Development of eDNA protocols for detection of four darter (Percidae: Etheostomatinae) species in central Kentucky streams

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Introduction

Environmental DNA (eDNA) has rapidly become a firmly established method for detecting organisms of research and conservation interest and promises to greatly increase the ease, efficacy, and scope of ecological studies. Recent works have highlighted the need for carefully tested assays for use in species specific marker studies and thorough vetting of eDNA primers using as many local sequences as available.

In this study, four darter species were chosen for assay development and testing*: E. flabellare* (Fantail darter), *E. caeruleum* (Rainbow darter*), E. blennioides* (Greenside darter)*, and P. caprodes* (Logperch). The developed assays were tested in silico with Mega X and Clustal W as well as in vitro with endpoint PCR before environmental water sample testing.

Table 1. Quantitative PCR assays developed for the four darter species.

Target species	Amplicon length (BP)	TM	Oligo	Sequence (5'- 3')
E. flabellare	**118**	**62**	**F**	**AAGCGAAGAAGCGAGTTAGG**
(fantail)		**62**	**R**	**GGTGCTACGGTCATCACTAATC**
		68	**P**	**CCCACATAAGGCACTGCAGAGAGT**
E. blennioides	135	64	F	TCTCCGCTCCATCCCAAATAAG
(greenside)		65	R	AATAGGAACTGTGAGAGAGGGCG
		68	P	CCTTGCTGGCCTCAATCTTGGTACT
E. caeruleum	**105**	**62**	**F**	**GAGTGAGGGTTGCGTTATCTAC**
(rainbow)		**62**	**R**	**GAGCCACAGTCATTACCAATCT**
		68	**P**	**AGAAGCCGCCTCAAATCCACTGAA**
P.caprodes	139	62	F	CTCCATCAGACAGGCTCAAATA
(logperch)		62	R	CGAATAGAGCGAGGGATGTTAG
		68	P	TAGGCTTCGCCGTTCTCCTTATTGC

Although none of the fish in this study are threatened or endangered Kentucky is home to five species of federally threatened or endangered fish, including three darter species. The results of this study should prove useful in the eDNA monitoring of these and other threatened and endangered fish species.

Figure 1. A male *Etheostoma caeruleum* (Rainbow darter) photo courtesy jforbes3, iNaturalist.

Methods

Tissue Collection of Target and Non-target Species
Tissue was collected from all target species from both the Cumberland and Kentucky River drainages. Additionally, tissue was collected from nontarget, sympatric species from both Cumberland and Kentucky drainages. Tissue DNA was extracted from each darter species using a DNeasy blood and tissue kit (Qiagen) according to the provided protocol.

Figure 2. *E. blennioides*, (greenside darter) photo courtesy of North American Native Fishes Association.

Sequencing of Target Species
Portions of cyt b were amplified from target species using published primers. Sequencing was completed by ACGT (ACTG inc) and conducted in duplicate.

Assay Development and Testing
Partial cyt b sequences were aligned with 10 potential sympatric Kentucky darter species using MegaX and Clustal W. F and R primer pairs were developed using PrimerQuest software (IDT) and aligned with sympatric or potentially sympatric species to verify specificity. All primers have at least three mismatches in the F or R primer. Tissue DNA from both Kentucky River and Cumberland River drainages were used.

Field Testing
Three different water samples were collected from Buck Creek. One liter of each sample was filtered and extracted. The developed primers were used to detect the presence of target DNA in the environmental sample.

Figure 3. *E. flabellare* (Fantail darter) photo courtesy of Emilio Concari, *Maryland Biodiversity.*

Results

Sequencing
Tissue-extracted DNA was used to create the bands for sequencing reactions. The amplicons were sequenced by ACGT (ACGT Inc). Target and non-target cyt b sequences were acquired for in silico analysis.

In Silico Testing
The developed assays were aligned with 10 potential sympatric Kentucky darter species using MegaX and Clustal W.

Table 2. Mismatches between *P. caprodes* oligos and 10 sympatric species. KY = Kentucky River drainage, CM = Cumberland drainage, UN = Unknown.

Sympatric species	Drainage	FP	RP	P	% sim.	Seq. acc. #
Percina caprodes	KY	0	0	0	-	KT880217.1
Etheostoma flabellare	KY	4	7	3	82.3	KT880219.1
Etheostoma blennioides	KY	3	6	4	81.2	KT880218.1
Etheostoma caeruleum	KY	6	6	4	81.8	KT880220.1
Etheostoma stigmaeum	CR	7	6	5	80.1	BC
Etheostoma camurum	CR	3	6	4	83.2	BC
Etheostoma sanguifluum	CR	3	6	5	82.1	BC
Etheostoma variatum	KY	5	7	4	82.9	AF289266.1
Etheostoma cinereum	CM	4	5	4	83.7	AY560356.1
Etheostoma spectabile	UN	4	5	4	81.4	AF045344.1
Etheostoma blennioides	CR	3	7	3		BC

In vitro testing
F and R primers were first evaluated via a temperature gradient approach to determine optimal annealing temperature. The optimal annealing temperature for each was then used for the specificity reactions.

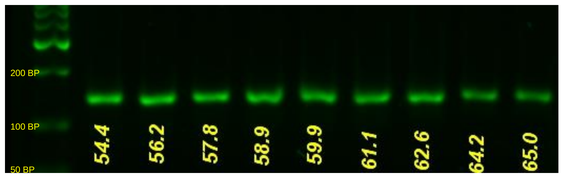

Figure 4. *P. caprodes* gradient reactions.

Specificity tests for each of the primers showed binding only with the target DNA. At optimal annealing temperature, the primers did not bind to the non-target sympatric species (Figure 5).

Results

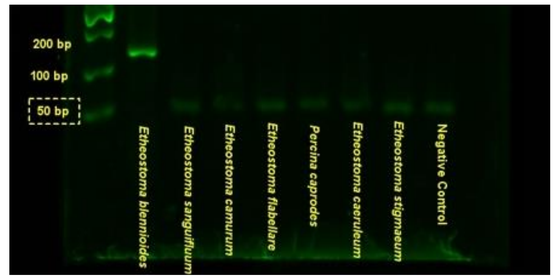

Figure 5. Specificity reactions for *E. blennioides*

Field Testing
Two of the three field collected samples have been processed at this time. *E. caeruleum* DNA was detected in both of the Buck Creek (Pulaski County) environmental samples*. P. caprodes* and *E. flabellare* DNA were each detected in one Buck Creek environmental sample while *E. blennioides* DNA was not detected in any sample.

Conclusions

- All darter assays exhibit significant mismatches with sympatric, but not target, species in *in silico* testing.
- All darter assays detect target species, but not sympatric species, in *in vitro* testing.
- Initial field testing indicates successful detection of darters in a manner consistent with field observations with the exception of *E. blennoioides*.
- Initial sequence analysis appears to indicate lack of detection of *E. blennoioides* in Buck Creek (Cumberland River) samples is the result of single nucleotide polymorphisms (SNP's) in Cytb in Cumberland versus KY River drainage (for which assays were created) specimens.

Bibliography

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