TECHNICAL NOTE: DEVELOPMENT OF DNA QUANTITATION AND STR TYPING SYSTEMS FOR *PANTHERA TIGRIS* SPECIES DETERMINATION AND INDIVIDUAL IDENTIFICATION IN FORENSIC CASEWORK

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ABSTRACT

The aim of this technical note is to provide an overview of methodical approaches used to develop molecular systems for species determination/DNA quantification called *Ptig Qplex* and individual identification called *Ptig STRplex* of *Panthera tigris* samples. Both systems will help to combat the illegal trade of endangered species and create a worldwide shared database of DNA profiles.

Keywords: CITES organism; DNA quantitation; forensic genetics; STR multiplex

Introduction

Human DNA identification for forensic purposes is a valuable tool for identifying suspects and proving guilt in criminal cases. Forensic genetics evolved significantly since the foundation of this scientific field in the mid-1990s (Jeffreys et al. 1985). The evolution is nicely documented on the polymorphic loci used at different times. The initial restriction fragment length polymorphism (RFLP) methodology (Bush 1984) was soon replaced by PCR-driven methods that used variable number of tandem repeats (VNTRs) (Budowle et al. 1990), short tandem repeats (STRs) (Fregeau and Fourney 1993), chromosomal single nucleotide polymorphisms (White et al. 1998), mtDNA typing (Butler and Levin 1998), or insertions-deletions (InDels) (Zhao et al. 2010). The majority of records in forensic DNA databases (Puri 2000; Khan 2021) consist of STR profiles; thus, we postulate that STRs are currently core polymorphisms used for DNA-based forensic identification. However, the set of STR loci has changed over the years toward a higher number of more informative loci (Hares 2015). Nonhuman DNA typing for forensic purposes was initially focused on species determination (Bataille et al. 1999) and identification of individual domestic animals, such as cats (Menotti-Raymond et al. 1997) and dogs (Hellmann et al. 2006), because animal hair serves as evidence in criminal cases (Pfeiffer et al. 2004). STR profiling of animal species has a wider range of applications, such as wildlife preservation (Sastre et al. 2009), veterinary public health protection (Workman et al. 2005), and food safety (Vallejo-Cordoba and González-Córdova 2010). The application of forensic genetics is specifically useful to track poaching and illegal wildlife trades. Methods of species identification are used to decipher the genetic composition of traditional Chinese medicine products (Votrubova et al. 2017) or animal parts (Panday et al. 2014), and individual STR-based identification is used to track ivory (Potoczniak et al. 2020), pangolin shells (Singh et al. 2020), and rhino horns (Harper 2021). The aim of this technical note is to provide an overview of methodical approaches used to develop molecular systems for species determination/DNA quantification (*Ptig Qplex*) and individual identification (*Ptig STRplex*) of *Panthera tigris* samples. Similar systems have not been developed for DNA quantification and multiplex STR analysis of *Panthera tigris* samples.

Material and Methods

Ptig Qplex DNA quantitation system

Molecular system for species determination/DNA quantification *Ptig Qplex* uses quantitative RT-PCR with TaqMan probes and primers highly specific for *Panthera tigris*. The assay requires only a single tube/well reaction.

Real-time monitoring of the reaction, including the detection of possible inhibition, was performed using internal amplification of internal positive control (IPC) DNA. IPC DNA, an artificially prepared sequence of 366 oligonucleotides, eliminates false negative results. Nuclear DNA concentrations were measured using the STR locus Pati01 (Wu et al. 2009). Newly designed primers are not *Panthera tigris* specific, but they also amplify the DNA of some other large cats, such as *Panthera leo* (see Fig. 1). The concentration of mitochondrial DNA was measured using primers and probes targeted to the D-loop of mtDNA *Panthera tigris*. The target sequence is highly specific for this species only. We detected no amplification products of related species. The composition of primers and probes in the *Ptig Qplex* amplification mix is shown in Table 1.

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Table 1 Primers and probes used in Ptig Qplex.

Primer/ probe name	Final concentration (µM)	PCR product size (*bp)	Specificity	TaqMan probe fluorescent label
qPtigM_f	0.75	121	*D-loop* (mtDNA)	–
qPtigM_r	0.75			–
qPtigM_p	0.25		probe	VIC
qPtigN_f	0.5	~215–260	STR locus *Pati01*** (nDNA)	–
qPtigN_r	0.5			–
qPtigN_p	0.25		probe	6-FAM
qPtigC_f	0.5	366	*IPC* (Internal Positive Control)	–
qPtigC_r	0.5			–
qPtigC_p	0.25		probe	NED

* bp = base pairs; ** Wu et al. 2009.

qPCR reaction set-up

The qPCR thermocycler QuantStudio 5 (Applied Biosystems) was employed in this study.

The following qPCR conditions were employed: initial denaturation at 95 °C followed by 50 cycles of 95 °C for 10 secs and 60 °C for 20 secs.

The qPCR reaction included the following: 2× *TaqMan* Multiplex Master Mix (Applied Biosystems); 20× qPtig Assay Mix for mtDNA, nuclear DNA and IPC DNA (Forensic DNA service, CZE). The final reaction volume was 10 µl and was prepared as described in Table 2.

Table 2 The qPCR reaction setup.

qPCR reaction composition	10 µl reaction	Final concentration in qPCR
2× TaqMan Multiplex Master Mix	5 µl	1×
20× qPtig mtDNA Assay Mix (20× qPtigM)	0.5 µl	1×
20× qPtig nDNA Assay Mix (20× qPtigN)	0.5 µl	1×
20× qPtig IPC DNA Assay Mix (20× qPtigC)	0.5 µl	1×
IPC DNA (0.1 pg/µl)	1 µl	0.1 pg
Template DNA	1 µl	different
H^2O	1.5 µl	

Ptig STRplex DNA profiling system

The molecular system *Ptig STRplex* enables the determination of the DNA profile from the sample of *Panthera tigris* based on the analysis of short tandem repeats (STRs). STRs used in the presented multiplexes contain tri- or tetranucleotide repetitions or complex repetitions (see Tables 3 and 4). The number of repeats within loci is highly variable. Fluorescently labeled amplified STRs were subsequently analyzed using capillary electrophoresis.

Ptig STRplex contains 11 pairs of fluorescently labeled primers divided into 2 multiplexes: *PtigPlex1* and *PtigPlex2*. The primers used in multiplexes have similar melting points and do not create primer dimers. Gender determination was performed using primers targeting the SRY gene (Zou et al. 2015). The ZFX/ZFY locus serves as a supplementary system for gender determination, where both X and Y chromosome sequences are amplified (inspired by (Pilgrim et al. 2005).

Table 3 STR multiplex *PtigPlex1 Panthera tigris.*

STR repeat name	Repeat structure	Size (bp*)	Primer concentration (µM)	5´ primer fluorescent label
Ptig3	(TATC)n	118–190	0.16	FAM
Ptig5	(TAGA)n	152–220	0.15	ATTO565
Ptig6	(TGGAA)n	129–180	0.082	ATTO550
Ptig15	(TGAGA)n (CGAGA)n (CAAGA)n	187–250	0.36	YAKYE
Ptig17	(TC)n (GATA)n (TAGAGA)n	266–410	1.2	FAM
Ptig18	(TGTC)n	110–140	0.073	ATTO565
Gender				
ZnfXY*	–	164, 167	0.4	YAKYE

* ZnfXY – males (YX) 164 bp and 167 bp; females (XX) 167 bp (Pilgrim et al. 2005).
* bp = base pairs

Table 4 STR multiplex *PtigPlex2 Panthera tigris*.

STR repeat name	Repeat structure	Size (bp*)	Primer concentration (µM)	5´ primer fluorescent label
Ptig8	(ATCTAT)n (ATC)n	109–220	0.12	ATTO550
Ptig9	(CTAT)n	131–200	1.3	YAKYE
Ptig10	(AC)n (GTAT)n	162–240	0.7	FAM
Ptig11	(TATC)n (ATC)n	119–200	0.15	ATTO565
Ptig16	(ATAA)n (ATAC)n	90–150	0.45	FAM
Gender				
SRY*	–	100	0.07	ATTO565

* SRY – male, 100 bp; female, no product (Zou et al. 2015).
* bp = base pairs

PCR set-up

PCR mix: Gold Star 10× buffer (Promega), *AmpliTaq Gold* DNA Polymerase (Applied Biosystems), and 10× *PtigPlex1* Primer Mix or 10× *PtigPlex2* Primer Mix (Forensic DNA service, CZE). The total volume of the PCR reaction is 12.5 µl. Details are provided in Table 5.

Table 5 PCR set-up.

PCR	12.5 µl reaction	Final concentration in PCR
Gold Star 10× buffer	1.25 µl	1×
10× PtigPlex1 Primer Mix or 10× PtigPlex2 Primer Mix	1.25 µl	1×
Template DNA	different	10 pg nuclear DNA
AmpliTaq Gold DNA polymerase	0.25 µl	2.5 U/PCR
H^2O	to 12.5 µl	

PCR thermocycler MasterCycler Nexus gradient (Eppendorf) was employed in this study.

The following PCR conditions were employed: initial denaturation at 95 °C for 11 mins followed by 32 cycles of 94 °C for 30 secs, 60 °C for 1 min 10 secs, and 72 °C for 1 min and 10 secs; final extension at 72 °C for 60 min, followed by 60 °C at 60 min.

Fragment analysis set-up

STR multiplex *PtigPlex1*: 0.5 µl PCR product + 12 µl deionized formamide (Hi-Di formamide) + 0.3 µl size standard (Size Standard LIZ600, Applied Biosystems)

STR multiplex *PtigPlex2*: 0.8 µl PCR product + 12 µl deionized formamide (Hi-Di formamide) + 0.3 µl size standard (Size Standard LIZ600, Applied Biosystems).

Capillary electrophoresis SeqStudio 3200 Genetic Analyzer (Applied Biosystems) was employed in this study. Raw data processing was done by GeneMapper5 software (Applied Biosystems).

Results

Examples of QuantStudio 5 (Applied Biosystems) quantitation and species determination outputs are shown in Fig. 1.

Examples of the resulting DNA profiles with PtigPlexes are shown in Fig. 2.

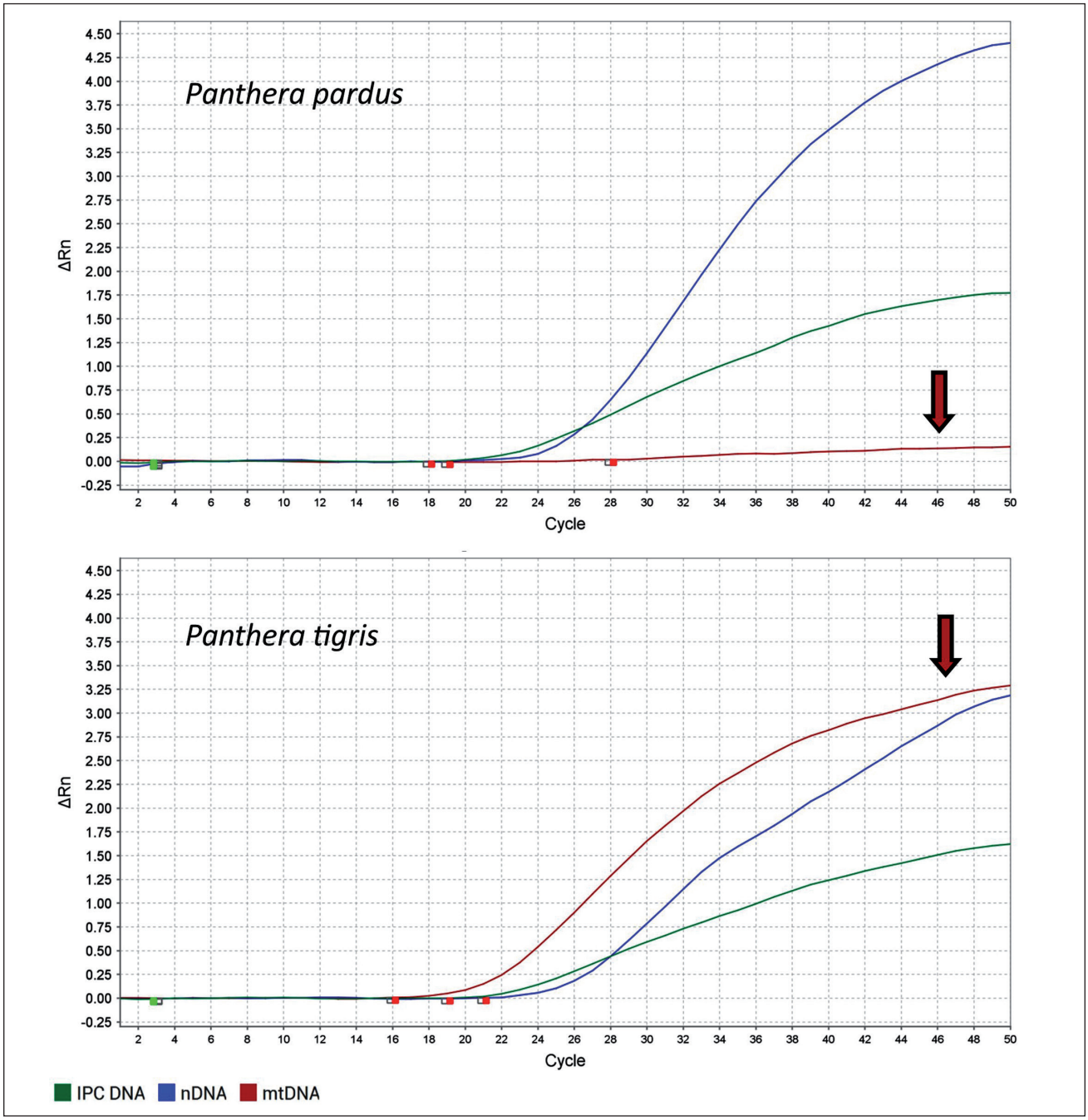

Fig. 1 The resulting qPCR plots generated using ***Ptig Qplex*** – comparison of nuclear DNA quantitation (blue curve) and mitochondrial DNA (red curve) in different cats. IPC DNA (green curve) serves as an internal positive control for qPCR. An arrow points to *Panthera tigris*-specific mtDNA amplification.

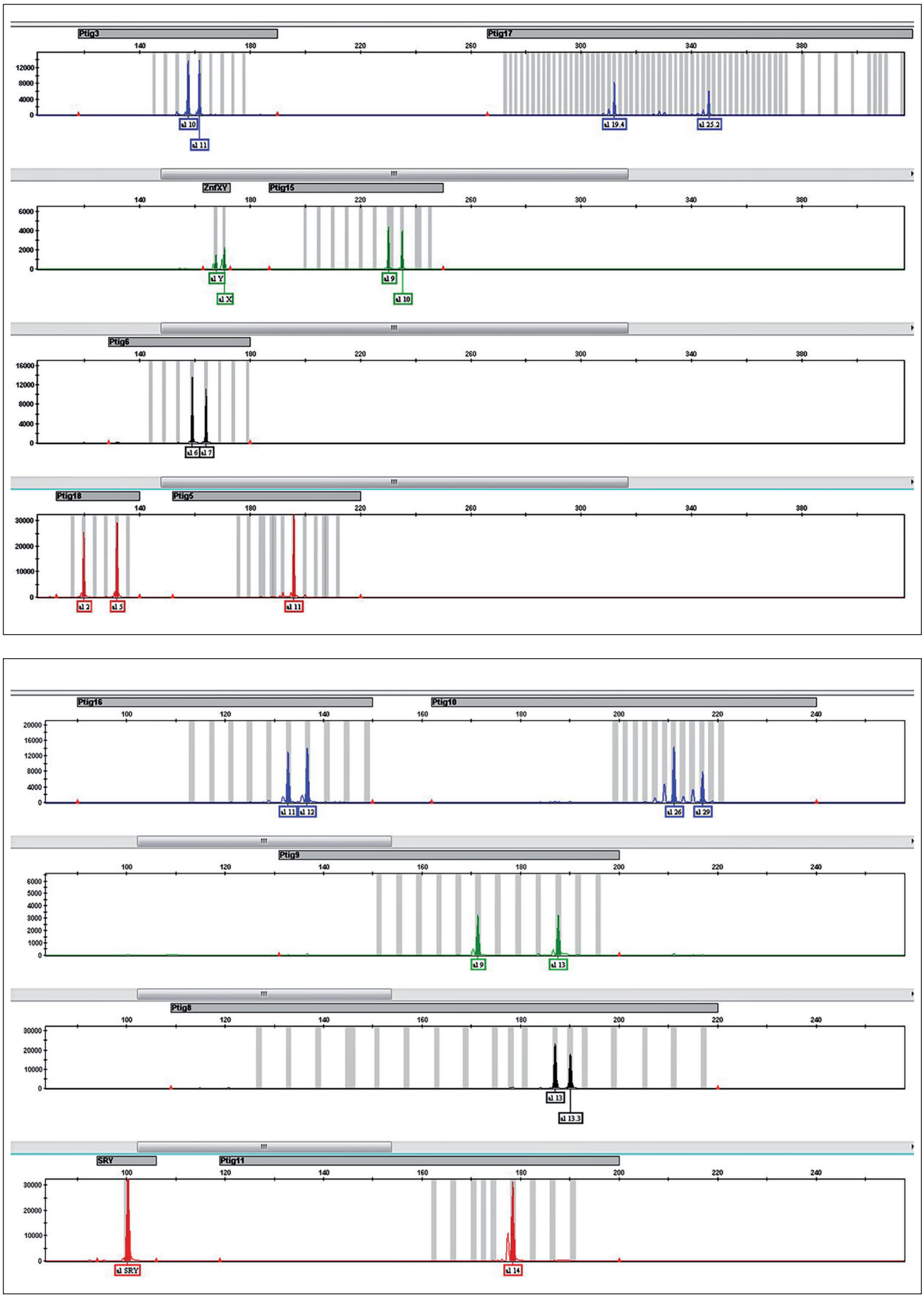

Fig. 2 Resulting electropherograms *PtigPlex1* (a) and *PtigPlex2* (b) with a DNA profile consisting of 11 STRs and sex (male) determination from samples obtained from *Panthera tigris*.

Conclusions

In July 2018, Czech Republic authorities raided premises in Prague and other locations, revealing a tiger slaughterhouse at the center of an international criminal trade ring. The raids, under the name Operation Trophy, were the culmination of two-and-a-half years of work and employed more than 200 enforcement officers from customs, police and the Czech Environmental Inspectorate. In the illegal slaughterhouse, they found a freshly killed tiger that was shot through the scull to leave its skin undamaged; a boiler for preparation of tiger glue; numerous tiger claws, bones and skins; and dozens of dead animals that were often in a state of decay. DNA analyses focused on species identification and individual identification of *Panthera tigris* samples. Genetic analysis performed using the above-described *Ptig Qplex* and *Ptig STRplex* identification systems enabled the identification of biological material from 7 different individuals of *Pathera tigris* and other CITES protected species. The illegal tiger trade seems to be more extensive than originally estimated. It exists not only in Asia but also in Europe. The offenders trade less recognizable types of goods, such as bones, claws, teeth, broth, paste, wine, and powder. Our participation in the case provided evidence that tigers from private breeders are used as a source for traditional Chinese medicine. The idea of our research team is to provide testing capacity and/or testing kits meeting the ISFG recommendations for nonhuman DNA typing (Linacre et al. 2011) to combat the illegal trade of endangered species and create a worldwide shared database of DNA profiles.

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