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METHODS AND RESOURCES ARTICLE

Characterization, optimization, and validation of environmental DNA (eDNA) markers to detect an endangered aquatic mammal

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Abstract Environmental DNA (eDNA) represents a sensitive and efficient method for noninvasively sampling rare or otherwise hard to monitor taxa, potentially making it a powerful tool for conservation management. Still, this novel method can be affected by sampling protocols, abiotic characteristics of the microhabitat, the focal taxa itself, and primer design. Here we designed 12 species-specific primers for the critically endangered Yangtze finless porpoise (*Neophocaena asiaeorientalis asiaeorientalis*) inhabiting the middle to lower Yangtze River in China to enable complementary population survey tools for conservation efforts. Representing primer pairs that amplify a range of DNA fragment sizes, we test these primers for sensitivity at amplifying finless porpoise DNA using conventional PCR from serial diluted blood samples, and from eDNA in aquaria and in the wild, including a nature reserve and a negative control site. We further investigated the capacity for these primers to detect finless porpoise DNA signals from water samples over a 30-day period. Our study presents which primers were successful at amplifying finless porpoise DNA from aquaria and in the wild, and further demonstrates no significant amplicon size effects on primer sensitivity or longevity. We summarize the impact primer design may have on eDNA applications in general and suggest future considerations for conservation efforts with the Yangtze finless porpoise.

Keywords Environmental DNA · eDNA · Primers · Detection sensitivity · Detection longevity · Amplicon length · Conservation management · Yangtze finless porpoise · *Neophocaena asiaeorientalis asiaeorientalis*

Introduction

Environmental DNA (eDNA) rely on the presence of DNA fragments that have been shed by organisms via metabolic waste, damaged or decomposing tissue, sloughed skin cells, and gametes into various habitats without having to isolate or invasively sample focal taxa (Kelly et al. 2014). The amount and geographical range of eDNA can thereby be used to infer species distributions and/or the conservation status of a population, and has been widely applied for ecological surveillance of rare, cryptic, or otherwise hard-to-study taxa, including endangered (Jerde et al. 2011; Thomsen et al. 2012b; Laramie et al. 2015), endemic (Fukumoto et al. 2015), and recently colonized invasive species (Mahon et al. 2013; Piaggio et al. 2014; Dejean et al. 2012; Hunter et al. 2015). Research consensus to date illustrates eDNA as a sensitive, efficient, effective and noninvasive method (Rees et al. 2014) well-suited for work in conservation management.

Despite recent research extolling the virtue of eDNA for surveying wild populations, the detection of eDNA signals may be ineffective for a number of reasons. For example, the probability of perceiving eDNA signals depends on

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population density, habitat-specific dilution and diffusion, temperature, microbial community composition, the longevity of signal or rate of DNA degradation (especially important in seasonally dynamic and/or migratory/geographically dispersing taxa) and the source genome (Bohmann et al. 2014; Pilliod et al. 2014). Combined, these factors indicate the need for species and habitat specific standardization (Rees et al. 2014). In particular, primer specificity and sensitivity to degraded fragments of DNA play a crucial initial role in optimizing eDNA detection protocols (Wilcox et al. 2013, 2015).

For animal taxa, eDNA protocol primarily targets mitochondrial DNA (mtDNA) rather than nuclear DNA because of its higher copy number in each cell, and in turn the environment (Rees et al. 2014). Commonly employed mtDNA genes include cytochrome *b* (cyt *b*; Piaggio et al. 2014), 12S ribosomal RNA (Deiner and Altermatt 2014) and cytochrome oxidase I (Deiner and Altermatt 2014), wherein amplified primer products (amplicons) are typically designed to target fragments of less than 150 base pairs (Rees et al. 2014). Targets for amplicon sizes stem from the tacit assumption that 'smaller is better' for optimizing the probability of DNA detection due to environmental degradation of DNA fragments (Deagle et al. 2006; Piaggio et al. 2014). However, studies fail to assess the relation between amplicon size and detection rate in complex aquatic environmental media (but see Piggot 2016), primarily relying on previous data collected from fecal analysis (Deagle et al. 2006) which encompasses disparate biotic and abiotic environments involved in DNA degradation. Thus the nuances of eDNA protocol, and understanding the limitations it presents, is imperative for conservation management application.

The Yangtze finless porpoise (*Neophocaena asiaeorientalis asiaeorientalis*, henceforth YFP) is endemic to the middle and lower reaches of the Yangtze River, China, and is the only fully freshwater porpoise species around the world (Mei et al. 2012). Escalating anthropogenic activities such as fishing, vessel traffic, sand mining, intensifying pollution, and habitat degradation in an already heavily deteriorated river system have resulted in a rapid decline of YFP populations (Wang 2009). From 1991 to 2006, YFP populations decreased from an estimated 2700 to 1800 (Zhang et al. 1993; Wei et al. 2002; Zhao et al. 2008). The most recent population estimates indicate an almost 50 % decline since 2006, implying less than 1000 individuals left in the wild (Wang et al. 2013). Now listed as Critically Endangered by the IUCN Red List (Wang et al. 2013), the loss of the YFP would also mean the disappearance of one of the Yangtze River's last top-level predators. Still, cogent conservation planning requires an accurate understanding of the distribution and abundance of current YFP populations. Traditional monitoring methods have utilized acoustic sonar detection and visual observation approaches by government and NGO officers alike, but these methods are often negatively affected by inclement weather and river conditions, time consuming, costly, and depend on ecological expertise by researchers (Kimura et al. 2009). Traditional methods could be improved and complemented with eDNA detection to yield a greater understanding of population demographics and spatial ecology for this imperiled species.

In this study we developed and optimized species-specific primers for YFP, and tested which primer pairs were most useful for genetic surveillance in the wild, including primer sensitivity in dilute aqueous environments, and the longevity of such signals due to the dispersal nature of this species. Specifically we: (1) designed species-specific primer pairs from mtDNA cytochrome *b* (cyt *b*) with amplicons ranging from 50 to 250 bp, testing the assumption that amplification of smaller DNA fragments will yield better detection rates; (2) tested the sensitivity of these primers using serial dilutions of blood samples extracted from multiple YFP individuals; (3) tested the longevity of eDNA detection using these primers, and (4) validated the ability of eDNA primers to detect YFP individuals in aquaria and the wild.

Materials and methods

Primer development

We focus on cyt *b* because this gene has been shown to be sufficiently variable to distinguish among closely-related species (Thomsen et al. 2012a) and GenBank already contains cyt *b* sequences for range-wide haplotypes of the YFP and its congeners (Benson et al. 2009; http://www.ncbi.nlm.nih.gov/genbank/). Using Primer-BLAST with default settings (Ye et al. 2012), we designed primer pairs based on 20 YFP complete cyt *b* sequences obtained from GenBank (accession numbers KJ472902, HM137083-HM137101) in four amplicon size range classes: 50–100, 100–150, 150–200, 200–250 bp. Primers were selected based on similarity of melting temperatures (Tm; 50–65 °C), balanced GC content (50–60 %), and lack of or low self-complementarity (tendency for primers to anneal to one another potentially causing primer-dimers; Ye et al. 2012). Once primer pairs were selected, we then checked their specificity in Primer-BLAST by comparing full genomes from all available sequences within the database and excluded primer pairs that also amplify species with adjacent geographic ranges (no closely-related species overlap their geographical distribution) with the YFP. Additionally, primers were tested using three East Asian finless porpoise (*Neophocaena asiaeorientalis sunameri*) samples, a subspecies to the YFP that inhabits the coastal waters of the Taiwan Strait

to the East China Sea (Wang and Reeves 2012), to confirm species-specificity and a lack of non-target amplification. Samples included the muscle tissue from one female and two male individuals acquired in April 2015 from Penglai Sea World (Shandong Province, China), and stored in a −80 °C freezer at the Wuhan Baiji Dolphinarium, Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China) until DNA extraction and amplification. As a positive amplification control, *N. a. sunameri* samples were amplified with cyt *b* primer HQ108395 (GenBank accession number; Li et al. 2011) alongside the 12 primers designed for YFP.

Primer testing and optimization

To test if these newly-designed primer pairs successfully amplify DNA from YFP, we proceeded to amplify total genomic DNA extracted from blood. Of the 6 YFPs currently housed at the Wuhan Baiji Dolphinarium (30°31′28 N, 114°22′58E, see Fig. 1) Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China) we obtained two blood samples: one from a male (Tao Tao, born in captivity in 5 July 2005, at Wuhan Baiji Dolphinarium) and one from a female (F7, imported from Poyang Lake of Jiangxi Province). Blood was drawn from the vein in the fluke by using a one-off, sterile and disposable syringe, anti-coagulated with EDTA-Na2 and frozen at −80 °C immediately. DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) in a final elution volume of 200 µL. DNA concentrations from blood were measured with a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Reaction volumes were 25 µL consisting of 1 µL of 1–20 ng/µL template DNA, 12.5 µL of 1 × Taq PCR Master Mix (BIO BASIC CANADA INC.), 1 µL each of 0.2 µM each of forward and reverse primer, and 9.5 µL of ddH_20. PCR amplification included the following thermal cycling profile: 94 °C initial denaturation for 4 min., followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 20 s, and one final extension step at 72 °C for 5 min. PCR products were visualized using electrophoresis on 2 % agarose gel, stained with 7 µL of 4S Red Plus Nucleic Acid Stain (BBI) per gel. All amplifications were repeated in triplicate (following Ficetola et al. 2015) and PCR products of the target sequences were sent to Sangon Biotech (Shanghai, China) utilizing an ABI 3730XL sequencer. To validate PCR products as indeed being from YFP, sequences were visualized using MEGA6 (Tamura et al. 2013) and then compared to all available sequences in GenBank using Primer-BLAST (Ye et al. 2012).

Presumably environmental samples will have much lower DNA concentrations compared to those extracted directly from blood, thus we tested concentration sensitivity of these primer pairs, diluting the DNA extracted from both blood samples with sterilized ddH_2O to 4 ratios: 1:2, 1:10, 1:100, 1:1000. We then replicated PCR amplification in triplicate and visualized electrophoresis results to note maximum detection sensitivity of each primer pair.

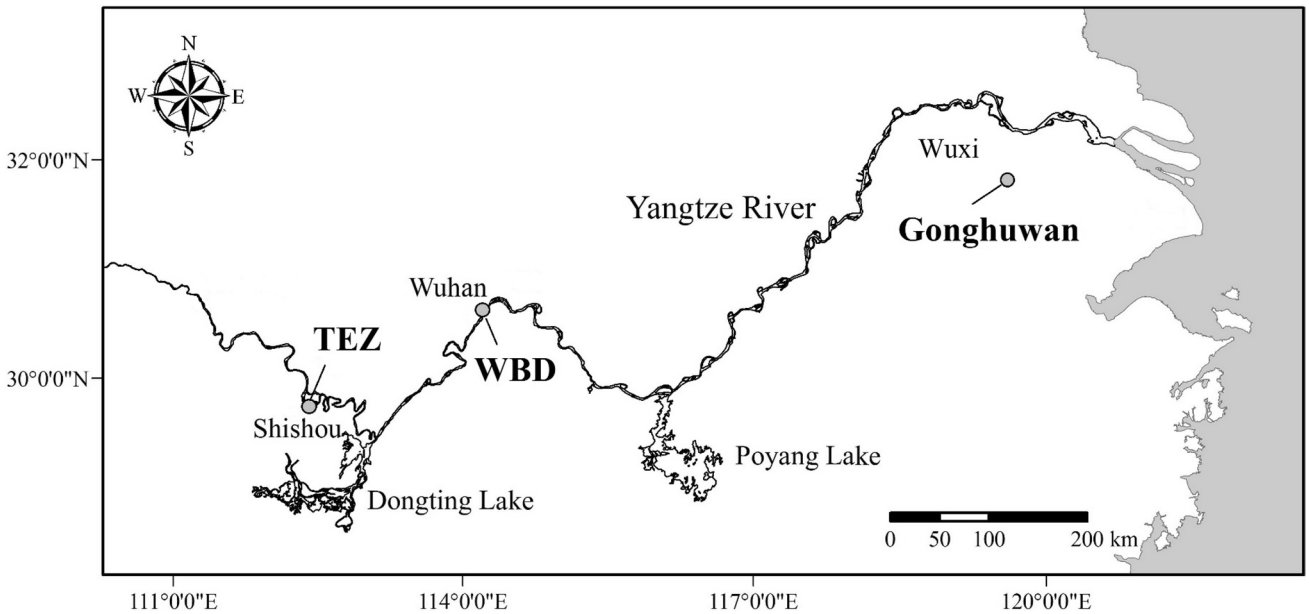

Fig. 1 Map of southeast China, illustrating sampling sites where water samples were collected. Sampling locales for the Wuhan Baiji Dolphinarium (WBD; 30°31′28 N, 114°22′58E), Tian e-Zhou Baiji National Nature Reserve (TEZ; 29°51′11 N, 112°35′15E), and Gonghuwan lake for the negative control (31°27′21 N, 120°20′E) are illustrated using a *grey circular marker*. Map has been modified with permission from Wan et al. (2016)

Validation in aquaria and in the wild

To authenticate the efficacy of these primer pairs, we tested whether these primers could amplify YFP eDNA from a location where we knew this species to be present. Thus, we collected 3 1-liter surface water samples in March 2015 from the Wuhan Baiji Dolphinarium at the Institute of Hydrobiology, housing 6 YFPs in three adjoining tanks (with a depth of 3.5 m and a total volume of around 900 m^3) where the water mixes homogenously among them.

Water samples were passed through a 47 mm diameter mixed cellulose ester (MCE) filter paper with 0.45 μm pore size, by means of a reusable but sterilized filter holder (Sterifil 47 mm Filter Holder, Millipore) employing a portable field peristaltic pump (Spectra Scientific Inc. Spectra Filed-Pro Professional Grade). To minimize clogging the filter paper with large suspended particles, we wrapped the inlet of the filter holder with sterilized, non-reusable medical-grade gauze. After filtering, we folded the filter paper using sterilized tweezers and placed the sample into an Eppendorf tube with 95% ethanol. Samples were then immediately stored in a −20 °C freezer until DNA extraction.

To check the longevity of detecting eDNA signals after YFP DNA deposition had ceased, we collected 8 1-liter samples of surface water from the Wuhan Baiji Dolphinarium which were then stored within closed, transparent sterile plastic containers indoors at 15 °C without direct contact with UV radiation. We filtered water samples at 1, 3, 5, 15, and 30 days after collection (as described above) and subsequently stored the filter papers in −20 °C until DNA extraction.

Frozen filter paper suspended in 95% ethanol was individually air dried at ambient temperature and DNA was then extracted using the PowerWater® DNA Isolation Kit (Mo Bio Laboratories, Inc) following manufacturer's protocol. After extraction, we amplified each sample in triplicate and tested for positive detection for all 12 primer pairs across the time series (see "Primer testing and optimization" section for details). The maximum number of days for detection was noted, making sure all amplifications were consistently positive until the maximum longevity measurement.

To test the efficacy of these primer pairs in the wild, we collected multiple field samples in March 2015; 3 1-liter samples from a location known to contain free-ranging populations of YFP (positive control), and 3 1-liter samples in a location where the species has historically, and currently remained absent (negative control). For the positive control location, we sampled at Tian-e-Zhou Baiji National Nature Reserve (29°51′11 N, 112°35′15E, see Fig. 1), a U-shaped oxbow lake parallel to the Yangtze River, in Hubei, China. The reserve is 20.9 km long, with a varying width between 1 and 2 km, and an average depth of approximately 4.5 m (the deepest reaches of the lake are between 15 and 25 m). At the time of sampling, the reserve contained a floating net cage (approximately15 × 15 m) with two YFPs being temporarily housed for breeding research. This area was connected to the rest of the oxbow reserve, where approximately 58 other YFPs live. For our negative control, we chose Gonghuwan (31°27′21″N, 120°20′E, see Fig. 1), an artificial lake in Wuxi, China, where porpoises are absent and water supply is not linked with the Yangtze River or any other waterway containing YFP. In both the positive and negative control sites, we collected and filtered water samples immediately on site and transported the filter papers (stored in 95% ethanol) on ice until samples could be frozen in the laboratory (within 24 h). As target-species DNA concentrations in natural environments are typically very low, we used a re-amplification PCR protocol for all amplifications (Tuvshintulga et al. 2015), using the diluted (1:10 with sterilized ddH_2O) amplicons as DNA template for a second round of PCR amplification. These final PCR products were visualized using electrophoresis on 2% agarose gel stained with 4S Red Plus Nucleic Acid Stain. All PCR products were sequenced at Sangon Biotech (Shanghai, China) utilizing an ABI 3730XL sequencer and visualized using MEGA6.

To avoid contamination, we utilized filtered pipette tips, separate clean-rooms for DNA extraction and PCR amplification, and all equipment was sterilized with 20% diluted bleach, rinsed with sterilized water, and then dried under UV light for 30 min. After each filtering session, the medical-grade sterilized gauze was replaced, and the filter holder and tweezers were washed in a 20% bleach dilution, rinsed with sterilized water, and allowed to be fully dry (Davy et al. 2015). To test for contamination during sample collection, we also filtered a 'blank sample' by replacing the environmental sample with distilled water which was subsequently extracted and amplified.

Statistical analysis

We performed one-way ANOVAs across primer pair categories (50–100, 100–150, 150–200, 200–250 bp) to assess whether various amplicon sizes demonstrated different sensitivity thresholds (serial dilutions) or detection longevity for eDNA amplification. All analyses were performed using JMP v.12.0 (SAS Institute Inc., Cary, NC, 1989–2007).

Results

Primer development, specificity, and sensitivity

We found a total of 82 candidate primer pairs using Primer-BLAST, 61 of which were eliminated for high self-complementarity and unbalanced G/C content, and another nine primers were eliminated because of non-specificity to the

YFP. Eventually, we selected 12 primer pairs (see Table 1), three for each amplicon size class, ranging from 76 to 249 base pairs.

All primer pairs successfully amplified DNA extracted from YFP blood samples (confirmed through bi-directional sequencing). Primer-BLAST alignment results showed that these primers are highly species-specific. The sequences of amplicons for ten primer pairs were 100 % identical with cyt *b* gene fragment of the YFP exclusively. DNA sequences of amplicons from two other primer pairs, FP233 and FP249, were 99 % identical with cyt *b* of the YFP. Two sister-species, the Indo-Pacific finless porpoise (*N. phocanoides phocaenoides*) and the East Asian finless porpoise (*N. a. sunameri*), showed sequence matches to approximately 94 % to that of the YFP, however neither species has ever been observed to geographically overlap in distribution with the YFP, remaining endemic to the Indian Ocean and South China Sea (Indo-Pacific finless porpoise), and the East China Sea from Taiwan to southern Japan (East Asian finless porpoise). All non-target *N. a. sunameri* samples further demonstrated no PCR amplification across all 12 primers designed for YFP, corroborating species-specificity.

Using the mean total DNA concentration extracted from YFP blood samples (3.1 ng/μL) as a baseline, our detection sensitivity experiment for both blood samples demonstrated FP147 to successfully amplify YFP DNA at 100 times diluted concentration (i.e. 0.031 ng/μL). Conversely, primers FP97 and FP104 were only found to be able to amplify undiluted DNA extracted from blood samples. In general, primers that amplified intermediate DNA fragment sizes showed a greater ability to amplify YFP DNA at greater dilutions (Table 1), though we found no significant

Table 1 Yangtze finless porpoise cyt *b* primers (including degenerate bases following IUPAC convention) developed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/)

Primer	Primer sequence (5′–3′)	Tm (°C)	GC%	Self 3′ comp.	Max. dilution	eDNA longevity (days)
FP76F	TTCGGCCCTTTAGCCARCTC	60.32	55	0	2×	At site
FP76R	GGCTGACCTCCGATTCATGT	59.82	55	2		
FP90F	TTTCGGCCCTTTAGCCARCT	59.89	50	1	10×	At site
FP90R	CGGGTGTTCTACAGGCTGAC	60.39	60	1		
FP97F	CAGAYACCTCTACCGCCTTTT	60.00	52	0	1×	–
FP97R	ATAGAGGCTCCGTTTGCGTG	60.46	55	0		
FP104F	ACACACCAGAYACCTCTACC	58.66	55	0	1×	–
FP104R	CATAGAGGCTCCGTTTGCGT	60.46	55	0		
FP133F	GTCATAGCCACCGCATTTGY	58.91	50	0	2×	15
FP133R	CGCCTCAGATCCACTCCACT	61.33	60	1		
FP147F	CGCAAACGGAGCCTCTATGT	60.46	55	3	100×	3
FP147R	ACGTAGCCTRCAAATGCGGT	59.75	50	0		
FP161F	ACGCAAACGGAGCCTCTATG	60.46	55	1	10×	30
FP161R	CCCTCAGGGTAGAACGTAGC	59.25	60	2		
FP162F	TTTCGGCCCTTTAGCCARCT	59.89	50	1	10×	–
FP162R	GCTRGCTGTTGGTATTAGCAC	59.6	52	3		
FP171F	ATAYCTCCACGCAAACGGAG	60.11	55	0	10×	15
FP171R	TGCCCTCAGGGTAGAACGTA	59.96	55	2		
FP221F	ACGCACTTATYGAWYTCCCC	59.33	55	0	2×	–
FP221R	CATAGAGGCTCCGTTTGCGT	60.46	55	0		
FP233F	CACCCGACCTTYTAGGTGAC	59.47	60	3	2×	15
FP233R	GAGKTGGCTAAAGGGCCGAA	60.32	55	0		
FP249F	TCGATAYCTCCACGCAAACG	60.46	55	2	2×	5
FP249R	TCCACTAGTGTGCTGCCAAT	59.31	50	3		

Primer names associate to amplicon lengths in bp (including primer) and the orientation of sequencing (*F* forward, *R* reverse). Primer melting temperature (Tm), GC nucleotide content (%), and self-complementarity are presented. The extent to which each primer pair could successfully detect and amplify Yangtze finless porpoise DNA is expressed at the maximum magnitude of dilution concentration for blood (Max. dilution) and for the maximum length DNA signals were detected after collection from the aquaria (eDNA longevity in days)

–, amplification failure

relationship between amplicon size and primer sensitivity ($F_{3,8} = 0.809$, $p = 0.52$).

Validation in aquaria and in the wild

Total eDNA concentration of the Wuhan Baiji Dolphinarium sample was 7.4 ng/μL. We were unable to detect YFP DNA from all primer pairs by filtering the aquarium water in Wuhan using the PCR protocol; in total, four primer pairs (FP97, FP104, FP162 and FP221) showed no DNA amplification extracted from aquarium water, despite this facility currently housing 6 YFP individuals. The other eight primer pairs however, did yield amplification products shown to be from the YFP after successful sequencing.

The mean total eDNA concentrations of the time serial samples from aquaria were 7.8, 8.9, 6.5, 9.1 and 7.6 ng/μL, from water filtered at 1, 3, 5, 15, and 30 days (respectively) after the samples were collected. Generally, amplicons of large or intermediate sizes showed a greater detection sensitivity from aquarium samples for longer periods of time after the cessation of DNA input. Of the eight primer pairs that successfully amplify eDNA from aquarium water filtered on site, only primer FP161 showed successful amplification of target eDNA 30 days after the sample was collected from the aquarium. Three other primers, FP133, FP171, and FP233, amplified target DNA 15 days after the aquarium sample was collected. Primer pairs FP76 and FP90 only demonstrated amplified DNA immediately upon collection but not from water samples that were stored (Table 1). Still, we found no significant relationship between amplicon size and eDNA longevity ($F_{3,8} = 1.21$, $p = 0.37$).

Across all primer pairs, negative control samples attained from Gonghuwan artificial lake (mean total eDNA concentration 5.6 ng/μL) showed no DNA amplification of YFP. The Tian e-Zhou sample (total eDNA concentration 10.1 ng/μL) however, showed positive amplification with FP133 to successfully detect the presence of YFP.

Discussion

Our study indicates that all 12 primer pairs are species-specific to the YFP. For the few primer pairs with closely matching sequences from taxa other the YFP, sequences were easily distinguishable based on data deposited within GenBank and originated from conspecifics inhabiting geographically and hydrologically distinct (marine vs. freshwater) aquatic habitats: the Indo-Pacific finless porpoise (*N. phocaenoides. phocaenoides*) and the East Asian Finless Porpoise (*N. a. sunameri*). DNA from the YFP subspecies (*N. a. sunameri*) further demonstrated a lack of amplification, corroborating all designed primer pairs as good candidates for use in eDNA surveillance. Species-specificity of these primers also suggests the application of eDNA protocols would likely not require sequencing, primarily in areas along the Yangtze River (and tributaries) where marine congeners are unable to survive, though possibly necessary around the Yangtze River estuary where occasional vagrant species might be encountered or detected.

Successful amplification of YFP DNA from positive control environments (the aquarium and the reserve) also demonstrated that YFP DNA can be detected in highly diluted and complex aquatic environmental samples, potentially a useful compliment to already existing traditional methods for YFP population censusing. Although traditional censusing efforts including visual and acoustic monitoring exhibit expensive and laborious application, positive eDNA detection does not necessarily equate to living (nor reproducing) individuals and thus should currently only be viewed as a complimentary practice. Furthermore, field tests would now be required to test the efficacy of these primers for eDNA assay use under varying population densities, hydrological and seasonal dynamics, and sampling regimes in order to determine optimal surveillance protocol for field managers.

Indeed, while our study illustrates designed primers can be used to survey YFP individuals under natural conditions, we anticipate and encourage further research circumscribing sampling protocol and environmental factors that influence detection levels. Considering the markedly distinct flow characteristics between the Tian e-Zhou Baiji National Nature Reserve (a lake) and the fast flowing Yangtze River where population surveillance is most needed, DNA concentrations and the sampling protocols required to attain measurable levels will likely require alteration due to environmental heterogeneity. For example, our study utilized conventional PCR to amplify eDNA signals which may represent a less-technical, quick, and cost-effective assay for managers, however quantitative PCR procedures may prove more favorable for future applications as they have been shown to be a more powerful tool at amplifying low concentrations of eDNA (Wilcox et al. 2013; Piggot 2016). In fact, some of our current YFP pilot studies using quantitative PCR illustrates positive amplification of a number of these designed primers that showed little to no amplification using conventional means (unpublished data). Certainly conventional PCR may still demonstrate utility (e.g. in Tian e-Zhou lake where DNA concentration are likely higher than in the Yangtze River, or for a more cost-effective precursory census) although a sampling approach where larger quantities of water are collected and filtered may prove necessary in riverine systems. Should conventional PCR be used by field managers for eDNA monitoring in this species, we encourage the use of primer pair FP133 which demonstrated positive amplification in the wild, in dilute solutions and for approximately 2 weeks post DNA input, rendering

it a good candidate for censusing the current presence and distribution of YFP individuals/populations.

Our study also underscored the importance of primer design for eDNA use, especially with regard to amplicon length. The tacit supposition that smaller DNA fragment amplification will be better in terms of primer pair sensitivity (Deagle et al. 2006), may not be universally accurate. We demonstrated amplicon size to have no significant effect at amplifying YFP eDNA under various concentrations and time (Table 1). While a disproportionate amount of eDNA studies use primers that amplify DNA fragments of 150 bp or less (e.g. Dejean et al. 2012; Takahara et al. 2012; Wilcox et al. 2013), amplicons ranging from as small as 62 bp (Foote et al. 2012) to as large as 650 bp (Foote et al. 2012; Egan et al. 2013) have proved successful at eDNA detection for aquatic sampling. In fact, the only study other than our own (to our knowledge) to assess amplicon size and detection rate of eDNA also found no relationship (although they only examined 3 markers of various sizes; Piggot 2016). Together, these findings suggest amplicons of less than 150 bp may not be the only, or best, solution for different eDNA sampling strategies. For example, while our primers were able to detect YFP eDNA spanning from days to weeks, a time-span reflecting similar values seen in other studies of aquatic fauna such as in fish and amphibians (Dejean et al. 2011), many environmental factors can potential interact with DNA to preserve or degrade it (Barnes et al. 2014); factors include the abundance of focal species, total eDNA present (from all organisms), and other abiotic and biotic factors, including temperature, pH, biochemical oxygen demand, and conductivity (Thomsen et al. 2012a; Barnes et al. 2014). However, there is also a relationship between DNA length and the particles it binds with within an environment (e.g. Ogram et al. 1994), and the way in which it interacts with the local microbial community (Lennon 2007), further suggesting some degree of habitat specificity to DNA degradation and thus optimal eDNA amplicon size ranges.

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