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SPECIAL SECTION: ANGLING FOR DINOSAURS

Primer Designs for Identification and Environmental DNA (eDNA) Detection of Gars

Nicole J. Farley and Adrian A. Vasquez
Department of Physiology, Wayne State University, Detroit, Michigan 48201, USA

Richard Kik IV
Belle Isle Aquarium, c/o Belle Isle Conservancy, 300 River Place Drive, Suite 2800, Detroit, Michigan 48207, USA

Solomon R. David
Nicholls State University, 906 East 1st Street, 112 Beauregard Hall, Thibodaux, Louisiana 70310, USA

Arjun S. Katailiha
Department of Biomedical Engineering, Wayne State University, Detroit, Michigan 48202, USA

Xavier N. Walker
Department of Physiology, Wayne State University, Detroit, Michigan 48201, USA

Jeffrey L. Ram*
Department of Physiology, Wayne State University, Detroit, Michigan 48201, USA; and
Belle Isle Aquarium, c/o Belle Isle Conservancy, 300 River Place Drive, Suite 2800, Detroit, Michigan 48207, USA

Abstract

Gars (family Lepisosteidae) play important roles as apex predators in freshwater ecosystems, helping to balance fish populations. Several gar species are exploited as food and game fish, and some species are classified as vulnerable due to habitat loss. New molecular techniques to detect, monitor, and identify environmental DNA (eDNA) from gars might help inform management and conservation efforts for these interesting fish. The goal of this project was to develop and test PCR primers for gars, using specimens of all seven gar species, which are on exhibit at the Belle Isle Aquarium in Detroit, Michigan. Focusing on the mitochondrial gene for cytochrome oxidase I, we first designed primers to amplify DNA from all species of gars ("universal gar primers") and confirmed their specificity in silico. These primers amplified DNA from all seven species, and species identities were confirmed by sequencing the PCR products. Only one of the three ostensibly Shortnose Gar *Lepisosteus platostomus* specimens sampled exhibited a Shortnose Gar matriline; two other specimens may be Longnose Gar *L. osseus* × Shortnose Gar hybrids. Genus-targeted primers were developed that amplified all *Atractosteus* species and two of four *Lepisosteus* species. Species-specific primers were developed for Longnose Gar, Shortnose Gar, and Tropical Gar *Atractosteus tropicus*. A primer set that targeted Alligator Gar *A. spatula* also amplified DNA from Cuban Gar *A. tristocheus*, but not other species. While the universal gar primers followed by sequencing confirmed the identities of all seven gar species at the Belle Isle Aquarium, PCR with the species-specific primers enabled direct detection of the presence of DNA from the targeted species in the water in which those species had been maintained. Designing these primers is the first step in developing eDNA markers for gars, which in turn could inform the conservation and management of gar populations.

*Corresponding author: jeffram@med.wayne.edu
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Gars (family Lepisosteidae) comprise seven extant species of freshwater fish native to North America, Central America, and Cuba (Dewey 2014). The family includes two extant genera, *Atractosteus* (which comprises Tropical Gar *A. tropicus*, Cuban Gar *A. tristocheus*, and Alligator Gar *A. spatula*) and *Lepisosteus* (which comprises Shortnose Gar *L. platostomus*, Longnose Gar *L. osseus*, Florida Gar *L. platyrhincus*, and Spotted Gar *L. oculatus*). Public opinion of gars has generally been negative, as gars are thought to compete with game fish for food (Scarnecchia 1992). Due to this perception, gars have earned a reputation as harmful, nuisance fish (Scarnecchia 1992; Spitzer 2012). Despite this, fisheries experts recognize the important roles of gars as apex predators in complex ecosystems (Walker et al. 2013). Gars also serve an ecosystem function as hosts for the parasitic glochidia of some native unionid mussels (Trdan and Hoeh 1982; Becker 1983; O'Dee and Waters 2000). Furthermore, some authors have suggested that gars do not have a significant impact on adult game fish populations (Dugas et al. 1976; Walker et al. 2013).

In the genus *Atractosteus*, the Tropical Gar is the most abundant (Aguilera et al. 2002; Alfaro et al. 2008). The Cuban Gar, however, has been listed as vulnerable since 1999 due to population declines resulting from overfishing and anthropogenic habitat alteration, though research on this species is scarce (Comabella et al. 2006). The distribution of Alligator Gars includes southern parts of the Mississippi River basin and parts of Mexico, with established populations in Louisiana and Texas and as far north as Arkansas and Oklahoma. Alligator Gars are classified as vulnerable primarily due to habitat loss across much of their native range (Jelks et al. 2008). Historically, Alligator Gars were found farther north, but they have been extirpated from much of their former northern range (Missouri Department of Conservation 2013). Currently, management and restoration programs are focusing on increasing populations and returning Alligator Gars to their previously known habitats in Illinois, Missouri, Tennessee, Alabama, Arkansas, and Kentucky (Echevarria 2015; Indiana Division of Fish and Wildlife 2016).

Among the *Lepisosteus* species, the Florida, Shortnose, and Longnose gars have stable populations and are not considered to be at risk (NatureServe 2013). However, peripheral populations of Spotted Gars in Ontario and the Great Lakes states are of special concern and conservation interest (Ohio Department of Natural Resources 2012; Committee on the Status of Endangered Wildlife in Canada 2015; David et al. 2015).

Detecting, monitoring, and identifying gars in the environment may help inform management and conservation efforts for vulnerable gar species and for assessing gar populations, regardless of conservation status. Traditional field capture and identification techniques are often costly and labor intensive. An alternative or supplement to traditional field capture and identification techniques is a promising new technique for detecting diverse types of organisms in the environment via the analysis of environmental DNA (eDNA) from water samples (Bohmann et al. 2014). In an aquatic environment, organisms are constantly leaving behind DNA through the sloughing off of cells and the processes of defecation and urination (Klymus et al. 2015). The DNA present in aquatic systems can be amplified by polymerase chain reaction (PCR) technology and the resulting PCR products can then be sequenced, indicating which organisms were present in a given water sample. Such eDNA analysis has been used in fisheries stock analyses (Baldigo et al. 2017) and has also been successful in detecting evidence of invading Asian carps, Silver Carp *Hypophthalmichthys molitrix* and Bighead Carp *Hypophthalmichthys nobilis* (Jerde et al. 2011) and elusive Spotted Gars (Boothroyd et al. 2016). Also, because juvenile gars are difficult to identify to species, another monitoring application for which PCR and sequencing may be useful is the identification of gars that are too immature to be identified by morphological criteria alone (Hardy et al. 2011; Loh et al. 2014; Murphy et al. 2017).

To identify the source of DNA using PCR, two general approaches may be used: (1) amplification of DNA using PCR primers that target conserved sequences which bracket species-specific regions of many organisms ("universal primers"), followed by sequencing of the diverse PCR products (Folmer et al. 1994; Simmons et al. 2016), and (2) amplification of DNA using primers targeted at species-specific regions of DNA ("species-specific primers"), such that only the DNA of a single species of interest will be amplified if it is present (Baldigo et al. 2017). In some cases, primers designed to be species specific may actually amplify the DNA of a few closely related species, in which case identification may depend either on taking into account the species likely to be present in the area or, more precisely, by sequencing the resultant PCR product to determine which species from that environment is present (Boothroyd et al. 2016). The present project aimed to facilitate the use of DNA in monitoring gar populations by designing and testing universal gar primers capable of amplifying DNA from all species of gars and species-specific primers capable of amplifying DNA only from individual gar species.

METHODS

Primer design.— Three types of primers were designed—those specific to the individual gar species, those that would amplify the DNA of multiple species within each genus, and those that would amplify the DNA of all gar species. To design the primer sequences of the cytochrome oxidase I (COI) gene in each of the gar species,

reference sequences were downloaded from GenBank. These sequences were aligned using MEGA7, and variable regions were used as potential species-specific primer annealing sites. Primer 3 was utilized to design hypothetical primers for each species of gar by targeting those variable regions. To achieve species specificity, we aimed to find primer sequence targets that differed from those of nontarget species by at least three to five base pairs, preferably with a mismatch at the final base pair at the 3′ end and/or the adjacent base pair. However, due to a high degree of similarity between the sequences of some gar species, many of the designed primers differed from those of nontarget species by only one or two base pairs. The efficacy of the primers was first tested on known tissues; once species specificity was achieved, the primers were tested on water samples from individual aquariums containing known species of gar and other fishes. Table 1 shows the sequences of the primers that were designed and empirically tested for this study, including primers designed to amplify the DNA of all Lepisosteidae, all *Atractosteus* spp., all *Lepisosteus* spp., and four of the seven individual gar species (Longnose, Shortnose, Tropical, and Alligator gars). Additionally, we tested the standard Folmer primers (Folmer et al. 1994; Hebert et al. 2003; Sparks and Smith 2004), which work on many fish species. Additionally, we searched in silico with BLAST for possible false-positive (species that matched both forward and reverse primers) and false-negative (sequences for target gar species in GenBank that differed from the designed primers) amplification targets among all GenBank sequences.

DNA isolation.— Tissue samples (fin clips) were collected from each of the seven gar species at the Belle Isle Aquarium, preserved in 90% ethanol, and stored at −20°C. Tissues were taken from three different Shortnose Gars and one fish from each of the other species. In addition, the tissues of several nontarget species (Lake Sturgeon *Acipenser fulvescens*, Bowfin *Amia calva*, and Round Goby *Neogobius melanostomus*) were similarly collected as nontarget negative controls. Tissue was also obtained from a juvenile gar collected in a lagoon of Belle Isle (Detroit River) as bycatch in another study. The DNA was purified from the tissue samples using DNeasy Blood and Tissue Kits (Qiagen, Hilden, Germany), as in previous studies from our laboratory (Vasquez et al. 2016). Briefly, small (~25-mg) pieces cut from the tissue samples were placed in 180 μL of Buffer ATL (catalog number, 19076; Qiagen). Proteinase *K* (20 μL) was then added, the mixture was incubated at 56°C for 2–18 hours, and DNA was purified from the resultant incubate with a spin column procedure according to the manufacturer's protocol (2006; https://www.qiagen.com/us/resources/resourcedetail?id=6b09dfb8-6319-464d-996c-79e8c7045a50&lang=en). Finally, 50 μL of Low tris–EDTA (Invitrogen, Carlsbad, California) was added, and the resultant DNA solution was stored at –20°C until used in the PCR reactions.

DNA amplification and sequencing.— Amplification of all DNA samples was done using a standard PCR protocol described by Vasquez et al. (2016), but with annealing temperatures adjusted to the specific primer set. The annealing temperatures varied among experiments based on the predicted annealing temperatures provided by Primer 3, and in some experiments we tried other temperatures to increase (or decrease) the specificity of primer annealing. For convenience in monitoring reactions and

TABLE 1. Primers developed in this study. Specificity indicates the design target. Nontarget species whose DNA was amplified in some cases are described in the text.

Specificity	Primer[a]	Sequence	Annealing temperature (°C)	Product length (bp)
Lepisosteidae	gsCOI62BF	CAGCCTGGAACCCTCCTTGG	57	263
	gsCOI324BR	ATGCTCCTGCGTGTGCGAG		
Atractosteus	AtrCOI133F	CCAGTTATAATTGGCGGGTTC	57	325
	AtropCOI457R	TGCGGGTGGTTTTATGTTTAGG		
Lepisosteus	LepCOI242F	CCTTCATTTCTTCTACTCCTA	57	293
	LepCOI534R	AGGACTGGCAGGGAGAGCAATAG		
Longnose Gar	LnCOI366F	CAGTTGATCTAACCATTTTCTCCCTT	59	252
	LnCOI518R	CGTAATACCTGCGGCTAGGA		
Shortnose Gar	SnCOI366F	TGATCTAACCATTTTTTCCCTG	59	252
	SnCOI518R	TGTAATACCTGCGGCTAGGA		
Tropical Gar	TrgCOI227F	TTCTCCCACCTTCCTTCCTG	62	215
	TrgCOI441R	TGCGGGTGGTTTCATGTTC		
Alligator Gar	AspCOI428F	TAAAACCACCCGCAGCTTCC	59	159
	AspCOI587R	CCTGCAGGGTCAAAGAAGGT		

[a]A final letter F designates a forward primer, a final letter R a reverse primer.

products, we ran the reaction on a BioRad iCycler real-time thermocycler with Universal SYBR Green Supermix (catalog number, 1725270). The results are presented as the relative fluorescence of each sample at the last amplification cycle, where fluorescence is relative to the internal ROX reference dye in the SYBR Green Supermix, which enables machine-to-machine comparisons to be made. This method of presenting the data shows a high relative fluorescence for successful amplification and near-zero relative fluorescence when no amplification has occurred (e.g., negative controls such as pure water or the DNA of nontarget species for successful species-specific primers).

All experiments included both positive and negative controls to ensure the reliability of the PCR results. The PCR products were sequenced to confirm their specificity and to detect false positives. Sanger sequencing was performed by Genewiz (South Plainfield, New Jersey), and the sequences were then compared towith reference sequences found in the GenBank database to determine their likely identity.

Additional procedures for Shortnose Gars.— Because the sequence of the first Shortnose Gar DNA extract that we tested did not confirm the visual identification (see Results), additional "blind" procedures were adopted to test DNA from two additional Shortnose Gars and to retest another tissue sample from the original fish. Fin clips in coded vials (i.e., fish samples unidentified to the DNA analysts) were provided from the Belle Isle Aquarium from the original Shortnose Gar specimen, two other Shortnose Gars, and negative control specimens (a Spotted Gar and a Lake Sturgeon). The three Shortnose Gars from the aquarium's collection were from Green Bay, western Wisconsin, and Kansas, respectively. All samples were transported in 90% ethanol and processed the same as the original specimens. Extracted DNA was amplified using our universal gar primer set and the PCR products were sequenced. The identity of the extracts was determined using BLAST, and only then were the codes identifying the fish revealed.

Aquarium water analysis.— Water samples were collected from tanks in the Belle Isle Aquarium where gars had been housed as well as from a tank without gars (Table 2). Using the protocol of Mahon et al. (2010), samples were collected in 473-mL containers. As a field blank, a bottle containing deionized water was brought to various aquarium locations and processed similarly to the tank water samples. Two hundred fifty mL of each water sample was filtered through a 1.2-μm polycarbonate track-etched filter on a vacuum filtration device (a hand-operated vacuum pump or a modified NUK Expressive Single Electric Breast Pump), and then filters were transferred to Longmire's preservation buffer and stored at room temperature until DNA extraction. DNA was extracted from the filters according to a protocol described by Turner et al. (2014).

RESULTS

The Folmer primers (Folmer et al. 1994; Hebert et al. 2003) did not amplify DNA from any gar, despite testing with a range of annealing temperatures, necessitating the design of primers that targeted the COI barcode region of all Lepisosteidae. These primers could then be used to amplify all gar species for verifying the barcodes of individual fish. As shown in Figure 1, our Lepisosteidae primers (Table 1) amplified the DNA of all gar species. The identities of all amplified species were verified by PCR sequencing, confirming that the amplified sequences (accession numbers MF357699, MF357700, MF357702, and MF357704–MF357707) matched the expected reference database sequence. With regard to specificity, the Lepisosteidae primers did not amplify Lake Sturgeon DNA (Figure 1), nor did they amplify Round Goby DNA (data not shown). Bowfin DNA intermittently (two PCRs out of more than five tests) gave a PCR product. However, in both cases the PCR product yielded sequences that could not be interpreted and did not match any known COI sequence by >95%. We also developed a primer set specific to *Atractosteus* (Figure 2), and although we have not developed a primer pair that specifically amplifies the DNA of all *Lepisosteus* spp., one primer pair amplifies that of two of the four species in the genus (Longnose Gar and Shortnose Gar; Table 1).

We were successful in designing species-specific primers that amplified the DNA of Shortnose, Longnose, and Tropical gars (Figure 3A–C). However, the Alligator Gar is very closely related to the Cuban Gar, and at this time the best "Alligator Gar-specific" primer is a primer pair that amplifies the DNA of both Alligator and Cuban Gars (Figure 3D).

TABLE 2. Fish species in the aquarium tanks that were sampled.

Tank	Species
2	Tropical Gar
	Spotted Gar
	Longnose Gar
	Shortnose Gar
	Florida Gar
5	Alligator Gar
	Tropical Gar
	Longnose Gar
9	Round Goby
	White Perch *Morone americana*
	Goldfish *Carassius auratus*

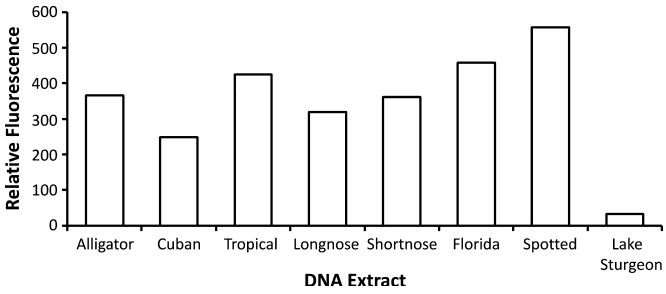

FIGURE 1. PCR results for amplification using the family-specific primers gsCOI324BR and gsCOI62BF. The data show relative fluorescence at the end of 40 cycles on a real-time BioRad thermocycler. All species of gar exhibit high relative fluorescence compared with Lake Sturgeon.

We analyzed all of the above primers in silico to determine whether they were likely to amplify the DNA of species other than those which they targeted (false positives; see Table 2). While one or the other of each primer pair was identical to sequences from several species of fish, in no case were both primers identical to sequences from the same nontarget species. Furthermore, out of 56 sequences in GenBank among all Lepisosteidae, there were only a few instances of sequences from gars that were not matched by the designed primers (potential false negatives; see Table 3).

Shortnose Gars with Longnose Gar COI Barcodes

Although the data in Figure 1 and its associated sequence data demonstrate the specificity of the designed primers and the expected sequence for Shortnose Gars, that result was, in fact, not the one initially obtained with a visually identified Shortnose Gar in the Belle Isle Aquarium's collection. The DNA of the first Shortnose Gar that we tested was amplified by the Lepisosteidae primers, but its PCR product sequence was identical to Longnose Gar reference sequences. To ensure that there had not been cross-contamination or other error resulting in the unexpected sequence, Belle Isle Aquarium provided five additional fish samples whose identities were blinded but included tissue from the first "Shortnose Gar" fish, two other Shortnose Gars, a Spotted Gar, and a Lake Sturgeon. The sample from the first Shortnose Gar again had the sequence expected of a Longnose Gar, as did that from one other Shortnose Gar (accession numbers MF357701 and MF357703). However, the Shortnose Gar in the Belle Isle Aquarium from western Wisconsin had the sequence expected for a Shortnose Gar (accession number MF357702). The Spotted Gar was correctly identified, and the Lake Sturgeon sample failed to amplify with the Lepisosteidae primers.

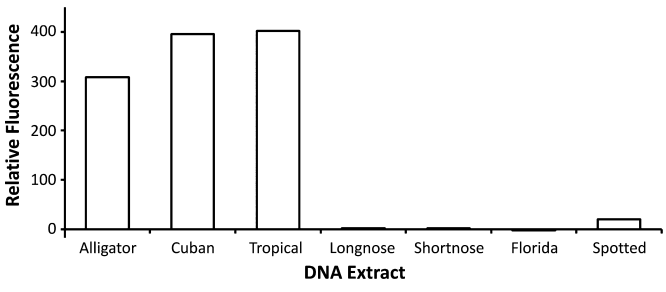

FIGURE 2. PCR results for amplification with the *Atractosteus*-specific primers AtropCOI457R and AtrCOI133F. Only members of the genus *Atractosteus* exhibited amplification.

Identification of a Juvenile Gar

The primers designed for this project were used to identify the species of a wild-caught juvenile gar that was too small to be identified to species by morphological criteria alone. We hypothesized that this fish was a Longnose Gar because that is the predominant gar species in the area, and Longnose Gar–specific primers confirmed this (Figure 4). DNA from the fish was also amplified by the Lepisosteidae primers, and sequencing the PCR products confirmed the sequence (accession number MF357708) as that of a Longnose Gar (100% agreement with Longnose Gar reference sequences), differing by 2% from Shortnose Gar and 5% from Spotted Gar sequences.

DNA Extracted from Belle Isle Aquarium Tanks

To determine whether these primers could be useful in an eDNA context, they were tested on water collected from aquarium tanks in which gars or other fishes were displayed at the Belle Isle Aquarium. Using eDNA capture and extraction protocols, Lepisosteidae primers and species-specific primers were tested using water samples taken from three tanks, two of which had various gar species in them and one of which did not (see Table 2). As shown in Figure 5A, amplification of DNA extracted from water from the gar-containing tanks showed positive PCR results with the Lepisosteidae primers, whereas the tank which contained no gars gave a signal only slightly above that of water alone and of pure water that had been subjected to the same DNA extraction procedure as the tank samples ("process water"). Sequencing the PCR products of the Lepisosteidae primers from tank 5 yielded complex chromatograms indicating that the sequences were from gars, but they difficult to identify to specific species due to the multiple bases at polymorphic positions (data not shown). The results of a separate experiment in which water from tank 5 (containing Longnose, Alligator, and Tropical gars) was sampled again and subjected to PCR with the Lepisosteidae, Longnose, Shortnose, Tropical, and Alligator–Cuban Gar primers are shown in Figure 5B. The DNA from this tank was successfully amplified by all primers except the Shortnose Gar–specific ones.

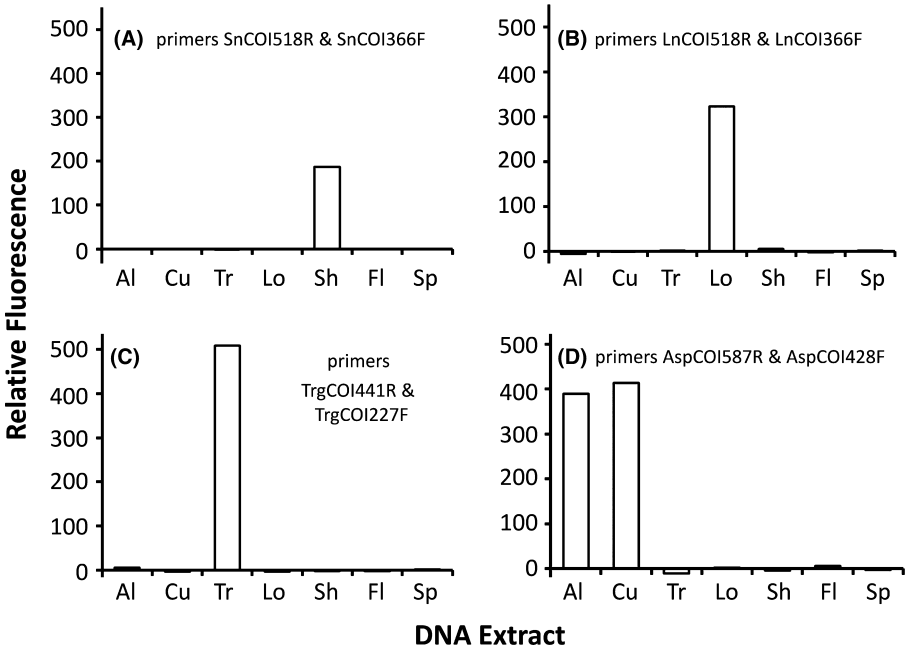

FIGURE 3. PCR results for amplification with various species-specific primer sets: **(A)** Shortnose Gar–specific primers SnCOI518R and SnCOI366F), **(B)** Longnose Gar–specific primers LnCOI518R and LnCOI366F, **(C)** Tropical Gar–specific primers TrgCOI441R and TrgCOI227F, and **(D)** "Alligator Gar–specific" primers AspCOI587R and AspCOI428F. Species abbreviations are as follows: Al = Alligator Gar, Cu = Cuban Gar, Tr = Tropical Gar, Lo = Longnose Gar, Sh = Shortnose Gar, Fl = Florida Gar, and Sp = Spotted Gar.

TABLE 3. Results of in silico tests for false positives and false negatives for the primers developed for this study.

		False positive analysis		Possible false negatives
Specificity	Primer	Number of nontarget taxa (100% match)	Number of nontarget taxa in common for F and R primers	Number of target taxon sequences not matched (not matched/total)
Lepisosteidae	gsCOI62BF	1	0	F: 1*/56, R: 0/56
	gsCOI324BR	20		*A single Shortnose Gar sequence differs by one base pair
Atractosteus	AtrCOI133F	1	0	F: 4*/15, R: 5*/15
	AtropCOI457R		2	*Multiple mismatches for Tropical Gars and one Cuban Gar
Lepisosteus	LepCOI242F	3	0	For Longnose Gars: 1/12; all 8 Shortnose Gars differ by one base pair from primer
	LepCOI534R	0		
Longnose Gar	LnCOI366F	7	0	1/12
	LnCOI518R	2		
Shortnose Gar	SnCOI366F	0	0	0/8
	SnCOI518R	28		
Tropical Gar	TrgCOI227F	4	0	2*/5
	TrgCOI441R	0		*R primer differs by 1 or 2 base pairs
Alligator Gar	AspCOI428F	3	0	0/8
	AspCOI587R	>500		

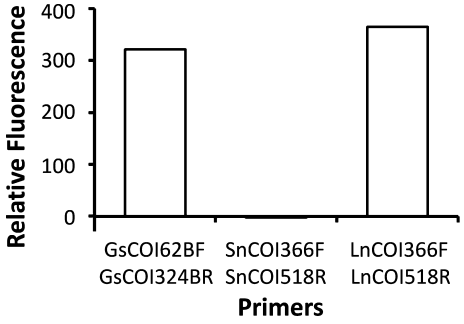

FIGURE 4. Analysis of the species identity of a juvenile gar. The three columns show the results for DNA extracted from the animal and subjected to PCR using Lepisosteidae, Shortnose Gar, and Longnose Gar primers.

DISCUSSION

The goal of this study was to develop species-specific primers for all seven species of gar. We developed species-specific primers for three species in two genera, Longnose Gar and Shortnose Gar (*Lepisosteus*), and Tropical Gar (*Atractosteus*). Primers with broader ranges, including ones for two species, the genus *Atractosteus*, and the family Lepisosteidae, were also developed. The reason that the Folmer primers did not work on gars is likely related to the large number of mismatches of the forward primer, which in comparison with Spotted, Longnose, Florida, Alligator, and Tropical gars has mismatches at nucleotides 3, 6, 12, 18, and 21, resp ectively. The reverse primer also has mismatches at bases 6 and 12.

The Lepisosteidae primers may be considered universal gar primers. The design of this family-specific primer set should make it amplify only the DNA of Lepisosteidae and, indeed, the DNA of nontarget species such as Lake Sturgeon (Figure 1) and Round Goby was not amplified by it; the failure of any other likely species to be amplified by these primers was confirmed by an in silico search of other potential target species. The DNA of Bowfins, which are classified with gar in the Holostei infraclass, appeared to be amplified by the Lepisosteidae primers; however, this occurred only sporadically, and our inability to recover Bowfin sequences under conditions in which correct gar sequences are recovered suggests (1) that the primers are indeed specific to Lepisosteidae and (2) that when Lepisosteidae primers return PCR products those products must be sequenced to confirm the presence of gars. The use of genus-specific and species-specific primers and the sequencing of PCR products can provide additional confirmation.

At the genus level, the design of primers that amplified the DNA of all *Atractosteus* species and no *Lepisosteus* species was achieved, but not a primer set that would amplify the DNA of all *Lepisosteus* species. A topic for future study could be development of a primer set that can amplify all *Lepisosteus* DNA without also amplifying *Atractosteus* DNA.

Although our goal was to develop primers specific to all seven gar species, up to this time we have focused on Longnose, Shortnose, Tropical, and Alligator gars. Primers designed specifically for Spotted and Florida gars have not been tested. The primers that were designed to amplify only Alligator Gars (the Asp primers) amplify DNA from both Alligator and Cuban gars. While these Alligator–Cuban Gar primers were not species-specific, the geographically distinct distributions of Alligator Gars and Cuban Gars make cohabitation unlikely except under captive conditions. Thus, the Alligator–Cuban Gar primer set may be useful if the locality of the species is taken into account. Despite our not having developed species-specific primers for Spotted and Florida gars, DNA from those

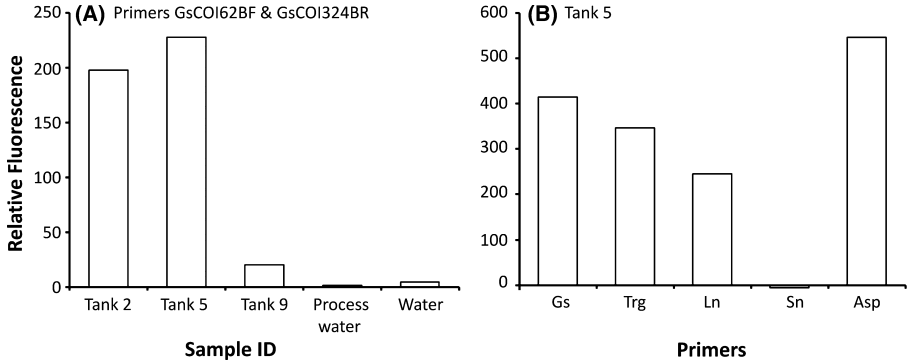

FIGURE 5. Detection and identification of waterborne gar DNA in Belle Isle Aquarium tanks. Panel **(A)** shows the results for tanks 2, 5, and 9 (see Table 2 for the species in each tank) tested with the GsCOI62BF and GsCOI324BR gar family primers. Also included are data from a "process water" sample (pure water subjected to the same DNA extraction method as the tank water) and a standard PCR negative control (pure water in place of template DNA). Panel **(B)** shows the results for another tank 5 sample with primers for Lepisosteidae (Gs) and specific gar species (Trg = Tropical, Ln = Longnose, Sn = Shortnose, and Asp = Alligator–Cuban), confirming the presence of Tropical Gar, Longnose Gar, and Alligator Gar and the absence of Shortnose Gar. The experiment also included appropriate positive and negative controls for all primers (not shown).

species can nevertheless be amplified by the Lepisosteidae primers and then identified by sequencing the PCR products.

In silico tests of whether the designed primers might fail to amplify the DNA of gars with known sequences that differ from the primers (false negatives) revealed several instances of sequences that were not identical to the designed primers. In one particular case, a sequence of the whole mitochondrial genome of a Longnose Gar (accession number DQ5364230) differed from 11 other Longnose Gar COI barcode sequences in GenBank, all of which were >99% identical to the barcode sequence that we obtained for Longnose Gars in the present study and whose primer regions were identical to the Lepisosteidae and Longnose Gar primers we designed. In comparison with many other Longnose Gar sequences, the Longnose Gar specimen from which the whole genome sequence was obtained seems to be from a much different strain of Longnose Gar. Tropical Gars also seem to have a number of variants that might prove elusive with the species-specific primers but whose DNA would nevertheless likely be amplified by the Lepisosteidae primers.

The experiments on tank water are considered to be interim steps toward the goal of using these primers to detect gar DNA in environmental waters. The experiments on water from the display tanks demonstrated that sufficient eDNA can be isolated to correctly identify the gar species in the tanks. Sequencing chromatograms of the PCR products of the universal gar primers on tank 5 indicated multiple bases at polymorphic positions, consistent with multiple species of gar being present in the tank. Sequencing such samples on a "next-generation" platform would be a way to identify all of the various DNA sequences that were amplified by these primers and might be a technique that can be applied to eDNA analysis in the future.

Despite the visual identification of three specimens in the Belle Isle Aquarium collection as Shortnose Gars, only one of them has COI sequences whose greatest alignment is with Shortnose Gar reference COI sequences, while the other two have COI sequences that align better with Longnose Gar reference sequences. Longnose Gar × Shortnose Gar hybrids have been suggested in previous studies of mitochondrial matrilines (Sipiorski 2011); however, to determine parentage, sequencing nuclear genes is necessary for assessing both the maternal and paternal genetic contributions (von der Heyden and Connell 2012; Wright et al. 2012). The mitochondrial gene that we utilized only informs us about the specimen's matriline.

Finally, identification of a juvenile gar that would otherwise have been difficult to identify to species demonstrated a technique that might be valuable for assessing populations of young gars. We hypothesized that the juvenile was a Longnose Gar, which is the most common species around Belle Isle. Another possibility had been Spotted Gar, since eDNA studies in nearby Canadian waters have suggested that Spotted Gars are present both upriver (i.e., in the Thames River, a tributary to Lake St. Clair, which is the source of the Detroit River) and downriver in Lake Erie (Boothroyd et al. 2016). In the present study, Spotted Gars might have been identified by amplification and sequencing of the product of the Lepisosteidae primers. Instead, the Longnose Gar–specific and Lepisosteidae PCRs followed by sequencing both indicated that the juvenile was a Longnose Gar.

The long-term goal of applying these primers to the detection, monitoring, and identification of gars is thus supported by our preliminary experiments on the detection of gar DNA in display-tank water at Belle Isle Aquarium, identification of possible hybrids in the aquarium's collection, and the use of the primers to identify a juvenile gar. Application of these primers and other gar species–specific primers that may be developed by similar techniques to eDNA is a logical next step toward their possible use in gar management and restoration.

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