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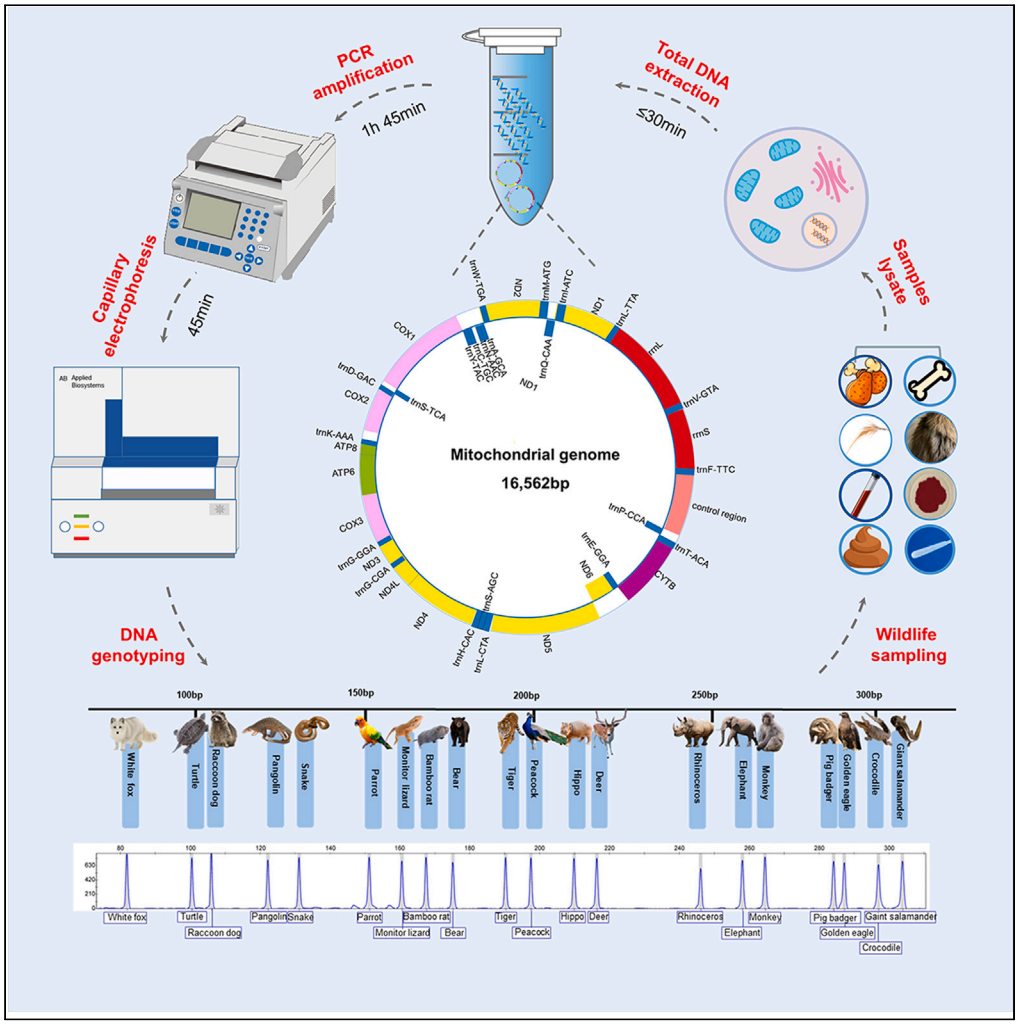
A multilocus DNA mini-barcode assay to identify twenty vertebrate wildlife species



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Highlights

The assay offers authentic and rapid identification of twenty vertebrate wild animals

DNA mini-barcodes were developed using mitochondrial genes for degraded samples

The assay was validated to be robust and practical for forensic casework

A feasible nondestructive identification method was proved by feather, hair, and fece

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A multilocus DNA mini-barcode assay to identify twenty vertebrate wildlife species

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SUMMARY

The world faces significant challenges in preserving the diversity of vertebrate species due to wildlife crimes. DNA barcoding, an effective molecular marker for insufficient nuclear DNA, is an authentic and quick identification technique to trace the origin of seized samples in forensic investigations. Here, we present a multiplex assay capable of identifying twenty vertebrate wildlife species utilizing twenty species-specific primers that target short fragments of the mitochondrial *Cyt b*, *COI*, *16S rRNA*, and *12S rRNA* genes. The assay achieved strong species specificity and sensitivity with a detection limit as low as 5 pg of DNA input. Additionally, it effectively discriminated a minor contributor ($\geq$1%) from binary mixtures and successfully identified of noninvasive samples, inhibited DNA samples, artificially degraded DNA samples, and case samples, demonstrating a sensitive, robust, practical and easily interpretable tool in screening, and investigating forensic wildlife crimes.

INTRODUCTION

The world faces significant challenges in preserving the diversity of vertebrate species due to factors such as overexploitation,[1] habitat destruction, poaching,[2] illegal wildlife trade, and smuggling.[3] Furthermore, the outbreak of zoonotic diseases has shed light on hidden threats that extend well beyond wildlife germplasm resources and ecological balance.[4,5] In addition to habitat protection,[6] accurate species identification based on DNA analysis plays a vital role in wildlife crime investigation and conservation efforts.[7]

Wildlife forensic science, a new subdiscipline of forensic science, employs molecular techniques that offer legal advantages comparable to traditional identification methods.[8,9] DNA-based identification systems have become widely adopted when morphological techniques fail to accurately identify species. These systems leverage the vast gene polymorphisms[9,10] and the high sensitivity of polymerase chain reaction (PCR) in recent years[11] to address the immense diversity of millions of species. Compared with the nuclear genome, mitochondrial DNA (mtDNA) is favored for serving as a valuable marker for species identification, phylogenetic studies, and biodiversity research in wildlife forensics.[12–14] MtDNA possesses a closed-circular molecular structure and mitochondrial membranes that protect it from degradation.[15,16] Moreover, the high copy number per cell of mtDNA[17,18] ensures a higher success rate of amplification for mtDNA markers compared to nuclear gene markers. Consequently, mtDNA is particularly advantageous for typing samples with limited nuclear DNA, such as feathers,[19,20] hairs,[21,22] bones,[23,24] and feces,[21,25,26] as well as severely degraded or processed samples.[27,28] Commonly employed molecular markers derived from the mitochondrial genome include cytochrome *b* (*Cyt b*),[29–31] cytochrome *c* oxidase I (*COI*),[32,33] 12S ribosomal RNA (*12S rRNA*),[34,35] and 16S ribosomal RNA (*16S rRNA*)[36,37] genes, collectively referred to as DNA barcodes.[38,39]

DNA barcodes are standard DNA regions that exhibit significant genetic variability and divergence at the species level, aiming to provide rapid and accurate species identification. Numerous studies focusing on a standardized short sequence of mtDNA, ranging from 400 to 800 bp in length, have demonstrated the potential of DNA barcodes as effective molecular markers for species identification in forensic science.[39–42] However, amplification may be impeded if degraded DNA fragments fall below the length spanned by the primers.[7,43] In such cases, DNA mini-barcodes rely on hypervariable DNA regions where inter- and intraspecific genetic distances differ, allowing for species delimitation.[7,44] This approach facilitates the identification of degraded DNA by amplifying short DNA fragments, exhibiting higher success rates compared to versatile primer sets traditionally employed for DNA barcoding due to the significantly reduced sequence length.[45–49]

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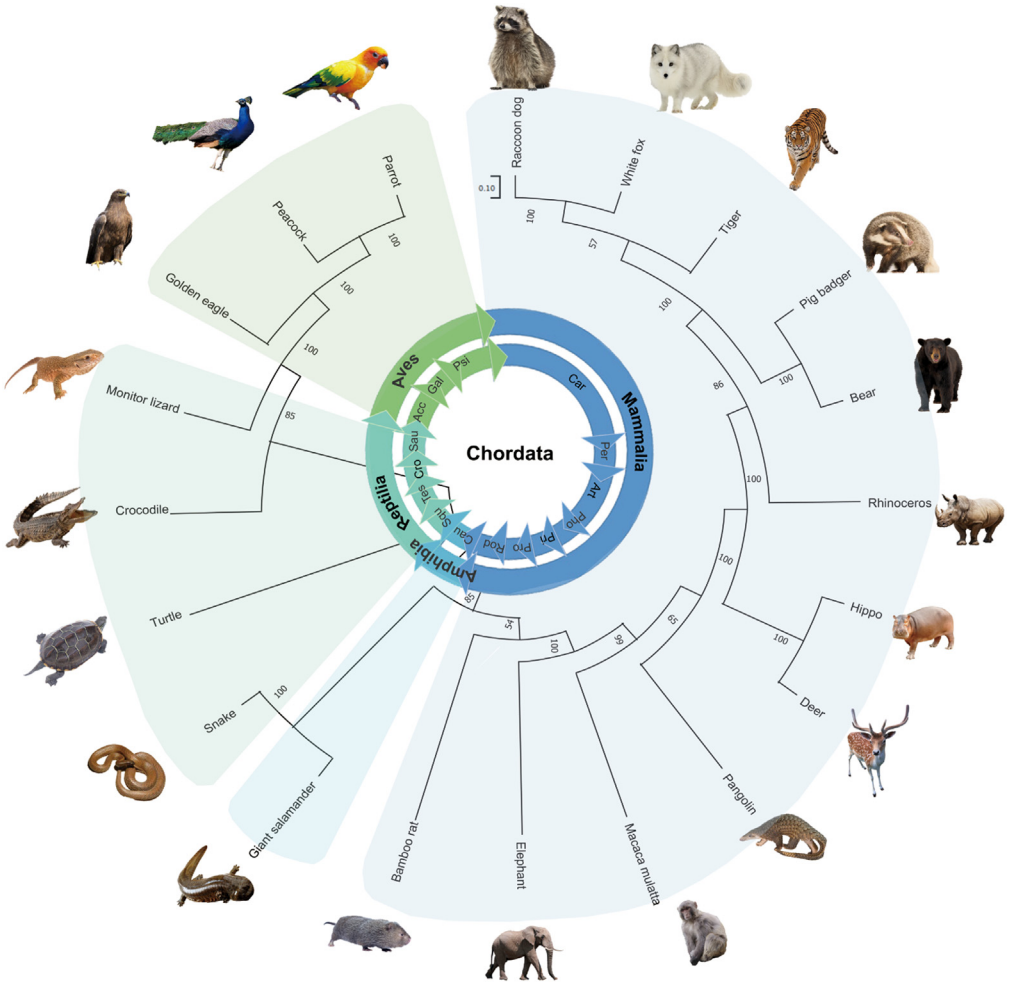

Figure 1. A phylogenetic tree based on the mtDNA sequences of 20 vertebrate wildlife species was constructed by the neighbor-joining method

The numbers represent confidence values assessed with the bootstrapping option. Scale bars represent branch length. The acronyms have the following meanings: **Car**: Carnivor; **Per**: Perissodactyla; **Art**: Artiodactyla; **Pho**: Pholidota; **Pri**: Primates; **Pro**: Proboscidea; **Rod**: Rodentia; **Cau**: Caudata; **Squ:** Squamata; **Tes**: Testudines; **Cro**: Crocodylia; **Sau**: Sauria; **Acc**: Accipitriformes; Falconiformes; **Gal**: Galliformes; **Psi**: Psittaciformes.

In this study, we developed a DNA mini-barcode assay ($\leq$304 bp) targeting twenty wildlife species (white fox, peacock, badger, monkey, deer, raccoon dog, turtle, snake, giant salamander, tiger, pangolin, crocodile, elephant, bamboo rat, rhinoceros, golden eagle, bear, monitor lizard, hippo, and parrot) that are often encountered in forensic investigations in southern China. We focused on short regions of four typical mitochondrial genes, namely, *Cyt b*, *COI*, *16S rRNA*, and *12S rRNA*. Under the guidelines of the Scientific Working Group on DNA Analysis Methods (SWGDAM),[50] a series of validations were conducted for species specificity, sample types, DNA mixtures, repeatability, stability, sensitivity, and degraded DNA and case samples.

RESULTS

Construction of the multilocus DNA mini-barcode PCR assay

Wildlife species selection and phylogenetic analysis

Twenty wildlife species frequently encountered in forensic investigations were chosen for this study. To analyze the evolutionary relationships among the target species, a phylogenetic tree was constructed (Figure 1; Table S1). These twenty wildlife species include twelve mammals (raccoon dog, white fox, tiger, pig badger, bear, rhinoceros, hippo, deer, pangolin, monkey, elephant, and bamboo rat), one amphibian (giant salamander), four reptiles (snake, turtle, crocodile, and monitor lizard), and three birds (golden eagle, peacock, and parrot).

Primer test and construction of the multiplex

Prior to the development of the multilocus DNA mini-barcode assay, preliminary uniplex PCR was conducted using DNA samples from twenty wildlife species to validate the specificity and amplification efficiency of each species-specific primer (Table 1). The expected size of the PCR

Table 1. List of GenBank accession numbers and the primer sets of twenty vertebrate wildlife species used in the multiplex

Common name	Scientific name	Accession number	Primer sequence (5′-3′)	Expected amplicon length (bp)	Target region
White fox	Vulpes lagopus	LT560062	F: TCCTAATTACAGCGGTTCTATTGC R: AAGGTTGCGATCCGTTAGAAGTAT	83	*COI*
Turtle	Astrochelys radiata	HQ329747	F: TCTGGCAGGTGTATCATCAATTCT R: ACGAATAAGGGTGTTTGGTACTGT	102	*COI*
Raccoon dog	Nyctereutes procyonoides	NC_013700	F: TGGTTGAGTGAATTTGAGGAGGTT R: TGTACTATTGCTAGTGCTGCGATA	109	*Cyt b*
Pangolin	Manis javanica	NC_026781	F: TCTGCCGAGATGTAAACTA R: CTCTTTGTAGGCAAAGGAT	128	*Cyt b*
Snake	Ptyas mucosa	KU529373	F: CTGGGCAGCGACCAAATCTT R: TGTCTGGTGCTCCAATTATTAGTG	133	*COI*
Parrot	Aratinga solstitialis	NC_026039	F: TGATGAAACTTCGGGTCCCTTCTA R: TTCGGATTAGCCAGCCATATTGT	154	*Cyt b*
Monitor lizard	Varanus bengalensis	MG670554	F: CGTCTTCCTTCACATCATACTCCT R: GCGAACGTAGTTAGGCATAGAAG	162	*Cyt b*
Bamboo rat	Rhizomys pruinosus	MH189045	F: TAGAATGGATTTGAGGTGGAT R: CGTCGGCGTTAGAGTTTAG	168	*Cyt b*
Bear	Ursus arctos	KF184267	F: GAGCGACTGTCATCACCAACCT R: AGGAACAATAGATGGACTGCTGCT	175	*Cyt b*
Tiger	Panthera tigris altaica	NC_010642	F: TGTGCTATTGTTTACGGTCAT R: GGAAGGCAAAGAATCGTGT	194	*Cyt b*
Peacock	Pavo cristatus	NC_024533	F: TTCTGAGGAGCAACTGTTATCACA R: TGAGCCTGATTCGTGGAGGAA	198	*Cyt b*
Hippo	Hippopotamus amphibius	AY011175	F: AAGCCTAACGAGCCTGGTGATAG R: AACGCTATCTTAATTGGTGGCTGC	212	*16S rRNA*
Deer	Cervus nippon	MZ047121	F: GCCTACTGATTCGTGCCG R: GAGGGAGGAGTCAAAAGC	219	*COI*
Rhinoceros	Ceratotherium simum	JF718874	F: ATCCTAGCACTACTCGCCCT R: GGAATATTATGCTTCGTTGTT	247	*Cyt b*
Elephant	Elephas maximus	FJ979617	F: GTCCACTTACCTCGCTATCAATAC R: AGAACATAACCTGTGGATCTACAC	258	*Cyt b*
Monkey	Macaca mulatta	JQ735464	F: CTGGCCGAGACGTTATT R: TCCTGGGTGGGAGAAGT	266	*COI*
Golden eagle	Aquila chrysaetos	MN356391	F: ACAATGAAACCCCAAGCAGC R: TGAGGGTTCGGCTACGGAT	285	*16S rRNA*
Pig badger	Arctonyx collaris	AB049810	F: TGGCTCTCTCCTCGGAATC R: CTATGAATGCGGTTGCTATTAC	290	*Cyt b*
Crocodile	Caiman yacare	NC_008795	F: ACTTCTCACCCAGCGATACC R: GATGGCGGATAGTAGGTTGGT	299	*Cyt b*
Giant salamander	Andrias davidianus	NC_004926	F: AGCCTTTTCTTCAGTT R: TTTCACCAGGTTGTCT	304	*Cyt b*
Universal primers	vertebrates		F: AAAGCTTCAAACTGGGATTA GATACC R: ACTGCAGAGGGTGACGGGC GGTGTGT	420–455	*12S rRNA*

fragments is depicted in Figure 2A. No overlap ensured unambiguous distinction from any other expected peak in the test. Additionally, an allelic ladder was constructed (Figure 2B) by balancing the ratios of plasmid amplicons (Table S2) to facilitate genotyping when compared with unknown samples.

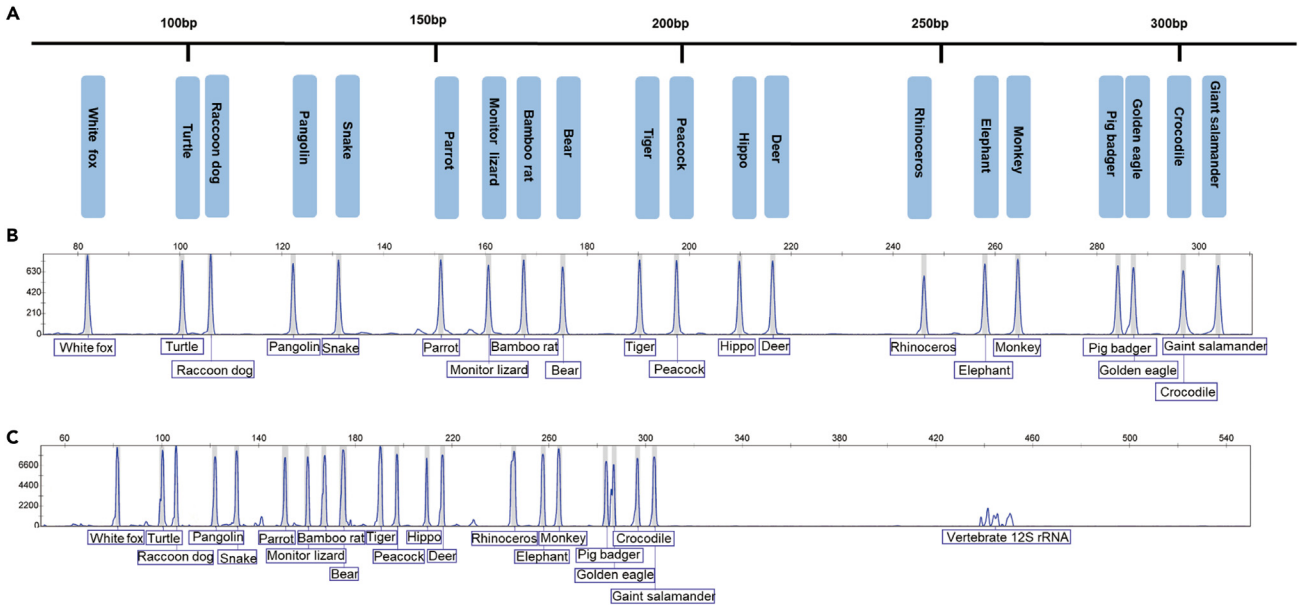

Figure 2. Multiplex identification of PCR amplicons of twenty wildlife species

(A) Fragment distribution diagram of twenty targeted PCR amplicons.

(B) An electropherogram of the allelic ladder.

(C) DNA typing of a DNA mixture of twenty wildlife species.

A stable and effective multiplex assay was subsequently developed (Figure 2C) by adjusting the proportion of the 20-plex primer combinations (Table S3) under the following conditions: an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 1 min, extension at 72°C for 50 s, and a final extension at 72°C for 10 min. The multiplex assay required DNA extraction, PCR amplification, and a standard capillary electrophoresis run to separate and detect the amplicons, which can be completed within 3 h. The results provided an easy-to-understand result presentation with direct yes or no. All of these were standard procedures that can be carried out in most genetics laboratories.

Developmental validation of the multiplex

Species specificity study

A single test of the 20 target species with the multiplex was conducted (Table S4). Each species, except snakes, simultaneously produced one specific and one universal PCR fragment, making the assay self-confirmatory (Figure S1). The lack of the *12S rRNA* peak did not hinder the accurate identification of the snake species using the newly developed assay.

Primer specificity evaluated using microbial DNA samples showed the absence of any amplification. In the case of domestic animals (Table S4) and human samples, a single PCR fragment of the universal primers was observed at approximately 420–455 bp outside the intended region, which is the discrimination of 20 wildlife species and nontarget animals (Figure S2). Furthermore, we examined the specificity of primers at the intergenus level using a limited collection of forty-six samples (Table S4) from closely related species (data not shown). The outcomes indicated a robust species specificity of the corresponding primers.

Sensitivity study

The multiplex assay demonstrated its ability to generate reliable DNA profiles from different amounts of DNA samples, thus determining the minimum detection limits. It successfully identified all species tested across a wide range of total DNA concentrations, starting from 1 ng down to 5 pg. For white fox, turtle, pangolin, snake, monitor lizard, bamboo rat, tiger, hippo, elephant, and pig badger, the detection limits were as low as 2 pg DNA input. Notably, greater sensitivity with 1 pg DNA of white fox, pangolin, and tiger was achieved (Figure 3).

Mixture study

Accurate DNA profiles from DNA mixtures are a typical challenge in forensics. The assay showed its capability to accurately identify all contributors within a 20-plex DNA mixture sample comprising twenty distinct species (Figure 2C). Figure 4 illustrated the successful identification ratios of all minor contributors from the three sets of binary mixtures between 1:500 and 100:1. Specifically, minor contributors, such as white fox and pangolin, were detected at a ratio of 2‰ (≥2 pg minor contributor). The DNA profiles revealed minor contributors of rhinoceros in the DNA mixtures at ratios of 1% (≥10 pg minor contributor).

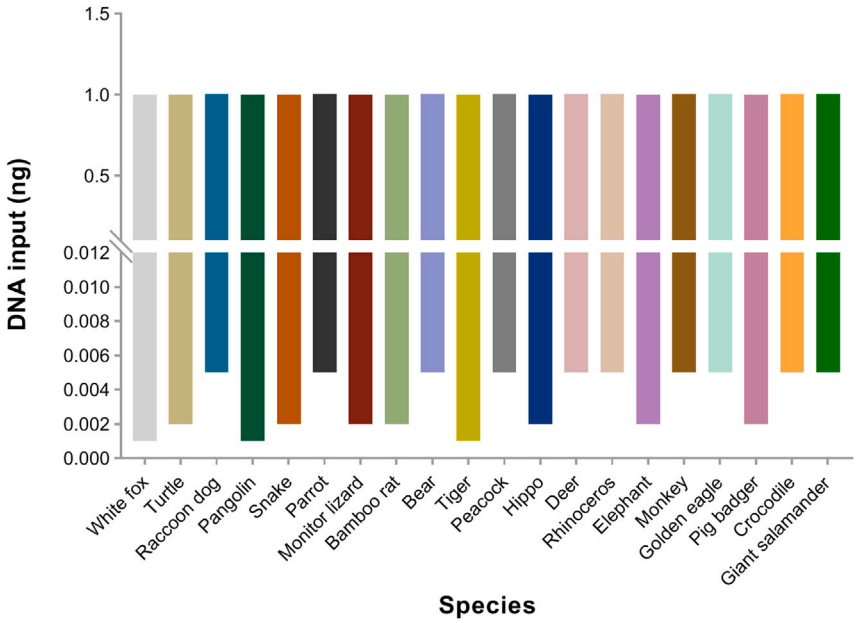

Figure 3. Sensitivity study, n = 4

Detection limits of twenty targeted PCR amplicons of twenty wildlife species with capillary electrophoresis.

Sample type testing

The present study successfully demonstrated that the multilocus DNA mini-barcode assay is capable of identifying a wide range of biological samples. These samples included tissue, blood, blood-spotted samples, feathers, hair bulbs and roots, fresh feces, saliva swabs, anal swabs, and processed samples (Table S4).

Repeatability study

Repeatability was assessed by conducting triplicate tests on one sample from each species, which consistently yielded the same typing results. To evaluate the sizing precision of fragment analysis, the standard deviations (SDs) of the peak sizes in the allelic ladder were calculated for each run. The observed SDs ranged from 0.02 bp to 0.15 bp (Figure 5A), all falling within the threshold of sizing precision (0.15 bp) specified in the performance check section of the 3130xL Genetic Analyzer User Guide.[51] The size measurements of the detection peaks were confined within a "window" of 1 bp around the allele ladder, enabling the assay to distinguish species with a size difference greater than 1 bp.[52] The reliability of PCR amplification was assessed by measuring the relative fluorescence units (RFUs) of each species (Figure 5B). The results demonstrated that the typing and repeatability were generally reliable and consistent.

Stability study

The multiplex assay exhibited enhanced tolerance to six commonly encountered forensic inhibitors, with notable resistance to hemoglobin, indigo, and calcium ions. Even at the highest concentration tested (6 mM) for hemoglobin and indigo, all twenty wildlife species

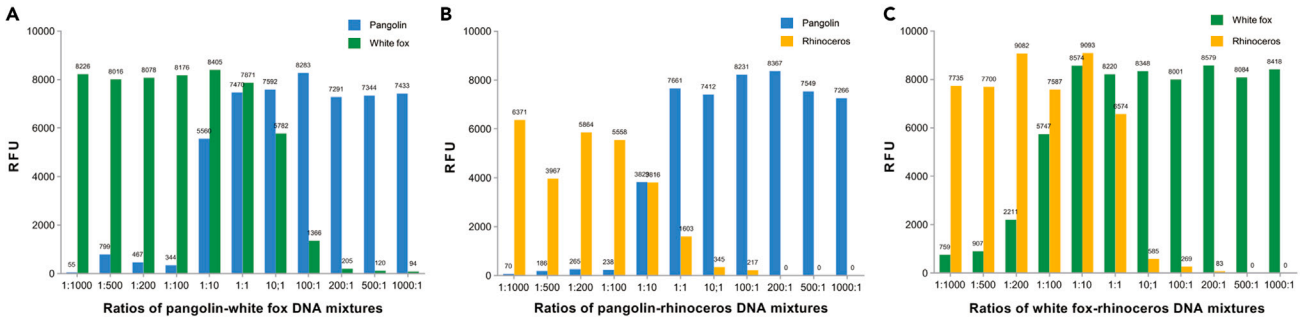

Figure 4. Mixture study, n = 4

(A–C) Relative fluorescence units (RFUs) of target peaks in the gradient ratios (1:1000 to 1000:1) from binary mixtures of pangolin-white fox, pangolin-rhinoceros, and white fox-rhinoceros.

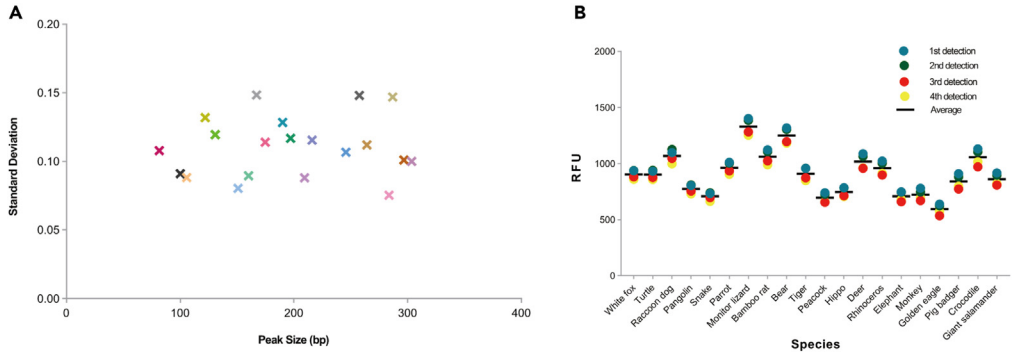

Figure 5. Repeatability study

The accuracy of capillary electrophoresis detection, n = 8 (A) and PCR amplification, n = 4 (B) The character "x" in A with different colors represents the corresponding amplification size of 20 wildlife.

could still be accurately identified. However, when the concentration of calcium ions reached 6 mM, only a few species were not detectable. Inhibition was observed for EDTA, humic acid, and ethanol at concentrations of 1.5 mM, 100 ng/μL, and 80%, respectively (Figure 6).

Degradation study

The impact of DNA degradation on detection was assessed by measuring the percentage of detected species when both the 20-plex DNA mixture and single DNA samples were treated with DNase I enzyme for different durations. The numbers of identified species from both decreased with increasing incubation time. Notably, the results indicated that the majority of DNA degradation occurred within the initial 20 min of enzyme incubation, after which the degradation rate slowed significantly. Despite simulating DNA degradation, the species detection rate remained above 60% throughout the experiment (Figure 7).

Case study

Fifteen additional case samples were reacted with the multiplex (Table S5). Samples 1, 5, 6, and 8–15 were identified as bamboo rat, parrot, peacock, turtle, deer, pig badger, tiger, pangolin, monkey, raccoon dog, and golden eagle by multiplex (Figure S3), which was further evidenced by the sequencing results of the *12S rRNA* gene. For fifteen unknown case samples, the resulting partial DNA sequences were compared with the *12S rRNA* genes of twenty wildlife species targeted in this study. A phylogenetic tree (Figure 8) showed that samples 1, 5, 6, and 8–15 were clustered with their corresponding species. For other species not included in the system, only a single peak was observed in the region of vertebrate *12S rRNA*, and the phylogenetic tree showed that these four unknown samples were clustered with turtles (*Astrochelys radiata*), golden eagles (*Aquila chrysaetos*), birds, and parrots (*Aratinga solstitialis*). Further sequence alignment led to the identification of sample 2 (*Chelonoidis carbonarius*), sample 3 (*Grus japonensis*), sample 4 (*Nycticorax nycticorax*), and sample 7 (*Otus bakkamoena*).

Additionally, eighteen mock case-type samples exposed to the laboratory environment (Table S6) were also successfully identified using the newly developed multilocus mini-barcode method to evaluate the amplification capability of short DNA fragments undergoing degradation.

DISCUSSION

Different species occupy different positions in the ecosystem. They restrict each other to jointly maintain the balance of ecosystems and biodiversity. The biological composition of local ecosystems is frequently quantified using species richness, which requires species identification techniques to help identify organisms. Through the results of species identification and classification, we can infer the biological interactions and evolutionary trends of animals and further explore the origin and formation of species, phylogenetic relationships, and biogeography. Therefore, species identification can help to explore the rules of the formation and evolution of regional ecosystems and put forward reasonable suggestions for environmental protection and transformation together with local ecology and zoogeography. However, the impact of climate change[53] and human-animal conflict[54] pose grave threats to wildlife, particularly those of at-risk species. The outbreak of zoonotic diseases[4,5,55] also revealed hidden threats that extend well beyond wildlife germplasm resources and ecological balance.

Keeping the previous concerns in mind, a cost-effective and rapid multilocus DNA mini-barcode method focusing on twenty vertebrate wildlife species was developed and validated, which offers several advantages in forensic applications. First, it allows for the simultaneous identification of multiple contributors within complex samples, which is valuable in screening and investigating forensic wildlife crimes. Additionally, multiplex PCR using species-specific primers overcomes the limitations of Sanger sequencing in mixture analysis and avoids the need for next-generation sequencing, making the process time-efficient and cost-effective. The assay only utilizes a single fluorescent label, 6-FAM,

Figure 6. Stability study, n = 4

(A–D) Relative fluorescence units (RFUs) of DNA profiles of twenty wildlife species with hemoglobin, indigo, calcium ions, and EDTA at distinct concentrations (0–6 mM).

(E) RFUs of DNA profiles of twenty wildlife species with humic acid at distinct concentrations (0–500 ng/μL).

(F) RFUs of DNA profiles of twenty wildlife species with ethanol at distinct concentrations (0%–100%). Concentrations of 0 mM, 0 ng/μL, and 0% inhibitor represent the positive controls of this study.

allowing for expanded capacity of adding loci by other fluorescent labels. The assay follows standard procedures of DNA extraction, PCR, and capillary electrophoresis, achieving rapid detection and enabling easy implementation in most genetics laboratories.

Four typical DNA barcodes, *Cyt b*, *COI*, *16S rRNA*, and *12S rRNA* genes with highly variable regions, were designed for species-specific primers to achieve the specific correlation between primers and the species. The results of species specificity tests and case sample tests in the present study succeeded in confirming the unambiguous species-specific identification of each primer in the assay. However, due to the difficulty and complexity of wildlife sampling, the number of wildlife samples collected in this study is limited. Therefore, more closely related species need to be collected to further test the specificity of primers in future studies. Universal primers have been widely used to identify the species of unknown samples. In this study, a pair of universal primers targeting the vertebrate *12S rRNA* region was used for self-confirmation of wildlife species when it was detected simultaneously with the specific fragments. Although universal primers may lack specificity for species such as snakes in our system, this did not hinder their accurate identification using specific primers. The identification of unexpected species from unknown samples is possible through the comparison of diagnostic DNA fragments amplified by vertebrate *12S rRNA* universal primers against reference DNA sequence databases. Moreover, species-specific testing with nontarget species showed that six domestic animals and humans only presented universal fragments, and microbial DNA showed the absence of any amplification. This indicates that the amplification of universal primers can be used as a basis for distinguishing wildlife from others, which is necessary during forensic investigation.

Forensic science is employed to determine if endangered species are present in seized samples but runs into problems due to three typical challenges: low levels of DNA, complex mixtures and degraded samples. The multiplex achieved high sensitivity, with a detection limit as low as 5 pg of total DNA, which makes it a very valuable tool for low levels of DNA according to the high copy number per cell of

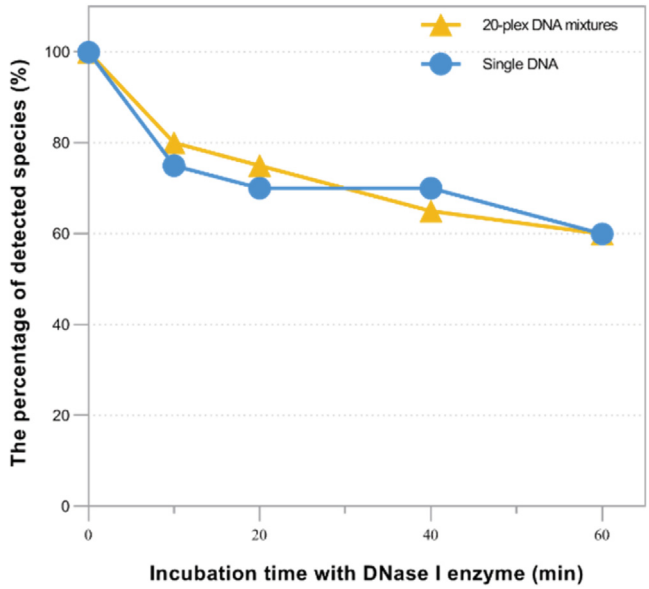

Figure 7. Degradation study, n = 4

The detection effect on degraded 20-plex DNA mixtures and single DNA incubated with DNase I enzyme for 10, 20, 40, and 60 min 0 min represents the positive control of this study.

mtDNA.[17,18] The assay's ability to identify species from 20-plex DNA mixtures and to discriminate a minor contributor (≥1% minor contributor) from binary mixtures was present in DNA mixture analysis. Minor contributors to the DNA mixture are susceptible to information loss due to the reduced amplification of random effects and shedding.[56,57] In addition, a phenomenon known as stochastic fluctuation could also occur when amplifying very low levels of DNA template in multiplex DNA mixtures.[58] A mixture study showed that the accurate discrimination of minor contributors in unbalanced DNA mixtures was as low as 1%. Moreover, the newly developed assay enables the identification of all species in a 20-plex sample. Although this is an unlikely combination to encounter in forensic science, it illustrates the potential for the test. Forensic samples are usually low levels of DNA and heavily degraded DNA, resulting in failed amplification of nuclear DNA[56] and long markers.[27,59] mtDNA analysis is an excellent alternative to nuclear genomic DNA identification due to the higher copy numbers per cell.[7,60,61] The main advantage of the newly developed multiplex assay is that these DNA mini-barcodes are designed for targeting short fragments and have been proven to be effective in identifying case samples and artificial case samples, making the assay applicable in forensic casework.

The encouraging findings in analyzing noninvasive samples (i.e., feathers, hairs, and fresh feces) make it a suitable alternative to insufficient nuclear DNA and a feasible nondestructive identification method for wildlife species given the difficulty of obtaining muscle tissue or blood during investigation and research from the complex and diverse living environments and habitats of wildlife. The ability of mini-barcodes in the assay to identify processed samples would be advantageous for criminal case filings involving processed wildlife products (meat, leather, powder, or some processed derivative) that cannot be identified by external morphology. Additionally, crime scene samples are susceptible to contamination by various chemical compositions and environmental factors, resulting in inefficient PCR amplification and failure to obtain a complete DNA profile. The high tolerance of the assay to six common inhibitors, hemoglobin, indigo, EDTA, calcium ions, humic acid, and ethanol, demonstrated a sensitive, robust, practical and easily interpretable tool of the assay as well as a repeatability study.

Limitations of the study

Our study could be limited by the quantification of DNA templates using the total DNA amount (ng). Although this is also a common method to measure the initial amount of DNA input, it may not provide a quantification of mitochondrial DNA content (copies). Although mtDNA barcoding technology has shown promising prospects in animal species identification, which has also been systematically demonstrated in this study, there are still shortcomings in the identification of unnatural hybrid species due to the maternal inheritance of mitochondria. Due to the difficulty and complexity of wildlife sampling, the number of wildlife samples collected in this study is limited. More wild animals and closely related species need to be collected to further improve its forensic application in future case investigations.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE

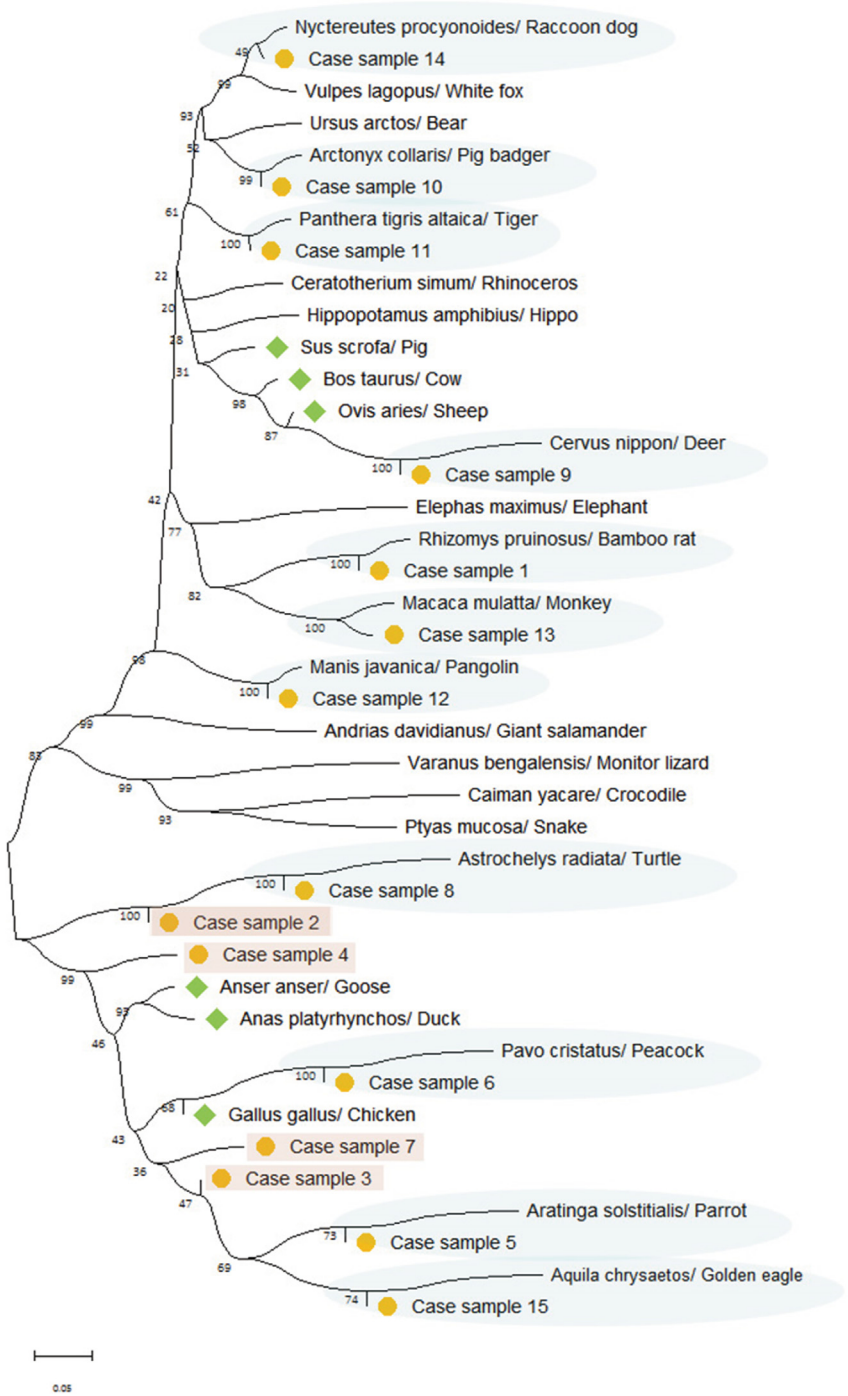

Figure 8. A phylogenetic tree based on the partial sequence of the *12S rRNA* gene was constructed by the neighbor-joining method

Confidence values for internal lineages were assessed with the bootstrapping option. Scale bars represent branch length. The green diamond annotations represent the domestic animals selected in this study; the yellow circle with light blue background annotations represents case samples compared to the target wildlife species, while the yellow circle with pink background annotations represents case samples outside the system.

- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.108275.

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AUTHOR CONTRIBUTIONS

Conceptualization, W.C., S.Q., L.C., and C.L.; methodology, X.L., W.D., C.W., Q.Y., S.Q., L.C., and C.L.; validation, X.L., W.D., and Y.Z.; formal analysis, X.L.; investigation, X.L., C.W., Y.W., and S.Q.; resources, C.W., Y.W., W.C., and H.L.; data curation, X.L.; writing - original draft, X.L., and W.D.; writing - review & editing, M.W., L.C., and C.L.; supervision, S.Q., L.C., and C.L.; project administration, C.L.; funding acquisition, H.L.

DECLARATION OF INTERESTS

The authors have a patent related to this work. All other authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Voucher samples for all species	See Table S4	See Table S4
Human DNA (9948 and 9947A)	AGCU ScienTech Incorporation, Wuxi, Jiangsu, China	Cat#500301-1; Cat#500003-4
Chemicals, peptides, and recombinant proteins		
Chelex-100 Resin	Bio-Rad Laboratories, Hercules, California, USA	Cat#142-2832
hot-start C-Taq enzyme	AGCU ScienTech Incorporation, Wuxi, Jiangsu, China	Cat#500201-2
Reaction Mix	AGCU ScienTech Incorporation, Wuxi, Jiangsu, China	Cat#500401-1
POP-4 Polymer	Thermo Fisher Scientific, South San Francisco, CA, United States	Cat# 4363752
Hi-Di formamide	Thermo Fisher Scientific, South San Francisco, CA, United States	Cat#4311320
AGCU Maker SIZ-500	AGCU ScienTech Incorporation, Wuxi, Jiangsu, China	Cat#500701-2
Critical commercial assays		
TIANamp Genomic DNA Kit	TIANGEN BIOTECH, Beijing, China	Cat#DP304
TIANamp Micro DNA Kit	TIANGEN BIOTECH, Beijing, China	Cat#DP316
TIANamp Stool DNA Kit	TIANGEN BIOTECH, Beijing, China	Cat#DP328,
Deposited data		
Sequences information	This paper	https://data.mendeley.com/drafts/f52cpwsz5w
Experimental models: Organisms/strains		
pMD18T	TaKaRa Biotechnology (Dalian) Co., Ltd. Dalian, Liaoning, China	Cat#6011
DH5α	Sangon Biotech (Shanghai) Co., Ltd. Shanghai, China	Cat#B528413
Oligonucleotides		
primers	This paper	See Table 1
Software and algorithms		
GeneMapper® ID v1.5.3	Thermo Fisher Scientific, South San Francisco, CA, United States	https://www.thermofisher.cn/document-connect/document-connect.html?url=https://assets.thermofisher.cn/TFS-Assets%2FLSG%2Fmanuals%2F100031704_GeneMapIDX_ver1_5_MS_GSG.pdf
MEGA11	Tamura K et al.[62]	https://www.megasoftware.net/
Oligo 7	Rychlik, W.[63]	http://www.oligo.net
Primer Premier 6	Premierbiosoft, San Francisco, CA, USA	http://www.premierbiosoft.com/primerdesign/

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Multiple Primer Analyzer	Thermo Fisher Scientific, South San Francisco, CA, United States	https://www.thermofisher.cn/cn/zh/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/multiple-primer-analyzer.html
GraphPad Prism 8	GraphPad Software Inc., San Diego, CA, USA	https://www.graphpad.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Dr. Liu (liuchaogzf@163.com).

Materials availability

The study did not generate new materials.

Data and code availability

- Sequence data generated by Sanger sequencing have been deposited at Mendeley Data (https://data.mendeley.com/drafts/f52cpwsz5w) and are publicly available as of the date of publication. The DOI is listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Ethics

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by Guangzhou Zoo & Guangzhou Wildlife Research Center, China (Project number GZZOO20210903A and Approval date: September 3, 2021).

Voucher samples and control DNA

Voucher samples for all species (Table S4) were sourced from the Guangzhou Zoo & Guangzhou Wildlife Research Center. Protocols for collection and research were approved by the Research Center. Additionally, human DNA (9948 and 9947A, AGCU ScienTech Incorporation, Wuxi, Jiangsu, China) and microbial DNA (microbial pool from *Escherichia coli, Lactobacillus acidophilus, Salmonella, Pseudomonas aeruginosa, Staphylococcus epidermidis, Streptococcus salivarius, Corynebacterium pyogenes, Saccharomyces cervisiae, Candida albicans, and Aspergillus flavus*) were provided by our laboratory.

The age and sex of the voucher samples used in this study were showed in Table S4. The age and sex information of fifteen case samples during collection was unclear or lacking, resulting in our inability to provide corresponding information. However, it did not influence the identification of these species using mtDNA.

METHOD DETAILS

Primer design

Mitochondrial DNA (mtDNA) sequences were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) (Table S1) and aligned using MEGA11 software.[62] For species-specific primers, the complete genome of the mitochondria for 20 target wildlife species and other species of the same genus were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), and multiple alignment among the same genus was performed by means of ClustalW using MEGA11 software. Initially, the DNA sequences for the *Cytb*, *COI*, *16S rRNA* and *12S rRNA* genes were extracted, and multiple oligos were selected as candidates according to each target DNA sequence by using Primer Premier 6 and considering the physical and structural properties of oligos (such as annealing temperature, G + C percentage, and self-complementarity) using Oligo 7 software.[63] In the next step, sequence similarity within and among species was further assessed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), and interactions such as the melting temperatures and complementarity between any two different primers of these candidates were carefully examined using Multiple Primer Analyzer (https://www.thermofisher.cn/cn/zh/home/brands/thermoscientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermoscientific-web-tools/multiple-primer-analyzer.html). Ultimately, a set of suitable primer pairs is obtained by selecting those with the least interaction between twenty different primer pairs. For vertebrate universal primers, sequences within *12S rRNA* genes located at the mtDNA were chosen as targets, showing the most informative sequences when different Class animals were taken into account. The universal primers used in this study

are modified versions of those given by Kocher et al.,[64] targeting an approximately 450 bp site of vertebrate *12S rRNA*. The highest similarity in priming sites, especially at the 3′ end, was confirmed by alignment of these genes among twenty wildlife species. All primers were ordered from Sangon Biotech (Sangon Biotech Co., Ltd. Shanghai, China) with forward primers labeled with 6-FAM dye. The primers were lyophilized and resuspended in sterile water to a concentration of 100 mM.

DNA extraction

Total DNA from tissue and blood samples was extracted using the TIANamp Genomic DNA Kit (Cat: DP304, TIANGEN BIOTECH, Beijing, China). The base of the calamus of freshly sampled feathers and hair bulbs and roots were treated using a TIANamp Micro DNA Kit (Cat: DP316, TIANGEN BIOTECH, Beijing, China). Following a standard preparation procedure[65] and the manufacturer's protocol of the TIANamp Stool DNA Kit (Cat: DP328, TIANGEN BIOTECH, Beijing, China), fecal samples were concentrated on the outer material to yield the least degraded DNA and the lowest concentration of PCR inhibitors.[66,67] Swab samples and blood-spotted FTA® cards were subjected to total DNA extraction using Chelex-100 Resin (Cat: 142-2832, Bio-Rad Laboratories, Hercules, California, USA).[68] All DNA solutions were quantified by measuring the DNA amount (ng) using a Nanodrop One Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). Subsequently, the concentration of each DNA sample was adjusted to 1 ng/μL with nuclease-free water before being stored in a refrigerator at 4°C until amplification.

PCR amplification

Unless otherwise specified, the standard PCR amplification and genotyping procedures followed the protocol outlined below. Multiplex PCR was conducted in a 10 μL reaction volume comprising 4.0 μL of Reaction Mix (Cat: 500401-1, AGCU ScienTech Incorporation, Wuxi, Jiangsu, China), 0.4 μL of hot-start C-Taq enzyme (Cat: 500201-2, AGCU ScienTech Incorporation, Wuxi, Jiangsu, China), 2.0 μL of primer mix (refer to primer concentrations in Table S3), 1 μL of DNA template (diluted to an initial concentration of 1 ng/μL), and nuclease-free water to a final volume of 10 μL. PCR amplification was carried out using an Eppendorf AG 22331 Hamburg PCR Amplifier (Eppendorf, Hamburg, Germany) with the following cycling conditions: initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 1 min, extension at 72°C for 50 s, and a final extension at 72°C for 10 min.

Allelic ladder

For the creation of an allelic ladder to be used in electrophoretic analysis, twenty amplicons of wildlife species were cloned and inserted into the pMD18T vector (Cat:6011, TaKaRa Biotechnology (Dalian) Co., Ltd. Dalian, Liaoning, China) and transformed into DH5α competent cells (Cat:B528413, Sangon Biotech (Shanghai) Co., Ltd. Shanghai, China). *E. coli* colonies successfully harboring the recombinant plasmid were sent to Sangon Biotech (Sangon Biotech (Shanghai) Co., Ltd. Shanghai, China) for sequencing to confirm the species identity. Subsequently, amplicons were generated using twenty purified plasmid DNAs as templates and mixed together (refer to the ratios of plasmid amplicons in Table S2). The resulting allelic ladder was stored at -20°C until use.

Electrophoresis and data analysis

Electrophoresis was carried out using the ABI 3130xL Genetic Analyzer (Thermo Fisher Scientific, South San Francisco, CA, United States) with 36-cm capillary arrays and POP-4 Polymer (Cat: 4363752, Thermo Fisher Scientific, South San Francisco, CA, United States). Prior to sample analysis, spectral calibration was performed using the 5-Dye Matrix standards. The PCR product (1 μL) was mixed with 9.5 μL of Hi-Di formamide (Cat: 4311320, Thermo Fisher Scientific, South San Francisco, CA, United States) and 0.5 μL of AGCU Maker SIZ-500 (Cat: 500701-2, AGCU ScienTech Incorporation, Wuxi, Jiangsu, China). The mixture was denatured at 95°C for 3 minutes and then placed in an ice bath for 3 minutes. The samples and allelic ladder were injected at 2 kV for 6 seconds and subjected to electrophoresis at 15 kV for 1500 seconds at 60°C.

Data from the 3130XL Genetic Analyzer were visualized as electropherograms using GeneMapper® ID v1.5.3. (Thermo Fisher Scientific, South San Francisco, CA, United States)[69] with customized panel and bin sets. One hundred relative fluorescence units (RFUs) based on signal-to-noise analyses of internally derived empirical data were established as an analytical threshold.[29,70,71]

Species specificity study

Biological samples from wildlife crime scenes are often contaminated with domestic animals and human and environmental DNA. Therefore, removing the interference of nontargeted DNA from mixed samples is the key to correct typing. The species specificity and intergenus species discrimination of the assay were assessed by analyzing eight nontarget species (chickens, ducks, geese, pigs, cows, sheep, humans, microbial pool) and forty-six closely related species (Table S4). One nanogram of DNA from each sample was input four times into the PCR, with nuclease-free water serving as a negative control.

Sensitivity study

To determine the sensitivity of the assay, serial dilutions of the DNA templates ranging from 1 ng to 1 pg (1 ng, 100 pg, 10 pg, 5 pg, 2 pg, and 1 pg) were amplified using the PCR amplification procedure described earlier. Each test was repeated four times.

Mixture study

Mixtures containing 20-plex DNA were used to evaluate the detection of each species from a complex sample.[72] Furthermore, binary mixtures with three species (pangolin, white fox, and rhinoceros) were designed at different concentration gradients ranging from 1000:1 to 1:1000 (1000:1, 500:1, 200:1, 100:1, 1:1, 1:10, 1:100, 1:200, 1:500, and 1:1000), allowing for the assessment of the ability to detect the minor contributor from an unbalanced DNA mixture.[73]

Repeatability study

The repeatability of the PCR amplification and capillary electrophoresis was evaluated to determine the consistency of the DNA typing results. For capillary electrophoresis sizing precision, the allelic ladder was injected onto the capillaries of the 3130xL Genetic Analyzer, and eight calculations were performed to assess the standard deviations (SDs) of peak size in each run. The stability and reliability of the PCR amplifier were also assessed by testing the RFUs of each sample, which was repeated four times.

Sample type testing

Different types of biological samples were tested to account for the complexity and specificity of wildlife investigation. In addition to tissues from injuries, peripheral venous blood and blood spots obtained during veterinary examinations, nondestructive samples such as feathers, hair, fresh feces, oral swabs, and anal swabs were primarily examined (Table S4). Additionally, processed samples from six domestic animals were also tested.

Stability study

The stability of the assay was determined by six common forensic inhibitors (hemoglobin, indigo, EDTA, calcium ions, humic acid, and ethanol). They were tested four times at different concentrations or ratios in combination with 1 ng standard DNA mixtures of twenty wildlife species. The four PCR inhibitors, hemoglobin, indigo, EDTA, and calcium ions, were diluted at six different ratios, 0, 0.5, 1, 1.5, 3, and 6 mM. Six ratios, 0, 25, 50, 100, 200, and 500 ng/μL, were set for humic acid. For ethanol, six different concentrations (0%, 10%, 20%, 40%, 80%, and 100%) were prepared. The positive controls represented the absence of inhibitors (0 mM, 0 ng/μL, and 0% concentration).

Degradation study

Deoxyribonuclease I (DNase I) is a nonspecific endonuclease with highly variable DNA cutting rates. The degradation study was designed by treating the 20-plex DNA mixture and single DNA samples with 1 U DNase I (Thermo Scientific, USA) for different durations (0, 10, 20, 40, and 60 minutes). The resulting PCR amplicons were analyzed by capillary electrophoresis to assess the detection capability of the assay for degraded DNA. The 0-minute time point represented the positive control.

Case study

To demonstrate the applicability of the multiplex assay in forensic casework, fifteen unknown case samples provided by Guangzhou Zoo & Guangzhou Wildlife Research Center were tested (Table S5). Additionally, a total of eighteen samples were exposed to the laboratory environment for six months to mock case-type samples (Table S6).

Phylogenetic analysis

A phylogenetic tree was constructed by the neighbor-joining method using MEGA 11 software.[62] Confidence values for internal lineages were assessed with the bootstrapping option with 1000 replicate runs labeled on the branches of the tree.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

The Mean and standard deviation (SD) were calculated by Excel. The exact numbers of replicates (n) are given in each figure legend. Statistical analyses were performed using Excel and GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA).