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The development and validation of a single SNaPshot multiplex for tiger species and subspecies identification—Implications for forensic purposes

Thitika Kitpipit [a,e], Shanan S. Tobe [a], Andrew C. Kitchener [b,c], Peter Gill [a,d], Adrian Linacre [e,*]

[a] Centre of Forensic Science, WestChem, Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow, G1 1XW, United Kingdom
[b] Department of Natural Sciences, National Museums Scotland, Edinburgh EH1 1JF, United Kingdom
[c] Institute of Geography, School of Geosciences, University of Edinburgh, Edinburgh, EH8 9XP, United Kingdom
[d] Institute of Legal Medicine, University of Oslo, Norway
[e] School of Biological Sciences, Flinders University, Adelaide 5001, South Australia, Australia

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ABSTRACT

The tiger (*Panthera tigris*) is currently listed on Appendix I of the Convention on the International Trade in Endangered Species of Wild Fauna and Flora; this affords it the highest level of international protection. To aid in the investigation of alleged illegal trade in tiger body parts and derivatives, molecular approaches have been developed to identify biological material as being of tiger in origin. Some countries also require knowledge of the exact tiger subspecies present in order to prosecute anyone alleged to be trading in tiger products. In this study we aimed to develop and validate a reliable single assay to identify tiger species and subspecies simultaneously; this test is based on identification of single nucleotide polymorphisms (SNPs) within the tiger mitochondrial genome. The mitochondrial DNA sequence from four of the five extant putative tiger subspecies that currently exist in the wild were obtained and combined with DNA sequence data from 492 tiger and 349 other mammalian species available on GenBank. From the sequence data a total of 11 SNP loci were identified as suitable for further analyses. Five SNPs were species-specific for tiger and six amplify one of the tiger subspecies-specific SNPs, three of which were specific to *P. t. sumatrae* and the other three were specific to *P. t. tigris*. The multiplex assay was able to reliably identify 15 voucher tiger samples. The sensitivity of the test was 15,000 mitochondrial DNA copies (approximately 0.26 pg), indicating that it will work on trace amounts of tissue, bone or hair samples. This simple test will add to the DNA-based methods currently being used to identify the presence of tiger within mixed samples.

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1. Introduction

Tigers once had a widespread geographical distribution, including southern and eastern Asia, ranging as far north as the Russian Far East to as far south as the Sunda Islands [1,2], and had an estimated population size of 100,000 in 1900 [2,3]. Owing to habitat loss and hunting/poaching, tiger populations are presently only a fraction of their previous size; recent estimates suggest there are only approximately 3200 wild tigers [4–8]. Up to nine subspecies of tiger are currently recognized: *Panthera tigris altica*, *P. t. amoyensis*, *P. t. tigris*, *P. t. corbetti*, *P. t. sumatrae*, *P. t. virgata*, *P. t. sondaica*, *P. t. balica* and *P. t. "jacksoni"* (N.B. This taxon is a *nomen nudum*, and if valid awaits a formal scientific description) [9]. Three of these putative subspecies are now extinct (*P. t. virgata*, *P. t. sondaica*, and *P. t. balica*) and *P. t. amoyensis* is no longer present in the wild and exists only in captivity [10], although it has been suggested recently that even these are subspecific hybrids with *P. t. corbetti* [11]. The remaining five extant tiger subspecies are critically endangered [5]; however there is a continuing discussion as to how many tiger subspecies can be recognized based on morphological, biochemical, and molecular genetic studies with different authors proposing diverse conclusions [11–14]. For the purposes of this study we assume that five existing tiger subspecies survive, with no further judgment on their taxonomic validity. As the subspecies *P. t. amoyensis* is no longer present in the wild, only the remaining four subspecies are likely to be poached from wild populations and are the focus of this report.

There are several governmental and charitable organizations that regularly produce reports on illegal wildlife trade [15–17]. Extreme poaching and the illegal trade in tiger skins and body parts are important factors in these reports and significantly contribute to the decline in the number of wild tigers [18] and is evident in surveys carried out by organizations such as TRAFFIC [19]. This has escalated to such an extent that tiger 'farms' have been proposed to breed tigers for commercial use of their parts [20]. Tiger parts are used in traditional Oriental medicine without any scientific

* Corresponding author. Tel.: +61 82012465; fax: +61 8201 3015.
E-mail address: adrian.linacre@flinders.edu.au (A. Linacre).

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support for a therapeutic effect. For example, tiger bone is used in Asian folk remedies or Traditional Chinese Medicine (TCM), and is normally mixed with materials derived from other animal species or herbal material for a compound recipe [21]. Tiger body parts, such as tail, whisker and eyeball, are also claimed to have medicinal properties [22]. Tiger skins are highly sought after as house and costume decoration in some countries such as Tibet [23]. All of these uses of tiger parts have resulted in a highly profitable market in many countries.

All five extant tiger subspecies are listed currently on CITES (Convention on International Trade in Endangered Species) Appendix I, which affords them protection at the highest level from international trade. All are on the International Union for the Conservation of Nature's Red List as Critically Endangered. International trade in tigers and tiger body parts is prohibited by signatories to CITES and many countries have national legislation preventing the ownership of tiger body parts. The role of a forensic science laboratory is to determine if seized material contains even traces of species for which trade is prohibited by CITES agreements or national legislation. Morphological features may aid in the identification of tiger skins and bones, but not their subspecies [24]. Recognizable parts are not present in many commercially available medicines and other products derived from the tiger. To aid in the prevention of trade in tigers and their parts, a molecular approach may be used to ensure whether or not examined samples contain tiger DNA. Owing to the potential for degradation of samples found in a forensic context, nuclear DNA is unlikely to yield results and, therefore, mitochondrial DNA may be used as an alternative means of species identification.

The use of genetic markers for tiger species identification has been previously reported using a number of different detection methods. These approaches employ traditional PCR amplification, using tiger species-specific primers designed for mitochondrial loci such as: cytochrome *b* (cyt *b*); NADH; cytochrome oxidase I (COI) and II (COII); 12s rRNA; and the control region [11,25–27]; RFLP-PCR [28]; and real-time PCR, using species-specific ARMs [29]. However, none of these techniques is able to distinguish between subspecies, and neither can they accurately assign an individual to a particular population. The use of single nucleotide polymorphisms (SNPs) has been used to try and separate tiger subspecies, but was never developed into a full functional or validated forensic test [11]. SNPs have been used in forensic wildlife investigations to identify a number of other protected or endangered wildlife species [30,31], including plants [32]. One of the major challenges of determining the presence of tiger in medicines is their very small quantities, which may have been pre-treated by various methods thus yielding trace quantities of potentially highly degraded DNA.

In this study we report on the identification of tiger-specific SNP haplotypes within the tiger mitochondrial genome, which can also be used to distinguish between two particular tiger subspecies. These SNP loci are the basis for a single multiplex SNaPshot assay. The development and validation of the assay is also demonstrated.

2. Materials and methods

2.1. Sample collection

Fifteen voucher tiger samples were obtained from the National Museums Scotland, Edinburgh Zoo, Dudley Zoo, Isle of Wight Zoo, and Auchingarrich Wildlife Park, all in the UK. These samples comprised hair and tissues from different and unrelated individual tigers. Of these samples, four samples were identified previously as *P. t. sumatrae*, four were from *P. t. tigris*, five samples were from *P. t. altaica*, and two samples were from *P. t. corbetti*. Hair samples were plucked randomly from the individual's body skin and then stored in a sterile bag. Sterile buccal swabs were used to collect forensically appropriate samples from muscle and other tissues. The hair was stored at room temperature and the swabs were stored at −20 °C until extraction.

2.2. DNA extraction

DNA was extracted using QIAamp DNA Mini Kit (QIAGEN, Crawley, UK), according to the manufacturer's protocols for the relevant sample type. The DNA extracts were stored at −20 °C for further analysis.

2.3. The identification of tiger species- and subspecies-specific SNPs

2.3.1. Tiger subspecies-specific SNP identification

The mitochondrial genome was sequenced for 8 tiger samples, comprising two from each of four subspecies. These new data were combined with 492 registered *P. tigris* sequences available on GenBank (www.ncbi.nih.gov). Five hundred and forty-four putative tiger DNA sequences were available although 52 sequences were not used as these data were incomplete or otherwise unsuitable. The remaining 492 DNA sequences were aligned using Clustal X2 [33] to confirm the authenticity of the tiger DNA sequences and minimize the potential for errors in the DNA sequence. The accession numbers, and other relevant information, for these eight new DNA sequences and the 492 DNA tiger sequences retrieved from GenBank data are shown in Table 1. DNA-sequence variability between tiger subspecies over the entire mitochondrial genome was determined. Any SNPs that were specific to a particular tiger subspecies were identified and recorded.

2.3.2. Tiger species-specific SNP identification

To examine tiger-species specific SNPs, 349 DNA sequences of the cyt *b* gene from mammalian species spanning a wide range of taxonomic groups were aligned with 71 tiger sequences, which included all five extant tiger subspecies. A list of mammalian species and their GenBank accession numbers are shown in the supplementary Table S1. These 420 DNA sequences were aligned using Clustal X2. A haplotype of the five SNPs was identified that is specific for all tiger subspecies and not present in other mammalian species. Within the haplotype it should be noted that other mammalian species share one or more of the SNPs with tiger subspecies, but no other species had all five.

2.4. Detection of tiger species and subspecies by SNaPshot assay

2.4.1. The multiplex of SNaPshot template amplification

After the tiger species- and subspecies-specific SNPs were identified, three primer pairs (P1, P2 and P3) were designed to amplify mitochondrial regions that included these SNPs. The physical parameters and secondary structure of the primers were checked using the same tools as primers for the amplification of an entire tiger mitochondrial genome as described previously [34]. The sequences of these three primer sets from 5′ to 3′ are: P1F ACTCAGGACAATGAACCGT; P1R TAAGTAGTGCTGTTATGGCTAG-TAG-TG; P2F ATCAACTCCATTAAACGTCTCTT; P2R GGAAGAT-GAGGTTGAGGTTG; P3F TTTGGCTCCTTACTAGGGGT; and P3R TTGGCGGGGATGTAGTT. The multiplex PCR amplifications were performed in a total volume of 20 μL, containing 200 μM of each dNTP, 1× PCR buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl), 2.0 mM $MgCl_2$, 2.0 units Platinum *Taq* polymerase (Invitrogen, Paisley, UK), 0.5 μM of primer P1 and P3, 1 μM of primer P2, and sterile water. The PCRs were conducted using a 2720 Applied Biosystems thermal cycler. Amplification conditions for these primers were as follows: 35 cycles of denaturation at 95 °C for 45 s, annealing at

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Table 1
Tiger DNA sequence information used in this study, including: accession number, total number of DNA sequences used, author of relevant paper, the year published.

Accession number	No.	Author, year	MtGene
JF357967–JF357974	8	Kitpipit et al., 2011	Complete mitochondrial genomes
EU661609–EU661691, FJ228452	84	Mondol, 2009	ND2, ND5, cyt *b*, CR
EU527874–EU527859	16	Sharma, 2009	12S rRNA, ND2, ND5, cyt *b*
FJ403464.1-67.1	4	Matrai, 2009	cyt *b*, CR
FJ895266.1	1	Yoo, 2009	cyt *b*
FN257739.1, FM999724.1	2	Doung/Din, 2009	ND2, cyt *b*
FJ469625.2, FJ694967.1-972.1, FJ461529.2-534.2, FJ608583.1-585.1	16	Shi and Zhang, 2009	COI, COIII, ND4
EU395630.1-645.1	16	Khan et al., 2009	ND5
EU184702–EU184691	12	Ryan, 2008	cyt *b*
FJ422145.1, FJ465508.1-511.1, FJ478155.1-158.1, FJ455122.1-125.1	13	Shi and Zhang, 2008	COI, COIII, CR
FM179470-71.1	2	Nagappa et al., 2008	12S rRNA
FJ185309.1	1	Ghosh et al., 2008	COI
EF375881.1	1	Rajput and Goyal, 2007	ND1
AB211408-11	4	Sugimoto et al., 2006	cyt *b*
EF179376–EF179357	20	Ryan, 2006	cyt *b*
AB193164.1	1	Nagata, 2006	cyt *b*
AY452097-99.1, AY452101, AY452111.1, 112.1,114.1-119.1	12	Zhang et al., 2006	ND5, CR
DQ111950-51.1	2	Wei et al., 2005	ND5
AY736559–AY736808	250	Luo et al., 2004	12S rRNA, ND1, ND2, COI, ND5, ND6, cyt *b*, CR
AY452110.1	1	Zhang et al., 2003	16S rRNA
AF053018–AF053051	34	Cracraft, 1998	cyt *b*
Total tiger sequences	500		

60 °C for 45 s, extension at 72 °C for 1 min 30 s, and a final extension at 72 °C for 20 min. The reaction was then held at 4 °C. From the final PCR, 2 μL were run on a 3% agarose gel to ensure that the amplifications were successful and provided all the expected fragments. The remainder of the PCR products were then purified using the QIAquick PCR purification kit (QIAGEN), following the manufacturer's protocol.

2.4.2. SNaPshot reaction and product analysis

Eleven SNaPshot extension primers were designed to bind adjacent to the SNPs either in the forward or in the reverse direction (Table 2). The SNaPshot primers were examined for their physical parameters and secondary structure as described previously [34]. Different-length tails (poly GACT) were added to the 5′ end of the primers to vary the primer sizes from 20 to 70 bp. The singleplex and multiplex SNaPshot reactions were performed using the SNaPshot multiplex kit (Applied Biosystems, Foster City, CA, USA) in a total volume of 10 μL, containing 5 μL reaction mix, 3 μL multiplex template, 1 μL sterile water, and 1 μL extension primer mix. For the singleplex reactions 0.2 μM of each extension primer was added, and the optimal concentrations of each primer added in the multiplex reaction are shown in Table 2. The SNaPshot PCRs were amplified in a 2720 Applied Biosystems thermal cycler using the following conditions: 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. SNaPshot products were then purified by Shrimp Alkaline Phosphatase (SAP) to remove unincorporated ddNTPs and primers, by adding 1 unit of SAP into the SNaPshot reaction mixtures. The mixture was incubated at 37 °C for 60 min and then 75 °C for 15 min to deactivate the enzyme.

From the SNaPshot-purified products, 2 μL were mixed with 15.5 μL of formamide and 0.5 μL of GeneScan-120 LIZ size standard (Applied Biosystems). The formamide mix was heated at 95 °C for 3 min and stored at 4 °C until ready for further analysis. The extended products were separated by capillary electrophoresis using the ABI Prism 310 Genetic Analyzer (Applied Biosystems), with a 36-cm length capillary and POP-4 polymer. The SNP typing results were analyzed using GeneMapper version 3.2.1.

2.5. The validation of the developed SNaPshot multiplex assay for tiger species and subspecies identification

The SNaPshot multiplex assay for tiger species and subspecies identification was validated for accuracy, sensitivity, specificity and blind-trial tests. The assay was used to analyze 15 tiger samples described in the sample collection method. Ten of fifteen voucher tiger samples were picked at random. Data were collected by using aliquots of the same PCR product which was injected onto the capillary 10 times to determine run variation. The average range and size of the fragments, and their standard deviation were calculated for all SNP peaks. For the sensitivity test, the *P. t. corbetti*

Table 2
The location, direction and sequence of the SNaPshot extension primer sequences used in this study. The size in nucleotide bases and the optimal primer concentration in the SNaPshot multiplex are also shown.

Position	Strand direction	Single based extension primer sequence (5–3)	Primer size (nucleotides)	[Opt.] (μM)
Tiger-15154	Forward	TTTGGCTCCTTACTAGGGGT	20	0.10
Tiger-15268	Reverse	gaTTGGCATGTAGATATCGGATAAT	25	0.50
Tiger-15385	Reverse	tgactgacTGACCGTAAACAATAGCACAAT	30	0.50
Tiger-15391	Forward	tgactgactgactAAACATGAAATATCGGGATTGT	35	0.20
Tiger-15673	Forward	gactgactgactgactgactAATAACCCCTCAGGAATGGT	40	0.15
TIG-5050	Forward	actgactgactgactgactgactAATGAACCGTATCAAAAGACCT	45	0.20
TIG-5533	Forward	actgactgactgactgactgactgacTATCCTACTGCTAATAATATCTCT	50	0.20
TIG-14618	Reverse	ctgactgactgactgactgactgactgactgactACCCGCACCATTAAACTTAAA	55	0.30
SUM-5608	Reverse	tgactgactgactgactgactgactgactgactTGTTATGGCTAGTAGTGTGGGTATTAT	60	0.12
SUM-15223	Forward	tgactgactgactgactgactgactgactgactgactgactACATCAGACACAATAACCGC	65	0.40
SUM-15743	Reverse	actgactgactgactgactgactgactgactgactgactgactGACGAGTAGTRTGAGGGTTAGGA	70	1.00

DNA was firstly quantified following the method described in [35] where copy number of mitochondrial DNA was obtained. This quantified tiger sample, with a starting concentration of 120,000 mitochondrial copies (equating to 2.06 pg) per μL, was used to make a two-fold serial dilution for 5 concentrations, 120,000, 60,000, 30,000, 15,000, and 7500 mitochondrial copies/μL. These serial dilutions were then analyzed by the developed assay. Fourteen other mammalian species were used to test the specificity of the assay. These mammalian species were cheetah (*Acinonyx jubatus*), jaguar (*Panthera onca*), lion (*Panthera leo*), leopard (*Panthera pardus*), clouded leopard (*Neofelis nebulosa*), puma (*Puma concolor*), domestic cat (*Felis catus*), bear (*Ursus thibetanus*), horse (*Equus caballus*), sika deer (*Cervus nippon*), domestic dog (*Canis familiaris*), cow (*Bos taurus*), goat (*Capra hircus*) and human (*Homo sapiens*). For the blind-trial test, 10 tiger and other mammalian samples were randomly picked from a total of 293 samples. Of these 293 samples, 250 samples are voucher non-*Panthera* mammalian DNA from 20 mammalian species, 15 samples are one of four tiger subspecies DNA, and 28 samples are voucher big cat DNA from 7 big cat species. The 10 blind samples were then analyzed by the developed SNaPshot assay.

3. Results and discussion

3.1. Tiger species- and subspecies-specific SNPs

The complete genome of eight tiger species was sequenced and the sequences registered with GenBank (Table 1). From a search of entire tiger mitochondrial genomes, a total of six tiger subspecies-specific SNPs were identified in three mitochondrial gene loci: ND2, ND6, and cyt *b*. Three of the six SNPs are specific to *P. t. sumatrae* and the rest are specific to *P. t. tigris*. SNPs specific to *P. t. altaica* or *P. t. corbetti* could not be identified over the entire mitochondrial genome. The position and location of SNPs on the mitochondrial genome are shown in Table 3. With the exception of the SNP at position 15223, the tiger species-specific SNPs are transition variants, which is consistent with nucleotide base substitutions biased in favor of transitions rather than transversions. Five of six

Table 3
Subspecies specific SNPs (shown in bold) for the tiger, with their position and location on the mitochondrial genome.

mtlocus	ND2			ND6	cyt *b*	
Position/17009[a]	5050	5533	5608	14618	15223	15743
P. t. altaica	C (3)	G (3)	C (3)	C (3)	T (20)	A (17)
P. t. tigris	**T** (29)	**A** (8)	C (8)	**T** (8)	T (10)	A (38)
P. t. sumatrae	C (10)	G (10)	**T** (10)	C (10)	**A** (11)	**G** (19)
P. t. amoy	C (1)	G (1)	C (1)	C (1)	T (1)	A (1)
P. t. corbetti	C (11)	G (12)	C (12)	C (11)	T (38)	A (15)

The number inside bracket is a number of tiger sequences used for the alignment.
[a] The position was determined by aligning tiger sequences with the cat sequences accession number U20753.

Table 4
Tiger species-specific SNPs and their position on the mitochondrial genome, including a list of the nucleotides of other mammalian species at the same base position.

Position/17009[a]	15154	15268	15385	15391	15673
Panthera tigris (Tiger)	G (43)	G (44)	G (69)	G (69)	G (70)
Melursus ursinus (Sloth bear)	. (2)	A (2)	C (2)	T (2)	A (2)
Ursus maritimus (Polar bear)	. (1)	A (1)	C (1)	T (1)	A (1)
Ursus arctos (Brown bear)	. (3)	A (3)	C (3)	T (3)	A (3)
Crossarchus obscurus (Long-nosed cusimanse)	. (2)	A (2)	A (2)	A (2)	A (2)
Panthera onca (Jaguar)	. (2)	A (2)	A (2)	A (2)	A (2)
Panthera onca (Jaguar)	. (1)	A (1)	–	–	–
Ovibos moschatus (Muskox)	C (4)	A (4)	. (4)	T (4)	A (4)
Tremarctos ornatus (Spectacled bear)	. (1)	A (1)	A (1)	T (1)	A (1)
Ailuropoda melanoleuca (Giant panda)	. (3)	A (3)	A (3)	T (3)	A (3)
Marmota caligata (Hoary marmot)	A (3)	A (3)	. (3)	T (3)	C (3)
Erignathus barbatus (Bearded seal)	C (3)	A (3)	C (3)	C (3)	. (3)
Fossa fossana (Malagasy civet)	A (2)	A (2)	. (2)	T (2)	A (2)
Herpestes naso (Long-nosed mongoose)	A (2)	A (2)	A (2)	C (2)	. (2)
Mungos mungo (Banded mongoose)	A (2)	A (2)	. (2)	C (2)	A (2)
Civettictis civetta (African civet)	C (2)	A (2)	A (2)	A (2)	. (2)
Martes zibellina (Sable)	C (2)	. (2)	T (2)	C (2)	C (2)
Microtus maximowiczii (Maximowicz's vole)	C (1)	A (1)	. (1)	C (1)	T (1)
Microtus arvalis (common vole)	T (2)	A (2)	. (2)	C (2)	C (2)
Lemmus trimucronatus (lemming)	T (1)	A (1)	. (1)	C (1)	C (1)
Crossarchus alexandri (Alexander's cusimanse)	A (1)	A (1)	A (1)	A (1)	. (1)
Prionailurus bengalensis (leopard cat)	A (1)	A (1)	A (1)	. (1)	A (1)
Meles meles (European badger)	C (1)	. (1)	C (1)	C (1)	C (1)
Proteles cristatus (Aardwolf)	C (1)	A (1)	. (1)	A (1)	A (1)
Tapirus indicus (Malayan tapir)	C (1)	A (1)	A (1)	C (1)	. (1)
Cynopterus brachyotis (Lesser short-nosed fruit bat)	C (1)	A (1)	. (1)	C (1)	A (1)
Talpa stankovici (Balkan mole)	C (1)	A (1)	. (1)	A (1)	A (1)
Dasypus novemcinctus (Nine-banded armadillo)	C (1)	A (1)	. (1)	C (1)	. (1)
Mystacina tuberculata (New Zealand lesser short-tailed bat)	C (1)	. (1)	A (1)	C (1)	C (1)
Oryctolagus cuniculus (European rabbit)	. (1)	A (1)	C (1)	C (1)	T (1)
Arctogalidia trivirgata (Small-toothed palm civet)	T (1)	A (1)	. (1)	A (1)	A (1)
Other mammals	A/C/T (281)	A (283)	A/C/T (280)	A/C/T (282)	A/C/T (283)

The number inside the bracket is the number of sequences showing the same nucleotide base from the same species.
'.' represents a nucleotide position showing the same nucleotide base as tiger.
[a] The position was determined by aligning these sequences with the cat sequences accession number U20753.

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SNPs, at positions 5050, 5533, 5608, 14618, and 15743, correspond to the only previously reported tiger subspecies-specific study [11]. In this previous report a further six subspecies-specific SNPs were recorded for the four tiger subspecies that we studied. However, these were not confirmed as subspecies-specific in our study as they were found in more than one subspecies.

Three hundred and forty-nine complete mammalian cyt *b* sequences from 169 mammalian species were aligned with 71 tiger sequences to search for SNPs. A haplotype of the five SNPs was identified that is specific for all tiger subspecies and not present in other mammalian species. Four of these SNPs are multi-allelic markers, with only one SNP position (15268) being a bi-allelic transition. Even though some mammalian species exhibit the same nucleotide base as the tiger at a particular SNP, the tiger can be distinguished by the remaining SNPs. The SNPs and their position on the mitochondrial genome, including the lists of mammalian species providing the same nucleotide base as tiger species for each SNP, are shown in Table 4. The positions of all eleven tiger species- and subspecies-specific SNPs are shown on a mitochondrial map (Fig. 1).

3.2. SNaPshot multiplex assay for tiger species and its subspecies-specific SNPs

The multiplex amplification using primer set P1, P2, and P3, which was designed to amplify mitochondrial regions surrounding all SNPs, was successfully performed on DNA samples of the four tiger subspecies. Each single subspecies sample provided the three expected PCR products of approximately 627, 656, and 683 bp for each of the primers P1, P2, and P3. These PCR products were separated on a 3% agarose gel. The purified PCR products of these samples were then used as a SNaPshot template for a multiplex SNaPshot reaction in the subsequent analysis.

Each of the eleven extension primers was used in a SNaPshot reaction to extend the primers by one base, using the purified PCR product template. The expected nucleotide base, dye color and fragment sizes for these tiger species- and subspecies-specific SNPs are shown in Table 5. The SNP typing for four tiger subspecies corresponded to the expected result, as shown in Fig. 2. It was noted that the observed peak size was slightly larger in mass than the

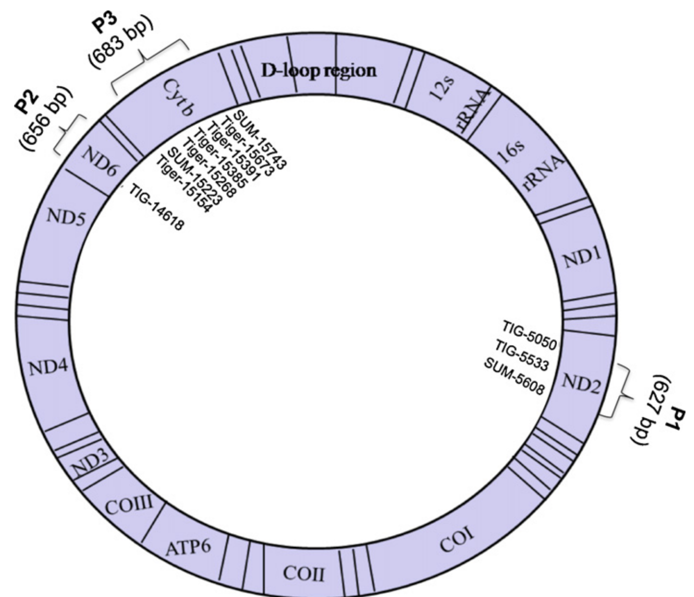

Fig. 1. The positions of all eleven tiger species- and subspecies-specific SNPs on the mitochondrial map. The SNPs are in the ND2, ND6 and cytochrome *b* genes.

actual size for all SNPs; this was most likely due to the influence of the dye attached to the ddNTPs, thus increasing their mass and affecting fragment mobility in the capillary electrophoresis. The other factors causing mobility shift of SNaPshot products are the nucleotide composition and fragment size; the shorter the fragment size, the greater the impact of the fluorescent dye that occurs [36].

3.3. SNaPshot assay validation

3.3.1. Accuracy test

The precision of the assay was tested with 15 voucher tiger DNA samples. This resulted in a 100% level of accuracy. Each sample also showed the expected SNP typing as predicted for its reported subspecies. For all single SNPs the observed range of peak sizes and

Table 5
The expected nucleotide base, dye color, expected and obtained fragment sizes, obtained peak size range, and standard deviation for the tiger species- and subspecies-specific SNPs.

Tiger subspecies	Tiger 15154		Tiger 15268		Tiger 15385		Tiger 15391		Tiger 15673		TIG 5050		TIG 5533		TIG 14618		SUM 5608		SUM 15223		SUM 15743	
	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C	B	C
P. t. tigris	G		C		C		G		G		T		A		T		G		T		T	
P. t. sumatrae	G		C		C		G		G		C		G		C		A		A		C	
P. t. corbetti	G		C		C		G		G		C		G		C		G		T		T	
P. t. altaica	G		C		C		G		G		C		G		C		G		T		T	
Expected peak size	20		25		30		35		40		45		50		55		60		65		70	
Observed peak size average	30.11		32.04		34.27		39.78		44.52		48.58		53.06		57.87		64.72		68.66		75.54	
Observed peak size-range (*n*=10)	29.53–30.81		31.57–32.66		33.91–34.64		39.51–40.13		44.27–44.87		47.97–49.62		52.22–54.50		57.46–58.83		64.23–65.57		68.04–69.11		73.58–76.23	
STD (*n*=10)	0.39		0.31		0.22		0.19		0.18		0.37		0.55		0.38		0.40		0.31		0.54	

B is nucleotide base/C is color.

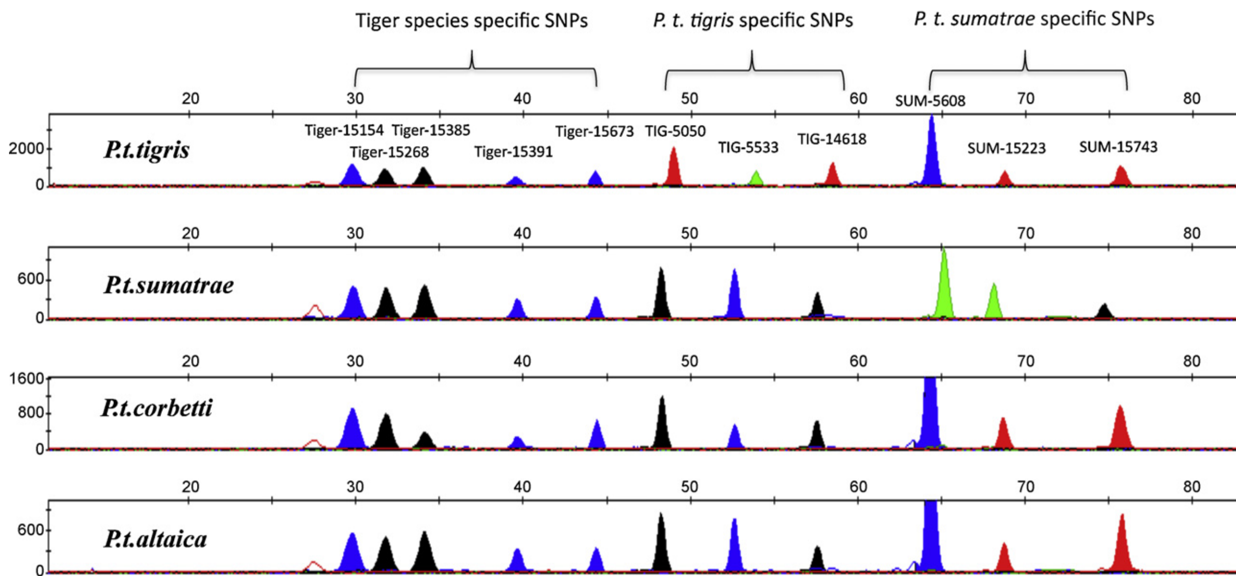

Fig. 2. The electropherogram showing SNP typing of four extant tiger subspecies (*P. t. tigris*, *P. t. sumatrae*, *P. t. corbetti*, and *P. t. altaica*) when analyzed with the single SNaPshot kit developed in this study for tiger species and subspecies identification.

standard deviations were calculated as shown in Table 5. The standard deviations of the observed fragment size are in the range of 0.18–0.55. The lowest values of standard deviation were found in the tiger species-specific SNPs of Tiger-15673 and Tiger-15391. Conversely, two of the tiger subspecies-specific SNPs, TIG-5533 and SUM-15743, reported the highest standard deviation, owing to the significant difference in fragment size between those for the species-specific assays and those for other tiger subspecies. However, this difference was still less than one base pair.

3.3.2. Sensitivity test

The sensitivity of the assay was determined by preparing five DNA concentrations from a two-fold serial dilution containing quantified tiger DNA. A full SNP profile can be obtained from as little as 15,000 copies of target mitochondrial DNA (equating to 0.26 pg), indicating that the test will work on trace amounts of tissue or hair samples. The PCR product amplified from this sample was also the lowest concentration of starting template from which a PCR product could be observed on an agarose gel when stained with ethidium bromide. The presence of an amplification product acted as a rapid screening test to indicate the potential success in obtaining a full SNP profile.

3.3.3. Specificity test

One tiger and fourteen mammalian samples were tested to determine the specificity of the assay. A full SNP profile was retrieved only for *P. tigris*, indicating that the developed assay is specific to this species. However, DNA from other cat species was also successfully amplified by P1, P2 and P3, including cheetah, jaguar, lion, leopard, snow leopard, clouded leopard, puma and domestic cat. All three expected multiplex PCR fragments were generated by the tiger sample as predicted. The band patterns and the intensities obtained for the cat species were different to those for the tiger samples as shown in Fig. 3.

A SNP specificity test was performed using 14 mammalian species, including closely related *Panthera* species, none of which produced products at the same positions as tiger SNPs; this new SNP test has the potential to identify other big cat species.

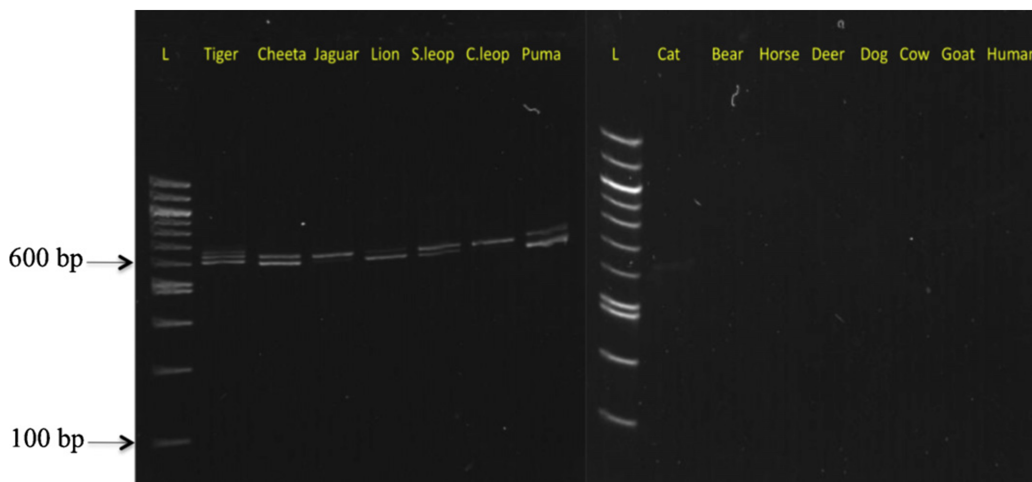

Fig. 3. The specificity test for the SNaPshot multiplex assay for tiger species and subspecies identification. Lanes 1 and 9 are 100 bp DNA ladders (100 bp is the smallest fragment shown); lane 2 is tiger (*Panthera tigris*); lane 3 is cheetah (*Acinoyx jubatus*); lane 4 is jaguar (*Panthera onca*); lane 5 is lion (*Panthera leo*); lane 6 is snow leopard (*Panthera pardus*); lane 7 is clouded leopard (*Neofelis nebulosa*); lane 8 is puma (*Puma concolor*); lane 10 is domestic cat (*Felis catus*); lane 11 is bear (*Ursus thibetanus*); lane 12 is horse (*Equus caballus*); lane 13 is sika deer (*Cervus nippon*); lane 14 is domestic dog (*Canis familiaris*); lane 15 is cow (*Bos taurus*); lane 16 is goat (*Capra hircus*); and lane 17 is human (*Homo sapiens*). The PCR products (10 μL/well) were separated on a 3% agarose gel and visualized with ethidium bromide staining.

Table 6
The result of blind trial testing in 10 DNA sample picked at random.

Result	Sample no.									
	1	2	3	4	5	6	7	8	9	10
Observed result	Other	Tiger *P. t. tigris*	Big cat	Big cat	Tiger *P. t. tigris*	Tiger *P. t. corbetti*	Other	Tiger *P. t. tigris*	Other	Tiger *P. t. altaica*
DNA source	Lamp	Tiger *P. t. tigris*	Jaguar	Puma	Tiger *P. t. tigris*	Tiger *P. t. corbetti*	Horse	Tiger *P. t. tigris*	Fox	Tiger *P. t. altaica*
Correct	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

3.3.4. Blind-trial test

Random single blind trials were performed to examine the SNaPshot multiplex effectiveness of the assay. Ten DNA samples were picked at random and amplified by the multiplex primers and run on a 3% agarose gel. Eight of ten samples were successfully amplified as noted by gel electrophoresis. The two samples that were not successfully amplified were identified as samples from other mammalian species, but were neither tiger nor other big cat species. The eight samples that were amplified successfully were then analyzed by SNaPshot multiplex assay. Of these samples, five provided full SNP typing as predicted if they were one of the tiger subspecies; this was later confirmed. The samples that showed no SNP peaks were later identified as a big cat species, other than tiger, again as predicted by the SNP testing. The blind-trial results for all samples revealed 100% accuracy when categorizing unknown samples into one of the three categories: tiger, other big cats, and other mammalian species, as shown in Table 6.

4. Conclusion

A common approach to species identification, using molecular methods, is to sequence a mitochondrial gene locus such as cyt *b* or COI; or to use species-specific primers [37]. In the case of subspecies identification, there are only a few signature bases that differentiate such closely related taxa. In the entire tiger mitochondrial genome, with a range of approximately 17,000 bp, only six SNP bases were identified that could distinguish between subspecies. Within the cyt *b* gene, one of the most commonly used gene loci in species identification [38], there were only five SNPs of value that separate any subspecies of *P. tigris* from other mammals. The development of a SNP test to identify these bases circumvents generating long DNA sequences that are of little or no diagnostic value. Further, the multiplex SNP assay allows SNPs from more than one gene to be identified in one reaction. We were able to identify and validate eleven tiger species- and subspecies-specific SNPs, spanning the entire tiger mitochondrial genome. The single SNaPshot multiplex assay developed to detect these SNPs was found to be reliable, accurate, specific, sensitive and robust. The assay will prove to be useful in forensic applications as it is able to detect and identify tiger species and subspecies in trace or degraded biological evidence.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsigen.2011.06.001.

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