

1 **Development and Testing of New Genetic Markers for the Detection of Invasive Bighead**  
2 **and Silver Carp (*Hypophthalmichthys nobilis* and *H. molitrix*) DNA in environmental water**  
3 **samples from North America**

4 Heather L. Farrington<sup>1,2</sup>, Christine E. Edwards<sup>1,3</sup>, Xin Guan<sup>1</sup>, Matthew R. Carr<sup>1</sup>, Kelly  
5 Baerwaldt<sup>4</sup>, and Richard F. Lance<sup>1,\*</sup>

6 <sup>1</sup>Environmental Laboratory, U.S. Army Engineer Research and Development Center, 3909 Halls  
7 Ferry Road, Vicksburg, MS, 39180, USA

8 <sup>2</sup> Current address: North Carolina Museum of Natural Sciences, 11 West Jones St., Raleigh,  
9 North Carolina, 27601, USA

10 <sup>3</sup>Current address: Center for Conservation and Sustainable Development, Missouri Botanical  
11 Garden, PO Box 299, St. Louis, MO, 63166, USA

12 <sup>4</sup> Current address: U.S. Army Corps of Engineers, Rock Island, IL 61201

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17 \*Corresponding Author: Richard F. Lance

18 Fax: 601-634-4017

19 Email: [richard.f.lance@usace.army.mil](mailto:richard.f.lance@usace.army.mil)

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21 Running Title: New eDNA Markers for Detection of Asian Carp

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25 **Abstract**

26 Invasive Asian bighead and silver carp (*Hypophthalmichthys nobilis* and *H. molitrix*) pose a  
27 substantial threat to North American waterways. Recently, environmental DNA (eDNA), the use  
28 of species-specific genetic assays to detect the DNA of a particular species in a water sample,  
29 has gained recognition as a tool for tracking the invasion front of these species toward the Great  
30 Lakes. The goal of this study was to develop new species-specific conventional PCR (cPCR) and  
31 quantitative (qPCR) markers for detection of these species in North American waterways. We  
32 first generated complete mitochondrial genome sequences from 33 bighead and 29 silver carp  
33 individuals collected throughout their introduced range. These sequences were aligned with  
34 other common and closely related species to identify potential eDNA markers. We then field-  
35 tested these genetic markers for species-specificity and sensitivity in environmental samples.  
36 Newly developed markers performed well in field trials, had low false positive rates and had  
37 comparable sensitivity compared to current markers. The new markers developed in this study  
38 greatly expand the number of species-specific genetic markers available to track the invasion  
39 front of bighead and silver carp, and can be used to improve the resolution of these assays.  
40 Additionally, the use of the qPCR markers developed in this study may reduce sample  
41 processing time and cost of eDNA monitoring for these species.

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## 45 **Introduction**

46 Invasive aquatic nuisance species pose a major threat to aquatic ecosystems worldwide.  
47 In North America, invasive Asian carps, particularly bighead carp (BHC; *Hypophthalmichthys*  
48 *nobilis*) and silver carp (SC; *H. molitrix*), have been very problematic in freshwater ecosystems.  
49 Asian carps were imported into the U.S. in the 1970s to control algae in Arkansas fish farms  
50 (Freeze and Henderson 1982). Flooding allowed them to escape and establish reproducing  
51 populations in the wild by the early 1980s. They have since been steadily dispersing upstream  
52 throughout the Mississippi River watershed (Freeze and Hendersen 1982; Tucker et al. 1996). At  
53 present, BHC and SC have been found in 23 states, and they have rapidly expanded their  
54 population sizes, with BHC and SC representing over 60% of the biomass in some portions of  
55 their North American Range (Garvey et al 2012). These filter-feeders cause significant  
56 ecological impacts by altering plankton communities at the base of the food chain and  
57 outcompeting native species for resources. There is considerable concern that these species will  
58 enter the Great Lakes through man-made shipping, sanitation and flood control canals, such as  
59 those of the Chicago Area Waterways System (CAWS). Should self-sustaining BHC or SC  
60 populations become established in the Great Lakes, these species could potentially cause  
61 dramatic ecosystem alterations, leading to negative effects on populations of native fishes and  
62 many threatened or endangered plant/animal species (Asian Carp Regional Coordinating  
63 Committee 2013). The impact of this invasion on Great Lakes fisheries is of particular concern.

64 Aquatic organisms shed biological materials (*e.g.*, scales, epithelial cells, slime coats,  
65 waste) containing DNA into their environments. This environmental DNA (eDNA) can persist in

66 aquatic environments for extended periods (Dejean et al. 2011, Thomsen et al. 2012), and the  
67 eDNA in water samples can be assayed using species-specific genetic markers to determine  
68 whether a species of interest may be present. Because eDNA can be detected in water when target  
69 species' populations are at low abundances, eDNA techniques may be particularly helpful in  
70 tracking changes in the distributions of aquatic invasive species (Ficetola et al. 2008; Dejean et  
71 al. 2012; Jerde et al 2011, Goldberg et al. 2013, Lance and Carr 2012) or identifying locations  
72 where threatened or endangered species may occur (Goldberg et al. 2011; Olson et al. 2012;  
73 Farrington and Lance, in prep).

74         Since 2009, eDNA monitoring has been used to track the invasion front of BHC and SC  
75 throughout the CAWS, Des Plaines River, and near-shore waters of Lake Michigan. The current  
76 eDNA monitoring program employs a single, species-specific genetic marker to detect each  
77 species (Jerde *et al.* 2011). The program utilizes conventional polymerase chain reaction (cPCR)  
78 analysis, whereby the presence or absence of eDNA is determined by PCR amplification of a  
79 target DNA fragment. The PCR-amplified product is then isolated by gel electrophoresis and the  
80 DNA is sequenced to confirm the species of origin. The Quality Assurance Project Plan (QAPP)  
81 for the Environmental DNA (eDNA) Monitoring of Invasive Asian Carp in the CAWS outlines  
82 the detailed procedures for the current planning, collection, filtering and processing of eDNA  
83 samples (USACE 2012).

84         The development of additional BHC and SC eDNA markers could provide a suite of  
85 assays to provide multiple lines of evidence or secondary verification for eDNA detections. In  
86 addition to cPCR markers, quantitative PCR (qPCR) may be used as an eDNA monitoring tool.  
87 The use of qPCR has several potential advantages relative to cPCR, including, typically, more

88 rapid PCR thermal-cycling programs, which can be important for large-scale sampling efforts, a  
89 reduced sensitivity in some cases to PCR inhibitors (personal observation; Barnes et al. 2014),  
90 and the ability to quantify, to some degree, the amount of DNA in a sample (taking into account  
91 inherent variations in DNA extraction recoveries and qPCR-based copy number estimates). Also,  
92 while conventional PCR requires specific oligonucleotide binding at *two* locations (the forward  
93 and reverse primers) in order to produce a PCR product, hydrolysis probe-based qPCR, which is  
94 one of two common qPCR methodologies, may often be a more stringent assay because it  
95 requires specific oligonucleotide binding at *three* locations (forward and reverse primers, as well  
96 as the internal hydrolysis probe) in order for the reaction to produce a product that emits a  
97 fluorescent signal.

98 Our objectives in this study were to: 1) sequence full mitochondrial (mtDNA) genomes  
99 from multiple BHC and SC throughout their North American range to represent the intraspecific  
100 genetic variation of each species, 2) use multiple sequence alignments of BHC, SC and other  
101 closely related species that may be present in aquatic ecosystems in the Midwestern U.S.A. to  
102 design species-specific cPCR and qPCR markers for the detection of BHC and SC in eDNA  
103 monitoring programs, and 3) test the specificity and sensitivity of these new markers in detecting  
104 BHC and SC in laboratory and eDNA field trials.

## 105 ***Methods***

### 106 **Sample Collection, DNA sequencing, and Alignment**

107 Tissue samples (fin clips or livers) were collected from silver and bighead carp  
108 populations throughout their introduced range within the Mississippi River watershed (Table 1;  
109 Fig 1). Total genomic DNA was extracted using DNeasy Blood and Tissue Kits (QIAGEN Inc.)

110 according to the manufacturer's instructions. DNA extractions were enriched for mitochondrial  
111 DNA using long PCR to amplify the mitochondrial genome as a single 16.6 kb fragment. Primer  
112 sequences were S-LA-16S-L 5'-CGATTAAAGTCCTACGTGATCTGAGTTCAG-3' and S-LA-  
113 16S-H 5'-TGCACCATTAGGATGTCCTGATCCAACATC-3' (Miya and Nishida 2000).  
114 QIAGEN LongRange PCR Kit reagents were used to formulate a 25  $\mu$ L PCR reaction mixture  
115 containing 1 $\times$  LongRange PCR buffer, 500  $\mu$ M dNTPs, 1.25 U LongRange PCR Enzyme mix,  
116 0.4  $\mu$ M of each primer, and 1  $\mu$ L of DNA template. Temperature cycling conditions began with  
117 an initial denaturation step of 93°C for 3 min, followed by 10 cycles of 93°C for 15 sec, 62°C for  
118 30 sec and 68°C for 18 min. An additional 29 cycles were then run adding 20 sec to the  
119 extension step for each cycle. Because amplification of a single fragment was not successful for  
120 all samples (likely due to degraded template DNA), we also attempted to amplify the  
121 mitochondrial genome in three shorter, overlapping fragments, using the same PCR chemistry  
122 and cycling conditions described above. Primer sequences were designed using Primer3 software  
123 (Rozen and Skaletsky 2000) based on BHC and SC complete mitochondrial genome sequences  
124 available on GenBank (accession numbers NC\_010194, EU343733, JQ231114, HM162839,  
125 EU315941, NC\_010156). The following primers were designed to amplify fragments of  
126 approximately 7.4, 7.0 and 3.0 kb, respectively: LC1-F and R (5'-  
127 GAATGGGCTAAACCCCTAAA -3' / 5'- TCGTAGTGAAAAGGGCAGTC -3'); LC2-F and R  
128 (5'- CAGGATTCCACGGACTACAC -3' / 5'- TTGGGGTTTGACAAGGATAA -3'; LC3-F  
129 and R (5'- CATGCCGAGCATTCTTTTAT -3' / 5'- CAACATCGAGGTCGTAAACC -3').  
130 When agarose gel electrophoresis revealed that all three of the shorter PCR reactions produced  
131 bands of the expected sizes, the reaction products were pooled for sequencing.

132 PCR products were purified using ZR-96 DNA Clean and Concentrator-5 kits (Zymo  
133 Research) and prepared for next-generation sequencing using Nextera DNA Sample Preparation  
134 Kits (Illumina, Inc.); Nextera Index Kits were used to pool up to 96 libraries into a run for  
135 sequencing. Sequencing was performed on the Illumina MiSeq system, using 150 bp paired-end  
136 reads. MiSeq Reporter Software was used to sort the resulting pool of sequences by the indices  
137 to identify the sequences arising from each sample. Mitochondrial genomes were assembled by  
138 aligning the reads of each individual to a reference sequence of the appropriate species from  
139 GenBank (see above for accession numbers) using Geneious software v.6 (Biomatters Ltd.,  
140 Auckland, New Zealand). Consensus sequences generated for each individual were exported and  
141 aligned, along with sequences of some related cyprinid fish species that may be present in the  
142 same North American regions as BHC and SC (common carp, grass carp and black carp;  
143 GenBank accession numbers NC\_010288.1, NC\_018035.1, NC\_018039.1, NC\_018036.1,  
144 NC\_011141.1). Alignments were carried out using the default settings in MUSCLE (Edgar  
145 2004) as implemented in Geneious V6.

#### 146 **Marker Design**

147 Marker loci were designed using the multiple sequence alignment of complete  
148 mitochondrial genomes of bighead carp, silver carp, and several related species (listed above).  
149 Potential PCR primer sites were chosen by identifying sequence regions that demonstrated no  
150 mismatches within the target taxa and that maximized differences between target and non-target  
151 taxa. Because eDNA may experience rapid degradation by environmental conditions, marker  
152 loci were designed to be short (<400 bp) to increase amplification probability. Primer3 (Rozen  
153 and Skaletsky 2000) was used to design cPCR primers and qPCR primer/probe sets with

154 preference for primers that contained 3'-end mismatches to homologous DNA in non-target  
155 species. All qPCR probes were labeled with 6FAM as the 5' fluorescent tag, and TAMRA as the  
156 3' quencher. Due to the limited genetic divergence between bighead and silver carp, we also  
157 developed a series of general BHC/SC markers that may detect both species.

### 158 **Marker testing for specificity and efficacy in eDNA field trials**

159 Unless otherwise noted, newly designed cPCR markers were tested using 25  $\mu$ L reactions  
160 containing 1 $\times$  Platinum<sup>®</sup> *Taq* PCR buffer (Invitrogen), 200  $\mu$ M dNTPs, 1.5 mM MgCl<sub>2</sub>, 0.2  $\mu$ M  
161 of each primer, 1.25 U Platinum<sup>®</sup> *Taq* polymerase (Invitrogen), and 1  $\mu$ L DNA template.  
162 Temperature cycling conditions began with an initial denaturation step of 94°C for 10 min,  
163 followed by 45 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min 30 sec, with a final  
164 elongation step at 72°C for 7 min. Amplification products of cPCR assays were purified using E-  
165 Gel SizeSelect Gels (Life Technologies) and sequenced using an ABI 3500XL Genetic Analyzer  
166 with BigDye chemistry and standard sequencing protocols. Resulting sequences were compared  
167 against BHC and SC reference DNA sequences and subjected to GenBank BLAST searches to  
168 identify the source species of the amplification product.

169 All qPCR reactions were run in 20 $\mu$ l volumes containing 1X TaqMan<sup>®</sup> Environmental  
170 Master Mix, 0.54  $\mu$ M of each primer, 0.125  $\mu$ M of the probe, and 1 $\mu$ L of DNA template.  
171 Temperature cycling began with an initial denaturation step at 95°C for 10min, followed by 40  
172 cycles of 95°C for 15 sec and 60°C for 1 min. qPCR reactions were run on a ViiA<sup>™</sup> 7 Real-  
173 Time PCR System (Applied Biosystems). qPCR reactions were considered positive if the  
174 amplification curve crossed the fluorescence detection threshold by the end of the 40 cycle qPCR  
175 run.



176 Both cPCR and qPCR markers were tested for: 1) species-specificity, 2) ability to  
177 amplify target species DNA from eDNA samples collected in areas of known BHC and SC  
178 presence, 3) false-positive amplification from eDNA samples that likely do not contain Asian  
179 carp DNA, and 4) limits of detection, or sensitivity, in targets species (*i.e.*, the minimum amount  
180 of starting DNA that can result in a detectable cPCR or qPCR product).

181 Species-specificity of both cPCR and qPCR assays was tested using a panel of genomic  
182 DNA (1 ng/ $\mu$ L) from individuals of the target species and 29 additional species likely to be  
183 present in the CAWS. This panel included closely related, non-target species such as shiners,  
184 common carp, goldfish, and grass carp (Table 2). If cPCR markers amplified non-target species,  
185 annealing temperatures were adjusted in an attempt to eliminate non-target amplification. The  
186 cPCR and qPCR markers that amplified the target species and showed little cross-amplification  
187 in non-target species were further tested using field-collected water samples from Steele Bayou,  
188 a backwater flood control area near the Yazoo River's confluence with the Mississippi River  
189 near Vicksburg, MS. U.S.A. Steele Bayou is locally known to have well-established BHC and  
190 SC populations with high densities (pers. commun., A. Katzenmeyer). We also tested for  
191 amplification of BHC and SC in water samples collected from a small tributary of Fishing Creek  
192 (Clinton County, PA, U.S.A), an area outside the introduced range of BHC and SC. These  
193 samples have all the typical components of environmental water samples, but were free of target  
194 DNA, and thus provided test cases to detect potential non-target amplification within naturally  
195 occurring DNA pools. In all cases, surface water samples were collected in 50 mL conical tubes.  
196 In the laboratory, tubes were centrifuged at maximum speed (4000 g) for 30 min at 4° C. The  
197 supernatant was poured off and DNA was extracted from the remaining pellet of material using a

198 modified cetyltrimethyl ammonium bromide (CTAB)/chloroform protocol (Doyle and Doyle  
199 1987). Each cPCR and qPCR marker was tested on a panel of 44 Steele Bayou and 44 Fishing  
200 Creek samples, with 4x replication of PCR reactions to evaluate the detection rate of these  
201 species from areas of known presence and the potential false positive rate from waters where  
202 they are absent. The performance of all new markers, as measured by rate of detection, was  
203 compared to the cPCR markers for BHC and SC from Jerde et al. 2012 (primers HN203-F &  
204 HN498-R and HMF-2 & HMR-2, respectively), which are currently used in the Asian carp  
205 monitoring QAPP (USACE 2012). We refer to these markers here as QAPP-SC and QAPP-  
206 BHC.

207         Markers with high detection rates and low false-positive rates in environmental samples  
208 were subjected to sensitivity testing. Genomic DNA of SC and BHC was extracted and the  
209 concentration of each was normalized to 1 ng/ $\mu$ L. A serial 1:10 dilution series was prepared and  
210 markers were tested across the concentration range of 0.1 ng/ $\mu$ L ( $10^{-1}$ ) through  $10^{-7}$ , with four  
211 replicate cPCRs or qPCRs at each concentration. A limitation to the use of genomic DNA in  
212 sensitivity testing for cPCR markers is that the number of marker copies present in the  
213 normalized DNA extractions, and therefore available for PCR amplification, is unknown. To  
214 estimate starting copy number in qPCR reactions, each qPCR marker was cloned into a bacterial  
215 plasmid vector using TOPO<sup>®</sup> Cloning kits (Life Technologies) as per the manufacturer's  
216 instructions. Successfully cloned bacterial colonies were cultured and plasmids extracted using  
217 Qiagen Miniprep plasmid extraction kits. The estimated number of plasmids in the resulting  
218 elutions was calculated using the combined base pair length of the plasmid and marker insert, a  
219 standard DNA base-to-Daltons conversion for double-stranded DNA (650 Daltons/base; Roche

220 Applied Science 2011), a Daltons-to-nanograms conversion, and DNA mass quantification of  
221 elutions using a NanoDrop 1000. A dilution series of the plasmid elution was then used to  
222 generate a standard curve for estimation of copy number in the qPCR reactions.

223 To test whether the throughput of eDNA screening methods could be increased by  
224 assaying for multiple markers simultaneously within single PCRs, several markers were  
225 combined in pairs for multiplex cPCR or qPCR reactions. For cPCR, the QAPP-SC and SC-1  
226 markers were combined. For qPCR, three primer sets were tested: BH-TM1/BH-TM2, SC-  
227 TM4/SC-TM5, and AC-TM1/AC-TM3. For qPCR multiplexing, the two markers utilized probes  
228 with different fluorescent labels (FAM or VIC). A genomic DNA dilution series and plasmid  
229 standards were again used for testing, with markers and standards run both individually and in  
230 combination in order to directly compare sensitivity in single versus multiplex reactions. qPCR  
231 reactions were prepared as described above, with both sets of primers and probes added to the  
232 reaction, and the same temperature cycling conditions.

### 233 **Results**

234 We generated complete mtDNA sequences for 33 BHC and 29 SC individuals (Table 1);  
235 all DNA sequences were submitted to GenBank (Accession numbers XXXX to XXXX).  
236 Average whole genome sequence coverage for the 62 haplotypes sequenced was 1595X (range  
237 3-11971X coverage). Total length of the aligned BHC and SC genomes was 16620 bp. There  
238 was very little sequence variation within species, with only 40 (0.24%) and 34 (0.20%) variable  
239 sites for BHC and SC, respectively. When species alignments were combined, there were a total  
240 of 823 (4.95%) variable sites across the mitochondrial genome. These genomes were aligned

241 with mtDNA genomes from closely related species obtained from GenBank, including common,  
242 grass and black carp for identification of potential species-specific eDNA markers.

243 Based on the alignment of mitochondrial genomes, we initially designed 12 SC, 11 BHC  
244 and 16 general BHC/SC cPCR markers. For TaqMan<sup>®</sup> qPCR, we initially designed five markers  
245 for BH, six for SC, and three general BHC/SC markers. Based on results from the initial cross-  
246 species screening, several markers amplified non-target species and were not further  
247 investigated, reducing the number of potential markers for testing to six for cPCR and eight for  
248 qPCR (Table 3). We focused all subsequent field and sensitivity testing on the markers with  
249 high affinity for the target species and little or no amplification of other species.

250 Assays of the 44 Steele Bayou samples with the established markers QAPP-SC and  
251 QAPP-BHC resulted in 28 (64%) positive SC detections and 0 (0%) positive BHC detections.  
252 All of the newly designed BHC markers performed better than the QAPP-BH marker, with the  
253 highest detection rate from the qPCR marker BH-TM2 (9 of 44, 20%). In comparison to the  
254 QAPP-SC marker, all newly-designed SC markers had similar or higher numbers of detections,  
255 with the highest detection rates from cPCR marker SC-1 and qPCR maker SC-TM5, both with  
256 32 positive samples (73%). Positive detection rates were 57-68% for the general (BHC/SC)  
257 markers. For the Fishing Creek samples, none of the new cPCR markers produced bands in the  
258 same size range as target species and none of the qPCR markers produced quantifiable  
259 fluorescence.

260 All the tested markers consistently yielded positive results from genomic DNA down to  
261 at least the 10<sup>-3</sup> dilution (0.001 ng/μL). Three SC (SC-5, SC-7 and SC-TM4) and one BHC/SC  
262 marker (AC-TM2) had consistent detections at 10<sup>-4</sup>, and nearly all markers had >50% detection

263 rates among the four replicates at the  $10^{-4}$  dilution level, which is estimated to have copy  
264 numbers in the single digits (Table 5). Detections became more stochastic at concentrations  
265 below  $10^{-4}$ , as expected for samples with extremely low copy number (average of  $\leq 1$  marker  
266 copy per reaction). Sensitivity of the new BHC and SC markers was comparable to the QAPP  
267 markers.

268 Multiplexing of cPCR markers was found to be unfeasible for high throughput processing  
269 and analysis using standard gel electrophoresis equipment in our lab. The new cPCR markers  
270 were all designed to be in the size range of 200-300 base pairs to increase the potential for  
271 amplification of degraded eDNA, therefore, amplicon base pair lengths were too similar for clear  
272 differentiation of bands on 2% agarose gels; gel-based isolation of fragments for sequencing  
273 would also be infeasible with this combination of amplicon lengths and electrophoresis  
274 equipment. Longer-running or higher density gels may have allowed more reliable separation of  
275 different cPCR marker bands but may somewhat negate the cost and time savings gained from  
276 multiplexing. Trials of multiplexed qPCR markers were successful for all combinations of  
277 markers tested, with no substantial reduction in marker sensitivity (limits of detection; Table 6).

## 278 *Discussion*

279 The large number of mtDNA haplotypes generated in this study allowed us to capture  
280 inter- and intra-species genetic variation in SC and BHC across their introduced, North American  
281 range. This information, along with comparisons to DNA sequences from related species found  
282 in the central United States, aided in the design of cPCR and qPCR markers specifically for  
283 eDNA testing for SC and BHC in their introduced range. Effective design of PCR-based assays  
284 for the differential or discriminatory detection of species requires that sequence differences

285 among taxa be clustered so that multiple differences among taxa are grouped into the length of a  
286 PCR primer and two or more of these areas are grouped within a few hundred base pairs.  
287 Because SC and BHC are closely related and have very low levels of sequence divergence across  
288 their mitochondrial genomes, a very limited number of sites demonstrated a sufficient number of  
289 clustered polymorphism to develop effective species-specific markers. Despite careful selection  
290 of markers to maximize differences among species, cross-amplification was observed in at least  
291 one non-target species for many markers, resulting in the elimination of nearly 70% of the  
292 originally designed markers. Despite these difficulties, we were able to design multiple cPCR  
293 and qPCR markers that specifically detect SC and BHC in field-collected water samples in North  
294 America.

295 In field trials, the new species-specific markers developed in this study generally had  
296 detection rates similar to or higher than the markers currently used to detect the presence of BHC  
297 and SC DNA in environmental water samples, with similar levels of sensitivity at low  
298 concentrations of target DNA. Further, confounding or efficiency-diminishing factors (e.g.,  
299 amplicons that result in gel bands of similar size to those obtained for the target species or  
300 nontarget fluorescence in qPCR trials) were not observed, indicating that these markers would be  
301 suitable as a high-throughput assays to detect the presence of BHC and SC from environmental  
302 water samples. Multiplexing of qPCR markers was successful in genomic DNA trials,  
303 suggesting that multiplexing may be feasible in eDNA screening, increasing throughput of the  
304 assays. However, performance of multiplexing reactions with field eDNA samples remains to be  
305 tested, and additional combinations of the various markers could be employed following further  
306 testing.

307           In addition to potential improvements in sensitivity and throughput by the markers  
308 developed in this study, the availability of multiple new cPCR markers for eDNA screening of  
309 SC and BHC in water samples may help increase the accuracy of eDNA monitoring programs.  
310 eDNA samples are largely comprised of randomly fragmented, low-abundance DNA targets.  
311 The current program uses a single marker locus to detect the presence of SC or BHC in  
312 environmental samples, which may be sensitive to random degradation of the single marker. The  
313 use of multiple marker loci would improve overall detection rates and provide stronger evidence  
314 for the presence of BHC and SC DNA in the water. Further, the addition of qPCR technology to  
315 eDNA screening provides the transition from simple presence/absence data provided by cPCR to  
316 the generation of data related to DNA concentration in field samples. This additional  
317 information *may* help estimate *relative* abundance or biomass of species of interest in the  
318 sampled waterway. qPCR may also reduce sample screening time by eliminating the need for gel  
319 electrophoresis and sequence verification. The new qPCR and cPCR markers developed in this  
320 study therefore represent a significant expansion of the tools available to detect the invasion of  
321 SC and BHC in North America and may improve the accuracy, resolution, and throughput of  
322 eDNA monitoring programs for these species in the future.

323

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### 331 **References**

332 Asian Carp Regional Coordinating Committee. 2013. FY 2013 Asian Carp Control Strategy and  
333 Framework. <http://www.asiancarp.us>

334 Baerwaldt K, Benson A, and Irons K (2013) Asian Carp Distribution in North America. Report  
335 to the Asian Carp Regional Coordinating Committee, April 2013.

336 Barnes M, Turner CR, Jerde CL, Renshaw MA, Chadderton WL, and Lodge DM (2014)  
337 Environmental conditions influence eDNA persistence in aquatic systems. *Environmental*  
338 *Science & Technology*, **48**,1819-1827

339 Dejean T, Valentini A, Miquel C, Taberlet P, Bellemain E, Miaud C (2012) Improved detection  
340 of an alien invasive species through environmental DNA barcoding: the example of the  
341 American bullfrog *Lithobates catesbeianus*. *Journal of Applied Ecology*, **49**, 953-959.

342 Doyle JJ, Doyle JL (1987) A rapid isolation procedure for small quantities of fresh tissue.  
343 *Phytochemical Bulletin*, **19**, 11–15.

344  
345 Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high  
346 throughput. *Nucleic Acid Research*, **32**, 1792-1797.

347  
348 Farrington HL, Lance RF (in prep). Development of genetic markers for environmental DNA  
349 (eDNA) monitoring of sturgeon. EMRRP Technical Notes Collection.

350 Ficetola GF, Miaud C, Pompanon F, Taberlet P (2008) Species detection using environmental  
351 DNA from water samples. *Biology Letters*, **4**, 423.

352 Freeze M, Henderson S (1982) Distribution and status of the bighead carp and silver carp in  
353 Arkansas. *North American Journal of Fisheries Management*, **2**, 197-200.

354  
355 Garvey JE, Sass GG, Trushenski J, Glover D, Charlebois PM, Levengood J, Roth B, Whitley  
356 G, Small BC, Tripp SJ, Secchi S (2012) Fishing down the bighead and silver carps:  
357 reducing the risk of invasion to the Great Lakes. Illinois Department of Natural  
358 Resources. <http://asiancarp.us>

359



- 360 Goldberg CS, Pilliod DS, Arkle RS, Waits LP (2011) Molecular detection of vertebrates in  
361 stream water: A demonstration using Rocky Mountain tailed frogs and Idaho giant  
362 salamanders. *PloS one*, **6**, e22746.
- 363 Goldberg CS, Sepulveda A, Ray A, Baumgardt J, Waits LP (2013) Environmental DNA as a  
364 new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*).  
365 *Freshwater Science*, **32**,792-800.
- 366 Jerde CL, Mahon AR, Chadderton WL, Lodge DM (2011) “Sight-unseen” detection of rare  
367 aquatic species using environmental DNA. *Conservation Letters*, **4**, 150-157.  
368
- 369 Lance RF, Carr MR (2012) Detecting eDNA of Invasive Dreissenid Mussels, Report on Capital  
370 Investment Project. ANSRP Technical Notes Collection. ERDC/TN ANSRP-12-2.  
371 Vicksburg, MS: U.S. Army Engineer Research and Development Center.
- 372 Li SF, Xu JW, Yang QL, Wang CH, Chen Q, Chapman DC, Lu G (2009) A comparison of  
373 complete mitochondrial genomes of silver carp *Hypophthalmichthys molitrix* and bighead  
374 carp *Hypophthalmichthys nobilis*: implications for their taxonomic relationship and  
375 phylogeny. *Journal of Fish Biology*, **74**, 1787-1803.
- 376 Miya M, Nishida M (2000) Use of mitogenomic information in teleostean molecular  
377 phylogenetics: a tree-based exploration under the maximum-parsimony optimality  
378 criterion. *Molecular Phylogenetics and Evolution*, **17**, 437-455.  
379
- 380 Olson ZH, Briggler JT, Williams RN (2012) An eDNA approach to detect eastern hellbenders  
381 (*Cryptobranchus a. alleghaniensis*) using samples of water. *Wildlife Research*, **39**, 629-  
382 636.
- 383 Roche Applied Science (2011) Nucleic Acid Isolation and Purification, 4<sup>th</sup> Edition. Roche  
384 Diagnostics GmbH, Mannheim, Germany.  
385
- 386 Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist  
387 programmers. In: Krawetz S, Misener S, editors. Bioinformatics Methods and Protocols:  
388 Methods in Molecular Biology. Totowa, NJ : Humana Press.  
389
- 390 Tucker JK, Cronin FA, Hrabik RA, Petersen MD, Herzog DP (1996) The Bighead Carp  
391 (*Hypophthalmichthys nobilis*) in the Mississippi River. *Journal of Freshwater Ecology*,  
392 **11**, 241-243.  
393
- 394 United States Army Corps of Engineers (2012) Quality Assurance Project Plan, eDNA  
395 Monitoring of Invasive Asian Carp in the Chicago Area Waterway System. USACE  
396 Chicago District, May 2012. [http://www.asiancarp.us/documents/USACE-eDNA-  
397 QAPP.pdf](http://www.asiancarp.us/documents/USACE-eDNA-QAPP.pdf)

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400 **Author Contributions**

401 H.L.F., C.E.E., R.F.L. designed study, performed sequence alignments, designed primers fro new  
402 markers. H.L.F., X.G., M.R.C. performed fieldwork and laboratory testing. K.B. participated in  
403 study design, sample collection, and project management. All authors participated in preparation  
404 of manuscript.

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408 **Data Accessibility**

409 DNA Sequences: Genbank accessions XXXX-XXXX

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415 **Table 1:** Origins of silver and bighead carp samples included in mtDNA genome sequencing and

416 alignment

<b>Location</b>	<b>Silver Carp (n)</b>	<b>Bighead Carp (n)</b>
East Lower Mississippi (Yazoo River, Steele Bayou, Big Sunflower River)	3	5
West Lower Mississippi (Red River, Atchafalaya River)	3	6
Arkansas River	3	3
Ohio River (at junction to Mississippi River)	3	3
Mississippi River (Knowlton Lake)	2	-
Illinois River (LaGrange Reach)	-	3
Illinois River (Marseilles Reach)	3	3
Illinois River (Starved Rock)	2	3
Mississippi River (Laketon, KY)	3	1
Upper Mississippi River (Pool 20)	1	3
Upper Mississippi River (Pool 26)	3	3
Missouri River (north of Omaha)	3	-

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421 **Table 2:** Panel of 29 non-target fish species collected from the CAWS used for testing of

422 primers for cross-species amplification.

<b>Common Name</b>	<b>Species name</b>
Brown Bullhead	<i>Ameiurus nebulosus</i>
Freshwater Drum	<i>Aplodinotus grunniens</i>
Goldfish	<i>Carassius auratus</i>
Quillback	<i>Carpiodes cyprinus</i>
Grass Carp	<i>Ctenopharyngodon idella</i>
Spotfin Shiner	<i>Cyprinella spiloptera</i>
Common Carp	<i>Cyprinus carpio</i>
Mirror Carp	<i>Cyprinus carpio</i> sp.
Gizzard Shad	<i>Dorosoma cepedianum</i>
Channel Catfish	<i>Ictalurus punctatus</i>
Smallmouth Buffalo	<i>Ictiobus bubalus</i>
Black Buffalo	<i>Ictiobus niger</i>
Brook Silverside	<i>Labidesthes sicculus</i>
Green Sunfish	<i>Lepomis cyanellus</i>
Pumpkinseed Sunfish	<i>Lepomis gibbosus</i>
Orangespotted Sunfish	<i>Lepomis humilis</i>
Bluegill	<i>Lepomis macrochirus</i>
Smallmouth Bass	<i>Micropterus dolmieu</i>
Largemouth Bass	<i>Micropterus salmoides</i>
White Perch	<i>Morone americana</i>
White Bass	<i>Morone chrysops</i>
Round Goby	<i>Neogobius melanostomus</i>
Golden Shiner	<i>Notemigonus crysoleucas</i>
Emerald Shiner	<i>Notropis atherinoides</i>
Yellow Perch	<i>Perca flavescens</i>
Bluntnose minnow	<i>Pimephales notatus</i>
White Crappie	<i>Pomoxis annularis</i>
Black Crappie	<i>Pomoxis nigromaculatus</i>
Flathead Catfish	<i>Pylodictis olivaris</i>

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**Table 3.** Primer sequences of markers used for field and sensitivity testing. For targeted species, BH=bighead carp, SC=silver carp, Both=primers that could potentially be used for non-specific detection of both BHC and SC. Names containing TM indicates TaqMan<sup>®</sup> qPCR markers.

Name	Target species	Forward	Reverse	Probe (TaqMan <sup>®</sup> markers only)	Length (bp)
SC-1	SC	GGACCCAGTACTATTAAGTCTCTA	TCCTAGGGCAAGGAGGGTA		171
SC-5	SC	TCCGATTACCGCCACAATTATAGCCTTAG	GATAGGGTTAGTGGAAAGAGAGGAC		161
SC-7	SC	ACTGAATAAACACACACATGTTTCGAT	ATCATCACCCGATTAGTAAAAATG		275
SC-TM4	SC	CCACTAACATCACCACGCAA	AGCCTTTTCCAGAGGCTTGG	TAACCCAGCTGCCAATACAA	168
SC-TM5	SC	CCACAACCTACCCTCCTTGCC	AAGGGTATTAATTTTTGTGGTGGGA	TCATGACATCCGCAGCATTCTC	98
BH-6	BH	CAATACCCTAGCAATTATCCCTTA	TGTAATTCCAAGGGCGGTTAG		375
BH-8	BH	GATGTAAACTATGGCTGGCTTATT	TGTAGAAAGAGGAGGTGTAGGAA		388
BH-TM1	BH	TAGACCTTCTAACAGGACTAATTC	AATCCACCTCATCCTCCAAC	CCGCCCTTGAATTACATCCACA	144
BH-TM2	BH	CCTTCGTCAAACAGACCTTAAATCC	CCCTCATGGGGTTTGGATTAGA	CCACATAGGACTTGTAGCGGGTGA	96
BH-TM4	BH	CCACTAACATCGCCACGTAG	AACCTTTTCCAGAAGCTTGG	TAGCCCAGCCGCCAACACAA	168
AC-6	Both	GTCCTAATCAGCACCTTAGTACTCT	AATTCGAAGGGATGGCAAG		156
AC-TM1	Both	GGCCGGAACAGGATGAACAGTT	TAATAGTTGTGGTGTGAAGTTAATTG	CACGCAGGAGCATCCGTAGACCT	145
AC-TM2	Both	CAATTAACCTCATCACCAACTATTA	TCCAGCAGCTAAAAGTGGTAAGG	AAACACCTCTCTTTGTTGAGCTGTGC	133
AC-TM3	Both	TTCATCGGCGTAAATCTTACAT	AGGGAAATAAGAGATCCGATAGA	ACCCAGATGCCTACGCCCTG	133
AC-TM3	Both	TTCATCGGCGTAAATCTTACAT	AGGGAAATAAGAGATCCGATAGA	ACCCAGATGCCTACGCCCTG	133

**Table 4:** Number of positive detections noted for each marker tested using 44 eDNA field samples. Steele Bayou samples were collected from an area of high concentrations of both BHC and SC, whereas Fishing Creek samples were collected from an area where carp are absent. QAPP-SC and QAPP-BH are the markers currently used for eDNA testing. Names containing TM indicate TaqMan<sup>®</sup> qPCR markers.

Marker	Steele Bayou	Fishing Creek
<b>Silver Carp:</b>		
QAPP-SC	28	0
SC-1	32	0
SC-5	25	0
SC-7	23	0
SC-TM4	26	0
SC-TM5	32	0
<b>Bighead Carp:</b>		
QAPP-BH	0	Not tested
BH-6	6	0
BH-8	6	0
BH-TM1	9	0
BH-TM2	7	0
BH-TM4	6	0
<b>Bighead and Silver Carp:</b>		
AC-6	30	0
AC-TM1	25	0
AC-TM2	30	0
AC-TM3	28*	0

\* - Tested with only 42 samples

**Table 5:** Sensitivity testing. Estimated marker copy numbers per dilution are based on averages calculated across all replicates of qPCR sensitivity trials using a plasmid DNA standard. AC markers were tested using SC dilutions. The number of positive detections out of four replicates is noted for each marker and dilution level. U=Undetermined copy number. Amplifications at these levels are likely due to stochasticity of PCR at such low DNA concentrations.

Dilution:	$10^{-1}$	$10^{-2}$	$10^{-3}$	$10^{-4}$	$10^{-5}$	$10^{-6}$	$10^{-7}$
<b>Silver Carp:</b>							
Estimated Copy Number:	6051	508	46	3.4	1.6	U	U
QAPP-SC	4	4	4	4	0	0	0
SC-1	4	4	4	3	0	0	0
SC-5	4	4	4	4	0	2	0
SC-7	4	4	4	4	0	0	0
SC-TM-4	4	4	4	4	1	1	0
SC-TM-5	4	4	4	3	2	1	1
<b>Bighead Carp:</b>							
Estimated Copy Number:	2193	230	16	2.2	1.1	U	U
QAPP-BH	4	4	4	4	1	1	0
BH-6	4	4	4	1	0	0	0
BH-8	4	4	4	3	0	1	0
BH-TM-1	4	4	4	3	1	0	0
BH-TM-2	4	4	4	3	0	0	0
BH-TM-4	4	4	4	3	0	1	0
<b>Bighead and Silver Carp:</b>							
Estimated Copy Number:	6051	508	46	3.4	1.6	U	U
AC-6	4	4	4	2	0	1	0
AC-TM-1	4	4	4	2	2	0	0
AC-TM-2	4	4	4	4	2	0	0
AC-TM-3	4	4	4	3	0	0	0



**Table 6:** Multiplexing of qPCR markers. Average Ct values (copy numbers) across 24 replicates for markers run individually and combined in a multiplex reaction.

Dilution:	$10^{-2}$	$10^{-3}$	$10^{-4}$	$10^{-5}$
<b>Silver Carp:</b>				
SC-TM4	30.6 (195-424)	34.1 (10-50)	37.3 (0-12)	38.7 (0-2.4)
SC-TM5	30.4 (872-1498)	33.7 (60-208)	37.4 (0-30)	38.1 (0-11)
Combined:				
SC-TM4	30.7 (203-422)	34.2 (11-50)	37.9 (0-5)	38.4 (0-3)
SC-TM5	30.4 (1145-1792)	33.9 (81-200)	37.5 (2-23)	38.4 (0-14)
<b>Bighead Carp:</b>				
BH-TM1	31.2 (107-244)	34.6 (8-28)	38.2 (0-4)	39.0 (0-1)
BH-TM2	31.2 (120-216)	34.8 (7-19)	37.8 (0-4)	39.3 (0-1)
Combined:				
BH-TM1	30.6 (118-263)	34.3 (8-23)	37.3 (0-5)	38.3 (0-1)
BH-TM2	31.1 (137-201)	34.6 (12-24)	38.1 (0-7)	38.7 (0-1)
<b>Bighead and Silver Carp:</b>				
AC-TM1	31.1 (241-358)	34.5 (16-65)	38.0 (0-12)	38.9 (0-3)
AC-TM3	29.1 (225-398)	32.3 (16-52)	36.0 (0-6)	37.7 (0-1)
Combined:				
AC-TM1	31.1 (283-536)	34.5 (22-91)	37.7 (0-16)	39.4 (0-1)
AC-TM3	29.1 (252-501)	32.4 (19-80)	35.9 (0-12)	37.6 (0-2)

Figure 1: Geographic distribution of sample collection for mitochondrial DNA sequencing

