Northern Australia Environmental Resources Hub

National Environmental Science Programme

Development of a revised eDNA assay for tilapia (*Oreochromis mossambicus* and *Tilapia mariae*)

Report

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Cover photographs:

Front cover: Mozambique tilapia (photo: Ammit Jack/Shutterstock.com)

Back cover: *Oreochromis mossambicus* and *Tilapia mariae* in captivity (photo: Centre for Tropical Water and Aquatic Ecosystem Research).

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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

Acknowledgements

Development of revised eDNA assay for tilapia (*Oreochromis mossambicus* and *Tilapia mariae;* Tilapia_v2_16S) was conducted as part of the Australian Government's National Environmental Science Program (NESP), Northern Australia Environmental Resources Hub Project 4.3: "The Northern Australia eDNA Program – Revolutionising Aquatic Monitoring and Field Surveys in Tropical Waters".

We thank Dr. Roger Huerlimann for extracted genomic DNA from Australian fish, frogs, and turtles used for *in vitro* validation.

Executive summary

A revised environmental DNA (eDNA) assay was developed for the detection of invasive tilapia (*Oreochromis mossambicus* and *Tilapia mariae*) in environmental water samples using Sybr-based quantitative real-time polymerase chain reaction (qPCR). Like the initial tilapia eDNA assay (Nobel, et al., 2014; Robson, et al., 2016), the revised eDNA assay (Tilapia_16S_v2) also targets mitochondrial *16S* ribosomal DNA. Here we present the *in silico*, *in vitro* and, *in situ* validations undertaken during development of Tilapia_16S_v2.

In silico, *in vitro* and, *in situ* validations confirmed that Tilapia_16S_v2 is specific to (i.e., detects only) *O. mossambicus* and *T. mariae* despite co-occurrence with native species. Limit of detection (LOD) was determined to be 1 copy per qPCR assay replicate under optimal qPCR conditions (60˚C annealing temperature, 500 nM each primer). Moreover, bidirectional Sanger sequencing confirmed all representative putative positive detections from *in vitro* and *in situ* validations (gDNA standards and Ross River water samples) to be positive for target species detection while all putative negative detections from *in vitro* validation (non-target species amplifications) were confirmed negative (i.e., false positives), respectively. As such, Tilapia_16S_v2 is considered to be fully validated and ready for application to environmental water samples to test for presence of eDNA from *O. mossambicus* and/or *T. mariae*.

Of note is that, compared to initial tilapia eDNA assay (Nobel, et al., 2014; Robson, et al., 2016), Tilapia_16S_v2 exhibits equal detectability of *O. mossambicus* but superior concurrent detectability of *T. mariae*. As such, surveys of waterways expected to harbour invasion fronts of either or both tilapia species should be (re)analysed using Tilapia_16S_v2 so as to ensure equal detectability of both invasive species.

1. Introduction

Mozambique tilapia (*Oreochromis mossambicus)* and spotted tilapia (*Tilapia mariae*) were both introduced to Queensland, Australia in the 1970s through the aquarium trade and have since spread widely. *Oreochromis mossambicus* are found in 20 of 76 catchments in Queensland, as well as WA and northern NSW (Webb, 2007; Bradford, et al., 2011; Russell, et al., 2012). *Tilapia mariae* are more restricted, only being found in the wet tropics region of north Queensland, although they have also recently established in the Walsh River, Gulf of Carpentaria, from which they are expected to spread much further in coming years. Both species are steadily expanding their range, largely due to human assistance and close monitoring is required to monitor their spread.

In order to accurately monitor Australian waterways for the invasion of these aggressively invasive species, a sensitive method for the concurrent detection of both *O. mossambicus* and *T. mariae* (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), provides such a method. eDNA can be captured and used to detect *O. mossambicus* and/or *T. mariae* in any waterbody known or suspected to have been inhabited by either invasive tilapia species.

An eDNA assay for tilapia in Australia has previously been developed (Nobel, et al., 2014; Robson, et al., 2016). This assay has been proven to sucessfully detect *O. mossambicus* (Robson, et al. 2016) but we found that it had limited resolution with *T. mariae* when using eDNA to monitor the arrival of this species in the Walsh River (Edmunds, et al., 2019). Thus, here we describe the development of a revised eDNA assay for tilapia that exhibits equal detectability of *O. mossambicus* eDNA and improved detectability of *T. mariae* eDNA compared to the previously developed assays. Development of this revised assay involved redesign of primers to be homologous to (i.e., no mismatches with) *O. mossambicus* and *T. mariae 16S* nucleotide sequences given that initial assay is homologous to *O. mossambicus* but contains three mismatches with *T. mariae* (Figure 1). Following primer redesign the revised eDNA assay was validated *in silico*, *in vitro*, and *in situ*.

2. Methodology

2.1 Primer design

The presence of multiple mitochondria within each cell makes mitochondrial DNA more abundant and thus more detectable than nuclear DNA within environmental water samples (Goldberg, et al., 2016). Moreover, mitochondrial *16S* is commonly targeted by barcoding studies and thus nucleotide sequence information from a broad range of species is available within the National Center for Biotechnology Information public database (GenBank; www.ncbi.nlm.nih.gov). As such, Geneious analysis software (version R11; Kearse et al., 2012) was used to obtain all available *16S* nucleotide sequences from Australian fish species (*n* = 86; Table 1) and subsequently align using ClustalW algorithm (Thompson, et al., 2003). Aligned *O. mossambicus* (*n* = 4) and *T. mariae* (*n* = 2) *16S* sequences were assessed by eye for regions wherein both *O. mossambicus* and *T. mariae* exhibited ≥ 2 base pair (bp) mismatches with potentially co-occurring species as well as human.

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 mitochonrial 16S *nucleotide sequences were obtained from GenBank (NCBI) and used to guide Tilapia_v2_16S assay development.*

Non-target Australian fish species		
Amatitlania nigrofasciata	*Giuris margaritacea*	*Nannoperca obscura*
Ambassis agassizii	*Glossamia aprion*	*Nannoperca variegata*
Ambassis agrammus	*Guyu wujalwujalensis*	*Nematalosa erebi*
Amphilophus citrinellus	*Hephaestus carbo*	*Neoceratodus forsteri*
Anabas testudineus	*Hephaestus fuliginosus*	*Neosilurus ater*
Anguilla australis	*Hypseleotris compressa*	*Oncorhynchus mykiss*
Anguilla bicolor	*Hypseleotris galii*	*Oreochromis aureus*
Anguilla obscura	*Kuhlia marginata*	*Oreochromis niloticus*
Anguilla reinhardtii	*Kuhlia rupestris*	*Ornechromis mossambicus*
Archocentrus nigrofasciatus	*Lates calcarifer*	*Oxyeleotris lineolatus*
Archocentrus citrinellum	*Leiopotherapon aheneus*	*Pangasius conchophilus*
Arius (Neoarius) berneyi	*Leiopotherapon unicolor*	*Paragalaxias eleotroides*
Carassius auratus	*Lepidogalaxias salamandroides*	*Paragalaxias julianus*
Craterocephalus eyresii	*Maccullochella ikei*	*Paragalaxias mesotes*
Cyprinus carpio	*Maccullochella macquariensis*	*Perca fluviatilis*
Eptatretus cirrhatus	*Maccullochella mariensis*	*Philypnodon grandiceps*
Eptatretus longipinnis	*Maccullochella peelii*	*Philypnodon macrostomus*
Gadopsis marmoratus	*Macquaria ambigua*	*Piaractus brachypomus*
Galaxias brevipinnis	*Macquaria australasica*	*Piaractus mesopotamicus*
Galaxias fuscus	*Macquaria colonorum*	*Plotosus lineatus*
Galaxias maculatus	*Macquaria novemaculeata*	*Porochilus obbesi*
Galaxias parvus	*Megalops cyprinoides*	*Retropinna semoni*

Galaxiella pusilla	*Melanotaenia fluviatilis*	*Rutilus rutilus*
Galaxias zebratus	*Melanotaenia splendida*	*Salmo trutta*
Galaxiella munda	*Mogurnda adspersa*	*Tandanus tandanus*
Galaxiella nigrostriata	*Mordacia mordax*	*Tinca tinca*
Galaxiella pusilla	*Nannoperca oxleyana*	*Toxotes chatareus*
Gambusia holbrooki	*Nannoperca vittata*	*Toxotes jaculatrix*
Geotria australis	*Nannoperca australis*	

2.2 *In silico* validation

Following design of forward (Tilapia_16S_v2-F) and reverse (Tilapia_16S_v2-R) primers, the combination (hereafter referred to as "Tilapia_16S_v2") 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 Tilapia_16S_v2 assay was tested for potential primer binding (Table 2). For this targeted search, all species with ≤ 5 base pair mismatches to either primer were documented (see Section 3.2). The subsequent *in silico* test used non-targeted PrimerBLAST (i.e., no species specified) to test Tilapia_16S_v2 assay against all species with nucleotide sequences deposited in NCBI "nr" database to ascertain which, if any, species might be amplified by the Tilapia_16S_v2 assay. For this non-targeted search, all species with ≤ 2 base pair mismatches to either primer were documented (see Section 3.2).

Following satisfactory compliance of Tilapia_16S_v2 assay with *in silico* tests (i.e., targeted PrimerBLAST confirmation that no specified species have 0 mismatches to either primer nor have an identical amplicon length of 101 bp as predicted by PrimerBLAST algorithm; Ye, et al., 2012; Table 3), standard desalted oligonucleotides were synthesized by Integrated DNA Technologies (IDT; New South Wales, Australia) and shipped pre-diluted to 100µM in low-EDTA TE buffer ("Lab Ready").

Table 2. Species against which Tilapia_v2_16S was tested in silico *using targeted PrimerBLAST search of humans 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 revised tilapia eDNA assay (Tilapia_v2_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')
Tilapia_16S_v2-F	57.6	55.39	50	101	AATGTCTTTGGTTGGGGC
Tilapia_16S_v2-R	56.8	54.82	40		TTCTGTTGCTTGGAGTTGTA

2.3 *In vitro* validation

Following confirmation of satisfactory *in silico* tests, the Tilapia_16S_v2 assay was tested empirically for species-specifity by attempting to amplify genomic DNA (gDNA) template extracted from non-target but potentially co-occurring species (i.e., *in vitro* validation; Table 4). More specifically, Tilapia_16S_v2 assay was empirically tested *in vitro* against five species of Australian rainforest frogs, six species of Australian freshwater turtles, and 31 species of Australian freshwater fishes (Table 4).

In vitro tests also included the generation of standard curves for verification of Tilapia_16S_v2 assay amplification efficiency and limit of detection (LOD). More specifically, the Tilapia_16S_v2 assay was tested against standard curves (see below) generated using both *O. mossambicus* (*n* = 1) and *T. mariae* (*n* = 2) gDNA to determine amplification efficiency and LOD of Tilapia_16S_v2 for both tilapia species. A similar standard curve was also generated using an artificial double stranded DNA replica (aDNA; gBlocks™, IDT Australia) of the 179bp region within *O. mossambicus 16S* nucleotide sequence [AY597335] wherein primers for both initial (Nobel, et al., 2014; Robson, et al., 2016) and revised (Tilapia_16S_v2) tilapia assays target (Figure 1). Top gDNA standards (1:145 and 1:100 for *O. mossambicus* and *T. mariae*, respectively) and stock aDNA were quantified in duplicate (ng/µL ± 99.7% CI) using the 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 an online calculator (http://www.endmemo.com/bio/dnacopynum.php).

To test Tilapia_16S_v2 assay efficiency and LOD for both *O. mossambicus* and *T. mariae* gDNA standard curves were generated by serial dilution (8-step $\log_{10}$). For *O. mossambicus*, neat gDNA from one individual was diluted 1:145 with MilliQ® water to generate Standard 1 (0.796 ± 0.017 ng/µL). For *T. mariae*, neat gDNA was pooled equally from two individuals and diluted 1:100 with MilliQ® water to generate Standard 1 (0.844 ± 0.157 ng/µL). Standard 1 for *O. mossambicus* and *T. mariae* was then serially diluted 1:10 ($\log_{10}$) with MilliQ® water 7 times (vortexed and spun-down between each dilution) to generate Standards 2–8 ($0.796 \pm 0.017 \times 10^{-1}$ – $0.796 \pm 0.017 \times 10^{-7}$ and $0.844 \pm 0.157 \times 10^{-1}$ – $0.844 \pm 0.157 \times 10^{-7}$ ng/µL), respectively. Tilapia_16S_v2 assay efficiency and LOD were also determined using *O. mossambicus 16S* aDNA fragment (Figure 1) by resuspending the dried pellet (IDT, Australia) in 50µL of 1x TE buffer following manufacturer's instructions, which yielded an aDNA stock concentration of 2.715 ± 0.23 ng/µL or 14,771,265,139 ± 1,267,662,902 copies/µL. Stock aDNA was then diluted 1:500 with MilliQ® water to generate Standard 1 ($2.95 \pm 0.254 \times 10^{7}$ copies/µL), which was then serially diluted $\log_{10}$ 7 times with MilliQ® water to generate Standards 2–8 ($2.95 \pm 0.254 \times 10^{6}$ – 2.95 ± 0.254 copies/µL, respectively). The number of *16S* amplicon copies generated from gDNA template of target and/or non-target species during *in vitro* Tests 2 and 3 (see below) was determined by extrapolation from aDNA standard curve run using the same chemistry and cycling conditions.

Quantitative real-time polymerase chain reaction (qPCR) was used for three separate *in vitro* tests: 1) determination of Tilapia_16S_v2 assay efficiency and LOD using 8-step $\log_{10}$ aDNA standard curve (Test 1), 2) determination of Tilapia_16S_v2 assay efficiency and LOD using 8-step $\log_{10}$ gDNA standard curves for both *O. mossambicus* and *T. mariae* (Test 2), and 3)

determination of Tilapia_16S_v2 assay amplification of gDNA from non-target but potentially co-occurring species (Test 3; see Table 4). 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), 0.5 µL forward primer (10 µM, 500 nM final; Table 3), 0.5 µL reverse primer (10 µM, 500 nM final; Table 3), 3 µL aDNA (Test 1; Figure 1) or 3 µL *g*DNA (Test 2) or 3 µL gDNA of non-target species (Test 3; 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 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 in Townsville, Queensland Australia.

For *in vitro* Test 1, 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) were loaded using a manual single channel P10 pipette (Eppendorf, Australia) fitted with Maximum Recovery filter tips (Axygene, Australia).

For *in vitro* Tests 2 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 of each tilapia gDNA standard (see above) and 3 µL gDNA template of each species (Table 4) was loaded using an Xplorer® electronic 12-channel pipette (Eppendorf, Australia) fitted with Maximum Recovery filter tips (Axygene, Australia), respectively.

Following aDNA or gDNA 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 60˚C for 1 min (ramp rate = 2.7˚C/sec) before terminal dissociation curve generation by transitioning from 60˚C to 95˚C (ramp rate = 0.15˚C/sec). *In vitro* Tests 1 and 2 (aDNA and gDNA 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 for all runs before export and analysis in Microsoft Excel.

All amplicons produced from gDNA standards template of both target species (Test 2) were considered putative positives. Representative amplicons from standard 1 of *O. mossambicus* and *T. mariae* gDNA standards (n = 2 and n = 2, respectively) were Sanger sequenced bidirectionally for verification. All aDNA standard amplifications were considered positive detection without Sanger sequencing confirmation given that aDNA template was specifically designed to be an exact replicate of targeted *16S* region (Figure 1). All amplifications from gDNA of non-target species (Test 3) that exhibited dissociation temperature (T_m) within

99.7% CI of aDNA and gDNA standards (Tests 1 and 2 respectively) were extrapolated using aDNA standard curve (Test 1) to determine amplicon copies generated per nanogram of gDNA template loaded (see Table 4). All non-target species that exhibited amplification were considered putative negatives because gDNA template from each non-target species generated only ≤ 0.2% of the total number of copies per nanogram that amplified from gDNA template of target species. Putative negative detections (individual or pooled technical qPCR replicates) for non-target species (*n* = 10) were Sanger sequenced bidirectionally for verification.

(A)

ATGGAGCTT(C)AGAC(G)CCAGAACAGACCATGTTAAGCACTCCTGAAATAAAGGATAA
AACTGATTGGCCCCTGTTCT**AATGTCTTTGGTTGGGGC**GACCGCGGGGAAACAAAAAA
CCCCCATGTGGACCGGGAGCACACTACTCCTACAAC**CCAGAG(T)**TACAACTCCAAGCA
ACAGAATT

(B)

Primer-BLAST Results

Input PCR template	none
Specificity of primers	Target templates were found in selected database: Nucleotide collection (nt) (Organism limited to Pelmatolapia mariae)
Other reports	▷Search Summary

Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CTTCAGACGCCAGAACAG	18	55.42	55.56	6.00	2.00
Reverse primer	GCTTGGAGTTGTAACTCTGG	20	56.07	50.00	8.00	2.00

Products on target templates

>JX910620.1 Pelmatolapia mariae isolate 101Tma 16S ribosomal RNA gene, partial sequence; mitochondrial

```
product length = 165
Forward primer  1    CTTCAGACGCCAGAACAG  18
Template        208  ...T....A.........  225

Reverse primer  1    GCTTGGAGTTGTAACTCTGG  20
Template        372  ............G......  353
```

>JX910612.1 Pelmatolapia mariae isolate 90Tmar 16S ribosomal RNA gene, partial sequence; mitochondrial

```
product length = 165
Forward primer  1    CTTCAGACGCCAGAACAG  18
Template        208  ...T....A.........  225

Reverse primer  1    GCTTGGAGTTGTAACTCTGG  20
Template        372  ............G......  353
```

>GQ168026.1 Tilapia mariae isolate J65 16S ribosomal RNA gene, partial sequence; mitochondrial

```
product length = 165
Forward primer  1    CTTCAGACGCCAGAACAG  18
Template        230  ...T....A.........  247

Reverse primer  1    GCTTGGAGTTGTAACTCTGG  20
Template        394  ............G......  375
```

Figure 1. (A) Artificial double stranded DNA (aDNA) replica of 179 bp region of O. mossambicus 16S *(GenBank accession [AY597335]) wherein initial (16S Oreo-F and 16S Tilapia-R; Nobel, et al., 2014; Robson, et al., 2016) and revised (Tilapia_16S_v2; this report) assay primers target. (B) PrimerBLAST of initial tilapia assay against* T. mariae 16S *sequences available within GenBank "nr" database. Bold underlined nucleotides (panel A) indicate binding sites for initial tilapia eDNA assay, with nucleotides in parentheses indicating base pairs homologous and non-homologous with* O. mossambicus *and* T. mariae 16S *(panel B), respectively. Bolded blue nucleotides (panel A) indicate binding sites for revised tilapia eDNA assay (Table 3). Note overlap of reverse primer binding site for initial and revised tilapia assays (black and blue underlined). Bolded green nucleotides (panel A) indicate 5' and 3' end extensions beyond primer binding sites included to promote efficient primer binding and amplification.*

Table 4. Non-target species against which Tilapia_v2_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 (*). Bolded species are concurrent targets of Tilapia_v2_16S.*

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 that Tilapia_16S_v2 assay had an acceptable qPCR amplification efficiency as per 8-point $\log_{10}$ aDNA and gDNA-based standard curves (90 - 105%, $R_2 > 0.990$; Edmunds, et al., 2015) and that Tilapia_16S_v2 assay did not amplify gDNA template of non-target species, the revised Tilapia_16S_v2 assay was put through two *in situ* validation tests using eDNA captured and extracted from water samples (15mL per replicate).

The first *in situ* validation (positive control validation; *in situ* Test 1) captured eDNA in water samples (*n* = 3) collected on 15 November 2017 and 7 August 2018 from *O. mossambicus* and *T. mariae* holding tanks, respectively, 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 *O. mossambicus* and *T. mariae* holding tanks are ≈ 800 and ≈ 700 L in volume and house ≈ 40 and ≈ 20 fish with complete turnover with UV sterilized water every ≈ 38 and ≈ 3 hours, respectively (Glenn Morgan and Anthony Squires, TropWATER Facility Technicians; personal communication). The second *in situ* validation (field validation; *in situ* Test 2) captured eDNA in water samples (*n* = 12) collected from a freshwater lotic system in Townsville, QLD Australia (Ross River under Nathan Street bridge: 19° 18' 21.96" S, 146° 45' 38.52" E) wherein *O. mossambicus* is known to occur (Russell, et al., 2012).

Water samples for *in situ* Tests 1 and 2 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 EDTA 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).

In situ Tests 1 and 2 were run in quadruplicate 10 µL technical qPCR using the same chemistry as *in vitro* tests (see Section 2.3) but with 3µL extracted eDNA from water samples as template (see above). Master mix for both *in situ* tests was loaded as per *in vitro* tests but with 3 µL eDNA template loaded in dedicated low copy DNA room within MEEL. Both *in situ* plates were sealed, vortexed, spun, run, and analysed as described above for *in vitro* tests.

Any single amplification products produced during *in situ* Test 1 that exhibited T_m within 99.7% CI of both species gDNA standards (see above) were considered putative positive detections for target species (*O. mossambicus* or *T. mariae*). Single amplification products generated during *in situ* Test 2 that exhibited T_m within 99.7% CI of *O. mossambicus* gDNA standards (see above) were considered putative positives for *O. mossambicus* detection. Representative putative positive detections from *in situ* Test 2 (*n* = 12) were Sanger sequenced bidirectionally for confirmation of positive *O. mossambicus* eDNA detection.

3. Results

3.1 Primer design

Optimal forward and reverse primer binding regions that are conserved across both *O. mossambicus* and *T. mariae 16S* nucleotide sequences were identified between base pairs 1273-1290 (Tilapia_16S_v2-F1) and 1377-1396 (Tilapia_16S_v2-R1; Table 3). Revised assay primers exhibited satisfactory compliance with all quality, accuracy, and efficiency parameters (see Section 2.1, Table 3).

Note that initial tilapia eDNA assay primers ("16S Oreo-F" and "16S Tilapia-R"; Nobel, et al., 2014; Le Port, et al., 2016; Robson, et al., 2016) have no mismatches with *O. mossambicus 16S* nucleotide sequence but do have three mismatches with *T. mariae 16S* nucleotide sequences (Figure 1).

3.2 *In silico* validation

Based on *16S* sequence alignments of *O. mossambicus* and *T. mariae* with Australian fishes, frogs, and turtles (Table 1) primers designed to target regions wherein no other Australian species are 100% homologous (i.e., no Australian species with zero mismatches to either primer).

Initial targeted *in silico* verification (see Section 2.2) confirmed that, other than tilapia species, no other specified species (Table 2) are identical (i.e., zero mismatches) to Tilapia_16S_v2 assay primers. 15 species on the targeted *in silico* list do exhibit two to five total mismatches with Tilapia_16S_v2 primers; however, all of these non-target species have predicted amplicon lengths smaller (1–5 bp) or larger (2–22 bp) than amplicon produced from target tilapia species (101 bp, i.e., discernible using dissociation curve analysis; Table 5).

Subsequent non-targeted *in silico* verification (see Section 2.2) returned 28 species with no mismatches and correct amplicon length predictions (i.e., undiscernible amplification expected if DNA present within sample); however, of these 28 species only *O. mossambicus* and *T. mariae* are known to occur in Australia (Table 6). Of the 60 species with one mismatch and 104 species with two mismatches only two (*Hypoatherina temminckii* and *Tilapia zillii*) and 12 (*Chelidonichthys kumu, Fistularia petimba, Lepidotrigla argus, Lepidotrigla papilio, Lepidotrigla spinose, Sciadonus galatheae, Anguilla bicolor bicolor, Entomacrodus decussatus, Microstoma microstoma, Nansenia ardesiaca, Platycephalus caeruleopunctatus,* and *Platycephalus speculator*) are known to occur within Australia (http://fishesofaustralia.net.au), respectively (Table 6).

Table 5. Species with ≤ 5 mismatches to Tilapia_v2_16S based on targeted PrimerBLAST search of Australian fish, frog, and turtle sequences in NCBI "nr" database. GenBank accession numbers provided in square brackets for two discrete Oreochromis niloticus *entries. Note that all species with ≥ 2 mismatches have a divergent predicted amplicon size compared to target species (0 or 1 mismatch).*

Forward mismatches	Reverse mismatches	Species	Predicted amplicon
0	0	*Oreochromis mossambicus*	101 bp
		Oreochromis niloticus (*Tilapia nilotica*)	101 bp
		Tilapia mariae (*Pelmatolapia mariae*)	101 bp
0	1	*Oreochromis niloticus* isolate J07 [**GQ167969.1**]	101 bp
		Oreochromis niloticus isolate Asejire_wild_tilapia_S2.4 [**MH567042.1**]	101 bp
1	1	*Anguilla bicolor bicolor*	98 bp
0	3	*Channa lucius*	96 bp
		Channa bankanensis	98 bp
1	2	*Oncorhynchus mykiss*	99 bp
		Channa marulius	98 bp
		Channa marulia	98 bp
		Anguilla obscura	98 bp
		Anguilla bicolor bicolor	98 bp
		Anguilla bicolor pacifica	98 bp
1	3	*Salmo trutta fario*	99 bp
		Salmo trutta macrostigma	96 bp
		Lates calcarifer	123 bp
		Amphilophus citrinellus	103 bp
2	2	*Anguilla bicolor bicolor*	98 bp
		Galaxias maculatus	100 bp
1	4	*Salmo trutta fario*	99 bp
3	2	*Megalops cyprinoides*	99 bp

Table 6. Species with ≤ 2 mismatches to Tilapia_v2_16S based on non-targeted PrimerBLAST search of entire NCBI "nr" database. Target species are indicated by highhat (^). Species endemic to or observed in Australia are indicated by asterisks (*). Note that Anguilla bicolor bicolor *is the only freshwater species.*

Forward mismatches	Reverse mismatches	Species	
0	0	*Bathybates ferox*	*Oreochromis tanganicae*
		Bathybates graueri	*Oreochromis variabilis*
		Chalinochromis popelini	*Pelmatochromis buettikoferi*
		Chilochromis duponti	*Pelmatolapia mariae^*
		Grammatotria lemairii	*Sarotherodon caudomarginatus*
		Konia eisentrauti	*Sarotherodon galilae*
		Ophthalmotilapia ventralis	*Sarotherodon galilaeus sanagaensis*
		Oreochromis andersonii	*Sarotherodon lohbergeri*
		Oreochromis aureus	*Sarotherodon melanotheron*
		Oreochromis esculentus	*Sarotherodon mvogoi*

		Oreochromis karongae	*Sarotherodon steinbachi*
		Oreochromis macrochir	*Stomatepia mariae*
		*Oreochromis mossambicus^*	*Tilapia mariae^*
		Oreochromis niloticus	*Tristramella simonis*
1	0	*Etia nguti*	
0	1	*Altolamprologus compressiceps*	*Orthochromis stormsi*
		Astatoreochromis alluaudi	*Pelmatolapia cabrae*
		Boulengerochromis microlepis	*Perissodus microlepis*
		Callochromis pleurospilus	*Peristedion gracile*
		Chalinochromis brichardi	*Petrochromis trewavasae*
		Coptodon bakossiorum	*Pseudocrenilabrus multicolor*
		Coptodon bemini	*Pseudocrenilabrus philander*
		Coptodon bythobathes	*Serranochromis robustus*
		Coptodon cameruensis	*Steatocranus tinanti*
		Coptodon deckerti	*Steatocranus bleheri*
		Coptodon snyderae	*Steatocranus casuarius*
		Ctenochromis horei	*Steatocranus gibbiceps*
		Ctenochromis pectoralis	*Steatocranus tinanti*
		Cyprichromis leptosoma	*Steatocranus ubanguiensis*
		Ectodus descampsii	*Tilapia brevimanus*
		Eretmodus cyanostictus	*Tilapia busumana*
		Gnathochromis permaxillaris	*Tilapia buttikoferi*
		Gobiocichla ethelwynnae	*Tilapia dageti*
		Helostoma temminckii	*Tilapia discolor*
		Heterotilapia buttikoferi	*Tilapia guineensis*
		*Hypoatherina temminckii**	*Tilapia louka*
		Hypoatherina tsurugae	*Tilapia zillii^*
		Iranocichla hormuzensis	*Trematochromis benthicola*
		Julidochromis regani	*Tropheus duboisi*
		Lamprologus callipterus	*Tropheus moorii*
		Lepidiolamprologus elongatus	*Tylochromis leonensis*
		Limnochromis abeelei	*Tylochromis polylepis*
		Neolamprologus pulcher	*Variabilichromis moorii*
		Orthochromis polyacanthus	*Xenotilapia ornatipinnis*
0	2	*Alcolapia alcalica*	*Lepidotrigla argus**
		Alticorpus geoffreyi	*Lepidotrigla cavillone*
		Aristochromis christyi	*Lepidotrigla papilio**
		Astatoreochromis alluaudi	*Lepidotrigla spinosa**
		Aulonocara baenschi	*Lethrinops lethrinus*
		Aulonocara stuartgranti	*Leuresthes tenuis*
		Bellator xenisma	*Limbochromis robertsi*
		Benitochromis batesii	*Maylandia estherae*
		Benitochromis nigrodorsalis	*Maylandia zebra*
		Betta falx	*Metriaclima zebra*
		Betta picta	*Neochromis rufocaudalis*
		Buccochromis nototaenia	*Neolamprologus brichardi*
		Cheilochromis euchilus	*Neolamprologus modestus*
		Chelidonichthys capensis	*Orthochromis kalungwishiensis*
		*Chelidonichthys kumu**	*Parananochromis brevirostris*

		Chelidonichthys lucernus	*Parananochromis longirostris*
		Chilotilapia rhoadesii	*Pelmatochromis nigrofasciatus*
		Chromidotilapia guntheri	*Pelvicachromis humilis*
		Chromidotilapia regani	*Pelvicachromis roloffi*
		Copadichromis mloto	*Pelvicachromis rubrolabiatus*
		Copadichromis quadrimaculatus	*Pelvicachromis signatus*
		Copadichromis trimaculatus	*Petrotilapia nigra*
		Cyathochromis obliquidens	*Placidochromis longimanus*
		Cynotilapia afra	*Platybelone argala*
		Cyphotilapia frontosa	*Platytaeniodus degeni*
		Cyphotilapia gibberosa	*Protomelas annectens*
		Cyrtocara moorii	*Pseudotropheus crabro*
		Dimidiochromis compressiceps	*Pseudotropheus zebra*
		Dimidiochromis kiwinge	*Ptychochromoides itasy*
		Dimidiochromis strigatus	*Pundamilia nyererei*
		Fistularia corneta	*Rhabdoblennius nitidus*
		*Fistularia petimba**	*Sciadonus galatheae**
		Floridichthys carpio	*Serranochromis robustus*
		Fossorochromis rostratus	*Steatocranus irvinei*
		Genyochromis mento	*Stenatherina panatela*
		Haplochromis burtoni	*Strongylura fluviatilis*
		Haplochromis ishmaeli	*Strongylura marina*
		Haplochromis piceatus	*Thoracochromis brauschi*
		Haplochromis simpsoni	*Tilapia ruweti*
		Helostoma temminkii	*Tilapia sparrmanii*
		Hemichromis fasciatus	*Tilapia tholloni*
		Hemitilapia oxyrhyncha	*Trematocranus placodon*
		Labidochromis caeruleus	*Trigloporus lastoviza*
		Laetacara thayeri	
1	1	*Anguilla bicolor bicolor**	*Nansenia boreacrassicauda*
		Brachymystax lenok	*Nansenia candida*
		Cleithracara maronii	*Nimbochromis linni*
		Coelotilapia joka	*Parahucho perryi*
		*Entomacrodus decussatus**	*Platycephalus caeruleopunctatus**
		Hypoatherina lunata	*Platycephalus speculator**
		Laetacara thayeri	*Tilapia nyongana*
		*Microstoma microstoma**	*Tilapia joka*
		*Nansenia ardesiaca**	

3.3 *In vitro* validation

The revised Tilapia_16S_v2 assay exhibited satisfactory efficiency and LOD based on both aDNA and gDNA standard curves (see Section 2.3).

In vitro Test 1 demonstrated that at 60˚C with 500nM each primer (optimal conditions) Tilapia_16S_v2 amplified standards 1–8 ($2.95 \pm 0.254 \times 10^7$ – 2.95 ± 0.254 copies/µL; see Section 2.3), respectively, with 96.666% efficiency ($R^2 = 0.998$) and minimal yet discernible

primer dimerization (dimer T_m > 10˚C from assay amplicon T_m). LOD was determined to be approx. 9 ± 1 copies loaded (Standard 8; Figure 2). Note that additional $\log_2$ serial dilutions were not run to empirically test aDNA detectability down to 1 copy loaded; however, the strong correlation coefficient of the 8-point $\log_{10}$ standard curve (R_2 = 0.998) permits confident extrapolation down to a LOD of 1 copy (i.e., only one $\log_{10}$ dilution or ≈ 3.3 cycles later than Standard 8).

In vitro Test 2 demonstrated that at 60˚C with 500nM each primer was also optimal for the amplification of both *O. mossambicus* and *T. mariae* gDNA standard curves (Figure 3). More specifically, Tilapia_16S_v2 assay amplified the top 6-points of the 8-point $\log_{10}$ *O. mossambicus* and *T. mariae* gDNA standard curves (see Section 2.3) with 102.6% (R_2 = 0.993) and 97.4% (R_2 = 0.997) efficiency (Figure 3) and minimal yet discernible primer dimerization (see above). LOD for *O. mossambicus* and *T. mariae* was gDNA Standard 6 (0.796 ± 0.017 x 10^{-5} and 0.844 ± 0.157 x 10^{-5} ng gDNA loaded) or one to two and one to four copies loaded based on aDNA standard curve extrapolation, respectively. Note that only two of three technical replicates for *O. mossambicus* Standard 6 amplified while no replicates from *O. mossambicus* or *T. mariae* gDNA Standards 7 or 8 amplified, which supports gDNA Standard 6 being exactly or within one order of magnitude (i.e., one $\log_{10}$ dilution or 3.3 qPCR cycles) of the lowest possible LOD of one copy loaded. Sanger sequencing of representative Standard 1 amplicons for *O. mossambicus* and *T. mariae* (*n* = 2 each) confirmed these to be positive detection (96.2–99% and 97.1–99% pairwise identity with GenBank accessions KU500883 and GQ168026) for target species, respectively.

In vitro Test 3 demonstrated that revised Tilapia_16S_v2 generates the same number of *16S* copies per ng of gDNA loaded for both *O. mossambicus* and *T. mariae* (45,066 ± 1,376 and 41,379 ± 3,847; two-tailed t-test *p* = 0.3301; average ± SD), respectively. Note that both initial (Nobel, et al., 2014; Robson, et al., 2016) and revised tilapia assays generate the same number of *16S* copies per ng of gDNA loaded for *O. mossambicus* (45,831 ± 464 and 41,379 ± 3,847; two-tailed t-test *p* = 0.2457) but significantly different *16S* copy numbers per ng of gDNA loaded for *T. mariae* (579 ± 16 and 45,066 ± 1,376; two-tailed t-test *p* = 0.00048), respectively. Sanger sequencing of non-target amplifications matched tilapia *16S* sequence and not *16S* sequence of tested non-target species (see Table 4), thus confirming that the observed amplifications for non-target species (Figure 4) to be false positives due to cross-contamination during *in vitro* plate loading (e.g., aerosolization).

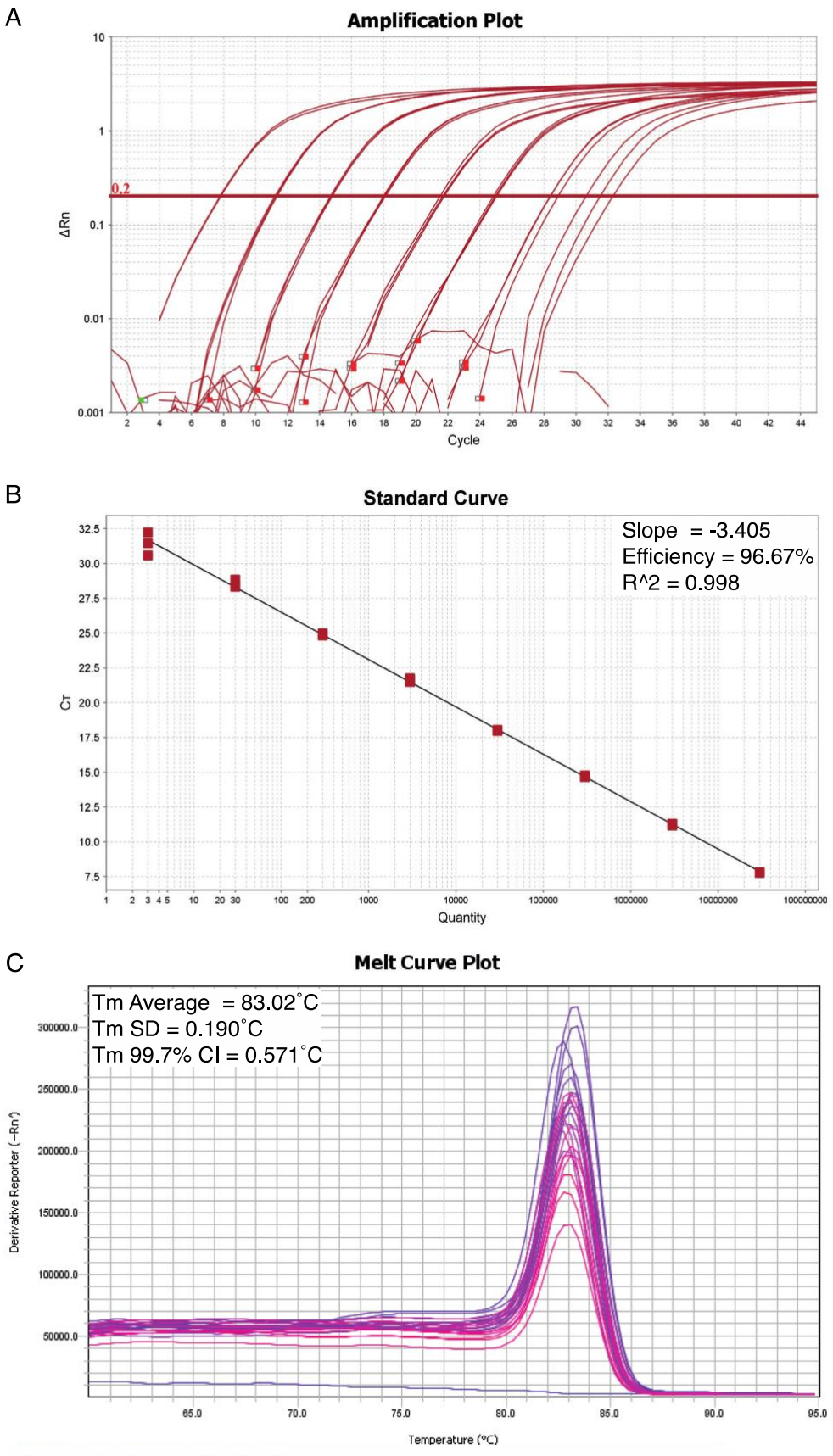

Figure 2. Amplification curves (A), 8-point log₁₀ aDNA standard curve linear regression (B), and amplicon dissociation temperature curves (T_m; C) generated by qPCR during in vitro *Test 1 of Tilapia_16S_v2 (see Section 2.3).*

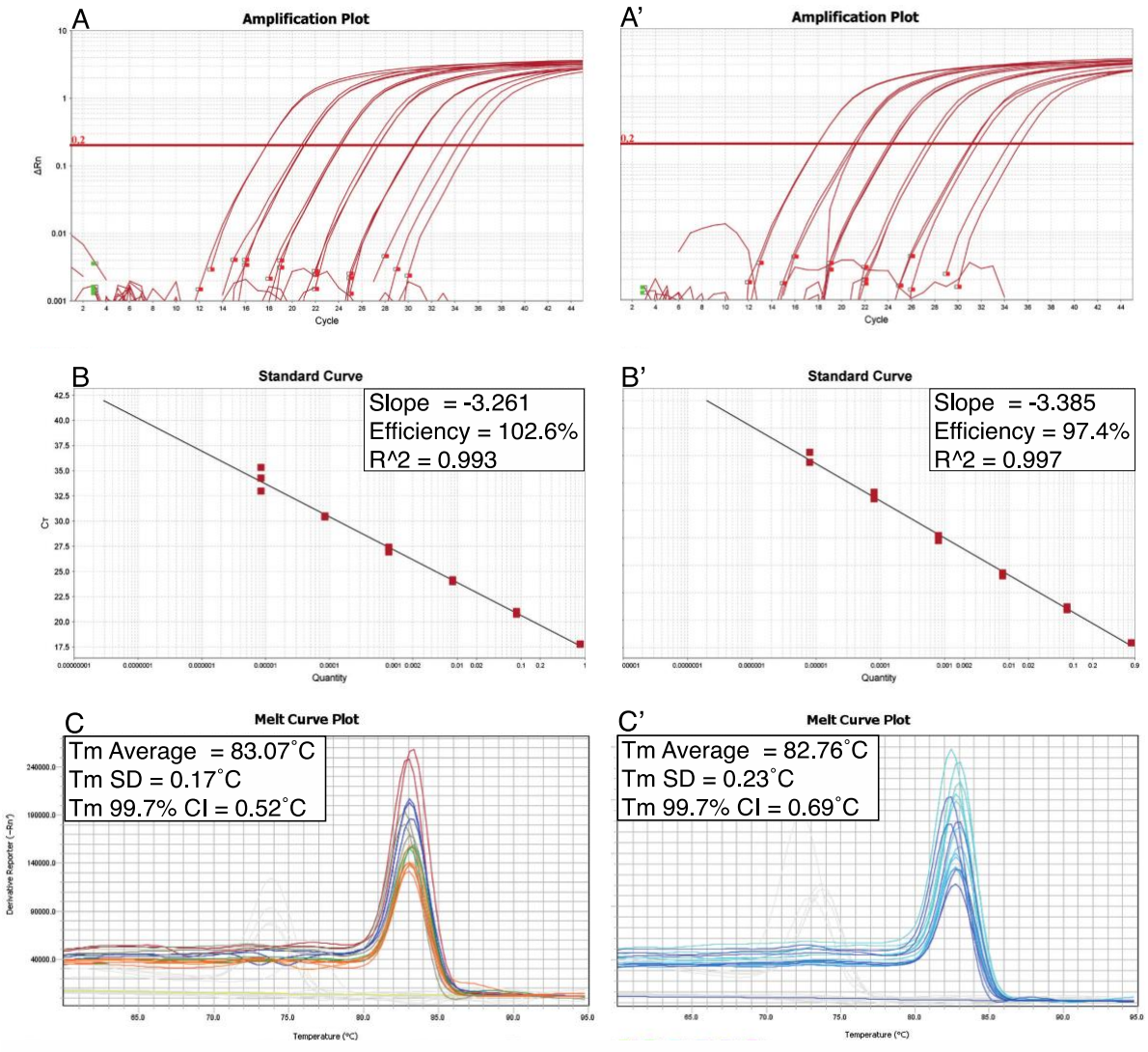

Figure 3. Oreochromis mossambicus *and* Tilapia mariae *gDNA standards amplification curves (A, A'), 6-point* log_{10} *gDNA standard curve linear regressions (B, B'), and dissociation temperature curves (*T_m*; C, C') generated by qPCR during* in vitro *Test 2 of Tilapia_16S_v2, respectively (see Section 2.3).*

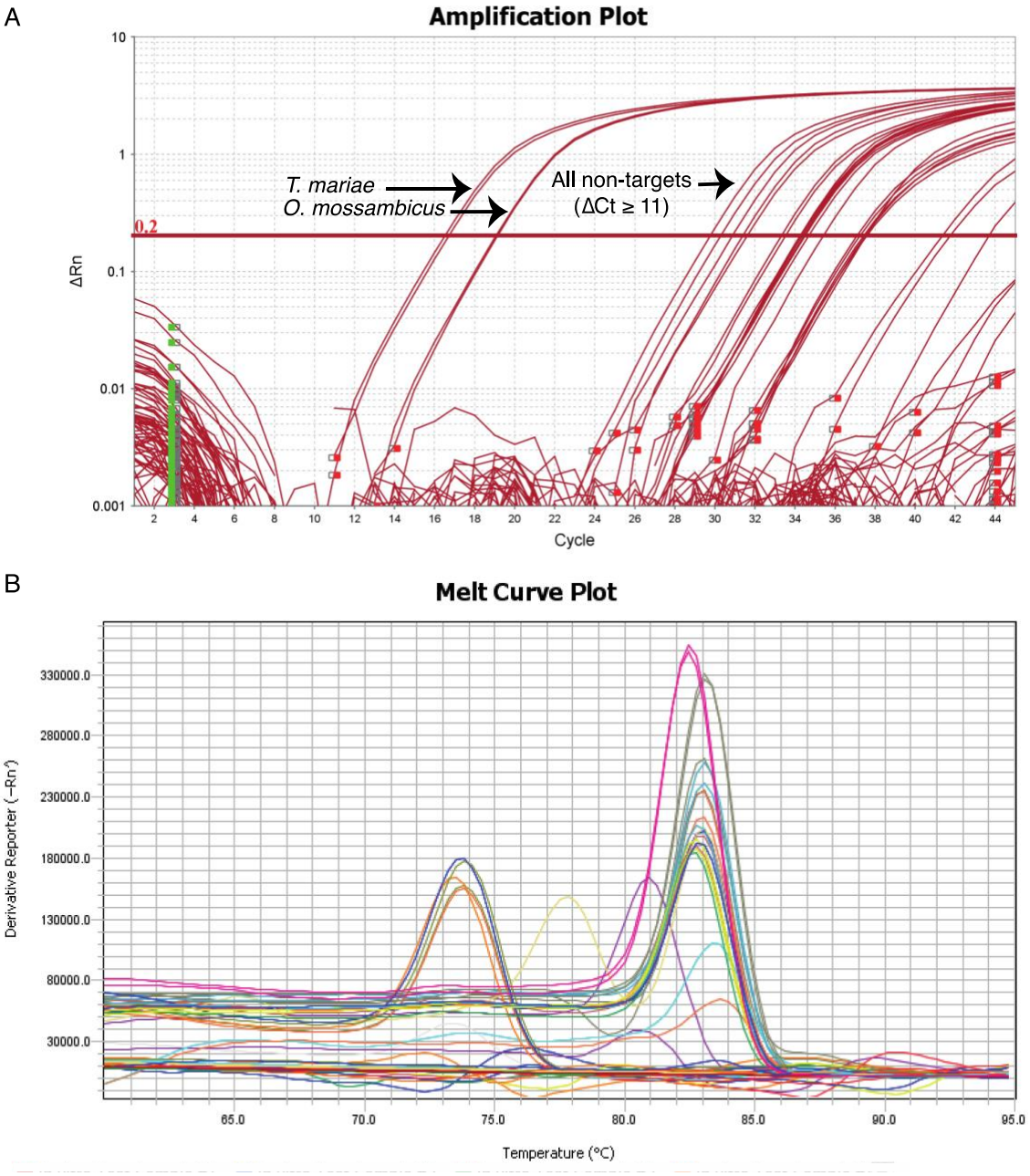

Figure 4. Amplification curves (A) and dissociation temperature curves (T_m; B) from Tilapia_16S_v2 in vitro *Test 3 (Sections 2.3 and 3.3; Table 4). Amplification was observed for both* O. mossambicus *and* T. mariae *gDNA during* in vitro *test (Table 4), as expected (2 main peaks; panel B). Amplification was also observed for five non-target fish species (*Craterocephalus stercusmuscarum, Glossamia aprion, Hephaestus carbo, Mogurnda adspersa*, and* Nematalosa erebi*); however, all observed non-target amplifications were ≥ 11 qPCR cycles later than target species amplification (panel A) and confirmed to be false positive detections by bidirectional Sanger sequencing (see Section 3.3).*

3.4 *In situ* validation

Both Tilapia_16S_v2 assay *in situ* validation tests (see Section 2.4) resulted in positive detections of tilapia eDNA (Figure 5 and Figure 6).

Tilapia_16S_v2 assay *in situ* validation Test 1 (positive control *in situ* validation; see Section 2.4) yielded 100% (*n* = 12/12) positive detection rate for tilapia eDNA from both *O. mossambicus* and *T. mariae* holding tanks with an average of ≈ 1,807 and ≈ 956 copies loaded per assay, respectively (Figure 5).

Tilapia_16S_v2 assay *in situ* validation Test 2 (field *in situ* validation; see Section 2.4) yielded an 89.58% (*n* = 43/48) positive detection rate for *O. mossambicus* eDNA (Figure 6) with an average of 5.487 ± 0.745 (± SEM) copies loaded per assay. The majority of Sanger sequenced representative amplicons (*n* = 10/12) exhibited 98.5-100% pairwise identity with GenBank accession KU500883. The remaining representative samples (*n* = 2/12) were of poor quality sequence and unable to be confirmed by NCBI BLAST but given amplicon T_m within 99.7% CI of *O. mossambicus* gDNA standards (like other representative amplicons Sanger sequenced) these two detections were considered positive for *O. mossambicus* eDNA detection.

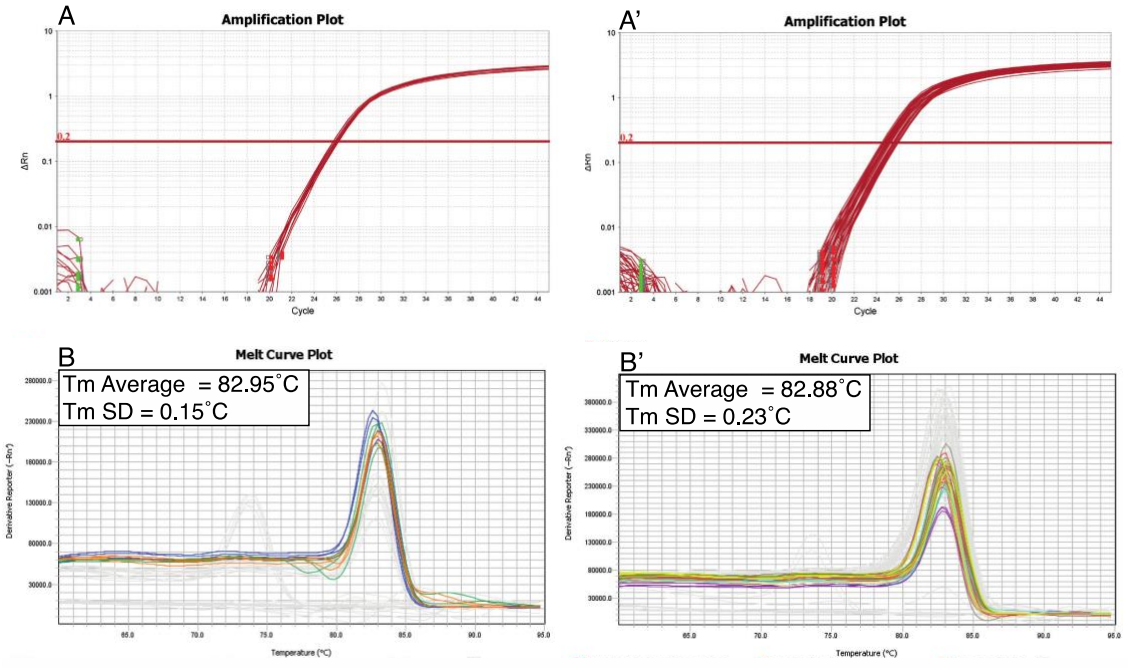

Figure 5. Amplification curves (A, A') and dissociation curves (T_m; B, B') resulting from in situ *validation Test 1 for* O. mossambicus *and* T. mariae *eDNA (positive control* in situ *validation; see Section 2.4), respectively.*

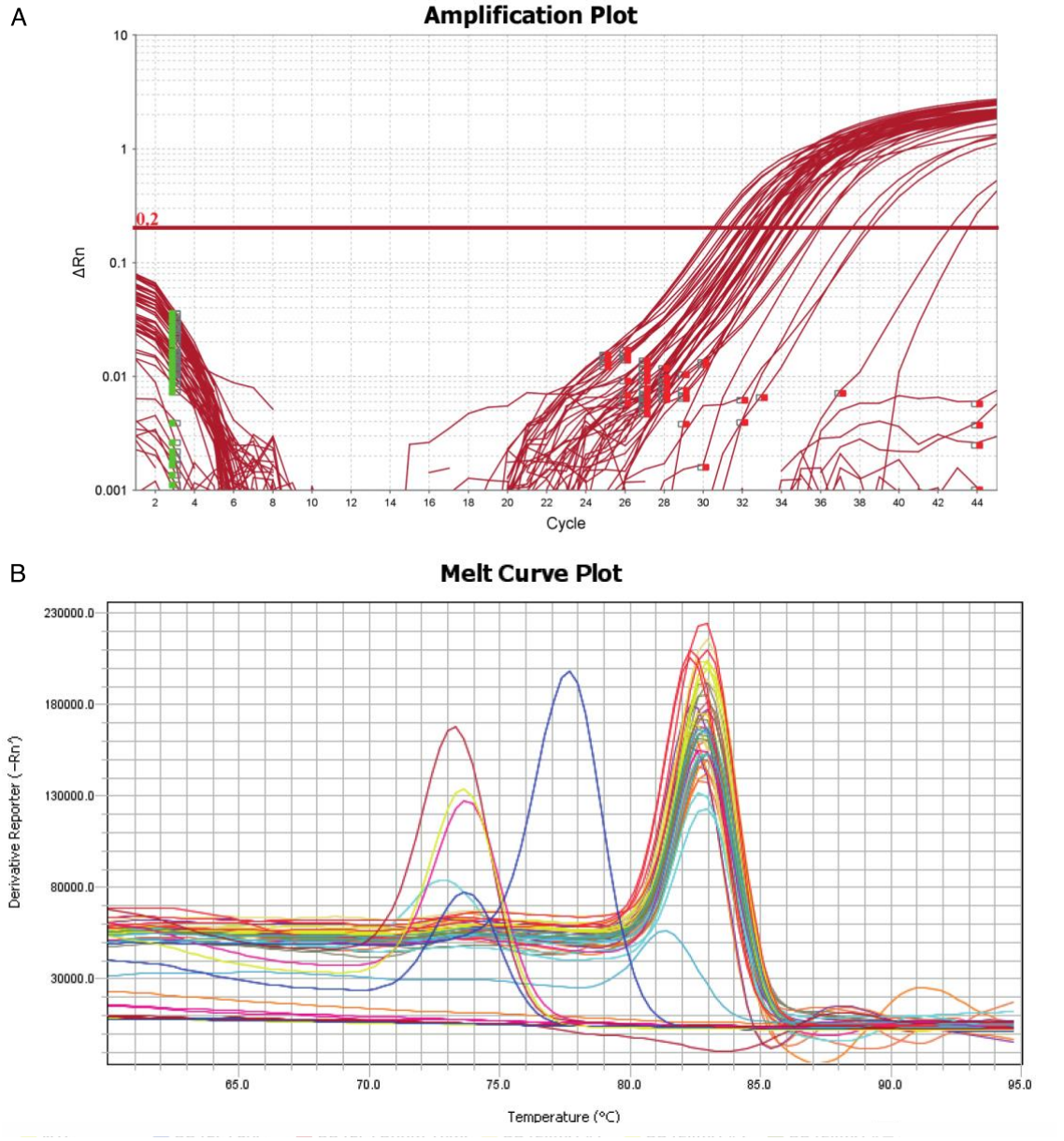

Figure 6. Amplification curves (A) and dissociation curves (B) resulting from Tilapia_16S_v2 in situ *validation Test 2 on eDNA captured from Ross River in Townsville Queensland Australia (see Section 2.4). All bidirectional Sanger sequenced representative amplicons were positive for* O. mossambicus *(see Section 3.4).*

4. Discussion

Two tilapia species – *Oreochromis mossambicus* and *Tilapia mariae* – are significant invasive fish pests in Australia. A new environmental DNA (eDNA) assay was developed for the detection of these species in water samples using Sybr-based quantitative real-time polymerase chain reaction (qPCR). This eDNA assay targets tilapia 16S gene.

Geneious analysis software (version R11; Kearse et al., 2012) was used to obtain all available *16S* nucleotide sequences from 86 Australian fish 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 the two tilapia target species by attempting to PCR amplify 16S from genomic DNA (gDNA) extracted from *O. mossambicus* and *T. mariae* and 42 non-target fish, frog, toad and turtle species. Finally an *in situ* test was performed using water samples from tanks and/or sites known to contain these tilapia species.

The revised Tilapia_16S_v2 assay that was developed and described herein passed all *in silico, in vitro,* and *in situ* validations. Accordingly, this revised eDNA assay for *O. mossambicus* and *T. mariae* can be used to confidently determine presence of either species within environmental water samples.

The revised Tilapia_16S_v2 assay can detect *O. mossambicus* eDNA as efficiently as initial tilapia assay (Nobel, et al., 2014; Robson, et al., 2016); however, and most notably, Tilapia_v2_16S is superior for concurrent detection of *T. mariae*. In light of Tilapia_v2_16S demonstrating efficient and concurrent detection of both tilapia species we recommend utilization of this assay for assessment of environmental water samples anticipated to contain spotted tilapia (*T. mariae*) or both tilapia species. Tilapia_v2_16S can be used to screen water samples collected from locations suspected of harbouring invasion fronts or following eradication efforts.

Lastly, Sanger sequencing confirmation is particularly recommended if environmental water samples are collected from freshwater sites wherein *Anguilla bicolor bicolor* is known to occur (e.g., north-western Australia; http://fishesofaustralia.net.au/home/species/1424) given the potential for cross-amplification; however, this is unexpected at 60˚C annealing (see Table 5).

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