Northern Australia Environmental Resources Hub

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Development of an eDNA assay for cane toad (*Rhinella marina*)

Report

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Cover photographs

Front cover: Cane toad *Rhinella marina* (photo: Peter Yeeles/Shutterstock.com).

Back cover: Cane toad *Rhinella marina* (photo: Cecilia Villacorta-Rath).

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Acronyms

BLAST..... Basic Local Alignment Search Tool

NESP..... National Environmental Science Program

Abbreviations

aDNA..... Artificial deoxyribonucleic acid

bp ..... Base pair

C_f ..... Final concentration

C_t ..... Threshold cycle

CI ..... Confidence interval

eDNA..... Environmental deoxyribonucleic acid

EDTA..... Ethylenediaminetetraacetic acid

gDNA..... Genomic deoxyribonucleic acid

IDT..... Integrated DNA Technologies

LOD ..... Limit of detection

MEEL..... Molecular Ecology and Evolution Laboratory

NaCl..... Sodium chloride

NCBI..... National Center for Biotechnology Information

qPCR..... Quantitative polyermerase chain reaction

SDS ..... Sodium dodecyl sulfate

TE ..... Trisaminomethane and ethylenediaminetetraacetic acid

T_m..... Melting temperature

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Executive summary

An environmental DNA (eDNA) assay (R.marina_16S) was developed for detection of invasive cane toads (*Rhinella marina*) in water samples using Sybr-based quantitative real-time polymerase chain reaction (qPCR). R.marina_16S targets 290 base pair (bp) region within mitochondrial *16S* gene that is unique to *R. marina* within Australia. Here we present *in silico*, *in vitro*, and *in situ* validations undertaken during R.marina_16S development.

In silico, *in vitro* and, *in situ* validations confirmed that R.marina_16S is specific to (i.e., detects only) *R. marina* within Australian waterways despite co-occurrence with native species. Amplification efficiencies for R.marina_16S using gDNA and aDNA template were 93.3% and 93.8% with limits of detection (LOD) of 1.80 x 10^{-4} nanograms and 9 copies loaded under optimal assay conditions (65˚C annealing temperature, 250 nM each primer), respectively. Bidirectional Sanger sequencing confirmed *in vitro* and *in situ* validations to be positive for *R. marina* (gDNA standards and Ross River water samples, respectively) while all non-target species amplifications observed during *in vitro* validation were confirmed negative (i.e., false positives). Collectively, these validations demonstrate the readiness of R.marina_16S for screening environmental water samples to detect low concentrations of *R. marina* eDNA.

In an effort to advance *R. marina* invasion front monitoring within Australia R.marina_16S was developed to permit cost-effective (Sybr) yet sensitive (≥ 9 copies loaded) eDNA detection in waterbodies known or suspected to contain cane toads. Moreover, the larger R.marina_16S amplicon (290 bp) permits direct Sanger sequencing for verification of putative positive detections.

Detection of *R. marina* eDNA within spatial and/or time-course water samples can help guide or monitor eradication efforts or range expansions. Invasive species management stands to benefit from the incorporation of eDNA into ongoing visual monitoring programs.

1. Introduction

Cane toads (*Rhinella marina*), a species native to Latin America, were introduced to north-eastern Australia in 1935 in an attempt to biologically control harmful sugarcane pest insects (Turvey, 2013; Tingley, et al., 2017). Since their introduction in 1935, *R. marina* has spread across >1.2 million km^2 of tropical and subtropical Australia (Urban, et al., 2008) and has had devastating impacts on native biodiversity, especially large anuran-eating predators (e.g., marsupial quolls, freshwater crocodiles, varanid and scincid lizards, and elapid snakes) due to their lethal endogenous toxin (Lentic, et al., 2008; Doody, et al., 2009; Shine, 2010; Jolly, et al., 2015; Fukuda, et al., 2016). The spread of *R. marina* throughout tropical and subtropical Australia has continued despite >$20 million invested by the Australian government between 1986 and 2009 toward eradication efforts (Commonwealth of Australia, 2011).

Initially, the *R. marina* invasion front was controlled by manually collecting and removing adult toads; however, this approach proved insufficient to stop the rapid spreading. More recently, a variety of molecular genetic approaches have been considered, which included: eradication by virally vectored autoimmune system disruption (Robinson, et al., 2006; Hyatt, et al., 2008; Pallister, et al., 2008; Pallister, et al., 2011), viral- and RNAi-based gene deletion (Shanmuganathan, et al., 2010), and artificial sex-biasing (Koopman, 2006; Mahony and Clulow, 2006); however, none of these molecular genetic approaches have been implemented due to both technical obstacles and ethical concerns about unintended or indirect consequences (Hyatt, et al., 2008; Shannon, et al., 2008). Instead, the Australian Commonwealth decided in 2011 to focus on protecting key biodiversity assets from the ever-expanding *R. marina* invasion front (e.g., offshore islands, native species, ecological communities; Commonwealth of Australia, 2011).

In order to achieve accurate monitoring a sensitive *R. marina* detection method 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 *R. marina* in any water source known or suspected to have been utilized by *R. marina* adults, juveniles, or tadpoles. Here we describe the development of the sensitive eDNA assay that can be used to detect *R. marina* DNA isolated from environmental water samples.

2. Methodology

2.1 Primer design

GenBank (NCBI) was mined for available *16S* nucleotide sequences from Australian frog species (*n* = 172; 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 (Goldberg, et al., 2016). However, seven species of interest (i.e., potentially co-occurring) did not have *16S* sequence(s) available on GenBank as of 17 April 2018 (*Crinia bilingual, Crinia remota, Litoria daviesae, Platyplectrum ornatum, Pseudophryne major, Uperoleia inundata,* and *Uperoleia lithomoda*). All available *16S* sequences were downloaded into Geneious analysis software (version R11) and subsequently aligned using the embedded ClustalW algorithm. Regions of conservation within alignment of *R. marina 16S* sequences (*n* = 27) were identified, annotated, and then assessed by eye for regions wherein *R. marina* exhibited ≥ 3 base pair mismatches with Australian frogs 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 frog species for which mitochonrial 16S nucleotide sequences were obtained from GenBank (NCBI) and used to guide R.marina_16S assay development.

Non-target Australian frog species		
Adelotus brevis	*Hylarana lepus*	*Litoria gracilenta*
Austrochaperina adelphe	*Hylarana luctuosa*	*Litoria inermis*
Austrochaperina adelphe	*Hylarana macrodactyla*	*Litoria infrafrenata*
Austrochaperina derongo	*Hylarana macrops*	*Litoria jervisiensis*
Austrochaperina guttata	*Hylarana magna*	*Litoria jungguy*
Austrochaperina palmipes	*Hylarana malabarica*	*Litoria latopalmata*
Austrochaperina rivularis	*Hylarana mangyanum*	*Litoria lesueurii*
Cophixalus crepitans	*Hylarana megalonesa*	*Litoria longirostris*
Cophixalus hosmeri	*Hylarana mocquardi*	*Litoria meiriana*
Cophixalus infacetus	*Hylarana moellendorffi*	*Litoria microbelos*
Cophixalus kulakula	*Hylarana montana*	*Litoria nannotis*
Cophixalus ornatus	*Hylarana nicobariensis*	*Litoria nasuta*
Cophixalus pakayakulangun	*Hylarana nigrovittata*	*Litoria nigrofrenata*
Cophixalus sp. *"Cape Melville"*	*Hylarana papua*	*Litoria nyakalensis*
Crinia deserticola	*Hylarana picturata*	*Litoria olongburensis*
Crinia parinsignifera	*Hylarana raniceps*	*Litoria pallida*
Crinia signifera	*Hylarana serendipi*	*Litoria pearsoniana*
Cyclorana alboguttata	*Hylarana siberu*	*Litoria peronii*
Cyclorana australis	*Hylarana signata*	*Litoria personata*
Cyclorana brevipes	*Hylarana similis*	*Litoria phyllochroa*
Cyclorana cryptotis	*Hylarana spinulosa*	*Litoria raniformis*

Cyclorana cultripes	*Hylarana sreeni*	*Litoria revelata*
Cyclorana longipes	*Hylarana taipehensis*	*Litoria rheocola*
Cyclorana maculosa	*Hylarana temporalis*	*Litoria rothii*
Cyclorana maini	*Hylarana urbis*	*Litoria rubella*
Cyclorana manya	*Limnodynastes convexiusculus*	*Litoria serrata*
Cyclorana novaehollandiae	*Limnodynastes dorsalis*	*Litoria subglandulosa*
Cyclorana verrucosa	*Limnodynastes dumerilii*	*Litoria tornieri*
Heleioporus australiacus	*Limnodynastes dumerilii dumerilii*	*Litoria tyleri*
Hylarana albolabris	*Limnodynastes dumerilii fryi*	*Litoria verreauxii*
Hylarana amnicola	*Limnodynastes dumerilii grayi*	*Litoria watjulumensis*
Hylarana aurantiaca	*Limnodynastes dumerilii insularis*	*Litoria wilcoxii*
Hylarana banjarana	*Limnodynastes dumerilii variegatus*	*Litoria xanthomera*
Hylarana baramica	*Limnodynastes fletcheri*	*Mixophyes carbinensis*
Hylarana caesari	*Limnodynastes lignarius*	*Mixophyes coggeri*
Hylarana cf. danieli	*Limnodynastes peronii*	*Mixophyes fasciolatus*
Hylarana cf. humeralis	*Limnodynastes salmini*	*Mixophyes schevilli*
Hylarana cf. leptoglossa	*Limnodynastes tasmaniensis*	*Neobatrachus pictus*
Hylarana cf. siberu	*Limnodynastes terraereginae*	*Neobatrachus sudelli*
Hylarana cf. taipehensis	*Litoria andiirrmalin*	*Notaden bennettii*
Hylarana cf. tytleri	*Litoria auae*	*Notaden melanoscaphus*
Hylarana chalconota	*Litoria aurea*	*Notaden melanoscaphus*
Hylarana doni	*Litoria barringtonensis*	*Nyctimystes dayi*
Hylarana erythraea	*Litoria bicolor*	*Pseudophryne bibroni*
Hylarana flavescens	*Litoria booroolongensis*	*Pseudophryne coriacea*
Hylarana galamensis	*Litoria brevipalmata*	*Rheobatrachus silus*
Hylarana glandulosa	*Litoria caerulea*	*Taudactylus acutirostris*
Hylarana gracilis	*Litoria chloris*	*Uperoleia altissima*
Hylarana grandocula	*Litoria citropa*	*Uperoleia fusca*
Hylarana guentheri	*Litoria coplandi*	*Uperoleia laevigata*
Hylarana hekouensis	*Litoria dahlii*	*Uperoleia littlejohni*
Hylarana indica	*Litoria dentata*	*Uperoleia martini*
Hylarana intermedia	*Litoria electrica*	*Uperoleia mimula*
Hylarana krefftii	*Litoria eucnemis*	*Uperoleia rugosa*
Hylarana labialis	*Litoria ewingii*	*Uperoleia stridera*
Hylarana laterimaculata	*Litoria fallax*	*Uperoleia trachyderma*
Hylarana latouchii	*Litoria freycineti*	
Hylarana leptoglossa	*Litoria genimaculata*	

2.2 *In silico* validation

Following design of forward (R.marina_16S-F) and reverse (R.marina_16S-R) primers, the *R. marina 16S* assay (hereafter referred to as R.marina_16S) 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 R.marina_16S was tested *in silico* (Table 2). For this targeted search, species with ≤ 5 base pair (bp) mismatches to

R.marina_16S were documented (see Section 3.2). Subsequent *in silico* test used non-targeted PrimerBLAST (i.e., no species specified) to test R.marina_16S against all species with nucleotide sequences deposited in NCBI "nr" database in order to ascertain which species are expected to amplify if present. For this non-targeted search, species with ≤ 1 bp mismatch to R.marina_16S were documented (see Section 3.2).

Following satisfactory compliance of R.marina_16S with *in silico* tests (e.g., targeted PrimerBLAST search returning no species with < 3 bp mismatches), 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 R.marina_16S was tested in silico *using targeted PrimerBLAST search of human and Australian fish, frog, and turtle* 16S *sequences present within NCBI "nr" database.*

Australian freshwater fish		
Amatitlania nigrofasciata	*Geotria australis*	*Nannoperca variegata*
Albula forsteri	*Glossamia aprion*	*Nannoperca vittata*
Albula oligolepis	*Hephaestus carbo*	*Nematalosa erebi*
Ambassis agrammus	*Hephaestus fuliginosus*	*Neoarius berneyi*
Ambassis marianus	*Homo sapiens*	*Neoceratodus forsteri*
Amphilophus citrinellus	*Hypseleotris compressa*	*Neosilurus* spp.
Anabas testudineus	*Kuhlia marginata*	*Neosilurus ater*
Anguilla australis	*Kuhlia rupestris*	*Neosilurus pseudospinosus*
Anguilla bicolor	*Lates calcarifer*	*Oncorhynchus mykiss*
Anguilla obscura	*Leiopotherapon unicolor*	*Oreochromis mossambicus*
Anguilla reinhardtii	*Lepidogalaxias salamandroides*	*Oreochromis niloticus*
Arius berneyi	*Maccullochella ikei*	*Perca fluviatilis*
Carassius auratus	*Maccullochella macquariensis*	*Percalates colonorum*
Channa spp.	*Maccullochella mariensis*	*Percalates novemaculeata*
Craterocephalus stercusmuscarum	*Maccullochella peelii*	*Philypnodon grandiceps*
Cyprinus carpio	*Macquaria ambigua*	*Philypnodon macrostomus*
Eptatretus spp.	*Macquaria australasica*	*Piaractus brachipomus*
Eptatretus cirrhatus	*Macquaria colonorum*	*Piaractus mesopotamicus*
Eptatretus longipinnis	*Macquaria novemaculeata*	*Plotosus lineatus*
Gadopsis marmoratus	*Megalops cyprinoides*	*Retropinna semoni*
Galaxias brevipinnis	*Melanotaenia fluviatilis*	*Rutilus rutilus*
Galaxias fuscus	*Melanotaenia splendida*	*Salmo trutta*
Galaxias maculatus	*Mogurnda adspersa*	*Syncomistes butleri*
Galaxias parvus	*Mogurnda mogurnda*	*Tandanus tandanus*
Galaxias zebratus	*Mordacia mordax*	*Tilapia mariae*
Galaxiella munda	*Mordacia praecox*	*Tinca tinca*
Galaxiella nigrostriata	*Nannoperca australis*	*Toxotes chatareus*
Galaxiella pusilla	*Nannoperca obscura*	*Toxotes jaculatrix*
Gambusia holbrooki	*Nannoperca oxleyana*	
Australian frogs		
Austrochaperina spp.	*Litoria* spp.	*Nyctimystes dayi*
Cophixalus spp.	*Mixophyes* spp.	*Pseudophryne bibroni*

Crinia spp.	*Neobatrachus pictus*	*Pseudophryne coriacea*
Cyclorana spp.	*Neobatrachus sudelli*	*Rheobatrachus silus*
Heleioporus australiacus	*Notaden bennettii*	*Taudactylus acutirostris*
Limnodynastes spp.	*Notaden melanoscaphus*	*Uperoleia* spp.
Australian freshwater turtles		
Carettochelys spp.	*Emydura* spp.	*Trachemys scripta*
Chelodina spp.	*Pelochelys bibroni*	*Wollumbinia bellii*
Elseya spp.	*Pseudemydura umbrina*	*Wollumbinia georgesi*
Elusor macrurus	*Rheodytes leukops*	*Wollumbinia latisternum*

Table 3. Primer information for R. marina eDNA assay (R.marina_16S). Asterisk (*) and highhat (^) indicate melting temperature as determined by Geneious (ver. R11) and PrimerBLAST (Ye, et al., 2012), respectively.

Primer name	Melt temp (˚C)*	Melt temp (˚C)^	GC content (%)	Amplicon (bp)	Oligonucleotide (5'–3')
R.marina_16S-F	64.4	62.28	63.2	290	AGCCTGCCCAGTGACCATG
R.marina_16S-R	62	60.04	55		TGTTATGCTCCGTGGTCACC

2.3 *In vitro* validation

Following confirmation of satisfactory *in silico* tests (see Section 2.2), R.marina_16S was tested empirically for species-specificity by attempting to amplify genomic DNA (gDNA) template extracted from non-target but potentially co-occurring species. More specifically, R.marina_16S was tested *in vitro* for cross-amplification of 5 species of Australian rainforest frogs, 6 species of Australian freshwater turtles, and 31 species of Australian freshwater fishes (Table 4).

In vitro tests included verification of R.marina_16S amplification efficiency and limit of detection (LOD) as nanograms of gDNA per reaction (ng loaded) and copies of artificial *R. marina 16S* double stranded DNA (317 bp; aDNA; gBlocks™, IDT Australia; Figure 1) per reaction (copies loaded). For precise quantification of equally pooled gDNA and stock aDNA (ng/µL ± 99.7% CI) each was measured in duplicate using QuantiFluor® fluorometer with QuantiFluor® ONE dsDNA System (Promega Co., Australia). Duplicate aDNA stock measurements were averaged ± 99.7% CI and converted to copies/µL ± 99.7% CI using the average ± 99.7% CI weights (ng) and specific nucleotide sequence of synthesized aDNA (Figure 1) using 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 21 individuals (n = 15 from Darwin NT and n = 6 from Townsville QLD). Neat gDNA of each individual (10.7–382.7 ng/µL, NanoDrop™; Life Technology Australia Ltd. Pty.) was diluted 1:10, 1:20, or 1:100 with MilliQ® water (depending on starting concentration) to normalize working gDNA stock to 1.1–7.2 ng/µL. Diluted gDNA was then pooled equally across all 21 individuals to generate Standard 1 ($6 \pm 0.1 \times 10^{-1}$ ng/µL), which was then serially diluted ($\log_{10}$) seven times to generate Standards 2–8 ($6 \pm 0.1 \times 10^{-2}$ – $6 \pm 0.1 \times 10^{-8}$ ng/µL), respectively.

To test assay efficiency and LOD using aDNA a 8-step serial dilution standard curve was generated by resuspending the dried pellet of *R. marina 16S* aDNA fragment (Figure 1) in 50µL of 1x TE buffer (IDT Australia) following manufacturer's instructions, which yielded a stock aDNA concentration of 4.92 ± 1.19 ng/µL or 15,118,550,489 ± 3,656,722,578 copies/µL. Stock aDNA was then diluted 1:500 with MilliQ® water to generate Standard 1 ($3.02 \pm 0.731 \times 10^7$ copies/µL), which was then serially diluted (log_{10}) seven times to generate Standards 2–8 ($3.02 \pm 0.731 \times 10^6$ – 3.02 ± 0.731 copies/µL), 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.

All four *in vitro* tests were assessed using qPCR: 1) determination of R.marina_16S efficiency and LOD using 8-step log_{10} gDNA standard curve, 2) determination of R.marina_16S efficiency and LOD using 8-step log_{10} aDNA standard curve, 3) determination of R.marina_16S amplification of gDNA extracted from NT and QLD individuals (see above), and 4) determination of R.marina_16S cross-amplification of gDNA from co-occurring non-target species (Table 4). *In vitro* Tests 1–4 were run as 10 µL reactions containing the following: 5 µL PowerUP® Sybr Green Master Mix (Thermo Fisher Scientific, Australia), 0.5 µL forward primer (5 µM, 250 nM final), 0.5 µL reverse primer (5 µM, 250 nM final), 3 µL *R. marina* gDNA (Test 1) or 3 µL *R. marina* aDNA (Test 2) or 3 µL gDNA from each NT and QLD individuals (Test 3) or 3 µL gDNA of non-target species (Test 4; see Table 4), 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 Multipette® Xstream electronic dispensing 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 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 room temperature pooled gDNA standard (1.8 ± 0.3 – $1.8 \pm 0.3 \times 10^{-7}$ ng loaded) was loaded using a manual single channel P10 pipette (Eppendorf, Australia) fitted with Maximum Recovery filter tips (Axygene, Australia).

For *in vitro* Test 2 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 ($9.07 \pm 2.193 \times 10^7$ – 9.07 ± 2.193 copies loaded) was loaded as described above for *in vitro* Test 1.

For *in vitro* Tests 3 and 4 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 NT and QLD individual (3.3–1.6 ng loaded) and gDNA template of each non-target species (Table 4) was loaded as described above for *in vitro* Test 1, respectively.

Following DNA loading, plates were sealed with an optical adhesive film (Life Technologies Australia Ltd. Pty.), 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 four *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 and 4 (target and non-target gDNA amplification, respectively) were 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 following each discretely run *in vitro* test.

Representative amplicons from gDNA standard 3 (n = 3) that exhibited T_m within 99.7% confidence internal (CI) of standards (Test 1) were bidirectionally Sanger sequenced for confirmation. No representative amplicons that exhibited T_m within 99.7% confidence internal (CI) of standards Tests 2 or 3 (aDNA standards or cane toad populations gDNA, respectively) were sequenced as these were considered to be redundant with Test 1 representatives sequenced. Single amplification products produced from R.marina_16S in vitro Test 4 (non-target species) that exhibited T_m inside 99.7% CI of standards were considered putative negatives (i.e., false positives) and confirmed by bidirectional Sanger sequencing.

AGGTCCAGCCTGCCCAGTGACCATGTTCAACGGCCGCGGTATCCTAACCGTGCGAAG
GTAGCGTAATCACTTGTTCTTTAAATGTGGACTAGTATGAATGGCATCACGAAGGTTATA
CTGTCTCCTTTTTCTAATCAGTGAAACTAATTTCCCCGTGAAGAAGCGAGGATATACTTA
TAAGACGAGAAGACCCTATGGAGCTTTAAACAACACAGCAATTACCCACTAAACTACAA
GGTTTCTGAACATTTTTAATCTTTTAAGTAATCTGACTGCAAGTTTTTGGTTGGGGTGAC
CACGGAGCATAACACAACCTC

Figure 1. Artificial double stranded DNA (aDNA) replica of the R. marina 16S *gene region wherein R.marina_16S targets. Total aDNA fragment length is 317bp. Blue nucleotides indicate location of forward (R.marina_16S-F) and reverse (R.marina_16S-R) primers, respectively (Table 3). Underlined regions indicate extensions beyond the primer binding sites for R.marina_16S-F and R.marina_16S-R, which were included to promote efficient primer binding to and amplification of aDNA fragment.*

Table 4. Non-target species against which R.marina_16S was tested empirically. Nanograms of gDNA template loaded into duplicate wells of in vitro *test provided within brackets. Non-native Australian species indicated by asterisks (*).*

Australian 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** (5.98)

*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** (1.025)
Macquaria ambigua (0.135)	*Xiphophorus maculatus** (0.405)
Australian frogs/toad	**Australian freshwater turtles**
Litoria dayi (5.31)	*Chelodina canni* (1.791)
Litoria lorica (5.13)	*Chelodina oblonga* (0.669)
Litoria nannotis (2.111^)	*Elseya lavarackorum* (1.449)
Litoria rheocola (2.922)	*Emydura subglobosa worrelli* (0.741)
Litoria serrata (4.92)	*Myuchelys latisternum* (1.134)
*Rhinella marina** (1.80)	*Rheodytes leukops* (1.248)

2.4 *In situ* validation

Following confirmation via *in vitro* Tests 1–4 that R.marina_16S had acceptable amplification efficiency and did not amplify gDNA template of non-target species (see Sections 2.3 and 3.3), the assay was put through one final empirical validation test using eDNA captured and extracted from environmental water samples (Goldberg, et al., 2016). For this *in situ* test eDNA was captured from Ross River in Townsville, QLD Australia (19° 18' 21.96" S, 146° 45' 38.52" E) wherein *R. marina* is known to occur (Trumbo, et al., 2016).

Water samples were collected by decanting 15 mL from a 50mL LoBind® (Eppendorf Inc.) falcon 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). 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, submitted). 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) so as to allow for rapid loading of eDNA template technical replicates using Xplorer® electronic 12-channel pipette (Eppendorf, Australia; see below).

The *in situ* test of R.marina_16S was run in quadruplicate 10 µL technical qPCR using the same chemistry as all four *in vitro* test but with 3µL extracted eDNA from Ross River water samples as template. Master mix for *in situ* test was loaded as per all four *in vitro* tests. 3 µL eDNA template from Ross River samples was subsequently loaded into each quadruplicate qPCR technical replicate on a 10% bleach and 70% ethanol cleaned bench in the dedicated low copy DNA room within MEEL using an Xplorer® electronic 12-channel pipette (Eppendorf, Australia) fitted with Maximum Recovery filter tips (Axygene, Australia). The loaded *in situ* plate was sealed, vortexed, spun, run, and analysed as described for *in vitro* tests.

Representative amplicons produced from R.marina_16S *in situ* test on Ross River eDNA that exhibited dissociation T_m within 99.7% CI of gDNA standards were considered putative positives and bidirectionally Sanger sequenced for confirmation (Trujillo-Gonzalez, et al., 2019).

3. Results

3.1 Primer design

Based on *16S* sequence alignments of *R. marina* with Australian frogs and turtles using Geneious software (Table 2) the genus *Litoria* and *Neobatrachus* were most similar. Primer binding regions were designed to possess ≥ 3 mismatches with both *Litoria* and *Neobatrachus* (see Section 3.2). Optimal forward and reverse primer binding regions were identified between base pairs 30–49 (R.marina_16S-F) and 319–338 (R.marina_16S-R) of *R. marina 16S*, respectively (Table 3). R.marina_16S-F exhibited the following characteristics: 1) T_m = 62.3–64.4, 2) GC content = 63.2%, 3) length = 19 bp, 4) self-dimer T_m = 0˚C, 5) hairpin T_m = 0˚C, 6) self-complementarity score = 4, and 7) 3' self-complementarity score = 4. R.marina_16S-R exhibited the following characteristics: 1) T_m = 60.0–62.0, 2) GC content = 55%, 3) length = 20 bp, 4) self-dimer T_m = 0˚C, 5) hairpin T_m = 0˚C, 6) self-complementarity score = 5, and 7) 3' self-complementarity score = 5.

3.2 *In silico* validation

Initial *in silico* test using targeted PrimerBLAST search of NCBI "nr" database confirmed that *Litoria rubella, Neobatrachus pictus*, and *N. sudelli 16S* sequence are the most likely to amplify with R.marina_16S given ≥ 3 bp mismatches to R.marina_16S-R (Table 5). Subsequent non-targeted in silico PrimerBLAST test of R.marina_16S returned ≤ 1 bp mismatch (i.e., PCR amplification expected) to multiple frog and toad species (*n* = 63); however, none of these species are native nor known to be present within Australia (Table 6).

Table 5. Species with ≤ 5 mismatches to R.marina_16S based on targeted PrimerBLAST search of Australian fish, frog, and turtle sequences in NCBI "nr" databse.

Forward mismatches	Reverse mismatches	Species
0	3	*Litoria rubella* *Neobatrachus pictus* *Neobatrachus sudelli*
1	3	*Litoria infrafrenata*
1	4	*Litoria meiriana*

Table 6. Species with ≤ 1 mismatch to R.marina_16S based on non-targeted PrimerBLAST search of entire NCBI "nr" databse. Note that none of the non-target species expected to amplify with R.marina_16S are native to Australia.

Forward mismatches	Reverse mismatches	Species
0	0	*Bufo achavali* *Bufo americanus* *Bufo arenarum* *Bufo crucifer* *Bufo ictericus*

		Bufo jimi
		Bufo longinasus
		Rhinella arenarum
		Rhinella henseli
		Rhinella limensis
		Rhinella marina
		Rhinella ornata
		Rhinella poeppigii
		Rhinella rubescens
		Rhinella schneideri
1	0	*Ansonia teneritas*
		Arthroleptis adelphus
		Barbarophryne brongersmai
		Bufo andrewsi
		Bufo gargarizans
		Bufo japonicus
		Bufo perplexus
		Bufo stejnegeri
		Bufo taitanus
		Bufo tibetanus
		Bufo tuberculatus
		Capensibufo tradouwi
		Gastrophryne olivacea
		Gastrophryne olivacea mazatlanensis
		Mertensophryne lindneri
		Mertensophryne taitana
		Poyntonophrynus fenoulheti
		Relictivomer pearsei
		Rhinella arequipensis
		Rhinella spinulosa
		Werneria mertensiana
		Werneria tandyi
		Wolterstorffina chirioi
		Wolterstorffina mirei
0	1	*Anaxyrus americanus*
		Anaxyrus baxteri
		Anaxyrus californicus
		Anaxyrus cognatus
		Anaxyrus fowleri
		Anaxyrus hemiophrys
		Anaxyrus houstonensis
		Anaxyrus kelloggi
		Anaxyrus microscaphus
		Anaxyrus punctatus
		Anaxyrus terrestris
		Anaxyrus woodhousii
		Bufo alvarius
		Bufo coniferus
		Bufo fastidiosus

	Bufo karenlipsae
	Bufo longinasus
	Bufo sp. DMP-2015
	Incilius alvarius
	Incilius coniferus
	Incilius fastidiosus
	Peltophryne lemur
	Peltophryne sp. SBH-2017
	Rhinella paracnemis

3.3 *In vitro* validation

R.marina_16S exhibited satisfactory efficiency and LOD based on gDNA and aDNA standard curves run at 65˚C with 250nM each primer (Table 3).

In vitro Test 1 demonstrated that R.marina_16S amplified the first 5-points of the $\log_{10}$ gDNA standard curve with 93.3% efficiency and no primer dimerization (Figure 2). This 5-point gDNA standard curve demonstrated the LOD of R.marina_16S to be $1.8 \pm 0.3 \times 10^{-4}$ ng gDNA loaded or 10–14 copies aDNA loaded (based on extrapolation from aDNA standard curve; Figure 3). Note that zero of three technical replicates amplified for Standard 6 ($1.8 \pm 0.3 \times 10^{-5}$ ng loaded or 1.2 copies predicted) and Standard 8 ($1.8 \pm 0.3 \times 10^{-7}$ ng loaded or 0.012 copies predicted). However, one of three technical replicates for Standard 7 ($1.8 \pm 0.3 \times 10^{-6}$ ng loaded or 0.12 copies predicted) did amplify but not in linear order so this Standard 7 replicate was not included in gDNA standard curve but rather was extrapolated using both 5-point gDNA standard curve (1.4×10^{-5} ng loaded) and 8-point aDNA standard curve (0.844 copies loaded) given its low copy number. The observed amplification of one Standard 7 replicate is likely due to the stochastic chance of capturing ≤ 1 copy (in 3µL of 300µL made for each Standard, i.e., only 1% of volume screened) whereas the ≥ 1 copies present in Standard 6 were simply missed by chance within the three 3µL technical replicates tested. Also, the lack of Standard 8 amplification suggests it being below LOD, whereas with more technical replicates *16S* detection would be predicted in both Standards 6 and 7. Sanger sequences of gDNA standards (*n* = 3; 304–305 bp) were positive for *R. marina 16S* (GenBank accession KF665157) with pairwise nucleotide sequence identity of ≥ 98.4%.

In vitro Test 2 demonstrated that R.marina_16S amplified all 8-points of the $\log_{10}$ aDNA standard curve with 93.75% efficiency and minor yet disenable primer dimerization (based on amplicon T_m shift relative to T_m of amplicons produced from aDNA standards). This 8-point aDNA standard curve demonstrated the LOD of R.marina_16S to be 9.07 ± 2.193 copies loaded (Figure 3). Note that the oblong dissociation curve is not due to multiple or incorrect sized products (Figure 4) nor is this oblong dissociation curve observed when amplifying *R. marina 16S* from gDNA template (Figure 2 and Figure 5) or eDNA template (Figure 6).

In vitro Test 3 demonstrated that R.marina_16S amplified gDNA template from all NT and QLD individuals (*n* = 21 total; Figure 5). Across the 21 individuals from NT and QLD the quantity of gDNA loaded (ng/µL) ranged from 0.179 to 1.335 while number of *16S* copies (copies/µL) ranged from 82,820–11,044. Sensitivity of R.marina_16S for target species

gDNA was further confirmed by detection of 1.6–2.3 x 10^{-4} ng/µL or 10–14 copies/µL in the two extraction blanks (approx. 800x less than lowest *R. marina* gDNA detections).

Sanger sequenced putative negative amplifications (i.e., false positives) from *in vitro* Test 4 (*n* = 8 non-target species; seeTable 4) were confirmed negative in that no Sanger sequences matched *R. marina 16S* sequence (i.e., all non-target species *in vitro* amplifications were false positive detections due to cross-contamination during plate loading).

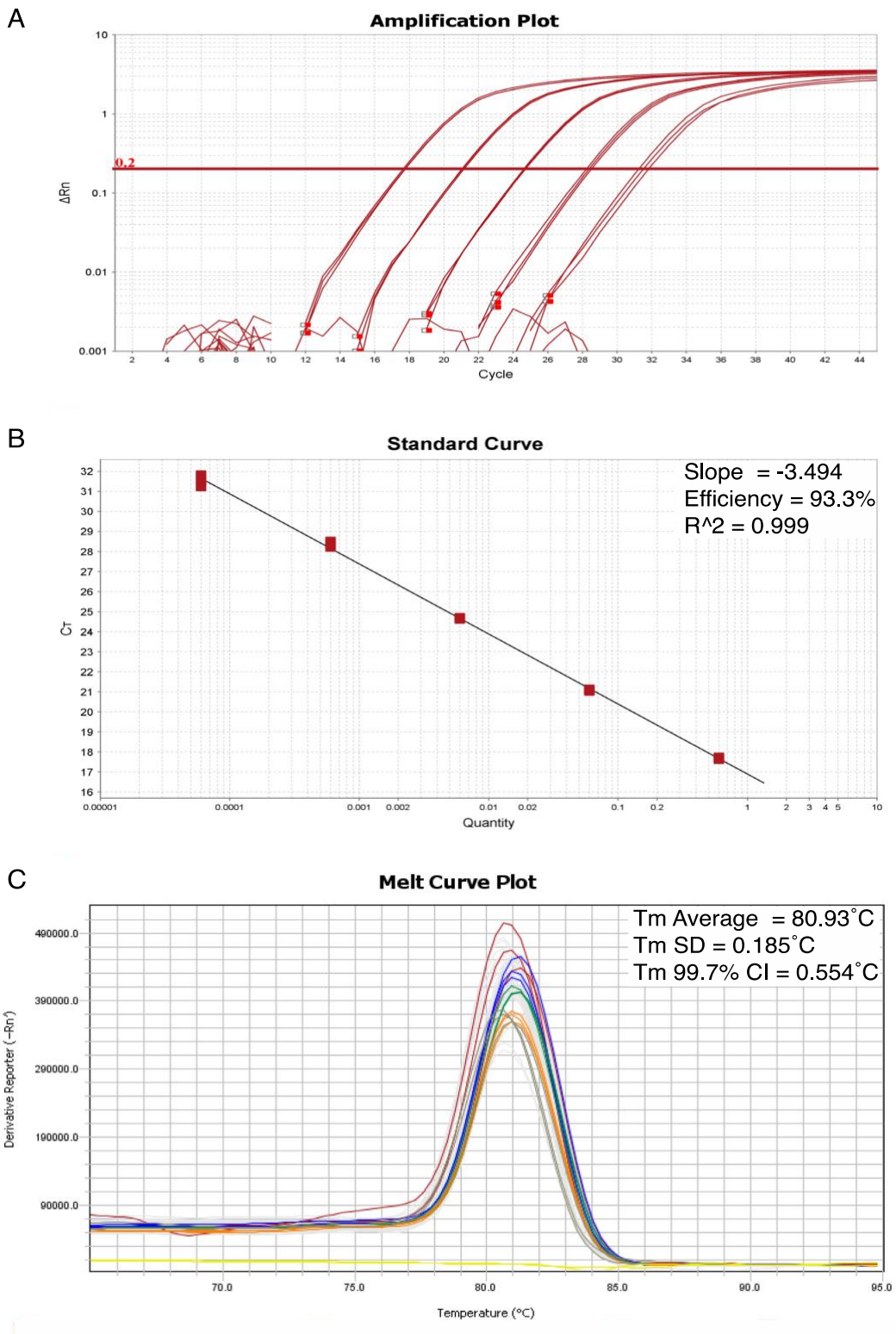

Figure 2. Amplification curves (A), linear regression of gDNA standard log_{10} serial dilution (B), and amplicon dissociation temperature curves (T_m; C) generated by qPCR during R.marina_16S in vitro *Test 1. Note absence of oblong amplicon dissociation curves in Panel C for gDNA template that were present for amplicons generated from aDNA template (see Figure 3).*

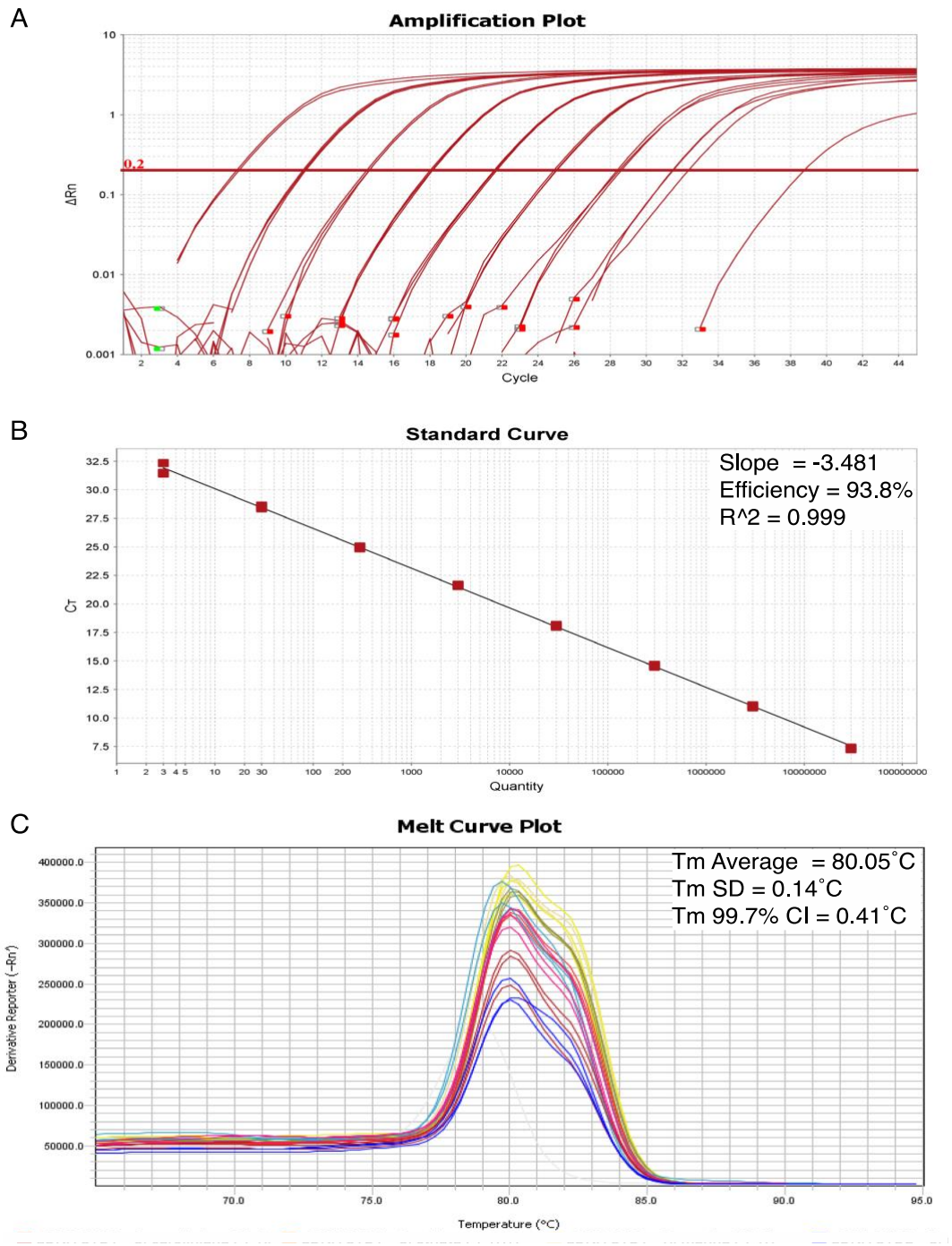

Figure 3. Amplification curves (A), linear regression of aDNA standard log$_{10}$ serial dilution (B), and amplicon dissociation temperature curves (T$_m$; C) generated by qPCR during R.marina_16S in vitro *Test 2. Note that oblong amplicon dissociation curve (C) is an artefact of aDNA template (e.g., tertiary structure) and that all amplicons generated from aDNA standards are single products of the correct size (Figure 4) based on expected length of 16S aDNA fragment (Figure 1). Moreover, oblong dissociation curves are not present when* R. marina *16S is amplified from gDNA (Figure 2 and Figure 5) or eDNA (Figure 7) templates.*

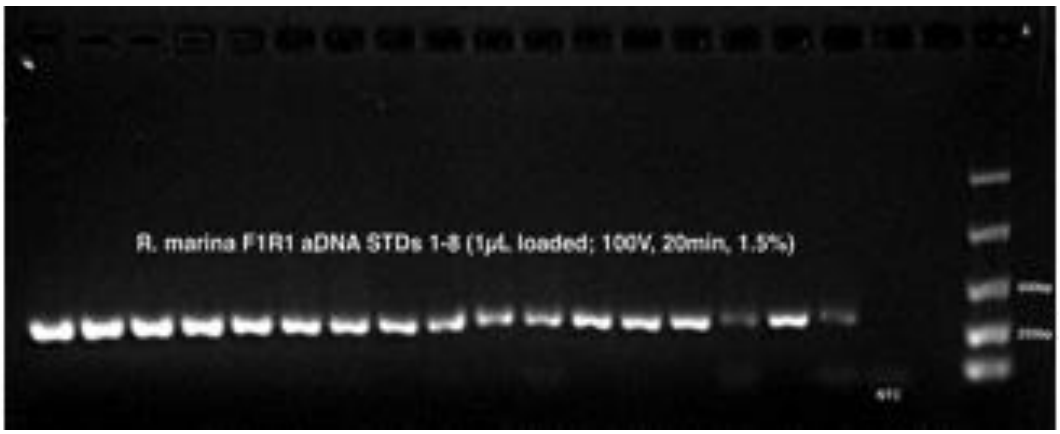

Figure 4. Gel visualization of amplicons generated from R.marina_16S aDNA standards that exhibited slightly oblong dissociation curves (Figure 2). 1.5% agarose gel loaded with 1µL of aDNA amplicons stained with 1:500 GelGreen®.

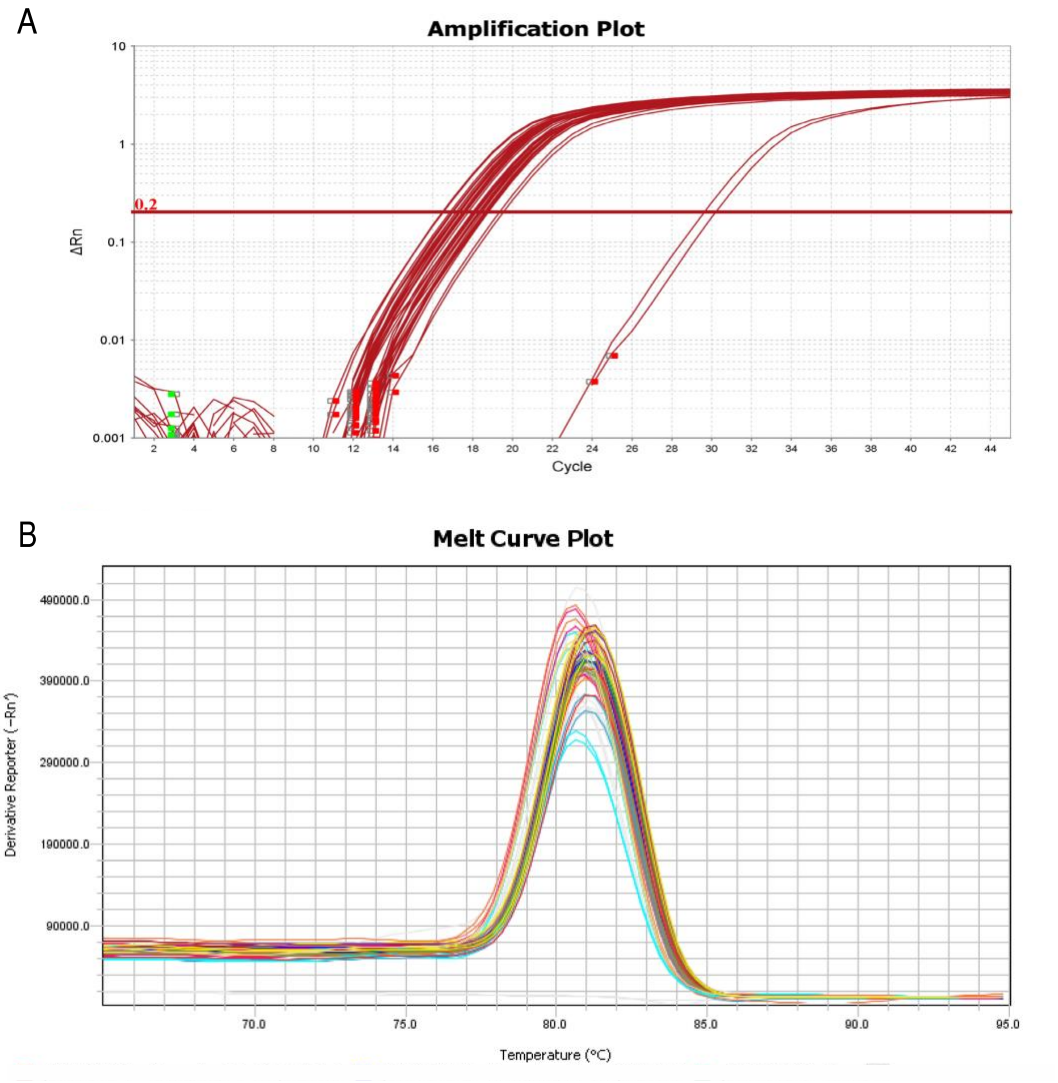

Figure 5. Amplification curves (A) and amplicon dissociation temperature curves (T_m; B) generated during R.marina_16S in vitro *Test 3. Note the absence of oblong amplicon dissociation curves from gDNA template (panel B) that were present for aDNA template amplicons (Figure 2). Amplification was observed in extraction blank (C_t = 30.18 and 29.61; panel A) with an aDNA standard curve extrapolated quantity of approx. 14 and approx. 10 copies/µL, respectively. Detection of trace* R. marina *gDNA due to extraction carry-over further supports the high sensitivity (i.e., low LOD) of R.marina_16S (Figure 2).*

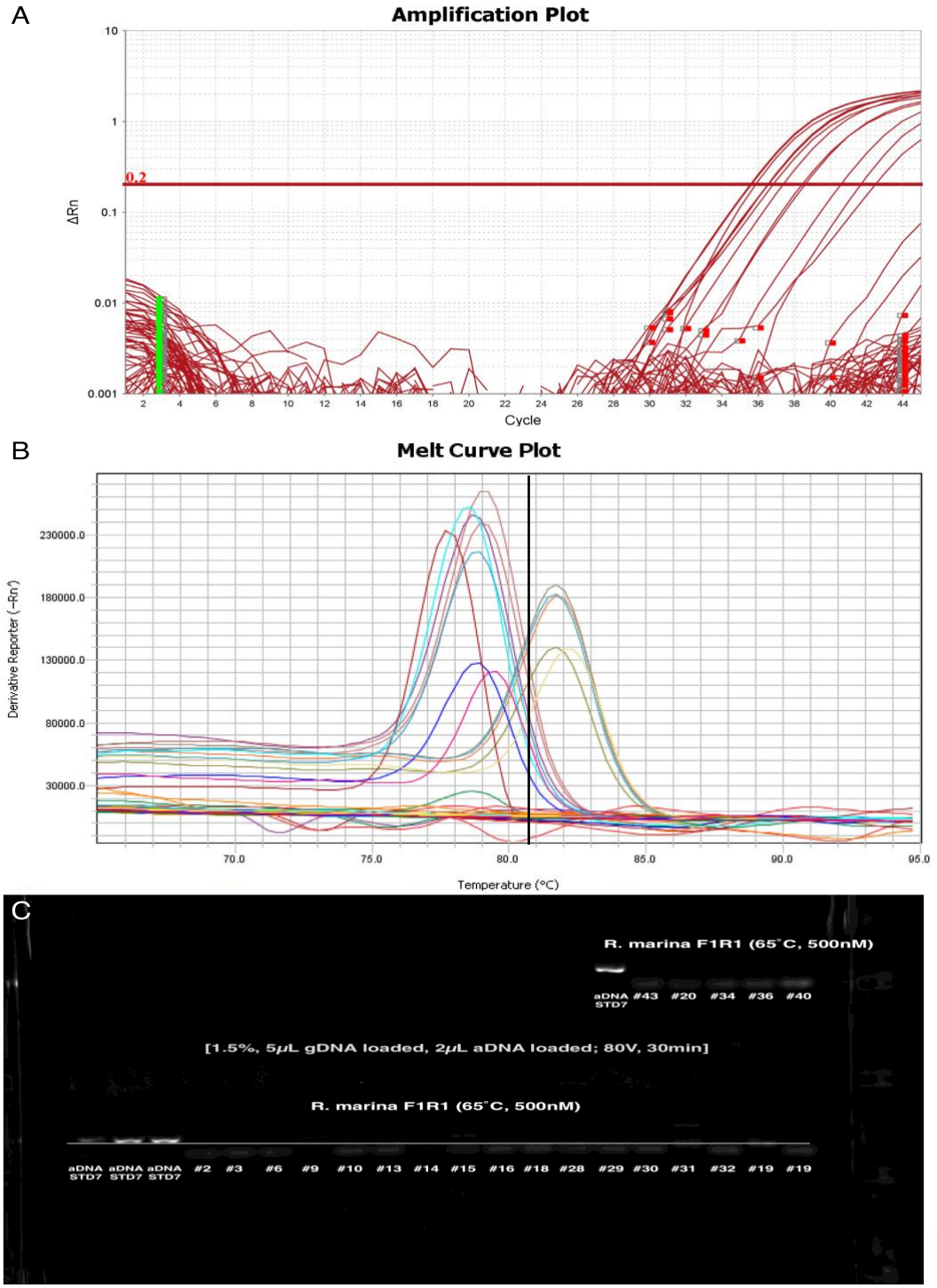

Figure 6. Amplification curves (A), amplicon dissociation temperature curves (T_m; B), and agarose check gel (C) from R.marina_16S in vitro *Test 4. Note that no amplicon exhibited T_m within 99.7% CI of* R. marina *gDNA standards (80.9 ± 0.55˚C) illustrated by vertical black line (B). Agarose check gel (C) is from an initial* in vitro *test run at 65˚C with 500nM each primer (note: in vitro Tests 2–4 run at 65˚C with 250nM each primer because primer dimerization present at 500nM is confounding). As such, R.marina_16S assay requires careful ΔT_m comparison to gDNA standards (within 99.7% CI) and Sanger sequencing verification to avoid false positive detections (i.e., discern from primer dimer).*

3.4 *In situ* validation

In situ validation of R.marina_16S using eDNA collected from Ross River (see Section 2.3) under optimal qPCR conditions (65˚C, 250nM) yielded successful amplification from all eDNA samples (Figure 7). Amplification of all Ross River eDNA samples was expected because *R. marina* tadpoles were visually abundant in the shallow water near the edge of Ross River where eDNA water samples were collected. Copies detected across eDNA extractions ranged from 56 to 15,218 depending on treatment. All Sanger sequenced representative eDNA amplicons (n = 12; 303–306 bp consensus) matched *R. marina 16S* sequence (GenBank accession KF665157) with pairwise identity of ≥ 98.4%.

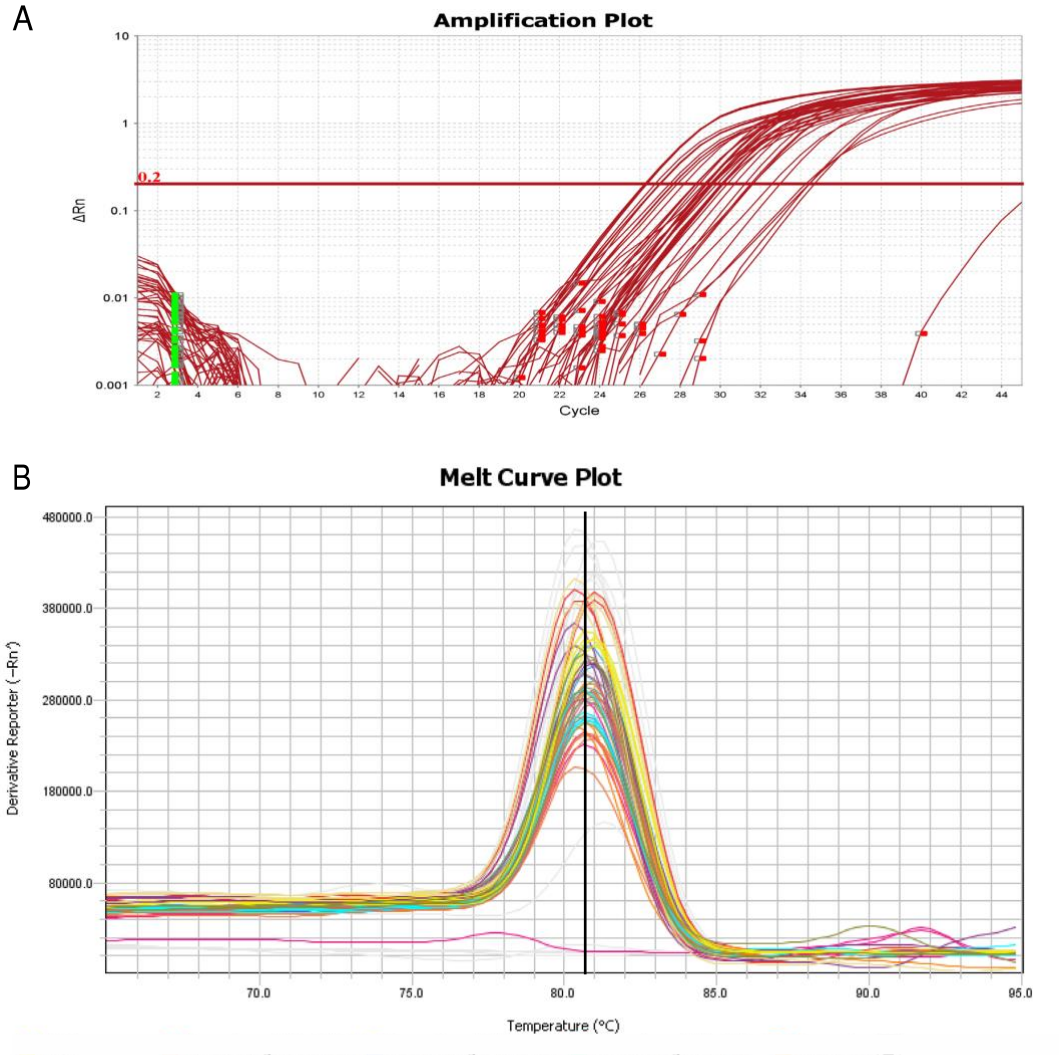

Figure 7. R.marina_16S amplification (A) and associated amplicon dissociation curves (B) generated during in situ *Test on Ross River eDNA samples (65˚C with 250nM each primer). Vertical black line represents average T_m of gDNA standards (80.9˚C ± 0.55˚C), which T_m of all Ross River eDNA amplicons are within. All Sanger sequenced representative amplicons (n = 12) were positive for* R. marina*.*

4. Discussion

The cane toad, *Rhinella marina* is a significant invasive pest in Australia. A new environmental DNA (eDNA) assay was developed for the detection of this species in water samples using Sybr-based quantitative real-time polymerase chain reaction (qPCR). This eDNA assay targets the *R. marina* 16S mitochondrial gene.

GenBank (NCBI) was mined for available 16S nucleotide sequences from 172 frog species. Then, 76 Australian frog, fish and turtle species against which the NCBI "nr" database was queried using PrimerBLAST, were tested *in silico*. Following satisfactory *in silico* tests we empirically tested for specificity to *R. marina* by attempting to PCR amplify 16S from genomic DNA (gDNA) extracted from *R. marina* and 63 non-target frog and toad species. Finally an *in situ* test was performed using water samples from a site known to contain *R. marina*.

The *in silico*, *in vitro*, and *in situ* validations undertaken during the eDNA assay development described herein demonstrate the readiness of R.marina_16S for screening environmental water samples. This validated assay can be used to monitor water samples taken from any location wherein *R. marina* is known to be present, suspected to be present (e.g., potential invasion front), or has previously been present (e.g., post-eradication monitoring).

Of note is the recent publication of a short (80 bp) TaqMan-based *R. marina* assay (Tingley, et al., 2019); however, putative positive detections were not confirmed by Sanger sequencing. We recommend using the longer Sybr-based R.marina_16S (290 bp; Table 3) presented here given as it will likely have a greater specificity.

Lastly, R.marina_16S should be used with caution in global regions wherein other *Bufo* toads are known to occur (e.g., South America) given potential for cross-amplification of several *Bufo* species (seeTable 6). Inherent 16S nucleotide variability between *R. marina* and non-target *Bufo* species should be sufficient to discriminate using ΔT_m analysis (Trujillo-Gonzalez, et al., 2019); however, this requires empirical testing and Sanger sequencing confirmation before employment of R.marina_16S within these global regions.

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