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Improving efficiency and reliability of environmental DNA analysis for silver carp

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ABSTRACT

Natural resource agencies have established surveillance programs which use environmental DNA (eDNA) for the early detection of bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix* before they establish populations within the Great Lakes. This molecular monitoring technique must be highly accurate and precise for confident interpretation and also efficient, both in detection threshold and cost. Therefore, we compared two DNA extraction techniques and compared a new quantitative PCR (qPCR) assay with the conventional PCR (cPCR) assay used by monitoring programs. Both the qPCR and cPCR assays were able to amplify the DNA of silver carp present in environmental samples taken from locations where mixed populations of bigheaded carps existed. However, the qPCR assay had substantially fewer PCR positive samples which were subsequently determined not to contain DNA of bigheaded carps than the cPCR assay. Additionally, the qPCR assay was able to amplify the DNA of bigheaded carps even in the presence of inhibitors that blocked amplification with cPCR. Also, the selection of an appropriate DNA extraction method can significantly alter the efficiency of eDNA surveillance programs by lowering detection limits and by decreasing costs associated with sample processing. The results reported herein are presently being incorporated into eDNA surveillance programs to decrease the costs, increase DNA yield and increase the confidence that assays are amplifying the target DNA. These results are critical to enhancing our ability to accurately and confidently interpret the results reported from monitoring programs using eDNA for early detection of invasive species.

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Introduction

Early detection is a vital part of any program seeking to manage invasive species, including the bighead carp *Hypophthalmichthys nobilis* (BHC) and the silver carp *Hypophthalmichthys molitrix* (SVC), collectively referred to as bigheaded carps. Native to Asia, both of these species were introduced to the United States to control plankton production in aquaculture and effluent ponds (Kolar et al., 2007). They subsequently escaped and have spread throughout the Mississippi River basin and currently threaten to invade the Great Lakes (Kolar et al., 2007). Until about 2009, methods for early detection of bigheaded carps relied on the capture of these fishes even though both species are known to avoid capture with conventional fishing gears (Lodge et al., 2012).

Significant effort has been expended by resource agencies to use molecular surveillance techniques to detect the presence of bigheaded carps (Darling and Mahon, 2011; Jerde et al., 2013; Lodge et al., 2012) as an alternative to capture with conventional gear. The primary molecular surveillance technique has been the analysis of water samples for the presence of fragments of environmental deoxyribonucleic acids (eDNA) shed from bigheaded carps. Conventional polymerase chain reaction (cPCR) techniques using markers targeting presumably species-specific sections of the mitochondrial DNA of BHC and SVC have been used to monitor for the presence of DNA from BHC and SVC throughout the Chicago Area Waterway System (CAWS), Des Plaines River, Upper Mississippi River and the western basin of Lake Erie. This technique was originally described for the detection of American bullfrog *Rana catesbeiana* in water samples (Ficetola et al., 2008) and was later applied to water samples taken from the CAWS as part of an attempt to evaluate the utility to detect the DNA of BHC or SVC (Jerde et al., 2011). Resource agencies subsequently implemented surveillance programs which processed DNA in environmental samples from the Great Lakes and Mississippi River basin using cPCR techniques to detect the DNA of BHC or SVC. Similarly, other researchers have used these techniques to detect the DNA of freshwater amphibians and mollusks and several species of marine fishes (Dejean et al., 2011, 2012; Foote et al., 2012; Goldberg et al., 2011, 2013; Jerde et al., 2011, 2013; Minamoto et al., 2012; Pilliod et al., 2013; Takahara et al., 2012, 2013; Thomsen et al., 2012a,b).

Current methods used for eDNA monitoring of invasive species have not been optimized to ensure consistent eDNA detection. For example, only about 60% of samples collected from waters with abundant,

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mixed populations of bigheaded carps result in the detection of the DNA of bigheaded carps (Jerde et al., 2011). This low probability of detection may be partially due to the indeterminate distribution of DNA in the waterway. However, low probability of detection could certainly be associated with inconsistent DNA extraction and inefficient PCR amplification, both of which can be controlled and optimized. As with any analytical technique, the methods used for chemical extraction should provide the maximum, consistent recovery, ideally with minimal extraction of confounding compounds. However, the current extraction technique applied for eDNA extraction in bigheaded carp eDNA surveillance programs and outlined in the 2012 Quality Assurance Project Plan (QAPP) (Mahon et al., 2010; USACE, 2012) has not been evaluated against other extraction methods. Similarly, the methods used to detect the chemical need to be accurate and precise and capable of detecting the chemical at levels that can be applied to management decision making processes. Neither the current cPCR techniques or the markers (Jerde et al., 2011) employed have been compared to the TaqMan® assay using quantitative PCR (qPCR), a method thought to be more sensitive than cPCR (Wilcox et al., 2013). Optimizing the performance of these techniques could improve the accuracy and precision of the methods used to detect eDNA and the efficiency of eDNA surveillance programs.

The goal of this project was to identify a robust qPCR assay and DNA extraction method that can be used to detect the DNA of silver carp in environmental samples. First, we determined the reliability of our qPCR assay by comparing its rate of detection of the DNA of silver carp extracted from environmental samples collected from waters with and without established populations of silver carp with the rate of detection when the same extracts were assayed using the standard cPCR monitoring assay described in the 2012 QAPP protocol (USACE, 2012). We further compared detection rates between these cPCR and qPCR assays by the analysis of environmental samples spiked with graded levels of genomic and mitochondrial DNA of silver carp. Finally, we used guidance from the U.S. Department of Health and Human Services Food and Drug Administration Center for Veterinary Medicine (http://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM052180.pdf) to conduct a blind round-robin study to compare the DNA extraction and cPCR analysis method defined in the 2012 QAPP against an alternative extraction kit paired with the new TaqMan® qPCR assay. Performance criteria for the alternative extraction kit and the new TaqMan® qPCR assay were established as having equal results (i.e. detection probability) as the current method (current extraction and cPCR protocol) regardless of the laboratory performing the analysis. This type of study is typically performed prior to the finalization of a regulatory method to detect and quantify a specific chemical constituent (e.g., a drug residue) in a sample matrix (e.g. the edible tissue of a food animal) but has rarely been completed to validate the methods used to detect the DNA of aquatic species. Results from this study are directly applicable to eDNA monitoring.

Material and methods

Because we did not directly use any vertebrate animals in this study, an IACUC or animal welfare protocol was not required for the study. The SVC DNA homogenate used was collected for a previous study (ACUC approval #AEH-12-eDNA-02). All environmental samples collected for these studies were from public waters and did not require any specific permission. Any use of trade, product, or company name is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Markers and PCR assays

cPCR — the presence of SVC DNA in 60 sample extracts was determined by cPCR using the previously reported SVC-specific primers (Jerde et al., 2011); these primers (Table 1) were used in the eDNA surveillance program operated by the U.S. Fish and Wildlife Service in 2013. Each run contained a positive and a negative PCR control, as well as a positive and a negative extraction control. All cPCR reactions (25 μL) were prepared using the reagents specified in the USACE QAPP (USACE, 2012). The PCR conditions used were initial denaturation at 94 °C for 10 min followed by 45 cycles of 94 °C for 1 min then 50 °C for 1 min then 72 °C for 1.5 min; there was a final extension at 72 °C for 7 min after the 45 cycles. The PCR products were held at 4 °C until the plate was removed from the thermal cycler. The PCR products were visualized by electrophoresis on a 2% agarose gel, stained with GelRed™ (Biotium Inc., Hayward, CA, USA) or ethidium bromide and visualized under ultraviolet light. The presence of a 191 bp band indicated a positive for SVC DNA. A sample was considered positive if a single reaction of replicate reactions was found to be positive for SVC DNA.

Table 1
Sets of primers and probe used to amplify DNA from silver carp by conventional PCR and quantitative PCR.

PCR type	Reference/ accession #	Primer	Sequence 5′-3′
Conventional PCR	Jerde et al., 2011	Sense	CCTGARAAAAGARKTRTTCCACTATAA
		Antisense	GCCAAATGCAAGTAATAGTTCATTC
Quantitative PCR	AB595924.1	Sense	GGTGGCGCAGAATGAACTA
		Antisense	TCACATCATTTAACCAGAT
		Probe	CCATGTCCGTGAGATTCCAAGCC

qPCR — a qPCR primer probe set was developed for SVC using the D-loop region of the published mitochondrial sequence (GenBank: AB595924.1). Using this sequence, unique and conserved regions of the D-loop of SVC were identified. This region of the mitochondrial genome was targeted since the current cPCR markers used in monitoring are designed within this region and this region is thought to be highly species-specific (Jerde et al., 2011). The new primers and internal probe were designed using Primer 3.0 v.0.4.0 (http://frodo.wi.mit.edu) for a TaqMan® qPCR. Initial sequence specificity was determined using Basic Local Alignment Search Tool (Altschul et al., 1997) against all sequences in GenBank using blastn. Only the primer-probe set that aligned only with the targeted SVC sequence was considered for further evaluation. The qPCR assay was then optimized for annealing temperature using gradient PCR and serially diluted SVC DNA. The annealing temperature that provided the greatest efficiency was used in all subsequent qPCR assays. This primer-probe set (Table 1) was then used in all subsequent qPCR reactions.

The presence of SVC DNA in 60 sample extracts was determined by qPCR in reactions (25 μL) which were comprised of: 12.5 μL TaqMan® Fast Universal Master Mix (Applied Biosystem, Foster City, California, USA), 500 nM of each primer, 125 nM probe, 1 uL DNA template and molecular-grade water. The PCR conditions were as follows: 94 °C for 2 min, then 45 cycles of 94 °C for 10 s, 58 °C for 15 s and 61 °C for 15 s to read the fluorescence. Plasmid DNA of the targeted sequence was used in serial dilution (100,000 copies to 1 copies per reaction) to verify PCR efficiency, to determine copy numbers and determine the detection threshold on each plate. The detection threshold was determined by using a no template control (negative control) to determine potential amplification drift. A sample was considered positive for SVC DNA if a single reaction amplified beyond the no template control.

Comparisons between extraction techniques

To compare DNA extraction techniques and assess the efficiency of the qPCR assay we developed for SVC DNA, we used a round robin study design with environmental samples spiked with known amounts of SVC DNA, environmental controls which were not spiked with SVC DNA, and incurred samples. Three hundred environmental samples (2 L) were collected from the Black River near La Crosse, WI. Though individuals of both BHC and SVC have been captured from the Mississippi River upstream of the collection site (the Black River and the Mississippi River comingle above the collection site to form Lake Onalaska), neither species was expected to be present in the location where samples were

collected. Each of 60 samples was spiked with 4000, 2000, 750 or 200 μg of SVC DNA. The SVC DNA was a homogenized mixture of SVC feces, mucous, scales and blood whose DNA content was determined by spectrophotometry; the remaining 60 samples were considered environmental controls and were not spiked with SVC DNA. Additionally, 60 incurred samples (2 L) were collected from a recirculating tank housing more than 500 juvenile SVC (<10 cm) in the invasive species holding facility at the U.S. Geological Survey Upper Midwest Environmental Sciences Center (UMESC). All water samples were collected and filtered using a 1.5 μm glass fiber filter within 6 h following procedures described in the 2012 QAPP (USACE, 2012). Immediately following filtration, filters were placed in 50 mL centrifuge tubes. All filters from a single sample were placed into a single tube. All tubes were immediately stored at −80 °C until shipped on dry ice or further processed.

Filters from 20 samples of each spiked concentration, the environmental controls and the incurred samples were provided to three independent laboratories: U.S. Army Corps of Engineers Engineer Research and Development Center (ERDC), U.S. Fish and Wildlife Service Whitney Genetics Laboratory and UMESC. Each laboratory extracted the DNA from filters from the samples received (spiked concentrations, environmental controls and incurred samples). Each laboratory used two DNA extraction protocols, the PowerWater® DNA Isolation Kit (PW; MO BIO Laboratories, Inc., Carlsbad, CA, USA) and the DNeasy Blood and Tissue Kit (Q; Qiagen Inc., Valencia, CA, USA). Samples (i.e. filters) of each spiked concentration, the environmental controls, and the incurred samples were randomly assigned to an extraction process at each laboratory so that each laboratory extracted the DNA from filters from 10 samples with each extraction kit. The DNA in samples assigned to the PW Kit was extracted following the procedures described in the QAPP (USACE, 2012). The DNA in samples assigned to the Q Kit was extracted according to the manufacturer's instructions with the following modification; individual filters were placed in a 2-mL centrifuge tube then 180 μL ATL buffer and 20 μL proteinase K were added. After samples incubated for 1 h at 55 °C, an additional 200 μL ATL buffer was added to each tube. Adding the additional solution volumes ensured that a small amount of liquid remained following centrifugation. The DNA extracted from multiple filters from a single water sample was pooled into a single sample extract. All samples were then diluted to 1 mL with molecular grade water. Total DNA concentration (μg/mL) of each sample processed by each laboratory was quantified by spectrophotometry (absorbance at 260 nm). All spectrophotometry was performed on a Nanodrop 8000 (Thermo Fisher Scientific Inc., Waltham, MA, USA) by WGL and ERDC or a BioPhotometer™ (Eppendorf NA, Hauppauge, NY, USA) by UMESC. Then the presence of SVC DNA was assessed using cPCR and qPCR as described above.

Mean DNA concentrations and copy numbers are reported for each spiked concentration for each laboratory. Linear regression analysis using an F-statistic in an ANOVA was used to test significance of: (1) total DNA concentration does not correlate to extraction kit; (2) SVC DNA detection from cPCR and qPCR does not correlate with extraction kit; and (3) SVC DNA copy numbers do not correlate with extraction kit. Total DNA concentration, rates of detection for cPCR and qPCR, SVC DNA copy number and their interactions were all dependent variables, while extraction technique, laboratory and level of spiked DNA were independent variables. Statistical analysis was performed with SYSTAT 10.2 (Systat Software, Inc., Chicago, IL, USA) and the significance level was set at $\alpha \leq 0.05$. Because both UMESC and WGL had 100% detection with samples extracted with the Q kit, we used samples extracted by ERDC to determine if a greater amount of inhibitors was extracted during the Q extraction. All DNA extracts were diluted with molecular grade water and cPCR reactions were repeated eight times.

Comparisons between conventional and quantitative PCR assays

The cPCR and qPCR assays were compared by UMESC and WGL in separate evaluations of environmental samples collected from locations where established, mixed populations of bigheaded carps are known to exist and where bigheaded carps are not expected to be present. First, UMESC compared the incidence of detecting SVC DNA with the qPCR assay described here with cPCR results obtained from environmental samples collected from the Mississippi River (Amberg et al., 2013). These samples had been collected as part of a monitoring program to detect the DNA of bigheaded carps and had been processed using the procedures outlined in the QAPP (USACE, 2012). In short, 50 surface water samples (2 L) had been indiscriminately collected from two sites by personnel from the Minnesota Department of Natural Resources and the University of Minnesota (UMN). The two locations selected were the Mississippi River below Lock and Dam 19 (near Keokuk, IA, USA), an area where SVC is abundant, and Square Lake (near Minneapolis, MN, USA), an inland lake that is not currently known to contain SVC. All sampling, filtration, storage and DNA extraction procedures, including the collection and handling of appropriate controls (e.g., 3 cooler blanks/site), followed the procedures specified in the QAPP (USACE, 2012). Samples were filtered by personnel from UMN then transported on dry ice to UMESC where they were stored at −80 °C until DNA was extracted using the PW Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). Conventional PCR and qPCR was performed on each sample in duplicate by each laboratory. All qPCR samples that appeared to be positive were visually analyzed using gel electrophoresis. Any sample with DNA of the appropriate amplicon length (i.e. 191 bp for cPCR and 110 bp for qPCR) present was considered presumptive-PCR positive. Presumptive-PCR positives from the MN sites were re-analyzed with additional replicates of cPCR, and those found to be positive were classified as determinative-PCR positive. Bands from selected determinative-PCR positive samples were eluted and purified from the gel and sequenced by the UMN Biomedical Genomics Center (St. Paul, MN, USA). Only samples with both the forward and reverse sequences matching ≥95% the targeted species in a BLAST search were designated as confirmed positive.

Second, the WGL separately processed 20 water samples (2 L) collected from the Peoria Pool of the Illinois River (just below Starved Rock Dam near LaSalle, IL, USA), an area where SVC are abundant, and Lake Neshonoc, an impoundment of the La Crosse River (near West Salem, WI, USA), an area where bigheaded carps are not expected to be present. Cooler blanks (one per site) were prepared at each site. Cooler banks consisted of tubes filled with well water and placed in the cooler to serve as a control to verify that the cooler did not get contaminated. Samples were collected by the U.S. Fish and Wildlife Service La Crosse Fish and Wildlife Conservation Office then filtered by WGL where the filters were stored at −80 °C. All sample collection and processing followed the QAPP (USACE, 2012) with the following exceptions: (1) only a single cooler blank was collected at each site instead of three cooler blanks per site; (2) DNA was extracted with the Q Kit according to the protocol above instead of the PW Kit. Conventional PCR and qPCR was performed in duplicate on each sample by each laboratory. Any sample with DNA of the appropriate amplicon length (i.e. ~190 and 110 base pairs) present was considered presumptive-PCR positive. Bands from the presumptive-PCR positives from selected samples from the Illinois River and Lake Neshonoc were gel-purified and sequenced at WGL on an Applied Biosystems 3500 (Life Technologies, Grand Island, NY, USA). Only samples with both the forward and reverse sequences matching ≥95% the targeted species in a BLAST search were designated as confirmed positive.

The relative efficiency of cPCR and qPCR to detect SVC DNA in environmental samples was evaluated through additional analysis of 10 of the 50 samples collected from the Mississippi River below Lock and Dam 19. Samples chosen include those that were confirmed positive for SVC, had no presumptive PCR-positives, and had presumptive PCR-positives but no determinative PCR-positives (Amberg et al., 2013). Each selected sample was analyzed in eight replicates using cPCR and qPCR according to the previously described conditions. The detections of SVC DNA by cPCR were compared to those by qPCR to determine

the relative efficiency of each method to detect SVC DNA in environmental samples.

Results

Extraction techniques

Extraction kit and laboratory explained a significant amount of the variation in total DNA extracted from spiked environmental samples, environmental controls and incurred samples ($F = 55.67$, $df = 7$, $p < 0.01$). The Q Kit extracted greater amounts of total DNA than the PW Kit ($p < 0.01$; Fig. 1). Although the amount of DNA extracted differed between laboratories for both kits ($p < 0.01$; Table 2), all laboratories extracted significantly more DNA using the Q Kit compared to the PW Kit ($p < 0.01$; Table 2).

Silver carp DNA was not detected in the environmental control samples regardless of extraction method with cPCR (Table 3). Although extraction method did not significantly alter the rate of detection of SVC DNA ($p = 0.07$), differences in the rates of detection were observed between labs ($p = 0.03$) processing spiked environmental samples and analyzed with cPCR. The UMESC and WGL were more likely to amplify SVC DNA when the Q Kit was used whereas ERDC was more likely to amplify the SVC DNA when the PW Kit was used. However, upon 5-fold dilution of the samples extracted with Q Kit, ERDC detected SVC DNA in 100% of all spiked samples. Interestingly, the rate of detection did not correspond to the amount of DNA spiked into the sample regardless of the lab (Table 3). Silver carp DNA was detected significantly more frequently when incurred samples were extracted with the Q Kit than with the PW Kit ($p < 0.01$; Table 3), regardless of laboratory. SVC DNA was detected in all positive controls and no SVC DNA was detected in any negative control by any of the three laboratories.

Quantitative PCR allowed for comparison of the rate of detection and copy number of SVC DNA (Fig. 2) among laboratory and DNA extraction protocol. The DNA of SVC was not detected in any of the environmental or process control samples. As a variable, testing laboratory did not explain a significant amount of the variation in either rate of SVC DNA detection or DNA copy number ($p = 0.42$ and 0.73, respectively). Extraction method, however, explained a significant amount of the variation ($F = 30.11$, $df = 7$, $p < 0.01$) in both rate of detection and DNA copy number (i.e., cycle threshold). The rate of SVC DNA detection from spiked environmental samples and from incurred samples was significantly higher ($p < 0.01$) when DNA was extracted with the Q Kit (100% detection) relative to the PW Kit (80% detection). The cycle threshold (CT) of spiked environmental samples and of incurred samples was significantly lower (i.e., higher DNA copy number; $p < 0.01$) for samples extracted with the Q Kit than samples extracted with the PW Kit. Additionally, the range of the coefficient of variation (CV) for spiked environmental samples and from incurred samples extracted with the Q Kit (0.02 to 0.04) was lower and narrower than the CV of samples extracted with the PW Kit (0.08 to 0.14).

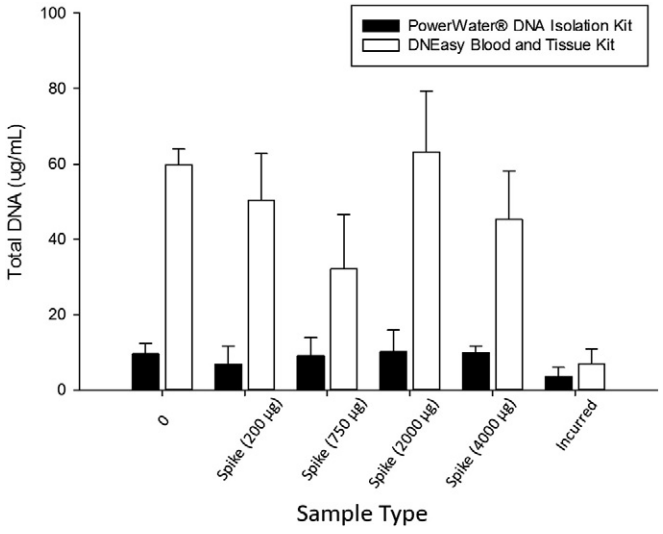

Fig. 1. Mean (±SE) total DNA extracted from samples using PowerWater® DNA Isolation Kit (dark bars) and DNeasy Blood and Tissue Kit (light bars). Means represent the average of ten replicates per spiked DNA concentration at each of the three laboratories; UMESC, WGL and ERDC.

Table 2
Estimates of total DNA and silver carp mtDNA quantity in six samples. Samples were extracted with PowerWater® DNA Isolation Kit and DNeasy Blood and Tissue Kit. Silver carp mtDNA quantity was determined by qPCR and the use of a standard curve. Means and CV were determined from ten replicates per group at each laboratory: U.S. Army Corps of Engineers Environmental Research and Development Center (ERDC), U.S. Fish and Wildlife Service Whitney Genetics Laboratory (WGL) and U.S. Geological Survey Upper Midwest Environmental Sciences Center (UMESC).

	SVC DNA spike level (ng)					Incurred
	0	200	750	2000	4000	
Total DNA (total µg DNA)						
PowerWater®						
ERDC	11.46	2.34	5.26	3.95	6.02	1.00
WGL	13.48	16.82	18.92	21.82	12.56	8.31
UMESC	3.56	1.05	2.97	4.67	10.97	1.40
Overall mean	9.50	6.74	9.05	10.15	9.85	3.57
CV	0.55	1.30	0.95	1.00	0.35	1.15
DNeasy						
ERDC	67.92	39.51	20.72	38.74	19.86	2.98
WGL	57.66	75.12	60.73	93.66	63.05	15.23
UMESC	53.90	36.15	14.97	56.91	52.59	2.34
Overall mean	59.83	50.26	32.14	63.10	45.17	6.85
CV	0.12	0.43	0.78	0.44	0.50	1.06
SVC DNA (copies)						
PowerWater®						
ERDC	0.0	83.7	795.2	1655.0	4383.7	3.7
WGL	0.0	16.2	249.7	347.9	15.9	5.0
UMESC	0.0	38.4	85.4	210.0	696.2	24.4
Overall mean	0.00	46.10	376.75	737.63	1698.59	11.03
CV	0.00	0.75	0.99	1.08	1.38	1.05
DNeasy						
ERDC	0.0	893.1	3192.0	9857.5	22,040.1	74.5
WGL	0.0	133.7	2391.2	3552.2	4782.3	9.2
UMESC	0.0	74.0	46.8	608.7	888.8	65.6
Overall mean	0.00	366.92	1876.67	4672.79	9237.08	49.77
CV	0.00	1.24	0.87	1.01	1.22	0.71

Comparison of qPCR and cPCR assay

The application of the qPCR protocol resulted in a lower presumptive PCR positive rate for samples collected from locations where SVC were presumed absent (Square Lake, Lake Neshonoc; Table 4) than the application of the cPCR protocol. The application of the cPCR and qPCR protocols to samples collected from the Mississippi River where SVC are abundant (below Lock and Dam 19) resulted in similar detection rates (Table 4). When these protocols were applied to samples collected from portions of the Illinois River (Peoria Pool) where SVC are abundant, 100% of the samples were characterized as presumptive PCR positive by qPCR but only 60% of samples were characterized as presumptive PCR positive by cPCR.

Ten samples previously collected from the Mississippi River (below Lock and Dam 19) and previously processed by cPCR to detect the presence of SVC DNA (Amberg et al., 2013) were randomly selected and processed in eight replicates by both cPCR and qPCR using the protocols described here. Similar rates of DNA detection (i.e., 7 of 10 samples identified as presumptive PCR positive; Table 5) existed between the cPCR and qPCR protocols. However, qPCR failed to detect SVC DNA in two of four samples which had previously been determined by Sanger sequencing to contain SVC DNA (Amberg et al., 2013) (Table 5). When processed by cPCR, Samples 2 and 3 (Table 5) had only 2 and 1 replicate

Table 3
The number of positive detections for silver carp DNA in samples using conventional PCR and quantitative PCR on samples extracted with PowerWater® DNA Isolation Kit and DNeasy Blood and Tissue Kit. Means represent the average of ten replicates per spiked DNA concentration at each of three laboratories: U.S. Army Corps of Engineers Environmental Research and Development Center (ERDC), U.S. Fish and Wildlife Service Whitney Genetics Laboratory (WGL) and U.S. Geological Survey Upper Midwest Environmental Sciences Center (UMESC).

	SVC DNA spike level (ng)					Incurred
	0	200	750	2000	4000	
Conventional PCR						
PowerWater®						
ERDC	0	8	10	10	7	4
WGL	0	5	10	8	7	6
UMESC	0	5	7	6	8	6
Overall mean	0.00	6.00	9.00	8.00	7.33	5.33
CV	0.00	0.29	0.19	0.25	0.08	0.22
DNeasy						
ERDC	0	7	6	0	7	10
WGL	0	9	10	10	10	10
UMESC	0	10	8	9	10	7
Overall mean	0.00	8.67	8.00	6.33	9.00	9.00
CV	0.00	0.18	0.25	0.87	0.19	0.19
Quantitative PCR						
PowerWater®						
ERDC	0	9	10	10	9	2
WGL	0	10	10	10	9	6
UMESC	0	5	6	8	10	6
Overall mean	0.00	8.00	8.67	9.33	9.33	4.67
CV	0.00	0.33	0.27	0.12	0.06	0.49
DNeasy						
ERDC	0	10	10	10	10	10
WGL	0	10	10	10	10	10
UMESC	0	10	10	10	10	10
Overall mean	0.00	10.00	10.00	10.00	10.00	10.00
CV	0.00	0.00	0.00	0.00	0.00	0.00

of the processed eight replicates, respectively, characterized as presumptive PCR positive, suggesting that the SVC DNA concentration may have been very low in these samples. In contrast, Samples 5 and 7 (also previously determined to contain SVC DNA) had 3 replicates characterized as presumptive PCR positive by cPCR but 8 of 8 replicates were identified as presumptive PCR positive when assayed by qPCR. Similarly, Sample 9 was characterized as presumptive PCR positive in 4 replicates by cPCR but 8 of 8 replicates were characterized to

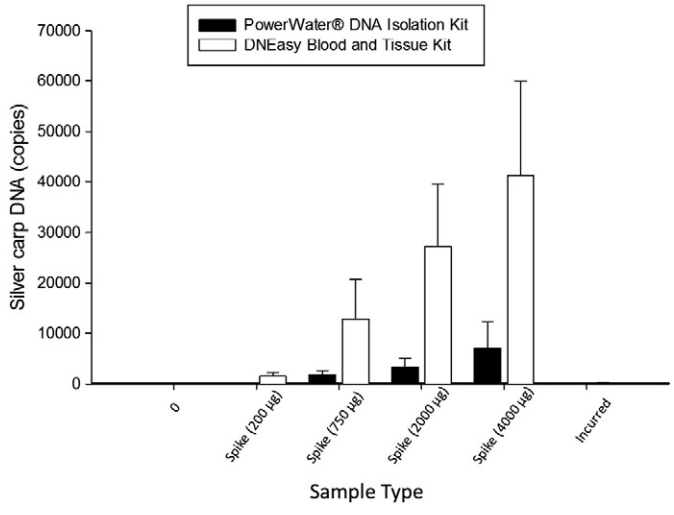

Fig. 2. Mean (±SE) number of copies of silver carp DNA extracted from samples using PowerWater® DNA Isolation Kit (dark bars) and DNeasy Blood and Tissue Kit (light bars). Means represent the average of ten replicates per spiked DNA concentration at each of the three laboratories; UMESC, WGL and ERDC.

Table 4
The number of conventional PCR (cPCR) and quantitative PCR (qPCR) presumptive-positive samples from sites where bigheaded carp were abundant (below Lock and Dam 19 on the Mississippi River; the Peoria Pool of the Illinois River) and not expected to be present (Square Lake, eastern Minnesota; Lake Neshonoc near West Salem, Wisconsin).

		Presumptive PCR positive	
Number of samples		cPCR	qPCR
Sites where bigheaded carp are absent			
Square Lake	50	11	2
Lake Neshonoc	20	1	0
Sites where bigheaded carp are abundant			
Lock and Dam 19	50	34	33
Peoria Pool	20	12	20

presumptive PCR positive when assayed by qPCR. When 2 or fewer replicates of a sample were described as presumptive PCR positive by cPCR, a similar number of replicates were identified as presumptive PCR positive by qPCR or DNA was not detected. Similarly, for those samples not characterized as presumptive PCR positive by cPCR, only 0 or 1 replicate was determined to be presumptive PCR positive by qPCR.

Amplicons from qPCR assays of 22 presumptive PCR positive samples (Mississippi River below Lock and Dam 19, n = 16; Illinois River from Peoria Pool, n = 6) were sequenced. BLAST analysis of the sequences from samples taken from the Illinois River aligned with the DNA of SVC on file in GenBank. Sequences (Table 6) could only be obtained from 12 of the 16 samples submitted from the Mississippi River and 11 of those 12 aligned with the DNA of SVC. The sequence for the amplicon from the Mississippi River that did not align with the DNA of SVC did align with the DNA of BHC.

Discussion

Programs that seek to monitor eDNA, whether for surveillance of invasive organisms or for other uses, should incorporate procedures that consistently recover DNA from environmental samples and analytical platforms that provide definitive, unambiguous results. Because eDNA monitoring programs are attempting to detect very low abundances of the target animal, it is critical that the DNA extraction techniques used consistently extract the greatest amount of DNA to maximize the opportunity to detect sequences of DNA that may be at very low levels of abundance, especially during the critical initial phases of invasion. The commercially available extraction kit (PW) specified in the QAPP (USACE, 2012) and Mahon et al. (2010) yielded much less total DNA than an alternative commercially available extraction kit (Q Kit). Differences in extraction efficiencies for various commercially available kits have been previously reported (Claassen et al., 2013; Piaggio et al.,

Table 5
Number of replicates characterized as presumptive PCR positive following conventional PCR (cPCR) or quantitative PCR (qPCR) of 8 replicates of 10 samples taken from a site where bigheaded carps are known to be abundant (Mississippi River below Lock and Dam 19).

Sample	Number of presumptive PCR positive samples	
	cPCR	qPCR
1	1	2
2[a]	2	0
3[a]	1	0
4	0	1
5[a]	3	8
6	0	0
7[a]	3	8
8	0	1
9	4	8
10	1	1

[a] Indicates samples were sequence confirmed.

Table 6
Sequences of the amplicons from quantitative PCR analysis of six presumptive PCR positives from the Illinois River (ILR) and 12 presumptive PCR positives from the Mississippi River (MSR). For comparisons, the sequence of the silver carp target sequence (SVC) has been included.

Sample	Sequence
SVC	TACTTGCATTTGGCTTGGAATCTCACGGACATGACTGTAAAATTCCACCCTC CATACATTATATCTGGCATCTGGTTAAATGATGTGA
ILR-1	TACTTGCATTTGGCTTGGAATCTCACGGACATGGCTGTAAAATTCCACCCTC CATACATTATATCTGGCATCTGGTTAAATGATGTGA
ILR-2	TACTTGCATTTGGCTTGGAATCTCACGGACATGGCTGTAAAATTCCACCCTC CATACATTATATCTGGCATCTGGTTAAATGATGTGA
ILR-3	TACTTGCATTTGGCTTGGAATCTCACGGACATGGCTGTAAAATTCCACCCTC CATACATTATATCTGGCATCTGGTTAAATGATGTGA
ILR-4	TACTTGCATTTGGCTTGGAATCTCACGGACAAGGCTGTAAAATTCCACCC TCCATACATTATATCTGGCATCTGGTTAAATGATGTGA
ILR-5	TACTTGCATTTGGCTTGGAATCTCACGGACATGGCTGTAAAATTCCACCCTC CATACATTATATCTGGCATCTGGTTAATGAAGTGAA
ILR-6	TACTTGCATTTGGCTTGGAATCTCACGGACATGGCTGTAAAATTCCACCCTC CATACATTATATCTGGCATCTGGTTAATGATGTGAA
MSR-1	TACTTGCATTTGGCTTGGAATCTCACGGACATGGCTGTAAAATTCCACCCTC CATACATTATATCTGGCATCTGGTTAAATGATGTGA
MSR-2	TACTTGCATTTGGCTTGGAATCTCACGGACATGGCTGTAAAATTCCACCCTC CATACATTATATCTGGCATCTGGTTAAATGATGTGA
MSR-3[a]	TACTTGCATCTGGTTTGGAATCTCACGGACATGGCTACAAAATTCCATCCCC GTTACATTATAACTGGCATCTGGTTAAATGATGTGA
MSR-4	TACTTGCATTTGGCTTGGAATCTCACGGACATGGCTGTAAAATTCCACCCTC CATACATTATATCTGGCATCTGGTTAAATGATGTGA
MSR-5	TACTTGCATTTGGCTTGGAATCTCACGGACATGGCTGTAAAATTCCACCCTC CATACATTATATCTGGCATCTGGTTAAATGATGTG
MSR-6	TACTTGCATTTGGCTTGGAATCTCACGGACATGGCTGTAAAATTCCACCCTC CATACATTATATCTGGCATCTGGTTAAATGATGTGA
MSR-7	TACTTGCATTTGGCTTGGAATCTCACGGACATGGCTGTAAAATTCCACCCTC CATACATTATATCTGGCATCTGGTTAAATGATGTGA
MSR-8	TACTTGCATTTGGCTTGGAATCTCACGGACATGGCTGTAAAATTCCACCCTC CATACATTATATCTGGCATCTGGTTAAATGATGTGA
MSR-9	TACTTGCATTTGGCTTGGAATCTCACGGACATGGCTGTAAAATTCCACCCTC CATACATTATATCTGGCATCTGGTTAAATGATGTGA
MSR-10	TACTTGCATTTGGCTTGGAATCTCACGGACATGGCTGTAAAATTCCACCCTC CATACATTATATCTGGCATCTGGTTAAATGATGTGA
MSR-11	TACTTGCATTTGGCTTGGAATCTCACGGACATGGCTGTAA MATTCCACCCTCCATACATTATATC
MSR-12	TACTTGCATTTGGCTTGGAATCTCACGGACATGGCTGTAAAATTCCACCCTC CATACATTATATCTGGCATCTGGTTAAATGATGTGA

[a] Indicates the sequence found to be most similar to Bighead carp.

2013; Podnecky et al., 2013; Poma et al., 2012). Piaggio et al. (2013) reported that the QAIamp DNA extraction kit (Qiagen Inc., Valencia, CA, USA) yielded more *Python bivittatus* DNA from water samples than did the PowerWater® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). However, no extraction kit is optimal for all organisms (Fredricks et al., 2005). Improved extraction efficiency has the potential to minimize the risk of falsely concluding that samples do not contain the DNA of the targeted species. Surveillance programs that depend on the detection of specific target DNA in environmental samples must use procedures and protocols to minimize the risk of falsely concluding that the target DNA is not present in samples processed. This is of specific import for those programs managing an aquatic invasive species where falsely concluding that the DNA of the target species is not present could allow the establishment of the invader into new habitats. Once established, it is extremely difficult and costly to control the invasive species (Pejchar and Mooney, 2009; Pimentel et al., 2005; Xu et al., 2006). As this field expands, improvements in our ability to efficiently extract DNA from water samples will decrease the risk of falsely concluding that the target DNA is absent when it is truly present.

We identified an accurate qPCR assay and an efficient and effective DNA extraction method with direct application to eDNA monitoring of bigheaded carps, including SVC. The sensitivity provided by the qPCR marker demonstrated comparable performance to the current cPCR marker in environmental samples in side-by-side comparison of samples from locations where bigheaded carps are abundant and where they are not known to exist. The qPCR assay yielded nearly identical detection rates of the DNA of SVC as cPCR for samples from the Mississippi River but outperformed cPCR in samples taken from the Illinois River. These rates of detection of SVC DNA are similar to those reported for the highly abundant common carp, *Cyprinus carpio*, in the CAWS (Mahon et al., 2013). Because both of these sites contained large, mixed populations of bigheaded carps that support commercial fisheries (Jones and Gritters, 2013; Roth et al., 2012), it was presumed that all samples from the Mississippi River below Lock and Dam 19 and from the Peoria Pool of the Illinois River had an equal probability (within a system) of containing the DNA of SVC. Although the number of samples characterized as presumptive PCR positive for the DNA of SVC did not differ when processed with qPCR or cPCR, more replicates of the processed samples were characterized as presumptive PCR positive when processed with qPCR. The 2-fold increase in the proportion of sample aliquots that were characterized as presumptive PCR positive by qPCR suggests that this qPCR assay may be more sensitive than the cPCR assay.

A greater portion of the samples from the Illinois River were characterized as presumptive PCR positive for SVC DNA than samples from the Mississippi River, probably because of the greater abundance of SVC in the Peoria Pool of the Illinois River than in Pool 19 of the Mississippi River. In 2012, more than 454,000 kg of bigheaded carps where harvested from the Peoria Pool whereas only 6800 kg of bigheaded carps were removed above Pool 19 (Jones and Gritters, 2013; Roth et al., 2012). While this study was not designed to compare detection probability based on target species abundance, the proportion of samples characterized as presumptive PCR positive relative to the abundance of the target species in the respective system generally agrees with other studies that suggest that detection correlates with animal abundance (Mahon et al., 2013; Takahara et al., 2012).

The qPCR assay reported here had fewer samples from sites where bigheaded carps are presumed absent that were characterized as presumptive PCR positive (i.e., non-targeted amplification) than cPCR. When previously processed by cPCR (USACE, 2012), 11 of 50 samples taken from Square Lake were characterized as presumptive PCR positive, requiring additional cPCR analysis and/or sequencing. When those same extracts were processed by the qPCR method reported here, only 2 of 50 were characterized as presumptive PCR positive and those amplifications were below the copy level of the lowest standard in the assay standard curve (10 copies per reaction). Further, the amplification plots of those two samples were dissimilar to those of reactions known to contain the DNA of SVC. The application of qPCR to the samples from Square Lake did not eliminate the need for confirmation of the amplified sequence of these non-target amplifications to confirm whether the DNA of SVC was present, but it did reduce the number of samples which had non-target amplification. The application of the qPCR assay reported here to samples such as these would likely result in fewer samples being falsely identified as positive, thus reducing the number of samples that require sequence confirmation. The qPCR assay did provide information not available from the cPCR assay for these non-target amplification in that the amplification plots were immediately characterized as suspect relative to the plots obtained from samples where SVC were known to be present. When applied to invasive species surveillance, the ability to use amplification plots and other information available from qPCR to characterize quickly amplifications as “probable positive” or “probable negative” even before sequencing results are available might shorten the response window to new invasions. Non-target amplification can further be avoided by increasing the specificity of a qPCR assay through careful design of primers and probes (Wilcox et al., 2013) and by thorough optimization and vetting prior to implementation within surveillance programs.

Quantitative PCR performed better than cPCR in detecting the SVC DNA in spiked environmental samples (Table 3). The DNA of SVC was detected in 90.0% of spiked samples when analyzed with qPCR compared to only 76.7% of spiked samples analyzed by cPCR. This difference may be due to the abilities of the *Taq* polymerase master mixes to

function in the presence of inhibitors. Inhibition is known to occur in PCR analysis of DNA from environmental samples (Gibson et al., 2012). Inhibition of a sample may result in falsely concluding that a sample or location does not contain DNA when in fact the target DNA is present, thus leading to the false conclusion that the target species was not present when it actually is. Application of the qPCR assay we report may lessen this risk, at least for the samples we processed.

In summary, we report that differences do exist in quantity of DNA extracted by commercial extraction kits with the Q Kit yielding greater quantities of DNA than the PW Kit. Additionally, the qPCR assay we report was more sensitive and had the potential to withstand greater amounts of PCR inhibition than the current cPCR assay. When combining improved extraction efficiency with the qPCR, more consistent results and improved interpretation of eDNA can be expected. This can provide greater opportunity for the detection of targeted DNA sequences. Results from our studies demonstrate that significant improvements can be made in the methods and procedures applied for eDNA monitoring that ultimately may provide managers more clarity when interpreting and evaluating eDNA results.

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