Validation of non-invasive genetic identification of two elusive, sympatric, sister-species – tiger (*Panthera tigris*) and leopard (*Panthera pardus*)

M. Bhavanishankar, P. Anuradha Reddy*, Digpal Singh Gour and S. Shivaji*

Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad 500 007, India

Population and conservation genetic studies of endangered species increasingly rely on non-invasively collected samples like hair and faeces. The extant genotyping errors in such samples can be further aggravated in studies on sympatric, closely related species like tiger (*Panthera tigris*) and leopard (*Panthera pardus*), where mistakes in species identification can lead to erroneous population estimation, structure and genetic diversity. In this study we assessed possible errors in identification of tiger/leopard faecal samples with published species identification markers. We further analysed a panel of cross-species, polymorphic microsatellite loci for individual identification of both tigers and leopards with an aim to understand the level of allelic overlaps between the two species and to identify a subset of loci useful in eliminating errors in species identification. Two out of four species-specific primers show cross-species amplification. The suite of microsatellite markers used in this study is informative enough to identify individuals and establish genetic diversity in both species. However, 11 out of 13 microsatellites loci exhibit overlapping alleles in tigers and leopards. One locus (Fca96) does not amplify in leopard, whereas only one locus (F124) shows different allelic ranges in the two species. Prior to taking up non-invasive genetic studies on one/multiple sympatric species, it is important to accurately identify the origin of samples. Further, we recommend correct identification of private alleles in each species which will help in eliminating errors in species identification.

Keywords: Genotyping errors, leopard, species-specific identification, sympatry, tiger.

RAPID and precise advancements in non-invasive genetics have now made it possible to analyse a large number of samples, like hair and faeces, to identify individuals and their gender, in elusive and threatened species. Multilocus microsatellite data can be used to assess individual movement, gene flow and population structure, and also to gain insights into mating systems and behaviour[1]. However data generated from non-invasively collected samples entail very high genotyping error rates which should be minimized by stringent laboratory protocols and by calculating quality indices[2–4]. Errors in microsatellite genotypes can have grievous consequences for studies attempting individual identification or paternity assignment, as they draw conclusions based on presence of mismatches between individuals[3]. These errors are further confounded in studies on sympatric, closely related species like tiger (*Panthera tigris*) and leopard (*Panthera pardus*), where mistakes in identity can lead to erroneous population estimation, structure and genetic diversity.

In the Indian subcontinent, tiger and leopard form a two-species solitary, stalk-and-ambush predator assemblage over large regions. Both are highly elusive and morphologically specialized to kill prey larger than themselves[5]. Few genetic studies carried out so far in India focus on either of these two species, although within overlapping landscapes/study areas and utilize similar sampling strategies[6–8]. Faecal samples of both these species are morphologically similar and are identified by species-specific PCR usually based on one or more mutations[9–11]. These samples are subsequently analysed for various species-based studies.

As part of a long-term study on tiger populations in India, we are in the process of assembling microsatellite genotypes from various tiger habitats of the country. As we rely heavily on DNA from faeces, here we assess possible errors in species identification of faecal samples. Further, we identify a panel of cross-species, polymorphic microsatellite loci which can be used for individual identification of both tigers and leopards, a few of which can also eliminate all potential errors in species identification.

Methodology

Sample collection

Fresh faecal samples (< 12 h old) were collected from captive tigers (n = 7) and leopards (n = 7) to evaluate the fidelity of published species identification primers. These animals are housed at the Nehru Zoological Park,

*For correspondence. (e-mail: anuradha@ccmb.res.in; shivas@ccmb.res.in)

Hyderabad and at the Laboratory for the Conservation of Endangered Species, Centre for Cellular and Molecular Biology, Hyderabad.

Six leopard blood and tissue samples, obtained from rescue centres and forensic cases, were used to assess 13 microsatellite loci used in tigers in our previous studies[7,12]. Six fresh faecal samples of captive leopards were also analysed with the same suite of microsatellite loci.

Between January and March 2010, fresh carnivore faecal samples were collected along all roads and trails, and around water bodies in Buxa Tiger Reserve (761 km^2), West Bengal, by forest department personnel. Samples were collected in two sessions with a gap of 30 days to allow for the deposition of fresh samples. All samples were collected in fresh, self-adhesive plastic bags (Ziploc covers) with silica beads and with their geographic locations appropriately recorded. Samples were transported to laboratory within one month and stored at –20°C till extraction. A similar exercise was carried out twice within the core area (392 km^2) and once in the buffer area (942 km^2) of Ranthambore Tiger Reserve, Rajasthan, between October and December 2010. These samples, however, were preserved by the two-step method[13], i.e. 24 h storage in ethanol followed by desiccation with silica. Permissions to collect carnivore faecal samples from Buxa and Ranthambore Tiger Reserves were granted by the Principal Chief Conservator of Forests and Chief Wildlife Warden of West Bengal and Rajasthan respectively (letter no. 893/26-5(1) dated 12 March 2010 and letter no. 5252 dated 17 May 2010).

DNA extraction

DNA was extracted from blood and tissue samples by standard phenol–chloroform method[14], and from fresh faecal samples, both captive and wild, by modified guanidinium thiocyanate–silica method[15], in a dedicated room free of PCR products to minimize contamination. Faecal samples were extracted in sets of ten along with an extraction control to monitor for contamination at the time of isolation.

Species identification

Faecal extracts from captive animals (n = 14) were analysed with four primer pairs for accuracy in species identification. Tiger-specific primers sets included TIF and TIR which amplify a region of cytochrome *b* gene[9]; Tig490F and Tig490R; Tig509F and Tig509R which amplify segments of NADH5 (ref. 10); while one set of leopard-specific primers amplifies a region of NADH4 (ref. 11). Amplicons were obtained following PCR protocols described in the respective papers and were visualized on 1.5–2% agarose gels depending on the amplicon size.

All wild samples were screened with TIF and TIR primers. Tiger-negative samples were further amplified with 16S rRNA felid primers[16]. Amplicons were visualized on 2% agarose gels and cleaned with QIA quick columns (Qiagen Inc., USA). Both strands were sequenced on ABI 3730 DNA Analyser.

Quantitative PCR of wild samples

Tiger and leopard faecal extracts from Buxa and Ranthambore were quantified by qPCR with CmycEx3-71F and CmycEx3-223R, which amplify 191 bp of exon 3 of *c-myc* proto-oncogene in felids[17]. Amplifications were performed in triplicates with 8 µl reaction mixture containing 4 µl SYBR Green (Invitrogen), 5 pM forward and reverse primers and 2 µl of faecal extract. PCR was carried out in an ABI 7900 HT Real Time PCR System. PCR conditions were – initial incubation at 50°C for 2 min and at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. This was followed by a final dissociation cycle of 95°C for 15 sec, 60°C for 15 sec and 95°C for 15 sec. Three no-DNA controls were included in each plate of extracts. DNA concentrations in the extracts were calculated from slope and Y-intercept (Y_{int}) of the trendline from standard curve, plotted as log of DNA concentrations versus C_{t} values: DNA concentrations = $10^{(C_t - Y_{int})/\text{slope})}$.

Individual identification

Tiger and leopard DNA extracts were genotyped at 13 microsatellite loci (Table 1). DNA was amplified, electrophoresed and analysed as described in Reddy *et al.*[17]. All allelic data were analysed in Microsoft EXCEL spreadsheets. Allele frequency analysis, estimates of probability of identity (P_{ID}) and P_{ID} (sib) were carried out using CERVUS version 3.0 (refs 18 and 19). Unique genotypes were identified by the Identity Test in CERVUS. Samples which matched at a minimum of eight loci were pooled to create consensus genotypes. Samples which had mismatches at up to three loci were re-examined by PCR for possible genotyping errors. PCR was also repeated in triplicates at the unamplified loci to obtain complete genotypes. All criteria for estimating genotyping errors were followed as described by Arandjelovic *et al.*[4].

Genetic diversity analysis

Observed and expected heterozygosities were calculated for 12 microsatellite loci for wild and captive leopard samples as implemented in POPGENE[20]. Observed and effective number of alleles[21] was also calculated using the same software. Polymorphic information content (PIC) of each locus was calculated using allelic frequencies (http://www.genomics.liv.ac.uk/animal/Pic1.html) to evaluate the informativeness of heterologous loci in leopards.

Table 1. Description of microsatellite loci used to screen tiger and leopard samples

Locus	Primer sequence	Repeat type	Reference	Size range and alleles in tiger	Size range and alleles in leopard
F37	CGCCTTTCTCACATTACCAT CACTGACAGATCTGATCCTG	Tetra	25	225–240 (**227***, 231, 235, 237)	215–230 (219, 223, **227**)
F42	CCCACGTGGACTAATCAAAT CACTGCACAAATTAAGAGGC	Tetra	25	200–240 (206, 210, 214, 218, **226**, **230**, **238**)	225–240 (**226**, **230**, 234, **238**)
F53	GTTGGGAGTAGAGATCACCT GAAAAAGACTCCTGCTTGCA	Tetra	25	165–190 (168, 172, 176, 180, 188)	150–175 (154, 158, 162, 166, 170, 174)
F115	CTCACACAAGTAACTCTTTG CCTTCCAGATTAAGATGAGA	Tetra	25	175–195 (179, 183, 187, **193**)	185–210 (185, **193**, 197, 199, 203, 209)
F124	TGCTGGGTATGAAGCCTACT ATTGCCTCAACTACCTAGGC	Tetra	25	200–220 (200, 204, 208, 212, 216, 220)	175–190 (178, 182, 186, 190)
F141	CATTCTGCTCTCAGAGCATG GTCTGGGTCTTGTAACTCCT	Tetra	25	255–285 (259, 263, **267**, **271**, **279**, 283)	265–280 (**267**, **271**, 275, **279**)
F146	TTACGGTCTCTCCACAAGTC GAACCAGGTGATGAGAACTG	Tetra	25	140–160 (146, 150, 154, 158)	145–155 (148, 152)
Fca391	GCCTTCTAACTTCCTTGCAGA TTTAGGTAGCCCATTTTCATCA	Tetra	25	195–220 (**198**, **206**, 210, **214**, 218)	190–215 (194, **198**, 202, **206**, **214**)
Fca424	TGGAAAAATGTGGAATACTGAA CCAATTTGTAGTGACATCCCC	Tetra	25	150–175 (154, **158**, **162**, **166**, **170**, **174**)	155–180 (**158**, **162**, **166**, **170**, **174**, 178)
Fca441	ATCGGTAGGTAGGTAGATATAG GCTTGCTTCAAATTTTCAC	Tetra	25	145–160 (**148**, **152**, **156**, 160)	135–160 (136, 144, **148**, **152**, **156**)
Fca96	CACGCCAAACTCTATGCTGA CAATGTGCCGTCCAAGAAC	Di	25	195–205 (197, 201, 203, 205)	No amplification
E6	CCTGGGGATAATAAAACTAGTA CATGAATGAATCTTTACACTGA	Tri	9	135–160 (138, 147, 150, 153, 156)	145–165 (148, 151, 154, 157, 160, 163)
E7	GCCCCAAAGCCCTAAAATAA GCATGTCGGACAGTAAAGCA	Di	9	135–150 (139, 141, **143**, 145, 149)	140–160 (**143**, 147, 151, 153, 155, 157)

*Alleles in bold are common in both species.

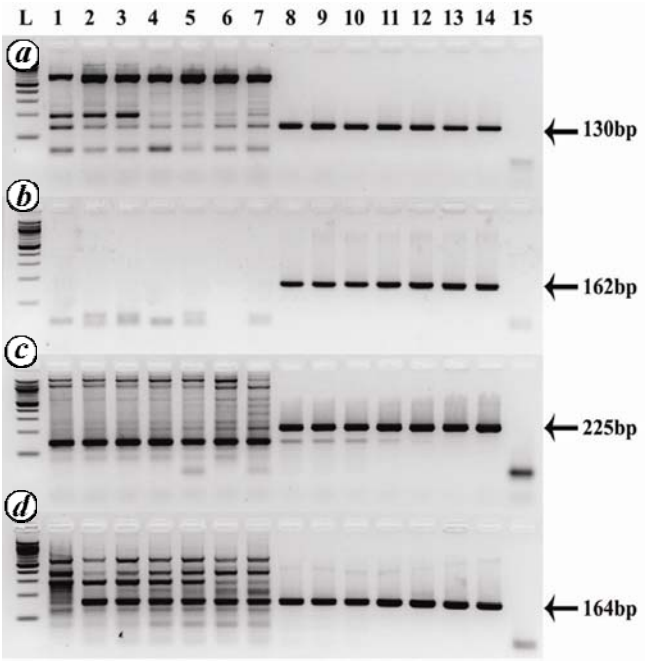

Figure 1. ***a***, Amplification with leopard-specific NADH4 primers (lanes 1–7, Tiger faecal extracts; lanes 8–14, Leopard faecal extracts). ***b***, Amplification with tiger-specific TIF and TIR primers (lanes 1–7, Leopard faecal extracts; lanes 8–14, Tiger faecal extracts). ***c***, Amplification with tiger-specific NADH5 Tig490 primers (lanes 1–7, Leopard faecal extracts; lanes 8–14, Tiger faecal extracts). ***d***, Amplification with tiger-specific NADH5 Tig509 primers (lanes 1–7, Leopard faecal extracts; lanes 8–14, Tiger faecal extracts). Lane L, 100 bp ladder and lane 15, PCR negative.

Results

Species identification

All four sets of primers accurately identified the target species in captive extracts. However, amplicons of desired sizes were also obtained in non-focal species (Figure 1) with Tig509 primers[10] and NADH4 (ref. 11).

Microsatellite amplification and analysis in leopards

Six leopard blood/tissue samples and six captive leopard faecal samples were used to screen 13 microsatellite loci routinely used in our analyses on tiger samples. One locus, Fca96, did not amplify in leopard samples. Only F124 showed an allelic range in leopard distinctly different from that in tiger (Table 1). The remaining 11 loci showed considerable overlap between the two species.

Out of a total of 304 faecal samples collected from Buxa and Ranthambore Tiger Reserves, 148 were of tiger origin and 72 were positively leopard samples. Following qPCR, 16 leopard samples with more than 20 pg/µl were analysed at 13 microsatellite loci. Amplification success rate, or the number of times PCR worked, at 12 loci (excluding Fca96) was 98.4%. Genotyping success was 97.2% and average dropout rate was 1.45%. A locus was accepted as heterozygous only if each allele occurred

Table 2. Allele and genetic diversity profiles of leopards

Locus	Observed number of alleles	Effective number of alleles*	Shannon's information index**	PIC***	Observed heterozygosity	Expected heterozygosity[a]	Nei's expected heterozygosity[a]	Heterozygosity deficiency[b]
F115	6	4.5714	1.6459	0.7499	1.0000	0.8065	0.7812	0.2399
Fca424	5	3.3835	1.4067	0.6666	0.4667	0.7287	0.7044	–0.3595
F124	4	3.0866	1.2241	0.6165	0.6429	0.7011	0.6760	–0.0830
Fca441	5	3.2611	1.3395	0.6408	0.5625	0.7157	0.6934	–0.2141
F146	2	1.6000	0.5623	0.3047	0.0000	0.3871	0.3750	–1.0000
F37	3	1.2659	0.4311	0.1985	0.1538	0.2185	0.2101	–0.2961
F141	4	1.9617	0.9429	0.4545	0.625	0.5060	0.4902	0.2352
E6	6	5.7528	1.7701	0.8018	0.6875	0.8528	0.8262	–0.1938
F53	4	3.7101	1.3463	0.6809	0.5625	0.7540	0.7305	–0.2540
E7	5	3.4595	1.3952	0.6646	1.0000	0.7339	0.7109	0.3626
F42	4	2.4038	1.0867	0.537	0.5000	0.6028	0.5840	–0.1705
Fca391	5	3.9130	1.4682	0.7035	0.6667	0.7701	0.7444	–0.1343
Mean	4.416	3.1975	1.2183	0.58494	0.5723	0.6481	0.6272	–0.1556
Standard deviation	± 1.1645	± 1.2704	± 0.4035	± 0.18136	± 0.2884	± 0.1881	± 0.1823	± 0.3532

*From Kimura and Crow[21]. **From Lewontin[26] . ***Polymorphic information content.
[a]Expected heterozygosities were computed using expected heterozygosity of Levene[27] and Nei[28].
[b]Heterozygote deficiencies were expressed as $D = (H_o - H_e)/H_e$.

twice in independent PCRs. With results from heterozygous loci, we estimated that three PCR replicates were sufficient to determine with more than 99% certainty that a given allele was homozygous and not a result of allelic dropout. The number of alleles in wild leopard samples ranged from 2 (F146) to 6 (F115, E6). The mean expected heterozygosity over all the loci analysed in CERVUS was 0.6481, while mean PIC was 0.5849. Individual probability of identity was 4.22E-0010, while the probability of identity of siblings was 1.32×10^{-4}, making it highly unlikely that two individual leopards could have identical genotypes. Various measures of genetic diversity are presented in Table 2. Observed number of alleles (4.416) across the loci was more than effective number of alleles (3.197). Shannon's information index and PIC showed that many of the loci were highly informative in leopards, with an overall mean polymorphism across the loci for Shannon's information index at 1.218 ± 0.403, and PIC at 0.585 ± 0.1813. Expected heterozygosity (H_e) ranged from 0.218 to 0.853 with mean of 0.648 ± 0.188, and observed heterozygosity (H_o) ranged from 0.000 to 1.000 with mean of 0.572 ± 0.288. Average expected gene diversity in the samples analysed ranged from 0.21 to 0.826, with an overall mean of 0.627 ± 0.182 (Table 2).

Discussion

Currently, several genetic studies on endangered species are based on our ability to get invaluable information from minute amounts of DNA from unlikely sources like hair, scales, faeces, urine and shed skin. Advanced PCR-based techniques are of enormous value, especially when studying elusive species that are difficult to locate in the field, but which leave traces that can be found relatively easily. Potential risks of working with such non-invasive samples are mainly due to poor quality and quantity of DNA[22]. False-positives and overlapping results can aggravate these risks with disastrous consequences while studying congeneric and co-habitating species like tiger and leopard.

The panel of cross-species microsatellite markers used in this study is as informative in identifying individuals and establishing genetic diversity in wild leopard populations as it is in tigers (Table 2). Both species, however, exhibit considerable similarity in alleles across most of the loci. This allelic overlap is not unusual as tiger and leopard are congeneric, and will occur even when species-specific microsatellite markers are employed. Isolation and characterization of microsatellite loci in each species is a complex, expensive and moderately long procedure, and unfortunately does not guarantee that the markers are unique to each species as often these loci are transferable between congenerics. Both E6 and E7 developed in tigers[9] here exhibit similar alleles in leopards (Table 1). Nevertheless, studies on closely related species[4], sub-species[23] and inter-species hybridization[24] employ common suites of cross-species microsatellite loci with remarkably valuable outputs.

We show the possibility of error in tiger or leopard faecal sample identification with reported species-specific mitochondrial markers in tiger and leopard (Figure 1). This inaccuracy can lead to erroneous interpretations when a panel of cross-species, polymorphic microsatellite markers is used in one species without parallel data in its congeneric species. Out of the 13 microsatellite markers used in our previous studies on tiger[7,12], only one (Fca96) did not amplify in leopards and one (F124) exhibited a different allelic size range (Table 1). Eight markers showed overlapping alleles in tiger and leopard and three loci (F53, F146, E6) exhibited one or two base differ-

ences in alleles in the two species although within similar size ranges. Even these differences can be mistaken for insertions or deletions within a single species, thereby leading to erroneous conclusions on population structure, migration or evolution.

India has a vast diversity of carnivores, both felids and canids, living in sympatry. Prior to taking up any non-invasive genetic studies on one/multiple species, it is important to accurately identify the origin of the samples. Only one study so far has reported a PCR–RFLP technique to assign wild faecal samples to the correct felid or canid species[16]. There is an urgent need to further develop species identification protocols which will accurately identify species with limiting amounts of DNA. While using cross-species microsatellite loci care should be taken to correctly identify private alleles in each species, which will help in eliminating errors in species identification.

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