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TECHNICAL NOTE

Scale samples from Temminck's ground pangolin (*Smutsia temminckii*): a non-invasive source of DNA

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Abstract Non-invasive sampling aims to obtain suitable samples without causing harm to the study organism, thus making it a more preferred sampling technique. Molecular methods have advanced in such a way that quality DNA can be obtained from less invasive mammal samples. Various PCR enhancing techniques have also been developed to aid in amplification of low quality DNA samples, to produce reliable results that can be used to monitor populations and species or for forensic applications. Temminck's ground pangolin is one of the few mammals to be covered in scales. In this study, we attempt to identify the most appropriate DNA isolation and PCR methods as well as determine whether PCR additives could be used to improve isolation of DNA and downstream amplification of mitochondrial DNA, from scale samples collected from these pangolins. In this study, it was determined that several different methods are required in order to successfully amplify DNA, and a decision tree rather than a single method should be followed.

Keywords DNA isolation · Non-invasive sampling · PCR optimization · Scale samples · Temminck's ground pangolin

Temminck's ground pangolins' entire body is covered in overlapping scales and when threatened, they roll into a ball with the overlapping scales protecting vulnerable parts of the body (Herklots 1937). Unrolling the animals to obtain blood samples can cause unnecessary stress, pain and harm (Pietersen 2013). Pangolin scales consist of non-living keratin (Atkins 2004) therefore taking scale clippings is considered to be non-invasive. Furthermore, in forensic cases where animal parts are confiscated in both Africa (Bräutigam et al. 1994) and Asia (Challender and Hywood 2012) the most likely material that will be obtained is scales. Non-invasive sampling has been reported to have several disadvantages including low quality and quantity of isolated DNA (Taberlet et al. 1996). Various DNA isolation kits and polymerase chain reaction (PCR) enhancing reagents such as bovine serum albumin (BSA) antibody or glycerol (Nagai et al. 1998) have been developed to increase PCR amplification yield. The objective of this study was thus to compare DNA extraction methods and PCR protocols in order to obtain high quality sequences of three mitochondrial (mt) DNA loci; *cytochrome c oxidase 1* (*COI*), *cytochrome b* (*Cytb*) and D-loop from pangolin scale samples that can be used in future to identify pangolin species in wildlife forensic cases worldwide.

Four pangolin scale samples were pulverised to powder using a dental electric micro-motor drill (Zhengzhou Xinghua Dental Equipment, Henan, China). A total of five different extraction methods were tested: (1) ZR Genomic DNA™—Tissue MiniPrep Kit (Zymo Research

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Table 1 DNA quality and quantity obtained for scale samples

Extraction method	A260/280 value/amount of DNA (ng/µl)			
	Sample 1	Sample 2	Sample 3	Sample 4
Zymo kit	1.38/3.49	0.29/0.25	0.52/0.66	1.75/2.65
Qiagen kit	1.84/21.56	1.85/10.36	1.34/8.88	1.61/18.95
Recoverall kit	1.86/6.35	0.13/2.09	−0.13/0.96	1.64/17.55
REPLI-g kit	1.87/435.32	1.88/398.23	1.89/283.61	1.88/360.31
MasterPure kit	1.63/394.56	1.11/43.65	1.56/65.36	1.60/70.48
PrepFiler kit	1.39/14.16	1.19/29.41	1.19/24.61	1.40/55.47

Corporation) using the manufactures protocol for hair, feathers or related samples; (2) QIAamp® DNA Investigator Kit (Qiagen), following the manufacturer's protocol for hair and nail clippings; (3) RecoverAll™ Total Nucleic Acid Isolation Kit (Applied Biosystems), following the manufacturers protocol for DNA extraction for tissue samples; (4) MasterPure™ Complete DNA and RNA Purification Kit (Illumina Company) and the general protocol as suggested by the manufacturers and (5) AutoMate Express™ Forensic DNA Extraction System (Applied Biosystems) along with the PrepFiler Express BTA Forensic DNA Extraction Kit (Applied Biosystems), using the manufacturer's protocols. Following extraction, the concentration of DNA was analysed on a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). The integrity of the isolated DNA (quality) was determined by a 0.7% agarose gel (Supplementary, Fig. 1). The PCR was performed to amplify three loci, namely COI, Cyt*b* and D-loop (Supplementary, Table 1). Various PCR protocols were tested (Supplementary, Table 2) namely: (1) Dream Taq™ Mastermix (Thermo Scientific), (2) Q-solution PCR protocol using the *Taq* DNA Polymerase Kit (Qiagen) and (3) REPLI-g Single Cell Kit (Qiagen) following the manufacturers protocol for amplification of purified genomic DNA along with the DNA isolated using the RecoverAll kit. In addition, different enhancers at various concentrations were employed, in the form of 0.25–0.5 µl BSA or 4.25 µl (5–10%) glycerol added to the DreamTaq PCR protocol. All PCR products were visualized on 2% agarose gels, followed by PCR purification using exosap (Thermo Scientific). Big Dye v3.1 Terminator Kit (Applied Biosystems) was used in a sequencing reaction and the ZR DNA Sequencing Clean-up™ Kit (Applied Biosystems) was used to remove remnants from cycle sequencing. Capillary electrophoresis was performed on an ABI3130 genetic analyser (Applied Biosystems).

The concentration and purity of DNA obtained in this study is presented in Table 1. High quality and quantity DNA from scale samples was obtained from the Qiagen, MasterPure, REPLI-g and PrepFiler kits. Low quality and quantity DNA was obtained from the Zymo and RecoverAll kit, however downstream applications were possible and sequencing was successful in the case of the Zymo kit. Amplification was not achieved using Q-solution. The remaining PCR protocols all produced sequences that were of high quality with varying success (Supplementary, Figs. 2–4). The average length of sequences obtained for the three gene regions ranged between 100 and 500 bp. In this study, an overall success rate of 89% (100/112) was achieved from four scale samples using five isolation techniques and eight PCR protocols. From this, we conclude that it is possible to amplify regions of the mtDNA genome from pangolin scale samples that is suitable for PCR. However, several different methods are required in order to successfully amplify DNA, and a decision tree (Fig. 1) rather than a single method should be followed. It was however noted in this study that the specific sample area of the scale can affect the success rate. Higher quality and quantity DNA was obtained from the base of the scale, where it is imbedded in the skin, compared to the edges of the scales. Nevertheless, the decision tree presented here can be used to amplify gene regions from non-invasive samples or low quality DNA from pangolins, for conservation research and for forensic applications and may prove successful for amplification of other types of non-invasive samples.

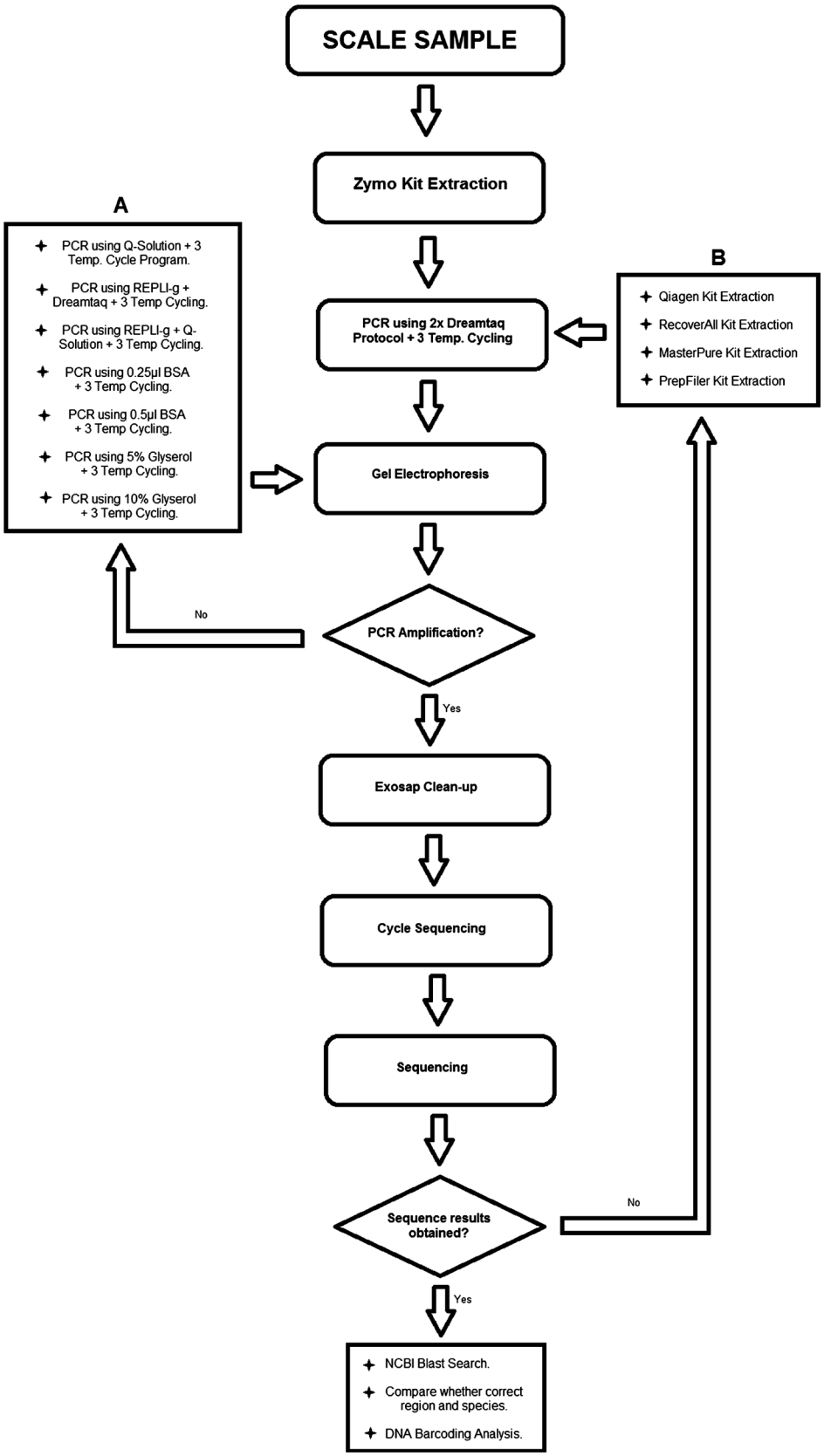

Fig. 1 Decision tree indicating suggested protocols for amplification of mtDNA gene regions using DNA obtained from non-invasive sources such as scale samples

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