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TECHNICAL NOTE

Development of quantitative PCR primers and probes for environmental DNA detection of amphibians in Ontario

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Abstract

DNA from environmental samples (eDNA) is increasingly being used to detect and monitor elusive species. We developed species-specific eDNA primers and probes for qPCR detection of 24 amphibian species native to Ontario, Canada. Cross-species testing confirmed their high specificity and low cross-species amplification, as well as their ability to detect DNA from target species at low concentrations. These detection tools should prove useful for monitoring at-risk amphibian species throughout their native ranges.

Keywords Amphibian · eDNA · Quantitative PCR

Lack of accurate data on amphibian distributions can hinder effective conservation and management. Traditionally, amphibian surveys involve capture of organisms via nets or traps, call surveys, visual encounter surveys or a combination of these (Vonesh et al. 2009). Intensive capture-based surveys risk injuring specimens and/or disrupting habitats, and survey success is often dependent on the skill of field crews in identifying species by call or appearance (Vonesh et al. 2009). Environmental DNA (eDNA) surveys have been established as sensitive, non-invasive and cost-effective tools for monitoring aquatic species including invasive and endangered amphibians (Biggs et al. 2015; Goldberg et al. 2011; Rees et al. 2014), and eDNA has been shown to be more sensitive than traditional sampling to document habitat occupancy by amphibians (Pilliod et al. 2013; Smart et al. 2015; Thomsen et al. 2012). Here we describe the development and optimization of species-specific eDNA primer and probe sets for 24 of Ontario's native amphibian species for use in monitoring.

Reference samples of target species were obtained as frozen or ethanol-preserved tissue from the Royal Ontario Museum (ROM), University of Guelph, and Laurentian University, or nonlethal toe clips from field specimens (Trent University animal care permit 23907). Genomic DNA was extracted using the E.Z.N.A. Tissue DNA kit (Omega Biotek) and eluted in 100–200 µL of TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0). All specimens were amplified and sequenced at the COI barcoding region using universal primers (Che et al. 2012; Folmer et al. 1994; Hebert et al. 2003; Smith et al. 2008) to verify species identity and use as reference sequences for primer design.

Species-specific primers and probes were designed to target small (65–171 bp) segments of the COI barcoding region (Hebert et al. 2003). Reference sequences for target species were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank) and BOLD (http://www.boldsystems.org), or generated in house and aligned in Geneious v.7.1.9 (http://www.geneious.com). Regions with interspecific divergence and little to no intraspecific variation were targeted for primer and probe design. Primers and probes for qPCR were designed manually or using Primer Express v.2.0 (Applied Biosystems).

Preliminary primer screening for amplification and annealing temperatures (T_a) was performed in 10 µL reactions containing 10 ng DNA, 1× PCR Buffer, 2 mM $MgCl_2$, 0.2 mM each dNTP, 0.3 mg/mL BSA (BioShop), 0.2 µM

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Table 1 Quantitative PCR primers, probes, and annealing temperatures (T_a) for 24 amphibian species in Ontario

Species Name	Common name	Forward primer	Reverse primer	Probe	Label	Length	T_a	R^2	Efficiency
Ambystoma laterale	Blue-spotted salamander	AACTAAGTCAACCCG GAGCTTTAC	TTCCGAATCCCCCGA TTATT	AGGTGATGACCAAAT CTACAA	6FAM	120	60	0.99	109.89
Ambystoma unisexuals	Unisexual mole salamander complex	CTGAGCTGGGATAGT TGGAACC	ATAGATTTGGTCGTC GCCTAGC	CGAGCAGAATTAAGCC	VIC	91	60	0.99	102.03
Ambystoma maculatum	Spotted salamander	CGCATTCGTAATGAT TTTCTTCAT	AAAGCTATATCTGGT GCCCCAAT	ATGCCTGTTATAATTGGC GGA	VIC	99	60	0.99	98.42
Ambystoma texanum	Small-mouthed salamander	GCTTTTGACTATTAC CCCCATCA	TCCGGCACCTGCTTC AAC	CTTCTTCTGTTAGCC TCCT	6FAM	68	60	0.99	115.75
Ambystoma tigrinum	Tiger salamander	GAACTGTATATCCCC CACTTGCA	AAACACCTGCTAAAT GAAGTGAAAAA	AACCTAGCCCATGCCG	VIC	90	60	0.99	101.19
Desmognathus fuscus	Northern dusky salamander	CCACGTATAAATAAT ATAAGCTTCTGATTA TTACC	TGAAGTGAAAAGATG GTTAAATCTACAGA	TATTTCCGGCTAAGGGC	6FAM	170	60	0.99	99.49
Desmognathus ochrophaeus	Allegheny Mountain dusky salamander	Same as *D. fuscus*	Same as *D. fuscus*	CCATATTTCCGGCTAGAG GA	VIC	170	55	0.99	86.56
Eurycea bislineata	Northern two-lined salamander	CATAATTGGTGGGTT TGGTAACTG	TTTATTCGTGGGAAG GCCATATC	CTGCCACTAATAATTGG	VIC	72	60	0.99	84.96
Hemidactylium scutatum	Four-toed salamander	CYGGCGCCTCAGTAG ATTT	TAGAGGTYGTGATAA AATTAAT	ACCATCTTTTCTCTT CACC	VIC	90	54	0.99	102.98
Necturus maculosus	Common mudpuppy	ATGCAGGTGCCTCTG TAGACTTAAC	TAGAGGGTGGTTTCA TATTGATGG	TTGGCGCAATTAAT	6FAM	114	60	0.99	90.44
Notophthalmus viridescens	Red-spotted newt (eastern)	GTACTTGCAGCAGGT ATTACAATGC	ACAGGGTCTCCTCCT CCAGC	ACAGATCGAAATCTA AAC	VIC	86	60	0.99	113.64
Plethodon cinereus	Eastern red-backed salamander	CCCCTTTAGCCGGAA ACC	GATAGATGATACTCC CGCAAGGTG	CGCACACGCCGGAG	6FAM	85	60	0.99	96.14
Acris crepitans blanchardi	Blanchard's cricket frog	GGACGGGCTGGACAG TATACC	AAGAAACCCCTGCGA GATGG	AGCACATGCTGGCCCA	VIC	102	60	0.99	97.49
Anaxyrus americanus	Eastern American toad	GCAGGACCATCAGTT GACTTAACC	GTGGTAATAAAATTA ATTGCCCCAAG	ACACCTGCTAGATGG AGG	6FAM	86	60	0.99	96.01
Anaxyrus fowleri	Fowler's toad	Same as *A. americanus*	Same as *A. americanus*	ACACCTGCTAAATGA AGA	VIC	86	60	0.99	102.04
Hyla versicolor	Eastern gray treefrog	GGAGCTGGAACAGGA TGAACTG	CAAATCGACTGATGG TCCAGC	CCCTCCGCTTGCC	6FAM	75	60	0.99	104.95
Lithobates catesbeianus	American bullfrog	CATCCTCAACTACAC AATACCAAACA	GGAGTATAGTAATTC CGGCAGCTARTA	CCTTTATTCGTCTGATCA GTT	VIC	111	60	0.99	98.13
Lithobates clamitans	Northern green frog	Same as *L. catesbeianus*	Same as *L. catesbeianus*	TCTATTTGTCTGATCAGT CTT	6FAM	111	60	0.99	93.77
Lithobates palustris	Pickerel frog	CGGGACTGGCTGGAC AGT	TGATACCCCCGCTAA ATGTAATG	ATCCCCCTTTAGCCGGT	VIC	103	60	0.99	104.09

Table 1 (continued)

Species Name	Common name	Forward primer	Reverse primer	Probe	Label	Length	T_a	R^2	Efficiency
Lithobates pipiens	Northern leopard frog	CACACAGTACCAAAC ACCCCTATTT	GAGTATAGTAATCCC TGCCGCTAGAA	TGTTTTGATCACCGC AGTT	VIC	100	60	0.99	102.45
Lithobates septentrionalis	Mink frog	ACCATCCTCAACCAC ACAATACC	TCCGGCAGCTAAGAC TGGAA	TATTCGTCTGGTCAG TTTT	6FAM	100	60	0.99	107.13
Lithobates sylvaticus	Wood frog	GCCCCTCAGTAGATT TAGCTATTTTC	AGRGGTGTTTGATAT TGTGTAGTTGATGA	CACCTAGCTGGTGTTTC	6FAM	130	60	0.99	96.75
Pseudacris crucifer	Spring peeper	CCATCTTTCCTTCTT CTCCTCG	TGCATGTGCTAAGTT CCCAGC	GTGTAGAGGCAGGTGC	6FAM	99	60	0.99	102.10
Pseudacris maculata	Boreal chorus frog	CTTGCTGGAAATTTA GCACACG	ATAGCTCCTAGGATT GAAGACACACC	GGCCCATCAGTTGAT	VIC	71	60	0.99	94.90

Quality of the qPCR assay is indicated by reaction efficiency and R^2 value of the standard curve

each primer, 0.25 U *Taq* polymerase (Promega), and ddH_2O. Thermal cycling conditions were 94 °C for 10 min, 40 cycles of 94 °C for 45 s, 55–68 °C for 45 s, and 72 °C for 45 s, and final extension of 72 °C for 10 min. Amplicons stained with SYBR Green (Cedarlane Laboratories) were visualized by electrophoresis on a 1.5% agarose gel. Positive amplification was recognized as a single band at the expected fragment size. Optimal T_a was determined by high DNA yield and amplicon quality.

Following primer screening, qPCR assays incorporating species-specific probes were performed to determine reaction efficiency, dynamic range, and limit of detection. For each species, the complete COI barcoding region was amplified as described above, and product yield quantified using a Qubit3 fluorometer (Invitrogen). The number of amplified target copies was calculated using the molecular weight of the known target sequence, and used to make 10-fold serial dilution standards from 10^6 to 10^0 amplicon copies per reaction. Standard curves for each species were then generated using 20 µL qPCR reactions consisting of 1× Taqman® Environmental Master Mix 2.0 (Life Technologies), 0.2 µM each primer, 0.2 µM probe (Life Technologies), and 5 µL of each dilution. DNA amplification and detection used the StepOnePlus™ Real-time PCR system (Applied Biosystems), with 10 min activation at 95 °C followed by 40 cycles of 95 °C for 15 s and T_a for 1 min. Analysis of run data was performed using StepOne™ software v2.3.

Optimized qPCR primers and probes were screened for species specificity using a known quantity of DNA from closely related and/or co-occurring species. For this to be manageable, primer/probe sets were primarily tested across species within genera (*Ambystoma*, *Desmognathus*, *Lithobates*, *Anaxyrus*) or families (Hylidae: *Acris, Pseudacris, Hyla*) as it is unlikely they would amplify across more divergent taxa due to the number of mismatches at primer and probe binding sites (Supplementary Table 1). Primer and probe sets for threatened and endangered Ontario species were also tested against common species with overlapping distributions.

All primer pairs successfully amplified DNA of the expected fragment lengths using tissue-derived DNA from the target species (Table 1). Results with qPCR and TaqMan probes were similar: all primer/probe sets successfully amplified individuals at 10^6–10^0 copies of target DNA. Threshold cycle (C_t) values were within the expected ranges of 16–21 for 10^6 target copies for all taxa, with $R^2 > 0.99$ for all species. All primer/probe sets detected down to 10 target copies reaction^{-1} in all replicates, and 39/52 trials were able to detect 1 target copy reaction^{-1}.

Cross-species testing showed very low amounts of cross-reactivity in both frogs (Supplementary Table 2) and salamanders (Supplementary Table 3). With the exception of the *L. palustris* assay, all instances of cross-reactivity

detected < 5 copies of non-target species when using 10^3 copies reaction^{-1} of control DNA. The *L. palustris* assay requires further optimization and/or redesigning before being implemented. These results indicate that the majority of these primer and probe sets could prove useful in detection of amphibian species in Ontario.

Mapping species occurrences and habitat occupancy are key conservation elements. This information will be particularly useful for biodiversity studies comparing current distributions to historical occurrence records (Vonesh et al. 2009). Using eDNA to detect rare and enigmatic species could increase accuracy and decrease costs of surveys (Smart et al. 2015), increase the number of sites sampled per unit effort, and refine distribution and extinction records (Biggs et al. 2015) with little risk to the species themselves (Goldberg et al. 2011).

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