An extremely sensitive species-specific ARMs PCR test for the presence of tiger bone DNA

Jon H. Wetton*, Carol S.F. Tsang, Chris A. Roney, Adrian C. Spriggs

Abstract

The survival of the tiger (Panthera tigris) is seriously threatened by poaching to provide raw materials for Traditional Chinese Medicines (TCMs). Most highly prized are the tiger’s bones, which are used in combination with other animal and plant derivatives in pills and plasters for the treatment of rheumatism and other ailments. Hundreds of patent remedies have been produced which claim to contain tiger bone, but proof of its presence is needed, if legislation prohibiting the trade in endangered species is to be enforced.

A highly sensitive tiger-specific real-time PCR assay has been developed to address this problem. Using primers specific to the tiger mitochondrial cytochrome b gene, successful amplification has been reliably achieved from blood, hair and bone as well as from a range of TCMs spiked with 0.5% tiger bone. Although capable of detecting fewer than 10 substrate molecules, the seven varieties of TCM pills and plasters tested showed no detectable trace of tiger DNA before spiking. Furthermore, sequencing several “tiger bone” fragments seized from TCM shops has shown that they actually originated from cattle and pigs. The potential effects of traditional bone preparation methods, evidence that much lower concentrations are used than alleged on TCM packaging, and substitution of bones from other species all suggest a low likelihood of detecting tiger DNA in patent medicines. Despite this, the basic methods have been thoroughly proven and can be readily applied to derivatives from other CITES protected species providing a rapid and highly sensitive forensic test for species of origin. Potential applications to the monitoring of wild populations are demonstrated by the successful identification of shed hairs and faecal samples.

Keywords: Species identification; Real-time PCR; LightCycler; Traditional Chinese medicines; CITES; Numt; Cytochrome b

1. Introduction

The tiger is warranted the highest level of protection by the Convention on International Trade in Endangered Species of Wild Fauna & Flora (CITES). Although as an Appendix I species all international trade is banned, a thriving global trade in tiger TCMs still persists [1–3]. Increased interest in traditional remedies and the rapid development of the Asian economies have raised demand dramatically in recent years. Consequently, the high value of tiger TCMs have led to greater rewards for those supplying the genuine article and a rising number of counterfeit patent remedies that have no tiger product within them at all.

Virtually, every part of the tiger is believed to have a therapeutic effect: whiskers relieve the pain of toothache, the eyeballs help to control epilepsy and tiger penis soup aids virility [1]. The bone in particular, is highly prized for its action against arthritis and for increasing male potency. Tiger bone products fall mainly into two categories: tablets for the treatment of a wide range of ailments, and plasters for relieving underlying muscular and joint pain. In each case the tiger bone is claimed to make-up only a small percentage (typically less than 10%) of the constituents, the remainder consisting of a highly variable range of animal and plant products with medicinal herbs comprising the greatest proportion.

Positive identification of the small amount of processed tiger derivative in a mixture of components of other plant and animal species is required before a prosecution is contemplated. DNA sequencing of the mitochondrial cytochrome b gene of which there may be between 100 and
10,000 copies per cell has been used to identify the species of origin of discrete items. Several researchers have used data from the 5′-end of the cytochrome *b* gene to reconstruct evolutionary trees and much of the data is accessible from the GenBank, EMBL and DDBJ databases, hence many species can be identified by sequencing this region with universal primers [4]. However, sequencing is limited both by its complexity and the difficulty of applying it to mixtures from different species. Alternative electrophoretic methods include restriction digestion of PCR products yielding species-specific patterns [5] and amplification with species-specific primers [6].

A faster, simpler and cheaper test is highly desirable for screening large quantities of TCMs. To meet these needs we have developed a single tube assay in which rapid thermocycling is combined with the simultaneous detection of a fluorescent double stranded DNA binding dye to detect the presence of DNA fragments matching tiger-specific primers without recourse to post-PCR processing. The assay was developed by sequencing the species most closely related to the tiger to identify discriminating base sequences and testing primers targeted against these bases for specificity. Using the ARMS principle, it is possible to design primers which have minimal potential for extension by ensuring that the 3′ base mismatches with non-target species, particularly when further destabilized by internal base mispairing with the template [7].

The potential for wider applications of this technology in the monitoring of wild populations was investigated through species identification of faecal samples, which may be useful in identifying the presence of tigers in remote locations where trace evidence is easier to obtain than visual sightings [8].

### 2. Methods

Blood samples collected during Home Office approved veterinary tests from most felid species captive in the UK, as well as representative species from other vertebrate groups were provided by zoos and analytical laboratories (see Table 1). Reference DNA samples were prepared using either chelex [9] or phenol chloroform extraction [10]. A 5′ region of the cytochrome *b* gene was amplified and sequenced with “universal” primer pairs b1 + b2 (5′-CCAATGATATGAAAAACCATCGTT-3′ + 5′-GCCCCCTCAGAATGATATTGTGCTCCT-3′), or cat-specific primers b6 + b9 (5′-ATGACCAACATTCGAAAATCAC-3′ + 5′-GAATATGGARGCTCCGTTGGC-3′) which were designed using published felid sequence data [11–14]. Short fragment primers b12 + b13 (5′-CGAAAAACCCACCCACTAAA-3′).

<table>
<thead>
<tr>
<th>Common name</th>
<th>Species</th>
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</tr>
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<tbody>
<tr>
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<td><em>Equus caballus</em></td>
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<tr>
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<td>Reptilia:Colubridae</td>
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<td><em>Anser cygnoides</em></td>
<td>Aves:Anatidae</td>
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<tr>
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<td><em>Gallus gallus</em></td>
<td>Aves:Phasianidae</td>
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AAAAT-3’ + 5’-AGGGAGCGAGTTTTCATCATGATG-
A-3’) were used to identify the origin of charred bones
confiscated from TCM practitioners. PCR reactions com-
prised 1 μl of extract, 0.25 μM of each primer, 1 x Taq Gold
Buffer, 1.25 U Taq Gold and 200 μM dNTPs in 50 μl. Cycle
parameters on a PE 9600 thermocycler were 95 °C for
20 min, then 35 cycles of 94 °C for 1 min, 50 °C for 1
min and 72 °C for 1 min, followed by an extension at
72 °C for 10 min prior to a 4 °C hold. Fifty nanograms of the
product was used for sequencing with BigDye Terminators
(Applied Biosystems) using the manufacturer’s recom-
pended protocol. Sequences were aligned in Sequence
Navigator and compared with published sequences. Primers
were targeted against tiger-specific bases common to the
to the three tested subspecies: Panthera tigris tigris, P. t. sumatrae
and P. t. altaica (Bengal, Sumatran and Siberian, respec-
tively) and tested against a range of reference samples for
species discrimination.

TCM pills were powdered prior to DNA extraction by
grinding in a liquid nitrogen cooled freezer mill, whilst
plasters were soaked in xylene to release the finely ground
coating from the fabric backing. Up to 1 g of powdered TCM
was added to 2 ml 0.5 M EDTA (pH 8.0), 200 μl Proteinase
K (10 mg/ml), 10 μl Tween 20 and 50 μl 1 M DTT and
incubated overnight at 37 °C. One extraction with phenol,
two with phenol/chloroform and a final wash with chloro-
form were followed by desalting through a Centriprep 30
column. Finally passage through a QIAquick column using
the manufacturer’s PCR purification protocol removed resi-
dual EDTA and other inhibitors. The purified DNA was
eluted from the column either in 30 μl 10 mM Tris–HCl, pH
8.5 or sterile deionised water.

PCRs comprising 0.5 μM primer Tg117F 5’-TTTG-
GCCTTACTAGGGGTG-3’ and Tg231R 5’-GCGATG-
TAGATATCGGATAATC-3’, 3 mM MgCl2, 1 x LightCycler
Master SYBR Green mix and sterile deionised water to a
volume of 19 μl were prepared in pre-cooled glass capil-
laries prior to addition of 1 μl of DNA extract. Target
detection was achieved using the following protocol on a
Roche LightCycler™ with fluorimeter gains set to F1 = 5.
After initial denaturation at 95 °C for 2 min, there were 40
cycles of 95 °C for 0 s, 60 °C for 10 s, 72 °C for 10 s and
80 °C for 2 s during which the fluorescence was read at
530 nm. A final melting profile was obtained in step mode
between 60–90 °C in 0.2 °C increments [15,16].

The specificity and sensitivity of the tiger primers were
assessed with DNA from the species listed in Table 1 and
serial dilutions of tiger blood DNA quantified with Pico-
Green on an Ascent Fluoroscan. Blood extracts yielded
25.5 ng of DNA/μl, each microlitre therefore contained
DNA derived from 4,250 nucleated cells assuming that each
cell contributes 6 pg DNA [17]. Mammalian leukocytes are
typically present at approximately this concentration and are
believed to contribute the most mtDNA, as the 1000-fold
more abundant erythrocytes lack nuclei and expel their
mitochondria within 1 or 2 days of entering the circulation
[18]. We assumed that each tiger leukocyte contained about
220 mitochondrial genomes based upon data for rabbit
macrophages [19]. The true mean number of amplifiable
mtDNA molecules (m) was derived by determining the
proportion of negative PCR replicates (z) from an input at
or near the predicted single molecule level using the Poisson
distribution where z = e\(^{-m}\) [20].

Samples of tiger DNA extracted from blood, hair and
bone were amplified as above to estimate relative DNA
content of the three sample types. Comparative data for
the same human tissue types was derived with control region
primers of similar Tm and amplicon length using the same
PCR parameters. Samples of TCMs were extracted as
described above along with equivalent samples spiked with
low concentrations (0.5, 1, 0.5, 0.1, 0.05 and 0.02%) of
tiger bone to detect potential inhibitory effects resulting
from components of the TCMs. Traditional TCM prepara-
tion methods were simulated to determine their effect on
DNA integrity, these involved boiling powdered bone and
extracted DNA in 2 ml screw top tubes for periods of 0, 0.25,
0.5, 1, 2, 4, 8 and 16 h, followed by extraction of all the tube
contents. Similar times were used on samples heated in an
oven to 200 °C in pig fat to simulate frying. All samples were
compared with a dilution series of a standard tiger DNA
extract so that the rate at which DNA was degraded could be
measured.

To determine the potential of faecal identification
samples from tiger, lion and wolf were stored at ambient
temperature for 48 h prior to freezing. DNA was extracted
using the QiaAmp stool kit following the manufacturer’s
protocol.

3. Results

Sequences generated from felid samples with primers b1
and b2 [12] were of the expected length (474 bp) except
those from lions in which two deletions of 18 and 4 bp were
observed, rather than 18 and 3 bp as reported by Janczewski
et al. [12]. The frameshifted sequence represents a non-
functional, and presumably nuclear embedded, copy of the
mitochondrial cytochrome b sequence. A full length and
presumably authentic mitochondrial cytochrome b sequence
was seen with primers b6 and b9 (see Fig. 1).

Tiger sequences also showed evidence of a nuclear copy
of the cytochrome b sequences with b1 and b2. Mixtures of
the nuclear and mitochondrial sequences published by Cra-
craft et al. [21] were detectable in electropherograms from
single individuals. The relative strengths of the two
sequences varied between individuals but were constant
in replicated extracts (see Fig. 2), possibly due to differences
in the repeat number of the nuclear copy. Interestingly, a
nuclear copy has been detected in the domestic cat (Felis
catus), with multiple repeats of a 7.9 kb fragment showing
5.1% sequence divergence from the genuine mtDNA
[22,23]. The 3’ end of the tiger-specific primers were
targeted against bases unique to the tiger mitochondrial sequence among the species surveyed (Table 2).

Using the tiger primers with typical tiger DNA inputs of 1–0.01 ng DNA, no primer dimer was seen and the sole product melted between 82–83°C (see Fig. 3—blue and black traces), minigel analysis confirmed that this corresponds to the expected 165 bp cytochrome b amplicon. Primer dimer was observed in both PCR and extraction negatives as a peak at about 73°C (Fig. 3—red trace) and also in tiger amplifications with inputs of less than 0.01 ng, the relative proportion of the 82°C peak decreasing as DNA input declines (Fig. 3—green trace).

The sensitivity of the technique was demonstrated when product peaks were seen in 10 out of 75 PCR replicates with a predicted mean input of 0.22 mtDNA molecules. Applying the Poisson distribution reveals the true mean number of amplifiable molecules as 0.14 (a 63% single molecule PCR efficiency). This implies that single molecule detection is possible and allows the calibration of the reference extract dilution series in terms of numbers of amplifiable molecules. Comparisons with the dilution series indicated that approximately 2,000,000 amplifiable molecules were recovered from 1 g of tiger bone, 7 cm of hair shaft and 0.6 ml of blood. Combining an estimate of 600,000 amplifiable molecules/cm of human hair [24] with our own data on the relative mtDNA content of human tissue extracts (see Table 3), would indicate a mtDNA content of the human bone just 10% lower than tiger. Despite considerable variation in DNA content within and between individuals, e.g. due to hair thickness and bone density and between primer pairs in PCR efficiency, the similarity between species provides further support for our calculation of DNA input and PCR sensitivity.

Other than primer dimer, the tiger-specific primers generated no detectable product from any other species except puma (see Table 1). Quantification of puma DNA relative to serial dilutions of extracted tiger DNA showed at least 50,000 times lower amplification efficiency. The mismatch between the terminal base of both forward and reverse primers alone are each expected to result in a greater than 1000-fold reduction in extension efficiency, which will be further affected by the internal mismatches [25]. Pumas have the longest region of base homology upstream of the 3’ terminal mismatch and this may provide the reason why this species can amplify (see Table 2).

Extracts from four varieties of TCM pills and three brands of plaster showed only primer dimer indicating the absence of amplifiable tiger cytochrome b fragments. The complex mixture of plant material and other products had some inhibitory effect as shown by comparisons between TCMs spiked with low concentrations of tiger and cow bone spiked to similar degrees. Successful amplification from bone mixtures was achieved down to inputs of 0.02% (approximately eight amplifiable molecules/µl of DNA extract). By contrast reliable amplification from TCM mixtures was achieved at 0.5% but was occasionally possible at inputs as low as 0.1%, presumably the lowered efficiency was due to a combination of inhibition and reduced recovery resulting from plant material clogging the extraction columns.

DNA extracted from several bone fragments seized from TCM traders in the UK, Far East and USA were identified as cow and pig bones using cytochrome b sequencing. Some showed signs of charring and required the use of primers b12 + b13 that by virtue of a small product size can amplify degraded DNA.

Fig. 1. Partial electropherograms of tiger and lion sequence (bases 175–209) generated with primers b1 + b2 and lion with primers b6 + b9 showing the differential amplification of nuclear (lion b1 + b2) and mitochondrial (tiger b1 + b2 and lion b6 + b9) cytochrome b sequences in the region of the 18 bp deletion.

Fig. 2. Partial electropherograms of tiger sequences generated with primers b1 + b2 from two individuals showing the preferential amplification of mitochondrial (tiger A) and nuclear (tiger B) cytochrome b sequences. The sequences shown here span bases 34–63.
Both the boiled and fried bone fragments showed a rapid decline in the amplification efficiency as shown by comparison against tiger DNA standards (Fig. 4). Following 8 h of heating at/or above 100 °C trace amounts of intact DNA remained but no amplification was detectable after further heating. In contrast tiger faeces produced a strong 82 °C peak, whilst only primer dimers were observed with wolf and lion faecal extracts.

4. Discussion

The initial approach of sequencing the 5' region of the cytochrome b gene revealed the presence of nuclear copies in pantherine cats such as the lion and tiger. Re-design of the primers reduced or eliminated this problem and allowed the design of tiger mtDNA specific primers that could be used for real-time PCR detection. This assay has a number of

<table>
<thead>
<tr>
<th>Species</th>
<th>5'-3' coding strand sequence differences within primer binding sites (bases 97-117 and 231-252)</th>
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<tr>
<td>tiger mtDNA</td>
<td>TTT GCC TCC TTA CTA GGG GTG G ATT ATC CGA TAT CTA CAT GCC</td>
</tr>
<tr>
<td>tiger numt</td>
<td>... ... C.G ... A ... A ... ... ... ... ... ... ...</td>
</tr>
<tr>
<td>lion mtDNA</td>
<td>... ... T ... T ... A ... A ... A ... C ... ... ... ... T</td>
</tr>
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<td>lion numt</td>
<td>... ... C ... T ... C ... ... ... ... ... ... ... T</td>
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<td>... ... C.G ... A ... A ... A ... A ... A ... A ... T</td>
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<td>jaguar</td>
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</tr>
<tr>
<td>sand cat</td>
<td>... ... C ... C ... ... ... ... ... ... ... ... ... ... ...</td>
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</table>

Table 2
Coding strand sequence of felid species highlighting nucleotide positions at which mismatches occur with the tiger-specific primers

Fig. 3. Typical results from a test for tiger material, the blue, black and green traces show the peak at 82 °C produced by progressively smaller inputs of tiger material, whilst the red trace which lacks the 82 °C peak but has a broad 73 °C peak is typical of a negative test in which there is no tiger DNA.
advantages over standard cytochrome b sequencing; the process is simpler, cheaper and faster, it works equally well with pure samples and mixtures, and presents a much reduced risk of contamination because capillaries containing replicated target DNAs are never opened. Minimizing the risk of contamination is particularly important when it is necessary to confidently distinguish samples containing very low concentrations of tiger DNA from others that contain none, given that a false positive test result can arise from a single contaminating molecule. Standard precautions for forensic analysis of bone DNA were followed including: extraction in a MicroSolve decontaminated Class II cabinet, UV irradiation of all consumables, and separate laboratories for PCR set-up, LightCycling and post-PCR analysis (mini-gel assay, etc.).

Testing a single sample including a co-amplifying positive control was discounted as it may outcompete traces of tiger DNA. However, inhibition problems caused by ingredients in patent TCMs can be identified by parallel testing of samples in which one is spiked with tiny quantities of pure tiger DNA to demonstrate the level of detection that can be achieved. In each of the TCMs tested, there was no evidence of tiger DNA at the levels expected given the claimed composition. Several possible explanations exist. The traditional methods for preparing bones range from soaking in water, which will have a limited effect, to frying or prolonged boiling for periods of 7–10 days, the latter is particularly common in South East Asia [3]. These methods are clearly damaging to DNA; our experiments have shown that after approximately 8 h boiling or frying, no detectable DNA remains (see Fig. 4.). Furthermore, species identification of “tiger” bone fragments recovered from traders showed that those with intact DNA actually originated from cattle and pigs. Enquiries within the TCM community suggest that in reality the amount of bone used is much less than is claimed on the label [3, 26], whilst examination of hundreds of TCMs by the USFWS Forensic Laboratory revealed no evidence of the minerals characteristic of bone (K. Goddard in litt.).

5. Conclusion

Real-time PCR using the LightCycler can provide a powerful means for rapidly screening mixed forensic samples [27]. Here, we have shown how it can identify trace quantities of tiger DNA in complex mixtures with many other species demonstrating a principle that could be applied to many different species. However, the potential treatments to which tiger bones are exposed combined with the use of bone at much lower concentrations than claimed, substitution with the bones of other species or the complete omission of bone, all reduce the likelihood of detecting tiger DNA in patent TCMs. Thus, the most valuable role of the test described here may be in the identification of raw materials and monitoring of elusive wild populations.

Table 3
Comparative data on the quantity of three tissue types in both human and tiger which yield similar numbers of amplifiable mtDNA molecules as detected by real-time PCR

<table>
<thead>
<tr>
<th>DNA content</th>
<th>Bone (g)</th>
<th>Blood (ml)</th>
<th>Hair shaft (cm)</th>
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<tr>
<td>Tiger 1</td>
<td>6</td>
<td>$6 \times 10^{-4}$</td>
<td>7</td>
</tr>
<tr>
<td>Human 1</td>
<td>2</td>
<td>$2 \times 10^{-4}$</td>
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Fig. 4. Results of boiling tiger DNA and frying tiger bone fragments. The number of amplifiable molecules declines rapidly, but an initial increase is seen with the fried bones, this may be due to easier grinding allowing more DNA to be extracted from the bone fragments.

![Graph showing the number of amplifiable molecules over time for boiling and frying](image-url)
Acknowledgements

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References


