Quantitative PCR assays for detection of five Alaskan fish species*: Lota lota, Salvelinus alpinus, Salvelinus malma, Thymallus arcticus,* and *Cottus cognatus* from environmental DNA

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Abstract

The North Slope of Alaska contains arctic fish populations that are important for subsistence of local human populations, and under threat from natural resource extraction and climate change. We designed and evaluated four quantitative PCR assays for detection of environmental DNA from five Alaskan fish species present on the North Slope of Alaska: burbot (*Lota lota*), *arctic char* (*Salvelinus alpinus*), *Dolly Varden* (*Salvelinus malma*), *arctic grayling* (*Thymallus arcticus*), and *slimy sculpin* (*Cottus cognatus*). All assays were designed and tested for species specificity and sensitivity, and all assays detected target species from filtered water samples collected from the field. These assays will enable efficient and economical detection of the above species from lakes and rivers. This in turn will provide managers with improved knowledge of current distributions and future range shifts associated with climate and development threats, enabling more timely management.

Introduction

The Arctic is warming faster than any other region on the globe (1) with a 2.1 °C increase in air temperature observed over the last 30 years (2). On the North Slope of Alaska (hereafter the North Slope), a vast region encompassing over 245,000 km^2 including the Arctic National Wildlife Refuge, Prudhoe Bay, and the National Petroleum Reserve-Alaska, climate change has, and will continue to have, a disproportionally large and rapid impact compared the continental U.S (3). Moreover, across the North Slope, native fish species are an important source of subsistence for local human populations including Inupiat and Nunamiut Eskimo communities (4, 5). If aquatic food webs do not respond proportionally to the warming climate, local extinctions of fish populations could occur (6). Additionally, many North Slope fish populations have high potential for negative effects from natural resource extraction, as the North Slope holds a large portion of North America's petroleum reserves (7). Thus, arctic fish and the local communities that depend on them are exceedingly vulnerable (8).

Given the ecological risks and array of conflicting human interests in this region, accurate monitoring and assessment of native North Slope fish populations is critically for effective management and policy decisions. Accurate assessment and monitoring of these fish species however is logistically challenging and expensive. In some areas, freshwater covers as much as 48% of the land surface (9), and sampling is generally restricted to summer months and sites are only accessible by air. Conducting surveys using traditional techniques such as gill or trap nets requires transportation of bulky gear as well as extensive man hours, severely limiting the number of sites visited in a season. Thus, there is a need for new, accurate techniques capable of rapidly detecting fish species of concern in order to increase the number of sites that can be sampled, and the amount of meaningful data that can be collected for these species.

Environmental DNA (eDNA) detection is a molecular technique that uses trace amounts of DNA from the water column to detect aquatic species including fish (10, 11). eDNA has been used for detection of many fish species worldwide, and has been shown to be more sensitive than traditional sampling (12, 13). In addition, an eDNA sample can be collected by a single person in less than 30 minutes, greatly increasing the number of sites that can be sampled in a single field season. Thus, this technique is ideal for remote sites such as the North Slope of Alaska where sampling is logistically difficult and expensive.

We developed and tested four highly sensitive eDNA assays to detect five fish species native to the North Slope of Alaska: burbot (*Lota lota), arctic char (Salvelinus alpinus),* Dolly Varden *(Salvelinus malma),* arctic grayling *(Thymallus arcticus)* and slimy sculpin *(Cottus cognatus)*. These assays use Taqman® based quantitative PCR (qPCR) to detect species from filtered water samples. Use of these assays will improve knowledge of species distributions on the North Slope as well as throughout the rest of Alaska and the arctic. In addition these assays will allow improved monitoring of distributional shifts,

and generation of presence/absence data can be used to inform models that predict species responses to natural resource extraction and climate change.

Methods

Ethics statement

Reference sample collection was conducted under State of Alaska Department of Fish and Game fish resources permit numbers SF2014-196, SF2015-177, CF2015-114, SF2014-123 and SF2015-162. Samples collected by personnel of Utah State University were collected in compliance with Institutional Animal Care and Use Committee Protocol Number 2369, Utah State University, which was approved 17 July 2014 prior to fish sampling work. Samples provided by the University of Alaska museum (UAM), the Bureau of Land Management (BLM) Alaska field office, and the United States Fish and Wildlife Service (USFWS) did not require IACUC approval. No fish were sacrificed specifically for the purpose of this study. In most cases fish were captured, anesthetized using Aqui-S prior to fin clipping, and released in good health to the lake from which they were captured. In some cases samples were collected from fish captured for consumption as part of a native subsistence fishery. No permits were required for collection of water samples.

Primer and probe development

Reference sequences from the mitochondrial genes cytochrome oxidase subunit 1 (COI) and cytochrome b (cytb) for *L. lota, S. alpinus, S. malma, T. arcticus, C. cognatus* and 22 other potentially sympatric fish species from Alaska (Supplementary Table S1) were obtained by sanger sequencing using primers and conditions from Crete-Lafreniere et al. (14), with the exception of cytb for *C. cognatus* which was sequenced using the custom designed primers Cco-F 5'- GCC AGC CTA CGA AAA ACC CA- 3' and Cco-R 5'- TCT ATT CAG CCT GCT ATT GGG A -3'. Reference samples were obtained from the

University of Alaska museum (UAM), the Bureau of Land Management (BLM) Alaska field office, the United States Fish and Wildlife Service (USFWS), and the Utah State University (USU) Fish Ecology Lab. Sequences have been deposited into NCBI Genbank with accession numbers in table S1. In addition, for *C. cognatus* assay design we included reference sequences from Genbank for two *Cottus* species present in coastal Alaska (but not on the North Slope) for which we could not obtain tissue samples: *C. aleuticus* (accession # AF549106), and *C. asper* (accession # AF549105).

Sequences from all species were aligned using Sequencher software (Gene Codes; Ann Arbor, MI). We used the online tool DECEPHIR (15) to select species specific primers that would amplify target species while excluding all sympatric non-target species. For *S. alpinus, and S. malma*, no primer sites could be identified with enough polymorphism for species specificity due to the fact that these two species have extremely little mitochondrial variation (justification for species delineation is based on morphology, ecology, and nuclear DNA (16)). Thus we designed one primer set that can detect both *S. alpinus and S. malma* but cannot distinguish between them, while still excluding all other species. For the remaining species, assays were designed to be species specific. We used ABI primer express software (applied Biosystems; Foster City, CA) to design Taqman® Minor Groove Binding qPCR probes with at least one mismatch to all non-target species, and also to modify primer length to meet melting temperature requirements for Taqman® qPCR where necessary. This resulted in an assay for *L. lota* that amplifies 91 base pairs of cytb, an assay for *C. cognatus* that amplifies 118 base pairs of cytb, an assay for *S. alpinus/S. malma* that amplifies 145 base pairs of cytb, and an assay for *T. arcticus* that amplifies 116 base pairs of COI. Primer and probe sequences are presented in Table 1.

Table 1. Primer and probe sequences and optimized primer concentrations for environmental DNA detection of 5 Alaskan fish species by taqman qPCR. All sequences are 5' to 3'.

Species	Forward primer	Reverse primer	Probe	Primer concentration
Lota lota	GCCGTAATACTCCTTGGCCTT	CAATCGGGTTAGCGGGTGTA	FAM-TGCCCTTGCCCTCTTCT-MGB-NFQ	F - 100nM R - 900nM
Cottus cognatus	GGAGGCGTCCTAGCCCTC	GAGTCCAAAATAGGAATTGGGTCAC	FAM-CATCCATCCTGGTGCTCAT-MGB-NFQ	F - 300nM R - 900nM
Salvelinus alpinus/ Salvelinus malma	CCGCCACAGTACTTCACCTTCTA	AGGCCAAGCAATATAGCTACGAAA	FAM-CCGACAAAATCTCATTCC -MGB-NFQ	F - 100nM R - 600nM
Thymallus arcticus	TGTGGGCTGTTCTGATTACCG	TGCTGGGTCAAAGAAAGTGGTATTA	FAM-CTTGCAGCAGGTATC-MGB-NFQ	F - 600nM R - 600nM

Assay specificity testing

Primer sets were first tested for target amplification, species specificity, and primer dimer formation with SYBR®-Green qPCR. Primer sets were tested on all corresponding target species samples from Table S1, as well on closely related non-target species (for salmonids, all other salmonids, for non-salmonids, all other non-salmonids from Table S1). qPCR reactions included 10 µl Power SYBR®-green Master Mix (Thermo-Fisher; Waltham, MA) 900nM of each primer, 4.4 µl of sterile H_2O and 0.2ng of template DNA in a total reaction volume of 20 µl. Cycling conditions were 95□ for 10 minutes followed by 45 cycles of

95□ for 15 seconds and 60□ for one minute, followed by a melt curve to test for primer dimers and off target amplification.

Next, both primer sets and probes were tested in Taqman® qPCR on all corresponding target species samples and on at least one sample from all non-target species from table S1. qPCR reaction included 10 µl Taqman® Environmental Master Mix (Thermo-Fisher; Waltham, MA), 900nM of each primer, 250nM of probe, 7 µl of sterile H_2O and 0.2ng of template DNA in a total reaction volume of 20 µl. Cycling conditions were 95□ for 10 minutes followed by 45 cycles of 95□ for 15 seconds and 60□ for one minute. Finally, primer concentrations were optimized by running permutations of forward and reverse primers at 100 nM, 300 nM, 600 nM and 900 nM, each in triplicate, on 0.1 ng per reaction of target species DNA. Primer concentrations with the greatest peak fluorescents and lowest C_t value were selected for further work (Table 1).

Assay sensitivity testing

To test assay sensitivity, we ordered MiniGene plasmids for each assay from Integrated DNA Technologies (Coralville, Iowa, USA) containing the assay sequence. The plasmid was suspended in 100 µL of IDTE (10 mM Tris, 0.1 mM EDTA) buffer, linearized by digestion with the enzyme Pvu1, and then purified with a PureLink PCR Micro Kit (Thermo-Fisher; Waltham, MA) following manufacturer protocols. The resulting product was quantified on a qubit fluorometer, and quantity was converted to copy number based on molecular weight (17). For sensitivity testing the product was then diluted to create quantities of 5, 10, 20, 50, and 100 copies/reaction. Each of these quantities was run in qPCR in 6 replicates to determine assay sensitivity.

Field Testing

All Taqman assays were tested on eDNA water samples collected by filtration from the Alaskan North Slope where target species were known to exist based on traditional surveys. Water was pumped through a 10 µm nylon net filter (EDM Millipore; Billerica, Massachusetts) using a peristaltic pump (Geotech Geopump; Denver, Colorado). This testing included samples from 5 known positive locations for *L. lota,* and 20 locations for *T. arcticus* from across the National Petroleum Reserve-Alaska, and *5* locations for C*. cognatus and S. alpinus* from near the Toolik Field Station. In addition, we tested each assay on a minimum of 3 samples collected from locations where each species was known to be absent (outside of the species range). All samples were run in triplicate.

Results

Assay specificity and sensitivity testing

All target samples amplified in SYBR®-Green qPCR with their corresponding primer set. All non-target samples either did not amplify, or amplified >11 C_t later than targets, a range which is suitable for specificity once a probe is added to the assay. The melt curve produced a single sharp peak in all instances indicating no primer-dimers or off target amplification.

All target samples ampified in Taqman® qPCR. Weak amplification was observed for two non-target samples (UAM:10523 and UAM:8066) for the *L. lota* assays, as well as two samples (UAM:6603 and UAM 9910) for the *S. alpinus/S. malma* assay. We aligned the primers and probe to the sequence data generated from these samples, and both had many mismatches in both the primers and the probe, including near the 3' end of primers, and thus true cross amplification is highly unlikely. Upon closer examination of the UAM database, we discovered that these fin-clip samples were collected directly after a target species sample, and thus cross contamination during fin-clip sample collection was highly

likely. We ran alternate samples for these same non-target species, and no amplification was observed. No amplification was observed in any other non-target samples. For all assays, all replicates amplified in qPCR down to 5 plasmid DNA copies per reaction. Thus we are confident that these assays are highly sensitive for detection of the target species.

Field Testing

Amplification was observed in at least one qPCR replicate from all locations where the target species was known to be present with the respective assay. No amplification was observed from any of the samples collected outside of the species range.

Discussion

We developed 4 qPCR assays capable of detecting 5 different fish species present on the North Slope of Alaska. In testing with tissue DNA, all assays were species specific within the North Slope fish community, with the exception of the Sal/Sma-cytb assay, which amplifies 2 closely related species, while excluding all others. In testing with known copy number plasmid DNA, all assays were highly sensitive. All assays also performed in the field for species detection from filtered water samples.

Although our Sal/Sma-cytb assay cannot distinguish between *S. alpinus* and *S. malma*, it should still be practical for most field applications due to the ecology of these two species. *S. alpinus* is almost exclusively found in lakes while *S. malma* is almost exclusively found in streams and rivers. Thus the location of detection should discriminate species in most cases, except where *S. alpinus* eDNA could potentially flow from a lake into an outlet steam. Studies of brook trout *Salvelinus fontinalis* (13, 18) found total eDNA transport distances of <1000m from caged fish in headwater streams, although downstream transport distances are likely to be dependent on many environmental factors such as flows level, stream morphology and eDNA starting concentration.

All of our assays were designed for use on the North Slope of Alaska; however they are likely to work in other arctic locations, other locations in Alaska and Canada, and may potentially work throughout the species' ranges. However, further specificity testing should be conducted if these assays are to be used in locations outside of the North Slope. As the majority of reference samples used to design and test our assays were from the North Slope, it is possible that different haplotypes from other locations could produce false negatives or false positives. Additionally, if research is to be undertaken in locations where species not tested in this study are present, these additional species should be empirically tested for cross-amplification. For example, for the *C. cognatus* assay we designed primers that should, based on primer mismatches, not amplify two other *Cottus* species (*C. aleuticus* and *C. asper)* present in other regions of Alaska. However empirical testing for cross-amplification of these two species should be conducted if studies using our assay are carried out in locations where either of these congeneric species may be present to ensure that they will not cause false positives.

Carim et al. (19) also designed an eDNA assay for detection of *Thymallus arcticus.* Their assay however, was designed for use in the upper Missouri river basin in Montana. Because the fish communities are quite different between the upper Missouri basin and the North Slope of Alaska (the far southern and far northern portions of the species range respectively), both assays are unlikely to be species specific in both communities. Having multiple assays for the same species however is beneficial as it provides managers with marker choices depending on what portion of the species range they wish to utilize eDNA for species detection.

In summary, we provide a valuable eDNA tool for detection of five Alaskan fish species that are important for subsistence fisheries in the North Slope. As North Slope populations of these species are under threat from climate change and petroleum extraction, improving knowledge of their current distributions in a timely manner is vital. eDNA methods will improve detection probability, especially for

rare or low abundance species or populations. Additionally, this molecular tool will help managers obtain these important data more efficiently and economically than methods currently available, which will allow rapid and informed management decisions in the face of impending threats.

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