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Development of HyBeacon® probes for the forensic detection of *Panthera*, rhinoceros, and pangolin species

Kimberley George, Alice Masters, Nick Dawnay*

School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom Street, Liverpool, L3 3AF, UK

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ABSTRACT

The Illegal Wildlife Trade (IWT) represents a multi-billion dollar black-market industry whereby wild species are illegally taken from their natural environment and sold. A common question asked by wildlife forensic scientists pertains to species and/or genus identity, which currently requires multi-step processing. Our work details the development of three HyBeacon® probes, used for the presumptive detection of rhinoceros, pangolin and key target species in the *Panthera* genus. The approach can be performed in a single tube using melt curve analysis and provide rapid assessment of sample identity. Using synthetic DNA of representative species, early data suggest the approach is sensitive enough to achieve species identification with < 10 cells. Future development and assay validation can allow the rapid screening of multiple seized items before confirmatory DNA sequencing.

The Illegal Wildlife Trade (IWT) is the fourth most lucrative form of illegal trade after guns and narcotics [1]. Three of the most highly trafficked groups of species include members of the *Panthera* genus, including the tiger (*P. tigris*) and Sumatran tiger (*P. tigris sondaica*), jaguar (*P. onca*), and leopard (*P. pardus*), where bone can be ground to powder for use in traditional medicines [2,3]; members of the Rhinocerotidae family, including the white (*C. simum*), black (*D. bicornis*), Indian (*R. unicornis*), Javan (*R. sondaicus*) and Sumatran (*D. sumatrensis*) rhinoceros, where the horn is ground into powder for use in traditional medicines [4]; and members of the Manidae family, including four species of Asian pangolin (*Manis spp*) and four species of African pangolin (*Phataginus spp* and *Smutsia spp*), where the meat is use in cooking and the scales are used in traditional medicines [5]. Currently, several molecular methods and techniques are used for forensic species identification, including Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) [6,7], Random amplified polymorphic DNA (RAPD) [8,9], melt curve analysis using intercalating dyes [10,11], and the gold standard, DNA sequencing [12,13]. HyBeacon probe technology offers promising results in the field of forensics and other scientific disciples including STR detection for forensic applications [14,15], SNP detection for personalised medicine [16], and species identification for food standard investigations [17]. HyBeacon probes work by emitting a fluorescent signal when hybridized to complimentary DNA sequences. The temperature at which the probes dissociates from the target is determined by the degree of complementarity between the probe and the sequence to which it is bound with data being observed as an end-point melt curve on a fluorescent Real Time PCR instrument. The probes can be labelled with different dyes allowing the multiplex detection of different DNA sequences [18].

In this study, mitochondrial Cytochrome Oxidase I (COI) sequences from rhinoceros and pangolin species, and Cytochrome *b* (Cytb) sequences for tiger, jaguar and leopard species were downloaded from the GenBank collection [19] together with representatives of other closely related species and aligned and edited to the same length using Clustal Omega [20]. The sequences were examined in MEGA 6.0 [21] to identify a ~30 bp region that showed a high degree of homology within each 'target' species but were heterologous to other, closely related, 'non-target' species. Once identified, the sequence was ordered in as a fluorescently labelled HyBeacon probe from LGC Biosearch (Table 1). Internal dt fluorophore labelling was used to allow multiplexing; rhinoceros (FAM), pangolin (JOE), *Panthera* (TAMRA). Due to difficulty in obtaining DNA samples from the species under study, both specificity and sensitivity studies used synthetic DNA constructs following the approach taken by Ref. [11]. The specificity of each probe to its target and non-target species was assessed through the use of 30bp long synthetic Reverse Complement (RC) oligos (Eurofins) which were designed for each species based on the downloaded GenBank sequences (Table 1). Multiplex reactions for testing specificity were performed in 20 μl volumes containing a final concentration of 0.15 μM each HyBeacon probe, 0.3 μM of the RC oligo under assessment, and 2x Phusion HF buffer (ThermoFisher). Three replicate reactions were performed for each species including negative controls following [22]. Melt curve

* Corresponding author.
E-mail address: n.m.dawnay@ljmu.ac.uk (N. Dawnay).

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Table 1
HyBeacon probe binding locations of target species and closely related species of rhinoceros, pangolin and *Panthera* species with the observed melt Temperature.

Scientific Name	Common Name	Oligo ID	Target DNA sequence (COI)	Observed Tm (°C)	Downloaded Sequence (n)	Species Coverage
Panthera Probe		–	5'- C C C T C A A A A A G G A C A T* T T G G C C T* C A T G G T A A G −3′	–	–	–
P. tigris	Tiger	RC0	5'- C T T A C C C A T G A G G G C C C A A A T G T* C T T T T T T G A G G G G −3′	59.8	100	100%
P. tigris sondaica	Sumatran tiger	RC2	5'- . . . . . A . . . C . . . . . A −3′	52.8	10	100%
Panthera onca	Jaguar	RC8	5'- . C . . . . . A . . . C . . . . . −3′	43.8	10	100%
Panthera pardus	Leopard	RC10	5'- . C . . . . . A . . . C . . . . . A −3′	43.8	10	100%
Rhinoceros Probe		–	5'- A T A A T T G T A G T* A A T A A A G T* T A A T G G C A C C −3′	–	–	–
Rhinoceros Reverse Complement Olgio		(RC 0)	5'- G G T G C C A T T A A C T T T A T T A C T A C A A T T A T −3′	–	–	–
Rhinoceros unicornis	Indian rhino	(RC 1)	5'- . . G . . . . . C . . . . . C . . . . G . . . . . −3′	46.2	2	100%
Rhinoceros sondaicus	Javan rhino	(RC 2)	5'- . . G . . . . C . . . . . −3′	55.5	2	100%
Dicerorhinus sumatrensis	Sumatran rhino	(RC 3)	5'- . . . . . C . . . . . −3′	57.5	17	100%
Ceratotherium simum	White rhino	(RC 4)	5'- . . . . . C . . T . . . . . −3′	49.2	4	100%
Diceros bicornis	Black rhino	(RC 5)	5'- . . . . . T . . . . . C . . . . . −3′	49.2	8	100%
Pangolin Probe		–	5'- G G A G G T T T* T A T G T T* A A T G A T A G T T* G T A A T −3′	–	–	–
Pangolin Reverse Complement Oligo		RC0	5'- A T T A C A A C T A T C A T T A A C A T A A A A C C T C C −3′	–	–	–
Manis pentadactyla	Chinese pangolin	RC1	5'- . . C . . . . C . . . . . C . . −3′	52.2	14	100%
Manis culionensis	Phillipine pangolin	RC2	5'- . . . . . A . . . . . C . . . . . C . . −3′	43.5	4	100%
Phataginus tricuspis	Tree pangolin	RC3	5'- . . . . . T . . . . . T . . . . . −3′	48.2	27	89%
		RC6	5'- . . . . . T . . . . . T . . . . . C . . −3′	44.2		11%
Phataginus tetradactyla	Long-tailed pangolin	RC5	5'- . . . . . A . . T . . . . . T . . . . . −3′	44.2	15	73%
		RC6	5'- . . . . . T . . . . . T . . . . . C . . −3′	44.2		27%
Smutsia gigantea	Gaint pangolin	RC3	5'- . . . . . T . . . . . T . . . . . −3′	48.2	6	100%
Smutsia temminckii	Ground pangolin	RC4	5'- . . C . . . . . C . . . . . −3′	53.2	8	100%

RC = Reverse complement oligo label. * In probe sequence denotes fluorophore position. "." Denotes same nucleotide as RC0 oligo. *Panthera* Forward Primer (Excess): 5′-TTGTTTACGGTCATGGCTACAGCCTT-3'; *Panthera* Reverse Primer (Limiting) 5′-GCTGACAGGAGGTTGGTGATTACG-3'; rhino Forward Primer (Excess) 5′-TTGACCTAACCATCTTCTCCCTACAC-3'; rhino Reverse Primer (Limiting) 5′-ATTGGGATATGGCTGGTGGTTT-3'; pangolin Forward Primer 1 (Excess) 5′-CCCTTCATTTGGCAGGTATCTCATCA-3'; pangolin Forward Primer 2 (Excess) 5′-CTCTCCACCTAGCAGGTATTTCCTCA-3'; pangolin Reverse Primer 1 (Limiting) 5′-CATACGAAC AATGGGGTTTGGTATTG-3'; pangolin Reverse Primer 2 (Limiting) 5′-CATACAAATAGTGGAGTTTGATATTG-3'.

analysis was performed on a Rotor-Gene Q 5plex HRM System (QIAGEN) with the following melt curve setting: 95 °C for 5 min, ramp from 95 °C to 30 °C, falling 1 °C each step, hold at 30 °C for 60 s and final melt from 30 °C to 80 °C rising by 1 °C each step. Temperature specific Analytical Thresholds (AT) were established for each species by measuring the fluorescence recorded at each species melt transition in relation to the negative control. This was done by calculating the average noise observed in the negative control samples at each species melt temperature plus three standard deviations. This threshold allowed for the unbiased differentiation between target peaks and negative controls. The melt temperatures of each species were recorded to establish if they could be uniquely identified.

The sensitivity of the proposed assay was assessed through the asymmetric PCR amplification of a synthetic DNA strand (100–150 bp long) that matched the species of interest (ThermoFisher GeneArt Strings). Species-specific primers (Eurofins) were designed to amplify the synthetic template DNA (Table 1). The known concentration of the synthetic DNA was used to calculate copy number before undergoing serial dilutions to obtain working solutions of 50,000; 5,000; 500; 500; 5 and 0.5 copies per µl. Asymmetric PCR [23] was performed in 20 µl volumes containing 0.15 µM each target species probe, 0.5 µM each species forward primer, 0.125 µM each species reverse primer, 200 µM dNTP, Phusion HF buffer (1x), 0.02 U/µl of Phusion hot start II DNA polymerase (ThermoFisher) and 2 µl synthetic template. Three replicate reactions were performed at each concentration including negative controls following [22]. Thermal cycling conditions were as follows: 98 °C for 30 s, followed by 40 cycles of 98 °C for 10 s, 60 °C for 2 s, 72 °C for 10 s. The melt was performed immediately after PCR following the same settings as that reported for the specificity study. The Limit of Detection (LOD) was established by performing a t-test to establish when there was no longer a significant difference between blank samples and those containing progressively lower amounts of input DNA.

Results show a differentiation between target signal and mean background noise (Fig. 1 a,c,e) which is supported by the results of a T-test showing a significant difference between signal observed fluorescence in the negative control (NTC) and fluorescence for all target species (*Panthera* $P = < 0.000$; rhinoceros $P = < 0.000$, pangolin $P = < 0.000$). This suggests that the probes do not form any secondary structures that may mask detection of target DNA. However, The signal:noise ratio of each probe showed some marked differences in quality with both the FAM labelled rhinoceros probe and the JOE labelled pangolin probe showing well defined melt transitions and derivative peaks while the TAMRA labelled *Panthera* probe was less well defined (Fig. 1 a,c,e). Such observations have been previously reported for TAMRA labelled HyBeacon probes and it is possible that an alternative probe label such at Cal-Fluor-610 would allow better signal to noise while still being detected in a third channel (LGC, personal communication).

When assessing the utility of probes for wildlife forensic species detection it is important to consider the protective legislation for the study species. Is species level identification required or is genus level or family level identification sufficient? The latter example allows some flexibility in probe design while the former example makes probe design more complicated. The melt derivative peaks obtained from the *Panthera* probe (Fig. 1b) show a clear differentiation between the two tested tiger subspecies species with the tiger (*P. tigris*) showing a distinct peak at 59.8 °C with the Sumatran tiger (*P. tigris sondaica*) showing a distinct peak at 52.8 °C. There were no temperature differences observed between the leopard (*P. pardus*) and the jaguar (*P. onca*) with both species displaying a peak at 42.8 °C. The inability to differentiate between the African leopard and the South America jaguar is unexpected given that there is an additional A/G transition observed in the leopard DNA sequence (Table 1). Despite this, the probe shows utility for the investigation of IWT as all the target species are CITES Appendix I listed and therefore illegal to trade [24]. The melt derivative peaks obtained from the rhinoceros probe (Fig. 1d) show a clear differentiation between the three Asian species with Indian rhinoceros (*Rhinoceros unicornis*) showing a distinct peak at 46.2 °C, the Javan rhinoceros (*Rhinoceros sondaicus*) showing a distinct peak at 55.5 °C and the Sumatran rhinoceros (*Dicerorhinus sumatrensis*) showing a distinct peak at 57.5 °C. There was no difference in melt temperature between the two African species with the black rhinoceros (*Diceros bicornis*) and the white rhinoceros (*Ceratotherium simum*) both showing a peak at 49.2 °C. The lack of differentiation between the African species is due to them both sharing a T/C transition albeit in a different place meaning there is no relative difference in melt temperature. However, given all the species tested are CITES Appendix I listed and therefore illegal to trade, the probe remains useful for the detection of the Rhinocerotidae family. The rhinoceros data also highlights the potential to differentiate between the two geographic regions (Asia and Africa) which, while not needed to level a criminal charge, may still provide useful forensic intelligence to investigators attempting to understand IWT routes and the composition of seizures [25]. Indeed, such work has been performed previously using microsatellite markers to determine the geographic origin of large shipments of ivory [26,27]. While a tantalising possibility, it is considered unlikely that there is sufficient variation in the mtDNA genome to allow a finer 'population specific' origin to be determined using the approach described. The melt derivative peaks obtained from the pangolin probe (Fig. 1f) show a clear differentiation between the two Asian species with Chinese pangolin (*Manis pentadactyla*) showing a distinct peak at 52.5 °C, and the Phillipine pangolin (*Manis culionensis*) showing a distinct peak at 43.5 °C. Of the African species, only the ground pangolin (*Smutsia temminckii*) also showed a species specific melt peak at 53.2 °C, with the remaining species, tree pangolin (*Phataginus tricuspis*), long-tailed pangolin (*Phataginus tetradactyla*) and the giant pangolin (*Smutsia gigantean*) showing some shared melt peak temperatures at 44.2 °C and 48.2 °C. The inability to differentiate between these remaining species does not invalidate the use of the probe for supporting the IWT investigations as all the species are CITES Appendix I listed [28] so the Manidae family detection remains useful.

The hypothetical Limit Of Detection (LOD) of the multiplex assay showed the LOD is 1000 copies of mtDNA for *Panthera* species (Fig. 2a), 100 copies of mtDNA for rhinoceros species (Fig. 2b), and 10,000 copies of mtDNA for pangolin species (Fig. 2c). It is possible that the lack of sensitivity displayed by the pangolin species is due to the use of two forward and two reverse primers, which were necessary given the diversity observed in the aligned pangolin sequences. The use of multiple primer sets, designed in the same region, increases the possibility that they may form hairpins effectively preventing them from amplifying DNA. Using a conservative estimate of 1000 copies of mtDNA in each cell [29,30] the level of sensitivity for the multiplex test is calculated at approximately 10 cells which matches the sensitivity requirements of a rapid wildlife forensic field-test [31].

The data presented represents the first attempt to develop HyBeacon probes for the detection of wildlife species subject to illegal trade and suggests that HyBeacon probes can offer advantages over gel based identification techniques with regards to single step processing and data quality. The probes described in the current study allowed the detection of individual species in some instances but were limited to providing genus/family level detection in others. However, given the protection status of the taxa described we believe that the probes described could be used to screen and triage samples before sending for confirmatory forensic analysis, an approach desired by wildlife forensic practitioners [31]. Further work could also look at the development of a single test for each species, which would allow for the design of additional species-specific probes based on other mtDNA regions which would allow species level detection rather than the genus/family level detection described here. Such work should look to design probes using an expanded mtDNA reference dataset, which limited the current study. Indeed, the lack of reference sequence data meant that certain species were not considered at all in the current study, including the Indian

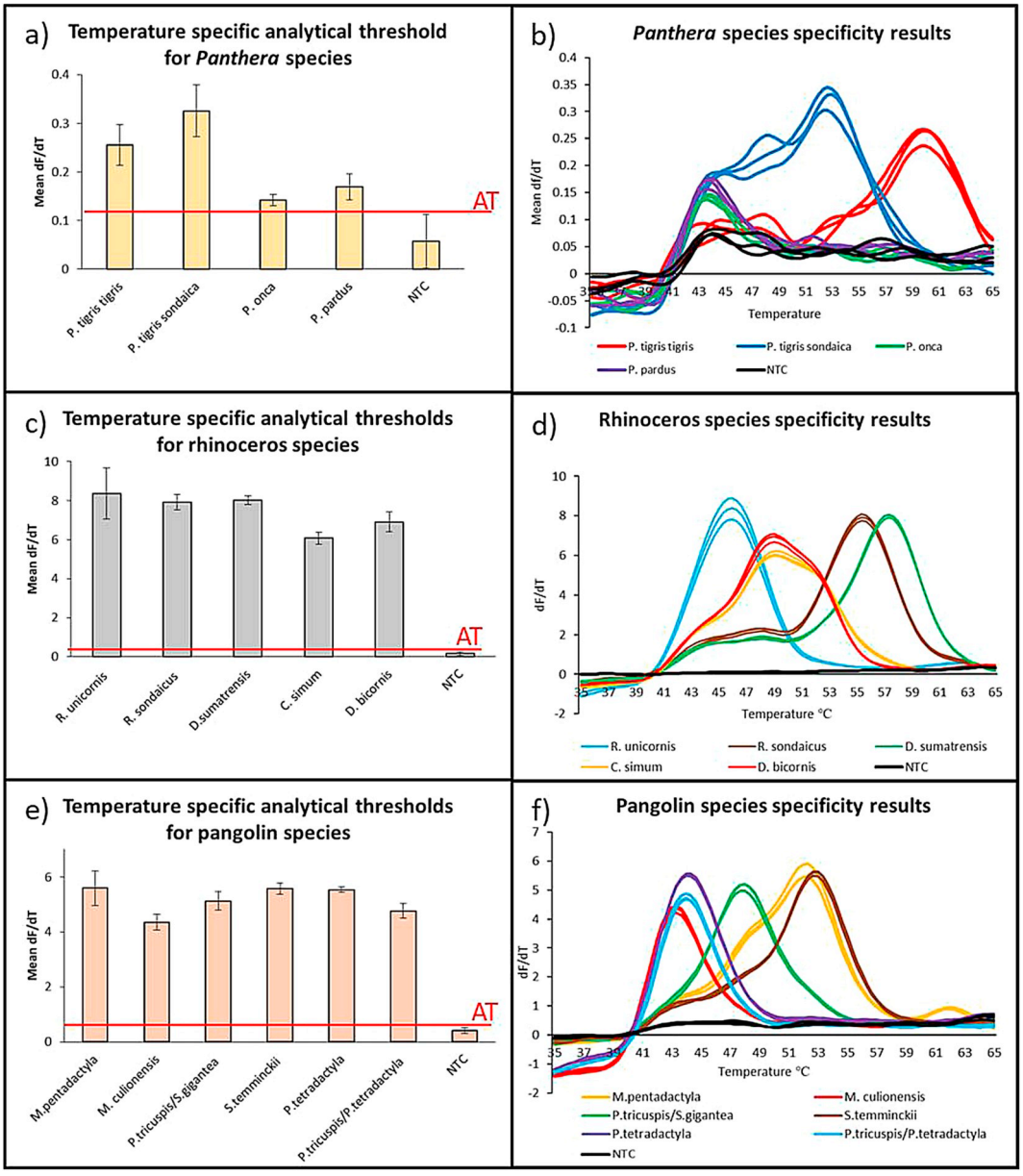

Fig. 1. HyBeacon fluorescence data showing a) differentiation between four species signals and negative control (NTC) for TAMRA labelled *Panthera* probe; b) melt derivative peaks showing melt temperatures for TAMRA labelled *Panthera* probe; c) differentiation between five species signals and negative control (NTC) for JOE labelled rhinoceros probe; d) melt derivative peaks showing melt temperatures for JOE labelled rhinoceros probe; e) differentiation between six species signals and negative control (NTC) for FAM labelled pangolin probe; f) melt derivative peaks showing melt temperatures for FAM labelled pangolin probe. Error bars in a,c,e represent 3 Standard Deviation. AT = Analytical Threshold.

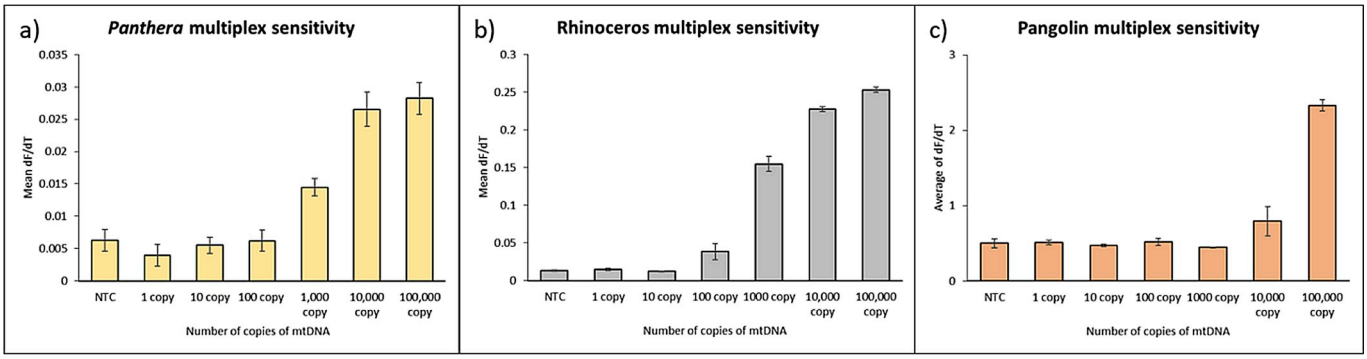

Fig. 2. Sensitivity data showing the Limit of Detection (LOD) of the multiplex PCR assay for a) *Panthera*, b) rhinoceros, and c) pangolin. Error bars represent 1 Standard Deviation.

pangolin (*Manis crassicaudata*), the Sunda pangolin (*Manis javanica*), the Bengal tiger (*Panthera tigris tigirs*), Malayan tiger (*Panthera tigris jacksoni*), Siberian tiger (*Panthera tirgris altaica*), the Indochinese tiger (*Panthera tigris corbetti*) and the South China tiger (*Panthera tigris amoyensis*). This is a problem that the wildlife forensic community is attempting to solve through the development of a dedicated database [32] which houses DNA sequence data from these forensically important species. Once completed, it is likely that more researchers will be able to use the resource to help develop molecular approaches to support wildlife forensic investigations leading to the development of field-based assays for rapid sample screening at points of seizure [31]. Finally, the development of wildlife forensic tests require extensive validation before application [12,33,34] and further development and optimisation is necessary before the approach described can be used in casework. Such work needs to increase the number of replicates used across validation studies, transition from using synthetic DNA to extracted species DNA, attempt detection of 'real world' samples, closely related 'non-target' species and mock casework samples to ensure PCR amplification and HyBeacon detection is consistent and reproducible. Future work will require collaboration with wildlife forensic laboratories to ensure accurate and reproducible results are obtained before the probes are used in criminal casework.

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