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METHODS AND RESOURCES ARTICLE

Developing quantitative PCR assays to detect threatened and invasive freshwater turtles in Hong Kong using environmental DNA

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Abstract

Environmental DNA (eDNA) techniques show promise in facilitating the study and conservation of threatened species. The advantages of high sensitivity and high efficiency have been demonstrated by studies across various taxa. Asian turtles, one of the most threatened vertebrate groups, are facing extinction due to overexploitation for use as traditional Chinese medicine, food, and pets. Most species are extirpated across their ranges, but Hong Kong is unique because wild turtle populations persist. Due to the rarity and secretiveness of turtles, data on the location and size of populations are incomplete, even for a small place like Hong Kong. The result is that research and conservation are hindered. In this study, quantitative PCR eDNA assays were developed for three turtle species: two native [big-headed turtle (*Platysternon megacephalum*) and Chinese softshell turtle (*Pelodiscus sinensis*)] and one invasive [red-eared slider (*Trachemys scripta elegans*)]. For each assay, we tested species specificity, optimized reactions, and benchmarked analytical sensitivity for eDNA surveys. The assays will help in the conservation of Hong Kong turtles by identify priority sites for protection and research, provide another tool for population monitoring, and clarify the impact of the invasive *T. s. elegans* on native species.

Keywords *Platysternon megacephalum* · *Pelodicus sinensis* · *Trachemys scripta elegans* · Invasive species · qPCR · TaqMan minor groove binder · Turtle conservation

Introduction

Turtles are one of the most threatened vertebrate group facing extinction. Over 80% of Asian turtle species are categorized as threatened (vulnerable, endangered, or critically endangered), of which over 50% are categorized as endangered or critically endangered (IUCN 2018; Rhodin et al. 2018). Overexploitation for use as traditional Chinese medicine, food, and pets are the major causes accounting for the population decline in Asian freshwater turtle species (Cheung and Dudgeon 2006). While Asian turtles are largely extirpated across their ranges, Hong Kong provides a unique opportunity for research and conservation of Asian turtles because wild turtle populations still exist. There are five native turtle species in Hong Kong: big-headed turtle (*Platysternon megacephalum*), three-banded box turtle (*Cuora trifasciata*), Reeves's turtle (*Mauremys reevesii*), Beale's-eyed turtle (*Sacalia bealei*), and Chinese softshell turtle (*Pelodiscus sinensis*). Of these five species, three (*Pl. megacephalum*, *S. bealei*, and *M. reevesii*) are categorized as endangered and one (*C. trifasciata*) is categorized as critically endangered (IUCN 2018).

Research and conservation of Asian turtles is hindered by their rarity and secretiveness; even in a small geographic region like Hong Kong (approximately 1100 km^2) (Land Department, HKSAR 2018) we do not have a clear picture of where remnant populations exist, let alone their health. Environmental DNA (eDNA) techniques show promise in facilitating the study of Asian turtles. Detecting target species using eDNA refers to the extraction and amplification of DNA deposited in the environment (air,

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soil, water) without first isolating it (Taberlet et al. 2012). Potential sources of DNA originate from skin fragments, saliva, gametes, faeces, and blood. eDNA methods have been applied to the detection of a wide range of species, including freshwater fish (Jerde et al. 2011; Takahara et al. 2012; Wilcox et al. 2013; Ikeda et al. 2016; Rodgers et al. 2017), amphibians (Ficetola et al. 2008; Goldberg et al. 2011; Lacoursiere-Roussel et al. 2016; Veldhoen et al. 2016; Spangler et al. 2017), freshwater turtles (Davy et al. 2015), marine fish (Thomsen et al. 2012), birds (Ushio et al. 2018), mammals (Padgett-Stewart et al. 2015; Ma et al. 2016), and invasive species (Bylemans et al. 2016; Hinlo et al. 2017). These studies demonstrate that eDNA approaches are highly sensitive, time-efficient, and cost-effective in detecting target species. In particular, eDNA methods are useful in detecting secretive species that are easily overlooked by traditional survey methods (Takahara et al. 2012) and potentially used for estimating population size (Takahara et al. 2012; Thomsen et al. 2012; Goldberg et al. 2013).

Asian turtle conservation is also affected by the issue of invasive species (van Dijk 2000). The red-eared slider (*Trachemys scripta elegans*) is one of the 100 worst invasive alien species (GISD 2018), and its competitive dominance over native species in Europe (Cadi and Joly 2004) and America (Pearson et al. 2015) has been documented. *Trachemys scripta elegans* is commonly found across Asia, and researchers have started to study its impact on native species (Chen 2006; Ramsay et al. 2007; Shen et al. 2011). In Hong Kong, *T. s. elegans* is present, with records of its presence becoming more common (Lau and Dudgeon 1999; Dahmer et al. 2001). As with the native turtle species in Hong Kong, the complete range of *T. s. elegans* is unknown.

In this study, we develop eDNA assays using quantitative PCR (qPCR) for two selected native (*Pl. megacephalum* and *Pe. sinensis*) and one invasive (*T. s. elegans*) turtle species in Hong Kong. *Platysternon megacephalum* and *Pe. sinensis* were chosen for two reasons. First, there are relatively recent records of these species in Hong Kong (Sung et al. 2013; Lau pers. comm.). Second, these species inhabit different habitat types—*Pl. megacephalum* inhabits rocky mountain streams while *Pe. sinensis* inhabits lowland areas and fish-ponds—allowing for testing of eDNA methods in different environments. In the future, these assays will be used to help clarify the current geographical distribution of these three species in Hong Kong, as well as tested to estimate their abundance. More specifically, the assays will play important roles in identifying priority sites for protection and further research, as well as understanding the impact of *T. s. elegans* on native species. Herein, we document the development of these eDNA qPCR assays and discuss their potential application.

Methodology

Primer pair and probe development

Primers and probes were developed for TaqMan minor groove binder (MGB) qPCR assays for the three target species (*Pl. megacephalum*, *Pe. sinensis*, *T. s. elegans*). A set of representative non-target species were selected for assay validation based on sympatry, relatedness (family and genus level), and availability of tissue samples for in vivo testing. Sequences from target and non-target species were downloaded from GenBank for five commonly sequenced loci: cytochrome c oxidase subunit 1 (*COI*), NADH-ubiquinone oxidoreductase chain 4 (*ND4*), cytochrome b (*CYTB*), 16S ribosomal RNA (*16S*), and 12S ribosomal RNA (*12S*). To account for genetic diversity in a species, we included all unique sequences per species per locus. When there were identical sequences, only one was retained. A list of these species and their GenBank accession numbers are found in Supplementary Table 1.

Sequences of each locus were aligned using MUSCLE (Edgar 2004) in Geneious R11 (Kearse et al. 2012). Candidate primer sets were selected to distinguish each target species by locating regions with mismatches between each target and non-target species. We aimed to place base pair mismatches towards the 3′-end of each primer to reduce non-target amplification (Wilcox et al. 2013). For each primer set, probe annealing sites were selected by targeting conservative regions of the target species.

Primer sets and probes were selected with optimum melting temperature (primers: 55–60 °C; probes: 68–70 °C), low tendency of secondary structure formation, and balanced GC content (30–80%), following suggestions from the Primer Express 3.0 protocol (Applied Biosystems; Thermo Fisher Scientific Inc; Waltham, MA, USA). Melting temperature and GC content of primers and probes were assessed using Primer Express 3.0 (Applied Biosystems; Thermo Fisher Scientific Inc; Waltham, MA, USA). The tendency of secondary structure formation was assessed using Multiple Primer Analyzer (https://www.thermofisher.com/hk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html) (Thermo Fisher Scientific Inc; Waltham, MA, USA).

Species specificity tests

Primer sets were tested using a four-step process. First, primer sets were screened in silico to identify potential non-target species amplification using Primer-BLAST

(Ye et al. 2012). We screened forward and reverse primer sequences in Primer-BLAST, looking for all GenBank entries with exact matches. Primer sets having an exact base pair match with all sequences of the corresponding target species were retained for further testing. If the Primer-BLAST result included non-target species, another comparison was conducted using the probe (on forward strand) and reverse primer sequences. If the result included the same non-target species, the primer set was discarded. Table 1 summarizes the number of base pair mismatch on the priming and probe regions between target and non-target species (used for in vivo testing).

Second, the specificity of primer sets was tested in vivo against DNA extracted from turtle tissue of target (Table 2) and non-target species (Supplementary Table 2) using conventional PCR, followed by gel electrophoresis. For most species, we included 2–5 individuals to test the primers against target and non-target intraspecific diversity. A variety of tissues (lung, tail clip, shell clip, mouth swab, blood; preserved in 95% ethanol or frozen at − 20 °C) were collected from captive individuals. Permits for possession of live turtles and their tissues for molecular work were granted by the Agricultural Fisheries and Conservation Department, Hong Kong Government (AF GR CON 09/51 Pt.6). DNA was extracted using a standard salt extraction protocol (Sambrook and Russell 2001). DNA concentration of target species samples were estimated using a standard curve in qPCR (See below) while that of non-target species were estimated by Qubit 3 Fluorometer (Invitrogen™; Thermo Fisher Scientific Inc; Waltham, MA, USA). Each PCR reaction included AccuPower® PCR PreMix (Bioneer Corporation; Daejeon, Republic of Korea), 0.5 µM forward primer, 0.5 µM reverse primer, 17 µL water, and 1 µL of DNA template in a total reaction volume of 20 µL. All water used for molecular work was autoclaved Milli-Q® water. To identify the ideal annealing temperature for each primer set, we performed PCR with a thermal gradient ranging from 50 to 65 °C, selecting the temperature with the strongest, locus-specific amplification. PCR was conducted in a T100™ Thermal Cycler (BIO-RAD; Hercules, CA, USA) with the following conditions: 95 °C for 3 min; followed by 35 cycles of 95 °C for 30 s, corresponding annealing temperature for 30 s and

Table 1 Species used in the in vivo testing of primer–probe sets using qPCR assays. Listed are the number of base pair mismatch between target and non-target species in the priming and probe regions. Bold indicate non-target amplification

	Pl. megacephalum ND4			*Pe. sinensis 16S*			*T. scripta elegans 12S*		
	Forward primer	Reverse primer	Probe	Forward primer	Reverse primer	Probe	Forward primer	Reverse primer	Probe
Platysternidae									
Pl. megacephalum	–	–	–	3–4	3	0–1	3–6	3–5	2–5
Geoemydidae									
C. trifasciata	2	9	7	0	4	0	10	12	15
M. reevesii	2	9	5	4	4	0	2	3	5
M. japonica	2	9	5	6–7	0	5	4	2	4–7
M. sinensis	1–2	9	5	9	Data deficient	Data deficient	2	3	4
S. bealei	3	8	5	Data deficient	4	Data deficient	4	3	2
S. quadriocellata	2	6	3	4	4	0	3	2	3
C. dentata	4	8	6	3	7	0	2	2	5–7
Tryonychidae									
Pe. sinensis	5	5–6	4	–	–	–	6	4	8
Pe. steindachneri	6	6	4	2–3	1	Data deficient	5	3	7–8
Emydidae									
T. scripta elegans	3	11	4	6	4	0	–	–	–
T. scripta scripta	3	11	4	Data deficient			**0–1**	**0**	**0**
G. pseudogeographica	3	11	4	10	5	3	3	0	3–4
G. ouachitensis	3	11	4	Data deficient			3	0	3

"Data deficient" means the difference of base pair mismatch is not calculable due to lack of comparable sequences

Table 2 Quantification of target species DNA samples used in this study. All estimates measured in number of DNA copies per µL

Species	Source	Replicates			Mean	R^2	Efficiency (%)	Y-intercept
		1	2	3				
Platysternon megacephalum	Tail clip	7.93E+06	1.47E+07	1.58E+07	1.28E+07	0.97	94.15	43.260
	Tail clip	4.11E+06	4.70E+06	4.10E+06	4.30E+06	0.97	94.15	43.260
	Tail clip	2.09E+07	3.31E+06	2.26E+07	1.56E+07	0.97	94.15	43.260
	Tail clip	2.05E+07	1.11E+07	1.41E+07	1.52E+07	0.97	94.15	43.260
	Tail clip	1.31E+06	2.78E+06	3.62E+06	2.57E+06	0.97	94.15	43.260
	Water filter	1.17E+05	1.48E+05	9.59E+04	1.20E+05	0.97	94.15	43.260
	Water filter	4.68E+02	6.80E+02	6.09E+02	5.86E+02	0.99	81.54	43.623
Pelodiscus sinensis	Shell clip	2.72E+06	8.77E+05	2.75E+05	1.29E+06	0.85	105.75	45.839
	Shell clip	4.71E+04	5.44E+04	2.94E+04	4.36E+04	0.85	105.75	45.839
	Shell clip	3.77E+05	1.68E+06	4.81E+04	7.02E+05	0.85	105.75	45.839
	Water filter	1.82E+04	1.53E+03	4.10E+03	7.94E+03	0.85	105.75	45.839
	Water filter	5.88E+03	8.24E+03	3.55E+02	4.82E+03	0.85	105.75	45.839
Trachemys scripta elegans	Tail clip	2.71E+06	2.02E+04	3.46E+04	9.22E+05	0.99	72.86	44.286
	Tail clip	1.35E+06	4.53E+05	1.09E+06	9.64E+05	0.99	72.86	44.286
	Tail clip	1.35E+06	2.59E+06	8.06E+05	1.58E+06	0.99	72.86	44.286
	Tail clip	9.14E+05	1.28E+06	2.30E+06	1.50E+06	0.99	72.86	44.286
	Tail clip	4.70E+06	1.89E+06	2.36E+06	2.98E+06	0.99	72.86	44.286
	Water filter	1.24E+02	1.68E+02	1.13E+02	1.35E+02	0.99	72.86	44.286
	Water filter	2.10E+04	2.02E+04	3.46E+04	2.53E+04	0.99	72.86	44.286

72 °C for 1 min. Negative controls using water in place of DNA were included to test for contamination. Gel electrophoresis was conducted using 2% agarose gels run at 150 V for 45 min. Primer sets exhibiting target species specificity were retained for further testing. Figure 1 is an example of two primer sets, one exhibiting species specificity, one not.

Third, the retained primer sets were tested against DNA extracted from water samples with known presence and absence of target species, following the same PCR and gel electrophoresis condition mentioned above. Water samples (200–750 mL) were collected from the enclosures of captive turtles (Table 2, Supplementary Table 2; except *M. japonica, C. dentata,* and *T. s. scripta* which no water samples were available) and filtered using 0.45 µL pore size, cellulose nitrate filters (Nalgene analytical test filter funnel; Thermo Fisher Scientific Inc; Waltham, MA, USA). Negative controls (filtering distilled water) were used through the entire process to detect contamination. DNA was extracted from half of the filter paper using a DNeasy Blood & Tissue Kit and QiaShredder (Qiagen GmbH; Hilden, Germany) following the protocol of Goldberg et al. (2011). DNA was eluted using 100 µL TE buffer and stored at − 20 °C. The remaining half of the filter paper was stored in 95% ethanol at − 20 °C as a spare and for future use. PCR products of expected size were sequenced for verification. One species-specific primer–probe set was selected for each of the three target

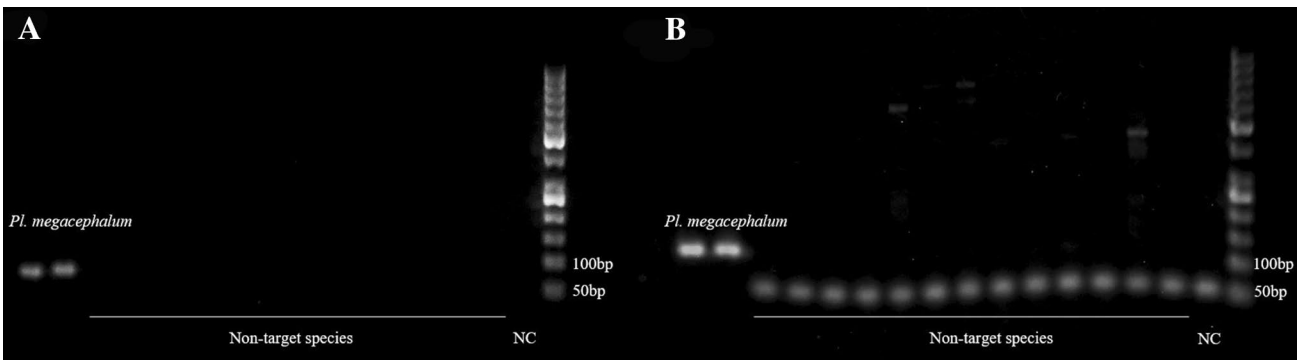

Fig. 1 Determination of species specificity of *Platysternon megacephalum* primer sets using conventional PCR and gel electrophoresis. **A** Results from a species-specific primer set. **B** Results from a non-species specific primer set. *NC* negative control

species for the TaqMan MGB non-fluorescent quencher qPCR assay.

Lastly, the specificity of primer sets was tested in vivo against the same DNA extractions in Table 2 and Supplementary Table 2 using qPCR in a StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific Inc; Waltham, MA, USA). Each assay included a probe labeled with 6-FAM at the 5′-end and an MGB non-fluorescent quencher (Thermo Fisher Scientific Inc; Waltham, MA, USA) at the 3′-end to increase the specificity (Kutyavin et al. 2000). Thermal cycler conditions are as follows: 50 °C for 2 min, then 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and the corresponding annealing temperature for 1 min. Assays were optimized by either ourselves or Thermo Fisher Scientific Inc. (Waltham, MA, USA). We optimized primer concentrations following Wilcox et al. (2015), varying primer concentration (100, 300, 600, 900 nM; n = 16 combinations) with a fixed probe concentration of 250 nM. The combination that exhibited the smallest C_q value was selected. For all optimizations by Thermo Fisher and us, primer concentrations of were 900 nM selected. The optimized qPCR reactions included 5 µL TaqMan® Environmental Master Mix (Thermo Fisher Scientific Inc; Waltham, MA, USA), 900 nM of each primer, 250 nM of probe, and 1 µL DNA template. TaqMan® Exogenous Internal Positive Control (VIC® Probe) (Applied Biosystems; Thermo Fisher Scientific Inc; Waltham, MA, USA) was included to test for inhibition within each reaction. Finally, water was added to make a final volume of 10 µL for each reaction. Each DNA template was tested in triplicate. Negative controls were included in all tests. All calculations were performed by StepOne™ Software v.2.3 (Applied Biosystems; Thermo Fisher Scientific Inc; Waltham, MA, USA).

Sensitivity tests

The analytical sensitivity of each assay was evaluated using qPCR. We made a single, synthetic gene fragment (Tech Dragon Limited; Hong Kong) for testing, containing the primer and probe binding sites for each of the three target species. The length of each target fragment remained constant as to maintain efficiency. To be able to detect contamination of qPCR assays from the synthetic gene fragment via DNA sequencing, intervening regions between the primers and probes were modified with respect to the target species' sequences. Each assay was tested against a 4-level dilution series (10,000, 1000, 100, and 10 copies per µL) of the synthetic target amplicon with 20 replicates for each concentration level. qPCR reaction mixture and thermocycling condition were the same as above. Negative controls were included in all tests.

Results

An assay for eDNA detection was developed for each target species (Table 3). Among the three assays developed, the assay for detecting *Pl. megacephalum* is species specific—there were no signs of non-target species amplification in all steps (in silico, tissue and water filter DNA for conventional PCR, tissue and water filter DNA for qPCR). For the assay of *Pe. sinensis*, it did not amplify any of the tissue and water filter DNA from the non-target species tested in both conventional PCR and qPCR, but we suspect this assay to work for at least two other *Pelodiscus* species (*Pe. axenaria* and *Pe. parviformis*) based on in silico results (See Discussion). For the assay of *T. s. elegans,* it amplified tissue DNA of only one of the non-target species tested (*T. s. scripta*), and it likely works for several other *Trachemys* species based on in silico results (See "Discussion"). In all validation tests, no amplification was seen in negative controls. The quantification of DNA samples can be found in Table 2 (Target species) and Supplementary Table 2 (Non-target species). The analytical sensitivity of each assay is summarized in Table 4.

Discussion

In this study, we developed and validated eDNA qPCR assays for three turtle species in Hong Kong: two native (*Pl. megacephalum*, *Pe. sinensis*) and one invasive (*T. s. elegans*). Through our multi-step testing, we verify the *Pl.*

Table 3 Primer and probe sequences for eDNA TaqMan MGB qPCR assays to detect target turtle species. Annealing temperature = 60 °C

Species	Locus	Forward primer	Reverse primer	Probe	Length
Pl. megacephalum	ND4	GCAAACACAAACTATGAG CGAAC	GTCCCCATTAGTGGCAGGAA	CGAACATTATTAATTGCC	83
Pe. sinensis[a]	16S	GCCCAGTGATAATATTYAACG	GCCTTTCATACGAGTCCTT	CGGTATCCTAACCGTGCAA	91
T. s. elegans[a]	12S	TCGCCAGCYTACCCYGT	CCTTGACCTGACTTGTTA ATGG	GGATACAAAAGTAAGCAAG	70

All sequences are listed from 5′ to 3′

[a]Suspected to amplify other species in the genus; see "Results" and "Discussion"

Table 4 Analytical sensitivity of assays developed in this study. (n = 20 for each concentration level)

Species	Locus	DNA concentration level (copies/μL)	Analytical sensitivity	Mean C_q value	Standard deviation
Platysternon megacephalum	*ND4*	10,000	1	26.56	0.18
		1000	1	29.76	0.20
		100	1	33.64	1.12
		10	0.95	34.24	2.59
Pelodiscus sinensis	*16S*	10,000	1	26.83	0.12
		1000	1	30.42	0.30
		100	1	34.05	0.98
		10	0.25	36.10	0.52
Trachemys scripta elegans	*12S*	10,000	1	28.53	0.14
		1000	1	32.07	0.25
		100	1	35.56	0.59
		10	0.2	38.08	0.89

megacephalum assay to be species specific, while the *Pe. sinensis* and *T. s. elegans* assays are likely to amplify other species in their respective genera (Supplementary Table 3). Despite the amplification of some non-target species, these assays will still be useful for our purposes of locating and monitoring turtle populations in Hong Kong. We detail these findings below.

Until recently, *Pe. sinensis* was considered to be the only extant species in the genus. Fritz et al. (2010) suggested *Pe. sinensis* represents a species complex, with subsequent work suggesting five species (*Pe. axenaria* [Zhou, Zhang and Fang, 1991]; *Pe. maackii* [Brandt, 1857]; *Pe. parviformis* Tang, 1997; *Pe. sinensis* [Wiegmann, 1835]; *Pe. variegatus* Farkas et al. 2019). Primer-BLAST results identified amplification of at least two non-target species, *Pe. axenaria* and *Pe. parviformis*. While some studies (Stuckas and Fritz 2011; Yang et al. 2011) recognize *Pe. axenaria* and *Pe. parviformis* to be valid species, relationships within the genus remain unresolved and complicated by translocation, large-scale breeding, and mixing of genetic lineages (van Dijk et al. 2000; Fritz et al. 2010; Turtle Taxonomy Working Group 2017). Until the evolutionary relationships within *Pelodiscus* are clarified, we are unable to determine the species-specificity of our assay. In Hong Kong *Pe. sinensis* is the only native *Pelodiscus* species. Since our goal of developing eDNA assays was to help in identifying sites with viable turtle populations in Hong Kong, this assays is still useful, despite the confusion surrounding *Pelodiscus* and the possibility of non-native *Pelodiscus* species being introduced into Hong Kong. Other researchers should take care when using this assay for other purposes, such as differentiating between or working in areas with multiple *Pelodiscus* species.

Davy et al. (2015) successfully developed an eDNA assay to detect *T. s. elegans* in the wild, demonstrating the potential of tracking invasive species. We tested their primer set and decided to develop a new assay for two reasons. First, while testing the specificity of the Davy et al. (2015) primer set using conventional PCR, we found it amplified DNA of two additional species (*G. pseudogeographic* and *G. ouachitensis*). Although *Graptemys* is not commonly found in Hong Kong as an introduced species, we targeted to develop a primer set specific to *T. s. elegans*. Second, Davy et al. (2015) did not use qPCR for their *T. s. elegans* assay. To extend the potential of invasive species monitoring to include quantification of eDNA, we included the development of a TaqMan probe. Based on Primer-BLAST results, our assay is suspected to amplify other species and subspecies from the genus *Trachemys*, including *T. callirostris callirostris, T. ornata*, *T. scripta*, *T. s. grayi, T. s. emolli,* and *T. s. scripta.* Of these non-target species, we only had access to tissue sample of one (*T. s. scripta*) to test, which successfully amplified during the qPCR assay. Conservatively, we assume that our assay work for at least a subset of other *Trachemys* species. Another non-target species, *Mauremys japonica* was suspected to work based on in silico test, but no amplification was detected in vivo. Despite lacking species specificity, this assay will be useful in tracking invasive species in regions like Hong Kong, where all *Trachemys* species are exotic. Further testing of this assay should be done if it is necessary to differentiate between *Trachemys* species.

Although eDNA techniques are being refined, they have already proven useful in detecting the presence of secretive species. Conservation often involves substantial financial and manpower investments, and we believe that the combination of eDNA techniques and traditional fieldwork will help with resource optimization. Fieldwork to physically catch a target species gives unambiguous results, but is time and labor intensive. eDNA techniques can aid fieldwork by identifying sites that the target species is likely present and worthy of additional field effort. With additional testing, we envision eDNA assays to be used for species monitoring,

providing a fast, inexpensive method to monitor a population over time. The assays developed in this study will be used directly for the study and conservation of Hong Kong turtle species, but we hope they can be used to study the same species across their geographic ranges in Asia, as well as serve as a template for developing eDNA assays for other endangered species.

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