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# DNA based method for determining source country of the short beaked echidna (*Tachyglossus aculeatus*) in the illegal wildlife trade

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ABSTRACT

The illegal trade in wild animals being sold as 'captive bred' is an emerging issue in the pet and zoo industry and has both animal welfare and conservation implications. DNA based methods can be a quick, inexpensive, and definitive way to determine the source of these animals, thereby assisting efforts to combat this trade. The short beaked echidna (*Tachyglossus aculeatus*) is currently one of the species suspected to be targeted in this trade. As this species is distributed throughout Australia and in New Guinea (currently comprising of five recognised sub-species), this project aimed to develop a DNA based method to definitively determine the source country of an echidna and explore the use of non-invasive sampling techniques. Here we use non-invasively sampled echidna quills and demonstrate the extraction of mitochondrial DNA and amplification of a region of the mitochondrial genome. Phylogenetically informative markers for analysis of a 322 bp segment of the D-loop region were developed, and subsequently validated, using animals with known source locations allowing us to reliably distinguish between echidnas from New Guinea, and Australia. This research presents the first validated forensic protocols for short beaked echidnas and will be an integral tool in understanding the movement of animals in this emerging trade.

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## 1. Introduction

The illegal trade in protected wildlife is a serious and widespread crime, and was estimated to be worth USD $7–23 Billion in 2014 [1]. Within this, the laundering of wild caught animals as 'captive bred', sometimes with falsified or no supporting paperwork or genetic data, has been identified as an emerging issue [2–6]. There is increasing evidence that monotremes, including the short beaked echidna (*Tachyglossus aculeatus* but with five recognised subspecies across Australian and Papua New Guinea) and the long beaked echidna (*Zaglossus sp.),* are at risk of becoming part of this trade [2,7,8]. Monotremes have a complex mode of reproduction, which is notoriously difficult to replicate within captivity [9–11]. Historic zoo records show that until 2009 there were less than ten short beaked echidnas born in captivity in Australia, with a low survival rate past 18 months of age [11,12]. The quotas from the Indonesian Captive Breeding Protection Plan [2,7] in recent years have included approximately 70 echidnas in 2014, 150 in 2015, and 50 in 2016, however based on the low breeding success recorded by Australian zoos, it is possible the large number of Indonesian captive bred animals were supplemented by wild poached individuals, potentially from the New Guinea subspecies of short beaked echidnas (*T.a. lawesii)* [2,7,11].

A vast number of animal and plant species are targeted in the illegal wildlife trade and, even in well-resourced countries, investment in enforcement and investigation of this trade is not at a commensurate level compared to 'human-victim' crimes [13]. Furthermore, in developing countries, there are often additional challenges in conducting adequate compliance investigations and appropriate forensic testing for prosecution [13]. Often tests or vouchered reference data do not exist to confirm biogeographic provenance or alleged pedigree, or even confirm species [13]. Providing data for evidentiary or intelligence purposes in a meaningful timeframe can assist in maintaining momentum within an investigation and give direction on what should be done with seized animals requiring rehoming. Therefore, the establishment of non-invasive sampling methods, PCR protocols, and analyses that can be performed quickly and inexpensively within the laboratory can be of significant benefit to an investigation in both developed and developing countries.

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Non-invasive sampling has many advantages, including reducing handling stress, and does not require anaesthetic or a vet to be present to take a sample. Previous studies have successfully extracted DNA using non-invasive methods from feathers, hair, scats and scales [14–19], these methods can however, be limited by quality of the DNA that is extracted [15,18,20]. In wildlife forensic science, non-invasive sampling is particularly useful as seizures of live animals often occur without the presence of veterinarians and it may not be possible or time efficient to take a blood sample from an animal in an investigation [19]. Echidna quills are made of keratin, similar to hair, and can be plucked, or are naturally shed [21]. Quills also have a larger surface area than hair, and therefore retain more epithelial cells for DNA extraction.

Analysis of mitochondrial DNA (mtDNA) is well established as an effective tool for species identification, phylogenetic studies, and determining source populations; an essential component of not only wildlife forensics, but species conservation [22]. Studies of mammalian evolutionary history, phylogenetics and phylogeography often utilise mtDNA analysis [23–25]. For trafficked species that have a broad geographic distribution covering multiple countries, it can be important to establish the source, so that the correct jurisdiction and legislation involved can be identified, for example whether local wildlife legislation or legislation enforcing CITES should be enacted [23]. Short beaked echidnas are documented as having both a New Guinea and Australian distribution, with 4 Australian subspecies (*T.a.aculeatus* (East coast of mainland Australia), *T.a. acanthion* (Western Australia), *T.a setosus* (Tasmania), *T.a. multiaculeatus* (Kangaroo Island)), and one New Guinea subspecies (*T.a. lawesii*) subspecies [26,27]. However, currently there is no published phylogeographic data to substantiate these delineations. Definitively identifying the country of origin of any traded echidna is the focus of this study, as this would benefit both investigative and enforcement efforts in order to identify the source of individuals poached from the wild. While the mtDNA regions CO1 and Cytochrome b are often used in wildlife forensics, because they provide useful interspecific resolution [28–32], to determine source populations we require a genetic region that provides sufficient intraspecific resolution. Our candidate region, the mtDNA 'control region' or D-loop, is a hypervariable region of the mitochondrial genome which is often used in intraspecific or phylogeographic studies [25,33,34].

This work aimed to evaluate DNA extraction methods using both plucked and shed quills of the short beaked echidna, utilising commonly available protocols and kits. Further, to strive for recommended best practice we aimed to develop validated protocols for amplification of the phylogenetically informative region of the D-loop that effectively differentiates between echidnas of New Guinea or Australian origin, and determine the source country of any trafficked and seized short beaked echidnas.

## 2. Methods

### 2.1. Quills – sampling, storage and gDNA extraction methods

Shed and plucked short beaked echidna quills were collected from echidnas in captivity by zookeepers, as well as from one Australian Museum specimen (M.47764.004). Within each extraction trial, five quills were used – two from M.47764.004 which had been preserved in ethanol upon collection, and three from captive echidnas; two plucked and one shed (for detailed sample information see Appendix A in Supplementary materials). The quills were transported to the laboratory in zip lock bags then frozen at −20 °C on arrival.

Four different commonly used and readily available extraction kits were trialled (Table 1.) to test the success of extracting DNA and the quantity of yield. Trials using purchased extraction kids were followed as to the manufacturer's instructions, and the salting out technique as to instructions from Sunnucks and Hale [35]. Approximately one centimetre from the root of the quill (the weakest and thinnest part) was cut using tin snips to use for the extraction (Fig. 1). DNA extractions were quantified using the Qubit 2.0 Fluorometer High Sensitivity Assay (Qiagen).

### 2.2. Samples for phylogeographic analysis

Due to the difficulty of obtaining quill samples from New Guinea echidnas, only quill samples from Australian animals were tested. However, four echidna tissue samples from New Guinea, specifically Papua New Guinea, were included as part of the phylogeographic validation, along with one sample from Moa Island, Torres Strait, and one sample from a captive animal from San Diego Zoo, obtained legally from an Indonesia Zoo, with suspected New Guinea parents. Forty-one additional tissue and blood samples were included to give a wider variety of Australian locations. A total of 50 quill, tissue, blood, skin, or gDNA samples, with known collection localities were used. These animals were sourced from both museum/university collections (30 samples)

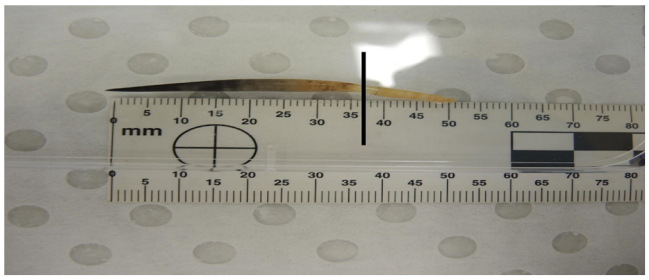


**Fig. 1.** Example of quill used. Black line depicts where the quill was cut (~1 cm from the root end of the quill).

**Table 1**
Methodology for each extraction trial.

| Trial | Method | Quill roots used per extraction |
|---|---|---|
| 1. | ISOLATE II Genomic DNA Kit (Bioline, Australia) animal tissue protocol. Automated extraction. | 2× M.47764.004; M.48044.001; M.48042.001; M.48045.001 |
| 2. | ISOLATE II Genomic DNA Kit (Bioline, Australia), animal tissue protocol with the addition of 20 μL of 1 M DTT during the lysis stage. | 2× M.47764.004; M.48044.001; M.48042.001; M.48045.001 |
| 3. | QIAamp DNA Investigator Kit (QIAGEN, Australia), following the Hair and Nails protocol. | 2× M.47764.004; M.48041.001; M.48040.001; M.48043.001 |
| 4. | Salting out technique [35], following protocol published in Sunnucks and Hale | 2× M.47764.004; M.48041.001; M.48040.001; M.48043.001 |

and zoo collections (20 samples) (Appendix A in Supplementary materials). For the reference samples, DNA extractions were carried out using either the ISOLATE II Genomic DNA Kit animal tissue protocol (Bioline, Australia), the ChargeSwitch Forensic DNA Purification Kit (Thermo Fisher, USA), or a salting out technique [35]. DNA concentrations were determined using Qubit 2.0 Fluorometer.

### 2.3. Amplification conditions

Quill extractions were initially checked by amplifying a region of Cytochrome B (14841–15149), using the following universal primers; M5 — AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA and M6 AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA [36]. Primers to amplify a 430 bp region (15408–15859) of the short beaked echidna D-loop were then designed using Oligo 7 [37] using the *Tachyglossus aculeatus* whole mitochondrial genome (AJ303116.1) as a reference. The Primer sequences developed as part of this study are as follows: TacCRF — 5′- ACCATCAGCTCCCAAAGCTGA-3′ and TacCRR — 5′- CTTGAAGGGGAATTACCAGAGG-3′.

One sample from PNG (M5966) and the sample from Moa Island, Torres Strait (M4954) were extracted from historic museum study specimens (Australian Museum Mammalogy Collection). Due to the age and storage conditions of these samples, extracted DNA was found to be highly degraded. To amplify these samples, two additional primer sets were designed that would amplify the required region in two overlapping segments (15498–15698 and 15696–15864). These primer sequences are TacCR2F — 5′- TGCATTCATCTTTTATCCCCATAC 3′, TacCR2R — 5′- TAATCTGTCAGAACCTCAATTATG′, and TacCR3F — 5′- AACATAATT-GAGGTTCTGACAG- 3′, TacCR3R – 5′- GTTCTTGAAGGGGAATTACC 3′.

Polymerase Chain Reactions (PCRs) were carried out in 25 μL reactions, containing 10 ng of DNA, 1× Bioline MyTaq Reagent Buffer, 10 μM for each primer, and 5U of Bioline MyTaq DNA polymerase. PCRs were carried out on an Eppendorf MasterCycler pro S under the following conditions: 3 min at 94 °C, 38 cycles of 94 °C (20 s) denature, 60 °C (40 s) annealing, and 72 °C (40 s) extension, with a final extension cycle of 72 °C for 5 min. To amplify the two skin samples, touchdown conditions 55 °C–50 °C (1 °C step down per cycle) were employed for the annealing step. PCR success was checked using a 1% Agarose Gel and reactions were cleaned using Affymetrix ExoSAP-IT. Sanger sequencing was carried out by the Australian Genome Research Facility (AGRF), Sydney on an AB3730 xl. Raw sequences were edited with reference to chromatograms using Sequencher version 5.3.

### 2.4. Analysis

D-loop sequences from all reference and quill samples were aligned using ClustalW in MEGA version 7.0 software [38]. To include all samples, sequences were edited to be the same length and analysis was conducted on a 322 bp fragment. The corresponding D-loop region from the western long beaked echidna (*Zaglossus bruijnii*; Accession number: AJ639865.1) was used as an outgroup. To perform phylogenetic analysis on all haplotypes generated, an appropriate model of evolution; T92 + G, was determined in MEGA 7.0, using the Bayesian Information Criterion. Maximum likelihood trees were built using MEGA 7.0, Statistical confidence was calculated using 1000 bootstrap resamples. Bayesian inference of phylogeny analysis was conducted in MrBayes 3.2 [39]. The analyses were run using default settings for priors. The chains were run for 10000000 generations and sampled every 1000 generations to obtain 2364 sampled trees. Tracer v1.6 [40] was used to check for chain convergence and adequate Effective Sample Size (>100). Posterior probabilities (decimals) were used to assess the level of branch support.

### 2.5. Validation

Validation was carried out based on the following characteristics: (1) reproducibility/repeatability, (2) limit of detection (3) specificity, (4) phylogenetic resolution (as described above).

To test for *repeatability*, 50 samples were amplified, on four different thermocyclers. A temperature validation was conducted with eight samples undergoing amplification with the annealing temperature altered to both ±1.5 °C of the regular annealing temperature (60 °C). A blind study conducted by a different analyst, using regular PCR and sequencing conditions was also carried out using four unknown samples (two from PNG and two from Australia). *Limit of detection* was tested using 1 in 10 (1 ng), 1 in 100 (0.1 ng), and 1 in 1000 (0.01 ng) serial dilutions of eight DNA samples, previously quantified using the Qubit 2.0 Fluorometer. Species *specificity* was analysed using the following species from the Australian Museum's Mammal, Bird and Fish collections; Western Long Beaked Echidna *(Zaglossus bruijnii)* (M.47975.001), Platypus *(Ornithorhynchus anatinus)* (M.35614.001), Bilby *(Macrotis lagotis)* (WGM118-186), Little Penguin *(Eudyptula minor)* (B10002), Agile Antechinus *(Antechinus agilis)* (AM205-7), Eastern Crevice Skink *(Egernia mcpheei)* (R.150174.001), White-browed Woodswallow (*Artamus superciliosus)* (O.71701.001), Queensland Grouper (*Epinephelus lanceolatus)* (I.39681.003) and Rock Cod (*Lotella rhacina)* (I.31253.130). These samples were chosen either because they were closely related species, or to represent a range of genera and potential contaminants for our laboratory. They were run with the regular PCR conditions as well as both ±1.5 °C of the optimal annealing temperature. All samples within the validation study were checked for PCR success using a 1% Agarose Gel and reactions were cleaned using Affymetrix ExoSAP-IT. Sanger sequencing was carried out by the Australian Genome Research Facility (AGRF), Sydney on an AB3730 xl. Raw sequences were edited with reference to chromatograms using Sequencher version 5.3 and species.

## 3. Results

### 3.1. Quill extractions

DNA was successfully extracted from all quills used in each trial. The Qubit quantification indicated that the extraction Trials 1 and 2 both produced comparable DNA concentrations, on average <1 ng/μL. Extraction Trials 3 and 4 both had quantification results that were higher than the previous two trials, with the average for Trial 3 = 24.2 ng/μL and the average for Trial 4 = 3.26 ng/μL. DNA was extracted successfully from both plucked and shed quills.

Comparison of sequences generated in this study to publicly available sequences to confirm species identification, returned 98–100% identity to the short beaked echidna (*Tachyglossus aculeatus* GenBank accession AJ303116.1) mitochondrial genome.

### 3.2. Phylogeographic analysis

A 322 bp region was sequenced from all quills and reference samples analysed. Three unique haplogroups were identified in the 50 samples; two Australian haplogroups and one PNG haplogroup. Four out of six samples from PNG and Moa Island shared the same haplotype, with the East PNG and Moa Island samples grouping closely with the West PNG samples. There were no control region haplotypes shared between samples of Australian and PNG origin. Both the maximum likelihood analysis and Bayesian Inference

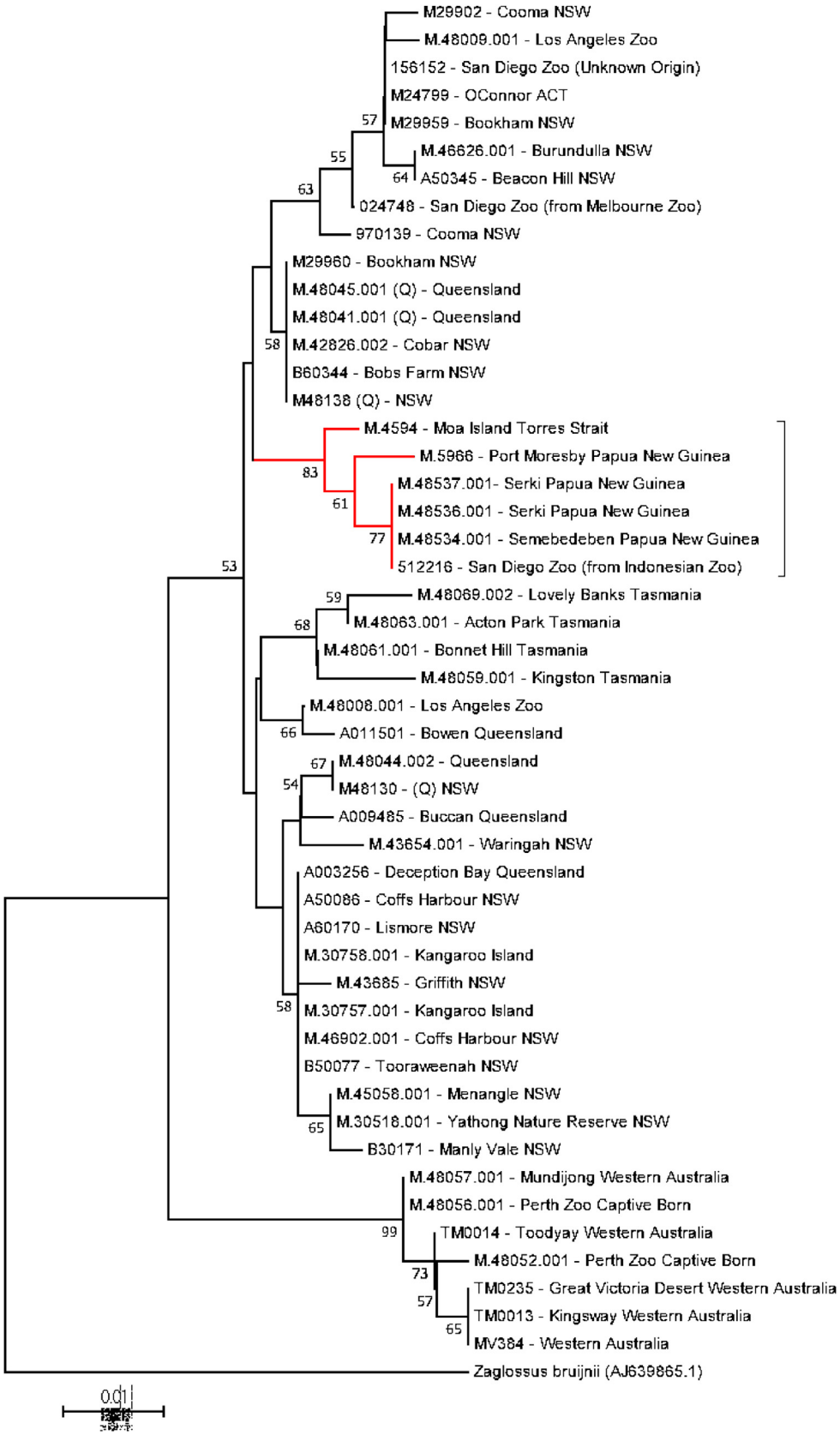


**Fig. 2.** Maximum likelihood phylogenetic tree representing D-loop sequences from both Australian and Papua New Guinea short beaked echidnas. Bootstrap values (percentage) is listed above the nodes and no values less than 50 were shown. PNG samples shown in red. Respective museum or zoo numbers are used for sample identification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

resolved trees with similar topologies showing low lineage resolution for most of the Australian reference samples, with the samples from PNG forming a distinct lineage. The samples from Western Australia also showed distinct lineage within in the sampling of this project, whereas the east coast, Tasmania, and Kangaroo Island samples show little variation. The bootstrap value for the clustering of the PNG samples is 83% shown in the Maximum Likelihood tree (Fig. 2), and 0.9901 in the Bayesian inference tree (Fig. 3) indicating moderate to strong support for this lineage.

### 3.3. Validation

Reproducibility/repeatability all samples were successfully amplified across the four different thermocyclers and both +1.5 °C and −1.5 °C annealing temperatures. The analyst conducting the blind test successfully determined the source country of all four unknown samples. *Limit of detection*: The 1 ng and 0.1 ng dilutions were also successfully amplified however the 0.01 ng dilution samples were not able to be amplified. *Specificity*: The western long beaked echidna sample was the only species that was

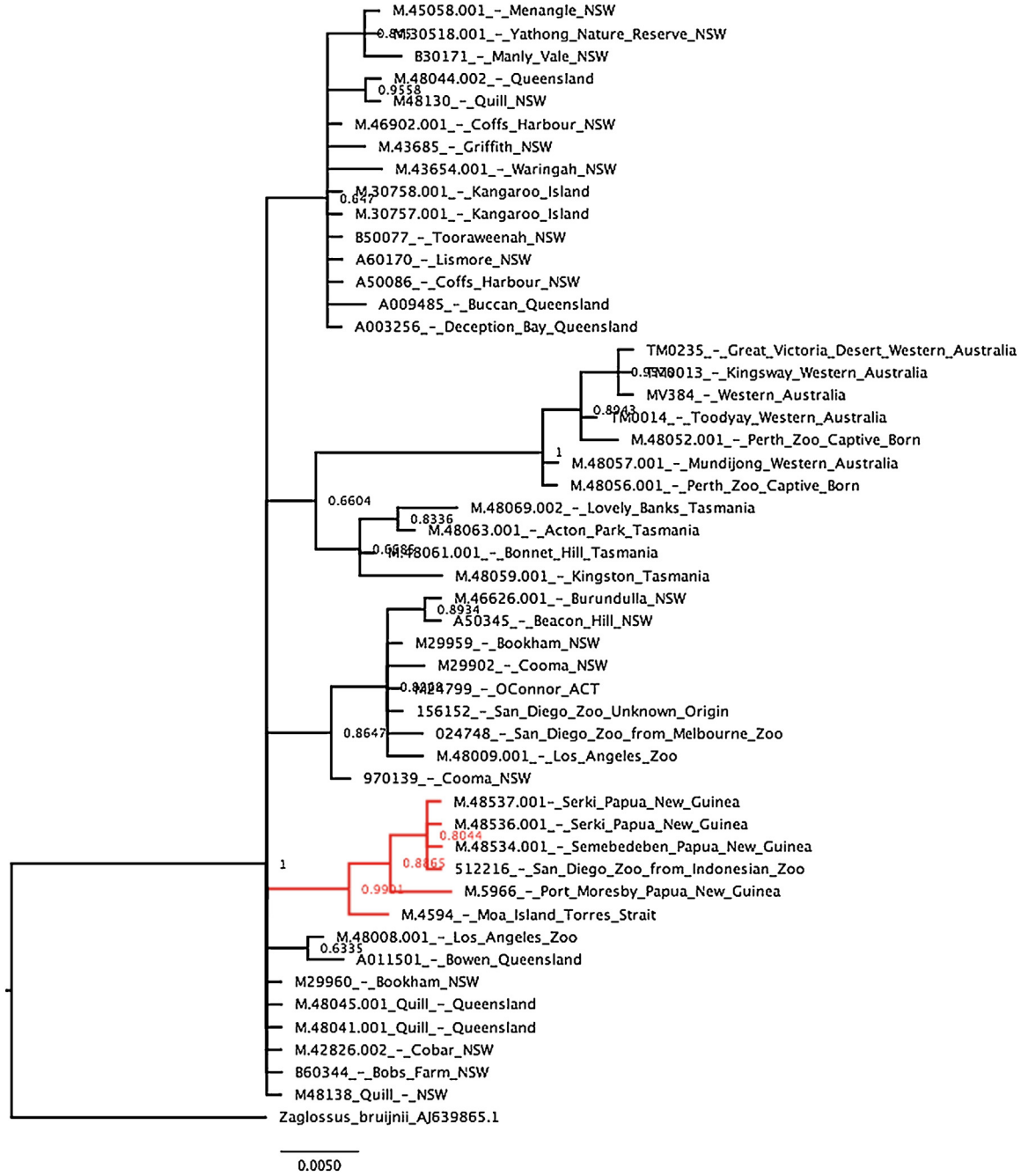


**Fig. 3.** Bayesian Inference representing D-loop sequences from both Australian and Papua New Guinea short beaked echidnas. Posterior probabilities (decimals) are listed above the nodes and no values less than 50% are shown. PNG samples shown in red. Respective museum or zoo numbers are used for sample identification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

amplified and generated a sequence using these primers. There is distinct phylogenetic differentiation between the two genera of echidna at this region so there is no chance of incorrect generic identification.

## 4. Discussion

This study represents the first validated forensic protocol to successfully determine source country of the short beaked echidna using a short mtDNA D-loop region. Additionally, we have evaluated several commonly used extraction methods to obtain mtDNA from echidna quills. Our results demonstrate this non-invasive method is suitable for extracting and sequencing phylogenetically informative mtDNA down to a limit of detection of 0.1 ng, which can be used for evidentiary or intelligence purposes to aid in combatting the illegal trade of the short beaked echidna.

The four extraction methods all produced suitable DNA quantity to successfully amplify the D-loop region. However, the Qiagen QIAamp Investigator Kit 'Hair and Nails' protocol (following manufacturer's instructions) produced the most consistent results across both shed and plucked quills. While this is one of the more costly kits, it is widely available, straightforward to use and produces consistent extracts with higher DNA concentrations on the whole. Importantly it would be the recommended kit for extraction from quills if blood or tissue were unavailable. With further optimisation, this kit has the potential to be used for nuclear DNA sequencing and genotyping, as has been shown with other non-invasive samples such as feathers, and preliminary results have indicated this (data not shown) [41,42]. In addition, there was also no remarkable quantitative difference between plucked and shed quills in the samples available for this study. Therefore, confirming that shed and non-invasively collected quills are appropriate to use for such a test, including those collected from enclosures containing seized animals. Further, sufficient template for mtDNA sequencing was recovered from both plucked and shed quills, however as these are from a zoo enclosure, further testing would be needed to investigate shed quills in a field context. This result provides insight into the benefit of this type of sampling, indicating there is the potential to collect shed quills from an echidna's natural habitat within a certain time frame. For many species in the illegal wildlife trade, including pangolins, tigers, and birds, non-invasive sampling has proved beneficial [19,43,44], and the methods presented here provide a sample preparation protocol that can also be implemented in better understanding the short beaked echidna trade.

Of the six samples analysed from PNG and Moa Island, four of the PNG samples shared the same haplotype. Three of these samples were from the same location in the Western Province of Papua New Guinea (in or around the village of Serki), but the fourth sample's specific location unknown because it was captive animal, with parents suspected to have come from New Guinea. The PNG sample from Port Moresby (East PNG) while not an identical haplotype, still consistently grouped closely with the Western PNG samples with strong support, as did and the Moa Island sample (Figs. 2 and 3). While we acknowledge that the sample size is small, the low haplotype diversity within these PNG samples suggests that, at least at the mtDNA level, there is limited structure in short beaked echidnas within the New Guinea population of short beaked echidnas, similar to what has been observed in eastern Australian echidnas (Fig. 4).

For further insight into the extent the Torres Strait poses as a biogeographic barrier, it would be highly desirable to obtain further samples from echidnas across their New Guinea distribution and from the northern region of the Cape York Peninsula in Australia. All attempts were made to obtain DNA from the one Cape York study specimen available in the Australian Museum Collection, however we were unsuccessful in extracting DNA from this study specimen, which is likely related to its age and preservation method [45].

**Fig. 4.** Map of samples used in phylogeographic analysis. PNG/Torres Strait (Moa Island) samples in red, and Australian in black. Location of sample SD-2 (San Diego Zoo with Indonesian parents) unknown so excluded from map. Map made in QGIS software [52]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

New Guinea and Australia have been isolated for approximately 8000 years, a separation which has acted as a significant biogeographic barrier for many species that have distributions throughout the two countries [46–49]. Phylogenetic research on the green python (*Morelia viridis*), a species also prominent in the illegal wildlife trade, was also successfully resolved using the D-loop region [48] and, as in our study, Australian and New Guinean pythons were able to be differentiated phylogeographically. The identification of geographic provenance of a species is often requested by enforcement agencies and can be of particular importance for CITES listed species with cross border distributions. It is of utmost importance to develop these tests that are validated if the end-point is to present this data in a legal context [23,50,51]. The validated work presented here is reproducible/repeatable, specific to the echidna group (genera *Tachyglossus* and *Zaglossus*), with a limit of detection of 0.1 ng [51]. While this test amplifies both echidna genera, because this test also requires sequencing, a simple BLAST search under carefully specified criteria, or phylogenetic analysis using sequences from vouchered reference material (GenBank accession AJ303116.1) can differentiate clearly between these two genera, and therefore does not interfere with determining species or source location (i.e. Australia or Torres Strait/PNG). Our phylogeographic analyses also indicate potential genetic variation between east and west Australian samples, however, this may be an artefact of sampling as we were unable to include many samples from central Australia. Identifying to state or population level within Australia may also be useful for state agencies, in cases where animals are found illegally in captivity within Australia. However, given that there was lack of variation along the east coast of Australia, this may need higher resolution data such as SNPs or microsatellites to obtain clarity, nor was it the focus of this study. We saw no phylogeographic structuring between the east coast, Tasmania, and Kangaroo Island populations to support their classification as separate subspecies (east coast: *T.a. aculeatus;* Tasmania: *T.a. setosus;* Kangaroo Island: *T.a. multiaculeatus*). Additional sample collection and analysis would be required to further investigate phylogeographic patterns of the Australian populations, and infer evolutionary history of the species.

This study demonstrated that non-invasive sampling, and subsequent DNA extraction from short beaked echidna quills is possible, and can be carried out using a range of commercially available DNA kits. Sample preparation from echidna quills can be useful for many cases, particularly in an illegal trafficking scenario where blood or tissue is unable, or unfeasible to be sampled. In addition, we were able to develop and validate phylogenetically informative markers to determine source country of any suspect individuals and direct any following enforcement actions. This also has potential to guide the establishment of presumptive indicators that could be used to flag the potential measures that could be put in place in areas where evidence shows higher levels of trafficking, as well as used by zoos to guarantee legitimate status of any captive echidnas that may come into their collections. Implementation of the methods presented here will provide important intelligence about this trade, and aid in analyses involving the illegal trafficking of this iconic species.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.forsciint.2018.11.019.

## References

[1] C. Nellemann, et al., The environmental crime crisis — threats to sustainable development from illegal exploitation and trade wildlife and forest resources, A UNEP Rapid Response Assessment. United Nations Environment Programme and GRID, United Nations Environment Programme (UNEP), Nairobi, Kenya, 2014.
[2] C. Beastall, C.R. Shepherd, Trade in "Captive Bred" echidnas, Traffic Bull. 25 (1) (2013) 16–17.
[3] G.J. Frankham, M.C. Hinds, R.N. Johnson, Development of 16 forensically informative microsatellite loci to detect the illegal trade of broad headed snakes (*Hoplocephalus bungaroides*), Conserv. Genet. Res. 7 (2) (2015) 533–535.
[4] E.R. Bush, S.E. Baker, D.W. Macdonald, Global trade in exotic pets 2006–2012, Conserv. Biol. 28 (3) (2014) 663–676.
[5] C.J. Hogg, et al., Stopping the spin cycle: genetics and bio-banking as a tool for addressing the laundering of illegally caught wildlife as 'captive-bred', Conserv. Genet. Res. 10 (2) (2017) 237–246.
[6] V. Nijman, C.R. Shepherd, Adding up the numbers: an investigation into commercial breeding of Tokay Geckos in Indonesia, TRAFFIC, (2015) Petaling Jaya, Selangor, Malaysia.
[7] J. Janssen, S.C.L. Chng, Biological parameters used in setting captive-breeding quotas for Indonesia's breeding facilities, Conserv. Biol. 32 (1) (2017) 18–25.
[8] C. Shepherd, E.Y. Sy, First record of international trafcking of Critically Endangered long-beaked echidnas (*Zaglossus* sp.), J. Indones. Nat. Hist. 5 (1) (2017) 22–26.
[9] P.D. Rismiller, M.W. McKelvey, Frequency of breeding and recruitment in the short-beaked echidna *Tachyglossus aculeatus*, J. Mammol. 81 (1) (2000) 1–17.
[10] G. Morrow, N.A. Andersen, S.C. Nicol, Reproductive strategies of the short-beaked echidna — a review with new data from a long-term study on the Tasmanian subspecies (*Tachyglossus aculeatus setosus*), Aust. J. Zool. 57 (4) (2009) 275–282.
[11] A. Wallage, et al., Advances in the captive breeding and reproductive biology of the short-beaked echidna (*Tachyglossus aculeatus*), Aust. J. Zool. 63 (3) (2015) 181.
[12] A. Ferguson, B. Turner, Reproductive parameters and behaviour of captive short-beaked echidna (*Tachyglossus aculeatus acanthion*) at Perth Zoo, Aust. Mammol. 35 (1) (2012) 84–92.
[13] S. Broad, T. Mulliken, D. Roe, The nature and extent of legal and illegal trade in wildlife, in: S. Oldfield (Ed.), The Trade in Wildlife: Regulation for Conservation, Earthscan Publications Ltd., London, 2003.
[14] M.P. Piggott, A.C. Taylor, Extensive evaluation of faecal preservation and DNA extraction methods in Australian native and introduced species, Aust. J. Zool. 51 (2003) 341–355.
[15] M.P. Piggott, Effect of sample age and season of collection on the reliability of microsatellite genotyping of faecal DNA, Wildl. Res. 31 (5) (2005) 485–493.
[16] E. Suenaga, H. Nakamura, Evaluation of three methods for effective extraction of DNA from human hair, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 820 (1) (2005) 137–141.
[17] L.P. Waits, D. Paetkau, Noninvasive genetic sampling tools for wildlife biologists: a review of applications and recommendations for accurate data collection, J. Wildl. Manage. 69 (4) (2005) 1419–1433.
[18] F.E. Hogan, et al., Optimizing the use of shed feathers for genetic analysis, Mol. Ecol. Resour. 8 (3) (2008) 561–567.
[19] Z. du Toit, et al., Scale samples from Temminck's ground pangolin (Smutsia temminckii): a non-invasive source of DNA, Conserv. Genet. Resour. 9 (1) (2016) 1–4.
[20] P. Taberlet, L.P. Waits, G. Luikart, Noninvasive genetic sampling: look before you leap, Trends Ecol. Evol. 14 (8) (1999) 323–327.
[21] V.J. James, Y. Amemiya, Intermediate filament packing in α-keratin of echidna quill, Text. Res. J. 68 (3) (1998) 167–170.
[22] R.N. Johnson, L. Wilson-Wilde, A. Linacre, Current and future directions of DNA in wildlife forensic science, Forensic Sci. Int. Genet. 10 (2014) 1–11.

[23] R. Ogden, A. Linacre, Wildlife forensic science: a review of genetic geographic origin assignment, Forensic Sci. Int. Genet. 18 (2015) 152–159.
[24] W.M. Brown, M. George, A.C. Wilson, Rapid evolution of animal mitochondrial DNA, Genetics 76 (4) (1979) 1967–1971.
[25] L.E. Neaves, et al., Impact of Pleistocene aridity oscillations on the population history of a widespread: vagile Australian mammal, Macropus fuliginosus, J. Biogeogr. 39 (8) (2012) 1545–1563.
[26] M.L. Augee, B.A. Gooden, A. Musser, Echidna Extraordinary Egg-Laying Mammal, CSIRO Publishing, Australia, 2006.
[27] M. Griffiths, The Biology of Monotremes, Academic Press, 1978.
[28] H.M. Hsieh, et al., Cytochrome b gene for species identification of the conservation animals, Forensic Sci. Int. 122 (1) (2001) 7–18.
[29] J.C. Lee, et al., A novel strategy for avian species identification by cytochrome b gene, Electrophoresis 29 (11) (2008) 2413–2418.
[30] L. Wilson-Wilde, et al., Current issues in species identification for forensic science and the validity of using the cytochrome oxidase I (COI) gene, Forensic Sci. Med. Pathol. 6 (3) (2010) 233–241.
[31] G. Ferri, et al., Species identification through DNA "barcodes", Genet. Test. Mol. Biomark. 13 (3) (2009) 421–426.
[32] W. Parson, et al., Species identification by means of the cytochrome b gene, Int. J. Legal Med. 114 (1–2) (2000) 23–28.
[33] L. Fumagalli, et al., Versatile primers for the amplification of the mitochondrial DNA control region in marsupials, Mol. Ecol. 6 (12) (1997) 1199–1201.
[34] B. Budowle, et al., Mitochondrial DNA regions HVI and HVII population data, Forensic Sci. Int. 103 (1) (1999) 23–25.
[35] P. Sunnucks, D.F. Hales, Numerous transposed sequences of mitochondrial cytochrome oxidase I-II in aphids of the genus sitobion (Hemiptera: Aphididae), Mol. Biol. Evol. 13 (3) (1996) 510–524.
[36] T.D. Kocher, et al., Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers, Proc. Natl. Acad. Sci. U. S. A. 86 (16) (1989) 6196–6200.
[37] W. Rychlik, OLIGO 7 primer analysis software, PCR Primer Des. 2007 (2016) 35–59.
[38] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (7) (2016) 1870–1874.
[39] F. Ronquist, J.P. Huelsenbeck, MrBayes 3: bayesian phylogenetic inference under mixed models, Bioinformatics 19 (12) (2003) 1572–1574.
[40] A. Rambaut, et al., Tracer v1.6, (2014) .
[41] K.M. Sefc, R.B. Payne, M.D. Sorenson, Microsatellite amplification from museum feather samples: effects of fragment size and template concentration on genotyping errors, Auk 120 (4) (2003) 982–989.
[42] C.-H. Chan, et al., Isolation and characterization of microsatellites in the kakerori (*Pomarea dimidiata*) using feathers as source of DNA, Conserv. Genet. 9 (4) (2007) 1067–1070.
[43] C.F. Speller, G.P. Nicholas, D.Y. Yang, Feather barbs as a good source of mtDNA for bird species identification in forensic wildlife investigations, Invest. Genet. 2 (2011) 16.
[44] Q.-H. Wan, S.-G. Fang, Application of species-specific polymerase chain reaction in the forensic identification of tiger species, Forensic Sci. Int. 131 (1) (2003) 75–78.
[45] G.J. Frankham, et al., Does the 'extinct' eastern quoll (Dasyurus viverrinus) persist in Barrington Tops, New South Wales? Aust. Mammal. 39 (2) (2017) 243.
[46] P. White, J.F. O'Connell, M. Koettig, A Prehistory of Australia, New Guinea, and Sahul, Academic Press, 1982.
[47] M.J. Osborne, L. Christidis, Molecular phylogenetics of Australo-Papuan possums and gliders (family Petauridae), Mol. Phylogenet. Evol. 20 (2) (2001) 211–224.
[48] L.H. Rawlings, S.C. Donnellan, Phylogeographic analysis of the green python Morelia viridis, reveals cryptic diversity, Mol. Phylogenet. Evol. 27 (1) (2003) 36–44.
[49] M. Malekian, et al., Molecular systematics and evolutionary origins of the genus *Petaurus* (Marsupialia: Petauridae) in Australia and New Guinea, Mol. Phylogenet. Evol. 54 (1) (2010) 122–135.
[50] A. Linacre, S.S. Tobe, An overview to the investigative approach to species testing in wildlife forensic science, Invest. Genet. 2 (2) (2011).
[51] A. Linacre, Forensic Science in Wildlife Investigations, CRC Press, 2009.
[52] Q.D. Team, QGIS Geographic Information System, (2017) Available from: http://qgis.osgeo.org/.