY and NY *H. scutatum* specimens showed amplification in each case, demonstrating the ability of the primer pair to recognize DNA from both KY and NY specimens.

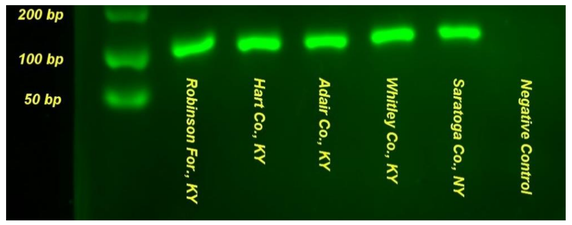

Figure 3. Amplicons produced using our environmental primers. Each reaction contains tissue-extracted DNA (1.0 µg/mL) from its respective *H. scutatum* specimen.

End-point PCR reactions using primers published in the NY study[5] (designed from the IGS region) showed amplification from the NY *H. scutatum* specimen but not the KY collected individuals.

Figure 4. Amplicon produced using environmental primers published in the NY study[5]. Each reaction contains tissue-extracted DNA (1.0 µg/mL) from its respective *H. scutatum* specimen.

Results

Specificity testing

End-point PCR reactions with our (KY) primers demonstrate only *H. scutatum* DNA amplification, confirming specificity.

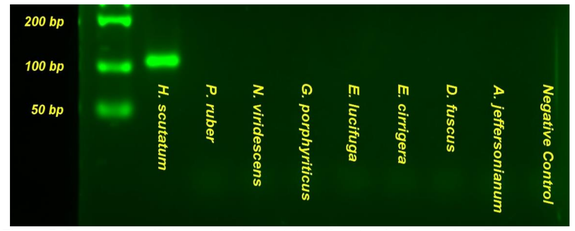

Figure 5. Species specificity test for *H. scutatum* (four-toed salamander). Each reaction contains tissue-extracted DNA of its respective species in a concentration of 1.0 µg/mL.

Field testing

qPCR analysis of 15 field-collected samples resulted in 10 positive detections for *H. scutatum* (Table 3).

Table 3. qPCR eDNA analysis from field-collected samples, tested in triplicate.

Site	Habitat Type	Vol. (ml) filtered	Coll. date	qPCR	*H. scutatum* field observations
1	LE	520	5/20/23	3/3	Hatched eggs
2	LE	535	5/20/23	0/3	Hatched eggs
3	LE	725	5/20/23	3/3	Hatched eggs
4	LE	925	5/20/23	3/3	Hatched eggs
5	LE	270	6/28/21	3/3	Possible breeding site but none observed
6	LE	300	6/28/21	0/3	Possible breeding site but none observed
7	LE	300	5/25/23	0/3	Larvae observed
8	LO	1000	3/16/23	3/3	Numerous adults observed on land
9	LO	1000	3/16/23	3/3	Numerous adults observed on land
10	LE	300	7/1/2021	0/3	Possible breeding site but none observed
11	LE	400	7/1/2021	3/3	Possible breeding site but none observed
12	LO	1000	3/12/23	3/3	Known breeding area but none observed
13	LO	1000	3/12/23	1/3	Larger stream, Site #12 is a trib. of this stream
14	LO	1000	3/12/23	0/3	Small, swift forest stream
15	LO	1000	3/12/23	1/3	Very small stream, adult collected in area

Conclusions

- *In silico* and *in vitro* testing indicate that our assay (KY) appears to be species-specific
- The 10/15 positive detections from field samples, mostly consistent with field observations, indicate our assay is capable of detecting *H. scutatum* eDNA *in situ*.
- Interestingly the assay (NY) targeting the IGS region only detected specimens locally but the assay (KY) targeting cytb detected specimens from two extremities of the range. This is consistent with our understanding of mitochondrial evolution rates in these two loci[6].
- Sequencing of both cytb and IGS regions from all specimens is in progress.

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