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Short Communication

Inferring presence of the western toad (*Anaxyrus boreas*) species complex using environmental DNA

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ABSTRACT

Western toads (species complex comprised of *Anaxyrus boreas*, *A. canorus*, *A. exsul*, and *A. nelsoni*) are widely distributed in the western United States but are declining, particularly in the southeastern extent of their range. The subspecies *A. b. boreas* is listed as a Species of Greatest Conservation Need in New Mexico, Colorado, Utah, and Wyoming. Reliable and sensitive methods for delineating distributions of western toads are critical for monitoring the status of the species and prioritizing conservation efforts. We developed two qPCR assays for detecting western toad DNA in environmental DNA samples. Both markers efficiently and reliably detect low concentrations of western toad DNA across their range in the conterminous U. S. without detecting non-target, sympatric species. To determine the optimal annual sampling period, we then tested these markers using repeated sampling in ponds where western toads were known to be present. Quantities of collected eDNA varied widely across samples, but sample-level detections across sites exceeded 80% for June sampling. In the later summer, detection dropped off sharply with only a single detection in the ten samples collected throughout August.

1. Introduction

Toads in the genus *Anaxyrus* represent a widely distributed and sometimes cryptic complex of species, subspecies, and lineages in North America (Frost et al., 2017). In the western United States, a number of forms are regarded as taxa of conservation concern because they are highly local endemics (Forrest et al., 2017; Gordon et al., 2017) or have undergone widespread declines leading to consideration for listing under the U.S. Endangered Species Act (U.S. Fish and Wildlife Service 2017). To prioritize conservation efforts for this species complex, reliable methods for assessing their presence and distributions are needed.

Environmental DNA (eDNA) has emerged as an efficient and useful tool for detecting rare or invasive aquatic species (Dejean et al., 2012; Wilcox et al., 2013; Sigsgaard et al., 2015) and delimiting distributions of rare species (Spear et al., 2015;

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McKelvey et al., 2016). By integrating minor-groove-binding probes into quantitative PCR (qPCR), eDNA sample analysis has proven effective in detecting low concentrations of targeted DNA and can be more sensitive than traditional PCR methods (Kutyavin et al., 2000; Turner et al., 2014). Development of eDNA assays, however, can be problematic for species complexes that exhibit incomplete lineage sorting or minor levels of divergence. In such cases, it may only be feasible to develop assays that are specific and effective for groups of species within a genus.

Here, we describe the development of two qPCR assays to detect DNA of the western toad species complex. We follow Goebel et al. (2009; also see Pyron and Wiens, 2011) and use western toad to collectively refer to *A. boreas*, *A. canorus*, *A. exsul*, and *A. nelsoni*. Multiple independent assays can be useful in validating eDNA detections as they increase the likelihood of positive detections while reducing the probabilities of false positive detections (Carim et al., 2016a).

2. Materials and methods

2.1. Assay development

To develop environmental DNA markers for western toad, we chose the cytochrome *b* (cytb) and cytochrome oxidase c subunit I (COI) regions of the mitochondrial genome because these regions provided the best combination of publically available reference samples for target and non-target species across a comprehensive geographic range. These regions were also used for *in silico* candidate primer and probe design because they provided sufficient nucleotide differences to distinguish western toads from most other species. After initially screening all publically available sequences for western toads and other closely related and sympatric toads, we found that sequences for all species within the western toad species complex were identical or nearly identical in these regions (Table A.1). Consequently, we pursued eDNA markers that would detect all species within the western toad species complex. We obtained candidate primers *in silico* using the *DECIPHER* package (Wright et al., 2014) in R v. 3.2.3 (R Core Development Team 2015), and aligned them with the genetic sequence data using MEGA 7.0 (Kumar et al., 2016). We then manually adjusted primer lengths and positions to optimize annealing temperatures and maximize base-pair mismatches with non-target species. Using the MEGA sequence alignments, we visually identified regions unique to western toad and designed TaqMan™ MGB probes (Applied Biosystems) with 6-carboxyfluorescein (FAM)-labeled 5' ends and minor-groove-binding, non-fluorescent quenchers (MGB-NFQ) within the target amplicon sequence of each gene (Table A.2). We assessed annealing temperatures for each primer-probe set in Primer Express 3.0.1 (Life Technologies; Table A.2) and screened each marker for secondary structures using IDT OligoAnalyzer (https://www.idtdna.com/calc/analyzer).

To test the specificity of each marker, we screened DNA extracted from 101 western toad tissues from 46 locations in the western U.S. (100 *A. boreas*, 1 *A. canorus*; Fig. 1), 9 non-target amphibian species, and 31 non-target fish species (Table A.3). All samples used in this study were from existing collections acquired under appropriate sampling permits. Toad tissues included toe clips, muscle tissues, and liver tissues from adult specimens and tail clips from tadpoles; fish tissues were fin clips. Tissues were extracted with the DNeasy Tissue and Blood Kit (Qiagen, Inc.) following the manufacturer's protocol. We screened each marker *in vitro* against tissue-derived DNA in a single qPCR reaction. Screening was performed on a StepOne Plus Real-time PCR Instrument (Life Technologies) in 15 μl reactions containing 7.5 μl Environmental Master Mix 2.0 (Life Technologies), 900 nM forward primer, 900 nM reverse primer, 250 nM probe, 4 μl DNA template (~0.12—0.88 ng), and 2.75 μl deionized water. Thermocycler conditions included 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. To minimize the risk of sample cross contamination, all qPCR tests were set up inside of a UV hood where consumables and pipettes were irradiated with UV light for 1 h prior to each test. Each test included a no-template control with distilled water used in place of DNA template.

We optimized primer concentrations following methods outlined in Wilcox et al. (2015; Table A.2). Using the optimized concentrations and cycling conditions above, we tested sensitivity of the markers by performing standard curve experiments created from western toad (specifically, *A. boreas*) qPCR product. For each marker, qPCR product was purified using PureLink™ PCR Micro Kit (Invitrogen) and quantified on a Qubit 2.0 fluorometer (ThermoFisher Scientific). From this stock, we prepared a six-level standard curve dilution series (6 250, 1 250, 250, 50, 10, and 2 copies per 4 μl) in sterile TE. We ran six replicates of each dilution.

Finally, we applied both assays to eDNA samples collected from two sites in Utah with known presence of western toads based on visual encounter surveys (Lower Pond at Left Fork Kanab Creek and Snow Lake; Table 1) and a site in Montana believed to be occupied by amphibians other than western toads (Hidden Lake, 47.14311 N, −113.57065 W). For the assay validation samples, eDNA was collected from a 300-ml water sample at Snow Lake on 11 September 2016, a 1000-ml water sample at Left Fork Kanab Creek on 31 August 2016, and a 5000-ml water sample at Hidden Lake on 28 October 2015 using a peristaltic pump and a 1.5 μm glass mircrofiber filter as described in Carim et al. (2016b). The sampling method for these differed from Carim et al. (2016b) in that sampling concluded when a filter was clogged, preventing water passage. The eDNA samples were then extracted with the DNeasy Blood and Tissue Kit (Qiagen, Inc.) using a modified protocol (Carim et al., 2016c) in a room dedicated solely to this practice where the work area, scissors, and forceps were cleaned with 50% bleach prior to extractions. Negative extraction controls were paired with eDNA extraction sets and filtered pipette tips were used for all laboratory based methods. All extracts were stored at −20 °C until analyzed. Environmental DNA samples and extraction controls were analyzed in 15 μl volumes using optimized concentrations (Table A.2) and the same PCR cycling conditions and recipe above except a TaqMan Exogenous Internal Positive Control (IPC; Life Technologies) consisting of 1.5 μl