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Development of eDNA assays for monitoring three endangered frog species (*Litoria dayi*, *L. lorica* and *L. nannotis*) in Australia's wet tropics

Report

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TropWATER
Centre for Tropical Water and Aquatic Ecosystem Research

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Cover photographs

Front cover: *Litoria dayi* (photo Trent Townsend/Shutterstock.com).

Back cover: *Litoria lorica* (left) and *L. nannotis* (right) *in situ* (photo: Conrad Hoskin).

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Acronyms

BLAST..... Basic Local Alignment Search Tool

NESP..... National Environmental Science Program

IUCN..... International Union for the Conservation of Nature

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

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We thank Dr. Conrad Hoskin for *Litoria* spp. tissue samples from Queensland populations for *in vitro* validation of each assay and for guiding field trip to Mt. Carbine area (*in situ* validations).

Executive summary

Three environmental DNA (eDNA) assays (Litday_COI, Litlor_COI, and LnannotisN&P_COI) were developed for discrete detection of three endangered Australian treefrogs (*Litoria dayi*, *L. lorica*, and *L. nannotis*) in water samples using Sybr-based quantitative real-time polymerase chain reaction (qPCR), respectively. Litday_COI, Litlor_COI, and LnannotisN&P_COI target species-specific 110, 166, and 119 base pair (bp) regions within mitochondrial cytochrome oxidase I (*COI*) gene, respectively. Here we present *in silico* and *in vitro* validations for all three assays plus *in situ* validations for Litlor_COI and LnannotisN&P_COI.

In silico, *in vitro* and, *in situ* validations confirmed that each assay is specific to (i.e., detects only) *L. dayi, L. lorica,* or *L. nannotis* despite co-occurrence with native species. Limit of detection (LOD) for Litday_COI, Litlor_COI, and LnannotisN&P_COI was determined to be 4.38×10^{-5}, 4.91×10^{-5}, and 4.83×10^{-5} nanograms gDNA (or 2 *COI* copies) loaded under optimal assay conditions (60˚C annealing, 500 nM each primer), respectively. Moreover, bidirectional Sanger sequencing confirmed all representative putative positive detections from *in vitro* or *in situ* validations (*Litoria* spp. gDNA or Mount Carbine area water samples), respectively, to be positive for target species while all putative negative detections from *in vitro* validation (non-target species amplifications) were confirmed negative (i.e., false positives). Collectively, these validations demonstrate the readiness of Litday_COI, Litlor_COI, and LnannotisN&P_COI for screening environmental water samples to detect low-copy eDNA shed from *L. dayi, L. lorica,* or *L. nannotis*, respectively.

Incorporation of eDNA monitoring can assist ongoing conservation efforts for endangered populations of *Litoria* spp. frogs in upland and lowland regions of northern Australia wet tropics. To help locate extant populations and prioiritise locations targeted by visual survey efforts rainforest waterways can be monitored for eDNA shed from all three *Litoria* spp. using Litday_COI, Litlor_COI, and LnannotisN&P_COI.

1. Introduction

Since the 1980s rainforest frogs endemic to eastern Australian highlands have undergone dramatic reductions in high elevation populations (≥ 90%; Laurance, et al., 1996). These declines have been linked to outbreaks of Chytridriomycosis caused by *Batrachochytrium dendrobatidis* fungus, which thrives in moist cool environments (Puschendorf, et al., 2011). Of the most impacted species are the lace-eyed tree frog (*Litoria dayi*), armored mist frog (*L. lorica*), and waterfall frog (*L. nannotis*), all of which are stream-dwelling and stream-breeding rainforest specialists endemic to lowland and upland regions of the northern Australia wet tropics (Paluma to Cooktown; Hero and Fickling, 1994).

Litoria dayi resides at altitudes between 0 and 1,200 meters (McDonald, 1992; Williams and Hero, 1998; 2001) and is an endangered species under International Union for the Conservation of Nature (IUCN; Berger, et al., 1999; AmphibiaWeb, 2008a). *Litoria dayi* has disappeared from upland sites and was last observed at Mt Spec State Forest in 1990 and Kirrama Range in 1989 (Richards et al., 1993). In mountainous areas *L. dayi* prefers fast-flowing rocky streams or slow-flowing waterways with ample vegetation (Czechura et al. 1987) while lowland populations favour rock soaks, narrow ephemeral streams, and rock outcrops in larger waterways (Czechura et al., 1987).

Litoria lorica resides at altitudes between 640 and 1,000 meters (McDonald, 1992; Hero and Fickling, 1994) and is a critically endangered species under IUCN (Berger, et al., 1999; AmphibiaWeb, 2008b). Previous to July 2008 sighting, *L. lorica* had not been observed since December 1991 and was thought to possibly be extinct (Cunningham, 2002); however, a newly discovered Mt. Carbine (Tablelands, Queensland) population shares habitat with a *L. nanno*tis population (AmphibiaWeb, 2008b). Moreover, *L. lorica* prefers fast flowing streams and generally resides on granite boulders in the splash zone (Davies and McDonald, 1979).

Litoria nannotis resides at altitudes between 180 and 1,300 meters (McDonald, 1992; Hodgkison and Hero, 2001) and is an endangered species under IUCN (Berger, et al., 1999; AmphibiaWeb, 2008c). The first *L. nannotis* population decline was noted in 1990 when it disappeared from most upland sites south of Daintree River; however, populations were observed at all lowland sites and at upland sites north of Daintree River in 1991-1992 (Richards et al., 1993). Moreover, *L. nannotis* is restricted to rocky stream habitats where there is fast flowing water, waterfalls, and cascades (Liem, 1974; McDonald, 1992). Of note is that, unlike most stream-breeding frog species that live in adjacent forest and use the stream habitat for breeding only, the stream constitues primary habitat for *L. nannotis* (Hodgkison and Hero, 2001).

To assist with on-going conservation efforts for endangered populations of *Litoria* spp. frogs in upland and lowland regions of northern Australia wet tropics we developed Litday_COI, Litlor_COI, and LnannotisN&P_COI assays to monitor for *L. dayi, L. lorica,* and *L. nannotis* eDNA in rainforest waterways, respectively. These assays help locate extant populations of frog species of conservation value and prioiritise locations for visual survey.

2. Methodology

2.1 Primer design

GenBank (NCBI) was mined for available *COI* nucleotide sequences from Australian frog species (*n* = 65; Table 1) given use of *COI* for barcoding studies and greater abundance within environmental samples (i.e., greater detectability) due to multiple mitochondria present within each cell (see Goldberg, et al., 2016). However, seven potentially co-occurring species did not have *COI* sequence(s) available on GenBank as of 5 June 2019 (*Crinia remota, Litoria daviesae, Litoria wilcoxii, Platyplectrum ornatum, Pseudophryne major, Uperoleia inundata,* and *Uperoleia lithomoda*). All available *COI* sequences were downloaded into Geneious Prime analysis software (ver. 2019.1.3) and subsequently aligned using embedded ClustalW algorithm. Regions of conservation within *COI* alignments of *L. dayi* (*n* = 4), *L. lorica* (*n* = 2), and *L. nannotis* (*n* = 36 total or *n* = 20, 7, and 9 for "Central", "Paluma", and "North" populations, respectively) were identified, annotated, and assessed by eye for regions wherein each *Litoria spp.* exhibited ≥ 1 base pair mismatch(es) with each other target *Litoria* spp., potentially co-occurring Australian frogs, and human *COI* 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 ≤ 2.5˚C difference between primer pair, 2) G/C content: 40 – 55%, 3) length: 18 – 22 bp. 4) amplicon size: 100 – 200bp, 5) self-dimer T_m: ≤ 25˚C, 6) hairpin T_m: ≤ 35˚C, 7) overall self-complementarity: PrimerBLAST score ≤ 7, and 8) 3' self-complementarity: PrimerBLAST score ≤ 5.

Table 1. Non-target Australian frog species for which mitochonrial COI nucleotide sequences were obtained from GenBank (NCBI) and used to guide development of Litday_COI, Litlor_COI, and LnannotisN&P_COI.

Non-target Australian frog species		
Amnirana darlingi	*Hylarana macrodactyla*	*Neobatrachus centralis*
Chalcorana eschatia	*Hylarana magna*	*Neobatrachus fulvus*
Clinotarsus curtipes	*Hylarana malabarica*	*Neobatrachus kunapalari*
Cophixalus cryptotympanum	*Hylarana montana*	*Neobatrachus pelobatoides*
Crinia bilingual	*Hylarana serendipi*	*Neobatrachus pictus*
Crinia tinnula	*Hylarana sreeni*	*Neobatrachus sudelli*
Humerana lateralis	*Hylarana taipehensis*	*Neobatrachus sutor*
Hylarana aurantiaca	*Hylarana temporalis*	*Neobatrachus wilsmorei*
Hylarana caesari	*Hylarana tytleri*	*Nyctimystes dayi*
Hylarana cf. danieli	*Hylarana urbis*	*Rana nigrovittata*
Hylarana cf. humeralis	*Indosylvirana aurantiaca*	*Rana latouchii*
Hylarana cf. leptoglossa	*Indosylvirana milleti*	*Rana spinulosa*
Hylarana cf. tytleri	*Litoria aurea*	*Rana guentheri*
Hylarana doni	*Litoria caerulea*	*Rana macrodactyla*
Hylarana erythraea	*Litoria eucnemis*	*Sylvirana cubitalis*
Hylarana eschatia	*Litoria genimaculata*	*Sylvirana faber*
Hylarana flavescens	*Litoria nannotis*	*Sylvirana guentheri*
Hylarana gracilis	*Litoria raniformis*	*Sylvirana latouchii*

Hylarana hekouensis	*Litoria rheocola*	*Sylvirana maosonensis*
Hylarana indica	*Litoria serrata*	*Sylvirana menglaensis*
Hylarana intermedia	*Neobatrachus albipes*	*Sylvirana nigrovittata*
Hylarana labialis	*Neobatrachus aquilonius*	

2.2 *In silico* validation

Following primer design for *L. dayi, L. lorica*, and *L. nannotis COI* assays (hereafter referred to as Litday_COI, Litlor_COI, and LnannotisN&P_COI, respectively), all three were tested *in silico* (i.e., virtual determination of potential PCR amplification of non-target species using specific primer pairs; Macdonald and Sarre, 2017) using both targeted and non-targeted searches of NCBI "nr" database via PrimerBLAST (Ye, et al., 2012). Initial targeted PrimerBLAST specified a list of fish, frog, and turtle species found in Australia against which each assay was tested *in silico* (Table 2). Subsequent non-targeted PrimerBLAST searches (i.e., no species specified) test each assay against all *COI* sequences in NCBI "nr" database to ascertain which, if any, species might be cross-amplified with Litday_COI, Litlor_COI, or LnannotisN&P_COI. Species with ≤ 7 and ≤ 2 base pair mismatches to Litday_COI, Litlor_COI, or LnannotisN&P_COI were documented for targeted and non-targeted in silico tests, respectively.

Following satisfactory compliance of Litday_COI, Litlor_COI, and LnannotisN&P_COI with both targeted and non-targeted *in silico* tests, 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 Litday_COI, Litlor_COI, and LnannotisN&P_COI were tested in silico *using targeted PrimerBLAST search of NCBI "nr" database for COI sequences of fish, frogs, and turtles found in Australian freshwater systems.*

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	*Hypseleotris compressa*	*Neoceratodus forsteri*
Amphilophus citrinellus	*Kuhlia marginata*	*Neosilurus* spp.
Anabas testudineus	*Kuhlia rupestris*	*Neosilurus ater*
Anguilla australis	*Lates calcarifer*	*Neosilurus pseudospinosus*
Anguilla bicolor	*Leiopotherapon unicolor*	*Oncorhynchus mykiss*
Anguilla obscura	*Lepidogalaxias salamandroides*	*Oreochromis mossambicus*
Anguilla reinhardtii	*Maccullochella ikei*	*Oreochromis niloticus*
Arius berneyi	*Maccullochella macquariensis*	*Perca fluviatilis*
Carassius auratus	*Maccullochella mariensis*	*Percalates colonorum*
Channa spp.	*Maccullochella peelii*	*Percalates novemaculeata*
Craterocephalus stercusmuscarum	*Macquaria ambigua*	*Philypnodon grandiceps*

Cyprinus carpio	*Macquaria australasica*	*Philypnodon macrostomus*
Eptatretus spp.	*Macquaria colonorum*	*Piaractus brachipomus*
Eptatretus cirrhatus	*Macquaria novemaculeata*	*Piaractus mesopotamicus*
Eptatretus longipinnis	*Megalops cyprinoides*	*Plotosus lineatus*
Gadopsis marmoratus	*Melanotaenia fluviatilis*	*Retropinna semoni*
Galaxias brevipinnis	*Melanotaenia splendida*	*Rutilus rutilus*
Galaxias fuscus	*Mogurnda adspersa*	*Salmo trutta*
Galaxias maculatus	*Mogurnda mogurnda*	*Syncomistes butleri*
Galaxias parvus	*Mordacia mordax*	*Tandanus tandanus*
Galaxias zebratus	*Mordacia praecox*	*Tilapia mariae*
Galaxiella munda	*Nannoperca australis*	*Tinca tinca*
Galaxiella nigrostriata	*Nannoperca obscura*	*Toxotes chatareus*
Galaxiella pusilla	*Nannoperca oxleyana*	*Toxotes jaculatrix*
Gambusia holbrooki		
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.
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 Litoria dayi, L. lorica*, and* L. nannotis *eDNA assays (Litday_COI, Litlor_COI, and LnannotisN&P_COI), respectively. 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')
Litday_COI_F	54.2	52.1	44.4	110	TCCGCCACAATAATCATC
Litday_COI_R	53.9	51.9	47.4		CCAAGAGCTCATAGTATGG
Litlor_COI_F	56.2	54.2	47.4	166	CCTGACCGGAATTGTCTTA
Litlor_COI_R	56.4	54.6	40.9		GGAGTGTAAAGAGTAACCAGTA
LnannotisN&P_COI_F	60.4	58.5	52.4	120	CCGAGCCTATTTTACCTCAGC
LnannotisN&P_COI_R	57.9	56.1	47.6		GCTCATAATATAGGTGCGTCC

2.3 *In vitro* validation

Following confirmation of satisfactory *in silico* tests, Litday_COI, Litlor_COI, and LnannotisN&P_COI (see Section 2.2) were each tested empirically (i.e., *in vitro* validation) for species-specificity by attempting to amplify genomic DNA (gDNA) template extracted from target and non-target species (Table 4) using standard cetyltrimethylammonium bromide methodology (Gomes, et al., 2017).

In vitro Test 1 tested Litday_COI, Litlor_COI, and LnannotisN&P_COI for amplification of Australian rainforest frogs (*n* = 1 target and *n* = 7 non-target), invasive toad (*n* = 1 non-target), Australian freshwater turtles (*n* = 6 non-target), and Australian freshwater fishes (*n* = 31 non-target; Table 4).

In vitro Test 2 verified Litday_COI, Litlor_COI, and LnannotisN&P_COI amplification efficiencies and limits of detection (LOD) using gDNA and artificial *COI* fragments (125, 176, and 275 bp; aDNA; gBlocks™, IDT Australia; Figure 1) per reaction (ng/µL and copies/µL), respectively (see Section 3.3). Quantification of gDNA and stock aDNA (ng/µL ± 99.7% CI) was determined in duplicate using QuantiFluor® fluorometer with QuantiFluor® ONE dsDNA System (Promega Pty Ltd, 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 (www.endmemo.com/bio/dnacopynum.php).

To determine gDNA efficiency and LOD a 7-point standard curve (4-point $\log_{10}$ and 2-point $\log_2$ off top standard) was made for *L. dayi*, *L. lorica*, and *L. nannotis* from pooled gDNA representing each discrete population. Pooled gDNA of each discrete population was quantified in duplicate using QuantiFluor® fluorometer with QuantiFluor® ONE dsDNA System (Promega Pty Ltd, Australia) and this quantity used as standard 1. For *L. dayi*, gDNA standards 1 - 7 for northern and southern populations spanned 3.35×10^{-1} – 8.375×10^{-6} ng/µL and 5.85×10^{-1} – 1.4625×10^{-5} ng/µL, respectively. For *L. lorica* gDNA standards 1 – 7 for northern population spanned 1.24 – 3.1×10^{-5} ng/µL. For *L. nannotis*, gDNA standard 1 – 7 for northern and southern populations spanned 6.3×10^{-1} – 1.575×10^{-5} ng/µL and 6.45×10^{-4} – 1.6125×10^{-5} ng/µL, respectively.

To determine aDNA efficiency and LOD a 10-point standard curve (7-point $\log_{10}$ and 2-point $\log_2$ off top standard) was generated by resuspending dried pellet of *L. dayi*, *L. lorica*, and *L. nannotis* aDNA fragments (Figure 1) in 50µL of 1x TE buffer following manufacturer's instructions (IDT Pty Ltd, Australia), which yielded stock aDNA concentrations of 1.193 ng/µL (9,293,633,827 copies/µL), 2.543 ng/µL (14,072,967,750 copies/µL), and 3.057 ng/µL (10,828,361,276 copies/µL), respectively. Stock aDNA of *L. dayi*, *L. lorica*, and *L. nannotis* was diluted 1:500 with MilliQ® water to generate Standard 1 (1.86×10^7, 2.82×10^7, and 2.17×10^7 copies/µL), which was then serially diluted nine times (seven times at $\log_{10}$ and two times at $\log_2$) to generate Standards 2 – 10 (1.86×10^6 – 0.47, 2.82×10^6 – 0.70, and 2.17×10^6 – 0.55 copies/µL), respectively. The number of *COI* copies generated from gDNA template of each *Litoria* spp. and non-target species (if amplified) during *in vitro* Test 3 (see below) was determined by extrapolation from species-specific aDNA standard curve run under the same conditions (60˚C, 500 nM each primer; Figure 2, Figure 3, Figure 4).

Quantitative real-time polymerase chain reaction (qPCR) was used for three *in vitro* tests run for Litday_COI, Litlor_COI, and LnannotisN&P_COI: 1) determination of gDNA efficiency and

LOD, 2) determination of aDNA efficiency and LOD, and 3) determination of cross-amplification of gDNA from co-occurring non-target species (Table 4). *In vitro* Tests 1 - 3 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), 0.5 µL reverse primer (10 µM, 500 nM final), 3 µL target species gDNA (*in vitro* Test 1) or 3 µL target species aDNA (*in vitro* Test 2) or 3 µL non-target species gDNA (*in vitro* Test 3), and 1 µL molecular grade water. Room temperature master mix (7 µL) was loaded into white 96-well plates (Life Technologies Inc., Australia) with an Multipette® Xstream electronic dispensing pipette (Eppendorf, Australia) fitted with 500 µL CombiTip ® (Eppendorf, Australia) in a UV-sterilized PCR cabinet (Esco, Australia) in dedicated pre-PCR (*in vitro* Tests 1 and 3) or post-PCR (*in vitro* Test 2) room within the Molecular Ecology and Evolution Laboratory (MEEL) at James Cook University (Townsville, Queensland Australia). For *in vitro* Tests 1 & 3 and 2 the 96-well plate containing 7 µL master mix was moved (unsealed) to a cleaned bench (wiped thoroughly with 10% bleach then water then 70% ethanol) in the dedicated pre- and post-PCR room within MEEL where 3 µL template was loaded using manual single channel P10 pipette (Eppendorf, Australia) fitted with Maximum Recovery filter tips (Axygene, Australia), respectively. Plates were sealed with 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 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 65˚C to 95˚C (ramp rate = 0.15˚C/sec). *In vitro* Tests 1 and 2 (gDNA and aDNA standard curves, respectively) were run in triplicate while *in vitro* Test 3 (non-target gDNA amplification) was run in duplicate. QuantStudio™ Design and Analysis Software (version 1.4.2; Life Technologies, Australia) was used to set the threshold fluorescence to 0.2 and analyse and export (Excel) data.

Representative amplicons that exhibited T_m within 99.7% confidence internal (CI) of gDNA or aDNA standards (*in vitro* Tests 1 and 2), respectively, were not verified by bidirectional Sanger sequencing as these were considered positive due to loading of species-specific templates. Amplicons generated during *L. dayi, L. lorica,* and *L. nannotis in vitro* Test 3 from both technical replicates of any non-target species that exhibited T_m inside 99.7% CI of corresponding species-specific gDNA standard were considered putative negatives (i.e., false positives) and confirmed by bidirectional Sanger sequencing.

>Litday_COI
ATTTCACCTCCGCCACAATAATCATCGCCATCCCCACCGGCGTAAAGGTTTTTA
GCTGACTAGCTACTATGCACGGAGGCGTAATCAAATGAGATGCGGCCATACTA
TGAGCTCTTGGTTTTATT

>Litlor_COI
ATGCACCTGACCGGAATTGTCTTATCCAACTCCTCATTAGACATTGTCCTTCAC
GACACTTATTATGTTGTAGCCCATTTCCATTATGTATTGTCTATAGGAGCTGTAT
TCGCCATTATAGCTGGCTTCGTTCACTGATTCCCGTTATTTACTGGTTACTCTTT
ACACTCCACATG

>LnannotisN&P_COI
GATACCCGAGCCTATTTTACCTCAGCCACAATAATTATTGCCATCCCAACCGGT
GTAAAAGTTTTTAGCTGACTGGCGACTATGCATGGGGGAATTGTTAAATGGGAC
GCACCTATATTATGAGCCATGGGCTTCATGTGTTGATGTACTATCGGGGGCCT
AACTGGAATTGTTTTATCTAACTCCTCATTAGATATCGTTCTTCACGATACTTACT
ACGTTGTAGCCCACTTTCACTATGTTTTATCAATAGGGGCTGTATTTGCTATTAT
AGC

Figure 1. Artificial double stranded DNA (aDNA) replicas of L. dayi, L. lorica, *and* L. nannotis COI *regions (125, 176, and 275 bp) targeted by Litday_COI, Litlor_COI, and LnannotisN&P_COI, respectively. Blue and green nucleotides indicate location of forward and reverse primers, respectively, while red nucleotides indicate 5' and 3' extensions included to promote efficient binding to and amplification of aDNA fragments. Bold underlined nucleotides within LnannotisN&P_COI fragment indicate sites where Guanine (G) was substituted for Thymine (T) to increase fragment complexity as per manufacturer's guidelines (IDT Pty Ltd, Australia). Note that LnannotisN&P_COI fragment is longer than Litday_COI and Litlor_COI fragments because a second primer pair was designed that included downstream COI region; however, given optimal performance of initial LnannotisN&P_COI primers these alternative primers were not validated.*

Table 4. Target and non-target species against which Litday_COI, Litlor_COI, and LnannotisN&P_COI were tested empirically. Nanograms of gDNA template loaded into duplicate wells of in vitro *test provided within brackets. Non-native Australian species indicated by asterisks (*).*

Freshwater fish		
Amatitlatina sp.* (1.107)	*Hypseleotris galii* (12.78)	*Neosilurus ater* (0.327)
Ambassis agrammus (0.636)	*Hypseleotris* sp. (0.636)	*Orechromis mossambicus** (5.85)
Amniataba percoides (0.45)	*Kuhlia marginata* (1.083)	*Oxyeleotris lineolatus* (0.759)
*Anabas testudineus** (1.026)	*Leiopotherapon unicolor* (0.579)	*Philypnodon grandiceps* (5.01)
Craterocephalus stercusmuscarum (1.221)	*Macquaria ambigua* (0.135)	*Philypnodon macrostomus* (2.337)
Giuris margaritacea (16.2)	*Macquaria australasica* (1.584)	*Stenogobius watsoni* (1.254)
Glossamia aprion (2.043)	*Melanotaenia splendida inornata* (0.711)	*Tandanus bellingerensis* (2.886)
*Haplochormis burtoni** (0.762)	*Mogurnda adspersa* (0.387)	*Tandanus tandanus* (17.4)
Hephaestus carbo (0.84)	*Nematalosa erebi* (3.84)	*Tilapia mariae** (0.564)
Hypseleotris compressa (8.73)	*Neosiluroides cooperensis* (0.702)	*Xiphophorus maculatus** (0.405)
Frogs/toad		
Litoria dayi (northern; 1.59)	*Litoria lorica* (southern; 3.39)	*Litoria serrata* (4.92)
Litoria dayi (southern; 3.12)	*Litoria nannotis* (northern; 2.79)	*Litoria wilcoxii* (3.33)

Litoria jungguy (3.63)	*Litoria nannotis* (Paluma; 0.821)	*Litoria xanthomera* (6.30)
Litoria lorica (northern; 5.13)	*Litoria rheocola* (2.92)	*Rhinella marina** (0.351)
Freshwater turtles		
Chelodina canni (1.791)	*Elseya lavarackorum* (1.449)	*Myuchelys latisternum* (1.134)
Chelodina oblonga (0.669)	*Emydura subglobosa worrelli* (0.741)	*Rheodytes leukops* (1.248)

2.4 *In situ* validation

Following *in vitro* Tests 1 – 3, Litday_COI, Litlor_COI, and LnannotisN&P_COI were put through a final empirical validation using eDNA captured and extracted from environmental water samples (i.e., *in situ* validation). For this *in situ* test, eDNA was captured in April 2019 from a flowing stream near Mount Carbine, Tablelands, Queensland Australia, where *L. lorica* and *L. nannotis* are known to occur and *L. dayi* is suspected to occur (Conrad Hoskin, personal communication). Water samples were collected by decanting 30 mL from 50mL LoBind® (Eppendorf Pty Ltd, Australia) tube into each of five replicate 50 mL LoBind® tubes (new water grab for each replicate) pre-loaded with 10 mL Longmire's Solution (0.1M Tris Base pH 8, 0.1M disodium ethylenediaminetetraacetate dihydrate pH 8, 0.01M sodium chloride, 0.5% (w/v) sodium dodecyl sulfate; Longmire, et al., 1992). Samples were transported back to MEEL at ambient temperature (≈ 24˚C) and eDNA was extracted from half of each sample (20 mL) using a novel eDNA workflow (Preserve, Precipitate, Lyse, Precipitate, Purify (PPLPP); Edmunds and Burrows, in review). Briefly, 20 mL of each sample was precipitated overnight (4˚C) with glycogen (final concentration (C_f) = 22.2 µg/mL), sodium chloride (C_f = 0.44M), and isopropanol (C_f = 40%), pelleted (6,750 x g for 10 min at 20˚C; Heraeus Megafuge 8R centrifuge with HighConicIII fixed-angle rotor; ThermoFisher Scientific Pty Ltd, Australia), resuspended in 600µL 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 = 111.1 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; Heraeus Megafuge 8R centrifuge with MicroClick rotor; ThermoFisher Scientific Pty Ltd, Australia), washed twice (1 mL 70% ethanol each wash), eluted in 100µL MilliQ® water, and purified of inhibitors (DNeasy PowerClean Pro Cleanup Kit; Qiagen Pty Ltd, Australia).

Litday_COI, Litlor_COI, and LnannotisN&P_COI *in situ* tests were run across 6, 12, and 6 technical replicates using same qPCR chemistry as *in vitro* Tests 1 - 3 (see Section 2.3) but with 3µL eDNA as template (18%, 36%, and 18% elution screened), respectively. Master mix for *in situ* test was loaded as per *in vitro* Tests 1 – 3 but with 3 µL eDNA loaded in dedicated MEEL eDNA lab on 10% bleach, water, and 70% ethanol cleaned bench (see Section 2.3) using the EZ Mate 601s liquid handling robot with APM 8-channel 50µL module (Arise Inc., USA) and robotic filter tips (Axygene Pty Ltd, Australia). The loaded *in situ* plate was sealed, vortexed, spun, run, and analysed as described above for *in vitro* Tests 1 – 3 (see Section 2.3). Representative amplicons from *in situ* tests that exhibited ΔT_m analysis within 99.7% CI of corresponding species-specific gDNA standards were considered putative positives and Sanger sequenced bidirectionally for confirmation (Trujillo-Gonzalez, et al., 2019).

3. Results

3.1 Primer design

Based on *COI* sequence alignments, regions of conversation within and divergence among target *Litoria* spp. as well as divergence from non-target co-occurring species were identified. Litday_COI, Litlor_COI, and LnannotisN&P_COI possessed ≥ 1 mismatch(es) with other *Litoria* spp. and non-target species (see Section 3.2). Litday_COI, Litlor_COI, and LnannotisN&P_COI target *COI* region spanned base pairs 187 – 296, 351 – 516, and 198 – 317, respectively (Table 3). Litday_COI-F, Litlor_COI-F, and LnannotisN&P_COI-F exhibited the following characteristics: 1) T_m = 52.1 – 54.2, 54.2 – 56.2, and 58.5 – 60.4; 2) GC content = 44.4%, 47.4%, and 52.4%; 3) length = 18, 19, and 21 bp; 4) self-dimer T_m = 0˚C, 14.7˚C, and 0˚C; 5) hairpin T_m = 0˚C, 33.8˚C, and 0˚C; 6) self-complementarity score = 4, 4, and 3; 7) 3' self-complementarity score = 2, 2, and 2, respectively. Litday_COI-R, Litlor_COI-R, and LnannotisN&P_COI-R exhibited the following characteristics: 1) T_m = 51.9 – 53.9, 54.6 – 56.4, and 56.1 – 57.9; 2) GC content = 47.4%, 40.9%, and 47.6%; 3) length = 19, 22, and 21 bp; 4) self-dimer T_m = 21.7˚C, 0˚C, and 0˚C; 5) hairpin T_m = 0˚C, 0˚C, and 0˚C; 6) self-complementarity score = 7, 3, and 5; 7) 3' self-complementarity score = 5, 2, and 0, respectively.

3.2 *In silico* validation

Initial *in silico* test using targeted PrimerBLAST search of NCBI "nr" database (see Section 2.2) confirmed that none of the specified species are predicted to amplify (i.e., zero mismatches) with Litday_COI, Litlor_COI, or LnannotisN&P_COI (Table 5). More specifically, Litday_COI exhibited 4 – 6 mismatches with *Carassius auratus auratus*, *Cyprinus carpio*, and *Myuchelys bellii* while Litlor_COI exhited zero mismatches to any specified species (Table 5). LnannotisN&P_COI exhibited 1 mismatch to *L. nannotis* "Central" population and 4 – 7 mismatches to *Chelodina mccordi*, *Myuchelys novaeguineae*, *Chelodina rugosa*, *Chelodina oblonga*, *Litoria raniformis*, and *Litoria serrata* (Table 5).

Subsequent non-targeted *in silico* PrimerBLAST of Litday_COI (see Section 2.2, Table 3) returned ≤ 2 mismatches (i.e., potential amplification) to multiple species (*n* = 93); however, only three native Australian bird species (*Trichoglossus rubritorquis, T. haematodus*, and *Melopsittacus undulates*) exhibited 2 mismatches (one in forward and one in reverse primer; Table 6). Non-targeted *in silico* PrimerBLAST of Litlor_COI and LnannotisN&P_COI returned no species with ≤ 2 mismatches (Table 6).

Table 5. Species with ≤ 7 mismatches to Litday_COI, Litlor_COI, and LnannotisN&P_COI based on targeted PrimerBLAST search of human and Australian fish, frog, and turtle COI *sequences available in NCBI "nr" databse. Note that no matches were returned for Litlor_COI because no* L. lorica COI *sequences are currently unavailable in GenBank "nr" database.*

Assay	Forward mismatches	Reverse mismatches	Species
Litday_COI	0	0	*Litoria (Nyctimystes) dayi*
	1	3	*Carassius auratus auratus*
	2	2	*Cyprinus carpio*
	2	3	*Carassius auratus*
			Cyprinus carpio 'wananensis'

	3	3	*Myuchelys bellii*
Litlor_COI	0	0	No matches
LnannotisN&P_COI	0	0	*Litoria nannotis LnD* ("North") *Litoria nannotis LnP* ("Paluma")
	0	1	*Litoria nannotis LnA* ("Central")
	3	1	*Chelodina mccordi*
	1	4	*Myuchelys novaeguineae*
	2	4	*Chelodina rugosa* *Chelodina oblonga*
	3	3	*Litoria raniformis*
	3	4	*Litoria serrata*

Table 6. Species with ≤ 2 mismatches to to Litday_COI, Litlor_COI, and LnannotisN&P_COI based on non-targeted PrimerBLAST search of entire NCBI "nr" databse. Native Australian species indicated with asterisks (*). Note that no matches were returned for Litlor_COI because no L. lorica COI *sequences are currently unavailable in GenBank "nr" database.*

Assay	Forward mismatches	Reverse mismatches	Species
Litday_COI	0	0	*Litoria (Nyctimystes) dayi* *Amazona pretrei* *Amazona aestiva* *Amazona auropalliata* *Amazona ochrocephala nattereri* *Amazona ochrocephala panamensis* *Amazona ochrocephala tresmariae* *Amazona ochrocephala* *Amazona autumnalis* *Amazona ochrocephala auropalliata* *Amazona ochrocephala belizensis* *Amazona ochrocephala ochrocephala* *Amazona pretrei* *Amazona oratrix belizensis* *Amazona auropalliata parvipes* *Amazona autumnalis lilacine* *Amazona autumnalis autumnalis* *Amazona auropalliata auropalliata* *Amazona aestiva xanthopteryx* *Amazona aestiva aestiva*
	1	0	*Amazona aestiva* *Amazona ochrocephala* *Luciobarbus capito* *Amazona farinosa virenticeps* *Amazona kawalli* *Amazona farinosa inornata* *Amazona farinosa guatemalae* *Amazona farinosa farinosa* *Amazona farinosa chapmani* *Amazona barbadensis barbadensis* *Amazona ochrocephala* *Amazona aestiva* *Amazona ochrocephala ochrocephala* *Amazona ochrocephala xantholaema* *Amazona aestiva xanthopteryx* *Amazona ochrocephala nattereri* *Amazona farinosa* *Amazona rhodocorytha* *Amazona guildingii* *Amazona farinosa guatemalae* *Amazona farinosa inornata*

			Amazona dufresniana *Amazona brasiliensis* *Amazona barbadensis*
	0	1	*Trachylepis occidentalis* *Pionopsitta pileata* *Amazona ochrocephala auropalliata* *Amazona vittata*
	1	1	*Dyscophus insularis* *Napothera epilepidota* *Amazona ventralis* *Caecilia tentaculata* *Trichoglossus euteles* *Eos squamata* *Eos semilarvata* *Oreopsittacus arfaki* *Psitteuteles iris* *Trichoglossus rubritorquis** *Trichoglossus ornatus* *Trichoglossus flavoviridis* *Eos cyanogenia* *Eos bornea* *Pionites leucogaster* *Melopsittacus undulatus** *Deroptyus accipitrinus* *Dopasia gracilis* *Caecilia tentaculata* *Puntius chelynoides* *Lutjanus vivanus* *Lutjanus purpureus* *Cacatua moluccensis* *Pyrrhura perlata* *Amazona festiva* *Psittacula roseata* *Psittacula krameri* *Eos histrio* *Amazona farinosa virenticeps* *Vini australis* *Trichoglossus haematodus** *Pseudeos fuscata* *Micropsitta finschii* *Melopsittacus undulatus* *Eos reticulata* *Deroptyus accipitrinus* *Bolbopsittacus lunulatus* *Melopsittacus undulatus* *Amazona barbadensis* *Amazona vinacea* *Amazona versicolor* *Amazona ventralis* *Amazona leucocephala leucocephala* *Amazona festiva bodini* *Amazona collaria*
Litlor_COI	0	0	No matches
LnannotisN&P_COI	0	0	*Litoria nannotis* LnD ("North") *Litoria nannotis* LnP ("Paluma")
	0	1	*Litoria nannotis* LnA ("Central")

3.3 *In vitro* validation

Litday_COI, Litlor_COI, and LnannotisN&P_COI exhibited satisfactory efficiency and LOD based on gDNA and aDNA standard curves (*in vitro* Tests 1 and 2; see Section 2.3).

In vitro Test 1 demonstrated that Litday_COI, Litlor_CO, and LnannotisN&P_COI successful amplify both geographically distinct populations of *L. dayi*, *L. lorica*, and *L. nannotis* (Figure 2**,** Figure 3**,** and Figure 4), respectively. Regarding non-target species amplification, Litday_COI, Litlor_COI, and LnannotisN&P_COI exhibited putative positive amplification of two (*L. serrata* and *L. xanthomera*), one (*L. wilcoxii*), and one (*L. dayi*) species. Sanger sequencing confirmed that Litday_COI and Litlor_COI amplifications of non-target species were due to cross-contamination with gDNA of *L. dayi* and *L. lorica*, respectively. LnannotisN&P_COI cross-amplification of *L. dayi* was considered a false positive because *L. nannotis* amplification was observed batch extraction blank (data not shown). Of note is that false positive amplification of non-target species has been observed during *in vitro* validation of other species-specific eDNA assays (see Edmunds and Burrows, 2019 a, b, c).

In vitro Test 2 demonstrated Litday_COI, Litlor_COI, and LnannotisN&P_COI gDNA amplification efficiencies of 97.6%, 78.8%, and 78.5% (Figure 2B, Figure 3B, and Figure 4B), respectively. Based on these gDNA standard curves LOD for northern and southern populations was determined to be 8.36×10^{-6} ng/µL and 1.46×10^{-5} ng/µL for *L. dayi*, 3.10×10^{-5} ng/µL and 1.64×10^{-5} ng/µL for *L. lorica*, and 3.15×10^{-5} ng/µL and 1.61×10^{-5} ng/µL for *L. nannotis*, respectively. Only *L. dayi* exhibited a discernible shift in amplicon T_m between northern and southern populations (2.45˚C; Figure 2C), which is driven by six nucleotide differences within 109 bp *COI* amplicon.

In vitro Test 3 demonstrated Litday_COI, Litlor_COI, and LnannotisN&P_COI aDNA amplification efficiencies of 95.3%, 97.6%, and 97.3% (Figure 2B', Figure 3B', and Figure 4B'), respectively. Based on aDNA standard curves the LOD of Litday_COI, Litlor_COI, and LnannotisN&P_COI was determined to be 0.6 copies/µL (i.e., 2 or 4 copies loaded into 10µL or 20µL assays, respectively).

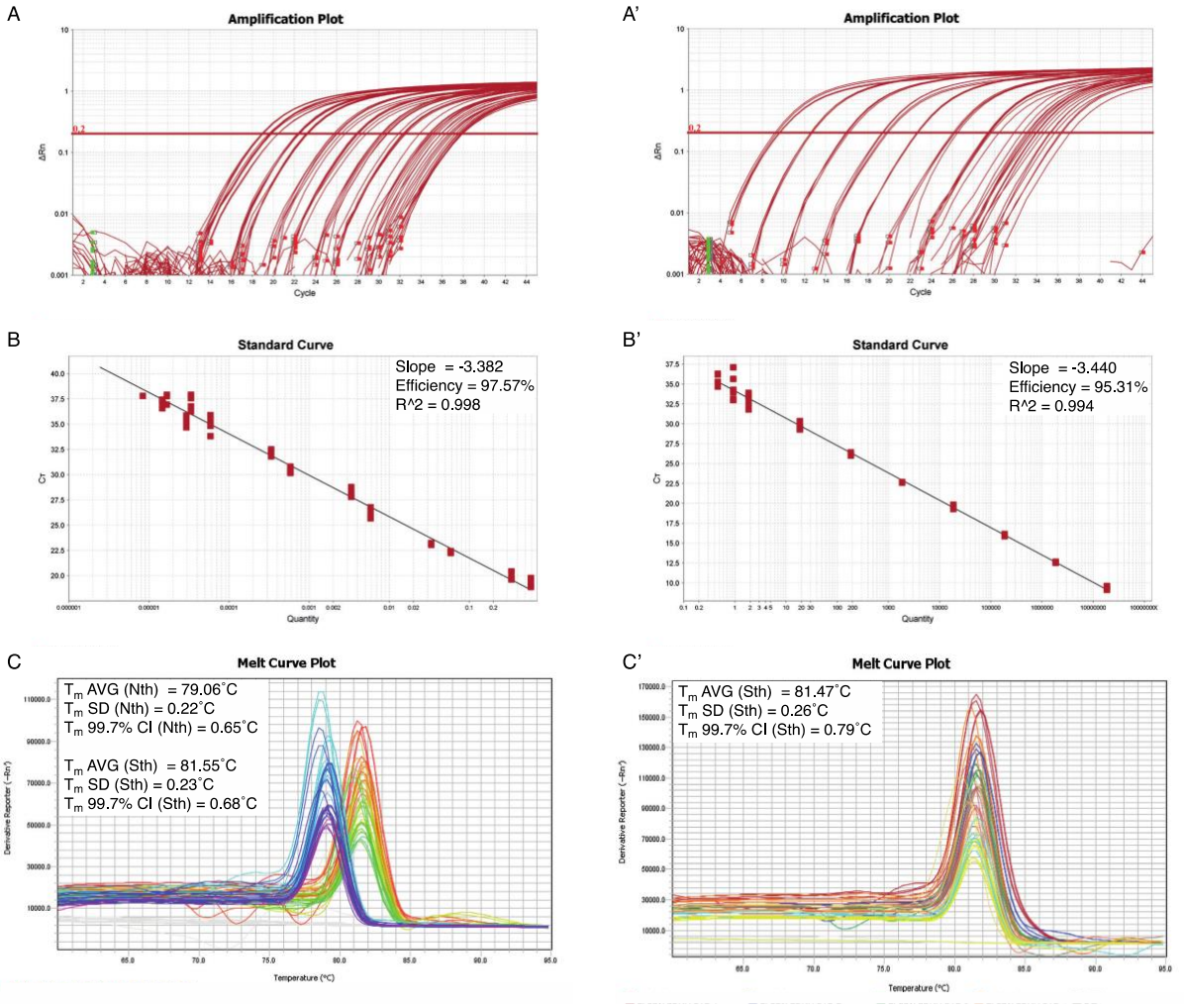

Figure 2. Genomic DNA (gDNA) and artificial DNA (aDNA) qPCR amplification curves (A and A'), standard curve linear regressions (B and B'), and amplicon dissociation temperature curves (T_m; C and C') generated during Litday_COI in vitro *Tests 1 and 2 on northern and southern populations, respectively (60˚C, 500nM each primer; see Section 2.3). Note that 2.49˚C shift in T_m between Litday_COI amplicons from northern and southern gDNA is due to presence of six nucleotide polymorphisms between these geographically discrete populations. Litday_COI gDNA amplification efficiency was determined for both northern and southern populations (A) but, given exhibited similarity, is presented as combination thereof (B and C). Synthesized aDNA fragment is replica of Southern* L. dayi COI *sequence given higher inherent GC content than northern* COI *sequence due to these six polymorphisms (Figure 1).*

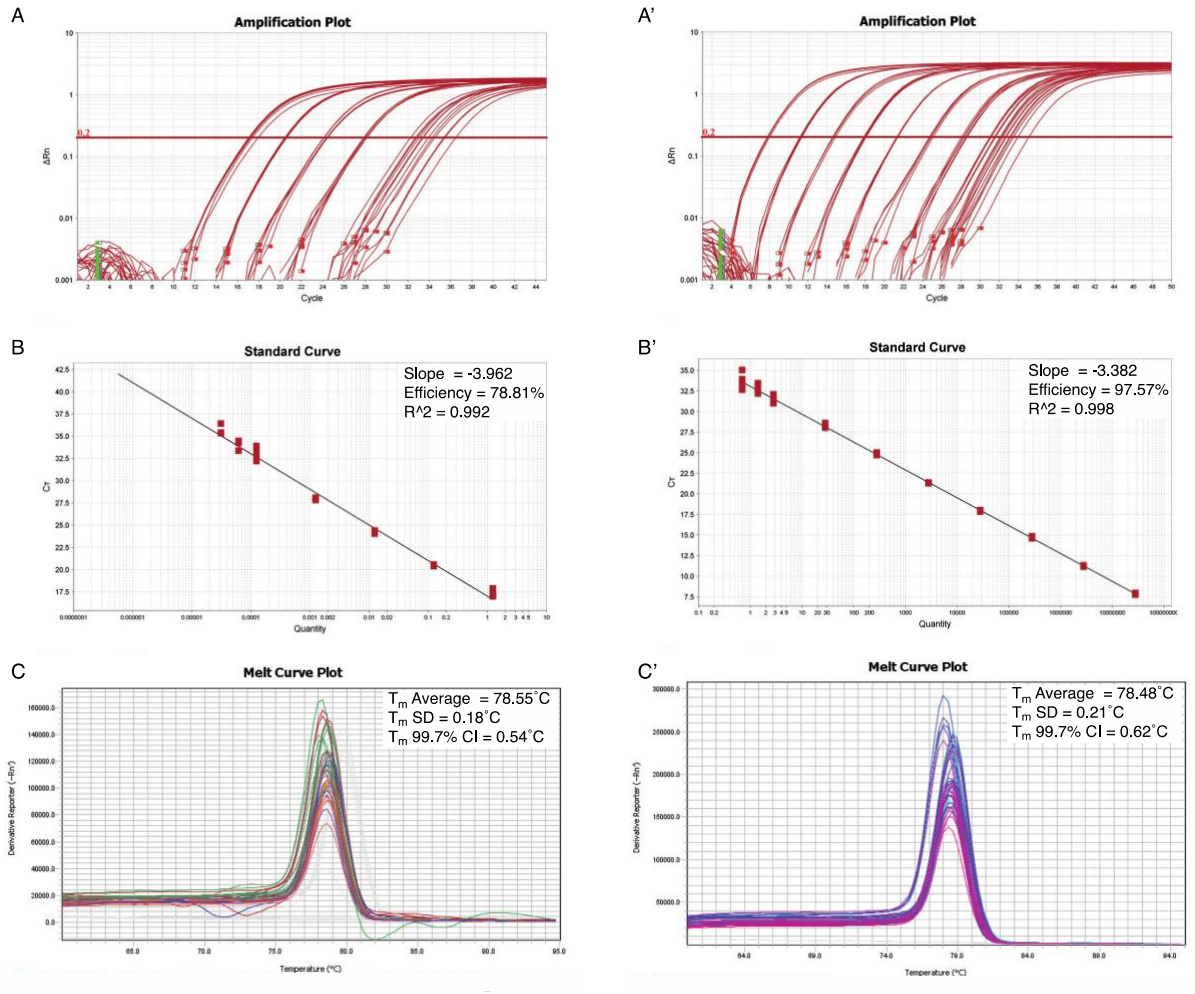

Figure 3. Genomic DNA (gDNA) and artificial DNA (aDNA) qPCR amplification curves (A and A'), standard curve linear regressions (B and B'), and amplicon dissociation temperature curves (T_m; C and C') generated during Litlor_COI in vitro *Tests 1 and 2 on northern and southern populations, respectively (60˚C, 500nM each primer; see Section 2.3). Note that gDNA and aDNA efficiencies (B and B') and associated T_m (C and C') are based on northern population only because high-quality gDNA and* COI *sequence from southern population were unavailable at time of assay development. Low gDNA efficiency (B) due to use of degraded gDNA samples; however, higher efficiency of aDNA (B') demonstrates true assay efficiency in presence of high quality template. Synthesized* L. lorica *aDNA fragment is replica of northern* COI *sequence only given that southern population* COI *sequences were unavailable at the time of assay development (Figure 1).*

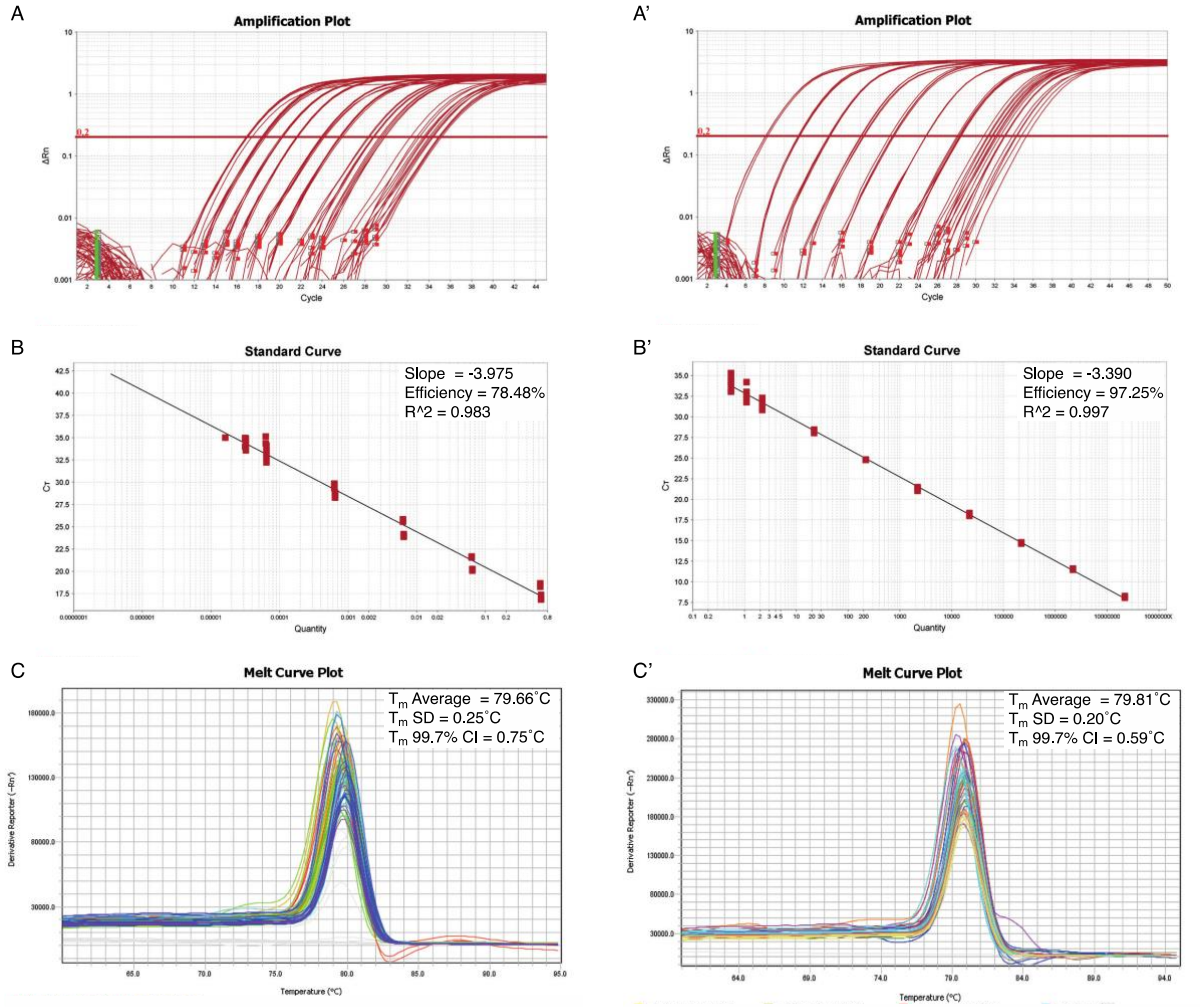

Figure 4. Genomic DNA (gDNA) and artificial DNA (aDNA) qPCR amplification curves (A and A'), standard curve linear regressions (B and B'), and amplicon dissociation temperature curves (T_m; C and C') generated during LnannotisN&P_COI in vitro *Tests 1 and 2 on northern and Paluma populations, respectively (60˚C, 500 nM; see Section 2.3). LnannotisN&P_COI gDNA amplification efficiency was determined for both northern and Paluma populations (A) but, given exhibited similarity, is presented as combination thereof (B and C). Low gDNA efficiency (B) due to use of degraded gDNA samples; however, higher efficiency of aDNA (B') demonstrates true assay efficiency in presence of high quality template. Synthesized* L. nannotis *aDNA fragment is replica of northern and Paluma consensus* COI *sequence (Figure 1).*

3.4 *In situ* validation

Water samples collected from Mount Cabine area (see Section 2.4) provided *in situ* validation for Litlor_COI and LnannotisN&P_COI. More specifically, Litlor_COI and LnannotisN&P_COI yielded 1.67% (1/60) and 36.67% (11/30) positive *COI* amplifications across all qPCR replicates (*n* = 6 or 12 technical replicates per *n* = 5 biological replicates; Figure 5). Extrapolation with Litlor_COI and LnannotisN&P_COI gDNA or aDNA standard curves (Figure 3 or Figure 4) revealed yields of 4.33×10^{-5} and $2.44 \pm 0.38 \times 10^{-5}$ ng/µL or 4.12×10^{-1} and $2.77 \pm 0.49 \times 10^{-1}$ copies/µL, respectively. The observed higher detection rate for *L. nannotis* than *L. lorica* eDNA was expected given occurance of higher and lower population densities in or near sampled flowing stream, respectively. No *L. dayi* detections were obtained within 18% elution volume screened using Litday_COI.

All Sanger sequenced representative Litlor_COI and LnannotisN&P_COI positive amplicons (*n* = 1 and 6) matched *L. lorica* (not on GenBank) and *L. nannotis* (GenBank accession AF304233) *COI* sequences with 100% and 97.2 ± 0.03% pairwise identity, respectively. These confirmed positive *in situ* detections verify that Litlor_COI and LnannotisN&P_COI can detect low-copy *L. lorica* and *L. nannotis* eDNA in 15 mL unfiltered environmental water samples preserved in Longmire's and extracted using PPLPP workflow (Edmunds and Burrows, in review).

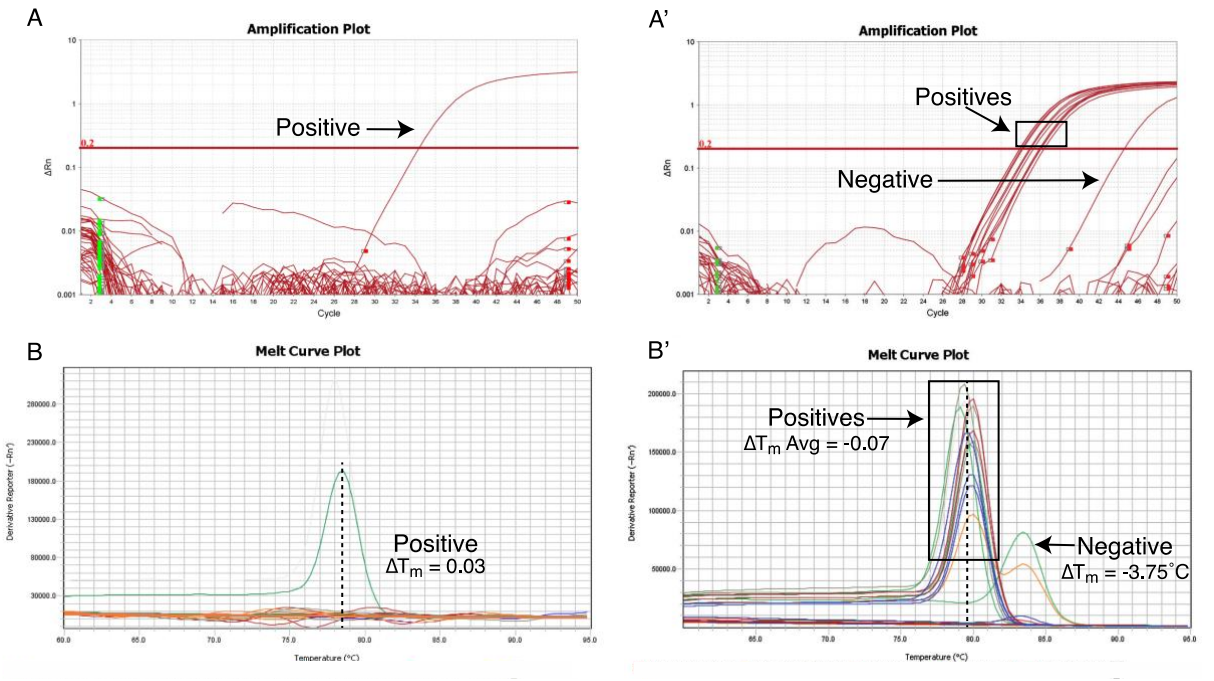

Figure 5. L. lorica *and* L. nannotis *amplification (A and A') and dissociation (T_m; B and B') curves generated during Litlor_COI and LnannotisN&P_COI* in situ *validations, respectively (see Section 2.4). Dashed vertical lines (B and B') denote average T_m of gDNA standards for Litlor_COI and LnannotisN&P_COI (78.45˚C and 79.66˚C), respectively. ΔT_m analysis for LnannotisN&P_COI* in situ *detections (B') accurately discriminated positive from negative eDNA detections (Trujillo-Gonzalez, et al., 2019).*

4. Discussion

In silico and *in vitro* validations confirm that Litday_COI, Litlor_COI, and LnannotisN&P_COI are species-specific, efficiently amplify gDNA and aDNA templates, and have low LODs. *In situ* validations also confirm that Litlor_COI and LnannotisN&P_COI can detect low-copy *L. lorica* and *L. nannotis* eDNA captured in unfiltered environmental water samples, which renders these two assays fully validated and ready for application to any situation where eDNA can assist with monitoring and conservation efforts for *L. lorica* and *L. nannotis*, respectively.

Litday_COI *in vitro* Test 2 demonstrated successful amplification of gDNA extracted from both northern and southern individuals and, in so doing, revealed that targeted *COI* region is divergent between *L. dayi* populations (Bell, et al., 2011). More specifically, six nucleotide polymorphisms are present within targeted *COI* region, which underlies the observed 2.49˚C difference in gDNA T_m observed for these geographically discrete populations. Synthesized aDNA fragement was based on southern *L. dayi COI* given higher inherent GC content due to these six polymorphisms, as per manufacturer's instructions. Litday_COI *in situ* validation was unsuccessful using unfiltered water samples from this particular Mount Carbine site; however, the lack of *L. dayi* detection was anticipated given distal upsteam distribution and discrete ecosystem utilization (Czechura et al., 1987).

Litlor_COI *in vitro* Test 2 demonstrated successful amplification of gDNA extracted from both northern and southern individuals, which revealed that targeted *COI* region is homologous across both *L. lorica* populations. No *L. lorica COI* sequences are currently available on GenBank but were, rather, obtained from colleagues (Conrad Hoskin, personal communication); however, initially obtained *L. lorica COI* sequences were for northern population only. Thus, *L. lorica* aDNA fragment is replica of northern *COI* concensus sequence despite subsequent attainment of southern population *COI* sequences and gDNA used for *in vitro* Test 2. Litlor_COI *in situ* validation was successful using unfiltered water samples collected from Mount Carbine despite low detection rate (1.67%) across high elution volume screened (36%) to obtain one low-copy *L. lorica* detection (approx. 1 copy loaded).

LnannotisN&P_COI *in vitro* Test 2 demonstrated successful amplification of gDNA extracted from both northern and Paluma individuals, which revealed that targeted *COI* region is homologous across both these *L. nannotis* populations. Given that L. nannotis conservation efforts are focused on northern and Paluma populations LnannotisN&P_COI was designed to concurrently target these populations due to lack of COI conservation across all three populations. LnannotisN&P_COI has not been emperically tested for amplifciation of central *L. nannotis* population gDNA athough this is likely given only one mismatch in reverse primer; however, a dedicated assay for detection of central *L. nannotis* eDNA was designed and is available upon request. Accordingly, *L. nannotis* aDNA fragment is based on northern and Paluma *COI* concensus sequence. LnannotisN&P_COI *in situ* validation was successful using unfiltered water samples collected from a flowing stream near Mount Carbine; however, despite higher detection rate (36.67%) across lower elution volume screened (18%), all detections (n = 11) were similarly low-copy to Litlor_COI *in situ* detection (approx. 1 copy loaded). The observed higher detection rate for *L. nannotis* than *L. lorica* was expected given known higher and lower population abundances within this regions of the Australian wet tropics (Conrad Hoskin, personal communication), respectively.

5. Recommendations and conclusions

Given that Litday_COI *in situ* validation was unsuccessful using these particular Mount Cabine stream water samples we recommend that a subsequent *in situ* validation attempt be undertaken using unfiltered water samples collected immediately downstream of alternative location(s) wherein *L. dayi* is known to occur.

We recommend that future conservation efforts for *L. lorica* and *L. nannotis* collect unfiltered water samples from both distal and proximal locations downstream of known populations to establish maximum eDNA detectability distance under tropical environmental conditions (e.g., temperature, flow rate, microbial community). This will inform the limitations of downstream eDNA monitoring for effective guidance of visual surveys to upstream locations harbouring known or unknown frog populations.

Species-specific biomass correlation has yet to be explored for Litlor_COI or LnannotisN&P_COI; however, small rehabilitation populations could provide ideal opportunities to investigate the ability of these assays to monitor growth over time (Arke, et al., 2019). Given the benefit of monitoring growth of rehabilitation populations over time using non-invasive eDNA, we recommend that future conservation studies targeting *L. lorica* and *L. nannotis* rehabilitation populations aim to incorporate collection of unfiltered water samples over time to determine utility of Litlor_COI and LnannotisN&P_COI for this specific purpose, respectively.

Lastly, given that both *L. lorica* and *L. nannotis* were detected *in situ* following eDNA extraction from half (15 mL) of collected 30 mL water samples, we recommend that rainforest frog conservation studies that incorporate eDNA monitoring collect ≥ 5 replicate 15 mL unfiltered water samples at each field site, preserve with 5 mL Longmire's, extract samples using PPLPP workflow (Edmunds and Burrows, in review), and screen at least 18% elution volume.

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