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Recovering trace reptile DNA from the illegal wildlife trade

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ABSTRACT

Purpose: The Illegal Wildlife Trade (IWT), aided by improved global transport, and the expansion of the internet, has facilitated the international demand for exotic reptiles. The risks associated with trafficking of live reptiles requires robust forensic techniques for detecting housed or transported animals. Detection of species of high IWT demand can be challenging due to the illicit nature of the trade, particularly when a specimen is missing. The ability to detect trace DNA in empty holdings and transport containers can be pivotal as a source of evidence.
Methods: Vivaria, containing either a corn snake (*Pantherophis guttatus*) or boa (*Boa constrictor*), were set up and monitored for 24 h simulating reptile holdings. Once removed, Diamond Nucleic Acid Dye™ (DD) was sprayed on experimental glass and plastic tiles recovered from within the vivaria, and trace DNA was visualized using a Polilight. Trace DNA was amplified using a novel reptile target specific qPCR assay and sequenced to identify both species.
Results: Movement patterns and scale imprints associated with reptile contact were visible on experimental tiles after DD-staining. Successful qPCR amplification and subsequent bi-directional Sanger sequencing confirmed the presence of both the species in the respective vivaria. DNA recovered from glass tiles had significantly greater amplification success than plastic tiles.
Conclusions: DD revealed valuable information about the presence, and movement, of reptiles in the absence of a specimen. Successful amplification of trace reptile DNA demonstrated that this approach could offer an effective tool for biosecurity staff to rapidly identify live reptiles in the IWT.

1. Introduction

Reptiles are among the most heavily trafficked live animals in the Illegal Wildlife Trade (IWT) [1,2]. Comprehensive monitoring and surveillance techniques are essential for detecting species circulating in the trade, and further reducing the risk of introducing diseases [3] or new invasive alien species (IAS) [4]. Despite targeted efforts towards curbing the illegal trade in high profile wildlife products (e.g., rhino horn [5], elephant ivory [6], and pangolin scales [7]), we still have limited knowledge of the live illegal trade in 'exotic' pet species, which includes reptiles [2]. This knowledge gap and the resulting lack of comprehensive onsite biosecurity checkpoint monitoring and surveillance techniques can have negative implications for biodiversity conservation [8], animal welfare [9], and the transport and introduction of IAS via the pet-release pathway [10].

Even with global enforcement dedicated to the protection of wildlife and seizing samples part of this illegal trade [11], prosecutions are limited, with misidentification occurring in over 70% of seized live animals and wildlife products [12]. Reliable species identification is required to facilitate downstream prosecution, yet it is often not possible in cases without specimen evidence [13]. This can lead to weakened biosecurity; particularly in cases where remnant trace evidence is the only evidence available. Additionally, onsite capacity for detecting trace evidence is often limited due to a lack of expertize and equipment, as well as complex requirements for sample analysis [14].

Fluorescent nucleic acid dyes have been increasingly used to visualize trace DNA deposited by human touch, providing an informed and targeted means of collecting human DNA samples [15]. While recent advancements in the direct detection and visualization of trace DNA have been instrumental in forensic human identification [16,17], these

Abbreviations: IWT, Illegal Wildlife Trade; IAS, Invasive Alien Species; DD, Diamond Nucleic Acid Dye.

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tools have yet to be widely applied to wildlife forensic research, practice, or enforcement. Regarding the IWT, visualization of trace DNA has been explored for detecting human touch and linking DNA of perpetrators to items such as traps, snares, or holdings [18], with the aim to assist authorities in identifying perpetrators and support prosecution. However, little is known about trace DNA deposition in non-human species, which are the target of the IWT.

Here, we have applied the concept of direct trace DNA detection using Diamond™ Nucleic Acid Dye (DD) to develop a pipeline to recover and characterize trace reptile DNA within empty enclosures encountered in the IWT. Two species common in the wildlife trade were used as models; both species are considered key IAS in Australia, *Pantherophis guttatus* (corn snake) and *Boa constrictors* (boa) [19,20]. We explored the capability to visualize reptile trace DNA, and successfully recover and amplify such DNA, ultimately providing accurate species identification. Our case study focussed on illegally owned reptiles housed in a glass vivarium to experimentally simulate cases common in the IWT or those encountered when prohibited animals are kept as pets in private collections. This case study was informed by off-record discussions with Australian wildlife enforcement officers, working in situations where specimen evidence has been removed or hidden by the time they issue a search warrant (P Cassey; personal observation).

2. Material and methods

2.1. Experimental set-up

The experiment was conducted at the Gorge Wildlife Park situated in Cudlee Creek, South Australia. This privately-owned wildlife sanctuary provided access to five corn snakes and four boas. The park's reptile keepers handled the reptiles during each stage of the project, moving them in and out of the experimental setting, weighing them, collecting buccal swabs from the inner cheek, and providing shed skin samples from juvenile corn snakes. Animal ethics approval was provided by The University of Adelaide Animal Ethics Committee (Science) approval number S-2020–024.

Four glass tanks (65 l) were established as experimental vivaria into which individual snakes were placed for 24-hours at separate times. These vivaria were sterilized with 10% bleach and wiped down with absolute ethanol before an animal was placed inside. In each of the four tank corners, either a glass (two) or plastic (two) 10 × 10 cm tile was placed, with paired glass and plastic tiles corresponding on the diagonal (Fig. 1). Glass and plastic tiles were chosen as experimental units to experimentally replicate the surfaces of permanent glass vivaria and temporary plastic storage and transport boxes, respectively (P. Cassey; personal observation). Each tile was sterilized using 10% bleach, followed by an absolute ethanol wipe down prior to use. Sterilization was conducted off-site, but once the tanks were set up at the park, an additional ethanol wipe down was conducted for both the tank and tiles to minimize the opportunity for any contamination introduced during transportation.

A Moultrie M-990I trail camera was mounted overhead on a tripod to monitor the reptile's movements during its 24-hour residence time. This camera was programmed to take an image at 10-minute intervals for a period of 24-hours, to monitor the contact with each tile. One individual snake was placed in the tank with the glass lid sealed at the edges using masking tape, and sufficient openings were left for air flow (Fig. 1). Once 24 h had elapsed, the individual was removed by the reptile keeper whilst wearing nitrile gloves and returned to its holding or display. The tiles were collected and stored in cardboard boxes upright using rubber dividers, to avoid contact, cross contamination, or DNA loss. The tank was then sterilized with 10% bleach and absolute ethanol wipe down.

This process was repeated for each corn snake, (C1-C5), and each boa, (B1-B4). We included offsite negative controls (two glass and two plastic tiles) to represent an environment where no reptiles had been present. For each individual, four tiles (two plastic and two glass) were collected and transported for storage at the Forensic DNA laboratory (Flinders University, SA).

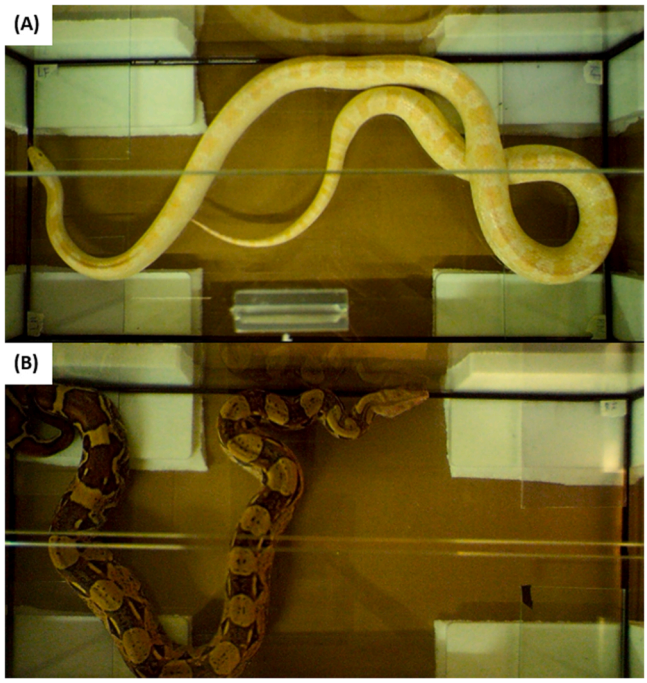

Fig. 1. Example of the experimental set-up. (A) Corn snake (*P. guttatus*; albino form) and (B) *Boa constrictor* during their 24-hour residence in the 65-litre vivarium. The two glass and two plastic tiles corresponding diagonally in each corner of the tank are labeled by their position, RF being right far, RN for right near, LF for left far and LN for left near.

2.2. Visualization

DD was used to visualize nucleic acids (single stranded and double stranded DNA and RNA) on the surface of the tiles. We used 20X DD solution, a 500-fold dilution of the 10,000X stock diluted in 75% ethanol (v/v) [21]. This was applied to each tile uniformly using a refillable pressurized spray can. Tiles were visualized and imaged using a Nikon D3400 with a 555 nm filter attached and a Rofin Polilight® (PL500) set at 490 nm. For each tile, an image was taken prior to DD staining to record the background material present on the tile and to account for autofluorescence (labeled as "Unsprayed", Fig. 2), and after spraying ("Sprayed").

2.3. DNA recovery

Each experimental 10 × 10 cm tile was subsequently sampled using four Copan Rayon tip swabs coated with 20 μL of 0.01X Triton-X, each swab targeting a 5 cm^2 area, designated using a clear plastic grid placed underneath each tile at the time of sampling. Each tile was continuously monitored throughout the swabbing process using the Rofin Polilight® ensuring maximal recovery of fluorescent material. Post swabbing, the swab head was removed using sterilized surgical scissors (sterilized with 10% bleach and absolute ethanol) and deposited in a 1.5 mL Eppendorf. Swab heads corresponding to the two left quarters of the tile were deposited in the same tube with a separate tube for the two swabs corresponding to the right quarters. Following swabbing, each tile was re-imaged using the Rofin Polilight® and camera as described above to assess recovery success ("post-swabbing").

Shed skin from the corn snakes, and buccal swabs from the boas were collected at the Gorge Wildlife Park and stored in ziplock plastic bags for use as positive controls. This was used to establish the standard curve for the qPCR assays and to confirm that the mtDNA ND2 sequences matched

the expected sequence for corn snake and boa. Shed skin samples were unavailable throughout the duration of the project for boas. As such, buccal swabs were an appropriate alternative [22]. For the available corn snakeskin and boa buccal samples, a single step Prep-and Go™ (Thermo Fisher Scientific, VIC, AU) lysis approach was used, which involved a one-hour incubation at 57 °C in 200 μL of Prep-n-Go™ Buffer. DNA concentration in each lysate was quantified in triplicate using the Invitrogen Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay Kit (Appendix B) [23].

2.4. Quantitative PCR

2.4.1. Primers and reference sequences

Primers were designed using Geneious Prime (2020.2.0) targeting the ND2 gene regions of the mitochondrial genome for both species. Mitochondrial genomes or sequences corresponding to these gene regions were acquired through the NCBI Nucleotide database and were as follows: *P. guttatus* (AM236349), *B. constrictor* mitochondrion (NC_007398). Primers were designed by eye considering parameters as described in [24] with an intended amplicon size of 200–300 bp. We included a ND2 sequence from humans (GU170821) to allow primer design to exclude amplification of human DNA that is likely to be present in IWT scenarios. Resulting primers were ordered through Sigma Aldrich at 50 μM. Primer sequences were ND2_F: 5′-CCGAAGCAGCWACAAAATAC-3′ and ND2_R: 5′-CCTGTGTGTGCGATTGATGA-3′.

2.4.2. Quantitative PCR set up

Quantitative PCRs were conducted in duplicate in Qiagen 0.1 mL strip tubes with a total volume of 20 μL, including 7.2 μL of DNA free H_20, 10 μL 2X KAPA SYBR FAST qPCR MasterMix (KAPA Biosystems), 0.4 μL of 10 μM Forward Primer, 0.4 μL 10 μM Reverse Primer and 2 μL of template DNA. For both species, a stepwise 1/10 dilution series of the positive control extracts (Appendix B) was used to construct species-specific standard curves (Appendix C). We note that the standard curves were based on genomic DNA concentrations (that include both nuclear and mitochondrial DNA) but the qPCR targeted only mtDNA. Therefore, we had to assume that nDNA:mtDNA ratios in the positive control DNA were the same as in the trace DNA swabs collected from the vivarium. All lab equipment was sterilized using 10% bleach and absolute ethanol wipe down pre- and post PCR set up, followed by 15 min of UV light radiation. All samples were run on the Qiagen Rotor-Gene Q 5plex HRM Platform with the green channel selected for fluorescence detection (excitation 470 ± 10 nm, emission 510 ± 5 nm). Cycling conditions were as follows: 1 × 94 °C for 4 min, 30 × 94 °C for 40 s, 56 °C 40 s, 72 °C for 60 s. Melt curve analysis was as follows: melt start at 72 °C hold for 90 s on the 1st step, hold for 5 s on the next steps, end 94 °C.

2.4.3. Material and species influence on amplification success

The output data obtained from Rotor-Gene Q 2.3.5 software were analysed using the R software environment (v 4.0.2 2020–06–22) for statistical and graphical computing [25]. Concentration data (in ng/μL) were graphed on a $\log_{10}$ scale due to the large variation (orders of magnitude) of values (Fig. 4). Every individual had two glass and two plastic tiles in the vivarium. Four measures of DNA concentration per tile (glass or plastic) corresponded to duplicate samples from the left and right sides of each tile. As such, every individual had eight corresponding measures of concentration per material type; where values were missing, amplification was not achieved.

Quantitative concentration outputs were converted to binary outcomes denoting either successful amplification or the lack thereof. This was based on the melt curve outputs. Successful amplification (presence) was denoted by a clean peak corresponding to the desired amplicon length. Amplification failure (absence) was denoted by a lack of an amplicon peak or a primer dimer peak with magnitude exceeding that of the target amplicon (Appendix D). To determine whether the probability of amplification success differed between material (i.e., glass or plastic) or species, binomial generalized linear mixed models were calculated, accounting for individual animal random effects and tile-specific random effects, using the R software package "lme4″ [26]. The estimated marginal means for both plastic and glass amplification probabilities were plotted with 95% confidence intervals [27]. Type II Wald Chi square tests were used to assess evidence against the null hypotheses of no differences in amplification between material types. Where there were clear differences, pairwise comparisons between material types for each species, with appropriate correction for multiple tests, were used to assess statistical differences in amplification probability.

For the glass tiles only, a generalized linear mixed effects model was used to assess probability of amplification success in response to non-zero concentrations, including random effects for each individual animal and tile.

2.4.4. Sanger sequencing for species level identification of trace DNA

Following qPCR amplification, a subset of PCR products generated from the tiles were selected for bi-directional Sanger sequencing at the Australian Genome Research Facility (AGRF). Selection was based on successful amplification of target amplicon, tile material type, and range of concentration for each species. For each species, two tiles, representing a different individual, were selected based on these criteria. Additionally, one positive control for each species and experimental negative controls were sequenced to validate the standards/assay and confirm primer dimer and null amplification scenarios, respectively.

Sequence data were analysed using Geneious (2020.2.0). The consensus sequence for each sample was BLAST searched against the NCBI nucleotide database. The NCBI nucleotide database was used in this instance as the premise of the study was to develop this tool and the related qPCR assay for the identification of reptiles from trace DNA evidence when the species is unknown to the practitioner.

3. Results

3.1. Visualization

All tiles revealed a visible increase in fluorescence after DD was applied (Fig. 2), including the negative controls. However, treatment tiles displayed much greater fluorescence: signs of movement patterns and distinct features for both snake species were recorded (Fig. 2). The fluorescence observed, and the visible deposition patterns, varied between species and individuals, with some tiles showing obvious signs of movement, visible patterns, or scale imprints, while others had less clear deposition patterns. Across both species, generally lower levels of fluorescence were observed for plastic tiles as opposed to glass (Appendix A). Swabbing efficiency, (i.e., recovery of trace material visible as fluorescence) was more challenging from the glass surfaces, with more fluorescence still visible post swabbing.

3.2. Quantitative PCR

3.2.1. Material and species influence on amplification success

Successful amplification for the reptile positive controls was achieved for the ND2 primer pair. The qPCR amplification for swab samples recovered from the experimental tiles using the novel primers was successful for both target species based on the combination of melt-curve and CT values. Trace DNA from the experimental tiles yielded positive amplification for tiles corresponding to each species with variable detected quantities of recovered DNA (Fig. 3). For the boa glass tiles, 6 out of 8 tiles resulted in at least one swab replicate with amplification, with a concentration range of 2.76E-4 –6.24 ng/μL. For the corn snake glass tiles, all tiles resulted in at least one swab replicate amplifying, with a concentration range of 0.01–3.49 ng/μL. Boa plastic

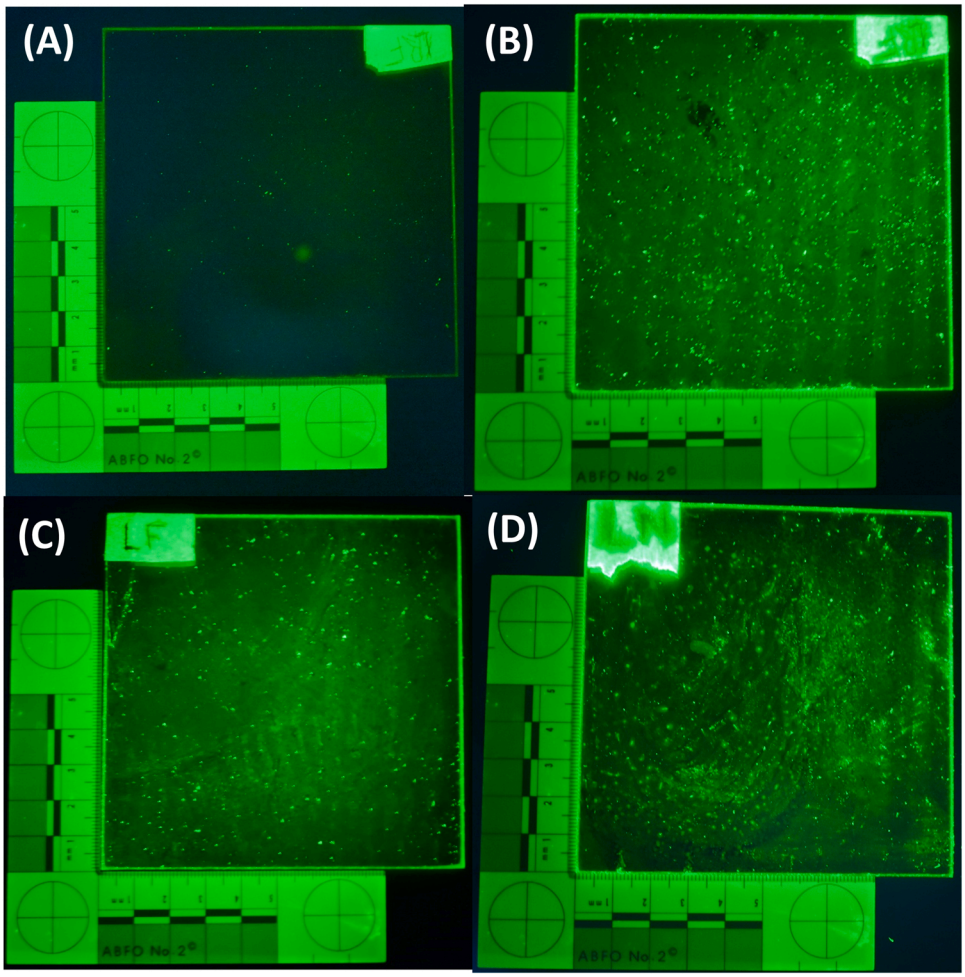

Fig. 2. Visualization of cellular material from glass tiles under the Rofin Polilight. (A) Tile from a *P. guttatus* trial imaged under the Polilight prior to Diamond Dye application indicating the lack of fluorescence; (B) the same tile after Diamond Dye application indicating the shift in fluorescence. (C) A *P. guttatus* tile; and (D) *B. constrictor* tile post spraying, highlighting examples of observable (C) linear-type scale imprints, and (D) curved movement patterns. All images captured under standardized conditions under the Rofin Polilight PL500 set to 490 nm using the Nikon 3400D with a 555 nm filter attached. See Appendix A for all sample tile images including negative controls.

tiles had lower amplification success with 4 out of 8 tiles resulting in at least one swab sample amplifying, with a concentration range of 2.48E-5–3.38E-3 ng/μL. Corn snake plastic tiles had the lowest amplification success with one tile swab sample resulting in amplification, with a concentration range of 3.01E-2–3.33E-2 ng/μL. Negative control swabs yielded melt-curves with no amplicon peak or a primer dimer peak with magnitude exceeding that of the target amplicon (Appendix D) and concentrations in the range 1.22E-2–2.20E-4 ng/μL.

The probability of amplification varied between species and material (Fig. 4), with DNA isolated from glass tiles amplifying significantly better than DNA from plastic tiles. There was a significant interaction between material and species ($\chi^2 = 5.403$, df = 1, p = 0.020). Pairwise comparisons indicated clear differences in the amplification success between glass and plastic surfaces. For corn snakes, the probability of amplification was 131.6 times greater for glass than plastic (95% CI lower = 124.7, upper = 138.6) and for boas, 4.7 times greater for glass than plastic (95% CI lower = 0.4, upper = 8.9).

For glass tiles, there was an increase in the probability of the amplification as $\log_{10}$ transformed concentrations increased. With a one unit increase on the $\log_{10}$ concentration scale, the odds of amplification increased by a factor of 4 (95% CI lower = 1.2, upper = 13.5) (Fig. 5).

3.2.2. *Sequencing results for confirmation*

The consensus sequences for each species resulted in correct species identification for the positive control samples, with the corn snake consensus BLAST returning the reference MG672879 with a 100% identity and query cover. The boa positive control yielded similar results returning the reference sequence AM236348 with a 100% identity and query cover. Negative controls failed to sequence, or returned primer dimer sequence only. As for the experimental samples, both C2 LN (Glass) and C5 RF (Glass) consensus sequences corresponded to the same reference sequence (MG672879) as the corn snake positive controls with 98.96% identity with 99% query cover, and 99.71% identity with 100% query cover, respectively. The same was true for DNA recovered from the boa tiles, B2 LN (Glass) and B3 RN (Plastic) consensus sequences yielded 99.19% identity with 100% query cover, and 98.91% identity with 100% query cover, respectively, with the AM236348 reference sequence.

4. Discussion

There are currently no methods for the visual detection of trace reptile DNA in cases devoid of specimen evidence. Application of DD has proven successful for visualizing human trace DNA from a variety of sources: including fingerprints [28], handprints [21] and lip prints [29]. We have successfully extended the use of this application to the IWT, with the detection of trace reptile DNA leading to successful PCR amplification and subsequent DNA sequencing correctly identifying two snake species. The concept of visual latent DNA detection and development of related technologies could thus have a real-world application to biosecurity enforcement for combatting illegal wildlife trafficking.

Detecting trace DNA presence on glass surfaces (and to a lesser degree on plastic), led to successful amplification for both corn snakes and boas with Sanger sequencing consensus reads corresponding to NCBI GenBank reference data. DD detected reptile trace DNA deposition with as little as 24 h of residence time for multiple individuals of each species.

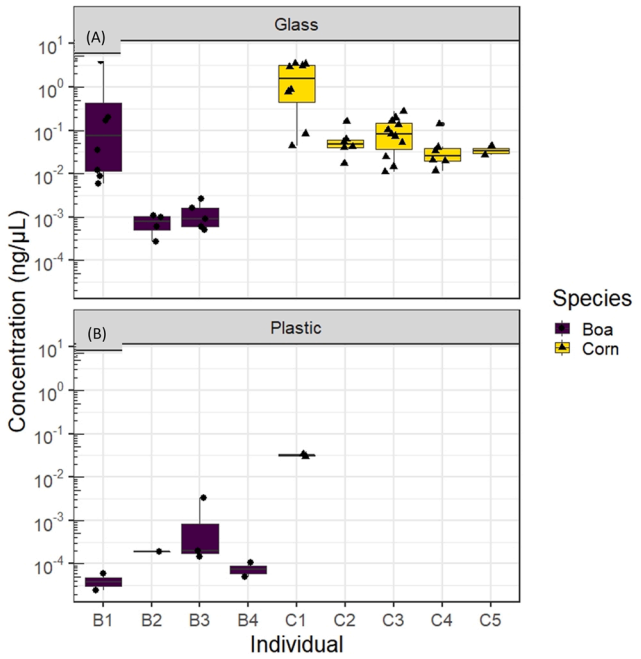

Fig. 3. Reptile DNA concentration recovered from (A) glass and (B) plastic tiles. Boxplots shown for every individual; including four measures in duplicate of recoverable reptile trace DNA for each. Each point represents an individual quantification value derived from a species-specific standard curve using the Rotor-Gene Q 2.3.5 software; where data points are missing amplification was not achieved.

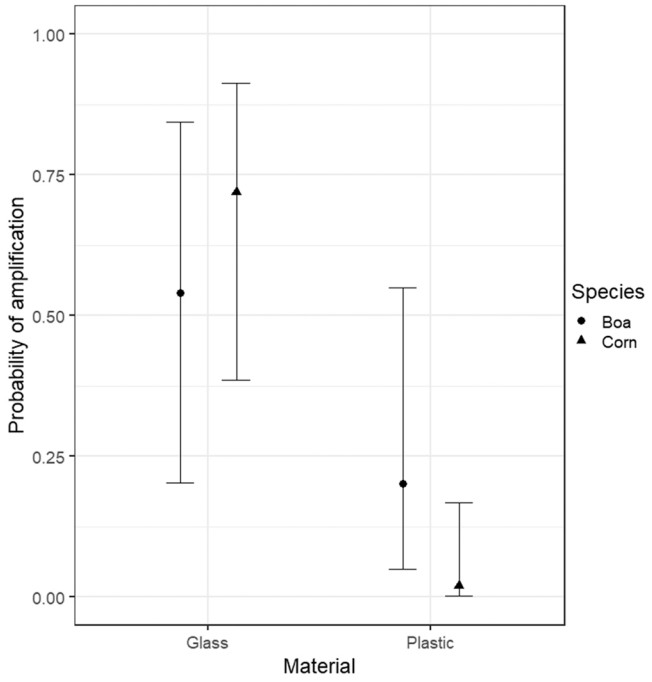

Fig. 4. Plot of the mean probability of amplification (points) for tile samples corresponding to species grouped by material, including the 95% confidence intervals as error bars. Binomial generalized linear mixed model used to determine the probability of amplification accounting for the aggregate individual, tiles, and duplicate tile-pair values.

Deposition resulted in visibly distinctive movement and scale imprints, in multiple trials. Our methodology requires limited specialist equipment, with materials constrained to those currently widely available in

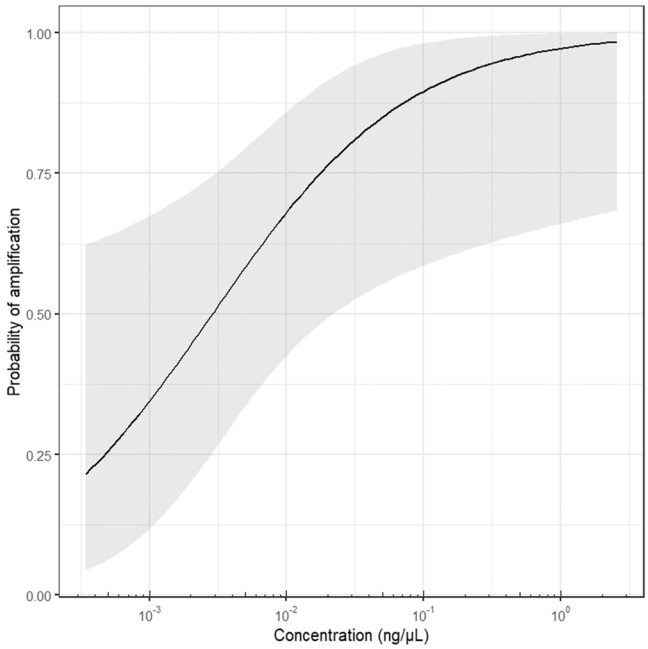

Fig. 5. Predicted probability of amplification versus $\log_{10}$ concentrations of initial target DNA. This includes only the glass tiles due to low amplification success for plastic tiles and only includes samples with positive amplification. The estimated curve and 95% Confidence Interval are calculated from a Binomial generalized linear mixed effect model, with ID as a random effect.

forensic genetics laboratories. Additionally, the described method incurs low financial costs, while detecting target species with high specificity.

4.1. *Reptile trace DNA visualization*

Application of DD highlights depositional patterns of movement and body form markings, such as scale imprints for reptiles. This is similar to the macroscale detection of human fingerprint patterns, which have previously been observed after DD had been applied [30], with comparable scale and movement observations for several corn snake and boa tiles.

One of our most significant findings was the difference that material played in both the visible deposition and the subsequent amplification probability (Fig. 4); as well as quantification (Fig. 3). We observed that plastic tiles generally had less intense fluorescence and fewer constructs present, which was confirmed by the amplification probability and quantification results. We recognize that the limited sampling of 10 × 10 cm tiles does not capture movement throughout the entire enclosure. At the vivarium wide scale, depositional movement patterns and scale imprints could better inform sampling effort as limited sampling of 10 × 10 cm tiles does not capture movement throughout the entire enclosure. Whereas the removeable tiles were a useful experimental component, for our proof-of-concept study, future adoption of the techniques should examine the entire vivarium, or enclosure.

We used a spray device based on the large surface area of the tile in contrast to previously published studies [31] and the fluorescence intensity achieved when using this in comparison to alternative options [30]. This approach worked well and led to informative visualization of trace DNA deposition seen as movement or scale patterns. However, there were cases in which this device led to excessive spray artefacts visible as droplets (Fig. 2). A specialized device optimized for the detection of cellular material could further improve the detection and subsequent identification of trace DNA deposition.

4.2. Visually informed DNA recovery

There are several factors that influence the transfer and persistence of trace DNA in humans, including: contact time, type of contact, pressure, friction, moisture, contact surface and the extent of previous contact before interaction with the item of interest [32]. Additionally, the shedder status of an individual has been shown to play a role in the amount of cellular deposition as a consequence of touch [15]. These factors identified as impacting trace DNA deposition were largely controlled throughout the reptile experiments in this project. Temperature and moisture remained consistent due to the climate-controlled room in which the experiment was conducted. The experimental contact surfaces consisted of two materials commonly encountered in the IWT (glass and plastic). However, contact time, shedder status, pressure and friction were more difficult to control or quantify. While the contact time was monitored using the trail camera, this only provided a rough estimate of contact as images were captured every 10 min. Previous contact and shedder status were factors that could not be accounted for. Reptiles were housed in their displays prior to each experimental trial and shedder status was unknown. The surface roughness of python skin is around one-third that of human skin [33], which may impact the transfer of cellular material. In addition, models of snake locomotion suggest that snakes dynamically distribute weight such that their belly is periodically loaded (pressed) in linear sections and unloaded (lifted) in curved sections, concentrating weight on specific points of contact [34]. The nature of the contact a snake had with a tile in terms of pressure, friction and type of contact was impossible to quantify from the contact monitoring footage.

We did not make any assumptions about the stage of an individual's shedding cycle, and any variability due to shedding was unaccounted for in our study. In preparation for shedding, snakes become anorexic and produce a lymph fluid which creates a white haze to the skin and eyes [35]. Photographing the snake's eye region and monitoring changes in weight in tandem to temporal experimental trials throughout the shedding cycle could determine whether shedding cycle plays a significant role in reptile DNA deposition. While an understanding of the effects of shedder cycle on reptile DNA transfer may be useful from a scientific perspective, from a biosecurity standpoint, the shedding cycle of individual reptiles will likely always be unknown and cannot be controlled. Species identification will rely solely on the detection of trace DNA irrespective of this factor.

4.3. DNA amplification and quantification

While our results present a successful proof of concept study, there are still several limitations to our assay, primarily concerning mixed samples composed of multiple reptile trace signatures. There are several ways of dealing with these problems, including further development towards a multiplex assay [36], or a short tandem repeat (STR) panel for the detection of reptiles common in the IWT, as available for other species of forensic significance [37]. Concerns surrounding trafficking of Australian reptiles has seen the development of '*OzPythonPlex*', which consists of three 11-plex assays targeting 24 STR loci for forensic profiling of the Australian carpet python [38]. The '*OzPythonPlex*' assay has been further applied to 12 other CITES listed Australasian python species of forensic significance, with 8 of the 12 species showing amplification success at ten or more loci, suggesting great potential in forensic investigations [39]. Further research towards a forensic profiling kit for reptiles of greatest concern in the pet trade, and related biosecurity or biodiversity threats, could provide a more comprehensive tool than the novel assay presented here.

4.4. Material and species influence on amplification success

Lower amplification success for plastic tiles suggests limited deposition or retention of cellular material prior to sampling. Touch DNA studies exploring recovery from human fingerprint samples on various substrates including glass and plastic found glass yielded the best results [40]. Glass or silica is common in DNA purification steps for certain extraction methods [41]. In the presence of chaotropic salts, glass microscopy slides can bind to DNA [42]. Since glass has an affinity for DNA at the molecular level, it is possible that small amounts of DNA bind to glass even without chaotropic salts present [41]. When dealing with low amounts of starting material this can impact DNA recovery and subsequently increase DNA profiling success [41]. The affinity of DNA to glass may have been an advantage here, as the transport of the tiles could have led to substantial loss of material prior to DD screening and sampling. Tiles were stored vertically at an acute angle using rubber dividers to avoid DNA loss. The slight movement during transport and low affinity of DNA for the PVC plastic tiles used, may have led to loss of cellular material prior to screening and swabbing. Polypropylene based plastics have been known to cause denaturation and absorption of DNA [43], yet multiple studies have indicated that plastic surfaces return some of the best DNA recovery and profiles in contrast to porous surfaces [44]. Conversely, glass has also been cited as having the highest rate of DNA recovery when subject to a range of environmental conditions in contrast to a range of other porous and non-porous materials excluding plastics [45].

Our findings similarly indicate that glass surfaces are highly desirable and should be preferentially targeted when illegal possession is suspected. The amplification probability was significantly greater for glass surfaces (Fig. 3) leading to better opportunities for downstream identification.

5. Conclusion

We have presented a best practise study for visually detecting reptile cellular deposition. Importantly, we have shown trace DNA amplification and species identification for experimentally simulated empty holdings used in the IWT of reptiles. The use of DD to indicate the presence of trace DNA prior to swabbing provides an informative and targeted means of sampling and reducing the likelihood of swabs devoid of trace DNA, which can be common in blind sampling scenarios. Visualization using DD in the enforcement pipeline highlighted depositional patterns of movement and body form markings such as scale imprints on some tiles. However, this was not visible to the degree at which human cellular deposition can be visualized. Distinct fingerprints with individual distinguishable cells have been observed in lab conditions across a range of substrates with varying success [46]. Additionally, finger marks have been observed at the macro-scale [30] after DD has been applied which is the scale most relevant for our IWT application. We found that depositional patterns and scale imprints could clearly indicate reptile contact at this scale previously invisible to the naked eye, better informing sampling effort.

Relatively few large-scale programs focus on the implementation of surveillance and enforcement management methods for reptiles [47] despite the hundreds of reptile species kept as pets globally [48]. The popularity of these species has led to increased invasive populations outside their native ranges with multiple direct impacts on biodiversity conservation and environmental biosecurity [49,50]. IWT is often faced with cases where visible evidence is absent and only trace material remains [18]. This study harnessed the power of DD to highlight cellular deposition as established in human forensic science, to provide information about reptile behavior and presences in cases where specimen evidence is no longer present. These emerging and effective forensic techniques have the potential to arm biosecurity staff and decision makers with tools for rapid identification of key new incursion species popular as live pets in illegal reptile collections.

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Authors' Contribution

ND, JMY, AL, PC designed the experiment; JA, SD provided specialist advice; ND wrote the manuscript; JMY, AL, JA, SD, and PC edited the manuscript.

Ethics Approval

This research was conducted under The University of Adelaide Animal Ethics Committee (Science) approval number S-2020–024.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Firstly, we would like to acknowledge this work was conducted on the traditional land of the Kaurna people and we recognize and respect their spiritual relationship with their country.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fsiae.2021.100040.

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