Northwest Science Notes

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A Non-Invasive Sampling Method for Detecting Non-Native Smallmouth Bass (*Micropterus dolomieu*)

Abstract

The smallmouth bass (*Micropterus dolomieu*) is a cool-water fish species native to central North America. Widespread introductions and secondary spread outside of its historical range have led to new recreational fisheries and associated economic benefits in western United States, but have also resulted in a number of ecological impacts to recipient ecosystems, including threats to Pacific salmon. Management of introduced smallmouth bass populations, now and into the future, relies on accurate detection and monitoring of this species. To address this need, we developed an environmental DNA assay that can detect smallmouth bass DNA extracted from filtered water samples in concentrations as low as 2 mtDNA copies per reaction. Field testing demonstrated that eDNA sampling produced results largely consistent with snorkel surveys, a traditional visual assessment, and gained a few additional positive detections. While this assay is robust against non-target detection, including the only other *Micropterus* in Pacific Northwest streams, largemouth bass (*M. salmoides*), the high genetic similarity within the sunfish family Centrarchidae made it unable to distinguish smallmouth bass from spotted bass (*M. punctulatus*) and some Guadalupe bass (*M. treculii*). The high sensitivity of this method and assay will be particularly useful for identifying the location of non-native smallmouth bass in the Pacific Northwest, quantifying its rate of spread, and aiding management actions.

Keywords: eDNA, qPCR, invasive species, non-invasive sampling, aquatic species monitoring

Introduction

Smallmouth bass (*Micropterus dolomieu*) are members of the sunfish family (Centrarchidae) and are native to large areas of the midwestern US and south-central Canada (Scott and Crossman 1973, Page and Burr 2011). Its popularity as a sport fish has led to widespread introductions, and it is now found in 41 US states and over 20 other countries (Loppnow et al. 2013). While these introductions have formed important recreational

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fishing industries across the country, they have also been associated with rapid declines in prey species (generally small fish and crayfish), with potential cascading effects throughout entire ecosystems (Jackson 2002, Vander Zanden et al. 2004, Loppnow et al. 2013). Within the northwestern US, the consequences of smallmouth bass predation on salmonids is of particular concern (Carey et al. 2011), since smallmouth bass have been shown to consume up to 35% of a single salmon run under certain conditions (Fritts and Pearsons 2004, Sanderson et al. 2009). The predicted warming of water temperatures are likely to benefit smallmouth bass, both creating more suitable habitat and increasing its metabolic efficiency throughout Pacific Northwest streams (Petersen and Kitchell 2001; Lawrence et al. 2014, 2015). Consequently, developing rapid, repeatable, and cost-effective techniques for assessing the distribution of non-native smallmouth bass is critical for targeting conservation and management activities (Brewer and Orth 2015).

Studies have repeatedly demonstrated that environmental DNA (eDNA) is a reliable, efficient and sensitive tool for identifying the presence and delimiting the distribution of aquatic species in low abundance (e.g., Dejean et al. 2012, Goldberg et al. 2013, Wilcox et al. 2013, Sigsgaard et al. 2015, Carim et al. 2016a, McKelvey et al. 2016). To assist management of non-native smallmouth bass, we developed an eDNA assay that detects low concentrations of smallmouth bass DNA extracted from filtered water samples.

Methods

To develop and validate the smallmouth bass eDNA assay, we considered four commonly sequenced regions of the mitome: cytochrome *b* (cyt*b*), cytochrome oxidase I (COI), mitochondrial control region (mtCR), and NADH dehydrogenase subunit 2 (ND2), and two nuclear regions: S7-ribosomal protein (S7-r) and rhodopsin. Of these COI provided the best combination of good geographic coverage of smallmouth bass, sufficient nucleotide differences to distinguish smallmouth bass from most non-target species, and sufficient samples associated both with sympatric bass and other non-target species. We therefore compiled publicly available DNA sequences of a fragment of the cytochrome oxidase I (COI) mitochondrial region of the smallmouth bass and 50 closely related or potentially sympatric taxa (Table 1). The smallmouth bass COI sequences (n = 38) were from fish originating in Alabama (n = 1), California (n = 1), Illinois (n = 2), Kentucky (n = 2), New Mexico (n = 2), New York (n = 2), Ohio (n = 1), and Pennsylvania (n = 1) in the United States; in Ontario (n = 10) and Quebec (n = 10) in Canada; in two locations in Japan (n = 2); and four of unknown origin. We screened these sequences *in silico* using the DECIPHER package (Wright et al. 2014) in R v. 3.0.3 (R Core Team 2013) and obtained candidate primers to amplify smallmouth bass DNA. We aligned the candidate primers with the sequence data in MEGA 6.0 (Tamura et al. 2013) and modified primer lengths (forward: 5'-CAGCTATTTCCCAGTATCAGACACC-3'; reverse: 5'-TTGAGGTTTCGATCCGTAAGRA-3') to optimize annealing temperatures in Primer Express 3.0.1 (Life Technologies; forward: 59.1 °C, reverse: 57.5–59.5 °C). Primers were designed to maximize nucleotide mismatches with non-target species and to amplify a 130-nucleotide fragment of the smallmouth bass COI region. Within this fragment, we designed a FAM-labeled, minor-groove-binding, non-fluorescent quencher (MGB-NFQ) probe (FAM-TTATCGCTCCCAGTCCT-MGBNFQ) that likewise minimized identity with non-targets. We assessed the annealing temperature of the probe in Primer Express 3.0.1 (69 °C) and screened the primer-probe set for secondary structures using IDT OligoAnalyzer (https://www.idtdna.com/calc/analyzer). To evaluate the specificity of the smallmouth bass assay, we compared primer and probe sequences with sequences in the NCBI database using a nucleotide BLAST search.

We then evaluated the eDNA assay *in vitro* by screening DNA extracted from 38 smallmouth bass tissues (from 10 locations) and 30 additional non-target species (Table 2). Smallmouth bass tissues from Oregon were collected under Oregon Scientific Take Permit 19450 for Fish and Freshwater Invertebrates; tissues from Colorado were collected from Cheesman Reservoir under written permission from the Denver Water Board.

All other tissues and DNA used in this study were from archived samples collected for other projects under appropriate state or federal permits. We extracted DNA from tissue using the DNeasy Blood and Tissue Kit (Qiagen, Inc.) following the manufacturer's protocol.

We tested smallmouth bass eDNA assay using a StepOne Plus Real-time PCR Instrument (Life Technologies) in 15-µl reactions containing 7.5 µl of Environmental Master Mix 2.0 (Life Technologies), 900 nM of each the forward and reverse primer, 250 nM of probe, and 4 µl of DNA template (~ 0.4 ng), with the remaining volume composed of PCR-grade water. Thermocycling conditions were as follows: initial denaturation at 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. We optimized primer concentrations using methods outlined in Wilcox et al. (2015), which resulted in a final concentration of 600 nM each for the forward and reverse primer, and 250 nM for the probe. We then tested the sensitivity of the marker by analyzing it with a seven-level standard curve dilution series (31 250, 6 250, 1 250, 250, 50, 10, and 2 copies per 4 µl) made from purified smallmouth bass PCR product diluted into sterile TE. Each dilution was run in sextuples using the aforementioned marker concentrations and cycling conditions. For all qPCR experiments, a reaction was considered positive if the amplification curve crossed the threshold during the exponential phase.

Finally, we tested the assay *in vivo* by analyzing environmental samples collected from 14 sites also surveyed for smallmouth bass via snorkeling (Table 3). Environmental DNA was collected from water samples using the protocol described in Carim et al. (2016b). Briefly, we filtered 5 l of subsurface water through a glass microfiber filter (pore size 1.5 µm) with a peristaltic pump and the filter was folded into quarters and immediately placed in a clean 1 l plastic bag with silica desiccant. We then stored the samples in a cool, dark location until shipping them to the lab for processing (see Carim et al. 2016b for details). After eDNA water samples were collected, snorkel surveys were conducted by two snorkelers on opposite shorelines of each river segment. Snorkelers surveyed a 200 m segment of river immediately upstream of the eDNA sampling location proceeding in an upstream direction and counting all bass observed within the segment. Where bass were present in numbers too large to accurately tally, the count was recorded as "abundant". The snorkel surveys were conducted as part of a larger study examining smallmouth bass occupancy across the Pacific Northwest.

Environmental DNA samples were extracted with the DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA) following a modified protocol (Carim et al. 2016c). Using optimized assay concentrations and the qPCR profile described above, we analyzed these eDNA samples in triplicate reactions and included a TaqMan Exogenous Internal Positive Control (1.5 µl of 10X IPC assay and 0.30 µl of 50X IPC DNA per reaction; Life Technologies) in place of some of the water to screen for inhibition. All analyses included a no-template control where distilled water was substituted for DNA.

Results

The *in silico* analyses revealed that smallmouth bass are divergent from the majority of Centrarchidae species that overlap in range and we do not expect to see cross amplification with largemouth bass (*M. salmoides*), the sole co-occurring *Micropterus* in Pacific Northwest streams (Table 1). However, smallmouth bass differed little from spotted bass (*M. punctulatus*; median difference = 2 nucleotides; range = 0–4 nucleotides) and 25 of 32 Guadalupe bass sequences (*M. treculii*; median difference = 3 nucleotides; range = 2–5 nucleotides) in the 623-nucleotide COI fragment considered for this assay. As a result, all spotted bass and most (25/32) Guadalupe bass sequences were identical to smallmouth bass in the primer-probe region and cross amplification would be expected (Table 1).

The *in vitro* analyses produced positive detections in all smallmouth bass samples, and negative results for all non-target samples. The standard curve analysis resulted in an amplification efficiency of 98.8% (r^2 = 0.997; y-intercept

TABLE 1. Species, number of sequences, and GenBank accession number for DNA sequences used for *in silico* marker development. Also included is the minimum number of nucleotide mismatches between each sequence and the forward primer (F), reverse primer (R), and probe (P).

Name		*n*	GenBank accession numbers	Nucleotide mismatches		
				F	R	P
Smallmouth bass	*Micropterus dolomieu*	38	AB378749, 750; EU524131, 810-828; HQ557267, 268; JN027219-227; KC819888; KF558298; KJ843438-440	0	0	0
Spotted bass	*Micropterus punctulatus*	9	HQ579041; JN027232-235; KJ843420-423	0	0	0
Guadalupe bass	*Micropterus treculii*	25	HQ557528, 529; KJ843386, 387, 393; KJ843396-415	0	0	0
		7[a]	KJ843388-392; KJ843394, 395	4	4	2
Alabama bass	*Micropterus henshalli*	4	KJ843374-377	4	2	2
Florida largemouth bass	*Micropterus floridanus*	12	HQ557526, 527; JN027228, 229; KC684999; KC789544-547; KJ843378-380	4	2	5
Largemouth bass	*Micropterus salmoides*	40	EU524132, 834-838; HQ557265, 266, 285, 286, 411; JN027236-241; KC819886; KF558299-301; KF930132, 133; KJ843416-419; KP112310-317; KR477066, 222; KT248859; KT307155; KX459325	4	2	3
Redeye bass	*Micropterus coosae*	10	HQ579042-044; JN027215-218; KJ843435-437	4	2	2
Shoal bass	*Micropterus cataractae*	9	JN027211-214; KJ843381-385	4	4	2
Suwanee bass	*Micropterus notius*	8	HQ557325; JN027230, 231; KJ843441-445	5	3	3
Choctaw bass[b]	*Micropterus cf. punctulatus*	52	KJ843424-434; KT806130-170	4	3	2
Brook lamprey	*Lampetra planeri*	1	KM286716	9	7	5
Brook trout	*Salvelinus fontinalis*	4	HQ960794; HQ961027; KM287121, 123	3	7	5
Brown trout	*Salmo trutta*	4	KC501168; KM287114, 116, 119	5	6	6
Bull trout	*Salvelinus confluentus*	4	EU522399, 401, 403; EU524365	4	5	5
Channel catfish	*Ictalurus punctatus*	2	EU524685; JN026912	7	5	4
Chinook salmon	*Oncorhynchus tshawytscha*	4	EU524234; FJ164931; HQ712706; KF558293	6	5	5
Common carp	*Cyprinus carpio*	2	KF929811; KM286637	5	4	7
Cutthroat trout	*Oncorhynchus clarkii*	4	EU524198, 201; HQ557150; JN027854	7	5	5
Dollar sunfish	*Lepomis marginatus*	4	JN027021-025	4	3	4
Dolly Varden	*Salvelinus malma*	4	EU522411, 413, 415, 417	4	5	4
European river lamprey	*Lampetra fluviatilis*	1	KM286704	9	7	5
Flathead chub	*Platygobio gracilis*	2	JN028256, 259	5	5	4
Flier	*Centrarchus macropterus*	4	JN024957-960	4	3	5
Freshwater drum	*Aplodinotus grunniens*	2	EU522444; EU523922	3	6	6
Goldeye	*Hiodon alosoides*	2	EU524650; KF929971	4	3	5
Green sunfish	*Lepomis cyanellus*	4	JN026981-984	4	4	3
Kern brook lamprey	*Entosphenus hubbsi*	1	HQ557301	9	7	5
Klamath lamprey	*Entosphenus similis*	1	JN025330	7	7	5
Longear sunfish	*Lepomis megalotis*	4	JN027035-037, 042	2	4	4
Mottled sculpin	*Cottus bairdii*	4	HQ557189; JN025020, 023, 026	5	5	5
Mountain whitefish	*Prosopium williamsoni*	2	HQ557336, 337	5	3	5
Muskellunge	*Esox maquinongy*	4	EU524600-602, 659	7	5	7

TABLE 1. *Continued*

Name		*n*	GenBank accession numbers	Nucleotide mismatches		
				F	R	P
Northern pike	*Esox lucius*	5	EU524589; HM563699; HQ961033; KM224846; KM286646	5	7	2
Olympic mud minnow	*Novumbra hubbsi*	4	HQ557339; JN027849-851	4	5	6
Pacific lamprey	*Entosphenus tridentatus*	2	GU440367; KF918874	7	7	5
Pit-Klamath brook lamprey	*Entosphenus lethophagus*	1	HQ579097	7	7	5
Rainbow trout	*Oncorhynchus mykiss*	4	FJ999086, 088, 090; KM373668	6	5	4
Redear sunfish	*Lepomis microlophus*	4	JN027043-046	4	3	3
River carpsucker	*Carpiodes carpio*	2	JN024862, 865	6	5	4
Sauger	*Sander canadensis*	4	EU524368-071	4	3	5
Shorthead redhorse	*Moxostoma macrolepidotum*	2	JN027298; KF930145	6	6	4
Shovelnose sturgeon	*Scaphirhynchus platorynchus*	2	JN028406, 07	7	6	6
Slimy sculpin	*Cottus cognatus*	3	JN025088, 097, 099	5	5	5
Sockeye salmon	*Oncorhynchus nerka*	4	EU524223, 225; FJ999233; HQ712703	5	6	4
Stonecat	*Noturus flavus*	2	JN027790, 97	6	3	3
Three-spined stickleback	*Gasterosteus aculeatus*	3	EU254634; HQ712384; KR862768	5	6	4
Walleye	*Sander vitreus*	4	EU524374-377	5	3	5
Western brook lamprey	*Lampetra richardsoni*	1	JN026960	8	8	5
Western silvery minnow	*Hybognathus argyritis*	2	EU524071, 074	5	6	5
White sucker	*Catostomus commersonii*	2	HQ579108; KF929688	5	7	4
Yellow perch	*Perca flavescens*	3	JX516993; JX517139, 165	5	5	6

[a]These seven samples are listed in Tringali et al. (2015) Figure 3 as "Lineage B"

[b]Samples in Genbank listed as *Micropterus cf. punctulatus* belong to a proposed species, the Choctaw bass (Tringali et al. 2015).

= 38.776; slope = –3.352), and DNA was detected in all six replicates at two copies per reaction, the lowest concentration tested. *In vivo* tests of the eDNA assay were consistent with the results of the snorkeling surveys. Specifically, smallmouth bass DNA was detected at all sites where smallmouth bass were observed by snorkelers. At two of these sites (MF John Day and Clark Fork Rivers), three bass were observed at each site; at the other three sites (Grande Ronde, Lochsa, and NF John Day Rivers), bass were abundant. Additionally, eDNA assays detected smallmouth bass at four sites where they were not visually observed (Table 3). There were no detections of DNA in the no-template controls.

Discussion

The eDNA assay we describe here efficiently and reliably detects low concentrations of smallmouth bass DNA present in filtered water samples, and will not amplify DNA of any non-target species likely to be present in the Pacific Northwest. While we did not evaluate the correlation between eDNA quantity and smallmouth bass abundance, this assay could be employed to do so in future studies. This correlation has been examined for

TABLE 2. List of species used for *in vitro* screening of the primers and probe in this study. Origin refers to the waterbody for smallmouth bass; for all other samples, origin is listed by state.

Species		*n*	Origin
Smallmouth bass	*Micropterus dolomieu*	2	Cheesman Reservoir, CO
		4	Clark Fork River, MT
		3	Missouri River, MT
		4	River Rock Pond, MT
		3	Seeley Lake, MT
		3	MF John Day River, OR
		13	NF John Day River, OR
		2	James River, VA
		2	Rockfish River, VA
		2	Upper Tye River, VA
Apache trout	*Oncorhynchus apache*	1	NM
Atlantic salmon	*Salmo salar*	1	F*
Bonneville cutthroat trout	*Oncorhynchus clarkii utah*	1	UT
Brook trout	*Salvelinus fontinalis*	1	VA
Brown trout	*Salmo trutta*	1	OR
Bull trout	*Salvelinus confluentus*	1	OR
Chinook salmon	*Oncorhynchus tshawytscha*	1	ID
Chum salmon	*Oncorhynchus keta*	1	OR
Coastal cutthroat trout	*Oncorhynchus clarkii clarkii*	2	OR
Coho salmon	*Oncorhynchus kisutch*	1	OR
Cutthroat trout	*Oncorhynchus clarkii*	1	WA
Dolly Varden trout	*Salvelinus malma*	1	AK
Gila trout	*Oncorhynchus gilae*	1	NM
Lake trout	*Salvelinus namaycush*	1	MT
Lake whitefish	*Coregonus clupeaformis*	1	MT
Lamprey	*Lampetra* sp.	1	OR
Largemouth bass	*Micropterus salmoides*	3	MT
Mountain whitefish	*Prosopium williamsoni*	1	MT
Muskellunge	*Esox maquinongy*	1	MN
Northern pike	*Esox lucius*	1	AK
Pacific lamprey	*Entosphenus tridentatus*	1	WA
Pink salmon	*Oncorhynchus gorbuscha*	1	OR
Pit-Klamath brook lamprey	*Entosphenus lethophagus*	1	OR
Rainbow trout	*Oncorhynchus mykiss*	3	ID, MT, OR
Sauger	*Sander canadensis*	1	WY
Sockeye salmon	*Oncorhynchus nerka*	2	MT, OR
Walleye	*Sander vitreus*	1	WA
Westslope cutthroat trout	*Oncorhynchus clarkii lewisi*	1	MT
Yellow perch	*Perca flavescens*	1	WA
Yellowstone cutthroat trout	*Oncorhynchus clarkii bouvieri*	1	WY

*F refers to a sample of farmed origin; location data not available for this sample.

TABLE 3. Collection information and detection results (Y = detected; N = not detected) of the snorkel surveys and eDNA samples used for *in vivo* validation of the smallmouth bass eDNA marker.

				Smallmouth bass presence	
Location	Latitude	Longitude	Date	Snorkel survey	eDNA sampling
Clark River, MT	47.32260	–114.89276	7/28/16	Y	Y
Lochsa River, ID	46.16267	–115.59100	7/27/16	Y	Y
Grande Ronde River, OR	45.38432	–117.92917	7/19/16	Y	Y
NF John Day River, OR	44.99097	–119.10401	7/20/16	Y	Y
MF John Day River, OR	44.82513	–119.01089	7/21/16	Y	Y
John Day River, OR	44.41832	–119.22548	7/22/16	N	Y
NF Malheur River, OR	43.75676	–118.06197	7/23/16	N	Y
Deschutes River, OR	45.38898	–120.87538	8/9/16	N	Y
Methow River, WA	48.04847	–119.92178	7/31/16	N	Y
Yakima River, WA	46.50535	–120.45632	7/18/16	N	N
Payette River, ID	44.07372	–116.11997	7/24/16	N	N
Salmon River, ID	45.35952	–113.94734	7/25/16	N	N
Selway River, ID	46.09872	–115.54492	7/26/16	N	N
Kootenay River, MT	48.60600	–116.04088	7/29/16	N	N

other species (Wilcox et al. 2016) and has shown promise as an assessment of species abundance in lakes (Lacoursiere-Roussel et al. 2016) and large streams and rivers (Doi et al. 2017). It is important to note that publicly available sequence data for spotted bass shows this species is nearly identical to smallmouth bass across the COI gene (Tringali et al. 2015), and identical in the primer and probe regions (Table 1). Therefore, the assay will lack specificity where these species co-occur, but can also be used to reliably detect spotted bass where smallmouth bass can confidently be assumed to be absent. Publicly available data for Guadalupe bass displayed a range of haplotypes at the COI gene, some of which are nearly identical to smallmouth bass, and others that are highly (8.99–9.31%) divergent (Tringali et al. 2015; Table 1). Thus, this assay will not provide reliable results for detection of smallmouth bass where they co-occur with Guadalupe bass, nor will it provide reliable detections of Guadalupe bass. Although this lack of specificity may limit application of the assay where both species are natively sympatric, it will be a reliable and effective tool for assessing the presence of nonnative smallmouth bass in sensitive regions such as the Pacific Northwest. This assay will facilitate the determination of smallmouth bass occurrence and spread, making it a powerful aid to any management actions.

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