รายงานวิจัยฉบับสมบูรณ์

การประยุกต์ใช้เทคนิคไดเร็คพีซีอาร์ในการระบุชนิดของสัตว์จากวัตถุพยานทางชีวภาพประเภทต่างๆ ในงานอาชญากรรมสัตว์ป่า

Forensic animal DNA analysis using economical two-step direct PCR

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1. ชื่อโครงการ: การประยุกต์ใช้เทคนิค direct PCR ในการระบุชนิดของสัตว์จากวัตถุพยานทางชีวภาพประเภทต่างๆในงานอาชญากรรมสัตว์ป่า
(Forensic animal DNA analysis using direct PCR)

2. คณะผู้วิจัย

2.1 หัวหน้าโครงการ

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3. กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบพระคุณมหาวิทยาลัยสงขลานครินทร์ ที่ให้การสนับสนุนทุนอุดหนุนการวิจัย ประเภทอาจารย์ ประจำปีงบประมาณ 2555 รหัสโครงการ SCI550385S นอกจากนี้ขอขอบพระคุณ คุณยิ่งยศ ลาภวงศ์ คุณสุคนธ์ ประดุจกาญจนา เจ้าหน้าที่สวนสัตว์สงขลาและเจ้าหน้าที่จากปางช้างเผือก หาดใหญ่ เป็นอย่างสูงที่ให้ความอนุเคราะห์ตัวอย่างจนทำให้งานวิจัยสำเร็จลุล่วงไปด้วยดี

4. บทคัดย่อ

การตรวจพิสูจน์ดีเอ็นในงานนิติวิทยาศาสตร์สัตว์ป่านั้น นิยมใช้ยูนิเวอร์แซลไพร์เมอร์ในการเพิ่มปริมาณดีเอ็นเอจากวัตถุพยานสัตว์ป่าต้องสงสัย ร่วมกับเทคนิคการหาลำดับนิวคลีโอไทด์ (DNA sequencing) และเปรียบเทียบกับลำดับเบสอ้างอิงในฐานข้อมูลออนไลน์เพื่อระบุชนิดของวัตถุพยานดังกล่าว เทคนิคนี้มีข้อเสียคือใช้เวลาในการตรวจพิสูจน์ค่อนข้างนานและมีค่าใช้จ่ายสูง งานวิจัยในครั้งนี้จึงพัฒนายูนิเวอร์แซลไพร์เมอร์จากบริเวณไซโตโครม

ออกซิเดส 1 (Cytochrome Oxidase I, COI) ไซโทโครม บี (Cytochrome *b*) และ 16S rRNA เพื่อใช้ร่วมกับเทคนิคไดเร็คพีซีอาร์แบบสองขั้นตอน (Two-step direct PCR) ในการตรวจพิสูจน์วัตถุพยานสัตว์ป่าทั่วไปจำนวน 12 ประเภท ได้แก่ ตัวอย่างคราบเลือด เนื้อเยื่อ กรงเล็บ ผิวหนัง ใบหู เส้นผมมีราก เส้นผมไม่มีราก เขาสัตว์ นอแรด อุจจาระ และปัสสาวะ รวมทั้งทดสอบในวัตถุพยานสัตว์ป่าที่ผ่านกระบวนการต่างๆและมีอายุนาน (Processed and aged sample) ผลการศึกษาพบว่าเทคนิคที่ได้พัฒนาขึ้น สามารถใช้วิเคราะห์ตัวอย่างทุกประเภทได้อย่างมีประสิทธิภาพด้วยวิธีการ pre-direct PCR dilution protocol โดยใช้สารละลาย PBS และชุดน้ำยา Phire® Hot Start II DNA polymerase ซึ่งจากผลการศึกษาด้วยวัตถุพยานทั้ง 12 ประเภทนั้นพบว่าวิธีการดังกล่าวมีอัตราความสำเร็จสูงถึง 92.5% (n=350) และให้ความเข้มข้นเฉลี่ยของผลิตภัณฑ์พีซีอาร์ที่ 220.71±180.84 นาโนกรัมต่อไมโครลิตร นอกจากนี้ยังพบว่าเทคนิคที่พัฒนาขึ้นนี้สามารถระบุชนิดและที่มาของตัวอย่างได้ถูกต้อง 100% แม้ว่าอัตราความสำเร็จและปริมาณผลิตภัณฑ์พีซีอาร์ที่ได้จากการวิเคราะห์จะขึ้นอยู่กับประเภทของตัวอย่าง

Wildlife forensic DNA analysis by amplification of a mitochondrial locus followed by DNA sequencing is routine yet suffers from being costly and time-consuming. To address these disadvantages we report on a low-cost two-step direct PCR assay to efficiently analyze 12 forensically relevant mammalian sample types without DNA extraction. A cytochrome oxidase I (COI), Cytochrome *b*, and 16S rRNA degenerated-universal primer pair was designed and validated for the developed assay and tested with 12 sample types , including processed and aged samples. The 12 sample types, which included bone, horn, feces, and urine, were amplified successfully by the assay using a pre-direct PCR dilution protocol. The average amplification success rate was as high as 92.5% (n=350), with an average PCR product concentration of 220.71±180.84 ng/μL. Differences in amplification success rate and PCR product quantity between sample types were observed; however, most samples provided high quality sequences, permitting a 100% nucleotide similarity to their respective species via BLAST database queries. The combination of PBS and Phire® Hot Start II DNA polymerase gave comparable amplification success rate and amplicon quantity with the proprietary commercial kits ($P > 0.05$, n=350) but at considerably lower cost. Stability of the assay was tested by

successfully amplifying samples that had been stored for up to 12 months. Our data indicate that the developed low-cost two-step direct amplification assay has the potential to be a valuable tool for the forensic DNA community.

5. บทสรุปผู้บริหาร (Executive Summary)

บทนำ

ในปัจจุบันปัญหาการลักลอบค้าสัตว์ป่ากลายเป็นปัญหาใหญ่ที่ส่งผลกระทบต่อมนุษย์และสิ่งแวดล้อมอย่างมาก หน่วยงานหรือองค์กรที่เกี่ยวข้องจึงเข้ามามีบทบาทในการคุ้มครองสัตว์ป่าดังกล่าวภายใต้กฎหมายของแต่ละประเทศและสนธิสัญญาระหว่างประเทศที่เรียกว่าไซเตส (CITES) นิติวิทยาศาสตร์จึงถูกใช้เป็นเครื่องมือในการพิสูจน์ข้อเท็จจริงเพื่อประโยชน์การบังคับใช้กฎหมายเหล่านั้น

การตรวจพิสูจน์ดีเอ็นเอในงานนิติวิทยาศาสตร์สัตว์ป่าโดยใช้ยูนิเวอร์แซลไพร์เมอร์ (Universal primer) หรือ ไพรเมอร์ที่ออกแบบมาจากบริเวณของดีเอ็นเอเป้าหมายที่มีลำดับเบสเหมือนกัน (Conserved region) ในสัตว์ชนิดต่างๆ วิเคราะห์ร่วมกับเทคนิคการหาลำดับนิวคลีโอไทด์ (DNA sequencing) และเปรียบเทียบกับลำดับเบสอ้างอิงในฐานข้อมูลออนไลน์เพื่อระบุชนิดของวัตถุพยานต้องสงสัยนั้น เป็นวิธีการที่น่าเชื่อถือและได้รับความนิยมอย่างแพร่หลายในห้องปฏิบัติการนิติวิทยาศาสตร์สัตว์ป่าหลายประเทศทั่วโลกรวมถึงประเทศไทย อย่างไรก็ตามเทคนิคนี้มีข้อเสียคือใช้เวลาในการตรวจพิสูจน์ค่อนข้างนานและมีค่าใช้จ่ายสูง

เทคนิคไดเร็คพีซีอาร์ (Direct PCR) คือ การเพิ่มปริมาณดีเอ็นเอโดยไม่ผ่านขั้นตอนการสกัดดีเอ็นเอ เป็นวิธีการระดับโมเลกุลที่กำลังได้รับความนิยมในงานนิติวิทยาศาสตร์ เนื่องจากสามารถลดค่าใช้จ่าย รวดเร็วและลดการปนเปื้อนดีเอ็นเอที่ไม่เกี่ยวข้องได้ ปัจจุบันมีรายงานการนำไปใช้ในการตรวจพิสูจน์ดีเอ็นเอมนุษย์ในงานนิติพันธุศาสตร์ แต่ยังไม่มีรายงานการประยุกต์ใช้เทคนิคดังกล่าวในงานด้านนิติวิทยาศาสตร์สัตว์ป่า ดังนั้นงานวิจัยครั้งนี้จึงมีวัตถประสงค์ คือ พัฒนายูนิเวอร์แซลไพร์เมอร์เพื่อใช้ร่วมกับเทคนิคไดเร็คพีซีอาร์แบบสองขั้นตอน (Two-step direct PCR) ในการตรวจพิสูจน์วัตถุพยานสัตว์ป่าจำนวน 12 ประเภท ได้แก่ ตัวอย่างคราบเลือด เนื้อเยื่อ กรงเล็บ ผิวหนัง ใบหู เส้นผมมีราก เส้นผมไม่มีราก เขาสัตว์ นอแรด อุจจาระ และปัสสาวะ รวมทั้งทดสอบในวัตถุพยานสัตว์ป่าที่ผ่านกระบวนการต่างๆและมีอายุนาน (Processed and aged sample)

วัตถุประสงค์

เพื่อพัฒนายูนิเวอร์แซลไพร์เมอร์เพื่อใช้ร่วมกับเทคนิคไดเร็คพีซีอาร์แบบสองขั้นตอน (Two-step direct PCR) ในการตรวจพิสูจน์วัตถุพยานสัตว์ป่าจำนวน 12 ประเภท ได้แก่ ตัวอย่างคราบเลือด เนื้อเยื่อ กรงเล็บ ผิวหนัง ใบหู เส้นผมมีราก เส้นผมไม่มีราก เขาสัตว์ นอแรด อุจจาระ และปัสสาวะ รวมทั้งทดสอบในวัตถุพยานสัตว์ป่าที่ผ่านกระบวนการต่างๆและมีอายุนาน (Processed and aged sample)

สรุป

รายละเอียดตามเอกสารผลงานตีพิมพ์จำนวน 3 เรื่อง ดังแสดงในภาคผนวก

6. ภาคผนวก

6.1 สำเนาบทความที่ได้รับการตีพิมพ์แล้ว จำนวน 3 เรื่อง ได้แก่

6.1.1 Forensic animal DNA analysis using economical two-step direct PCR

6.1.2 Direct PCR-FINS: Wildlife species identification without DNA extraction

6.1.3 Low-cost direct PCR for aged and processed wildlife sample analysis

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Forensic animal DNA analysis using economical two-step direct PCR

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Abstract Wildlife forensic DNA analysis by amplification of a mitochondrial locus followed by DNA sequencing is routine, yet suffers from being costly and time-consuming. To address these disadvantages we report on a low-cost two-step direct PCR assay to efficiently analyze 12 forensically relevant mammalian sample types without DNA extraction. A cytochrome oxidase I degenerate-universal primer pair was designed and validated for the developed assay. The 12 sample types, which included bone, horn, feces, and urine, were amplified successfully by the assay using a pre-direct PCR dilution protocol. The average amplification success rate was as high as 92.5 % ($n = 350$), with an average PCR product concentration of 220.71 ± 180.84 ng/μL. Differences in amplification success rate and PCR product quantity between sample types were observed; however, most samples provided high quality sequences, permitting a 100 % nucleotide similarity to their respective species via BLAST database queries. The combination of PBS and Phire® Hot Start II DNA polymerase gave comparable amplification success rate and amplicon quantity with the proprietary commercial kits ($P > 0.05$, $n = 350$) but at considerably lower cost. The stability of the assay was tested by successfully amplifying samples that had been stored for up to 12 months. Our data indicate that this low-cost two-step direct amplification assay has the potential to be a valuable tool for the forensic DNA community.

Keywords Cytochrome oxidase I · COI · Direct PCR · Forensic DNA · Non-human

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Introduction

DNA testing of seized materials can aid in the investigation of illegal mammalian wildlife trafficking. Typical sample types include body parts and their derivatives such as hairs, tissues, bones, ivory, and horns [1–3]. Biological tissues and samples such as blood, feces, and urine are also commonly encountered in such investigations [4–6].

Mitochondrial DNA (mtDNA) is employed for forensic animal DNA analysis as it has multiple copy numbers, high stability, and a high mutation rate [7]. Variations in some mitochondrial coding regions exhibit high inter-species variation but also low intra-species variation [7]. Many mitochondrial genes have been suggested for species identification, such as the widely used loci cytochrome *b* (cyt *b*) [2, 3, 8], and cytochrome c oxidase subunit 1 (COI) [9, 10]. Several mtDNA-based technologies have been reported for forensic animal species identification [1, 11–17]. DNA amplification with universal primers followed by sequencing is frequently used in non-human forensic laboratories, as it requires no prior information of the species within the sample and can also be universally applied to many species and biological sample types, including degraded or processed samples [16]. A range of universal primers have been proposed for this technique [8, 18–21].

Table 1 Details of the fourteen collected species including their scientific and common names, number of individual, sample type collected from each individual, sample origin, and voucher or laboratory designation

No.	Species		Individual	Sample type collected from each individual	Obtained from[a]	Voucher no.[b]
	Common name	Scientific name				
1	Domestic dog	*Canis lupus familiaris*	1	Hair root, Hair shaft, Blood	ID	PSU: CF1, CF2
			2	Urine		
2	Domestic cat	*Felis catus*	1	Hair root, Hair shaft	ID	PSU: FC1, FC2
			2	Urine		
3	Sheep	*Ovis aries*	1	Muscle	ID	PSU: OA1, OA2
			2	Horn		
4	Goat	*Capra aegagrus*	1	Muscle	ID	PSU: CA1, CA2
			2	Horn		
5	Asian elephant	*Elephas maximus*	1	Feces	CEC	PSU: EM1, EM2
			2	Urine		
6	Domestic cow	*Bos taurus*	1	Feces	ID	PSU: BT1
7	Horse	*Equus callubus*	1	Feces	ID	PSU: EC1
8	Tiger	*Panthera tigris*	1	Hair root, Hair shaft, Muscle, Bone, Claw, Ear, Skin	PSU-MS	PSU: PT1
9	Water buffalo	*Bubalus bubalis*	1	Hair root, Hair shaft	ID	PSU: BB1
10	White rhino	*Ceratotherium simum*	1	Muscle, Bone, Horn, Ear, Skin	PSU-MS	PSU: CS1
11	Lion	*Panthera leo*	1	Blood	SZ	PSU: PL1
12	Tapir	*Tapirus indicus*	1	Blood	SZ	PSU: TI1
13	Bornean orangutan	*Pongo pygmaeus*	1	Bone	PSU-MS	PSU: PP1
14	Muntjac	*Muntiacus muntjak*	1	Bone, Antler, Horn	PSU-MS	PSU: MM1

[a] ID is individuals donation; PSU-MS is the Princess Maha Chakri Sirindhorn Natural History Museum, Thailand; SZ is Songkhla Zoo, Thailand; CEC is Chang-Puak Elephant Camp, Thailand

[b] Voucher numbers for zoo and museum samples were designated by the Princess Maha Chakri Sirindhorn Natural History Museum. Other samples' identification numbers were designated at the laboratory

Direct PCR is a technique for amplification of target DNA from samples without prior DNA extraction. It has the potential to be faster and cheaper than conventional methods as the extraction process is omitted. Higher sensitivity is achieved, as no DNA is lost during the extraction steps. The technique is possible due to genetically modified DNA polymerases and proprietary additives in the reaction buffer [22, 23]. In recent years, direct PCR has been used to shorten the analysis time for human forensic samples [24] and detection of microbes from contaminated environmental, food, and clinical samples [25–27]; however, it has never been applied to forensic animal DNA analysis. Commercial direct amplification kits with proprietary buffers for animal DNA analysis and polymerases are currently available, but their high price limits widespread adoption in developing countries, where illegal trades are often more severe [28]. A low-cost two-step direct PCR assay utilizing commonly available laboratory chemicals would reduce both analysis time and cost.

In this study, we aimed to develop a two-step direct PCR assay and demonstrate that it efficiently amplified various forensically relevant animal samples. A comparison of chemicals that could be used as pre-direct PCR buffers as well as a comparison of DNA polymerases suitable for direct PCR was carried out. The effect of sample type and sample storage time on the assay were also evaluated.

Materials and methods

Animal sample collection and preparation

Twelve sample types (hair root, hair shaft, muscle, blood, bone, feces, urine, claw, horn, ear, skin, and antler) relevant to alleged mammalian wildlife investigations were collected from voucher specimens of 14 commonly traded or domestic species (Table 1). Guard hairs and claws were plucked from the skin, rinsed in sterile water, and dried before storing in a sterile bag. Sterile buccal swabs (Puritan Medical Products Co., USA) were used to collect EDTA-mixed bloodstains. Fresh urine was collected in a sterile bag around the time of excretion. Feces samples were collected from the field (in an elephant camp) and kept in a sterile bag. Small pieces of bone samples, horns, and aged antlers were obtained from individual donations and the Princess Maha Chakri Sirindhorn Natural History Museum,

Table 2 Details of species used for primer design

Order	Species	Common name	Accession number
Carnivora	*Panthera tigris*	Tiger	EF551003.1
	Helarctos malayanus	Malayan bear	FM177765.1
	Canis lupus familiaris	Domestic dog	U96639.2
	Felis catus	Domestic cat	U20753.1
Chiroptera	*Rhinolophus luctus*	Woolly horseshoe bat	NC_018539.1
Perissodactyla	*Ceratotherium simum*	White rhino	NC_001808.1
Proboscidea	*Elephas maximus*	Asian elephant	AJ428946.1
Primates	*Hylobates lar*	Gibbon	HQ622771.1
Pholidota	*Manis tetradactyla*	Long-tailed pangolin	AJ421454.1
Diprotodontia	*Macropus robustus*	Eastern wallaroo	Y10524.1

Thailand, ground using a sterile pestle and mortar, and then stored in a 1.5 mL microcentrifuge tube. Soft tissues, skins, and ears were incised from the specimens and then stored in a sterile bag. All samples were stored at −20 °C until further analysis.

Primer design

Cytochrome oxidase I (COI) sequences of 10 mammalian species spanning through seven mammalian orders were downloaded from GenBank (www.ncbi.nih.gov). Table 2 shows the details of these species and their accession number. Universal primers were designed from conserved regions based on the sequence alignment and suitable for degraded DNA samples. The candidate primers were checked to have suitable physical parameters and absence of secondary structures using the bioinformatics web-based tool Oligo Calc (http://www.basic.northwestern.edu/biotools/OligoCalc.html). Primers were synthesized from Macrogen Inc., Korea.

Development of low cost two-step direct amplification

Comparison of protocols for direct amplification

Two protocols of direct amplification and direct amplification incorporating a dilution step were performed using the Phire® Animal Tissue Direct PCR Kit (Thermo Scientific, Germany) following the manufacturer's protocols. Using the direct protocol, approximately 3–5 hair roots or 5–10 pieces of 2 mm hair shafts; 0.5–1.0 mm^2 each tissue sample (bloodstain swabs, ears, skins, and claws); 0.15–0.35 mg bones, horns, and antler powder; urine pellet prepared by centrifuging 300 μL urine; and 1 mm^2 feces diluted in a sterile water (1:30–100) were placed directly into the PCR. Using the dilution protocol, the same amount of sample was placed into 20 μL of proprietary dilution buffer followed by the addition of 0.5 μL DNARelease™ additive. The solution was mixed and incubated at room temperature for 2–5 min and then at 98 °C for 2 min. The tubes were briefly centrifuged and the supernatant was then added to the PCR. Human DNA acted as a positive control throughout this work. Negative controls were performed alongside all the amplifications.

The PCRs were performed in a total volume of 20 μL, containing 10 μL Phire® Animal Tissue PCR Buffer (with dNTPs and $MgCl_2$), 1.0 unit Phire® Hot Start II DNA Polymerase, 0.5 pmol of each primers, 1.5 μL dilution sample (only for the dilution protocol), and sterile water. A newly-designed COI universal primer set amplifying 520 bp (Forward 5′-CCYATYATRATYGGAGGGTTYGGHAAYTG-3′ and Reverse 5′-CDGGGTGTCCRAARAAYCARAATARGTGTTG-3′) was used. Ten replicates were performed for each treatment and sample type using a T100™ Bio-Rad thermal cycler. Amplification conditions were as follows: initial denaturation at 98 °C for 5 min; 35 cycles of denaturation at 98 °C for 5 s, annealing and extension at 60 °C for 25 s; and a final extension at 72 °C for 1 min. The reactions were stored at 4 °C until further analysis.

Comparison of chemicals and DNA polymerase for low-cost direct amplification

Five readily available chemicals (PBS (Sigma-Aldrich, USA), proteinase K (Invitrogen, USA), sterile water, 1 % w/v SDS (Invitrogen, USA), and DNAzol® (Invitrogen, USA) and two commercial buffers (Phire® Animal Tissue Direct PCR kit's pre-direct PCR buffer (Thermo Scientific, USA) and Terra™ Direct PCR Genotyping kit's pre-direct PCR buffer (Clontech, USA)) that could be used for cell lysis were compared as a pre-direct PCR buffer for the dilution protocol. The PCRs were also performed using eight different commercial PCR kits (containing six different DNA polymerases) according to their manufacturers' protocols.

These commercial kits were Phire® Animal Tissue Direct Kit (Thermo Scientific, USA), Terra™ PCR Direct Genotyping Kit (Clontech, USA), Phire® Hot Start II DNA polymerase (Thermo Scientific, USA), Phusion® Hot Start II DNA polymerase (Thermo Scientific, USA), Pfu DNA polymerase (Thermo Scientific, USA), Q5® DNA polymerase (NEB, USA), TopTaq DNA polymerase (Qiagen, USA), and Platinum® Taq DNA polymerase (Invitrogen, USA). Their amplification efficiency in terms of success rate and DNA quantity were compared to determine if there was a cheaper alternative to specialized direct PCR kits.

DNA separation, detection, and purification

PCR products were visualized on a 2 % agarose gel to observe if the amplifications provided the expected fragments. The mass of DNA in the bands on the gel was estimated using a GelAnalyzer2010a (http://www.gelanalyzer.com). The PCR products were purified using the ExoSTAR™ reagent (GE Healthcare, USA) following the manufacturer's protocol.

Validation of low-cost two-step direct amplification assay

The low-cost direct PCR assay was validated for its accuracy in identification by sequencing both strands of all the purified PCR products using an ABI 3130xl Genetic Analyzer (Applied Biosystems, USA). Subsequent DNA sequences were edited and compared using FINCH TV Version 1.4.0 (Geospiza Inc, USA) and Mega 5 [29] to identify and then correct any ambiguous bases. The amended DNA sequences were used to identify their species or origin by comparison to data on the NCBI GenBank database using the nucleotide BLAST (blastn) with the default parameters [30].

The low-cost direct PCR assay was also validated for its robustness using the 12 sample types listed in the Secion "Animal sample collection and preparation". All samples were prepared with PBS and the pre-direct PCR buffer was stored at −20 °C for a duration of 1, 2, 4, 8, 10, and 12 months. The samples were then analyzed as described previously to evaluate the effect of storage time on rate of successful amplification. Each sample type was amplified in triplicate.

Statistical analysis

The successful amplification of a PCR product at the expected size and the quantities of PCR product were tabulated in Microsoft Excel and exported to R (http://www.R-project.org/) for statistical analysis. Comparisons were made between pre-direct PCR buffers, DNA polymerases, and sample types. Tukey's Honest Significant Differences method was used as a post hoc analysis to adjust for multiple comparisons after analysis of variance. P value of less than 0.05 was deemed statistically significant.

Results and discussion

The comparison of protocols for direct amplification

Amplification efficiency using the direct amplification protocol and the method incorporating a dilution step were compared to determine the most suitable procedure. The comparison was performed using 12 sample types obtained from 14 animal species commonly related to wildlife investigations.

Incorporating a dilution step

For the protocol that incorporated a dilution step (Fig. 1 top), all sample types from the 14 species and the positive control were successfully amplified using the new COI primers and generated the expected 520 bp PCR products. No PCR product was obtained from the negative control. The overall success rate in amplifying all 350 amplifications (10 replicates performed for each sample type within each species; each species contributed varying sample types (Fig. 1)) was as high as 92.5 %, with an average PCR product concentration of 220.71 ± 180.84 ng/μL. The high variability in PCR product concentration was due to the differences between sample types (Fig. 1). All but one sample type exhibited more than a 90 % amplification success rate, with urine being the exception (73.3 %). Although seven sample types (urine, feces, horn, hair shaft, antler, hair root, and bone) showed lower amplification success rate, the quantity generated was sufficient for subsequent sequencing reactions.

Direct PCR results

Using the direct protocol (Fig. 1 bottom), three sample types (feces, horn, and bone) failed to amplify while very low amplification success rates were observed in antler (20 %) and urine (6.7 %) samples. The overall amplification success rate was only 41.8 % ($n = 350$) generating an average PCR product concentration of 228.98 ± 174.73 ng/μL. Higher success rates were found with blood (90 %), ear (85 %), and claw (80 %) while hair root (60 %), hair shaft (60 %), muscle (55 %), and skin (45 %) were less reproducible. Positive control gave an expected 520 bp PCR product while no PCR product was obtained from the negative control.

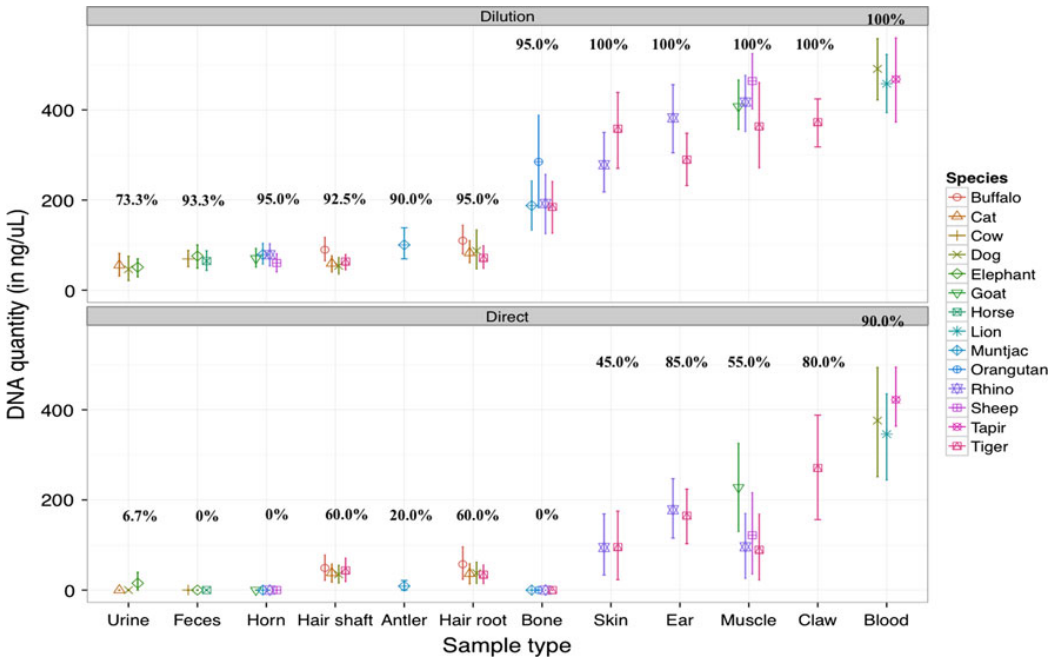

Fig. 1 The two-step amplification results in terms of DNA quantity and PCR success rate for both direct and dilution protocol of all 12 sample types from 14 animal species commonly related to crimes. Each mean and 95 % confidence interval is derived from 10 amplifications (total number of amplifications = 350)

Comparison of the two methods

The dilution protocol provided a higher efficiency and amplified a PCR product from more of the sample types, including aged bone and antler obtained from a museum, when compared to the direct protocol. This could be due to the pre-direct PCR buffer helping to lyse cells and release DNA from the samples. The dilution protocol is also sufficiently robust to overcome all inhibitors compared to the direct protocol. It is assumed that the proprietary dilution buffer and DNARelease^{TM} additive assist in the release of DNA [31, 32]. Another possible explanation for the higher success of the dilution protocol is that DNA was also diluted to the optimal range for amplification. Based on published data, and our unpublished initial results, samples diluted in a buffer were more likely to generate a PCR product and that excess DNA template decreases amplification efficiency [33]. The dilution protocol also enables limited samples to be amplified multiple times, as is the case with extracted DNA, but without having to repeat the DNA extraction. Only a few minutes incubation in the dilution buffer was sufficient for successful amplifications, even with difficult samples, e.g., bones, that would have needed additional sample preparation steps with conventional PCR. A newly designed COI primer pair was used in this study as this target length gave high identification confidence and higher amplification success rates than barcode primers (data not shown).

Development of low cost two-step direct amplification assay

In the previous experiment, the dilution protocol provided a higher success rate than the direct amplification in all sample types. Readily available laboratory chemicals were explored as a substitute for the proprietary pre-direct PCR buffer. Alternative DNA polymerases were then tested to develop a low-cost direct PCR assay as shown below.

Comparison of chemicals as a pre-direct PCR buffer

Seven chemicals were tested as a sample preparation buffer used for direct amplification with the dilution protocol. These data are shown in Fig. 2. PBS, proteinase K solution, and sterile water gave comparable amplification success rates and amplicon quantity with the proprietary commercial buffers ($P > 0.05$, $n = 120$ for each buffer (10 replicates $\times$ 12 sample types)). Amplification success rates of each sample type were not significantly different among the three chemicals and commercial buffers ($P > 0.05, n = 120$ for each buffer (10 replicates $\times$ 12 sample types)). This may

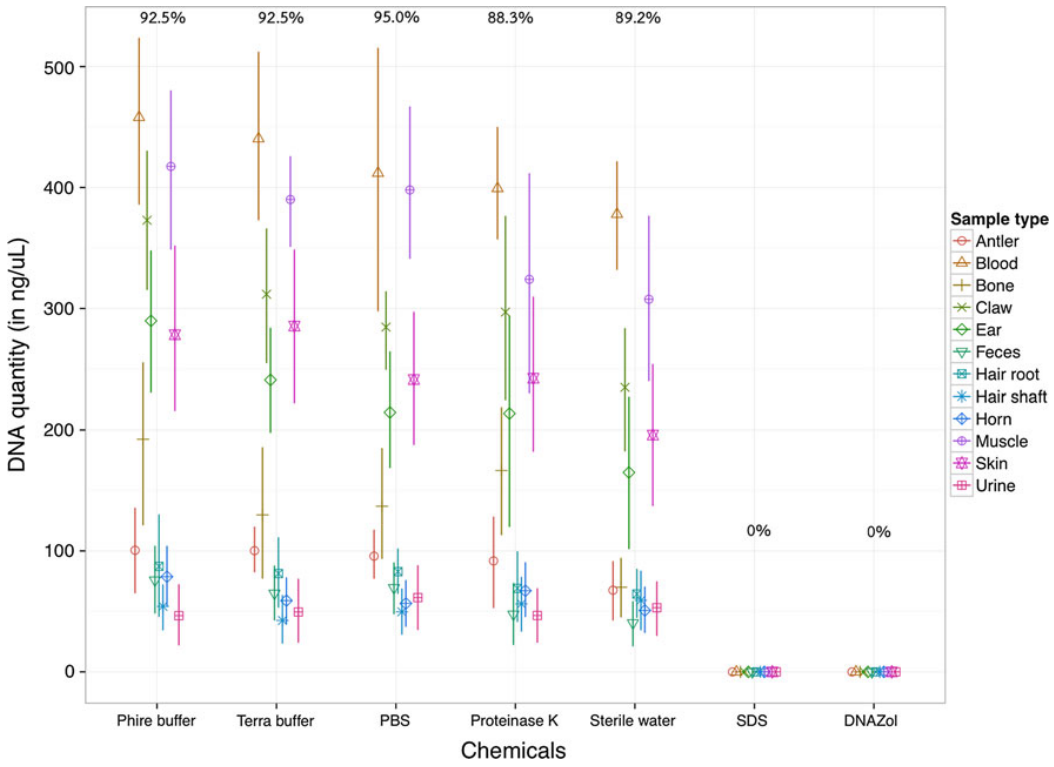

Fig. 2 The amplification success rates and PCR product concentrations of various samples prepared from 12 forensically relevant sample types. $N = 120$ for each pre-PCR buffer (10 replicates × 12 sample types). One species was used as a representative for each sample type: *Muntiacus muntjak* for antler; *Canis familiaris* for blood, hair root, hair shaft, and urine; *Ceratotherium simum* for bone, ear, and horn; *Panthera tigris* for claw, muscle, and skin; *Elephas maximus* for feces

be because the commercial buffers contain PBS, proteinase K, sterile water and/or chemicals with similar functions.

The short incubation step at 98 °C of the dilution protocol should denature cellular proteins including cytoplasmic enzymes. PBS, proteinase K, and sterile water aid direct PCR in different ways: PBS is non-toxic to cells and can maintain the pH of the PCR; proteinase K aids cell lysis and degrades nucleases, thereby minimizing DNA degradation; and sterile water results in cell lysis through osmotic shock. Surprisingly, the highest amplification success rate was found in the samples that were treated with PBS (95 %); thus it was chosen for further analysis.

All SDS and DNAzol dilution samples that had been expected to aid in the release of DNA from cells yielded no PCR products. This may be because the two chemicals contain PCR inhibitors [34] or alter the solution's pH, affecting the activity and activation of DNA polymerase [35].

The data suggest that it is not necessary to use the proprietary dilution buffers supplied with commercial direct PCR kits for direct amplification of commonly found forensic samples. Using readily available chemicals as an alternative decreases the analysis cost and could promote wider adoption of direct PCR protocols if funds are limited.

Comparison of DNA polymerases

Eight commercial PCR kits containing six different DNA polymerases were tested with 12 sample types prepared in PBS (Fig. 3). Variations in amplification success rate and amplicon quantity between polymerases and sample types were observed. Three DNA polymerases (Phire® Hot Start II DNA polymerase, Pfu DNA polymerase and Q5® DNA polymerase) produced PCR products with high amplification success rates and were not significantly different from the two commercial direct PCR kits ($P > 0.05, n = 120$ for each polymerase (10 replicates × 12 sample types)). This suggests that these three DNA polymerases could be an economical alternative to specialized direct PCR polymerases. No difference between the specialized Phire® Direct Kit and the normal Phire® polymerase, both available from Finnzymes, were observed. These data support the claim by the producers that Phire® polymerase exhibits high inhibitor resistance. Q5® DNA polymerase contains a processivity-enhancing Sso7d DNA binding domain [36] which may aid in overcoming PCR inhibitors. Substituting the commercial direct PCR polymerase with the three more economical polymerases reduces the cost per reaction by three to ten

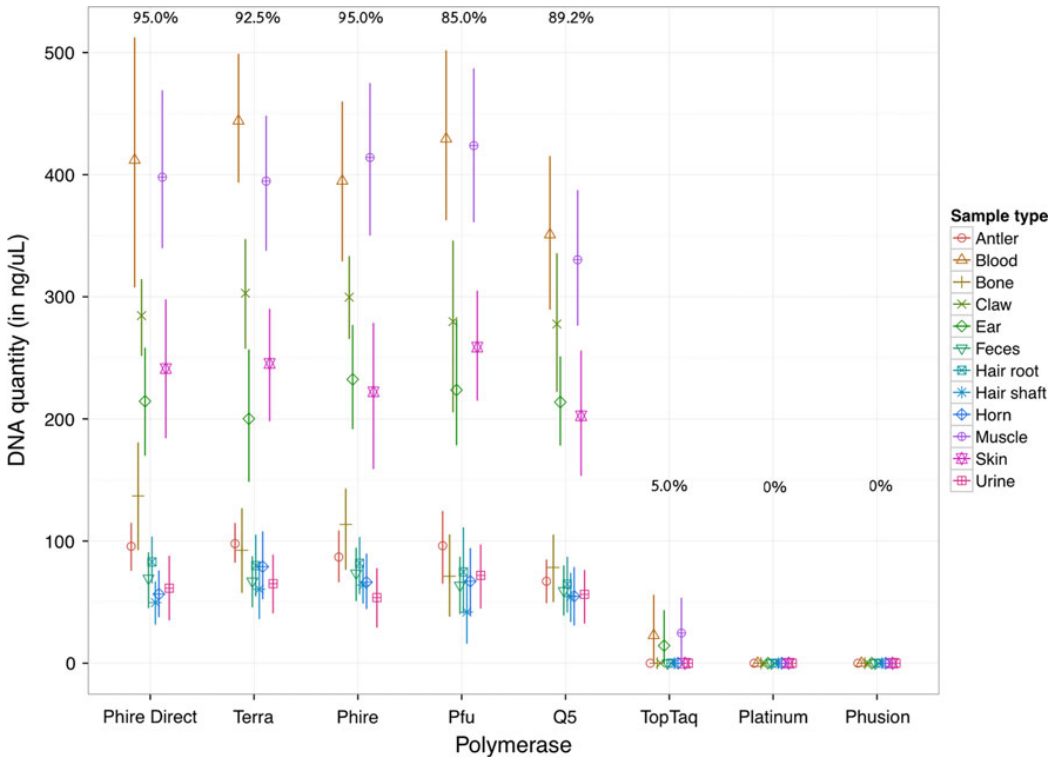

Fig. 3 The amplification success rates and PCR product concentrations from 12 samples types prepared with PBS buffer and directly amplified using eight polymerases. $N = 120$ for each polymerase (10 replicates × 12 sample types). One species was used as a representative for each sample type: *Muntiacus muntjak* for antler; *Canis familiaris* for blood, hair root, hair shaft, and urine; *Ceratotherium simum* for bone, ear, and horn; *Panthera tigris* for claw, muscle, and skin; *Elephas maximus* for feces

times. Among the three polymerases, Phire® Hot Start II DNA polymerase provided the highest success rate (95 %).

The Phusion® and two modified *Taq* polymerases (TopTaq and Platinum®) consistently failed to amplify all PBS-dilution samples, suggesting that they are not as robust as the other novel polymerases. The Phusion® result contradicts the manufacturer's claim that it performs well in the presence of PCR inhibitors [37]. Overall, these findings concur with earlier reports that genetically modified polymerases can overcome PCR inhibitors better than *Taq* [22, 38–40]. Even inhibitor-rich samples such as bone and feces were successfully amplified and sequenced.

Although the combined use of PBS and Phire® polymerase was applicable to all 12 sample types, the amplification efficiency of each type was variable. Amplification success rates could have varied due to variation in (1) DNA amount in each sample type [41] and (2) the concentration of any PCR inhibitor. Blood samples had the highest amplification efficiency, possibly due to the lack of tough extracellular matrix in blood, resulting in more efficient lysis. Also, the modified DNA polymerase used in this study might have been more tolerant to PCR inhibitors commonly found in blood [42]. Ear, skin, muscle and claw showed similar reproducibility. These are all cell-dense tissues [41]; therefore, they contain more DNA than other less dense tissues such as bone and ivory. Claw, contrary to expectation, displayed similar success rates with blood and cell-dense tissues. Claw samples used include both an outer keratin layer and an inner dermis layer containing once actively dividing cells and blood supply [41]. The other seven sample types (bone, horn, antler, feces, urine, hair root, and hair shaft) showed low PCR product concentration, which could be due to the combination of low amount of DNA and high PCR inhibitors present: calcium (bone, horn, and antler) [43], bilirubin and plant polysaccharides (feces) [42], and melanin (hair shaft) [42].

Validation of the low-cost direct amplification assay

Species identification by BLAST

All PCR products obtained from direct amplification (from samples diluted in pre-direct PCR buffer) using the low-cost assay provided high quality sequences. DNA sequences obtained from the same individual were aligned and no

Table 3 Accession number, BLAST E-value, percent nucleotide similarity (%NS) and the top matched species of the sequences obtained in this study

Species	Sample type (Individual no.)	NCBI no.	E-value	%NS	Matched species (Accession no.)
1. *Canis lupus familiaris*	Hair root, Hair shaft, Blood (1)	KF385956	0.0	100	*Canis lupus familiaris* (JF342822.1)
	Urine (2)	KF385957			
2. *Felis catus*	Hair root, Hair shaft (1)	KF385958	0.0	100	*Felis catus* (U20753.1)
	Urine (2)	KF385959		100	*Felis catus* (JX426133.1)
3. *Ovis aries*	Muscle (1)	KF385960	0.0	100	*Ovis aries* (HM236177.1)
	Horn (2)	KF385961			
4. *Capra aegagrus*	Muscle (1)	KF385962	0.0	100	*Capra aegagrus hircus* (AB736109.1)
	Horn (2)	KF385963			
5. *Elephas maximus*	Feces (1)	KF385964	0.0	100	*Elephas maximus* (EF588275.2)
	Urine (2)	KF385965			
6. *Bos taurus*	Feces (1)	KF385966	0.0	100	*Bos taurus* (FJ971088.1)
7. *Equus ferus*	Feces (1)	KF385967	0.0	100	*Equus ferus* (KC203031.1)
8. *Panthera tigris*	Hair root, Hair shaft, Muscle, Bone, Claw, Ear, Skin (1)	KF385968	0.0	100	*Panthera tigris* (JF357970.1)
9. Bubalus bubalis	Hair root, Hair shaft (1)	KF385969	0.0	99	*Bubalus bubalis (JN632607.1)*
10. *Ceratotherium simum*	Muscle, Bone, Horn, Ear, Skin (1)	KF385970	0.0	100	*Ceratotherium simum* (Y07726.1)
11. *Panthera leo*	Blood (1)	KF385971	0.0	100	*Panthera leo* (KC834784.1)
12. *Tapirus indicus*	Blood (1)	KF385972	0.0	99	*Tapirus terrestris* (JF444473.1)
13. *Pongo pygmaeus*	Bone (1)	KF385973	0.0	100	*Pongo pygmaeus* (AY972696.1)
14. *Muntiacus muntjak*	Bone, Antler, Horn (1)	KF385974	0.0	99	*Muntiacus muntjak* (AY225986.1)

Only one representative sequence from each individual was uploaded to the database, as alignment of multiple sequences from different samples types did not differ

difference between them was observed. One of these sequences was used as representative of the data, annotated, and compared to data on GenBank. Table 3 shows the GenBank accession number and BLAST results of these sequences.

The results support the use of a two-step low-cost direct amplification using dilution protocol in alleged cases of wildlife crimes, as all relevant sample types, including difficult and aged samples such as bone, antler, horn, and feces, were assigned to their expected species. Using the primers designed, the amplicon contained sufficient sequence information to provide confident match with appropriate species and short enough to efficiently amplify a target fragment from degraded samples commonly found in biological evidence. COI was chosen because its database sequences are increasing rapidly due to the Barcode of Life project and reported ability to distinguish species [10]. The two species that did not result in a 100 % similarity (*Bubalus bubalis* and *Muntjacus muntjak*) exhibited a 99 % similarity with their correct species. This indicates that either there is 1 % intraspecies variation or a species yet to have their sequence for this locus on GenBank shares 99 % similarity for the DNA sequence. Another 99 % match was found for *Tapirus indicus,* yet according to GenBank there is no sequence for this species lodged. The 99 % match was to a closely related species (*Tapirus terrestris*) within the same genus. This underlies the need to have relevant taxonomic knowledge and be able to account for either intraspecies or interspecies variation. The degree of confidence in species assignment is dependent on the data available and the reliability of the data. GenBank is a wealth of knowledge but is known to have a few errors. Greater confidence in species identification will occur if there is confidence in the coverage of GenBank for the genus to which the species may originate and by generating phylogenetic trees. If this is not possible, for whatever reason, the scientist should state caveats that either the 99 % similarity is due to intraspecies variation, and hence the questioned sample is from this species, or it may be from another member of the same genus with a higher than expected interspecies variation, or it may be from a species yet to be identified.

Estimation of storage time for dilution sample

Thirty-six dilution samples (three samples for each of the 12 sample types) stored in PBS at −20 °C for 1, 2, 4, 8, 10, and 12 months were amplified by the developed low-cost direct PCR (using the Phire® polymerase). Triplicate amplifications of seven sample types (hair root, hair shaft,

Table 4 The effect of storage time on ability to amplify a PCR product using the developed two-step direct PCR with PBS as the dilution buffer and Phire® polymerase

Sample type	Storage time (month)					
	1	2	4	8	10	12
Hair root, Hair shaft, Blood, Muscle, Skin, Ear, Claw	+++	+++	+++	+++	+++	+++
Bone	+++	+++	+++	+−+	+−−	−−−
Horn, Antler, Urine	+++	+++	+++	+++	++−	++−
Feces	+++	+++	+++	+++	+++	+−+

A plus (+) denotes a successful amplification and a minus (−) denotes a failed amplification. Three samples were tested at each time-point

blood, muscle, skin, ear, and claw) generated PCR products after storage for up to 12 months (Table 4). Feces, horn, antler, and urine were also amplifiable up to 12 months storage, but there were some failed amplifications. None of the three PCRs from bone stored for 12 months were successfully amplified.

The ability to amplify a PCR product at different storage times for each sample type appears to be associated with (1) the initial template amount, (2) the amount of inhibitors and microorganisms, and (3) the frequency of freezing and thawing. As expected, five sample types (blood, muscle, skin, ear, and claw) with a high initial DNA level [44] (as reflected by the PCR amplicon quantity shown in Fig. 2) could be stored the longest. Although hair root and shaft produced a low amount of amplicon, the samples were amplifiable after up to 12 months storage. This is probably because hair roots and shafts contain less inhibitors and microorganisms when compared with bone, horn, antler, urine, and feces.

Conclusion

This is the first time that a two-step direct PCR assay has been evaluated and applied for all forensically relevant sample types, including difficult samples such as bone, hair shaft, and feces. The dilution protocol using PBS in combination with Phire® Hot Start II DNA polymerase and COI universal primer generated the best data based on the maximum amplification efficiency. The assay is up to ten times cheaper than a commercial direct PCR kit. No complex pre-PCR treatments, no DNA extraction, and no quantitation are required, saving at least 2–4 h of time in the DNA analysis process. This rapid assay will be a beneficial and valuable tool for the non-human forensic DNA community, particularly if funds are limited. The test is not only limited to forensic purposes but may be adopted by field studies in the area of wildlife conservation and management of biodiversity where fecal and hair samples are commonly encountered.

Key points

1. This is the first time direct PCR has been applied for forensic animal DNA analysis. All 12 sample types, including samples such as bones and feces which frequently contain trace or compromised DNA, were successfully amplified without the need for complex pre-PCR treatment.
2. A novel low-cost two-step direct PCR assay using a new cytochrome oxidase I universal primer pair was developed and optimized. The new COI primers were suitable for forensic analysis as it produces a short PCR product for degraded samples and yet contains sufficient DNA data to permit species identification.
3. Seven chemicals and six polymerases were evaluated as a substitute for commercial direct PCR kits in order to keep the cost of analysis low. PBS with Phire® DNA polymerase performed comparably to commercially available direct PCR kits.
4. All samples amplified using this assay gave correct species identification using BLAST queries with high confidence. Samples prepared using the proposed dilution protocol could still be amplified after 12 months of storage.

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Direct PCR-FINS: Wildlife species identification without DNA extraction

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ABSTRACT

Forensically informative nucleotide sequencing (FINS) is commonly used in forensic laboratories worldwide for wildlife species identification. Recently direct PCR – DNA amplification without prior DNA extraction – has been gathering attention in the human forensic community due to its high efficiency, but it has never been applied to non-human forensic investigation. Therefore, we aimed to evaluate the possibility and efficiency of using direct PCR-FINS for species identification from various types of forensically relevant animal samples. Eight biological evidence types (hair, muscle tissue, bloodstain, bone, ear, skin, urine, and feces) from three common crime-related animals (tiger, white rhino, and Asian elephant) were analyzed with the three-step direct PCR-FINS method: direct PCR amplification of target DNA with 16S rRNA and cytochrome *b* universal primers, sequencing, and database searching (BLAST). All sample types were successfully amplified using direct PCR-FINS method and provided the expected PCR products. Each amplicon sequence and its relevant animal species were completely matched using BLAST, meaning that the species of the samples were correctly identified. In conclusion, the 'direct PCR-FINS method' is rapid, reliable, and meets the requirement of international forensic organizations.

1. Introduction

Species identification using mitochondrial DNA analysis is a valuable tool for proving illegal possession of evidence from protected-wildlife species. Forensically informative nucleotide sequencing (FINS) is one of many DNA-based approaches commonly used in wildlife forensic laboratory worldwide for this purposes. However, the method is costly and labor-intensive, which are the most significant obstacles in routine forensic work. Recently, direct amplification method has proved to be efficient for human forensic DNA analysis with several advantages: quick, simple, and requires only a small amount of sample.

Therefore, in this study, we aimed to evaluate the possibility and efficiency of using direct PCR-FINS method for wildlife forensic investigation in the aspect of species identification from eight forensically relevant sample types.

2. Materials and methods

Eight sample types (hair, muscle tissue, bloodstain, bone, ear, skin, urine, and feces) commonly found in wildlife crime scenes were collected from voucher samples of three commonly traded species (tiger, white rhino, and Asian elephant). Each samples type was decontaminated or cleaned before performing direct amplification with 16S rRNA and cytochrome *b* universal primers using the Phire® Animal Tissue Direct PCR Kit (Thermo Scientific, Germany) following the two manufacturer's protocols – direct and dilution protocol. Optimal amounts for each sample type were determined.

The PCR products were quantified using a MaestroNano Spectrophotometer (Maestrogen, USA) and purified using the QIAquick gel extraction kit (QIAGEN, USA) or the ExoSTAR™ reagent (GE Healthcare, USA) following their manufacturer's protocols. All purified PCR products were sequenced using an ABI 3130xl Genetic Analyzer (Applied Biosystems, USA) in both the forward and reverse directions. DNA sequences were edited and compared using FINCH TV Version 1.4.0 (Geospiza Inc, USA) and Mega 5 [1] to correct ambiguous bases. The corrected sequences were then used to identify their species origin by matching with the closest reported sequence uploaded to the NCBI Genbank database using the Basic Local Alignment Search Tool (BLAST) [2].

3. Results and discussion

The amplification result showed that direct PCR could be used for wildlife DNA analysis as the expected products of 240 and 1140 bp were generated for the 16S rRNA and cyt *b* primers,

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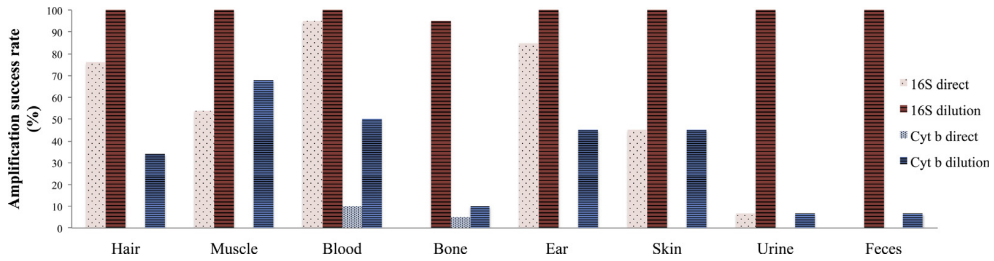

Fig. 1. Average amplification success rates (%) from ten amplifications categorized by sample type. Color bars indicate the method used (pink for 16S direct protocol, red for 16S dilution protocol, light blue for cyt *b* direct protocol, and dark blue for cyt *b* dilution protocol).

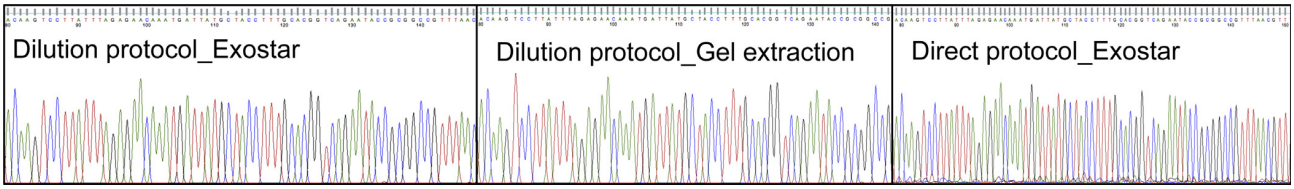

Fig. 2. The DNA sequencing quality of tiger muscle sample amplified using the 16S rRNA universal primer. Good quality sequences were obtained regardless of protocol and purification method.

respectively. The dilution protocol provided a higher success rate and amplified more sample types when compared to the direct protocol for both primer sets (Fig. 1). Using the dilution protocol with 16S rRNA universal primers, all samples were successfully amplified and provided an average success rate as high as 99% (ten replicates per sample type). The successes obtained in this study are attributed to genetically modified DNA polymerases, which have high inhibitor tolerance, and proprietary additives in the reaction buffer [3,4]. Amplicon length is one of the factors affecting the amplification competence, as success rates of 16S rRNA amplification were much higher than cyt *b*. The failure in amplifications is probably due to PCR inhibitors above the tolerance limit of the PCR buffer and DNA polymerase system.

All PCR products provided high quality DNA sequences regardless of the PCR purification methods and PCR product concentration. Some samples from the direct protocol had higher background noise when compared with the dilution protocol; however, the noise was low enough not to interfere with the base calls (Fig. 2). Most BLAST queries using the sequence data in this study reported a 100% match with the correct species. Some sequences resulted in less than 100% match, which is due to (1) no reference sequence for that species is available in Genbank and (2) intra-species variation within the queried region.

4. Conclusion

Direct PCR assay can be used for wildlife DNA analysis. The assay is rapid, robust, reliable, and applicable to identify all forensically relevant unknown and casework sample types. The procedure of the dilution protocol with 16S rRNA universal primers is recommended, because it provided the maximum amplification success rate and PCR product concentration.

Role of funding

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Conflict of interest

None declared.

Acknowledgements

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Low-cost direct PCR for aged and processed wildlife sample analysis

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ABSTRACT

Direct PCR has been used successfully in wildlife forensic DNA analysis from several types of biological samples using specialized, commercial direct PCR kits. This is attributed to the proprietary chemicals provided in the kits such as pre-PCR buffer and modified DNA polymerases. These reagents can be expensive, thereby limiting their widespread adoption in developing countries, where wildlife crimes are often encountered. We report on a study to evaluate the possibility of using low-cost direct PCR assay for degraded and processed wildlife sample analysis. Phire® and Q5® polymerases were used, due to their relatively low cost, for direct amplification of six aged and processed sample types (dried skin, 30-year old hair, muscle tissue, bone, trace blood mixed in vodka, and dried soft antler). The result indicated that Phire® Hot Start II DNA polymerase and Q5® DNA polymerase performed similarly to commercially available direct PCR kit. The low-cost amplification could efficiently identify species origin from all aged and processed samples. We observed a rate of more than 80% amplification success and high PCR product concentrations sufficient for further sequencing. The assay proved to be cost effective and robust; thus, we expect it to be adopted by wildlife forensic laboratories in developing countries.

1. Introduction

Wildlife forensic science frequently requires to analyse samples to determine if controlled or protected species are present. The evidence in such cases may include both body parts and their derivatives such as hairs, tissues, and bones [1,2]. Degraded or processed biological evidence are also commonly encountered [3,4]. Generally the species identification process is divided into three steps – DNA extraction, PCR amplification and purification of PCR products, and sequencing followed by phylogenetic analysis or database searching.

Direct PCR using a dilution protocol with a commercial kit has been proven to be efficient for DNA analysis from various types of samples attributed to wildlife crimes (authors' unpublished data). This increased efficiency is attributed to the proprietary chemicals provided in the kits such as pre-PCR buffer and modified DNA polymerases. However, the commercial assays are expensive, limiting their widespread adoption in developing countries where wildlife crimes are often encountered. Other novel, less expensive polymerases are available but their performance in direct amplification with aged and processed wildlife samples has never been evaluated. Thus, in this study, we compared the performance of two novel polymerases with a specialized direct PCR kit on aged and processed sample types encountered in wildlife crimes.

2. Materials and methods

Dried skin and muscle tissue of tiger, bone of rhino, and 30-year-old leopard hair were collected from museum voucher specimens. A product labelled as vodka mixed with trace deer blood and dried soft antler were bought from a local deer farm. Guard hairs were plucked from the skin, rinsed in sterile water, and dried before storing in a sterile bag. Dried skin and muscle tissue were incised and kept in sterile bags while bone sample was ground using a sterile pestle and mortar and then stored in a 1.5 mL tube. Small amounts of these samples were separately added with 20 μL sterile water and incubated at 98 °C for 2 min. The supernatant was used for direct amplification.

The PCRs were performed using a commercial direct PCR kit – Phire® Animal Tissue Direct Kit (Thermo Scientific, USA) – and two economical PCR kits – Phire® Hot Start II DNA polymerase (Thermo Scientific, USA) and Q5® DNA polymerase (NEB, USA),

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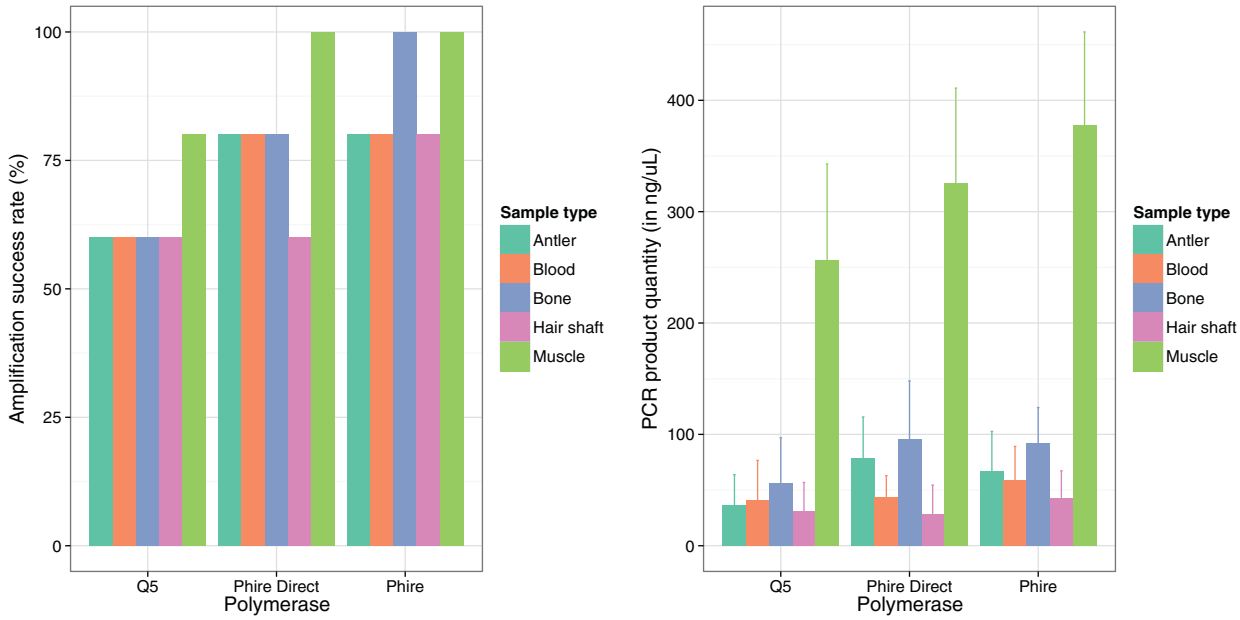

Fig. 1. Left: the average amplification success rate (%) of six sample types (either aged or processed samples) commonly found in wildlife crimes using Phire® Animal Tissue Direct Kit, Phire® Hot Start II DNA polymerase, and Q5® DNA polymerase. Right: the PCR product quantity (in ng/μL) of the same samples. Error bars denote 95% bootstrapped confidence intervals. Differences in amplification success rates and PCR product quantities were apparent.

according to their manufacturer's protocols and using a cytochrome oxidase I universal primer pair following our unpublished method. Five amplifications were performed for each sample.

3. Results and discussion

The amplification result showed that both commercial (Phire® Animal Tissue Direct Kit) and low cost direct PCR assays (Phire® Hot Start II and Q5® DNA polymerase) were able to amplified aged and degraded wildlife samples and generated the expected products of 520 bp. The average amplification success rate of direct amplification using the commercial Phire® Animal Tissue Direct Kit, Phire® Hot Start II DNA polymerase, and Q5® DNA polymerase were 80%, 88%, and 64%, respectively. These data indicate that the low-cost direct PCR assay using both Phire® Hot Start II and Q5® DNA polymerase showed amplification success rates comparable to commercially available direct PCR kit in the analysis of aged and degraded wildlife biological samples (Fig. 1). Even though PCR product concentrations of most samples were low, DNA sequences of sufficient quality were obtained and found to report a 100% match with the correct species using BLAST (data not shown). Omitting the sample preparation step and DNA extraction has the potential to be faster by 1 h and save at least 5–10 USD per sample, assuming a DNA extraction kit is used. The use of the less expensive DNA polymerases saves up to three times in analysis cost.

Although the low-cost direct PCR assay was applicable to all aged and degraded sample types, the amplification success rate (reproducibility) of each type was different. Amplification success rates in each sample types varied due to (1) variation in DNA amount; (2) PCR inhibitors in the samples, whether from naturally occurring sources (e.g. heme in blood) or from sample processing steps; and (3) sample storage approach. The highest amplification success rate and PCR product concentration were found in aged muscle samples. This could have been because muscle is a cell-dense tissue, thereby containing more mtDNA copies and also because the muscle samples had always been kept at −20 °C during storage. Other sample types were not as efficiently amplified as muscle tissues or had less initial DNA template, resulting in a much lower PCR product concentration.

4. Conclusion

Low-cost direct PCR using Phire® Hot Start II DNA polymerase and Q5® DNA polymerase with the dilution protocol proved to be efficient in the analysis of aged or processed wildlife samples commonly found in wildlife crime scenes. It therefore can be a valuable tool for wildlife forensic DNA community, particularly in developing countries.

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Conflict of interest

None.

Acknowledgements

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6.2 ข้อคิดเห็นและข้อเสนอแนะสำหรับการวิจัยต่อไป

เทคนิคไดเร็คพีซีอาร์ (Direct PCR) ด้วยวิธีการที่พัฒนาได้จากการศึกษาในครั้งนี้ สามารถนำไปประยุกต์ใช้สำหรับผลงานวิจัยในระดับโมเลกุล (Molecular genetics) ได้หลากหลายสาขา โดยมีแนวโน้มว่าสามารถใช้เพิ่มปริมาณดีเอ็นเอจากสิ่งมีชีวิตอื่นๆนอกเหนือจากสัตว์ป่า เช่น มนุษย์ พืช จุลชีพ หรืออื่นๆ ได้สำเร็จ รวมทั้งอาจนำไปใช้ในการทดสอบตัวอย่างที่ปรากฏตัวยับยั้งปฏิกิริยาพีซีอาร์ (PCR inhibitors) ในปริมาณที่สูงได้อีกด้วย

แบบวิจัย 5/รายได้

รายงานสรุปแสดงรายการค่าใช้จ่ายเงินอุดหนุนการวิจัย
จากเงินรายได้มหาวิทยาลัยสงขลานครินทร์

ชื่อโครงการ .....การประยุกต์ใช้เทคนิคไดเร็คพีซีอาร์ในการระบุชนิดของสัตว์จากวัตถุพยานทางชีวภาพประเภทต่างๆในงานอาชญากรรมสัตว์ป่า.....
ชื่อหัวหน้าโครงการ...ผศ.ดร.ฐิติกา กิจพิพิธ.....ภาควิชา...วิทยาศาสตร์ประยุกต์.....คณะ...วิทยาศาสตร์...
ได้รับทุนอุดหนุนการวิจัยจากเงินรายได้ประจำปีงบประมาณ...2555.....ประเภท...ดรุณาจารย์.....
จำนวน.....460,000.....บาท (.....สี่แสนหกหมื่นบาทถ้วน.....) และได้เบิกเงินอุดหนุนไปแล้ว จำนวน...394,000.....บาท (...สามแสนเก้าหมื่นสี่พันบาทถ้วน.....) เพื่อเป็นค่าใช้จ่ายในการวิจัย และได้ใช้จ่ายเงินทั้งโครงการไปแล้วทั้งสิ้น จำนวน...460,338.53.....บาท (รวมเงินงวดสุดท้ายที่ยังไม่ได้เบิกจากมหาวิทยาลัยด้วย ยกเว้นทุนประเภทโครงงานของนักศึกษา) ดังรายละเอียดต่อไปนี้

หมวดค่าใช้จ่าย	บาท	สต.
1. หมวดค่าใช้สอย	**34,086**	**10**
1. ค่า sequencing service	28,123	10
2. ค่าบริการการใช้ Autoclave	150	00
3. ค่าถ่ายเอกสาร	813	00
4. ค่าประกอบเครื่อง UV	5,000	00
2. หมวดค่าตอบแทน	**264,000**	**00**
1. ค่าตอบแทนนักวิจัยหัวหน้าโครงการ	168,000	00
2. ค่าตอบแทนนักวิจัยพี่เลี้ยง	96,000	00
3. หมวดค่าวัสดุและสารเคมี	**162,252**	**43**
1. ค่าวัสดุ	60,382	80
2.ค่าสารเคมี	101,869	63
รวมเงิน สี่แสนหกหมื่นสามร้อยสามสิบแปดบาทห้าสิบสามสตางค์	**460,338**	**53**

ข้าพเจ้าขอรับรองว่าข้อความดังกล่าวข้างต้นเป็นความจริงทุกประการ

(ลงชื่อ).....ฐิติกา กิจพิพิธ.....
(ผศ.ดร.ฐิติกา กิจพิพิธ)
หัวหน้าโครงการ
...11.../...12.../...57...

(ลงชื่อ).....
(ผศ.อดุลย์ เที่ยงจรรยา)
หัวหน้าภาควิชา
11.../...12.../...57....

C:\wan\ว5/รายได้