Development of an eDNA assay for invasive Channa striata (snakehead murrel)

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Richard C. Edmunds and Damien Burrows

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Executive Summary

We developed an environmental DNA (eDNA) assay to permit detection of invasive snakehead murrel (*Channa striata*) in water samples using Sybr-based quantitative real-time polymerase chain reaction (qPCR). This eDNA assay targets a mitochondrial *16S* gene region that is unique six *Channa* species (*C. asiatica, C. diplogramme, C. melasoma, C. micropeltes*, and *C. striata*), of which *C. striata* is the only species considered an invasion threat to Australia. Here we present *in silico* and *in vitro* validations undertaken during development of C.striata_16S.

Based on genomic DNA (gDNA) or artificial DNA (aDNA) standard curves (94.7% or 94.1% efficiency) C.striata_16S limit of detection (LOD) was determined to be 1.47 x 10^{-5} ng/µL or 1 copy/µL under optimal assay conditions (65˚C annealing, 600nM each primer), respectively. *In vitro* validations confirmed that none of the empirically tested fish, frog, or turtle species found in Australia freshwater systems empirically (n = 40) cross-amplify. Moreover, bidirectional Sanger sequencing confirmed that representative gDNA standards (n = 3) were positive for *C. striata* despite gDNA extraction from a taxonomically unverified tissue sample. Lastly, given that C.striata_16S was developed pre-emptively (i.e., no Australian waterbodies yet invaded by *C. striata*), *in situ* validation was not possible; however, successful *in vitro* validations provide sufficient evidence that C.striata_16S can detect adequately low-copy *C. striata* eDNA. As such, C.striata_16S is considered ready and suitable for screening environmental water samples wherein *C. striata* is suspected to occur (i.e., invasion front monitoring).

In light of invasion fronts consisting of only a few individuals, waterways previously invaded or prone to invasion should be regularly monitored for *C. striata* eDNA using C.straiata_16S so as to detect an invasion as early as possible. If detected, C.striata_16S can be utilized in conjunction with spatial water sampling to guide *C. striata* eradication efforts.

1. Introduction

Channa striata (Actinopterygii, Perciformes, Channidae), or commonly known as stripped snakehead, common snakehead, chevron snakehead, or snakehead murrel, is an eel-like fish that is native to and economically important in Asia (Pakistan to Thailand and south China; Vidthayanon, C., 2002) that can grow up to 150 cm in length (Yamamoto and Tagawa, 2000) and 3 kg in weight (IGFA, 2001). *Channa striata* has a sub-cylindrical body, depressed head, rounded caudal fin, no dorsal spines, no anal spines, 38 – 43 soft dorsal rays, and 23 – 27 soft anal rays (Allen, 1991). Morphologically, *C. striata* has a large head (reminiscent of a snake head) with deeply-gaping and fully toothed mouth that is covered in large scales, which are a combination of black and ochre on dorsal surface and sides while white on underside (Yamamoto and Tagawa, 2000).

Adult *C. striata* are known to inhabit ponds, swamps, streams, brooks, and rivers, with a preference for stagnant and muddy water of freshwater plains or sluggish flowing canals with 1 - 2 meters of still water (Taki, 1978; Menon, 1999; Vidthayanon, C., 2002). For breeding, *C. striata* prefers ditches, ponds, and flooded paddy fields wherein juveniles shoal at the surface under guard of male parent hidden below the surface (Rahman, 1989; Ng, 2004). Interestingly, *C. striata* survive the dry season by burrowing into the muddy bottom of lakes, canals, and swamps because they can survive as long as skin and air-breathing apparatus remain moist (Davidson, 1975). The diet of *C. striata* consists of fish, frogs, snakes, insects, earthworms, tadpoles and crustaceans (Rahman, 1989; Allen, 1991). *Channa striata* typically migrate laterally from the Mekong mainstream (or other permanent water bodies) to flooded areas during the wet season (Sokheng, et al., 1999).

Recently, *C. striata* has begun to spread outside Asia (e.g., Pacific Island) through the hobbyist aquarium trade (Torres Strait – A New Frontier for Freshwater Fish Invasions into Australia, accessed 3 February 2019). In light of this increasing international aquarium trade traffic, efforts are being made to prevent *C. striata* from invading Australian waterways (Torres Strait – A New Frontier for Freshwater Fish Invasions into Australia, accessed 3 February 2019).

Environmental DNA (eDNA), or the DNA shed by all living organisms into their local environment (Goldberg, et al., 2016), can be used to monitor *C. striata* presence in locations suspected of invasion. As such, we developed an assay that targets the mitochondrial *16S* gene for sensitive detection of *C. striata* eDNA (C.striata_16S) to advance invasion front monitoring. Here we present *in silico* and *in vitro* validations of C.striata_16S.

2. Methods

2.1 Primer Design

GenBank (NCBI) was mined for available mitochondrial *16S* rDNA nucleotide sequences from *C. striata* (*n* = 9) and a range of non-target Australian fish species (*n* = 86; Table 1). *16S* was chosen as target gene given common use in barcoding studies (i.e., 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 (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 *C. striata 16S* sequences were identified, annotated, and then assessed by eye for regions wherein *C. striata* 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˚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˚C, 6) hairpin T_m: < 30˚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 C.striata_16S primer design.

Amatitlania nigrofasciata	*Oreochromis niloticus*
Ambassis agassizii	*Galaxiella pusilla*
Ambassis agrammus	*Galaxias zebratus*
Amphilophus citrinellus	*Galaxiella munda*
Anabas testudineus	*Galaxiella nigrostriata*
Anguilla australis	*Galaxiella pusilla*
Anguilla bicolor	*Gambusia holbrooki*
Anguilla obscura	*Geotria australis*
Anguilla reinhardtii	*Giuris margaritacea*
Archocentrus nigrofasciatus	*Glossamia aprion*
Archocentrus citrinellum	*Guyu wujalwujalensis*
Arius (Neoarius) berneyi	*Hephaestus carbo*
Carassius auratus	*Hephaestus fuliginosus*
Craterocephalus eyresii	*Hypseleotris compressa*
Cyprinus carpio	*Hypseleotris galii*
Eptatretus cirrhatus	*Kuhlia marginata*
Eptatretus longipinnis	*Kuhlia rupestris*

Gadopsis marmoratus	*Lates calcarifer*
Galaxias brevipinnis	*Leiopotherapon aheneus*
Galaxias fuscus	*Leiopotherapon unicolor*
Galaxias maculatus	*Lepidogalaxias salamandroides*
Galaxias parvus	*Maccullochella ikei*
Maccullochella mariensis	*Maccullochella macquariensis*
Maccullochella peelii	*Ornechromis mossambicus*
Macquaria ambigua	*Oxyeleotris lineolatus*
Macquaria australasica	*Pangasius conchophilus*
Macquaria colonorum	*Paragalaxias eleotroides*
Macquaria novemaculeata	*Paragalaxias julianus*
Megalops cyprinoides	*Paragalaxias mesotes*
Melanotaenia fluviatilis	*Perca fluviatilis*
Melanotaenia splendida	*Philypnodon grandiceps*
Mogurnda adspersa	*Philypnodon macrostomus*
Mordacia mordax KY798444	*Piaractus brachypomus*
Nannoperca oxleyana	*Piaractus mesopotamicus*
Nannoperca vittata	*Plotosus lineatus*
Nannoperca australis	*Porochilus obbesi*
Nannoperca obscura	*Retropinna semoni*
Nannoperca variegata	*Rutilus rutilus*
Nematalosa erebi	*Salmo trutta*
Neoceratodus forsteri	*Tandanus tandanus*
Neosilurus ater	*Tinca tinca*
Oncorhynchus mykiss	*Toxotes chatareus*
Oreochromis aureus	*Toxotes jaculatrix*

2.2 *In silico* Validation

Following primer design for *C. striata* eDNA assay (hereafter referred to as C.striata_16S), virtual assessment for potential cross-amplification of non-target species was undertaken (i.e., *in silico* validation; 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 fish, frogs and turtles known to occur within Australian freshwater systems (i.e., endemic and exotic) against which C.striata_16S was validated *in silico* (Table 2). Subsequent *in silico* validation used non-targeted PrimerBLAST (i.e., no species specified) to determine which species, if any, might also be amplified by C.striata_16S. Targeted and non-targeted searches documented all species with ≤ 5 and ≤ 2 base pair mismatches to C.striata_16S, respectively.

Following satisfactory *in silico* compliance (e.g., targeted PrimerBLAST search returning no species with < 3 base pair mismatches to C.striata_16S), standard desalted 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 C.striata_16S was tested *in silico* using targeted PrimerBLAST search against all available *16S* nucleotide sequences in GenBank (NCBI) "nr" database for freshwater fish, frogs, and turtles known to occur within Australia.

Fish	
Amatitlania nigrofasciata	*Neosilurus ater*
Albula forsteri	*Galaxias maculatus*
Albula oligolepis	*Galaxias parvus*
Ambassis agrammus	*Galaxias zebratus*
Ambassis marianus	*Galaxiella munda*
Amphilophus citrinellus	*Galaxiella nigrostriata*
Anabas testudineus	*Galaxiella pusilla*
Anguilla australis	*Gambusia holbrooki*
Anguilla bicolor	*Geotria australis*
Anguilla obscura	*Hephaestus carbo*
Anguilla reinhardtii	*Hephaestus fuliginosus*
Arius berneyi	*Homo sapiens*
Carassius auratus	*Hypseleotris compressa*
Channa spp.	*Kuhlia marginata*
Cyprinus carpio	*Kuhlia rupestris*
Eptatretus spp.	*Lates calcarifer*
Eptatretus cirrhatus	*Leiopotherapon unicolor*
Eptatretus longipinnis	*Lepidogalaxias salamandroides*
Gadopsis marmoratus	*Maccullochella ikei*
Galaxias brevipinnis	*Maccullochella macquariensis*
Galaxias fuscus	*Maccullochella mariensis*
Macquaria ambigua	*Maccullochella peelii*
Macquaria australasica	*Neosilurus pseudospinosus*
Macquaria colonorum	*Oncorhynchus mykiss*
Macquaria novemaculeata	*Oreochromis mossambicus*
Megalops cyprinoides	*Oreochromis niloticus*
Melanotaenia fluviatilis	*Perca fluviatilis*
Melanotaenia splendida	*Percalates colonorum*
Mogurnda adspersa	*Percalates novemaculeata*
Mogurnda mogurnda	*Philypnodon grandiceps*
Mordacia mordax	*Piaractus brachipomus*

Mordacia praecox	*Piaractus mesopotamicus*
Nannoperca australis	*Plotosus lineatus*
Nannoperca obscura	*Retropinna semoni*
Nannoperca oxleyana	*Rutilus rutilus*
Nannoperca variegata	*Salmo trutta*
Nannoperca vittata	*Syncomistes butleri*
Nematalosa erebi	*Tandanus tandanus*
Neoarius berneyi	*Tinca tinca*
Neoceratodus forsteri	*Toxotes chatareus*
Neosilurus spp.	*Toxotes jaculatrix*
Frogs	
Austrochaperina spp.	*Neobatrachus sudelli*
Cophixalus spp.	*Notaden bennettii*
Crinia spp.	*Notaden melanoscaphus*
Cyclorana spp.	*Nyctimystes dayi*
Heleioporus australiacus	*Pseudophryne bibroni*
Limnodynastes spp.	*Pseudophryne coriacea*
Litoria spp.	*Rheobatrachus silus*
Mixophyes spp.	*Taudactylus acutirostris*
Neobatrachus pictus	*Uperoleia* spp.
Turtles	
Carettochelys spp.	*Pseudemydura umbrina*
Chelodina spp.	*Rheodytes leukops*
Elseya spp.	*Trachemys scripta*
Elusor macrurus	*Wollumbinia bellii*
Emydura spp.	*Wollumbinia georgesi*
Pelochelys bibroni	*Wollumbinia latisternum*

Table 3. Primer information for *C. striata* eDNA assay (C.striata_16S). *Melting temperature determined by Geneious (ver. R11). ^Melting temperature determined by PrimerBLAST (Ye, et al., 2012).

Primer Name	Melt Temp (˚C)*	Melt Temp (˚C)^	GC Content (%)	Amplicon (bp)	Oligonucleotide (5' - 3')
Channa_16S-F	60.8	59.18	41.7	313	TAAATGAAGACCCGTATGAATGGC
Channa_16S-R	60	58.23	43.5		CTTTGCCGGATCTTTAGGTTAGA

2.3 *In vitro* Validation

Following confirmation of satisfactory *in silico* tests (see Section 2.2), C.striata_16S amplification efficiency and limit of detection (LOD) were determined using both gDNA (*in vitro* Test 1) and aDNA (*in vitro* Test 2) standard curves (ng/µL and copies/µL), respectively. Genomic DNA (gDNA) and stock artificial DNA (aDNA) were quantified (ng/µL ± 99.7% CI) by measuring each in triplicate using QuantiFluor® fluorometer with QuantiFluor® ONE dsDNA System (Promega Co., Australia). Triplicate aDNA stock measurements were averaged ± 99.7% CI and converted to copies/µL ± 99.7% CI using the average ± 99.7% CI weight (ng) and specific nucleotide sequence of synthesized aDNA (Figure 1) using an online calculator (http://www.endmemo.com/bio/dnacopynum.php).

In vitro Test 1 assessed C.straia_16S gDNA amplification efficiency and LOD using a standard curve generated by serial dilution of gDNA extracted from a frozen fillet of one *C. striata* individual using standard cetyltrimethylammonium bromide (CTAB) methodology (Gomez, et al., 2017). More specifically, extracted stock gDNA was diluted 1:100 with MilliQ water, quantified, and then serially diluted across four $\log_{10}$ steps (Standards 1 – 5: 1.29 – 1.29 x 10^{-4} ng/µL) and two $\log_2$ steps (Standards 6 - 7: 6.45 x 10^{-5} – 3.23 x 10^{-5} ng/µL).

In vitro Test 2 assessed C.straia_16S aDNA amplification efficiency and LOD using a standard curve generated by serial dilution of synthetic aDNA replica (gBlocks™, Integrated DNA Technologies Pty Ltd, Australia) of *C. striata 16S* gene region wherein C.striata_16S targets (330 bp; Figure 1). More specifically, dried aDNA pellet was resuspended in 50 µL 1x TE buffer following manufacturer's instructions (Integrated DNA Technologies Pty Ltd, Australia), which exhibited stock aDNA yield of 2.45 ± 0.33 ng/µL or 7,231,512,036 ± 974,040,397 copies/µL (± 99.7% CI). Standard 1 was generated by diluting stock aDNA 1:500 with MilliQ water (1.44 ± 0.195 x 10^7 copies/µL) followed by seven $\log_{10}$ serial dilutions (Standards 2 – 8: 1.44 ± 0.195 x 10^6 – 1.44 ± 0.195 copies/µL) and two $\log_2$ serial dilutions (Standards 9 – 10: 0.723 ± 0.097 - 0.362 ± 0.049 copies/µL). The number of *16S* copies generated from gDNA template (ng/µL) during *in vitro* Test 1 (see above) and Test 3 (see below) were determined by extrapolation from aDNA standard curve run under the same cycling conditions.

In vitro Test 3 empirically tested C.striata_16S for species-specificity by attempting to amplify gDNA template extracted from non-target but potentially co-occurring species within Australia (Table 4). More specifically, C.straita_16S was empirically tested against frogs (*n* =

5 species), turtles (n = 6 species), and fish (n = 31) known to occur in Australian freshwater systems (Table 4).

In vitro test 4 used environmental water samples from a tropical freshwater system wherein a broad diversity of non-target species are present to further verify C.striata_16S species-specificity (i.e., *in vitro in situ* Test). More specifically, 15 mL unfiltered water samples were collected from Ross River in Townsville, Queensland Australia (19° 18' 21.96" S, 146° 45' 38.52" E) wherein several empirically tested species occur (e.g., *Oreochromis mossambicus* and *Rhinella marina*; Table 4; Edmunds and Burrows, 2019 a, b) yet *C. striata* does not to occur. Ross River water samples were collected by decanting 15 mL from a 50mL LoBind® (Eppendorf Inc.) tube into each of three replicate 50 mL LoBind® falcon tubes (new water grab for each replicate) pre-loaded with 5 mL 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). Samples were transported back to MEEL at ambient temperature (≈ 24˚C) and eDNA was extracted using a novel eDNA workflow (Preserve, Precipitate, Lyse, Precipitate, Purify (PPLPP); Edmunds and Burrows, in review). Briefly, 20 mL samples were precipitated overnight (4˚C) with glycogen (final concentration (C_f) = 4.4 µg/mL), sodium chloride (C_f = 0.44M), and isopropanol (C_f = 40%) then pelleted (3,270 x g for 90 min at 20˚C; Allegra X12R centrifuge with SX4750 swinging-bucket rotor; Beckman Coulter Pty Ltd, Australia), resuspended in lysis buffer (30 mM Tris-HCl pH 8, 30 mM EDTA pH 8, 800 mM guanidium hydrochloride, 0.5% TritonX-100, pH 10; Leaver, et al., 2015), frozen (≤ -20˚C, ≥ 30 min), thawed (≥ 30 min, room temperature), incubated (50˚C, ≥ 3 hours), precipitated overnight (4˚C) with glycogen (C_f = 55.5 µg/mL) and 2 volumes polyethylene glycol (PEG) precipitation buffer (30% PEG in 1.6M NaCl), pelleted (20,000 x g for 30 min at 20˚C; 5430R centrifuge with FA-45-30-11 rotor; Eppendorf Pty Ltd, Australia), washed twice (1 mL 70% ethanol each wash), and purified of inhibitors (OneStep PCR Inhibitor Removal Kit; Zymo Research Inc., USA). Extracted eDNA was eluted in 100 µL water and split equally four-ways when transferred into 96-well plate (Axygene, Australia) to permit rapid template loading into technical qPCR replicates using Xplorer® electronic 12-channel pipette (Eppendorf Pty Ltd, Australia).

All four *in vitro* Tests assessed C.striata_16S performance using quantitative real-time polymerase chain reaction (qPCR) with 10 µL reactions containing: 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 gDNA or aDNA (see above), and 1 µL DNA-free 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 Pty Ltd, Australia) fitted with 500 µL CombiTip® (Eppendorf Pty Ltd, Australia) in a UV-sterilized PCR cabinet (Esco Pty Ltd, Australia) in the dedicated low-copy DNA room within Molecular Ecology and Evolution Laboratory (MEEL) at James Cook University (Townsville, Queensland Australia).

For *in vitro* Tests 1 and 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 gDNA template (see above) was loaded using a manual single channel LTS series P10 pipette (Mettler-Toledo Pty Ltd, Australia) fitted with Maximum Recovery filter tips (Axygene Pty Ltd, Australia). For *in vitro* Test 2 the 96-well plate containing 7 µL 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 aDNA template (see above) was loaded using a manual single channel LTS series P10 pipette fitted with Maximum Recovery filter tips. For *in vitro* Test 4 the 96-well plate containing 7 µL master mix was moved (unsealed) to a cleaned bench (wiped thoroughly with 70% ethanol) in the dedicated low-copy DNA room within MEEL where 3 µL eDNA template (see above) was loaded using a manual single channel LTS series P10 pipette fitted with Maximum Recovery filter tips. Following template loading, all qPCR plates were sealed with an optical adhesive film (Life Technologies), briefly vortexed (10 sec), pulse spun (10 sec), loaded onto opened QuantStudio3 Real-Time PCR System (Life Technologies Inc., Australia) tray, 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 run.

In vitro Tests 1 – 3 or 4 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 or 60˚C for 1 min (ramp rate = 2.7˚C/sec) before terminal dissociation curve generation by transitioning from 65˚C to 95˚C or 60˚C to 95˚C (ramp rate = 0.15˚C/sec), respectively. QuantStudio™ Design and Analysis Software (version 1.4.2; Life Technologies, Australia) was used to set the common threshold fluorescence to 0.2, analyse standard curves (for reaction efficiency and LOD determination), and export data to Microsoft Excel for subsequent ΔT_m analysis (Trujillo-Gonzalez, et al., 2019; Edmunds and Burrows, 2019 a, b, c).

Representative amplicons from gDNA standards (n = 3) that exhibited ΔT_m within 99.7% CI of gDNA standards (*in vitro* Test 1) were bidirectionally Sanger sequenced for

confirmation given unconfirmed taxonomic identification of frozen fillet from which gDNA was extracted (see above). No representative amplicons from aDNA standards (*in vitro* Tests 2) were sequenced given the known species-specificity of synthesized replica of targeted *C. striata 16S* region. For C.striata_16S *in vitro* Test 3 and 4 amplifications of non-target species that occurred across multiple technical replicates and exhibited ΔT_m inside 99.7% CI of gDNA standards were considered putative positive detections and Sanger sequenced for confirmation, otherwise detections were considered false positives (e.g., amplification in only one of two *in vitro* Test 3 replicate wells or ΔT_m outside 99.7% CI of gDNA standards).

TTGTCTTT**TAAATGAAGACCCGTATGAATGGC**ATAACGAGGGCCTAACTGTCTC
CTTTTTCAAGTCAATGAAATTGATCTCCCCGTGCAGAAGCGGGGATAAAACCAT
AAGACGAGAAGACCCTATGGAGCTTTAGACACCAGAGCAGACCTTGTTAAACAC
CCCCAAATAAAGGACAACAACCAAAAGGACCCTGCCCTAATGTCTTTGGTTGGG
GCGACCGCGGGGAACACAAAACCCCCATGTGGAATGGGAGAACTTCTCCCACA
ACCAAGAGCCACAGCTCTAAGCAATAGAATT**TCTAACCTAAAGATCCGGCAAA**
GCCGATCA

Figure 1. Artificial double stranded DNA (aDNA) replica of *C. striata 16S* gene region wherein C.striata_16S targets. Total aDNA fragment length is 330 bp. Blue nucleotides indicate the binding region for C.striata_16S-F1 and C.striata_16S-R (Table 3). Underlined regions indicate extensions beyond C.striata_16S-F1 and C.striata_16S-R binding sites included to promote efficient aDNA fragment amplification.

Table 4. Non-target species against which C.striata_16S was tested empirically. Nanograms of gDNA template loaded into duplicate wells of *in vitro* test provided within brackets. *Non-native Australian species. ^Species known to inhabit Ross River in Townsville Queensland Australia (see Sections 2.3 and 3.3).

Freshwater Fish	
Amatitlatina sp.* (1.107)	*Macquaria australasica* (1.584)
Ambassis agrammus (0.636)	*Melanotaenia splendida inornata* (0.711)
Amniataba percoides (0.45)	*Mogurnda adspersa* (0.387)
*Anabas testudineus** (1.026)	*Nematalosa erebi* (3.84)
Craterocephalus stercusmuscarum (1.221)	*Neosiluroides cooperensis* (0.702)
Giuris margaritacea (16.2)	*Neosilurus ater* (0.327)
Glossamia aprion (2.043)	*Orechromis mossambicus*^* (5.85)
*Haplochormis burtoni** (0.762)	*Oxyeleotris lineolatus* (0.759)
Hephaestus carbo (0.84)	*Philypnodon grandiceps* (5.01)
Hypseleotris compressa (8.73)	*Philypnodon macrostomus* (2.337)
Hypseleotris galii (12.78)	*Stenogobius watsoni* (1.254)

Hypseleotris sp. (0.636)	*Tandanus bellingerensis* (2.886)
Kuhlia marginata (1.083)	*Tandanus tandanus* (17.4)
Leiopotherapon unicolor (0.579)	*Tilapia mariae** (0.564)
Macquaria ambigua (0.135)	*Xiphophorus maculatus** (0.405)
Frogs/Toad	
Litoria dayi (Northern; 1.005)	*Litoria nannotis* (Paluma; 1.11)
Litoria dayi (Southern; 1.755)	*Litoria rheocola* (2.92)
Litoria jungguy (3.63)	*Litoria serrata* (4.92)
Litoria lorica (Northern; 3.72)	*Litoria wilcoxii* (3.33)
Litoria lorica (Southern; 2.289)	*Litoria xanthomera* (6.30)
Litoria nannotis (Northern; 1.89)	*Rhinella marina*^ (0.35)
Freshwater Turtles	
Chelodina canni^ (1.791)	*Emydura subglobosa worrelli* (0.741)
Chelodina oblonga (0.669)	*Myuchelys latisternum* (1.134)
Elseya lavarackorum (1.449)	*Rheodytes leukops* (1.248)

3. Results

3.1 Primer design

Based on *16S* sequence alignments of *C. striata* with Australian fish (Table 2) the genus *Piaractus* was most similar. Primer binding regions were designed to possess ≥ 5 mismatches to *Piaractus* species (see Section 3.2).

Optimal forward and reverse primer binding regions were identified between base pairs 131-154 (C.striata_16S-F) and 449-475 (C.striata_16S-R) of *C. striata 16S*, respectively (Table 3). C.striata_16S-F exhibited the following characteristics: 1) T_m = 59.2 – 60.8, 2) GC content = 41.7%, 3) length = 24 bp, 4) self-dimer T_m = 0˚C, 5) hairpin T_m = 0˚C, 6) self-complementarity score = 2, and 7) 3’ self-complementarity score = 2. C.striata_16S-R exhibited the following characteristics: 1) T_m = 58.2 – 60.0, 2) GC content = 43.5%, 3) length = 23 bp, 4) self-dimer T_m = 10.4˚C, 5) hairpin T_m = 0˚C, 6) self-complementarity score = 4, and 7) 3’ self-complementarity score = 1.

3.2 *In silico* Validation

Initial targeted *in silico* PrimerBLAST search of NCBI "nr" database (see Section 2.2) confirmed that C.striata_16S targets (i.e., no mismatches) C. striata *16S* as well as *C. asiatica*, *C. diplogramme*, *C. melasoma*, and *C. micropeltes* (**Table 5**). Moreover, C.striata_16S only exhibits potential cross-amplification (i.e., ≤ 5 base pair mismatches) of other *Channa* spp. (*n* = 9), none of which are known to be present within Australian freshwater systems (**Table 5**). Subsequent non-targeted *in silico* PrimerBLAST test revealed that C.striata_16S could potentially cross-amplify (i.e., 2 base pair mismatches) *Lophiogobius ocellicauda* (Table 6). However, this goby species is endangered and endemic to China and South Korea with no confirmed sightings in Australia.

Table 5*. Species with ≤ 5 mismatches to C.striata_16S based on targeted PrimerBLAST search against all available* 16S *nucleotide sequences in GenBank (NCBI) "nr" database for freshwater fish, frogs, and turtles known to occur within Australia. *Target species.*

Forward Mismatches	Reverse Mismatches	*Species*
0	0	*Channa asiatica*
		Channa diplogramme
		Channa melasoma
		Channa micropeltes
		*Channa striata**
0	1	*Channa asiatica*
		Channa aurantimaculata
		Channa bankanensis
		Channa bleheri
		Channa gachua
		Channa lucius
		Channa stewartii
1	0	*Channa orientalis*
0	2	*Channa asiatica*
		Channa barca
0	3	*Channa argus*
		Channa lucius
1	2	*Channa gachua*
		Channa lucius
		Channa sp. CIARI/FF02
		Channa sp. PJ-2016
0	4	*Channa argus*
		Channa maculata

		Channa maculata x Channa argus
2	2	*Channa gachua*
0	5	*Channa punctata*

Table 6. Species with ≤ 2 mismatches to C.striata_16S based on non-targeted PrimerBLAST search against all available 16S *nucleotide sequences in GenBank (NCBI) "nr" database. *Target species.*

Forward Mismatches	**Reverse Mismatches**	***Species***
0	0	*Channa asiatica*
		Channa diplogramme
		Channa melasoma
		Channa micropeltes
		*Channa striata**
0	1	*Channa aurantimaculata*
		Channa bankanensis
		Channa bleheri
		Channa gachua
		Channa lucius
		Channa stewartii
1	0	*Channa orientalis*
1	1	*Channa barca*
		Lophiogobius ocellicauda

3.3 *In vitro* Validation

C.striata_16S exhibited satisfactory efficiency and LOD based on gDNA and aDNA standard curves run at 65˚C with 600nM each primer (see Section 2.3).

In vitro Test 1 demonstrated that C.striata_16S amplified the first 6-points of $\log_{10}$ gDNA standard curve with 94.69% efficiency ($R^2 = 0.980$) and no primer dimerization (Figure 2). This 6-point gDNA standard curve demonstrated the LOD of C.striata_16S to be 1.47×10^{-5} ng/µL or 1 copy/µL based on gDNA or aDNA standard curve extrapolations, respectively. Standard curve included five replicates for Standard 1, six technical replicates for Standards 2 – 6, and no replicates for Standard 7. Based on gDNA standard curve extrapolation the one excluded replicate from Standard 1 contained 3.8×10^{-1} ng/µL (instead of 1.29 ng/µL) while the three excluded Standard 7 replicates contained 1.06×10^{-4}, 1.13×10^{-4}, and 1.47×10^{-5} ng/µL (instead of 3.23×10^{-5} ng/µL). Sanger sequences of representative amplicons from

gDNA standard curve (258 – 276 bp) were confirmed positive given 100% pairwise identity with *C. striata 16S* nucleotide sequence (GenBank accession KC200558).

In vitro Test 2 demonstrated that C.striata_16S amplified aDNA standard curve (see Section 2.3) with 94.09% efficiency ($R^2 = 0.985$) and no primer dimerization with an LOD of 2 ± 1 copies loaded (Figure 3). Note that all three technical replicates for Standard 5 (4,338.9 ± 584.42 copies loaded) and Standard 7 (43.4 ± 58.4 copies loaded) amplified but only one technical replicate was included in standard curve for each because it amplified in linear order while other two replicates were extrapolated (1,327.4 ± 47.1 and 53.4 ± 184.3 copies loaded), respectively. Only one of five technical replicates amplified for Standard 9 (2.17 ± 0.29 copies loaded) within expected linear order so it was included in aDNA standard curve (Figure 2). Two of five technical replicates amplified for Standard 10 (1.09 ± 0.15 copies loaded) but were outside expected linear order (e.g., poor mixing or pipetting error) so both replicates were excluded from aDNA standard curve and extrapolated instead (5.49 and 6.69 copies loaded or 5 - 6x more than expected).

In vitro Test 3 revealed potential cross-amplification of one non-target Australian fish (*Neosiluroides cooperensis*) with C.striata_16S (Figure 4). However, this *N. cooperensis* amplification was considered false positive without Sanger sequence verification because only one of two replicate reactions exhibited amplification (Figure 4A) and ΔT_m analysis was outside 99.7% CI of gDNA standards (Figure 4B; see Section 2.3). Moreover, similar amplifications were observed during *in vitro* validation for other invasive species eDNA assays and confirmed to be false detections via Sanger sequencing (Edmunds and Burrows, 2019a, b, c).

In vitro Test 4 confirmed the absence of *C. striata* eDNA in unfiltered Ross River water samples screened under less stringent cycling conditions (60˚C, 500nM each primer; see Section 2.3) despite the known presence of *Oreochromis mossambicus* and *Rhinella marina* within these same Ross River water samples (Edmunds and Burrows, 2019 a and b), respectively. The lack of *C. striata* detection in Ross River water samples (Figure 5) confirms C.striata_16S species-specificity demonstrated by *in vitro* Test 3 (Table 4; Figure 4). Moreover, the lack of *C. striata* amplification from Ross River eDNA samples was not due to suboptimal assay performance under these less stringent qPCR conditions used (see Section 2.3) but rather confirms C.striata_16S species-specificity given that several empirically tested species (Table 4) do occur within Ross River (Edmunds and Burrows, 2019 a, b). *In situ in vitro* confirmation of C.striata_16S species-specificity using Ross River eDNA samples was

not re-run under optimal conditions (65˚C with 600nM each primer) given higher stringency associated with these cycling conditions (no amplification expected at 65˚C if no amplification was observed at 60˚C).

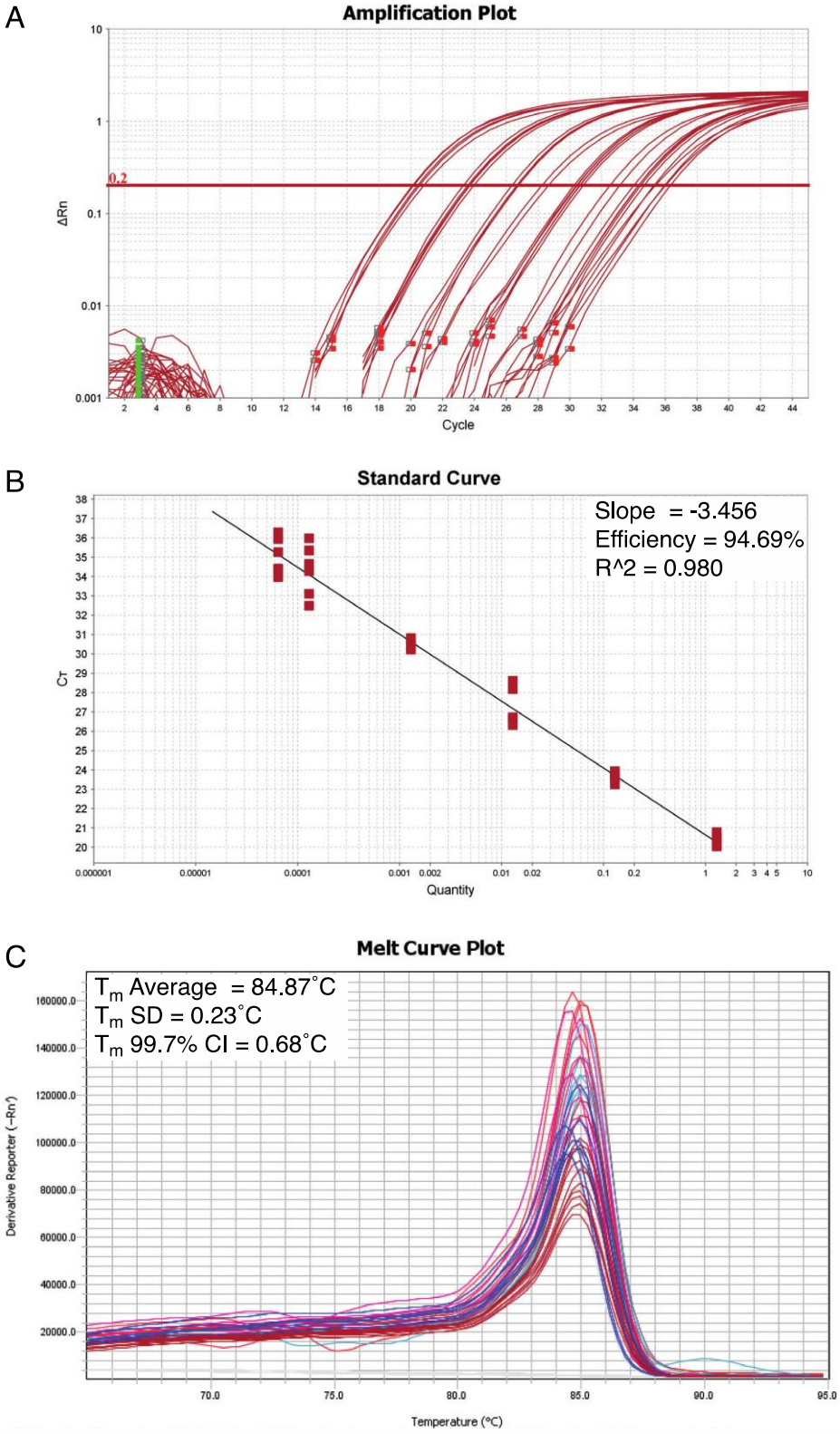

Figure 2. Amplification curves (A), linear regression of gDNA standard curve (B), and amplicon dissociation temperature curves (T_m; C) generated by qPCR during C.striata_16S *in vitro* Test 1 (65˚C, 600nM each primer; see Section 2.3).

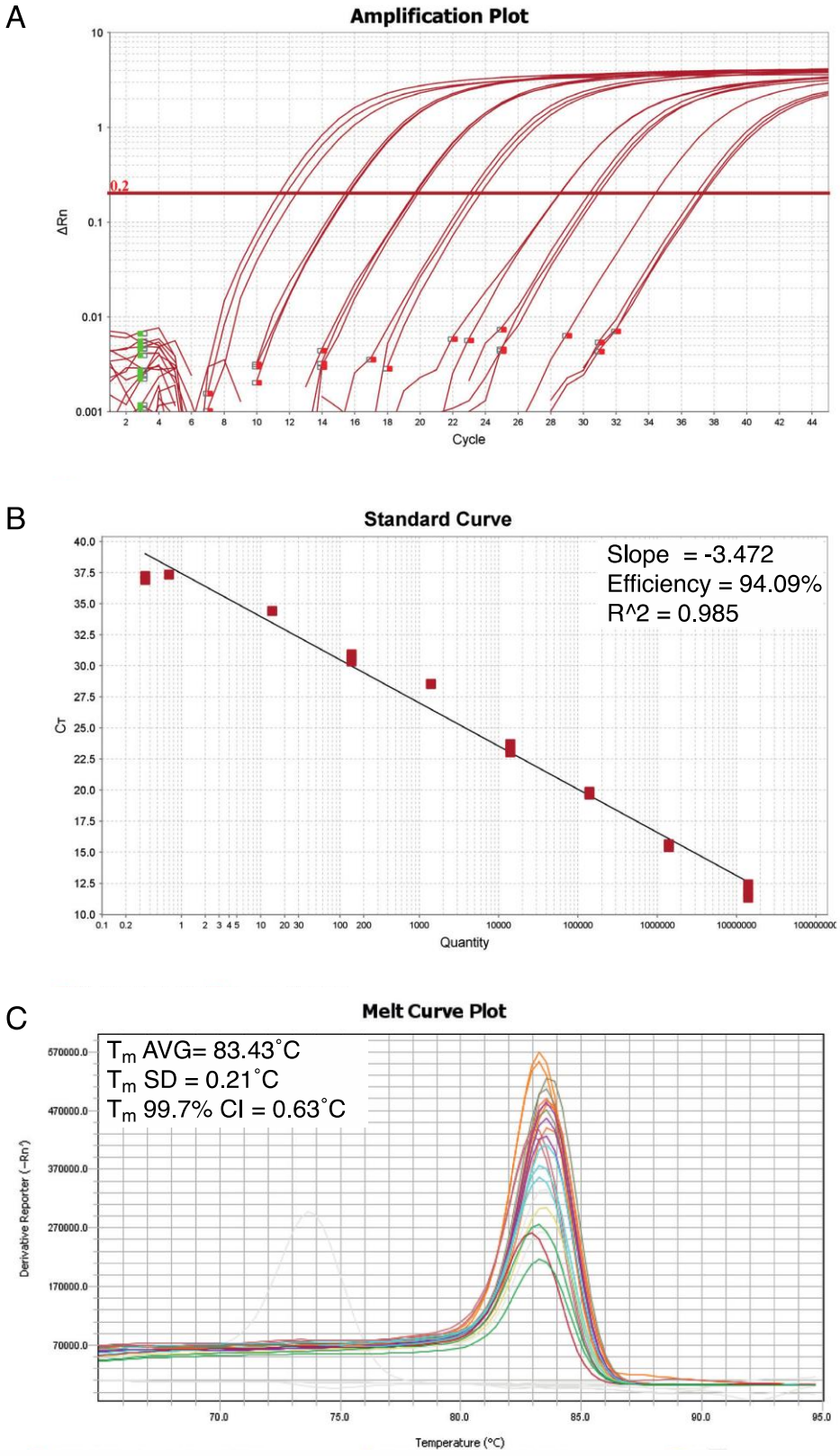

Figure 3. Amplification curves (A), linear regression of aDNA standard curve (B), and amplicon dissociation temperature curves (T_m; C) generated by qPCR during C.striata_16S *in vitro* Test 2 (65˚C, 600nM each primer; see Section 2.3).

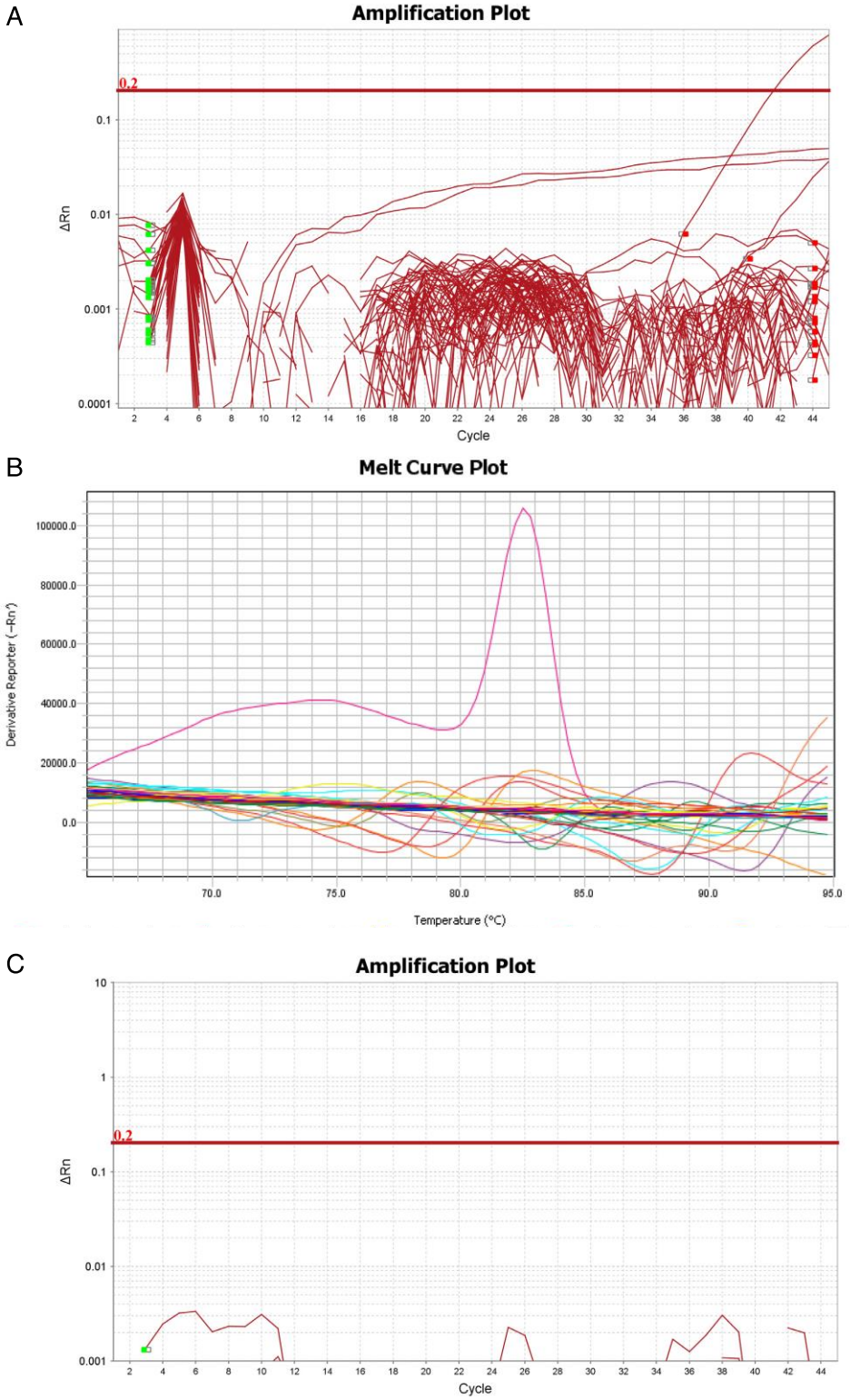

Figure 4. Amplification curves (A) and amplicon dissociation curves (T_m; B) from C.striata_16S *in vitro* Test 3 (see Section 2.3; Table 4). Note that *Neosiluroides cooperensis* amplification was only observed in one of two replicate reactions and that T_m of generated amplicon (82.49˚C) was outside 99.7% CI of *C. striata* gDNA standards (84.87 ± 0.68˚C; Figure 2). Moreover, *N. cooperensis* did not amplify during previous C.striata_16S *in vitro* test conducted at 60˚C with 500nM primers (C). Taken together, observed *N. cooperensis* amplification is considered false positive due to cross-contamination of *N. cooperensis* gDNA with *C. striata* gDNA, which has been previously observed during *in vitro* validations (see Section 3.3).

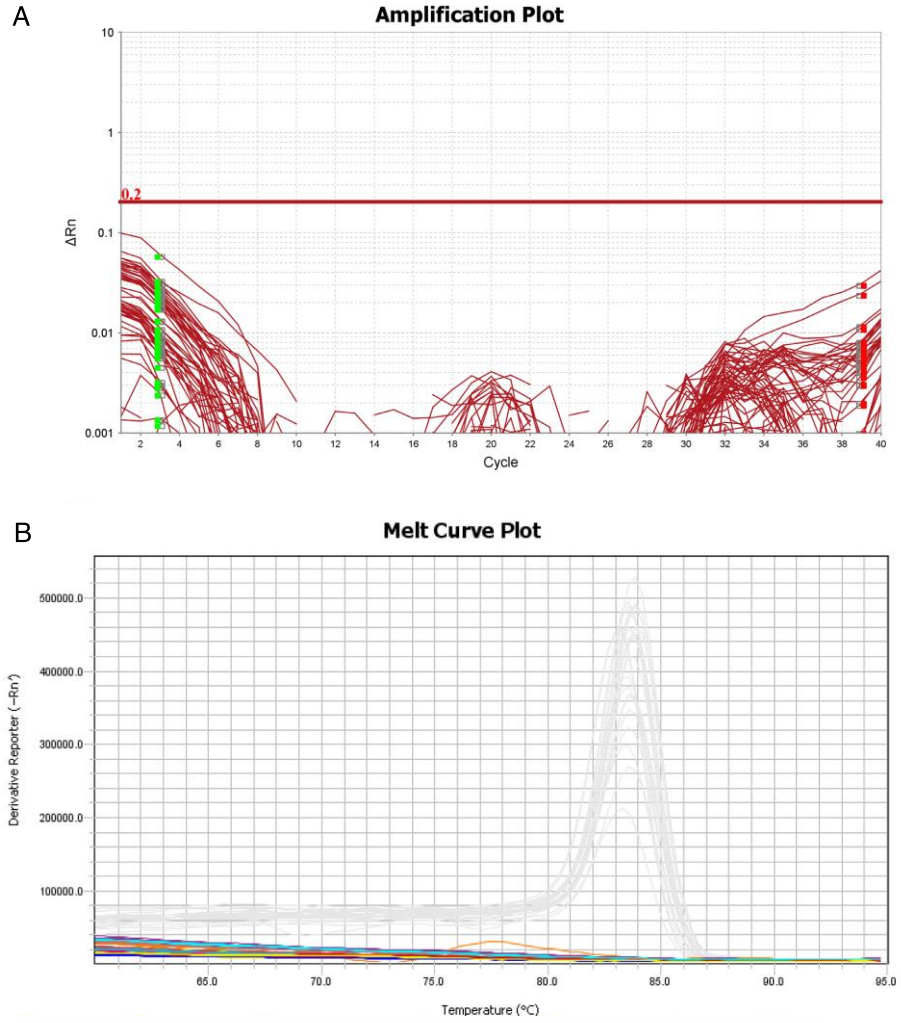

Figure 5. Expected lack of amplification (A) and associated lack of amplicon dissociation curves (B) generated during C.striata_16S *in situ* confirmation of species-specificity using unfiltered Ross River water samples (see Section 2.3 and 3.3). Note that this *in situ* confirmation was run under less stringent conditions (60˚C, 500nM each primer) than C.striata_16S *in vitro* Tests 1 – 3 (see Sections 2.3 and 3.3).

4. Discussion

The *in silico* and *in vitro* validations undertaken during C.striata_16S development demonstrate readiness for screening environmental water samples for *C. striata* eDNA (i.e., *in situ* validation).

Given the pre-emptive development of C.striata_16S, the first field application will effectively provide *in situ* validation given that no freshwater systems within Australia currently harbour *C. striat*a; however, northern Torres Straight islands are considered to be of high invasion risk. Despite the lack of *in situ* validation, which for this species is not available within Australia, C.striata_16S it is considered suitable for invasion front monitoring (i.e.,

screening water samples from locations wherein *C. striata* is suspected to have invaded) given satisfactory gDNA amplification efficiency and LOD (Figure 2). Future screening of environmental water samples for *C. striata* eDNA should utilize optimal cycling conditions (65˚C, 600nM each primer) to ensure non-target species are not cross-amplified (see Section 2.3).

Regardless of assay amplification efficiency and LOD, failure to detect eDNA from target species cannot be interpreted as conclusive evidence of target species absence (Roussel, et al., 2015). Accordingly, *C. striata* eDNA is not detected in 15 mL unfiltered environmental water samples collected during initial monitoring of high invasion risk waterbodies (i.e., invasion front) then routine eDNA monitoring is recommended. Following *C. striata* detection, C.striata_16S can be utilized to monitor eradication efforts (Edmunds, et al., 2019). Moreover, unfiltered water samples can be increased to 30 mL (plus 10 mL Longmire's for preservation) if low *C. striata* eDNA abundance is anticipated (Edmunds, et al., 2019).

Lastly, C.striata_16S should be used with caution in global regions wherein other *Channa* species are known to occur (e.g., Asia) given co-amplification of *C. asiatica*, *C. diplogramme*, *C. melasoma*, and *C. micropeltes* (i.e., no mismatches; Table 5) and potential amplification of *Lophiogobius ocellicauda* (i.e., 2 mismatches; Table 6). Given the length of C.striata_16S amplicon (≈ 315 bp), ΔT_m analysis (Trujillo-Gonzalez, et al., 2019, Edmunds and Burrows, in review) should provide accurate discrimination between *C. striata* amplification and *C. asiatica*, *C. diplogramme*, *C. melasoma*, *C. micropeltes*, or *L. ocellicauda* detection; however, we recommend empirical testing and Sanger sequencing confirmation before C.striata_16S is utilized to detect *C. striata* eDNA in environmental water samples collected within Asia.

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