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Effectiveness of DNA barcoding for identifying piscine prey items in stomach contents of piscivorous catfishes

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Abstract Introduced predators pose ecological impacts upon prey species and receiving ecosystems. Understanding such ecological interactions creates technical challenges including species-specific identification of partially digested prey items in the stomachs of piscivorous predators. We present the first evaluation of DNA barcoding to identify piscine prey in the stomachs of North American catfishes (Family Ictaluridae). Fish prey items of non-native Blue Catfish *Ictalurus furcatus* and Flathead Catfish *Pylodictis olivaris* were obtained by gastric lavage and ranked as lightly, moderately, or heavily digested. We used an established cocktail of universal fish primers (FishF2_t1, FishR2_t1, VF2_t1, and FR1d_t1) to amplify the cytochrome oxidase I (*COI-3*) region of mitochondrial DNA from these samples. Amplification products were subjected to Sanger sequencing, and edited sequences were compared to entries in GenBank. Eighty-six percent of the sequences generated for lightly or moderately digested samples and 66 % of those for heavily digested samples could be assigned to the species level based on similarity with archived *COI-3* sequences. While traditional morphological identification led to species-level identification of 65 % of fish prey items, addition of DNA barcoding resulted in identification to species of 88 % of fish prey items overall. Diet items identified by DNA markers included anadromous Striped Bass *Morone saxatilis* and herrings and shads *Alosa spp*. that are the focus of fishery restoration programs in these rivers. We found DNA barcoding to be an efficient and cost-effective addition to diet studies of non-native predators.

Keywords Non-native catfishes · Predation · Clupeidae · DNA barcoding · MtDNA · Universal fish primers

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Introduction

Piscivorous populations are usually limited by classical predator-prey dynamics and other ecological regulatory processes (Pombo et al. 2005; Beauchamp et al. 2007). However, when introduced into a non-native ecosystem, invasive predators can impose dramatic impacts upon native fish assemblages (Zaret and Paine 1973; Curio 1976; Stein 1979; Oguto-Ohwayo 1990; Cambray 2003). Determining the scale of these predatory impacts upon native species is highly important in assessing impacts upon the structural diversity and functional integrity of an ecosystem (Simberloff 2005); diet studies often are used to understand these critical trophic relations (Garvey and Chipps 2012). One major obstacle to

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effectively using a diet study to assess these interactions is identifying highly digested fish prey items in the stomachs of predatory fishes (Hardy et al. 2010; Carreon-Martinez et al. 2011; Jo et al. 2013).

Blue Catfish, *Ictalurus furcatus*, and Flathead Catfish *Pylodictis olivaris* were introduced into tidal rivers of Virginia in the eastern United States during the 1970s to create recreational and commercial fisheries (Jenkins and Burkhead 1994; Greenlee and Lim 2011). Suitable habitat and abundant prey resources supported rapid population growth and expansion of range for Blue Catfish (Greenlee and Lim 2011; Schloesser et al. 2011), although little is known of the population dynamics of Flathead Catfish. Both non-native catfish species exhibit ontogenetic shifts to piscivory, often growing to adult sizes in excess of 45 kg (Jackson 1999; Brown et al. 2005; Baumann and Kwak 2011; Greenlee and Lim 2011; Schloesser et al. 2011). Past diet studies in other drainages have shown that these non-native catfishes create negative impacts on native species of fisheries management concern, such as American Shad *Alosa sapidissima*, Blueback Herring *Alosa aestivalis*, and Alewife *Alosa pseudoharengus* (Guier et al. 1984; Chandler 1998; Pine et al. 2005; Schloesser et al. 2011). An area of technical difficulty facing those trophic studies was identification of highly digested piscine prey items. That is, although many fish prey items were effectively identified using morphological analysis, the approach proved ineffective when identifying highly digested samples of species with similar morphometric characteristics, e.g., clupeids, centrarchids, and cyprinids. Partially digested unidentified fish (PDUF) items generally have been classified as "unidentified fish", which often constitute 25 %-30 % of prey items (Guier et al. 1984; Chandler 1998; Baumann and Kwak 2011; Schloesser et al. 2011). This outcome represents a considerable amount of valuable information lost, can obscure observation of predation upon rare species, and can yield biased results if differential digestion is causing certain species to become unidentifiable more quickly than others (Hyslop 1980). Hence, demonstration of another cost-effective identification technique is warranted so that fisheries scientists can fully assess the trophic impacts of non-native catfishes.

Application of DNA barcoding utilizes species-specific DNA sequences in a taxonomic database (Herbert et al. 2003; Kress and Erickson 2012) as a reference for subsequent identification and analysis (BOL 2015; Ivanova et al. 2007). Genetic identification of fishes is best accomplished through amplification and sequencing of the cytochrome oxidase I – subunit 3 (*COI-3*) gene of mitochondrial DNA (mtDNA; Ivanova et al. 2007; Weigt et al. 2012). The *COI-3* region can be amplified with either universal or specially constructed species-specific primers using the polymerase chain reaction (PCR; Ivanova et al. 2007). DNA sequencing results then are compared to entries in the Barcode of Life (BOL) or GenBank databases using the Basic Local Alignment Search Tool (BLAST; Kress and Erickson 2012). DNA barcoding has proven useful in fisheries applications, including identification of stomach contents and diet diversity of piscivorous predators in the Laurentian Great Lakes (Carreon-Martinez et al. 2011), determining stomach contents of invasive Lionfish *Pterois sp.* in Florida (Cote et al. 2013), identifying members of complex fish assemblages on Australia's coral reefs (Ward et al. 2005), and characterizing the diets of Giant Squid *Archituthus dux* (Deagle et al. 2005), Largemouth Bass *Micropterus salmoides* (Jo et al. 2013), and other pelagic predators (Smith et al. 2005). Here, we assessed whether DNA barcoding is an effective and cost-efficient technique for identifying otherwise unidentifiable prey items in the stomach contents of non-native ictalurids in the context of an ecological invasion impact assessment study.

Methods

Sample area and field collection of specimens

Non-native catfish were collected during fall 2012, summer 2013, spring 2014, and summer 2014 from the tidal James, York, and Rappahannock river systems (Fig. 1) using low-frequency electrofishing. Pulsed gastric lavage (Waters et al. 2004) was used to non-lethally collect stomach contents from 740 Blue Catfish and 126 Flathead Catfish. Of these 966 non-native catfish, 508 fish prey items were obtained, frozen, and maintained at −20 °C. Using modified methods of Carreon-Martinez et al. (2011),

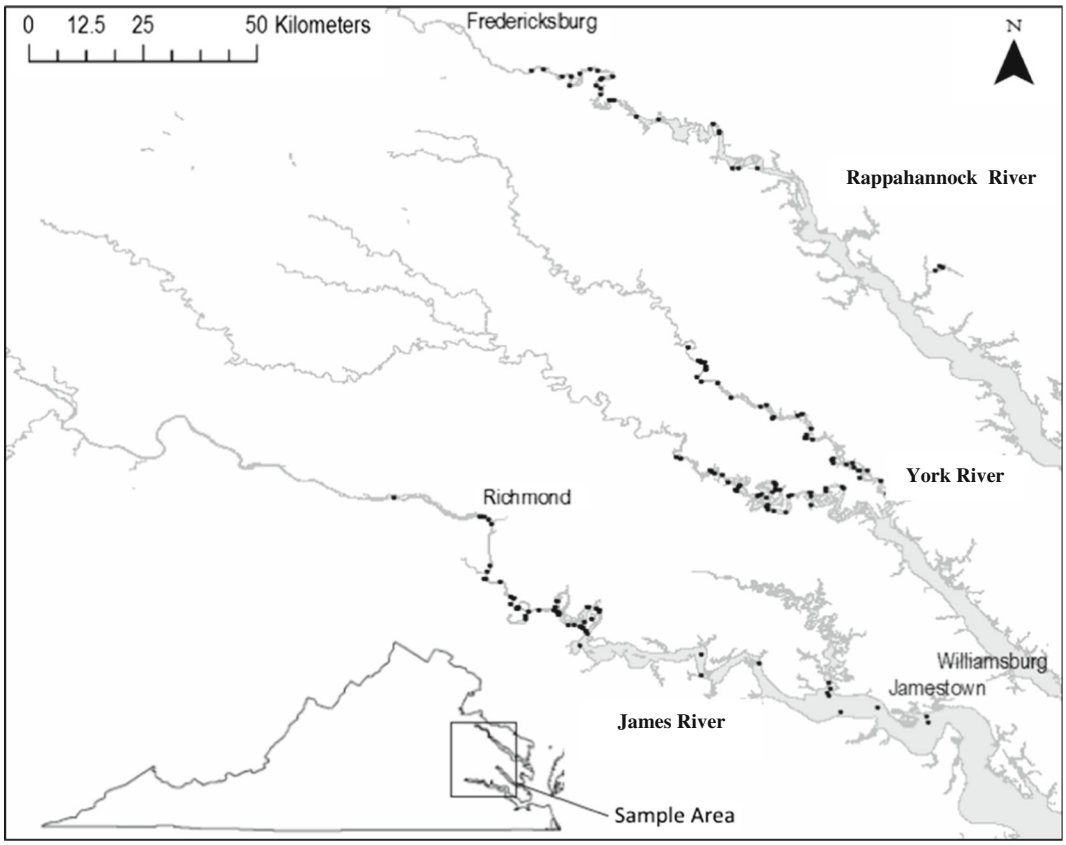

Fig. 1 Collection sites for non-native catfishes on the James, Mattaponi, Pamunkey, and Rappahannock rivers in Virginia, USA

fish prey were ranked as: 1. Lightly digested and easily identified to the species level, 2. Moderately digested, maintaining key morphological characteristics such as spines or a well-formed skull, or 3. Heavily digested with very little tissue remaining.

Lysis and DNA extraction

Prior to lysis, samples were defrosted and rinsed using ethanol to remove any chyme that may have remained from the host stomach. Using sanitized tweezers and scalpel, a 10–25 mg sample of tissue was excised and transferred to a sterile microcentrifuge tube. Samples were incubated with lysis reagents at 56 °C, and DNA extraction was carried out using the DNEasy Blood & Tissue kit (Qiagen) using the manufacturer's protocols.

DNA amplification and sequencing

Targeted *COI-3* mitochondrial DNA sequences were amplified using a cocktail of four primers (FishF2_t1, FishR2_t1, VF2_t1, and FR1d_t1) and protocols developed for fish by Ivanova et al. (2007) with minor modifications. PCR reactions had a total volume of 12.5 μL, which included 6.25 μL of 10 % trehalose, 2.00 μL of ultrapure water, 1.25 μL 10xPCR buffer (10 mM KCl, 10 nM $(NH_4)_2SO_4$, 20 mM Tris-HCl (ph 8.8), 2 mM $MgSO_4$, and 0.1 % Triton X-100), 0.625 μL $MgCl_2$ (50 mM), 0.125 μL of each primer (0.01 mM), 0.0625 μL of each dNTP (10 mM), 0.0625 μL of *Taq* DNA polymerase (New England Biolabs) and 2.0 μL of DNA template (mean conc. 74 μg/mL). The PCR reaction was conducted on a BioRad MyCycler with the following thermocycling conditions: initial denaturation at 94 °C for 2 min; followed by 35 cycles of 94 °C for 30 s, 52 °C for 40 s, and 72 °C for 1 min; with a final extension step at 72 °C for 10 min.

PCR amplification products were sequenced using the BigDye Terminator Cycle Sequencing Kit v 3.1 on an ABI3730 DNA sequencer at the Smithsonian Environmental Research Center (SERC) or the Virginia Bioinformatics Institute (VBI). Sequencing

reactions were initiated using the C_FishF1t1 or C_FishR1t1 primers of Ivanova et al. (2007).

Data analysis

Mitochondrial COI sequences were visualized and edited using Bioedit (Hall 2013) and Sequencher v4.5 (Gene Code Corporation). Edited sequences then were identified to species using the BLAST program (Altschul et al. 1990) and the National Center for Biotechnology Information database (NCBI 2007). Species were identified based on high quintile scores from % Identification, % Query Cover and Maximum Identification scores as previously demonstrated (Carreon-Martinez et al. 2011; Jo et al. 2013). DNA sequences of less than 300 base-pairs were not analyzed (Martinez et al. 2011; Jo et al. 2013).

Logistic regression analysis was used to determine the binary probability of successfully identifying prey as a function of predator species (Blue Catfish or Flathead Catfish) and level of digestion (lightly, moderately, and highly digested). Logistic regression analysis was conducted using a generalized linear model with a logit link function and a binary error distribution (Goodnight et al. 1982). Predator species and level of digestion were initially incorporated as nested explanatory variables. Samples were pooled among predator species after no significant difference was detected. Level of digestion was a significant factor, so a *post-hoc* Tukey's multiple contrast test was used to determine which levels of digestion led to significant differences in frequency of identification (Zar 1999); an alpha level of 0.05 was used for all significance testing. All analyses were conducted in JMP®, Version 11.0 (SAS Instiute 2013).

Results

Using traditional taxonomic analysis, we were able to identify 330 (65 %) of 508 fish prey items collected from the stomachs of non-native Blue Catfish and Flathead Catfish, leaving 192 (35 %) of fish prey items unidentified. Against this background, we assessed the utility of DNA barcoding for identifying those 192 partially digested fish prey items to species. Of the DNA sequences amplified from these samples, 139 (72.4 %) could be assigned to the species level based upon similarity with archived *COI-3* sequences (Fig. 2). Using traditional taxonomic analysis, only 14 (7.3 %) of these partially digested samples had been confidently identified to species (Fig. 2). That is, using DNA barcoding, we identified 127 (66.1 %) more fish prey items than we had been able to identify using morphological analysis alone, leading us to assign species to 88 % of fish prey items overall. Using DNA barcoding, we identified 25 fish species among prey items, including obligate freshwater (e.g., cyprinids and centrarchids), euryhaline (Hogchoker and Spot), and anadromous (e.g., moronids and clupeids) fishes (Table 1). Critically, many of the highly digested samples were identified as species of conservation and management concern, including Striped Bass and White Perch or American Shad, Alewife, and Blueback Herring that are difficult to distinguish when partially digested.

Results of logistic regression analysis revealed a significant difference, among levels of digestion, in percent of diet items identified ($P = 0.009$; $F_{2,189} = 9.530$; odds ratio = 3.218), and multiple contrast tests revealed that highly digested samples had a significantly lower probability of being identified than moderately digested samples. While no significant difference was detected between lightly and highly digested prey items, limited numbers ($N = 14$) of lightly digested samples were analyzed, reducing the power of this comparison.

Discussion

In past studies, partially digested fish prey items have been identified by observing and identifying taxonomically informative morphological structures, such as otoliths, spines, or scales (Hyslop 1980; Recchia and Read, 1989; Prime and Hammond 1990; Pierce et al. 1993). Identifying partially digested fish prey items to species using these morphological structures can prove difficult, as they degrade during digestion (Schooley et al. 2008; Legler et al. 2010). Examination of morphological structures to identify highly digested fish prey items in catfish stomachs often leaves 25–30 % of items unidentified (Guier et al. 1984; Chandler 1998; Baumann and Kwak, 2011; Schloesser et al. 2011). Because non-native catfishes prey upon a variety of fish taxa that are morphologically similar and of conservation concern, particularly clupeids, a more robust identification technique is needed (Guier et al. 1984; Graham 1999; Chandler 1998; Baumann and Kwak, 2011; Schloesser et al.

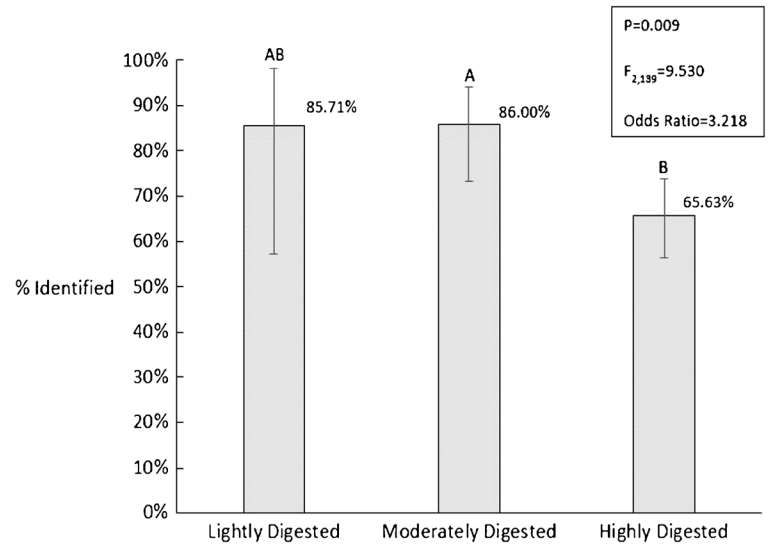

Fig. 2 Percent of fish prey identified with DNA barcoding based on three levels of digestion: lightly digested ($N = 14$), moderately digested ($N = 50$), and highly digested ($N = 128$). Error bars represent 95 % confidence intervals, and columns with differing letters differed significantly (Tukey's HSD; $\alpha = 0.05$)

Table 1 Blue and Flathead Catfish prey items successfully identified using DNA barcoding

Family	Common name	Species name	No. Observations	Accession number
Atherinopsidae	Inland Silverside	*Menidia beryllina*	2	JQ841925.1
Clupeidae	Alewife	*Alosa pseudoharengus*	17	EU523899
	American Shad	*Alosa sapidissima*	7	EU523903
	Blueback Herring	*Alosa aestivalis*	12	KC015128
	Menhaden	*Brevoortia tyrannus*	3	AP009618.1
	Hickory Shad	*Alosa mediocris*	4	AP009132.1
	Threadfin Shad	*Dorosoma petenense*	1	AP009136
	Gizzard Shad	*Dorosoma cepedianum*	20	EU366583.1
Cyprinidae	Spottail Shiner	*Notropis hudsonius*	12	JN027553.1
	River Chub	*Nocomis micropogon*	1	EU524924.1
Ictaluridae	White Catfish	*Ameiurus catus*	2	KF558302.1
	Blue Catfish	*Ictalurus furcatus*	9	KF929995.1
	Channel Catfish	*Ictalurus punctatus*	2	KF558290.1
	Flathead Catfish	*Pylodictus olivaris*	1	EU525113.1
	Brown Bullhead	*Ameiurus nebulosus*	1	EU524431
Moronidae	White Perch	*Morone americana*	32	HQ024969.1
	Striped Bass	*Morone saxatilis*	2	HM447585.1
Sciaenidae	Spot	*Leiostomus xanthurus*	2	HQ024954.1
Catostomidae	Shorthead Redhorse	*Moxostoma macrolepidotum*	1	EU524903
Centrarchidae	Blue Spotted Sunfish	*Enneacanthus gloriosus*	1	JN026073.1
	Pumpkinseed	*Lepomis gibbosus*	1	JQ979163
	Bluegill	*Lepomis macrochirus*	2	KM220892.1
	Largemouth Bass	*Micropterus salmoides*	1	EU524837
Percidae	Tessellated Darter	*Etheostoma olmstedi*	2	EU524050
Achiridae	Hogchoker	*Trinectes maculatus*	1	JN02843.1

2011). While past diet studies have used DNA barcodes to identify prey of piscivorous predators (Carreon-Martinez and Heath 2011; Carreon-Martinez et al. 2011; Jo et al. 2013), no studies have investigated the effectiveness of DNA barcoding for identifying fish prey items of North American ictalurids, warmwater species presumably with correspondingly rapid digestive rates.

Our results showed that non-native catfishes in the tidal rivers of Virginia consume a varied fish diet (25 fish species identified from PDUF in this investigation alone, $N = 192$, Table 1), including many economically important and taxonomically similar species of conservation concern (Guier et al. 1984; Graham, 1999; Chandler 1998; Baumann and Kwak, 2011; Schloesser et al. 2011). Considerable management resources having been expended to remove barriers to migration and to reduce fishery mortality, recovering populations of anadromous fishes now face high levels of predation by invasive catfishes, which is the subject of ongoing study. Demonstration of this viable DNA marker-based means of identifying catfish prey items was a development within the larger study.

Many of the catfish diet items that we collected were highly digested, making morphological identification difficult or impossible. Use of DNA barcodes allowed us to confidently identify 66 % of otherwise unidentifiable fish prey items to species level, contributing to an 88 % identification of fish prey items overall. Without using DNA barcoding, many of these prey items would have been classified as partially digested unidentified fish (PDUF) and would have resulted in lost information or false identification, and lesser understanding of the trophic impacts of non-native catfishes. DNA barcoding also proved cost-effective, costing US$ 8.47 to identify each sample to species level within our cost structure (Supplemental Table 1).

Our reduced level of success when using DNA barcodes for highly digested samples was similar to that of Carreon-Martinez et al. (2011), who similarly found that breakdown of tissue, and subsequent reduced DNA identification success occurred within a 24-h time period. This reduction could be due to degradation of DNA by digestive enzymes in stomachs and by the prior state of the prey item if ingested as scavenged material. Because gastric evacuation rate is species-specific and influenced by temperature, we believe that a well-controlled study assessing the effects of digestion time in ictalurids upon subsequent DNA barcoding success is needed. This information would inform the amount of effort and cost that should be allocated to identifying a highly digested sample.

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