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Development of *Anabas testudineus* (climbing perch) eDNA Assay: Technical Report

Richard C. Edmunds, Ph.D., Roger Huerlimann, Ph.D., Prof. Damien Burrows

Report No. 19/10

March 2019

Development of *Anabas testudineus* (climbing perch) eDNA Assay

Technical Report

TropWATER Report No. 19/10

March 2019

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Executive Summary

A novel environmental DNA (eDNA) assay was developed for detection of invasive climbing perch (*Anabas testudineus*) in water samples using Sybr-based quantitative real-time polymerase chain reaction (qPCR). This eDNA assay targets a region within the mitochondrial *16S* gene that is unique to *A. testudineus*. Here we present *in silico*, *in vitro*, and *in situ* validations undertaken during assay development.

In silico, *in vitro* and, *in situ* validations confirmed that this eDNA assay is specific to (i.e., detects only) *A. testudineus* despite co-occurrence with native species. Limit of detection (LOD) for this assay was determined to be 9 ± 3 copies per qPCR assay under optimal assay conditions (65°C annealing temperature, 600nM each primer). Moreover, bidirectional Sanger sequencing confirmed all representative putative positive detections from *in vitro* and *in situ* validations (gDNA standards and TropWater Facility holding talk water samples) to be positive for *A. testudineus*, respectively. Lastly, none of the non-target species tested during *in vitro* validation ($n = 40$) amplified. Collectively, these validations demonstrate the readiness of this assay for screening environmental water samples for determination of *A. testudineus* eDNA presence.

In light of invasion fronts consisting of only a few individuals, waterways previously invaded or prone to invasion should be regularly monitored for *A. testudineus* eDNA using this assay so as to detect invasion front(s) as early as possible. If detected, this eDNA assay can be subsequently utilized in conjunction with spatial water sampling to guide *A. testudineus* eradication efforts.

1. Introduction

Anabas testudineus (Actinopterygii, Perciformes, Anabantidae), or commonly known as climbing perch or climbing gouramis, is a small fish (up to 25 cm in length) native to Asia (India east to China and to the Wallace Line); however, *A. testudineus* has established invasive populations in both eastern Indonesia and Papua New Guinea (PNG). The *A. testudineus* invasion front is believed to be advancing towards Australia, with *A. testudineus* discovered in late 2005 on Saibai Island and another small Australian island in the Torres Strait south of PNG (Pearlman, 2015).

Anabas testudineus most commonly occur in canals, lakes, ponds, swamps, estuaries, medium to large rivers, brooks, flooded fields and stagnant water bodies including sluggish flowing canals and areas with dense vegetation (Taki, 1978; Rainboth, 1996; Menon, 1999; Vidthayanon, 2002). Moreover, *A. testudineus* is a unique fish in that it can tolerate extremely unfavourable water conditions (Pethiyagoda, 1991), possess an accessory air-breathing organ (Allen, 1991), is able to “walk” on pectoral fins (Herre, 1935), and can survive for several days to weeks out of water as long as their air-breathing organ is kept moist (Rahman, 1989). During the dry season *A. testudineus* tend to remain buried in the mud of pools associated with submerged woods and shrubs (Rahman, 1989, Sokheng, et al., 1999).

In order to accurately monitor Australian waterways for the invasion of this aggressive invasive species, a sensitive method for the detection of *A. testudineus* (across all life history stages) is needed. Environmental DNA (eDNA), or the DNA shed by all living organisms into their local environment (Goldberg, et al., 2016), allows for the detection of *A. testudineus* in any water source known or suspected to have inhibited by *A. testudineus* during any life history stage. Here we describe the development of a sensitive quantitative real-time PCR (qPCR) based eDNA assay that can be used to detect *A. testudineus* DNA inherent in environmental water samples.

2. Methods

2.1 Primer Design

GenBank (NCBI) was mined for available *16S* nucleotide sequences from Australian fish species ($n = 86$; Table 1) given the common use of this gene for barcoding studies (i.e., *16S* nucleotide sequence available for wide range of species) and greater abundance within environmental samples (i.e., greater detectability) due to multiple mitochondria present within

each cell (i.e., each cell shed into environment contains multiple copies of mitochondrial genome; Goldberg, et al., 2016). All available *16S* sequences were downloaded into Geneious analysis software (ver. R11) and subsequently aligned using the embedded ClustalW algorithm. Regions of conservation within alignment of *A. testudineus* *16S* sequences ($n = 13$) were identified, annotated, and then assessed by eye for regions wherein *A. testudineus* exhibited ≥ 3 base pair mismatches with Australian fish and Human *16S* sequences.

Primers were assessed for quality and probability of accuracy and efficiency based on the following parameters: 1) melting temperature (T_m): 55 – 65°C with $< 4^\circ\text{C}$ difference between primer pair, 2) G/C content: 40-80%, 3) length: 16-25 bp. 4) amplicon size: 80 - 350bp, 5) self-dimer T_m : $< 30^\circ\text{C}$, 6) hairpin T_m : $< 30^\circ\text{C}$, 7) overall self-complementarity: PrimerBLAST score < 6 , and 8) 3' self-complementarity: PrimerBLAST score < 6 .

Table 1. Non-target Australian fish species for which *16S* nucleotide sequences were obtained from GenBank (NCBI) and used to guide *Anabas testudineus* eDNA assay primer design.

<i>Amatitlania nigrofasciata</i>	<i>Galaxiella pusilla</i>	<i>Maccullochella mariensis</i>	<i>Ornithodoros mossambicus</i>
<i>Ambassis agassizii</i>	<i>Galaxias zebratus</i>	<i>Maccullochella peelii</i>	<i>Oxyeleotris lineolatus</i>
<i>Ambassis agrammus</i>	<i>Galaxiella munda</i>	<i>Macquaria ambigua</i>	<i>Pangasius conchophilus</i>
<i>Amphilophus citrinellus</i>	<i>Galaxiella nigrostriata</i>	<i>Macquaria australasicica</i>	<i>Paragalaxias eleotroides</i>
<i>Anabas testudineus</i>	<i>Galaxiella pusilla</i>	<i>Macquaria colonorum</i>	<i>Paragalaxias julianus</i>
<i>Anguilla australis</i>	<i>Gambusia holbrooki</i>	<i>Macquaria novemaculeata</i>	<i>Paragalaxias mesotes</i>
<i>Anguilla bicolor</i>	<i>Geotria australis</i>	<i>Megalops cyprinoides</i>	<i>Perca fluviatilis</i>
<i>Anguilla obscura</i>	<i>Giuris margaritacea</i>	<i>Melanotaenia fluviatilis</i>	<i>Philypnodon grandiceps</i>
<i>Anguilla reinhardtii</i>	<i>Glossamia aprion</i>	<i>Melanotaenia splendida</i>	<i>Philypnodon macrostomus</i>
<i>Archocentrus nigrofasciatus</i>	<i>Guyu wujalwujalensis</i>	<i>Mogurnda adspersa</i>	<i>Piaractus brachypomus</i>
<i>Archocentrus citrinellum</i>	<i>Hephaestus carbo</i>	<i>Mordacia mordax KY798444</i>	<i>Piaractus mesopotamicus</i>
<i>Arius (Neoarius) berneyi</i>	<i>Hephaestus fuliginosus</i>	<i>Nannoperca oxleyana</i>	<i>Plotosus lineatus</i>
<i>Carassius auratus</i>	<i>Hypseleotris compressa</i>	<i>Nannoperca vittata</i>	<i>Porochilus obbesi</i>
<i>Craterocephalus eyresii</i>	<i>Hypseleotris galii</i>	<i>Nannoperca australis</i>	<i>Retropinna semoni</i>
<i>Cyprinus carpio</i>	<i>Kuhlia marginata</i>	<i>Nannoperca obscura</i>	<i>Rutilus rutilus</i>
<i>Eptatretus cirrhatus</i>	<i>Kuhlia rupestris</i>	<i>Nannoperca variegata</i>	<i>Salmo trutta</i>
<i>Eptatretus longipinnis</i>	<i>Lates calcarifer</i>	<i>Nematalosa erebi</i>	<i>Tandanus tandanus</i>
<i>Gadopsis marmoratus</i>	<i>Leiopotherapon aheneus</i>	<i>Neoceratodus forsteri</i>	<i>Tinca tinca</i>
<i>Galaxias brevipinnis</i>	<i>Leiopotherapon unicolor</i>	<i>Neosilurus ater</i>	<i>Toxotes chatareus</i>
<i>Galaxias fuscus</i>	<i>Lepidogalaxias salamandroides</i>	<i>Oncorhynchus mykiss</i>	<i>Toxotes jaculatrix</i>
<i>Galaxias maculatus</i>	<i>Maccullochella ikei</i>	<i>Oreochromis aureus</i>	
<i>Galaxias parvus</i>	<i>Maccullochella macquariensis</i>	<i>Oreochromis niloticus</i>	

2.2 In silico Validation

Following design of primers (one forward: “F1”, one reverse: “R1”) the combination (F1-R1) was tested *in silico* (i.e., virtual determination of potential PCR amplification of non-target species using specific primer pairs; Goldberg, et al., 2016) using both targeted and non-targeted searches of NCBI “nr” database via PrimerBLAST (Ye, et al., 2012). Initial targeted PrimerBLAST specified a list of Australian freshwater fish, frogs and freshwater turtles against which to test F1-R1 (Table 2). For this targeted search, all species with ≤ 5 base pair mismatches to *A. testudineus* F1 or R1 were documented (see Section 3.2). Subsequent *in silico*

test used non-targeted PrimerBLAST (i.e., no species specified) to test F1-R1 against all species with nucleotide sequences deposited in NCBI “nr” database so as to ascertain which species are expected to amplify in *A. testudineus* 16S assay. For this non-targeted search, all species with ≤ 2 base pair mismatches to *A. testudineus* F1 or R1 were documented (see Section 3.2).

Following satisfactory compliance of F1 and R1 sequences with *in silico* tests (e.g., targeted PrimerBLAST search returning no species with < 3 base pair mismatches to *A. testudineus* F1 or R1), standard desalting oligonucleotides were ordered from and synthesized by Integrated DNA Technologies (IDT; New South Wales, Australia) and shipped pre-diluted to 100µM in low-EDTA TE buffer (“Lab Ready”; Table 3).

Table 2. Species against which *Anabas testudineus* primer pairs were tested *in silico* using targeted PrimerBLAST search of Australian fish, frog, and turtle sequences in NCBI “nr” database

Australian Freshwater Fish			
<i>Amatitlania nigrofasciata</i>	<i>Galaxias maculatus</i>	<i>Macquaria ambigua</i>	<i>Neosilurus pseudospinosus</i>
<i>Albula forsteri</i>	<i>Galaxias parvus</i>	<i>Macquaria australasica</i>	<i>Oncorhynchus mykiss</i>
<i>Albula oligolepis</i>	<i>Galaxias zebratus</i>	<i>Macquaria colonorum</i>	<i>Oreochromis mossambicus</i>
<i>Ambassis agrammus</i>	<i>Galaxiella munda</i>	<i>Macquaria novemaculeata</i>	<i>Oreochromis niloticus</i>
<i>Ambassis marijanus</i>	<i>Galaxiella nigrostriata</i>	<i>Megalops cyprinoides</i>	<i>Perca fluviatilis</i>
<i>Amphilophus citrinellus</i>	<i>Galaxiella pusilla</i>	<i>Melanotaenia fluviatilis</i>	<i>Percalates colonorum</i>
<i>Anabas testudineus</i>	<i>Gambusia holbrookii</i>	<i>Melanotaenia splendida</i>	<i>Percalates novemaculeata</i>
<i>Anguilla australis</i>	<i>Geotria australis</i>	<i>Mogurnda adspersa</i>	<i>Philyodon grandiceps</i>
<i>Anguilla bicolor</i>	<i>Hephaestus carbo</i>	<i>Mogurnda mogurnda</i>	<i>Piaractus brachipomus</i>
<i>Anguilla obscura</i>	<i>Hephaestus fuliginosus</i>	<i>Mordacia mordax</i>	<i>Piaractus mesopotamicus</i>
<i>Anguilla reinhardtii</i>	<i>Homo sapiens</i>	<i>Mordacia praecox</i>	<i>Plotosus lineatus</i>
<i>Arius berneyi</i>	<i>Hypseleotris compressa</i>	<i>Nannoperca australis</i>	<i>Retropinna semoni</i>
<i>Carassius auratus</i>	<i>Kuhlia marginata</i>	<i>Nannoperca obscura</i>	<i>Rutilus rutilus</i>
<i>Channa</i> spp.	<i>Kuhlia rupestris</i>	<i>Nannoperca oxleyana</i>	<i>Salmo trutta</i>
<i>Cyprinus carpio</i>	<i>Lates calcarifer</i>	<i>Nannoperca variegata</i>	<i>Syncomistes butleri</i>
<i>Eptatretus</i> spp.	<i>Leiopotherapon unicolor</i>	<i>Nannoperca vittata</i>	<i>Tandanus tandanus</i>
<i>Eptatretus cirrhatus</i>	<i>Lepidogalaxias salamandroides</i>	<i>Nematalosa erebi</i>	<i>Tinca tinca</i>
<i>Eptatretus longipinnis</i>	<i>Maccullochella ikei</i>	<i>Neoarius berneyi</i>	<i>Toxotes chatareus</i>
<i>Gadopsis marmoratus</i>	<i>Maccullochella macquariensis</i>	<i>Neoceratodus forsteri</i>	<i>Toxotes jaculator</i>
<i>Galaxias brevipinnis</i>	<i>Maccullochella mariensis</i>	<i>Neosilurus</i> spp.	
<i>Galaxias fuscus</i>	<i>Maccullochella peelii</i>	<i>Neosilurus ater</i>	
Australian Frogs		Australian Freshwater Turtles	
<i>Astrochaperina</i> spp.	<i>Neobatrachus sudelli</i>	<i>Carettochelys</i> spp.	<i>Wollumbinia bellii</i>
<i>Cophixalus</i> spp.	<i>Notaden bennetti</i>	<i>Chelodina</i> spp.	<i>Wollumbinia georgesi</i>
<i>Crinia</i> spp.	<i>Notaden melanoscaphus</i>	<i>Elseya</i> spp.	<i>Wollumbinia latisternum</i>
<i>Cyclorana</i> spp.	<i>Nyctimystes dayi</i>	<i>Elusor macrurus</i>	
<i>Heleioporus australiacus</i>	<i>Pseudophryne bibroni</i>	<i>Emydura</i> spp.	
<i>Limnodynastes</i> spp.	<i>Pseudophryne coriacea</i>	<i>Pelochelys bibroni</i>	
<i>Litoria</i> spp.	<i>Rheobatrachus silus</i>	<i>Pseudemydura umbrina</i>	
<i>Mixophyes</i> spp.	<i>Taudactylus acutirostris</i>	<i>Rheodytes leukops</i>	
<i>Neobatrachus pictus</i>	<i>Uperoleia</i> spp.	<i>Trachemys scripta</i>	

Table 3. *Anabas testudineus* 16S eDNA assay primer information.

Primer Name	16S Region	Melt Temp (Tm; °C)*	Melt Temp (Tm; °C)†	GC Content (%)	Length (bp)	Amplicon (with R1)	Tm*	Tm*	Self-comp.▲	Self-comp.▲	3' Self-comp.▲	Oligonucleotide (5' - 3')
16S-Anate1-F	1168-1187	59.6	57.59	50	20	178	0	0	5	3		AGCAGCTCTTGTCATAACCC
16S-Anate1-R	1326-1345	60.2	58.23	50	20		14.7	0	4	3		ACCGGATCGTTATGGTCAGA

*: Determined by Geneious (ver. R11); †: Determined by PrimerBLAST

2.3 In vitro Validation

Following confirmation of satisfactory *in silico* tests F1-R1 was tested empirically for species-specificity by attempting to amplify genomic DNA (gDNA) template extracted from non-target but potentially co-occurring species using *A. testudineus* 16S primers (i.e., *in vitro* validation; Table 4). More specifically, 5 species of Australian rainforest frogs, 6 species of Australian freshwater turtles, and 31 species of Australian freshwater fishes were tested *in vitro* using *A. testudineus* 16S primer pairs (Table 4).

In vitro tests included verification of *A. testudineus* 16S assay amplification efficiency and limit of detection (LOD) as nanograms of gDNA per reaction (ng loaded) and copies of artificial *A. testudineus* 16S double stranded DNA (190 bp; aDNA; gBlocksTM, IDT Australia; Figure 1) per reaction (copies loaded). For precise quantification of pooled gDNA and stock aDNA ($\text{ng}/\mu\text{L} \pm 99.7\% \text{ CI}$) each was measured in duplicate using QuantiFluor[®] fluorometer with QuantiFluor[®] ONE dsDNA System (Promega Co., Australia). Duplicate aDNA stock measurements were averaged $\pm 99.7\% \text{ CI}$ and converted to $\text{copies}/\mu\text{L} \pm 99.7\% \text{ CI}$ using the average $\pm 99.7\% \text{ CI}$ weights (ng) and specific nucleotide sequence of synthesized aDNA (Figure 1) using an online calculator (<http://www.endmemo.com/bio/dnacopynum.php>).

To test assay efficiency and LOD using gDNA an 8-step \log_{10} serial dilution was made from genomic DNA that was pooled equally across 10 individuals sourced locally from TropWater compound on James Cook University Campus in Townsville QLD. Neat gDNA ($528.5 \pm 68.98 \text{ ng}/\mu\text{L}$, average $\pm \text{ SEM}$; NanoDropTM; Life Technologies Australia, Ltd. Pty.) was pooled equally (2 μL each) and diluted 1:50 with MilliQ[®] water to generate Standard 1 ($2.14 \pm 0.17 \text{ ng}/\mu\text{L}$; average $\pm 99.7\% \text{ CI}$; QuantiFluor[®]; Promega Australia). Standard 1 was then serially diluted (\log_{10}) 7 times to generate Standards 2 – 8 ($2.14 \pm 0.17 \times 10^{-1} – 2.14 \pm 0.17 \times 10^{-7} \text{ ng}/\mu\text{L}$), respectively. To test assay efficiency and LOD using aDNA a 10-step serial dilution standard curve was generated by resuspending the dried pellet of *A. testudineus* 16S aDNA fragment in 50 μL of 1x TE buffer (IDT Australia) following manufacturer's instructions, which yielded an aDNA stock concentration of $4.10 \pm 1.63 \text{ ng}/\mu\text{L}$ or $21,016,428,100 \pm 16,761,882,899 \text{ copies}/\mu\text{L}$. Stock aDNA was then diluted 1:500 with MilliQ[®] water to generate Standard 1 (stock aDNA divided by 500 or $4.20 \pm 1.67 \times 10^7 \text{ copies}/\mu\text{L}$), which was then serially diluted 1:10 (i.e., \log_{10}) 7 times to generate Standards 2 – 8 ($4.20 \pm 1.67 \times 10^6 – 4.20 \pm 1.67 \text{ copies}/\mu\text{L}$, respectively). Standard 8 was then diluted 1:2 (i.e., \log_2) twice to generate Standards 9 and 10 (2.1 ± 1.6 and $1.05 \pm 1.6 \text{ copies}/\mu\text{L}$,

respectively). The number of *16S* amplicon copies generated from gDNA template (ng/µL) of target and/or non-target species during *in vitro* Test 3 (see below) was determined by extrapolation from the aDNA standard curve run under the same conditions.

Quantitative real-time polymerase chain reaction (qPCR) was used for three separate *in vitro* tests: 1) determination of *A. testudineus* *16S* assay efficiency and LOD using 8-step log₁₀ gDNA standard curve under optimal assay conditions (Test 1), 2) determination of *A. testudineus* *16S* assay efficiency and LOD using 10-step log₁₀ aDNA standard curve under optimal assay conditions (Test 2), and 3) determination of *A. testudineus* *16S* assay amplification of gDNA from co-occurring non-target species (see Table 4) under optimal assay conditions (Test 3). All three *in vitro* tests were run as 10 µL reactions containing the following: 5 µL PowerUP® Sybr Green Master Mix (Thermo Fisher Scientific, Australia), 0.6 µL forward primer (10 µM, 600 nM final), 0.6 µL reverse primer (10 µM, 600 nM final), 3 µL *A. testudineus* gDNA ($6.42 \pm 0.51 - 6.42 \pm 0.51 \times 10^{-7}$ ng template; Test 1) or 3 µL *A. testudineus* aDNA ($12.60 \pm 5.01 \times 10^7 - 3.15 \pm 5.01$ copies template; Test 2) or 3 µL gDNA of non-target species (see Table 4; Test 3), and 1 µL molecular grade water. Room temperature master mix (7 µL) was loaded into white 96-well plates (Life Technologies Inc., Australia) with an epMotion® multi-dispensing electronic single channel pipette (Eppendorf, Australia) fitted with 500µL CombiTip ® (Eppendorf, Australia) in a UV-sterilized PCR cabinet (Esco, Australia) in the dedicated low copy DNA room within the Molecular Ecology and Evolution Laboratory (MEEL) at James Cook University's Australian Tropical Science and Innovation Precinct (ATSIP, Building 145) in Townsville, Queensland Australia.

For *in vitro* Test 1 the 96-well plate containing 7µL master mix was moved (unsealed) to a cleaned bench (wiped thoroughly with 70% ethanol) in the dedicated pre-PCR room within MEEL where 3 µL of each gDNA standard (see above) was loaded using a manual single channel P10 pipette (Eppendorf, Australia) fitted with Maximum Recovery filter tips (Axygen, Australia).

For *in vitro* Test 2, due to the high cross-contamination risk posed by high-copy aDNA standards, the 96-well plate containing 7µL master mix was moved (unsealed) to a cleaned bench (wiped thoroughly with 70% ethanol) in the dedicated post-PCR room within MEEL where 3 µL of each aDNA standard (see above) was loaded as described above for *in vitro* Test 1.

For *in vitro* Test 3 the 96-well plate containing 7µL master mix was moved (unsealed) to a cleaned bench (wiped thoroughly with 70% ethanol) in the dedicated pre-PCR room within

MEEL where 3 µL room temperature gDNA template of each non-target species (see Table 4) was loaded as described above for *in vitro* Test 1.

Following gDNA or aDNA loading each plate was sealed with an optical adhesive film (Life Technologies), briefly vortexed (10 sec), pulse spun (10 sec), loaded onto opened tray of QuantStudio3 Real-Time PCR System (Life Technologies Inc., Australia), and wiped thoroughly with nonabrasive Kimwipe® to ensure a complete removal of any transparency obstructions present on optical seal (e.g., smudges or dust) before closing QuantStudio3 tray and commencing qPCR run.

All three *in vitro* tests were run under the following qPCR cycling conditions: initial UDG incubation at 50°C for 2 min then initial denaturation at 95°C for 2 min followed by 45 cycles of 95°C for 15 secs and 65°C for 1 min (ramp rate = 2.7°C/sec) before terminal dissociation curve generation by transitioning from 65°C to 95°C (ramp rate = 0.15°C/sec). *In vitro* Tests 1 and 2 (gDNA and aDNA standard curves, respectively) were run in triplicate while *in vitro* Test 3 (non-target gDNA amplification) was run in duplicate. QuantStudio™ Design and Analysis Software (version 1.4.2; Life Technologies, Australia) was used to set the threshold fluorescence to 0.2 and analyse and export (Excel) data from *A. testudineus* *in vitro* Tests 1-3.

Three representative amplicons from gDNA standard curve were Sanger sequenced for confirmation (*in vitro* Test 1) whereas no amplicons from aDNA standard curve (*in vitro* Test 2) were Sanger sequenced given that aDNA fragment is exact replica of target *16S* region (i.e., gDNA standards sequenced to ensure assay targeted *16S* from total DNA extractions). Any amplicons produced from non-target species (*in vitro* Test 3) that exhibited T_m within 99.7% CI of standards were considered putative negatives (i.e., false positives) and Sanger sequenced for verification.

CACTAAAGCAGCTTGTCAATACCC TAAGAATAATTAGACTAAACTTAGGAA CCCTGCTTCATGTCTTGGTGGGGCGACCACGGGGAAGTAAGTAACCCCCCG CGCGGAATAAGAGAACATCTCGCACCAAGAGCTTCTGCTCTAACAGA ATA <u>TCTGACCATAACGATCCGGT</u> AAGACC
--

Figure 1. Artificial double stranded DNA (aDNA) replica of the *A. testudineus* *16S* gene region wherein designed primers target. Total aDNA fragment length is 190 bp. Blue nucleotides indicate the binding region for the forward (F1) and reverse (R1) primers. Underlined regions indicate extensions beyond the primer binding sites for F1 and R1, which were included to promote efficient primer binding to and amplification of aDNA fragment.

Table 4. Non-target species against which *Anabas testudineus* F1-R1 primer pair was tested *in vitro* using Sybr-based qPCR.

Freshwater Fish	
<i>Amatitlalina</i> sp.* (1.107)	<i>Macquaria australasica</i> (1.584)
<i>Ambassis agrammus</i> (0.636)	<i>Melanotaenia splendida inornata</i> (0.711)
<i>Amniataba percoides</i> (0.45)	<i>Mogurnda adspersa</i> (0.387)
<i>Anabas testudineus</i> * (1.026)	<i>Nematalosa erebi</i> (3.84)
<i>Craterocephalus stercusmuscarum</i> (1.221)	<i>Neosiluroides cooperensis</i> (0.702)
<i>Giuris marginitacea</i> (16.2)	<i>Neosilurus ater</i> (0.327)
<i>Glossamia aprion</i> (2.043)	<i>Orechromis mossambicus</i> (5.85)
<i>Haplochormis burtoni</i> * (0.762)	<i>Oxyeleotris lineolatus</i> (0.759)
<i>Hephaestus carbo</i> (0.84)	<i>Philypnodon grandiceps</i> (5.01)
<i>Hypseleotris compressa</i> (8.73)	<i>Philypnodon macrostomus</i> (2.337)
<i>Hypseleotris galii</i> (12.78)	<i>Stenogobius watsoni</i> (1.254)
<i>Hypseleotris</i> sp. (0.636)	<i>Tandanus bellingerensis</i> (2.886)
<i>Kuhlia marginata</i> (1.083)	<i>Tandanus tandanus</i> (17.4)
<i>Leiopotherapon unicolor</i> (0.579)	<i>Tilapia mariae</i> * (0.564)
<i>Macquaria ambigua</i> (0.135)	<i>Xiphophorus maculatus</i> * (0.405)
Frogs	
<i>Litoria dayi</i> (5.31)	Freshwater Turtles
<i>Litoria lorica</i> (5.13)	<i>Chelodina canni</i> (1.791)
<i>Litoria nannotis</i> (2.111 [^])	<i>Chelodina oblonga</i> (0.669)
<i>Litoria rheocola</i> (2.922)	<i>Elseya larvackorum</i> (1.449)
<i>Litoria serrata</i> (4.92)	<i>Emydura subglobosa worrelli</i> (0.741)
	<i>Myuchelys latisternum</i> (1.134)
	<i>Rheodytes leukops</i> (1.248)

*: Not native to Australia; ^: Average of individuals representing 3 different populations

Numbers in brackets are amount of gDNA template (ng) loaded into *in vitro* test

2.4 In situ Validation

Following confirmation via *in vitro* Tests 1-3 that *A. testudineus* eDNA assay had acceptable PCR amplification efficiency and did not amplify gDNA template of non-target species (see Sections 2.3 and 3.3), the *A. testudineus* 16S eDNA assay was put through one final empirical test using eDNA captured and extracted from pseudo-environmental water samples (i.e., *in situ* validation). For this *in situ* test eDNA was captured from an *A. testudineus* holding tank (i.e., pseudo-environmental positive control) located at the TropWATER Facility at James Cook University in Townsville QLD Australia (19° 19' 39" S, 146° 45' 39.24" E). More specifically, the *A. testudineus* holding tank is ≈ 800 L in volume and houses ≈ 40 fish with complete turnover with UV sterilized facility water every ≈ 3 hours (Glenn Morgan and Anthony Squires, TropWATER Facility Technicians; personal communication).

Water samples were collected by decanting 15 mL from a 50mL LoBind® (Eppendorf Inc.) falcon “measurement” tube into each of three replicate 50 mL LoBind® falcon tubes (new water grab for each replicate) pre-loaded with 5mL Longmire’s Solution (0.1M Tris Base pH

8, 0.1M disodium ethylenediaminetetraacetate dihydrate pH 8, 0.01M sodium chloride, 0.5% (w/v) sodium dodecyl sulfate; Longmire, et al., 1992; Renshaw, et al., 2015; Williams, et al., 2016). Samples were transported back to MEEL at ambient temperature ($\approx 24^{\circ}\text{C}$) and eDNA was extracted using a novel eDNA workflow (“Preserve, Precipitate, Lyse, Precipitate, Purify (PPLPP)”; Edmunds, et al., *final prep*). Extracted eDNA from each sample (100 μL) was split equally four-ways when transferred to a 96-well plate (Axygen, Australia) so as to allow for loading of eDNA template using Xplorer® electronic 12-channel pipette (Eppendorf, Australia; see below). *In situ* test of *A. testudineus* 16S assay was run in quadruplicate 10 μL technical qPCR using the same chemistry as *in vitro* tests (see Section 2.3) but with 3 μL eDNA extracted from *A. testudineus* holding tank water samples (see above) as template.

Master mix for *in situ* test was loaded as per *in vitro* tests with 3 μL eDNA template loaded into each qPCR replicate from 96-well plate containing Zymo purified eDNA on a 10% bleach and 70% ethanol cleaned bench in the dedicated low copy DNA room within MEEL (see Section 2.3) using an Xplorer® electronic 12-channel pipette (Eppendorf, Australia) fitted with Maximum Recovery filter tips (Axygen, Australia). The loaded *in situ* plate was sealed, vortexed, spun, run, and analysed as described above for *in vitro* tests (see Section 2.3).

Representative amplicons produced from *A. testudineus* *in situ* test on pseudo-environmental eDNA that exhibited T_m within 99.7% CI of gDNA standards (see above) were Sanger sequenced for confirmation.

3. Results

3.1 Primer design

Based on 16S sequence alignments of *A. testudineus* with Australian fish (Table 2) there was no genus of Australian fish that was similar. Primer binding regions were designed to possess ≥ 5 mismatches to the 16S nucleotide sequence of all endemic and invasive freshwater Australian fish (see Section 3.2 for results of *in silico* test of primer design accuracy).

Optimal forward and reverse primer binding regions were identified between base pairs 1168-1187 (F1) and 1326-1345 (R1) of *A. testudineus* 16S, respectively (Table 3). Both primers exhibited parameters in line with those expected for assay quality, accuracy, and efficiency (see Section 2.1, Table 3).

3.2 In silico Validation

Initial targeted *in silico* PrimerBLAST search of NCBI “nr” database (see Section 2.2) confirmed that no other species specified (Table 2) are similar to *A. testudineus* 16S F1-R1 (Table 5). Subsequent non-targeted *in silico* PrimerBLAST test of *A. testudineus* F1-R1 (see Section 2.2) returned *A. testudineus*, *A. cobojius*, *Anabas* sp. AT8090J, and *Anabas* sp. VRK-2017 as having 0 base pair mismatches to F1 or R1 (i.e., PCR amplification expected, Table 6).

Table 5. Species with \leq 5 mismatches to *Anabas testudineus* primers based on targeted PrimerBLAST search of Australian fish, frog, and turtle sequences in NCBI "nr" database.

Primer pair	Forward Mismatches	Reverse Mismatches	Species
F1-R1	0	0	<i>Anabas testudineus</i>

Table 6. Species with \leq 2 mismatch to *Anabas testudineus* primers based on non-targeted PrimerBLAST search of entire NCBI "nr" database.

Primer pair	Forward Mismatches	Reverse Mismatches	Species
F1-R1	0	0	<i>Anabas cobojius</i>
			<i>Anabas</i> sp. AT8090J
			<i>Anabas</i> sp. VRK-2017
			<i>Anabas testudineus</i>

3.3 In vitro Validation

Designed *A. testudineus* primer pairs exhibited satisfactory efficiency and LOD based on both gDNA and aDNA standard curves (see Section 2.3).

In vitro Test 1 demonstrated that at 65°C with 600nM each primer (optimal conditions) the F1-R1 assay amplified all points of the 8-point \log_{10} gDNA standard curve (see Section 2.3) with 103.43% efficiency ($R^2 = 0.995$) and no primer dimerization (as evidenced by a lack of signal in “no template controls”) with an LOD of 0.0000038 ng gDNA per reaction or \geq 2 copies of 16S template per reaction (aDNA standard curve extrapolation; Figure 2). Note that all three technical replicates for Standard 5 (0.0006 ng loaded) amplified but only one technical

replicate was included in the standard curve due to amplifying in linear order (efficiency calculation) while remaining two due remaining technical replicates (out of linear order due to poor mixing and/or pipetting error) had concentrations extrapolated from gDNA standard curve (0.00018 and 0.00012 ng loaded). As for Standards 7 and 8 (0.0000064 and 0.00000064 ng loaded, respectively), only one of three technical replicates amplified for each; however, Standard 7 amplified in linear order so was included in standard curve (Figure 2) whereas Standard 8 did not amplify in linear order so was not included in gDNA standard curve but rather was extrapolated using the 7-point gDNA standard curve (0.0000038 ng or 6.3x more gDNA template loaded than expected). Sanger sequenced representatives from gDNA standard 1 ($n = 3$; consensus length = 177 bp) matched GenBank accession KC774722 with pairwise identity of 99.4%.

In vitro Test 2 demonstrated that at 65°C with 600nM each primer (optimal conditions) the F1-R1 assay amplified standards 1 - 9 (4.20×10^7 – 2.10 copies/ μ L; see Section 2.3) with 87.609% efficiency ($R^2 = 0.995$) and no primer dimerization (as evidenced by a lack of signal in “no template controls”) with an LOD of 6.3 ± 2.5 aDNA copies loaded (Figure 3). Note that zero of five technical replicates for standard 10 (3.2 ± 1.3 copies loaded) amplified, thus absolute LOD is between 1.9 and 8.8 copies loaded under these assay conditions (i.e., lower LOD possible at lower annealing temperature; however, reducing annealing temperature reduces assay specificity and could lead to false positive detections via amplification on co-occurring non-target species fly-speckled hardyhead; see Section 2.4).

In vitro Test 3 demonstrated that the *A. testudineus* F1-R1 16S assay does not amplify gDNA from any co-occurring non-target species of Australian fish, frogs, or turtles (Figure 4, Table 4) under optimal assay conditions (65°C, 600nM; see Section 2.4). Sanger sequencing of three gDNA standard replicates confirmed correct amplification with 99.7% pairwise identity between each amplicon sequence and *A. testudineus* mitochondrial genome sequence (GenBank accession KX950694).

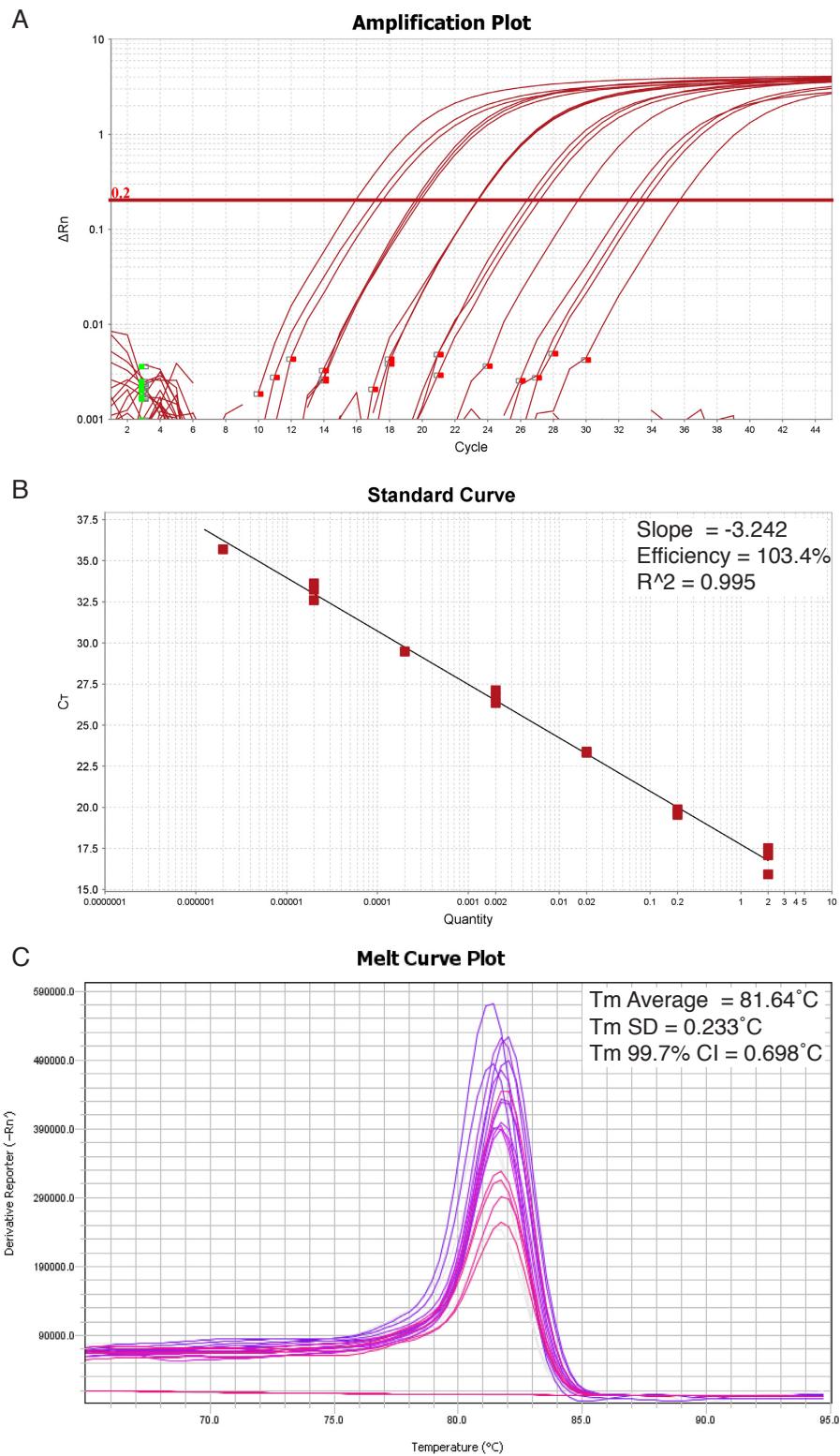


Figure 2. Amplification curves (A), linear regression of gDNA standard \log_{10} serial dilution (B), and amplicon dissociation temperature curves (T_m ; C) generated by qPCR during *A. testudineus* 16S assay *in vitro* Test 1 at 65°C with 600nM each primer (see Section 2.3).

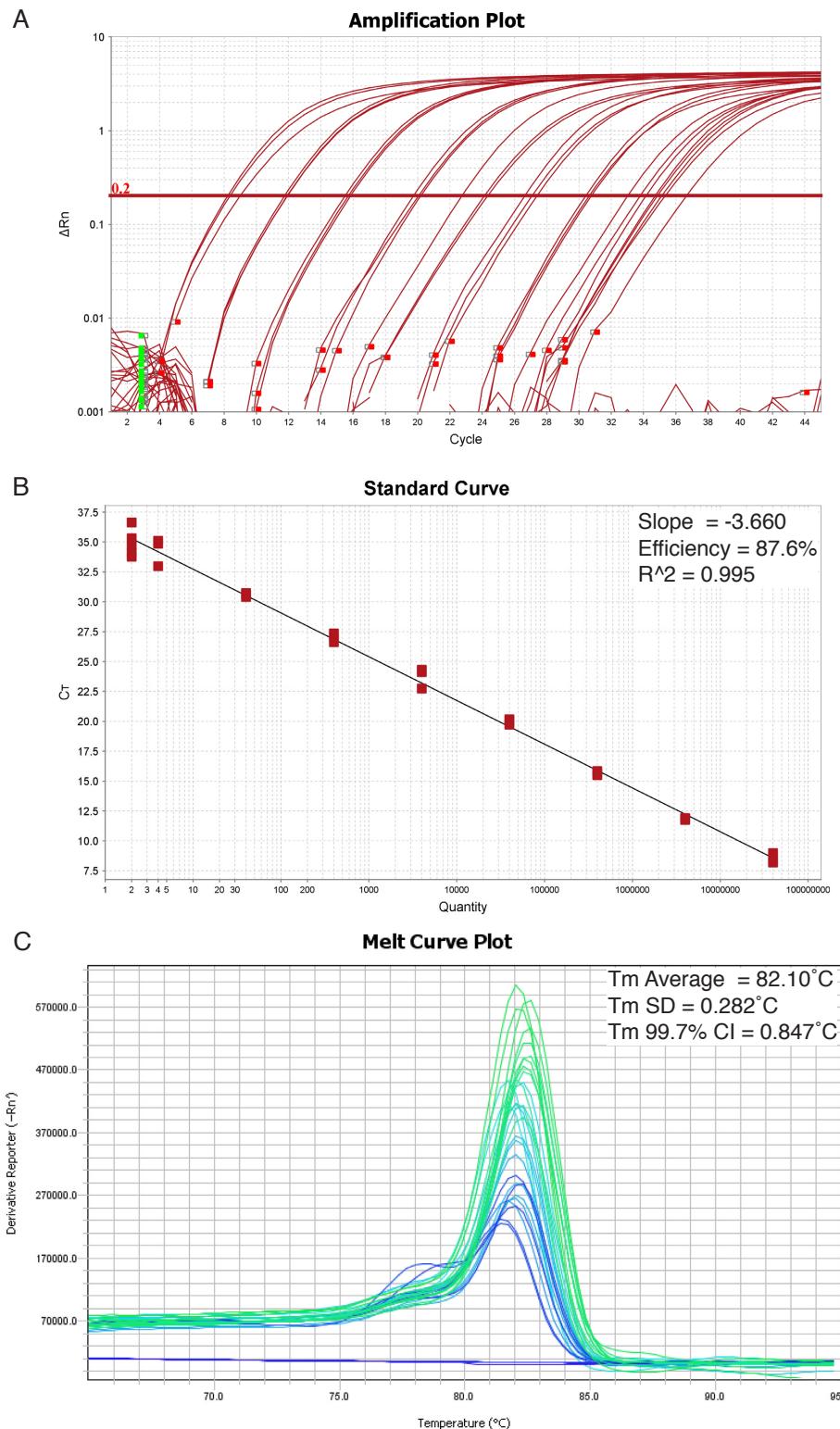


Figure 3. Amplification curves (A), linear regression of aDNA standard \log_{10} serial dilution (B), and amplicon dissociation temperature curves (T_m ; C) generated by qPCR during *A. testudineus* 16S assay *in vitro* Test 2 at 65°C with 600nM each primer (see Section 2.3).

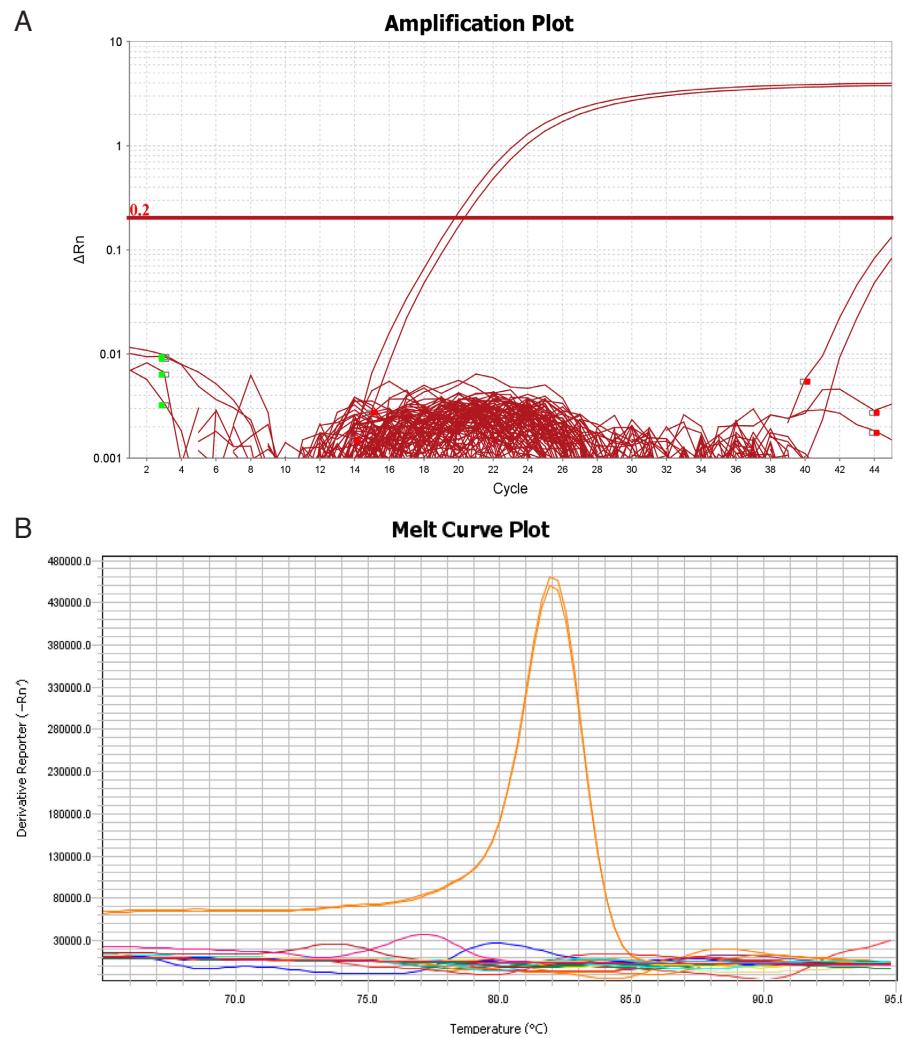


Figure 4. Amplification curves (A) and amplicon dissociation temperature curves (T_m ; B) from *A. testudineus* 16S assay *in vitro* test against both target and non-target species gDNA (Table 4) at 65 $^{\circ}\text{C}$ with 600nM each primer (see Sections 2.3 and 3.3). Amplification was observed for *A. testudineus* (target) gDNA included on *in vitro* assay plate, as expected, but not observed for gDNA from any non-target Australian fishes, turtles, or frogs also tested *in vitro* (Table 4).

3.4 In situ Validation

The *A. testudineus* F1-R1 assay demonstrated 100% putative positive detection across all technical replicates run for each water sample collected from TropWater Facility (Figure 5; see Section 2.4). All amplicons exhibited T_m within 99.7% CI of combined gDNA and aDNA standards ($81.911 \pm 1.05^\circ\text{C}$; Figure 5). Sanger sequencing confirmed the amplification of *A. testudineus* 16S for all 18 validated *in situ* replicates with $\geq 97.9\%$ pairwise identity with *A. testudineus* mitochondrial genome sequence (GenBank accession KX950694).

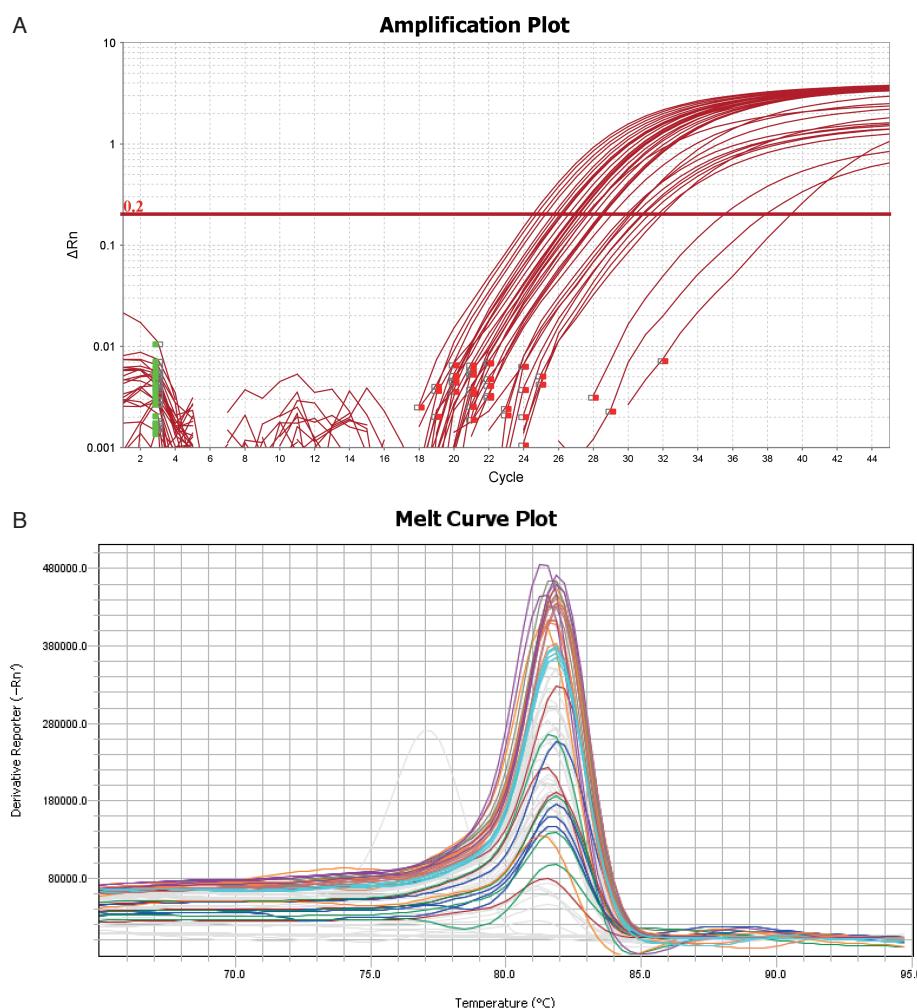


Figure 5. Amplification (A) and associated amplicon dissociation curves (B) generated during *A. testudineus* F1-R1 assay “positive control” *in situ* test on eDNA captured from pseudo-environmental (holding tank) water samples (see Section 2.4). All Sanger sequenced representative amplicons ($n = 18$) were positive for *A. testudineus* (see Section 3.4).

4. Discussion

The *in silico*, *in vitro*, and *in situ* validations undertaken during the development of the novel *A. testudineus* eDNA assay described herein demonstrate the readiness of this sensitive eDNA assay for screening environmental water samples. This validated assay can be used to monitor water samples taken from any location wherein *A. testudineus* is known to be present, suspected to be present (e.g., potential invasion front), or has previously been present (e.g., post-eradication monitoring).

The lack of *16S* sequence similarity to or assay amplification of (i.e., *in silico* or *in vitro* tested, respectively) this novel *A. testudineus* eDNA assay provides confidence that this validated assay can be utilized to test water samples collected from anywhere within Australian. Moreover, being that the only observed potential for non-target species amplification during *in silico* tests (see Tables 5 and 6) was the climbing gourami *A. cobojius* that is native to Bangladesh and India (Table 6), the *A. testudineus* eDNA described herein is likely useful for testing waterways anywhere in the world wherein *A. cobojius* is not known or suspected to occur. However, being that many fish species have yet to be sequenced for mitochondrial genes (e.g., *16S*), confirmation of all putative positive *A. testudineus* detections by Sanger sequencing is strongly advised.

Lastly, failure to detect eDNA from target species cannot be interpreted as conclusive evidence of target species absence (Roussel, et al., 2015). If no eDNA of target species is detected in initial surveillance samples then regular monitoring of waterbodies should be undertaken so as to detect *A. testudineus* presence as soon as logistically possible. Alternatively, eDNA can be used to monitor the efficiency and consistency of *A. testudineus* eradication efforts.

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