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TECHNICAL NOTE

An environmental DNA assay for detecting Arctic grayling in the upper Missouri River basin, North America

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Abstract The upper Missouri River basin in the northwestern US contains disjunct Arctic grayling (*Thymallus arcticus*) populations of conservation concern. To assist efforts aimed at understanding Artic grayling distribution, we developed a quantitative PCR assay to detect the presence of Arctic grayling DNA in environmental samples. The assay amplified low concentrations of Arctic grayling DNA consistently, and did not amplify non-target species, including sympatric salmonid fishes.

Keywords *Thymallus arcticus* · eDNA · Invasive species · qPCR

The Arctic grayling (*Thymallus arcticus*) is a freshwater salmonid widely distributed across northern North America and Asia. This species has been extirpated from portions of its distribution in the conterminous US, with a few small, disjunct populations remaining in the headwaters of the Missouri River basin in Montana. The species is a conservation priority and restoration efforts aimed at protecting and expanding populations of Arctic grayling are underway. Consequently, rapid and reliable methods to monitor Arctic grayling distribution within this region are needed. Environmental DNA (eDNA) sampling is gaining popularity as a sensitive and reliable method for detection of rare or invading aquatic species (Biggs et al. 2014; Dejean et al. 2012; Rees et al. 2014; Siggsgaard et al. 2015; Wilcox et al. 2016) and for delimiting species distributions (McKelvey et al. 2016). Here, we describe a quantitative PCR (qPCR) assay for eDNA-based detection of Arctic grayling from the upper Missouri River basin.

To develop a primer set specific to Arctic grayling, we first compiled DNA sequences from GenBank of the cytochrome *c* oxidase 1 (*COI*) mitochondrial gene for Arctic grayling and seven other salmonids commonly found in this basin (Table S1). We used these sequences and the *DECIPHER* package (Wright et al. 2013) in *R* v. 3.0.1 (R Core Team 2014) to develop primers specific to Arctic grayling: forward primer 5′-CCTTTCCCCGAA-TAAATAACATGAG-3′; reverse primer 5′-ATACTGTC-CACCCTGTCCCG-3′.

We designed a hydrolysis probe (5′-CTCTTAGCCT-CATCTGG-3′) by aligning the GenBank sequences in MEGA 6.0 (Tamura et al. 2011) and identifying a region specific to Arctic grayling. The probe matches three Arctic grayling reference sequences obtained from GenBank and has four basepair mismatches with published sequences of lake whitefish (*Coregonus clupeaformis*), cisco (*Coregonus artedi*), and mountain whitefish (*Prosopium williamsoni*), the most closely related salmonids in the Upper Missouri River Basin. To more generally test for specificity, we performed a primer BLAST search (Ye et al. 2012) in GenBank. Other than European grayling (*Thymallus thymallus*), where all published sequences vary at ≤2 BP mismatches (all in the middle of the forward primer), BLAST results indicated enough dissimilarity across the primer and probe sequences that non-target amplification is unlikely. Using Primer Express 3.0.1 (Life Technologies), we assessed the melting temperatures of the primers (both

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at 60 °C) and probe (68 °C). We screened the primers and probe for secondary structures using IDT OligoAnalyzer (https://www.idtdna.com/calc/analyzer).

We extracted DNA from 28 Arctic grayling tissues from 14 different locations, as well as 12 non-target species using the Qiagen DNEasy® Blood and Tissue Kit and following the manufacturer's protocol (Table 1). Prior to optimization, the assay was tested using these 40 tissues 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 (diluted 1:100 from extract to roughly ~0.5 ng/µl), and 2.75 µl deionized water. The PCR conditions were slightly modified from StepOne Plus Real-time PCR Instrument (Life Technologies) standard conditions by removing the initial 50 °C incubation (which is not necessary for the Environmental Master Mix) and by increasing the number of cycles to 45 to increase probability of detecting low levels of amplification. The final cycling conditions were: 95 °C/10 min (95 °C/15 s, 60 °C/60 s) × 45. DNA from all Arctic grayling tissues successfully amplified, and there was no amplification of DNA from non-target tissues.

We then optimized primer concentrations following methods outlined in Wilcox et al. (2015) for final concentrations of 600 nM for both the forward and reverse primer, respectively. We tested assay sensitivity by creating a six-level standard curve dilution series (6250, 1250, 250, 50, 10, and 2 copies per 4 µl). This standard curve was created by quantifying PCR product (Arctic grayling tissue amplified with the assay) from the above analysis on a Qubit Fluorometer (Thermo Fisher Scientific) and diluting the DNA in TE to the desired concentrations. We ran six replicates of each dilution (using optimized primer concentrations) to determine the standard curve slope with a resulting amplification efficiency of 100.5 % and $r^2 = 0.988$. We observed 100 % detection across six replicates with 2 copies per 4 µl.

Finally, the assay was tested using eDNA samples collected from one where Arctic grayling were known to be present and four where Arctic grayling were absent

Table 1 Species used for in vitro testing of the primers and probe

Common name	Species name	Probe testing sample size	Origin
Arctic Grayling	*Thymallus arcticus*	2	Deep Creek, MT
		1	La Marche Creek, MT
		1	Steel Creek, MT
		1	Pintlar Creek, MT
		2	Axolotl Lake, MT
		2	Deer Lake, MT
		2	Emerald Lake, MT
		2	Hyalite Reservoir, MT
		2	Grayling Lake, MT
		2	Mussigbrod Lake, MT
		2	O'Dell Lake, MT
		4	Ruby River, MT
		2	Bobcat Lake, MT
		3	Big Hole Lake, MT
Brown trout	*Salmo trutta*	3	MT
Brook trout	*Salvelinus fontinalis*	2	ID, QC
Bull trout	*Salvelinus confluentus*	3	ID, MT
Chinook salmon	*Oncorhynchus tshawytscha*	2	H, ID, MT
Coho salmon	*Oncorhynchus kisutch*	2	AK
Lake trout	*Salvelinus namaycush*	3	MT
Lake whitefish	*Coregonus clupeaformis*	2	MT
Mountain whitefish	*Prosopium williamsoni*	1	MT
Northern pike	*Esox lucius*	3	MT
Rainbow trout	*Oncorhynchus mykiss*	3	H, ID, MT
Westslope cutthroat trout	*Oncorhynchus clarkii lewisi*	4	MT
Yellowstone cutthroat trout	*Oncorhynchus clarkii bouvieri*	9	ID, MT

Origin refers to the Montana waterbody for Arctic grayling samples. For all other samples origin is listed as state or "H" for hatchery origin

Table 2 Collection and species assemblage information for eDNA samples used to test the Arctic grayling assay

Waterbody (state)	Latitude	Longitude	Collection date	Salmonid species present	Arctic grayling detected?
Big Hole River, MT	45.869923	−113.224756	11/22/2015	ARG, MWF, RBT	Y
Big Creek, ID	45.081292	−115.339288	10/15/2014	BUT, CHK, RBT, WCT	N
Little Blackfoot River, MT	46.399051	−112.484441	10/14/2015	WCT	N
West Fork Lolo Creek, MT	46.700550	−114.539697	8/12/2014	WCT, MWF	N
Rattlesnake Creek, MT	46.945721	−113.945222	4/7/2015	BNT, BRK, BUT, RBT, WCT	N

AGR arctic grayling, *BNT* brown trout, *BRK* brook trout, *BUT* bull trout, *CHK* chinook salmon, *MWF* mountain whitefish, *RBT* rainbow trout, *WCT* westslope cutthroat trout

(Table 2). Five-liter samples were collected following methods described in Carim et al. (2015), and DNA extracted using the Qiagen Kit as above with modifications to the protocol (Supplement 2). These samples were tested using optimized primer concentrations and PCR conditions as above, but replacing 1.8 µl of water in each reaction with TaqMan® Exogenous Internal Positive Control Reagents to screen for PCR inhibition. The assay amplified Arctic grayling DNA in samples where the species was known to be present, and no amplification was observed where the species was absent.

This assay was designed for detection of Arctic grayling and was tested on samples from within the upper Missouri River basin. Other lineages of Arctic grayling may contain polymorphisms in the primer or probe regions leading to either lower efficiency or failure (cf. Wilcox et al. 2015). To ensure reliable detection of other Arctic grayling lineages, we recommend screening this assay against locally-derived tissues prior to eDNA application. Because sequence differences between Arctic grayling and European grayling are limited to ≤2 BP in the middle of the forward primer, this assay will likely also detect European grayling (see Wright et al. 2013), though perhaps with reduced efficiency.

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