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ORIGINAL PAPER

Early detection of a highly invasive bivalve based on environmental DNA (eDNA)

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Abstract Management of non-indigenous invasive species (NIS) is challenging owing in part to limitations of early detection and identification. The advent of environmental DNA (eDNA) techniques provides an efficient way to detect NIS when their abundance is extremely low. However, eDNA-based methods often suffer from uncertain detection sensitivity, which requires detailed testing before applying these methods in the field. Here we developed an eDNA tool for early detection of the highly invasive golden mussel, *Limnoperna fortunei*, based on the mitochondrial cytochrome *c* oxidase subunit I gene (COI). Further, we tested technical issues, including sampling strategy and detection sensitivity, based on a laboratory experiment. We then applied the method to field samples collected from water bodies in China where this mussel has or is expected to colonize. Results showed that the detection limit varied extensively among our newly developed primer pairs, ranging from 4×10^{-2} to 4×10^{-6} ng of total genomic DNA. Laboratory detection was affected by the availability of eDNA (i.e., both mussel abundance and incubation time). Detection capacity was higher in laboratory samples containing re-suspended matter from the bottom layer versus that collected from the surface. Among 25 field sites, detection was 100% at sites with high mussel abundance and as low as 40% at sites with low abundance when tested using our most sensitive primer pair. Early detection of NIS present at low abundance in nature requires not only sensitive primers, but also an optimized sampling strategy to reduce the occurrence of false negatives. Careful selection and detailed testing of primer pairs ensures effective eDNA-based species detection in surveillance and management programs.

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Introduction

Non-indigenous invasive species (NIS) are a leading cause of ecological, economic and health harm, and thus have received the attention of both scientists and policy makers (e.g., Walsh et al. 2016). Decisions regarding management should be made early to enhance the likelihood of success, though this is largely dependent upon early detection (Brown et al. 2016; Holden et al. 2016; Xiong et al. 2016). Traditionally, morphological trait-based methods were utilized to identify species. However, these methods have limitations when dealing with cryptic species (Heinrichs et al. 2011), and species with ontogenetic stages or exhibiting extreme phenotypic plasticity (e.g., Kekkonen and Hebert 2014). These methods are also increasingly challenged by the long-term erosion in systematics expertise (e.g., Xiong et al. 2016). Furthermore, detecting rare NIS may require very extensive sampling to maximize species retrieval (e.g., Harvey et al. 2009).

The advent of the DNA barcode created a novel way to conduct species identification that bypasses morphological traits, allowing researchers lacking taxonomic expertise to nevertheless identify species (Hebert et al. 2003, 2004; Ikeda et al. 2016). Environmental DNA (eDNA) refers to DNA shed into the environment, and its presence in aquatic systems allows for efficient and sensitive identification of target species from bulk water samples (e.g., Bohmann et al. 2014). For rare species, such as newly introduced NIS or endangered species, eDNA usually yields a higher detection rate than traditional methods such as trapping and seining (Dejean et al. 2012; Dougherty et al. 2016; Schmelzle and Kinziger 2016). In addition, eDNA-based metabarcoding usually recovers more species in a community than morphological methods (Zhan and MacIsaac 2015; Valentini et al. 2016).

The bio-fouling golden mussel (*Limnoperna fortunei*) is native to Southeast Asia but has spread widely there (Nakano et al. 2015) as well as in eastern South America (see Boltovskoy 2015). The species poses serious biofouling problems to water supply and drainage systems and is an "ecosystem engineer" in many invaded ecosystems, altering planktonic and benthic communities and changing nutrient cycling (see Boltovskoy 2015). The species is similar to *Dreissena* species (i.e., zebra and quagga mussels) in terms of physiological traits and ecological impacts, though it possesses broader tolerance to many environmental conditions and thus has the potential of a wider distribution (Karateyev et al. 2007). The species has many avenues for dispersal, including as veliger larvae in actively transported water (ballast, live wells; Ricciardi 1998) or in currents and water diversion channels (Zhan et al. 2015), while adults may disperse fouled on external surfaces of vessels or floating debris (see Boltovskoy 2015). Despite the negative impacts of this species in both Asia and South America, heretofore early detection has not been well prioritized though it had been molecularly identified (Pie 2006; Pie et al. 2017).

In this study, we developed an eDNA-based early detection method and conducted a sensitivity test using a serial dilution of total genomic DNA. Subsequently, we explored species detectability in laboratory and field experiments using our most sensitive primers. We also optimized a field sampling strategy to minimize false negatives.

Materials and methods

To obtain robust primers to detect the golden mussel from environmental samples, we first designed 13 primer pairs based on the mitochondrial COI gene. Subsequently, we conducted a sensitivity test using a serial dilution of the total genomic DNA to screen these primer pairs. The two most sensitive primer pairs were tested further based on laboratory aquarium experiments for sensitivity validation using controlled numbers of animals. Finally, we chose our most sensitive primers for detecting this mussel based on environmental samples collected from a variety of water bodies using an optimized sampling method.

Primer design and laboratory sensitivity validation

To design species-specific primers, we downloaded all 56 available mitochondrial COI sequences (>500 bp) of *L. fortunei* in the NCBI GenBank

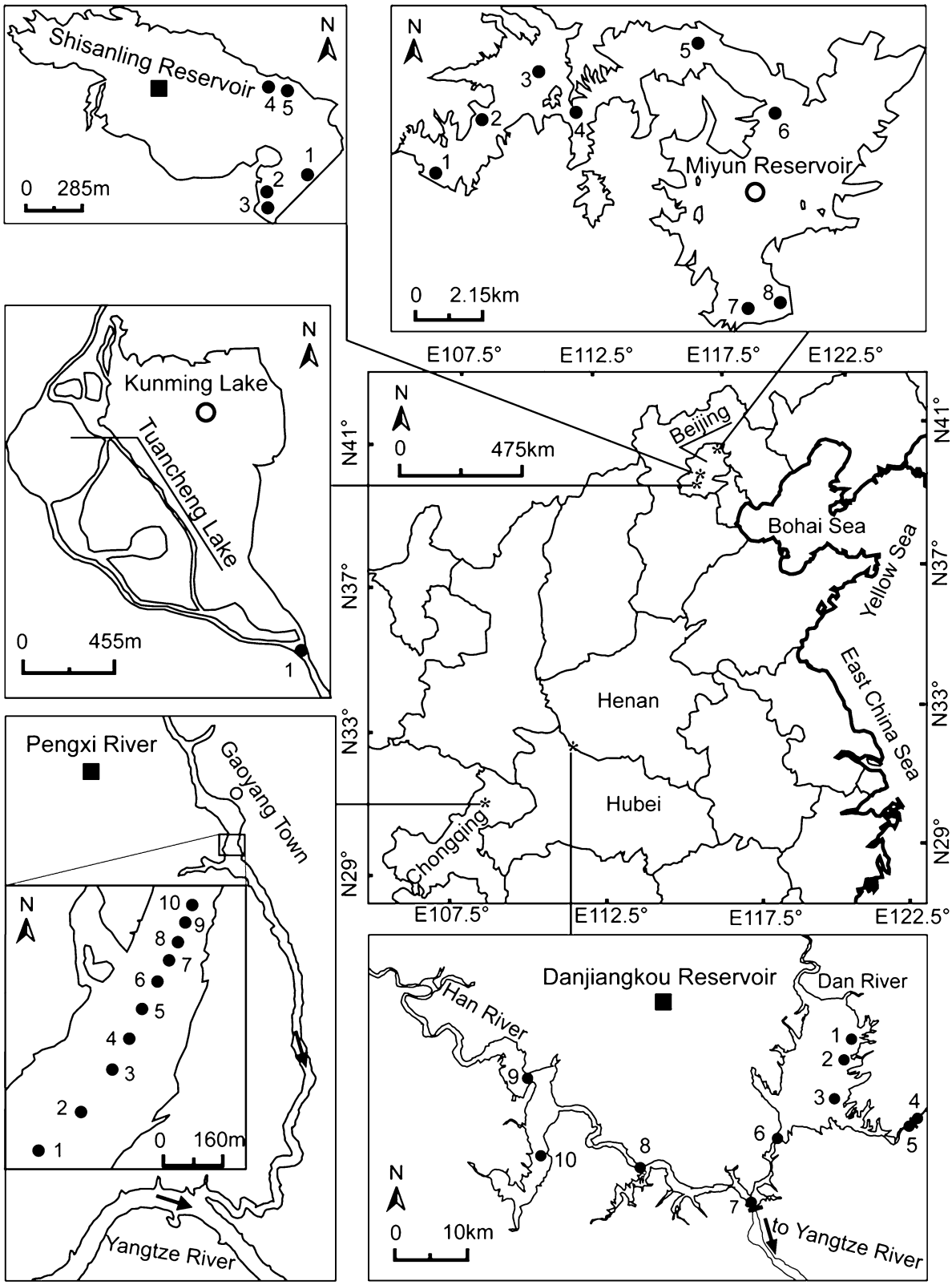
(https://www.ncbi.nlm.nih.gov/) and retrieved representative COI sequences with high similarity (>70%) to *L. fortunei*. These sequences were aligned in MEGA (version 6.06) and inspected to determine conserved regions for our target species (*L. fortunei*) but sufficiently variable in related species to avoid cross-species amplification and false positive results. Given that DNA in aquatic ecosystems is usually highly degraded, we designed primers with fragment length ranging from 127 to 299 bp using Primer Premier 5 (PREMIER Biosoft) based on suggestions from other studies (Bohmann et al. 2014; Dougherty et al. 2016). To ensure species specificity, all 13 primer pairs were compared with available mitochondrial COI sequences of five bivalve species (family Unionidae: *Bellamya purificata*, *Semisulcospira cancellata*, *Unio douglasiae* and *Sinanodonta woodiana*; family Cyrenidae: *Corbicula fluminea*) which may be recovered in our sampling areas. Also, we tested the primers against the total genomic DNA extracted from tissues of these species and results showed no positive PCR amplification. As *L. fortunei* is the only freshwater species in genus *Limnoperna* and the order Mytiloida, we did not consider other related species in the order Mytiloida when conducting the species-specificity test.

The sensitivity of a primer pair was characterized by the detection limit (i.e., the lowest amount of genomic DNA that can be amplified) in a 25 μL PCR reaction. A lower detection limit equates to higher sensitivity for the species. We performed tenfold serial dilutions of genomic DNA from an initial concentration of 40.0 ng/μL to achieve a series of concentrations from 4 to 4×10^{-8} ng/μL. A total of 10 replicates were carried out for each concentration. To reduce biased PCR amplification, we defined the detection limit of each primer pair as the lowest amount of genomic DNA that could be successfully amplified in five or more replicates.

Animals used for the laboratory validation test were collected from the Pengxi River, a tributary of the Yangtze River near Chongqing, China (Fig. 1). A submerged brick covered with golden mussels was collected and brought in a cooler with wet towels back to the laboratory and acclimated at 26 °C in a 60 L aquarium tank. We prepared single animals and animal aggregates with five and 15 medium-sized (shell length ~15 mm) individuals by carefully cutting byssal threads, following which single animals were allowed to re-attach on a glass slide during acclimation. During acclimation, animals were fed every 2 days with 50 mL commercial *Chlorella sp.* (~10^8 cells/L). Three treatments with one, five and 15 animals and five replicates for each treatment were used in the laboratory validation. The average wet weight of animals for the three treatments was 0.994, 4.301 and 10.554 g, respectively. Prepared animal aggregates (or slides with one animal on each) were taken from the acclimation tank and flushed by double-distilled water to eliminate chemical traces prior to deployment in experiment tanks (10 L target-DNA-free water therein). Water used in the acclimation tank and experiment tanks was well-aerated tap water, and the water was detected as target DNA-free throughout the whole experiment. Healthy animals typically began to filter within 5 min after transfer. A 250 mL water sample for mussel eDNA was collected from each tank after 20, 40, 60, 90 and 120 min exposure in the tanks and an additional sample was collected at 10 and 30 min, respectively, for the treatment with 15 animals. Animals were not fed during the 2-h experiment. The tank was mixed thoroughly before water collection and each sample was filtered onto a 0.45-μm pore size cellulose acetate microporous membrane filter. Each filter was preserved in a 2 mL centrifuge tube and stored under −20 °C until DNA extraction. Detection time (i.e., time between animal deployment and the point of first detection from the aquarium water sample) of each replicate was recorded, while detection time of each treatment was reported when ≥50% of replicates had successful detections.

We conducted both laboratory and field experiments to optimize the sampling method before the field application. We tested the hypothesis that water samples containing particulate matter re-suspended from the bottom layer were more likely to test positive for the species than those without re-suspended particulate matter. We examined water samples collected by two sampling methods using the most sensitive primer pair: (1) collecting only surface water, without disturbing the bottom layer; and (2) collection of surface water after the bottom layer was disturbed and particulates re-suspended.

In the laboratory experiment, we combined water from the five 15-animal treatment tanks, which experienced an additional 7 days culture after the 2-h of laboratory validation experiment to accumulate


Shisanling Reservoir
N
0 285m
1
2
3
4
5
Miyun Reservoir
0 2.15km
6
7
8
Kunming Lake
Tuancheng Lake
0 455m
E107.5°
E112.5°
E117.5°
E122.5°
N41°
N37°
N33°
N29°
0 475km
Beijing
Bohai Sea
Yellow Sea
East China Sea
Henan
Hubei
Chongqing
Pengxi River
Gaoyang Town
0 160m
Yangtze River
Danjiangkou Reservoir
Han River
Dan River
0 10km
to Yangtze River

◀**Fig. 1** Map of the study sites in the field test of the two most sensitive primers, showing water bodies inhabited (*solid squares*) or uninhabited (*open circles*) by the golden mussel

eDNA into a new tank. We allowed the tank (~40 cm depth) to remain undisturbed for 5 days to let any particulate matter sink to the bottom. Subsequently, we carefully siphoned three 250 mL water samples from the surface layer (~5 cm) prior to collecting the same amount of water after thoroughly mixing the tank. We repeated the same two sampling methods at three field sites in Shisanling Reservoir (Fig. 1: Sites 1–3). Specifically, we collected water samples from the surface layer (~25 cm) and then we disturbed the bottom of the same site, followed by immediate collection of the water containing re-suspended particulates at the depth ~50 cm above the bottom. We examined these samples using primer pair B by detecting golden mussels in their eDNA extracts from the water samples and serial dilutions thereof, with the expectation that more positive detections would be observed at a higher dilution rate from the samples collected from the disturbed water column than those without disturbance.

Field application

From June to July 2015, we sampled a total of 22 field sites in three water bodies where golden mussels have been reported [i.e., Pengxi River and Danjiangkou Reservoir in South China and Shisanling Reservoir (sites 4 and 5) in Beijing in North China; Fig. 1], and nine sites in two systems where golden mussels have not been reported (i.e., Miyun Reservoir and Kunming Lake in Beijing; Fig. 1). Sampling sites at each location were randomly determined. All water bodies were lentic systems except Pengxi River, where we sampled from downstream to upstream to avoid cross-contamination. For the other water bodies, we disturbed the bottom layer before collecting water when depth permitted. Three 250 mL subsamples were collected at each site and those samples were then shipped to the laboratory in a cooler with ice and processed within 12 h after collection. Water was filtered and stored until DNA extraction. To reduce false positives, we considered a positive detection if ≥2 of subsamples at a site detected the species.

Pengxi River and Danjiangkou Reservoir are located in the native region of golden mussels in South China. While we did not quantify the abundance of golden mussels at these sites, the density is higher at the former than the latter. Shisanling Reservoir is outside of the mussel's native region but was previously invaded (Ye et al. 2011), thus we conducted a field survey in May 2015 (prior to the formal sampling in July 2015) and observed a very low density of animals on hard surfaces near the shoreline. Sites that have not been colonized by golden mussels were sampled twice in August 2014 and May 2015 (Miyun Reservoir) by field observation, and verified by interviews of local residents and reservoir managers.

DNA extraction and PCR

Genomic DNA was extracted from animals collected from Pengxi River (Fig. 1), and extracted DNA was quantified by Nanodrop 2000 spectrophotometer (Thermo Scientific). The eDNA on the filter was extracted following a modified protocol based on a published method (Waters et al. 2000). Specifically, digested solution was transferred to a new 2 mL tube before centrifugation and the air-dried DNA pellet was dissolved in 30 μL pure water; proteins were precipitated by 7.5 M ammonium acetate and DNA was precipitated by 100% ethanol.

We conducted gradient temperature PCR for each primer pair prior to formal sensitivity testing to determine the optimized annealing temperature for each primer pair. PCR was conducted in a 25 μL reaction volume containing 1 × PCR buffer, 1 μL DNA extract, 0.05 mM of each dNTP, 0.4 mM of each primer, 2.0 mM of Mg^{2+}, 1 unit of *Taq* Polymerase (Takara Bio Inc.). PCR amplification was performed in a Mastercycler (Eppendorf) with a thermal profile consisting of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 35 s and extension at 72 °C for 30 s, with a final extension at 72 °C for 7 min. PCR products were visualized and analyzed using an automatic gelatin image analysis system, by loading 5 μL of PCR products in each well of 2% agarose gels stained with ethidium bromide. Positive reactions were identified for *L. fortunei* by a single, distinct band at 197 and 286 bp for primer pairs B and I (Table S1 in Appendix), respectively.

To avoid cross-contamination, all bottles used for sample collection were new. During each sampling trip, two bottles were filled with deionized water and

placed with sampling bottles as sampling blanks. Vessels were regularly maintained by cleaning hull and checked before sampling to ensure that no mussels colonized. Non-disposable tools (e.g., forceps and glassware) used in sample filtration and DNA extraction were immersed in 10% commercial bleach for 10 min to destroy residual DNA before reuse. Aerated tap water was filtered for laboratory validation experiment tanks and deionized water was filtered for sample filtration equipment (e.g., filters and glassware) as equipment controls throughout laboratory analyses. In each batch of PCR, a positive (total genomic DNA) and negative control (deionized water) were applied to assure good practice.

Results

Detection limit varied widely across the 13 primer pairs tested, ranging from 4×10^{-2} to 4×10^{-6} ng of total genomic DNA (Table S1 in Appendix). Primer pair B was the most sensitive and could amplify genomic DNA as low as 4×10^{-6} ng. Another three primer pairs (G, I and L) also had relatively high sensitivity ($\sim 4 \times 10^{-4}$ ng). These primer pairs exhibited a similar capacity to amplify aquarium eDNA in a pilot test prior to our laboratory, validation experiment, so we subsequently utilized primer pairs B and I for our formal validation experiment.

For primer pairs B and I, the time required for three out of five replicates detected was inversely related to animal abundance in aquaria (Fig. 2). Specifically, detection with primer pair B occurred in 60, 40 and 20 min, using one, five and 15 animals, respectively, while the less sensitive primer pair I required 90, 90 and 60 min. Primer pair B was more sensitive to amplification of aquarium eDNA samples than primer pair I and detection occurred 30, 50 and 40 min earlier than those for primer pair I with one, five and 15 animals, respectively.

Golden mussels were detected when using both sampling methods in the laboratory experiment (Table 1) by primer pair B. Specifically, eDNA extracted from the water surface layer could be detected at 10^{-2} dilution fraction, while samples from the mixed water column were detectable at 10^{-4} dilution fraction. Among samples collected in Shisanling Reservoir, no positive detections were observed for surface layer samples, though mixed water samples recorded positive detections at two sites. Specifically, mixed samples from sites 2 and 3 detected presence of golden mussel at 20 and 10% dilution fraction, respectively (Table 1). All equipment controls and sampling blanks yielded negative tests throughout this study.

Primer pairs B and I detected golden mussels in natural waters inhabited by the species, while the samples never tested positive for all areas where the species has never been reported (Fig. 3). Similar to our laboratory validation, primer pair B was more sensitive than primer pair I in the field, yielding four more positive detections in Danjiangkou Reservoir, though both primer pairs yielded a 100% hit rate in Pengxi River (10 sites). In Danjiangkou Reservoir, primer pair B successfully detected the species at 70% of sites (i.e., sites 1–3 and 5–8), while primer pair I detected at only 30% of sites (i.e., sites 6–8). In Shisanling Reservoir, all five sites were examined by primer pairs B and I, though only sites 4 and 5 were sampled during the formal field sampling. Primer pair B detected the species at 40% of sites (i.e., site 2 and 3) while primer pair I did not detect at any site. Also, we performed Sanger sequencing for PCR products randomly selected from sites 6 and 7 from Danjiangkou Reservoir using primer pairs B and I, both of which correctly identified the presence of golden mussels.

Discussion

The golden mussel is rapidly expanding its distribution in both South America and Southeast Asia (Boltovskoy 2015). Early detection is, therefore, an essential component of the species' management, though this aspect has not been extensively studied. Recently, Pie et al. (2017) developed a COI-based method to detect this species, which was sensitive to DNA levels as low as 2.25×10^{-4} ng. In this study we screened 13 primer pairs, the most sensitive of which (primer pair B) could detect down to 4×10^{-6} ng. Indeed, primer pair B was highly sensitive to eDNA samples from both laboratory and field (i.e., river, reservoir, and lake). It is not surprising that the detection limit of the former was poor relative to primer pair B, as our study was specifically designed to identify a highly sensitive primer pair.

Enormous numbers of samples may be required to detect NIS when they are present at low population

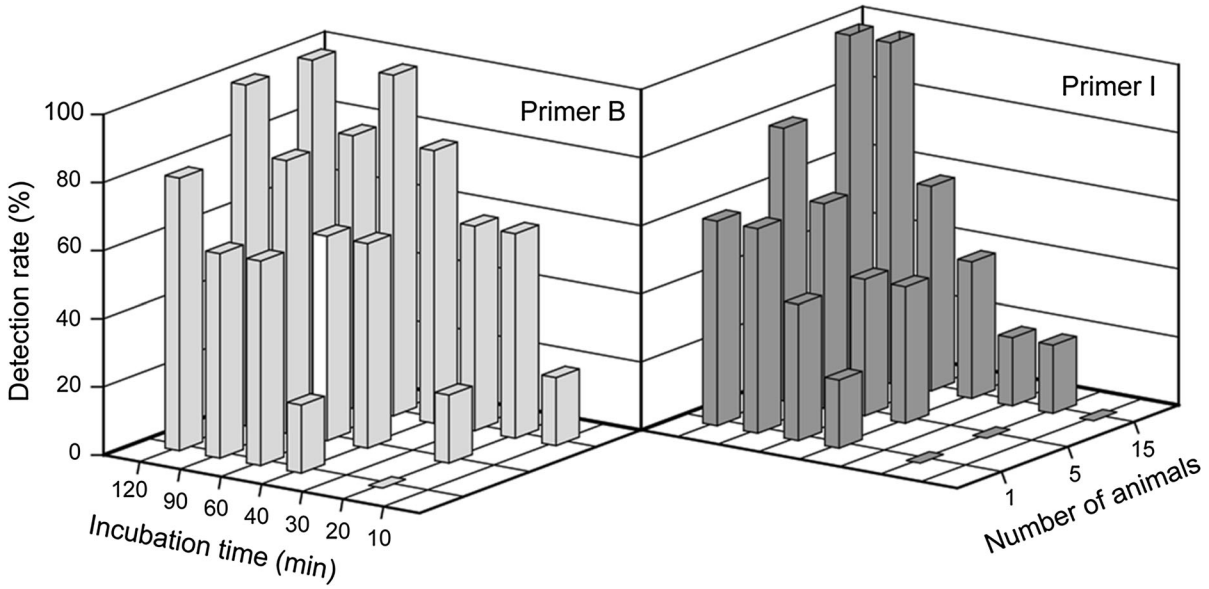


Fig. 2 Detection results of the laboratory validation, showing detection rate as a function of exposure time and number of mussels present

Table 1 Detectability of primer pair B against water samples from surface layer and mixed water column, respectively, collected from both laboratory aquarium tanks and Shisanling Reservoir

Sample source	Surface layer	Mixed water column
Aquarium tank (replicate 1)	+ (10^{-2})	+ (10^{-4})
Aquarium tank (replicate 2)	+ (10^{-2})	+ (10^{-5})
Aquarium tank (replicate 3)	+ (10^{-2})	+ (10^{-4})
Shisanling Reservoir (site 1)	− (ND)	− (ND)
Shisanling Reservoir (site 2)	− (ND)	+ (0.2)
Shisanling Reservoir (site 3)	− (ND)	+ (0.1)

+, positive amplification; −, negative amplification; numbers in brackets referred the lowest dilution fraction from the original eDNA extracts that could be successfully amplified by primer B; ND, not detected

abundance in order to preclude false negatives (i.e., Harvey et al. 2009; Hoffman et al. 2011). Judicious selection of primers in this study dramatically improved detection sensitivity to low levels of eDNA. Screening primer pairs resulted in two benefits. First, sensitive primer pairs could be identified that allow for early detection in field applications (Fig. 2). This is critical for some groups of NIS which have long lag times before populations exceed traditional detection thresholds. Second, utilization of a sensitive primer pair on field-collected samples should reduce the likelihood of false negative results, a major limitation of traditional field sampling for rare species (e.g., Wilcox et al. 2016; Schultz and Lance 2016) or when species release only small amounts of extracellular DNA (Tréguier et al. 2014). For example, we detected the presence of golden mussels in Shisanling Reservoir using primer pair B though primer pair I failed to detect the species (false negative; Fig. 3). The use of highly sensitive primer pairs can reduce the occurrence of such false negatives. We did not, however, observe performance difference between the two primer pairs for Pengxi River (Fig. 3), where mussel abundance (and presumably eDNA concentration) is much higher.

Both conventional PCR and quantitative real-time PCR (qPCR) have been widely utilized for species detection based on eDNA in environmental samples.

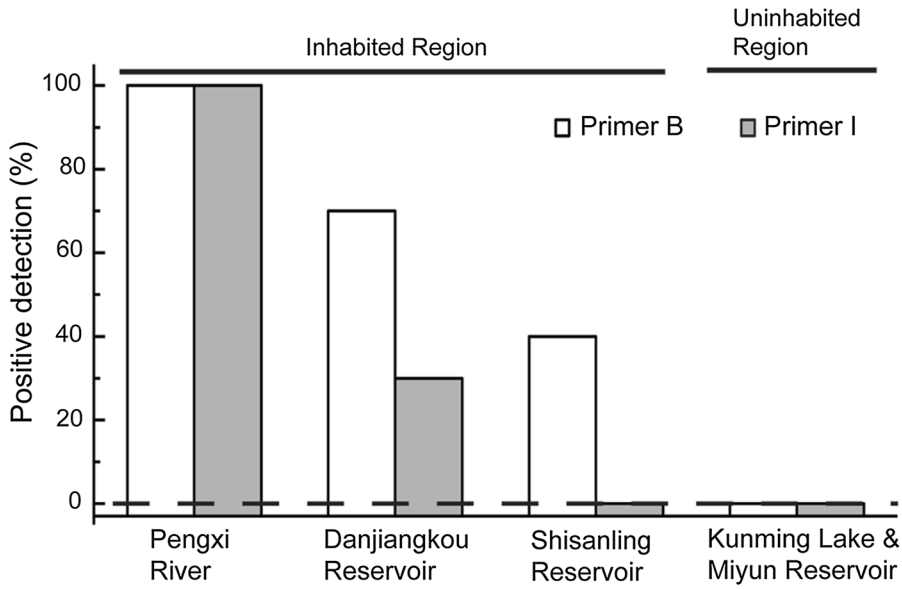


Fig. 3 Species detection in samples collected from water bodies inhabited (or not) by the golden mussel. Results of Shisanling Reservoir consist of the first sampling (sites 1–3) and the second (field application) sampling (sites 4–5). *Dash line* indicates no detection

Both methods can be used to infer species presence/absence, but the latter is more sensitive and informative and can provide additional abundance information of target DNA template (Wilcox et al. 2013; Balasingham et al. 2017). We used conventional PCR in this study as this method is cost-efficient and can be conducted in most laboratories. A critical concern using conventional PCR in eDNA studies is the potential for false positives, which may result from either low specificity of primers or from contamination (Ficetola et al. 2016). No false positives were recorded with these primer pairs in any tested water bodies from which *Limnoperna* has never been reported (Fig. 3), nor in any negative controls or blanks used during sample processing. This suggests that the high specificity of our primers, coupled with careful sample collection and handling protocols, precluded false positives. In addition, we randomly selected and sequenced several PCR products to further confirm positive detections. If the primers developed here were utilized in other areas, specificity tests against sympatric species could be conducted to assess the possibility of false positives. As conventional PCR is not quantitative and cannot provide abundance information, qPCR-based methods appear superior in this regard.

DNA may be degraded due to a variety of factors, notably high water temperature (Taberlet et al. 2012; Barnes et al. 2014). Inhibitors present in eDNA samples may also impede species detection (McKee et al. 2015). The detection limit of primers in this study was determined using the total genomic DNA, while the sensitivity against the water samples was not known although we expected primer pair B to perform best. Results indicate that the capacity of a primer pair to detect species in environmental samples was influenced by its detection limit, indicating that screening primer pairs can increase sensitivity for detection purposes.

We utilized the mitochondrial COI gene, as it has proven reliable in animal species discrimination (e.g., Pečnikar and Buzan 2014; Jiang et al. 2016). In addition, the abundance of mitochondrial genes is higher than nuclear ones, as there are usually multiple copies of the former in somatic cells. Consequently, mitochondrial genes have a higher probability being detected than nuclear ones in a single, complex animal eDNA sample (Taberlet et al. 2012). Degradation of eDNA requires that amplicon size of candidate primer pairs be constrained to a small size. Thus, designing more candidate primer pairs with relatively small amplicons, either from single or multiple genes (Pečnikar and Buzan 2014), may increase the probability of finding highly sensitive primers. However, further studies are required to explore the relationship between the number of

candidate primer pairs and the highest sensitivity achieved.

To maximize the efficiency of eDNA-based methods in rare species surveillance, knowledge of target DNA sources is important to ensure that DNA is collected from environmental samples (e.g., Lacoursière-Roussel et al. 2016). The golden mussel is a filter-feeder that produces feces and pseudofeces, aggregates of which collect on the sediment surface (see Boltovskoy 2015). This material provides a good opportunity to collect eDNA. In addition, the microenvironment of the sediment surface allows the eDNA to be better preserved than upper layer water column due to organic matter and minerals (Tréguier et al. 2014; Turner et al. 2015) as well as reduced UV exposure (Strickler et al. 2015). Thus water samples containing stirred up sediments may yield a higher detection probability for target species than those collected strictly at the surface. Results from sampling of both aquaria and field sites demonstrated that water samples contained resuspended particulate matter were a better eDNA source than surface waters only (Table 1). In the field, areas with lower disturbance may experience a higher particle settling rate, thereby capturing available eDNA and resulting in lower eDNA levels in the surface layer (Turner et al. 2014). An optimized sampling method was developed for crayfish *Procambarus clarkii* surveillance in ponds with these issues in mind (Tréguier et al. 2014). One concern is that positive detections from sediment samples may reflect historic DNA from animals no longer present at the site or DNA transported from another location. However, eDNA is relatively short-lived in common aquatic environments (Turner et al. 2014; Strickler et al. 2015) and for sedentary organisms like golden mussels, we expect this issue to be negligible in most cases.

Harnessing a sensitive primer can improve rare species detection (Zhan et al. 2013, 2014). Our study demonstrated that sensitivity against genomic DNA varied among primers, even though all primers used were designed based on a single gene with similar amplicon size. However, the sensitivity of primer pairs used in eDNA methods applications are often not tested or stated (e.g., Dejean et al. 2012), with comparatively few studies conducting the preliminary step of screening these primer pairs before use (e.g., Dougherty et al. 2016). Indeed, many researchers utilize primer pairs recommended by primer design software (e.g., Rees et al. 2014) or those available in the literature (e.g., Egan et al. 2013). The lack of information pertaining to sensitivity may unwittingly expose these studies to an elevated false negative rate, which can be problematic if the goal of a project is the detection of a NIS or a threatened species. Goldberg et al. (2016) summarized a number of critical aspects that should be considered when designing eDNA-based methods to detect aquatic species. We add that careful screening of primers is an essential step that ought to be coupled with these considerations and be employed in all such studies.

In conclusion, eDNA-based methods are increasingly used in rare species detection, though the sensitivity of applied genetic markers and their corresponding primers is rarely tested. In this study, we found that the sensitivity of different primer pairs varied widely, and urge that primer sensitivity should be known (or tested) before use in the field. When combined with an optimized sampling strategy and good field and laboratory practices, the use of highly sensitive primers can reduce false negative results.

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