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Developing Loop Mediated Isothermal Amplification (LAMP) assays for rapid, presumptive DNA detection of an invasive reptile (*Boa constrictor*)

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ABSTRACT

Context. Wildlife trade is a prominent pathway for invasive species introductions into novel environments. Deliberate or accidental release of exotic pets can result in the establishment of alien populations, with damaging impacts for native species and environmental assets. This process is well documented for reptiles globally and is of considerable biosecurity concern in Australia. *Boa constrictor* is one species at high risk of establishment in Australia, and has insufficient biosecurity detection and post-border control capacity. **Aims.** We aimed to develop rapid DNA-based presumptive testing capacity for detecting *B. constrictor*, with appropriate sensitivity and specificity to operate in a trace DNA biosecurity context. **Methods.** Loop Mediated Isothermal Amplification (LAMP) is an emerging biosecurity tool that provides highly specific, sensitive, low-resource methods for detection of trace DNA in the absence of physical evidence. We developed colourimetric and fluorescent LAMP assays targeting the mitochondrial DNA control region of *B. constrictor*. We tested and validated these assays against synthetic DNA fragments, as well as DNA extracted from: (1) vouchered museum *B. constrictor* tissue; (2) shed *B. constrictor* skin samples; (3) a range of non-target species to test specificity; and (4) trace DNA recovered from glass tanks post *B. constrictor* presence. **Key results.** We successfully detected synthetic target DNA down to 1 fg and genomic *B. constrictor* DNA from tissue and shed skins down to <10 pg in under 30 minutes with our fluorescence-based LAMP assay. Additionally, we were able to detect *B. constrictor* trace DNA following 24 h of presence utilising a traditional laboratory-based DNA extraction method (approximately 180 min) and a rapid lysis step (approximately 8 min). **Conclusions.** Both colourimetric and fluorescent assays show promise for the specific detection of *B. constrictor* in biosecurity contexts, including post-border enforcement and compliance checks in the domestic illicit wildlife trade. **Implications.** Our findings greatly strengthen the ongoing development of biosecurity tools for trace DNA detection of commonly traded and trafficked species (i.e. reptiles) in wildlife enforcement contexts, advancing both preparedness and surveillance.

Keywords: Biosecurity, *Boa constrictor*, DNA detection, invasive species, Loop Mediated Isothermal Amplification (LAMP), reptiles, wildlife forensics, wildlife trade.

Introduction

Wildlife trade is a prominent driver of extinction (Scheffers *et al.* 2019; Hinsley *et al.* 2023) that affects species from across the tree of life (Fukushima *et al.* 2020), including terrestrial (Morton *et al.* 2021) and aquatic vertebrates (Ripple *et al.* 2019), invertebrates (Purcell *et al.* 2014; Lassaline *et al.* 2023), plants (Margulies *et al.* 2019), and fungi (Sills *et al.* 2021). The scale of wildlife trade is increasing in the face of ongoing globalisation, with vertebrate trade involving over a quarter of all terrestrial species (Scheffers *et al.* 2019). One group that is particularly prominent in the global illegal wildlife trade is reptiles (Class: Reptilia) (Marshall *et al.* 2020). The reason for this popularity is often attributed to their uniqueness, with certain species harbouring patterns and colourations that are valuable in international markets (Heinrich *et al.* 2022). The importation of alien reptile species as pets

has been identified as a major source of invasive species (García-Díaz *et al.* 2017), which is a key extinction driver when pets are released or escape into the wild (Lockwood *et al.* 2019). In addition to intentional trade, herpetofauna also present serious biosecurity concerns due to incursions as stowaways (García-Díaz and Cassey 2014; Pili *et al.* 2023).

Several biosecurity measures have been recommended or implemented to detect and monitor live reptile trade. These include DNA based methods for species identification and detection (e.g. Short Tandem Repeat (STR) assays for the detection of carpet python trafficking (Ciavaglia and Linacre 2018)), the use of stable isotope analysis to determine whether animals are released pets or wild individuals (e.g. *Trachemys scripta elegans* (red eared slider) as either pet release or wild caught individuals (Hill *et al.* 2020)), and recent developments in 3D X-ray technologies for imaging and detecting cases of reptile trafficking (Pirotta *et al.* 2022). Predictive modelling of future incursions (Stringham *et al.* 2021*a*) and web scraping of existing trade have also advanced biosecurity preparedness (Stringham *et al.* 2021*b*). Most recently, a forensically validated genetic toolkit has been developed for *Tiliqua rugosa* (shingleback lizards), which shows great promise for detection of illegal wildlife trafficking (Brown *et al.* 2023).

One component of the biosecurity toolkit that currently requires greater adoption is targeted invasive species presumptive detection from trace DNA samples. This is particularly true for situations in which reptiles have been hidden, moved, or destroyed, and only trace evidence remains, as commonly encountered in elusive illegal trade pathways. In addition, there is growing interest in detecting reptiles from environmental DNA (eDNA) in a variety of contexts, including biodiversity surveys (Kyle *et al.* 2022), invasive species detection (Hunter *et al.* 2015), and threatened species detection (Katz *et al.* 2021). Limited attention has been directed towards rapid DNA-based detection of traded reptiles, with some previous work conducted on trace DNA visualisation (Deliveyne *et al.* 2022). Loop Mediated Isothermal Amplification (LAMP) has recently emerged as a promising biosecurity tool (Deliveyne *et al.* 2023). LAMP provides robust DNA or RNA detection with limited processing time and apparatus required, as well as simple visualisation of results (Notomi *et al.* 2015). This contrasts with conventional Polymerase Chain Reaction (PCR), which often requires significant time, resources, and external expertise (Lee 2017). Current applications of LAMP have primarily addressed the detection of invertebrate species as stowaways crossing transnational boundaries by air and sea (Blaser *et al.* 2018; Rako *et al.* 2021), detection of adulterated meat products (Nikunj and Vivek 2019), and illegitimate fur products (Yu *et al.* 2019). Currently, LAMP has not been applied to the detection of live illegal wildlife trade (Deliveyne *et al.* 2023); however, the method has been proposed as a rapid field-based detection system in studies addressing interrelated issues (Wimbles *et al.* 2021), and for biosecurity screening, including the detection of one reptile species, the Asian House Gecko, which is of key biosecurity concern in Australia (Marks 2022).

Several alien reptile species warrant development of advanced DNA detection methods in Australia, including those that are frequently detected in Australian border-level seizure and post border seizure records. One such species is *Boa constrictor*, with 89 incidents and 176 animals recovered in post-border seizures between 1999 and 2016 (Toomes *et al.* 2020). Climate matching indicates high suitability for establishment across the northern half of Australia for *B. constrictor*, with sufficient introductions (Henderson *et al.* 2011). Consequently, *B. constrictor* is a biosecurity risk and is included on the Australian Department of Agriculture, Fisheries and Forestry (DAFF) national vertebrate pest priority list as a species of highest concern (DAFF 2023). Additionally, *B. constrictor* is a host of Inclusion Body Disease (IBD) (Schumacher *et al.* 1994), a disease mainly of the family Boidae – but cases have also been diagnosed in captive Australian pythons (Carlisle-Nowak *et al.* 1998). There are concerns this disease could become established in native Australian species, which presents substantial risks due to very high mortality rates (Chang and Jacobson 2010).

Our aim was to develop LAMP assays for the rapid biosecurity detection of *B. constrictor* from trace DNA sources. We focused our efforts on development and optimisation of LAMP methods validating the specificity, sensitivity, and efficacy of these methods for detecting trace DNA. This included: (1) primer design validated *in silico*; (2) *in vitro* optimisation using synthetic DNA, tissue samples, and shed skin as trace DNA proxies; and (3) experimental conditions designed to mimic reptile holdings used to hold *B. constrictor*. We successfully detected *B. constrictor* DNA from a range of samples using the developed LAMP assays without considerable cross-reactivity for other reptiles common in the domestic Australian pet trade. In this article we discuss further development, necessary steps, and current obstacles to the implementation of the described *B. constrictor* assay, to deliver a rapid, presumptive, and cost-effective detection tool for an emerging biosecurity threat in Australia.

Materials and methods

Primer design and *in silico* validation

LAMP assay development followed a systematic approach (Fig. 1), with mitochondrial DNA genome sequences for *B. constrictor* obtained from GenBank, (accession numbers, NC_007398, and AB177354) and the tRNA-lle, control region (accession number D84260). The mitochondrial DNA control region was selected, and the New England Biolabs (NEB) LAMP Primer Design Tool (https://lamp.neb.com/#!/) was used to develop LAMP primer sets. Additionally, primer binding sites were investigated for mismatches at primer target sites for Australian snakes common in the legal pet

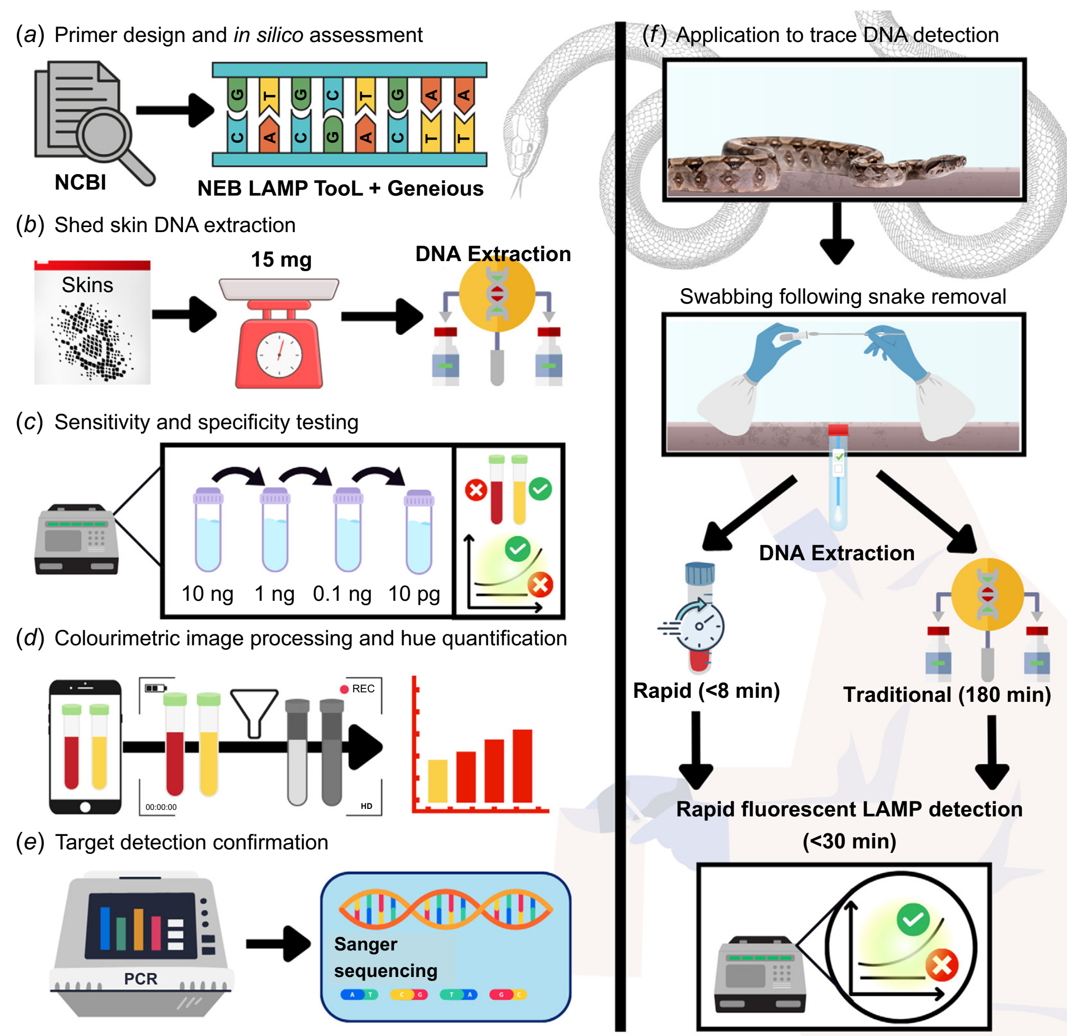


Fig. 1. Schematic outlining the methodological approach to *B. constrictor* Loop Mediated Isothermal Amplification (LAMP) assay development. (*a*) *B. constrictor* sequences were collected from NCBI nucleotide, with the New England Biolabs LAMP Primer Design Tool used for primer design in tandem with Geneious. (*b*) DNA was extracted from tissue and a range of reptile shed skins and (c) serially diluted 1:10 in tandem to synthetic DNA matching the target region, to assess the sensitivity and specificity. (*d*) Colourimetric reactions were assessed quantitatively as the hue component of colour and (e) LAMP target validation was conducted by PCR amplification using outer most LAMP primers followed by Sanger sequencing. (*f*) The applicability to trace DNA was assessed for two extraction methods, a rapid approach and a more traditional laboratory method, with detection facilitated by *B. constrictor* fluorescence LAMP.

trade, based on previously published python mtDNA sequences (Supplementary Appendix A) (GenBank accession numbers EF545015–107) (Rawlings *et al.* 2008). Primers were designed using the normal default parameters and selected based on the largest delta G for dimerisation and with end stability of less than −4 kcal/mol at the 3′ end of the F2 region, 5′ end of the F1c region, 3′ end of the B2 region, and 5′ end of the B1c region. Additionally, all primers were designed to have GC content between 40 and 60%. Species-specific LAMP primer sequences were imported into Geneious Prime 2023.1 (Biomatters, New Zealand) for visual inspection against *B. constrictor* mtDNA sequences, and Megablast searches of the nucleotide collection (nr/nt) database were conducted for the target regions. As an assessment of primer pair specificity, the FastPCR software was used to test cross-species amplification *in silico*. FastPCR was used because this tool allows for linked searches of multiple primer pairs (Kalendar *et al.* 2017). In FastPCR the LAMP primers were uploaded, and the *in silico* PCR function was used to assess potential amplification for the top 20 Megablast hit table results. Based on these primer design conditions, a 207-bp fragment of the mitochondrial D-loop control region was ultimately selected for *in vitro* testing. LAMP primers were ordered from Sigma Aldrich, USA, with the synthesis scale

of 0.025 μmole, desalt purification, at a concentration of 100 μM in TRIS solution.

Synthetic gBlock development and use in validation

The LAMP target region for *B. constrictor* mitochondrial control region was used to design synthetic DNA ordered as gBlocks™ Gene Fragments (IDT, USA) to provide positive controls of known concentration, and for assessing reaction sensitivity (Agarwal *et al.* 2020; Rako *et al.* 2021; Starkie *et al.* 2022). Developing gBlocks™ with slight differences in annealing derivative temperature can facilitate differentiation between sample and synthetic positive control when separating priming regions by triplicate GGG or CCC (Agarwal *et al.* 2022). Thus, we designed two gBlocks™, BC.MCR that matched the target region for which the primers were designed and BC.CR.P that includes each primer binding site separated by CCC.

BC.MCR: 5 – AT TGT GTC CCT TAA TTC TGC CCT TCC CGT GAA ATC CTC TAT CCT TTC ATA CAT GCT AAC AGT CCT GCT TTT CAC GTC CAT ATA ATG TAA CCC CTCCCT ACT GTA CTT TCC AAG ACC ACT GGT TAC ACC TTC AAG TCC ATT TCA ACG GCC CGG AAC CAT CCC TCC CTA CTT GCT CTT TCC AAG ACC TTT GGT CGCACC CTT TAT TTA AGT AC – 3

BC.CR.P: 5 – CC TGT GTC CCT TAA TTC TGC CCC CAT CCT CTA TCC TTT CAT ACA TGC CCC TAA CAG TCC TGC TTT TCA CGT CCC ATA ATG TAA CCC CTC CCT ACTGTA CCC AGA CCA CTG GTT ACA CCT TCA CCC TCC ATT TCA ACG GCC CGG CCC ACT TGC TCT TTC CAA GAC CCC CGT CGC ACC CTT TAT TTA AGT CCC – 3

BC.MCR was used as a positive control and serially diluted from 1 ng/μL to 1 fg/μL as standards used for assessing the limit of detection. BC.CR.P was developed and utilised to test primer binding affinity and as an additional synthetic control.

In vitro validation of LAMP primers

Samples

Positive control DNA was extracted from a *B. constrictor* liver sample (10 mg), collected on 13 June 1997 (ex-Adelaide Zoo) and obtained from the Australian Biological Tissue Collection (ABTC accession #74730, South Australian Museum). Samples used for *in vitro* validation included shed skins from *B. constrictor* and non-target reptiles common in the legal Australian reptile trade covering several genera (*Morelia carinata*, *Morelia spilota*, *Antaresia childreni*, *Antaresia maculosa*, *Liasis olivaceus*, *Liasis fuscus*, *Aspidites ramsayi*, *Aspidites melanocephalus*, *Simalia kinghorni*), and those identified as key incursion species (*Pantherophis guttatus* and *Python brongersmai* (Stringham *et al.* 2021*a*). Shed skins were selected because they are morphologically indistinguishable without expert training but provide a reliable source of DNA (Bricker *et al.* 1996). These samples were sourced from the Gorge Wildlife Park, South Australia, and some shed skins were received from members of the public. DNA extraction was conducted in duplicate using the QIAamp DNA Investigator Kit (Qiagen, USA), alongside extraction blank controls. Initial DNA yields were quantified using the Quantus™ Fluorometer and QuantiFluor dsDNA System (Promega, USA) and then serially diluted 1:10 to assess the limit of detection for LAMP assays.

Colourimetric LAMP reactions

The WarmStart® Colourimetric LAMP 2x Master Mix (New England Biolabs, USA) was used to amplify synthetic gBlock DNA, ABTC reference DNA, and DNA from shed skin extracts. Reactions were conducted for *B. constrictor* and all non-target species following the manufacturer's protocol. LAMP primers were combined in a 10x primer mix at two different concentrations: 'standard' – containing 16 μM FIP/BIP, 2 μM F3/B3, and 4 μM LF/LB; and 'high' – 20 μM/2 μM/10 μM. LAMP reactions were conducted using 12.5 μL 2x WarmStart MasterMix, 2.5 μL 10x primer mix (standard or high), 1 μL of DNA, and dH_2O to a total volume of 25 μL, using an Eppendorf Mastercycler X50 at 65°C for 30–75 min.

Sensitivity was assessed by performing serial 1:10 dilutions of the synthetic gBlock DNA ranging from 1 ng/μL to 0.1 fg/μL, and for DNA extracted from the ABTC *B. constrictor* positive control ranging from 63 ng/μL to 0.63 pg/μL. We included 12 technical replicates at each concentration (eight in 12.5 μL, and four in 25 μL reaction volumes). Samples were photographed following 30, 45, and 60 min of incubation at 65°C, to assess colour transition. We defined the limit of detection (LOD) using a discrete threshold of 100% positive amplification for all replicates at the lowest concentration (Klymus *et al.* 2020), without detectable primer-dimer (NTC transitioned to yellow).

Specificity assessments were conducted for all non-target Australian snakes with four technical replicates of each DNA extract, followed by an additional four technical replicates for 1:10 dilutions of DNA extracts that transitioned away from pink towards yellow.

Colour component analysis

Colour component analysis was conducted on all colourimetric LAMP reactions to determine the earliest point of colour change, colour change sensitivity, and conditions for colour change, including initial template concentration and primer concentrations. This analysis was conducted in the Fiji version of ImageJ (Schindelin *et al.* 2012). Images were collected under standardised conditions by use of a lightbox placed underneath samples after incubation for 30, 45, and 60 min, halting at the earliest point of non-specific amplification. At each time interval an image was captured using a Xiaomi Pocophone F1 using the manual mode, with the shutter speed locked at 1/500 and the ISO set to 400.

The images were imported into ImageJ and the image type was changed to HSB stack. A circular region of interest was placed under the meniscus of each sample with a 40-unit

height and width encapsulating 1264 pixels. For each region of interest, the mean hue values were recorded (Fig. 2). Hue is an established indicator for colourimetric LAMP transition, exemplified in studies concerning human body fluid identification (Scott *et al*. 2020; Layne *et al*. 2021). The hue values for each of the reactions were plotted using ggplot2 (Wickham 2011) package in the R software environment (v 1.3.1073) for statistical and graphical computing (R Core Team 2023) to provide an objective and quantitative assessment of colourimetric transition success. This approach was used to establish upper and lower bounds for positive LAMP reactions.

Fluorescence-based LAMP

In parallel, we tested fluorescence-based real-time LAMP detection. We followed the LAMP user guide published by Optigene to develop our assay and optimise primer concentrations (Optigene 2017). We firstly tested fluorescence LAMP reactions in 25 μL volumes containing 15 μL of the ISO-004 GspSSD2.0 Isothermal Master Mix (Optigene, UK), 2.5 μL of the 'high' primer mix, 1 μL sample, and 6.5 μL water. We also tested 'low' primer concentrations (FIP/BIP = 0.8 μM, F3/B3 = 0.2 μM, LF/LB = 0.4 μM), in 12.5 μL final volumes containing 7.5 μL of the ISO-004 GspSSD2.0 Isothermal Master Mix (Optigene, UK), 1.25 μL primer mix, 1 μL sample, and 2.75 μL dH_2O. All real-time isothermal LAMP incubations were conducted at 65°C for 30 min, with measurements of fluorescence recorded at 90 intervals of 20 s on the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, USA). Additionally, melt curve analysis was conducted starting at 98°C for 15 s, 70°C for 60 s, 0.05°C/S ramping to 98°C for 15 s.

Sensitivity of the fluorescent LAMP approach was assessed by including 12 technical replicates of synthetic gBlock DNA diluted at concentrations ranging from 1 ng/μL to 0.1 fg/μL. Similarly, DNA extracted from the ABTC *B. constrictor* sample was diluted in series 1:10 ranging from 63 ng/μL to 0.63 pg/μL, with 12 technical replicates at each concentration. We used a discrete threshold approach to determine the LOD, defined here as 100% positive replicates (Klymus *et al*. 2020). Specificity was assessed for all non-target species in at least triplicate, and additional testing was conducted for genera including *Morelia*, *Antaresia*, *Liasis*, and *Aspidites*. This included 10 technical replicates per species belonging to each genus, because these species were identified as common native Australian snakes kept as pets.

PCR amplification and Sanger sequencing to confirm species specificity

Following LAMP testing, *B. constrictor* DNA extracts from shed skin underwent PCR amplification using the species specific F3/B3 LAMP primers. PCRs were conducted containing PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, USA), 0.4 μM of forward and reverse Primer, 1 μL of DNA extract, and dH_2O to a volume of 20 μL. Thermocycling conditions on the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, USA) were as follows. Initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 15 s, annealing at 54°C for 20 s, extension at 72°C for 60 s. This also included a melt curve following amplification with the following conditions: 95°C for 15 s, 60°C for 60 s, 0.05°C/S ramping to 95°C for 15 s.

Subsequent bi-directional Sanger sequencing of PCR amplicons was conducted by the Australian Genome Research Facility (AGRF, Australia) to confirm correct target amplification. Sequences were analysed in Geneious Prime (Biomatters, New Zealand), with ends trimmed at the 3′ and 5′ ends and an error probability limit of 0.05. De novo assemblies were created using the reference sequence NC_007398, F3 and B3 primers and the bi-directional Sanger sequencing data. The consensus sequence was subject to a BLAST search.

DNA detection from swabbed tanks

Following optimisation of LAMP assays using synthetic DNA and DNA extracted from tissue and shed skins, we tested the detection efficacy of the colourimetric and fluorescence assays on detection of remanent trace DNA left behind post *B. constrictor* presence in glass tanks (following The University of Adelaide animal ethics approval ID S-2020–024). Prior to reptile exposure, 65-L glass tanks were sterilised using 10% bleach followed by a wipe down with absolute ethanol. Tanks were placed in a constant-temperature room (25°C) next to one another for the duration of each experimental trial. For each of five replicates and one negative control (*B. constrictor* absence), one *B. constrictor* individual was placed in a 65-L glass tank for 24 h, the snake was then removed, and the inside surface of the base was swabbed with five different swabs, one targeting each corner and one targeting the centre of the tank. Copan FLOQSwabs pre-wetted with 40 μL of 0.1% Triton X-100 (Bio-Rad, USA) in DNA-free water were used for sample collection. DNA was extracted from the tank swabs using QIAamp DNA Investigator Kit (Qiagen, USA) and quantified using the Quantus™ Fluorometer and QuantiFluor dsDNA System (Promega, USA).

We also tested a rapid lysis DNA extraction method QuickExtract (Lucigen, USA) as an alternative to the more time-consuming QIAamp DNA Investigator Kit, facilitating rapid detection. An additional six replicate tanks, including a negative control, were set up and swabbed as previously described. Each swab was placed in 250 μL of the QuickExtract buffer and incubated following the manufacturer's instructions. DNA was quantified using the Quantus™ Fluorometer and QuantiFluor dsDNA System (Promega, USA).

Results

LAMP primer development

LAMP primers were successfully developed targeting a 207-bp portion of the *B. constrictor* mitochondrial control

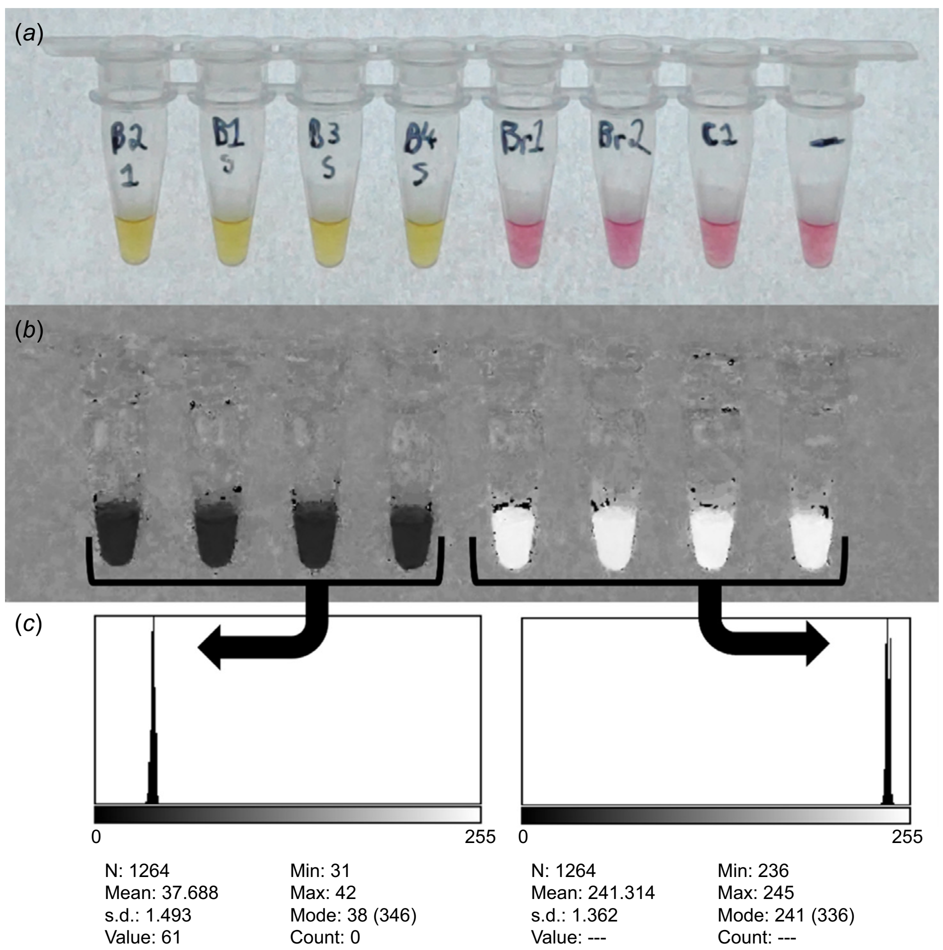


Fig. 2. Image capture, transformation, and hue quantification of LAMP colourimetric detection of *B. constrictor* DNA. (*a*) Reaction series captured under standardised conditions using a light pad placed underneath the samples, captured using a Pocophone F1 with a shutter speed of 1/500 s and ISO locked to 400. (*b*) The same image is then converted to a HSB stack. (*c*) A circular selection of 1264 pixels is made under the meniscus of the reaction, and the hue mean is quantified.

region (Table 1). BLAST searches of this 207-bp sequence showed the highest non-target percentage identity was 91.09%, corresponding to *Eunectes notaeus* (yellow anaconda, JN967236.1). A linked search conducted in FastPCR (Kalendar *et al.* 2017) indicated no cross amplification concerns for the LAMP primers, returning only *B. constrictor* (D84260, and AB177354) sites when queried against the top 20 Megablast hits.

Table 1. LAMP primer sequences (5′ > 3′) for species-specific detection of *B. constrictor*.

Species	Regions	Primers
B. constrictor	Mitochondrial DNA control region	F3: TGTGTCCCTTAATTCTGCC
		B3: ACTTAAATAAAGGGTGCGAC
		FIP: TACAGTAGGGAGGGGTTACATTAT ATCCTCTATCCTTTCATACATGC
		BIP: AGACCACTGGTTACACCTTCA GGTCTTGGAAAGAGCAAGT
		LF: ACGTGAAAAGCAGGACTGTTA
		LB: TCCATTTCAACGGCCCGG

Primers target a 207-bp fragment of the mitochondrial control region with each primer in the 5′ to 3′ direction.

Primer and reaction condition optimisation for *B. constrictor* colourimetric LAMP assay

Greater specificity, and shorter reaction time to positive detection (45 min), was achieved with 'high' primer concentrations (Fig. 3*a*). No cross amplification *of P. guttatus* DNA was observed. *B. constrictor* template transitioned to yellow (positive) with input DNA ranging from 83 ng to 83 pg after 75-min incubation, when using the 'standard' primer concentrations (Fig. 3*b*). Cross amplification (mild colour change) was detected for *P. guttatus* containing 16 ng of

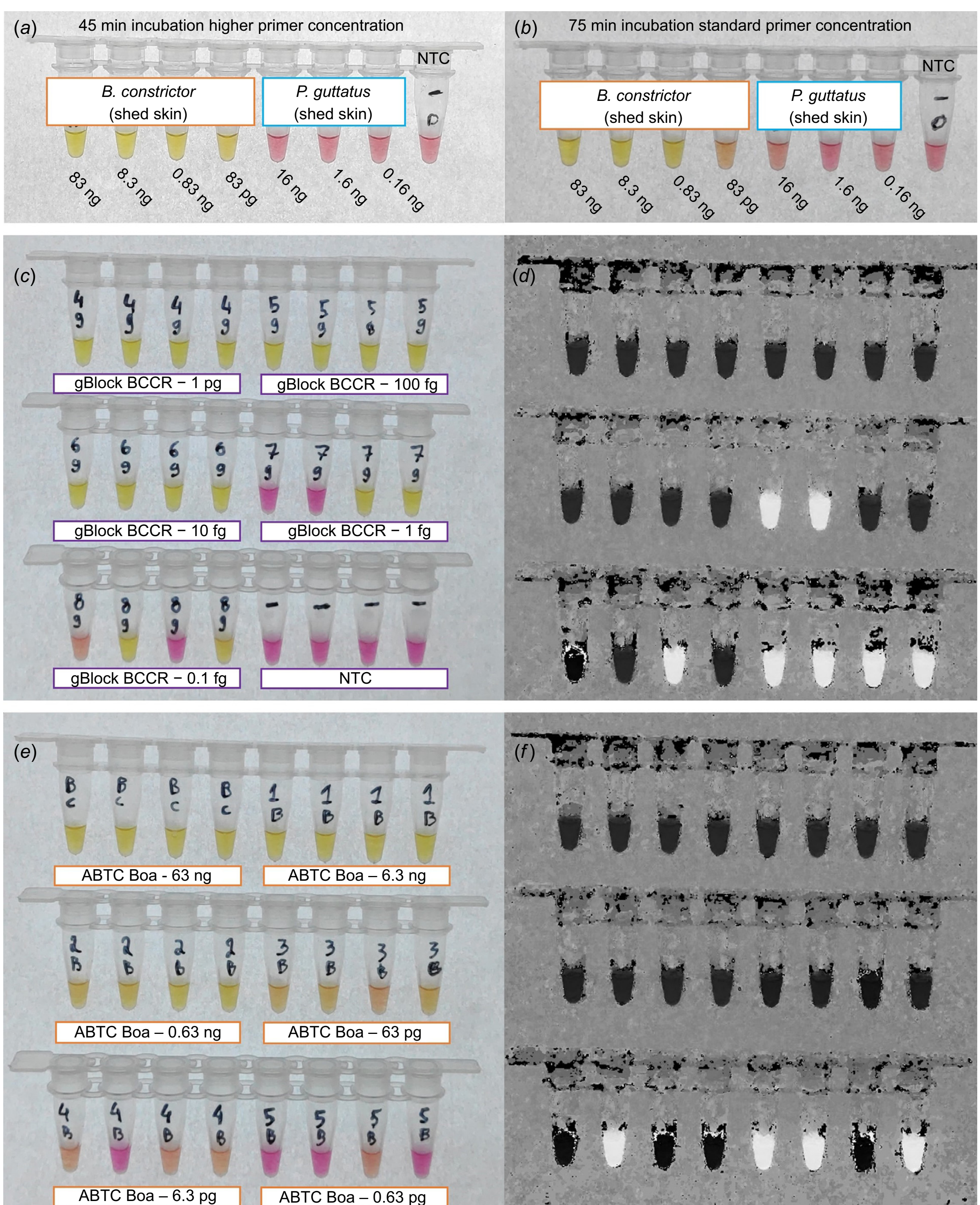


Fig. 3. Colourimetric *B. constrictor* LAMP assay with varied primer concentrations, incubation periods, and constant 65°C incubation temperature. (*a*) Results of higher primer concentrations post 45 min of incubation, with *B. constrictor* and *P. guttatus* DNA included in the reactions. (*b*) Reaction series incubated for 75 min including standard primer concentration, all reactions containing *B. constrictor* template (83 ng–83 pg) transitioning to yellow. (*c, d*) Sensitivity testing, for high primer concentrations, incubated for 45 min, including 1:10 dilution series of synthetic DNA (BC.MCR) with four replicates ranging from 1 pg–0.1 fg, shown as the raw image and the HSB transformed image. (*e, f*) Sensitivity testing, with high primer concentrations for a 1:10 dilution series including genomic *B. constrictor* DNA (ABTC Boa) ranging from 63 ng–0.63 pg, incubated for 45 min.

input DNA after 75-min of incubation (Fig. 3*b*). Maintaining the higher primer concentrations, LAMP reactions containing synthetic DNA diluted down to 10 fg (100% of replicates) transitioned to yellow after 30 min (Fig. 3*c*, *d*), including the primer gBlock (BC.CR.P). Colour change for the ABTC *B. constrictor* liver DNA extract (i.e. down to 63 pg) was achieved after 45 min (Fig. 3*e*, *f*).

Maintaining the higher primer concentration while testing specificity led to promising results for native Australian snake species and new incursion species in the pet trade (Fig. 4). We did not detect cross amplification for *M. carinata*, *M. spilota*, *A. childreni*, *A. maculosa*, *L. olivaceus*, *P. guttatus*, *P. brongersmai*, or *S. kinghorni* (Fig. 4*a*–*d*). Mild colour transitions were observed for *L. fuscus*, *A. ramsayi*, and *A. melanocephalus* with DNA inputs >10 ng (Fig. 4*a*–*d*). Reducing the template to <10 ng following 1:10 dilutions reduced the severity of cross amplifications, as clearly indicated on the hue-transformed image, with none of the reactions transitioning to solid black (Fig. 4*f*). Incubating reactions for 60 min resulted in far greater cross amplification, visible as stronger transitions to yellow, and is thus strongly recommended against.

Colour component analysis

Quantifying colourimetric reactions as the hue component of colour provided an objective quantitative delimitation of positive and negative reactions, establishing the optimal primer concentrations, incubation times, and limits of detection. The resulting optimal reaction conditions utilised the 'high' primer concentration with 45-min incubation at 65°C. Assessing the selected reaction conditions as the hue component of colour for gBlock fragments BC.MCR and BC.CR.P, the ABTC *B. constrictor* DNA extract and several snakeskin extracts (B1, B2, B3, B5, B6) serially diluted 1:10 ranging from 83 ng to 35 pg led to positive detection for all LAMP reactions containing *B. constrictor* target DNA after 45 min of incubation at 65°C (Fig. 5). The positive detection range (orange dashed lines) spanned mean hue values of 20–60 units (Fig. 5) and defined the quantitative range for target detection. Incubating the reactions for 60 min resulted in occasional colour change in the NTC's (primer-dimer) and is thus strongly recommended against.

Fluorescent LAMP detection

Synthetic *B. constrictor* gBlock DNA and genomic DNA from the ABTC reference sample and shed skin samples were detected using the fluorescent LAMP assay, at two different primer concentrations (0.8 μM/0.2 μM/0.4 μM and 2.0 μM/0.2 μM/1.0 μM for FIP/BIP, F3/B3, and LF/LB respectively). At higher primer concentrations we detected primer dimer using the DNA 1000 kit (Agilent, USA) on the 2100 Bioanalyser system, so we opted for lower primer concentrations. Serial 1:10 dilutions of BC.MCR, *B. constrictor* ABTC reference DNA, and shed skin samples led to the positive detection of synthetic DNA down to 1 fg and genomic *B. constrictor* DNA extracts down to <10 pg (Fig. 6*a*, *b*). The LOD for synthetic DNA was 1 fg as at 0.1 fg only 5/12 replicates amplified, and *c*. 1 pg for genomic *B. constrictor* DNA as at 0.63 pg only 2/12 replicates amplified. Additionally, the melt curves confirmed the target LAMP amplicons were generated for synthetic DNA (BC.MCR, BC.CR.P), the *B. constrictor* ABTC reference sample, and skin extracts (Fig. 6*c*).

Based on the amplification curves and melt curve temperatures, no cross amplification was detected for most of the native Australian snakes common in the pet trade. However, cross amplification was detected for one native snake species, *L. olivaceus* (99 ng), and one invasive species, *P. guttatus* (16 ng), at high template DNA inputs. Each cross amplification event was limited to only one out of each triplicate reaction. Thus, only results with two or more positive amplification curves crossing the fluorescence threshold were further considered positive detections for *B. constrictor*.

Additional dilution series to assess cross amplification sensitivity for species where cross amplification was detected initially resulted in no amplification for two shed skin extracts of *L. olivaceus* (L.oli1 99 ng–99 pg and L.oli2 at 78 ng–78 pg). Additional cross amplification assessed for *P. guttatus* (C3 and C4 at 32 ng–32 pg) led to cross amplification for one reaction within a technical triplicate with 32 ng of input *P. guttatus* DNA. Reactions including less than 10 ng of non-target DNA did not lead to conclusive amplification.

We identified high initial DNA template (>10 ng) as a potential source of undesirable cross amplification. Further cross amplification testing conducted for two species of each genus of common Australian pet snake (*M. carinata*, *M. spilota*, *A. childreni*, *A. maculosa*, *L. olivaceus*, *L. fuscus*, *A. ramsayi*, and *A. melanocephalus*) at concentrations of input DNA between 10 and 4 ng did not lead to any cross amplification for 10 technical replicates of each species.

PCR and bi-directional Sanger sequencing

Synthetic gBlocks and *B. constrictor* skin extracts were successfully amplified using the F3 and B3 primers. Bi-directional Sanger sequencing confirmed the intended target PCR amplicons, with the gBlock BC.MCR amplicon matching the NC_007398 reference with 100% query cover and percentage identity. Similarly, DNA extracts derived from shed snakeskin of *B. constrictor* (B4) also matched reference samples AB177354 and D84260, with PCR reactions including 37 ng of template returning 100% Query cover and 98.08% identity and 37 pg of template returning 100% query cover and 97.60% identity.

DNA detection from swabbed tanks

The colourimetric LAMP assay successfully detected *B. constrictor* DNA from swab samples retrieved from empty

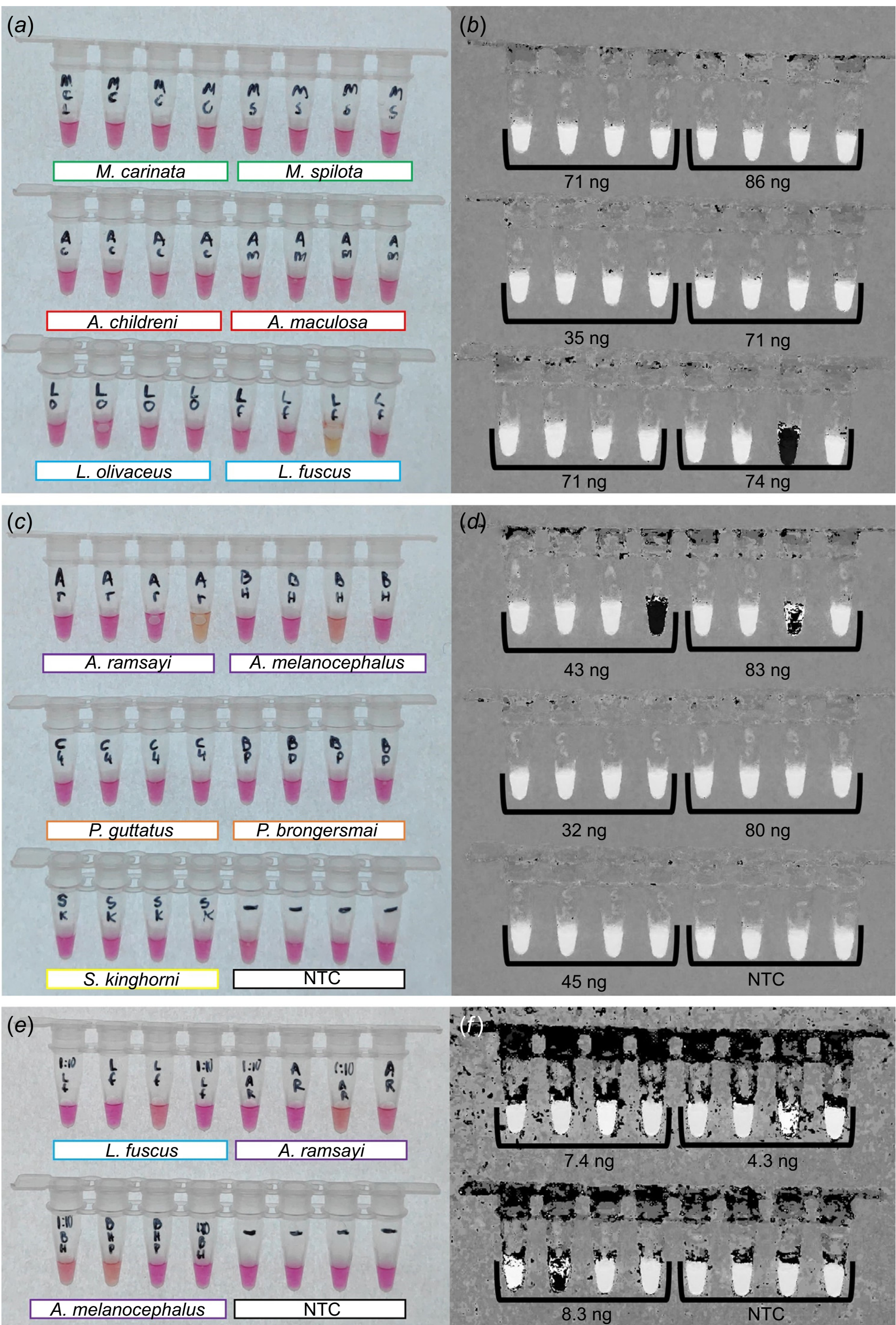


Fig. 4. Cross amplification testing for colourimetric LAMP assay targeting a 207-bp section of the mitochondrial D-loop of *B. constrictor*. All reactions were incubated at 65°C for 45 min with F3/B3, FIP/BIP, and LF/LB primers included at 2.0 μM/0.2 μM/1.0 μM respectively. (*a*, *b*) Colourimetric transitions for *M. carinata*, *M. spilota*, *A. childreni*, *A. maculosa*, *L. olivaceus*, and *L. fuscus*, shown as a raw image and HSB transformed image, including amount of added DNA. (*c*, *d*) Colourimetric transition for *A. ramsayi*, *A. melanocephalus*, *P. guttatus*, *P. brongersmai*, *S. kinghorni,* and the no template control (NTC), shown as a raw image and the HSB transformed image, including DNA input amounts. (*e*, *f*) Colourimetric transitions of 1:10 diluted *L. fuscus*, *A. ramsayi*, and *A. melanocephalus*, and NTC reactions to re-assess the colour transition at lower concentrations.

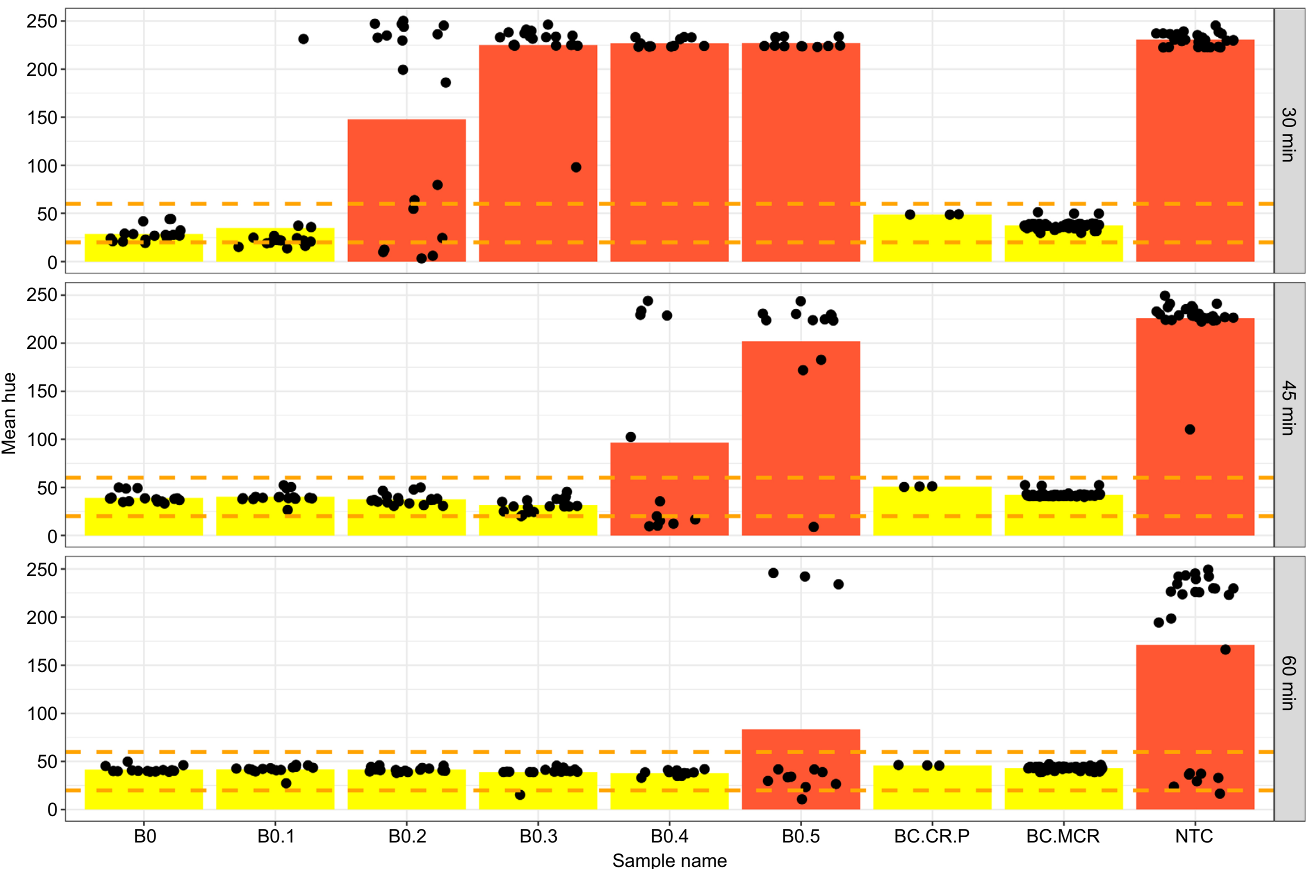


Fig. 5. Hue component of colour assessed at different time points during the progress of the LAMP reaction for several samples with F3/B3, FIP/BIP, and LF/LB primers included at 2.0 μM/0.2 μM/1.0 μM respectively. Points indicate different individual sample hue measurements, with bars indicating the average hue for each category. Category B0 includes ABTC liver DNA sample at 63 ng/μL and *B. constrictor* shed skin DNA extracts B1, B2, B3, B5, B6 ranging from 89 to 35 ng/μL of input DNA. Categories B0.1–B0.3 showcase a serial 1:10 dilution of the B0 samples. Synthetic gBlock DNA fragments BC.MCR, BC.CR.P, and a no-template control (NTC) are included for reference. Orange dashed lines indicates the range of positive detection.

tanks (following 24-h *B. constrictor* presence). However, repeatability and colourimetric transition were low and inconsistent, so we abandoned this approach for the specific application of trace DNA detection from contact samples. The fluorescent assay resulted in less time to detection (<30 min in contrast to <45 min for colourimetric LAMP), greater repeatability (consistent amplification in triplicate), and an order of magnitude greater sensitivity. As such, the remainder of *B. constrictor* touch samples from glass tanks were amplified using the fluorescent assay.

Initial testing of trace DNA detectability

Initial testing conducted for sample swabs extracted using QIAamp investigator kit retrieved post removal after 24-h of *B. constrictor* presence yielded positive results, with at least one swab extract amplifying in duplicate for each tank replicate (Table 2*a*). Swabs retrieved from Tank 1 had a minimum of four swabs with at least duplicate amplification. Swabs retrieved from Tank 2 led to positive triplicate amplification for a minimum of three swabs. Tank 3 led to positive amplification for two swabs in at least duplicate. Tank 4 led to positive amplification of a single swab in duplicate. Tank 5 led to positive amplification for one swab in triplicate. No amplification was detected in the negative control tank.

QuickExtract integration for rapid detection of touch samples

Testing conducted for tank swabs subject to QuickExtract DNA extractions led to positive detections for a minimum of two swabs in duplicate for each tank (Table 2*b*). Swabs retrieved from Tank 1 led to at least duplicate amplification for two swabs. Tank 2 led to the recovery and amplification of two swabs in triplicate. Swabs recovered from Tank 3 led to triplicate amplification for two swabs. Tank 4 included four swabs with at least duplicate amplification. Tank 5 included two swabs that amplified in at least duplicate. We did not observe any amplification from the negative control tank.

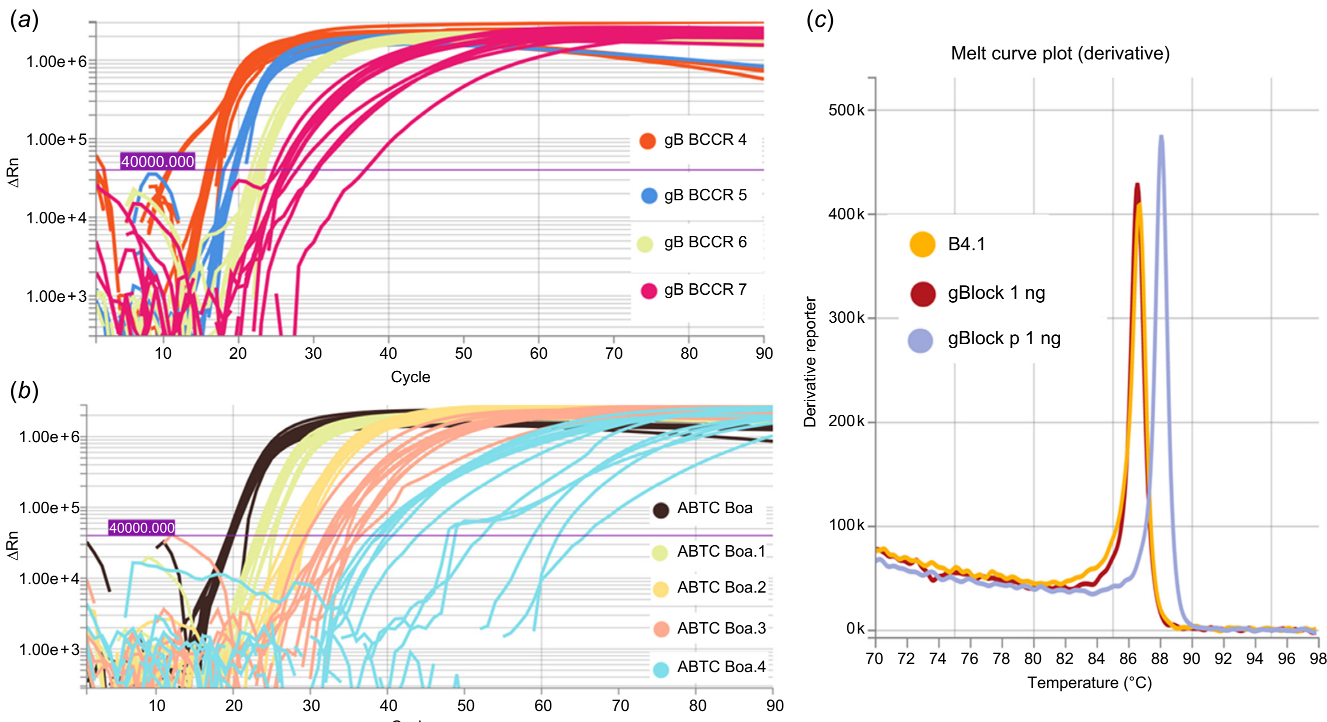


Fig. 6. Amplification curves for LAMP reactions conducted for 30 min at an incubation temperature of 65°C. (*a*) Synthetic gBlock designed to match priming regions diluted 1:10 (1 pg/μL–1 fg/μL) and (*b*) template DNA extracted from *B. constrictor* liver sample diluted 1:10 (63 ng/μL–6.3 pg/μL). (*c*) Melt curve plot indicating the LAMP amplicons for synthetic (gBlock BC.MCR) and *B. constrictor* shed skin DNA (B4.1) extracts match, with a different anneal derivative temperature for the synthetic primer gBlock (BC.CR.P).

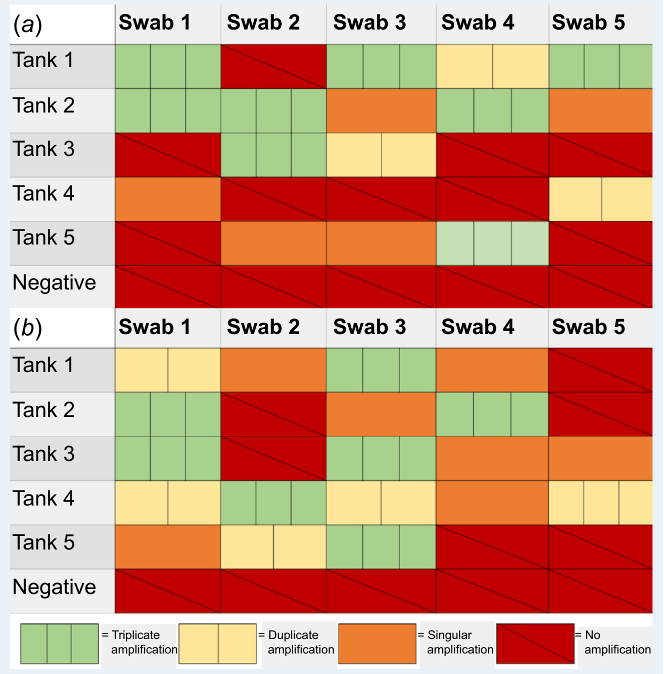
Discussion

Colourimetric and fluorescent LAMP assays are a promising novel tool for detecting reptile species of highest biosecurity concern in Australia. Both assays detected the presence of target DNA with high specificity under optimal primer concentrations and showed appropriate sensitivity to operate in a trace DNA environment, detecting as little as 1 fg of synthetic template and less than 10 pg of genomic *B. constrictor* DNA extracted from reference tissue and shed skins. Additionally, LAMP detection for both assays was possible within 45 min. We present promising solutions to field testing of trace DNA samples due to the low expertise, equipment, and time required to carry out the set-up and incubation of LAMP reactions. As such, we have provided an important first step to developing field-based detection capacity for biosecurity risk reptile trafficking.

Several additional steps and considerations could be taken to improve and build on our work presented here. These improvements would primarily involve further increasing the specificity, sensitivity, and robustness of the outlined detection protocol, including considerations such as comparisons with alternative amplification methods and field-ready apparatus.

Our fluorescence LAMP assay, developed using the Optigene GspSSD2.0 Isothermal Master Mix (ISO-004), provided the best diagnostic capacity. We confidently detected described gBlock fragments, DNA extracts from tissue, shed snakeskin, and trace DNA recovered from glass tank surfaces. The fluorescent assay also provided a faster time to detection, with incubation limited to 30 min. The difference in assay performance could be due to the polymerase involved in the reactions with the colourimetric approach utilising the *bst 2.0* polymerase, as opposed to the *GspSSD2.0* used in the fluorescent assay. Differences in polymerase performance have been observed in the presence of inhibitors (Jevtuševskaja *et al.* 2017). Additionally, this could be due to fundamental differences in the chemistries of the two approaches, with the colourimetric approach relying on a change in pH due to the by-product magnesium pyrophosphate, resulting in a colour change from pink to yellow (Tanner *et al.* 2015). The fluorescent approach utilises an intercalating dye that binds to amplified DNA and thus increases over time as amplification progresses (Hardinge and Murray 2019). The sensitivity of the QuantStudio may also play a role due to its capacity for detecting small changes in fluorescence, as opposed to naked-eye or hue component analysis at the end point of the reaction. This gap could potentially be bridged by use of a spectrophotometer, with additional potential for quantification (Nguyen *et al.* 2019). However, such specialised equipment is commonly confined to a dedicated lab so is not well suited to rapid *in situ* biosecurity detection. Fluorescent real-time detection also allowed us to assess the amplification

Table 2. Tabulated LAMP amplification results for each empty tank and the five swabs used to sample following *B. constrictor* presence and removal after 24 h. (*a*) DNA was extracted using the QIAamp DNA Investigator Kit (Qiagen). (*b*) QuickExtract (Lucigen, USA) was used to extract DNA prior to DNA amplification. Each swab from every tank is indicated as either triplicate, duplicate, singular, or no amplification.



(*a*)	Swab 1	Swab 2	Swab 3	Swab 4	Swab 5
Tank 1	Triplicate	No	Triplicate	Duplicate	Triplicate
Tank 2	Triplicate	Triplicate	Singular	Triplicate	Singular
Tank 3	No	Triplicate	Duplicate	No	No
Tank 4	Singular	No	No	No	Duplicate
Tank 5	No	Singular	Singular	Triplicate	No
Negative	No	No	No	No	No

(*b*)	Swab 1	Swab 2	Swab 3	Swab 4	Swab 5
Tank 1	Duplicate	Singular	Triplicate	Singular	No
Tank 2	Triplicate	No	Singular	Triplicate	No
Tank 3	Triplicate	No	Triplicate	Singular	Singular
Tank 4	Duplicate	Triplicate	Duplicate	Singular	Duplicate
Tank 5	Singular	Duplicate	Triplicate	No	No
Negative	No	No	No	No	No

= Triplicate amplification; = Duplicate amplification; = Singular amplification; = No amplification

curves and corresponding melt curves. This was a useful tool in determining whether amplification followed the expected sigmoidal pattern and melt temperature corresponded to the synthetic and positive ABTC control sample. End point detection using the colorimetric approach does not offer this benefit, leading to limited capacity to assess and exclude false positives due to non-target amplification.

Suboptimal results encountered when applying the colourimetric LAMP to tank samples may stem from the quality of DNA retrieved from swabs. This template DNA could have been degraded or low quality because it was indirectly retrieved from surfaces using swabs. DNA extracted directly from shed skins may be of higher quality because it is from a direct source (Bricker *et al.* 1996; Fetzner 1999; Horreo *et al.* 2015). As such, we can only recommend the application of our colourimetric assay to the detection of *B. constrictor* DNA from high-quality sources such as tissue, saliva swabs, and shed skins. These key sample types still present areas that require rapid biosecurity detection due to disease concerns, including IBD and scenarios in which the only remaining evidence of trafficking are morphologically indistinguishable shed skins. A similar issue was encountered when extracting DNA from snakeskin using the QuickExtract (Lucigen, USA) solution; this drastically impacted colour transition and resulted in inconclusive results, further demonstrating the requirement for high-quality DNA.

The primary advantage of the colourimetric approach is the limited resources required to conduct the experiment and the ease of interpretation for results. Fluorescent real-time detection requires a fluorometer, which is perhaps the largest barrier to biosecurity integration because these are traditionally benchtop qPCR machines confined to a dedicated laboratory space. This limitation can be overcome with the integration of field-ready LAMP apparatus, such as the Genie III developed by Optigene. The integration of in-field amplification and detection of biosecurity risk species has been demonstrated on several occasions (Rako *et al.* 2021; Agarwal *et al.* 2022), illustrating the suitability of a fluorescence LAMP assay in situations without overbearing financial concerns.

In addition to the greater sensitivity, repeatability, and capacity for field integration, specificity of our fluorescent LAMP assay could be increased with the integration of molecular probes. We encountered some non-specific amplification, which was limited to one technical replicate within each non-target triplicate. All non-target amplification was observed when relatively high DNA (>10 ng) was added into the reaction, which is much greater than what we would expect to encounter from trace DNA samples. Nonetheless, this presents a source of potential false positives. Consequently, we recommend diluting DNA and limiting input to less than 10 ng to avoid false positive detection. Alternatively, integrating molecular probes or beacons has previously led to increased specificity, reducing the risk of false positives due to non-specific amplification eliminating this issue altogether (Liu *et al.* 2017; Harding and Murray 2019). The molecular probe-based methods could additionally facilitate multiplexing, allowing for the detection of multiple biosecurity risk reptiles within one reaction (Tanner *et al.* 2012). The advantages for field integration using emerging in-field thermocyclers and the capacity for molecular probe integration means fluorescent LAMP methods are well poised for biosecurity screening, despite the higher financial costs in contrast to colourimetric LAMP.

The lack of amplification observed for some swabs retrieved from tanks subject to *B. constrictor* presence could stem from factors external to assay robustness, such as the shedding cycle of the individual present within the tank, the activity level of the individual while in the tank, or the lack of appropriate contact with the tank surfaces. Touch DNA is not distributed evenly or consistently, and deposition can vary due to a range of factors. DNA deposition dynamics is an understudied topic for reptiles, with a recent review highlighting the limited literature regarding terrestrial reptiles in contrast to other taxa (Nordstrom *et al.* 2022). A previous study has explored trace environmental DNA deposition, accumulation, and degradation for snakes in controlled lab and field environments (Kucherenko *et al.* 2018). In contrast to previous work, our study looks at recovery directly from

surface contact samples as opposed to sand or soil. Additionally, our accumulation time (24 h) is far shorter compared with the 7-day accumulation and degradation assessed for *P. guttatus* by Kucherenko *et al.* (2018). This reflects the context to which we envision our work best applies – short term transportation and rapid compliance checks of existing collections. In the context of our study, temperature, activity level, position in shedding cycle, and time of last meal (and their interactions) are likely to drastically alter the movement of individuals within their enclosure and thus impact DNA deposition and downstream recovery and amplification. These factors will almost always remain unknown in a realistic biosecurity scenario; however, we predict they will play a large role in detectability.

Confirming LAMP amplification by quantitative PCR led to successful amplification of gBlock fragments and extracts from snake skins. This approach illustrates the utility of LAMP for presumptive testing because the results can easily be confirmed by use of the F3 and B3 primers for PCR amplification followed by Bi-directional Sanger sequencing for target confirmation. Presumptive positive LAMP detection provides the end-user with a rapid test (LAMP) that will reduce the number of samples sent off for more exhaustive laboratory confirmation testing (PCR followed by sequencing). The qPCR amplification protocol used throughout had an overall thermocycling period of 1 h and 40 min, indicating this approach would not be well suited for in-field detection of *B. constrictor* because it is time consuming and requires specialist equipment. It would, however, be beneficial to conduct a comparative assessment of qPCR and related methods such as TaqMan PCR against our developed LAMP approach to ensure the most appropriate method for any given biosecurity scenario can be recommended. This has been conducted for *Trogoderma granarium* (Khapra beetle) in biosecurity incursion scenarios, highlighting important concerns about LAMP and the lack of suitability for highly degraded sources of trace DNA (Trujillo-González *et al.* 2022). Assessing factors such as speed of detection, sensitivity, specificity, cost, resource requirements, and ease of integration in a comparative study across the most likely sample types to be encountered would be highly beneficial when contrasting the suitability of LAMP against PCR, qPCR, TaqMan PCR, and alternative forms of isothermal amplification, including recombinase polymerase amplification (Hsu *et al.* 2021). We have focused our efforts on developing methods for trace DNA detection in a biosecurity and compliance context, but our described assays could also be applied to field environmental DNA biomonitoring, if or when *B. constrictor* establishes in Australia. We addressed this somewhat by including *S. kinghorni* in our cross amplification testing – a species with a range that overlaps the climate matching conducted for *B. constrictor* (Freeman and Freeman 2009; Henderson and Bomford 2011). Implementation following the best-practice guidelines for Australian and New Zealand eDNA researchers and end-users will aid preparedness given the possibility and risk of establishment (De Brauwer *et al.* 2023). This includes accounting for *B. constrictor* sub-species (Card *et al.* 2016) and population-level differences in the mitochondrial control region, which will impact primer binding, LAMP efficiency, and broad applicability. This also extends to the detection of sub-species and populations for which trafficking has been identified as a high risk at the source. Having high-quality vouchered specimens as positive controls for individuals from these populations will be necessary to assess LAMP applicability, particularly if mismatches are present in primer-binding regions. International applications of our assays will also require the careful considerations of non-target native species common in local pet-trades, to minimise potential cross amplification.

In summary, we have developed two LAMP assays, one that relies on colourimetric chemistry and another that is based on fluorescence detection. We critically and rigorously tested both the described assays for key sample types, including synthetic DNA, DNA extracts from tissue, shed skins, and trace DNA recovered from glass surfaces. Both assays could be used to detect *B. constrictor* trace DNA in biosecurity contexts, with our fluorescence-based approach most broadly suited to all sample types with acceptable sensitivity, specificity, and robustness. We recommend further optimisation of sample recovery, DNA extraction, specificity, and comparison with alternative methods to best address current unknowns and weaknesses.

Supplementary material

Supplementary material is available online.

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