



LJMU Research Online

George, KP, Masters, A and Dawnay, N

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

<http://researchonline.ljmu.ac.uk/id/eprint/11415/>

Article

Citation (please note it is advisable to refer to the publisher's version if you intend to cite from this work)

George, KP, Masters, A and Dawnay, N (2019) Development of HyBeacon® probes for the forensic detection of Panthera, rhinoceros, and pangolin species. Molecular and Cellular Probes. ISSN 0890-8508

LJMU has developed [LJMU Research Online](#) for users to access the research output of the University more effectively. Copyright © and Moral Rights for the papers on this site are retained by the individual authors and/or other copyright owners. Users may download and/or print one copy of any article(s) in LJMU Research Online to facilitate their private study or for non-commercial research. You may not engage in further distribution of the material or use it for any profit-making activities or any commercial gain.

The version presented here may differ from the published version or from the version of the record. Please see the repository URL above for details on accessing the published version and note that access may require a subscription.

For more information please contact researchonline@ljmu.ac.uk

<http://researchonline.ljmu.ac.uk/>

1 **Short communication:** Development of HyBeacon[®] probes for the forensic detection of
2 *Panthera*, rhinoceros, and pangolin species.

3

4 Kimberley George^a, Alice Masters^a, Nick Dawnay^{a,*}

5

6 ^a School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, Byrom

7 Street, Liverpool, L3 3AF

8 * Corresponding author: E-mail address: n.dawnay@ljmu.ac.uk

1 **Abstract**

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

12

13 **Key Words:** Species detection; endangered species; HyBeacon; wildlife forensics;
14 presumptive detection; rapid screening.

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

23 In this study, mitochondrial Cytochrome Oxidase I (COI) sequences from rhinoceros and
24 pangolin species, and Cytochrome b (Cytb) sequences for tiger, jaguar and leopard species
25 were downloaded from the GenBank collection [19] together with representatives of other

26 closely related species and aligned and edited to the same length using Clustal Omega [20].
27 The sequences were examined in MEGA 6.0 [21] to identify a ~30 bp region that showed a
28 high degree of homology within each ‘target’ species but were heterologous to other, closely
29 related, ‘non-target’ species. Once identified, the sequence was ordered in as a fluorescently
30 labelled HyBeacon probe from LGC Biosearch (Table 1). Internal dt fluorophore labelling was
31 used to allow multiplexing; rhinoceros (FAM), pangolin (JOE), *Panthera* (TAMRA). Due to
32 difficulty in obtaining DNA samples from the species under study, both specificity and
33 sensitivity studies used synthetic DNA constructs following the approach taken by [11]. The
34 specificity of each probe to its target and non-target species was assessed through the use of
35 30bp long synthetic Reverse Complement (RC) oligos (Eurofins) which were the designed for
36 each species based on the downloaded GenBank sequences (Table 1). Multiplex reactions for
37 testing specificity were performed in 20 µl volumes containing a final concentration of 0.15
38 µM each HyBeacon probe, 0.3 µM of the RC oligo under assessment and 2x Phusion HF buffer
39 (ThermoFisher). Three replicate reactions were performed for each species including negative
40 controls following [22]. Melt curve analysis was performed on a Rotor-Gene Q 5plex HRM
41 System (QIAGEN) with the following melt curve setting: 95°C for five minutes, ramp from
42 95°C to 30°C, falling 1°C each step, hold at 30°C for 60 seconds and final melt from 30°C to
43 80°C rising by 1°C each step. Temperature specific Analytical Thresholds (AT) were
44 established for each species by measuring the fluorescence recorded at each species melt
45 transition in relation to the negative control. This was done by calculating the average noise
46 observed in the negative control samples at each species melt temperature plus three standard
47 deviations. This threshold allowed for the unbiased differentiation between target peaks and
48 negative controls. The melt temperatures of each species were recorded to establish if they
49 could be uniquely identified.

50 The sensitivity of the proposed assay was assessed through the asymmetric PCR amplification
51 of a synthetic DNA strand (100-150 bp long) that matched the species of interest
52 (ThermoFisher GeneArt Strings). Species-specific primers (Eurofins) were designed to
53 amplify the synthetic template DNA (Table 1). The known concentration of the synthetic DNA
54 was used to calculate copy number before undergoing serial dilutions to obtain working
55 solutions of 50,000; 5,000; 500; 50; 5 and 0.5 copies per μl . Asymmetric PCR [23] was
56 performed in 20 μl volumes containing 0.15 μM each target species probe, 0.5 μM each species
57 forward primer, 0.125 μM each species reverse primer, 200 μM dNTP, Phusion HF buffer (1x),
58 0.02 U/ μl of Phusion hot start II DNA polymerase (ThermoFisher) and 2 μl synthetic template.
59 Three replicate reactions were performed at each concentration including negative controls
60 following [22]. Thermal cycling conditions were as follows: 98°C for 30 seconds, followed by
61 40 cycles of 98°C for 10 seconds, 60°C for 2 seconds, 72°C for 10 seconds. The melt was
62 performed immediately after PCR following the same settings as that reported for the
63 specificity study. The Limit of Detection (LOD) was established by performing a t-test to
64 establish when there was no longer a significant difference between blank samples and those
65 containing progressively lower amounts of input DNA.

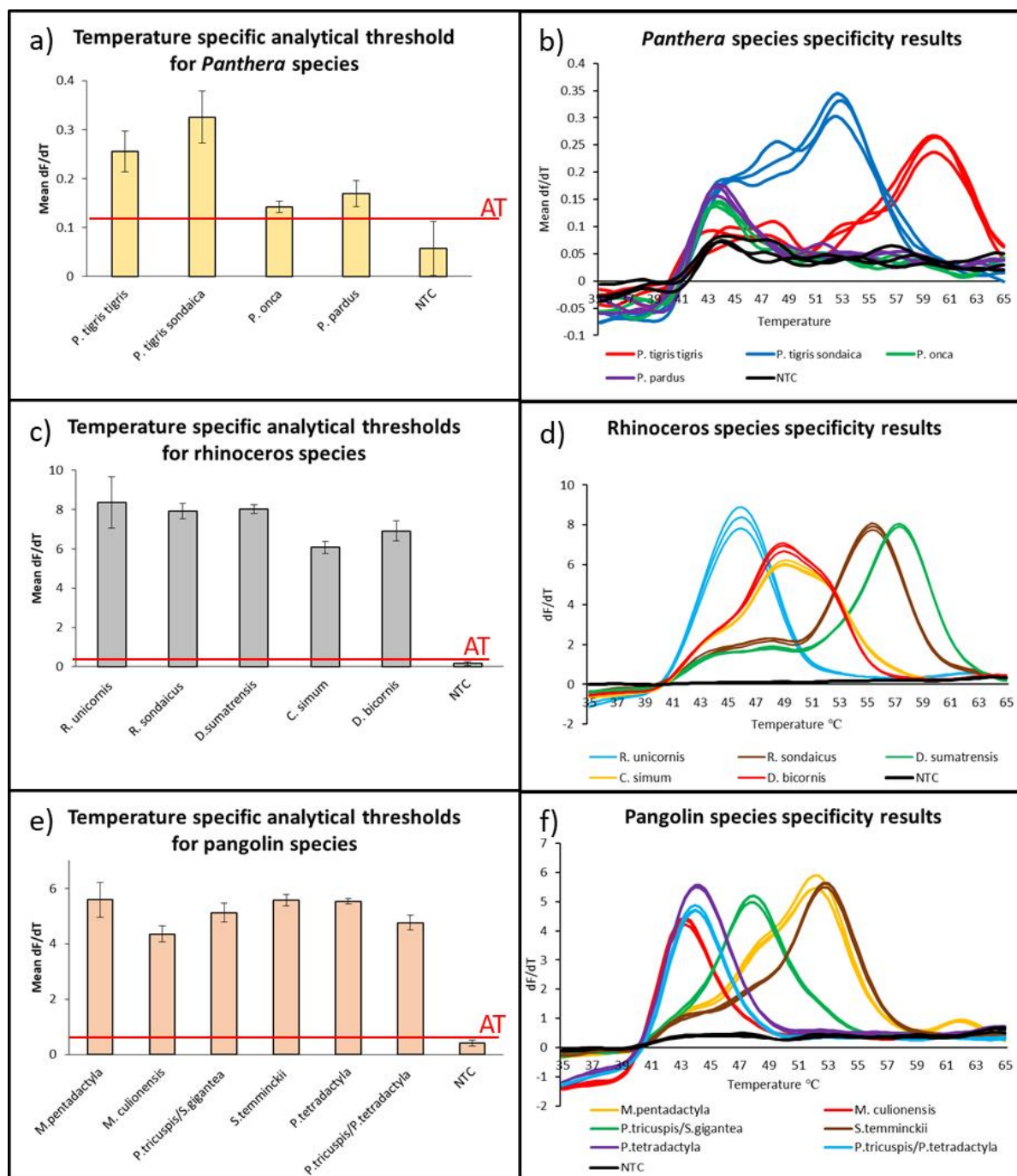
Table 1. HyBeacon probe binding locations of target species and closely related species of rhinoceros, pangolin and *Panthera* species with the observed melt Temperature.

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 A C A T* T T G G C C T* C A T G G T A A G -3'	-	-	-
<i>P. tigris tigris</i>	Tiger	RC0	5'- C T T A C C A T G A G G C C A A A T G T* C T T T T T G A G G G -3'	59.8	100	100%
<i>P. tigris sondaica</i>	Sumatran tiger	RC2	5'- A C A -3'	52.8	10	100%
<i>Panthera onca</i>	Jaguar	RC8	5'- . C A C -3'	43.8	10	100%
<i>Panthera pardus</i>	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 Oligo		(RC0)	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'	-	-	-
<i>Rhinoceros unicornis</i>	Indian rhino	(RC1)	5'- . . G C C G -3'	46.2	2	100%
<i>Rhinoceros sondaicus</i>	Javan rhino	(RC2)	5'- . . G C -3'	55.5	2	100%
<i>Dicerorhinus sumatrensis</i>	Sumatran rhino	(RC3)	5'- C -3'	57.5	17	100%
<i>Ceratotherium simum</i>	White rhino	(RC4)	5'- C . . T -3'	49.2	4	100%
<i>Diceros bicornis</i>	Black rhino	(RC5)	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'	-	-	-
<i>Manis pentadactyla</i>	Chinese pangolin	RC1	5'- . . C C C -3'	52.2	14	100%
<i>Manis culionensis</i>	Phillipine pangolin	RC2	5'- A C C -3'	43.5	4	100%
<i>Phataginus tricuspis</i>	Tree pangolin	RC3	5'- T T -3'	48.2	27	89%
		RC6	5'- T T C -3'	44.2		11%
<i>Phataginus tetradactyla</i>	Long-tailed pangolin	RC5	5'- A . . T T -3'	44.2	15	73%
		RC6	5'- T T C -3'	44.2		27%
<i>Smutsia gigantea</i>	Gaint pangolin	RC3	5'- T T -3'	48.2	6	100%
<i>Smutsia temminckii</i>	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'-GCTGACAGGAGTTGGTGA TTACG-3'; rhino Forward Primer (Excess) 5'-TTGACCTAACCATCTTCTCCCTACAC-3'; rhino Reverse Primer (Limiting) 5'-ATTGGGATATGGCTGGTGGTTT-3'; pangolin Forward Primer 1 (Excess) 5'-CCCTTCA TTTGGCAGGTA TCTCA TCA-3'; pangolin Forward Primer 2 (Excess) 5'-CTCTCCA CCTAGCAGGTA TTTCTCA-3'; pangolin Reverse Primer 1 (Limiting) 5'-CATACGAACAATGGGGTTTGTA TTG-3'; pangolin Reverse Primer 2 (Limiting) 5'-CATACAAAATGTGGAGTTTGATATTG-3'. *Manis javanica* and *Manis crassicaudata* not represented in the GenBank database

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

63



64

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

73

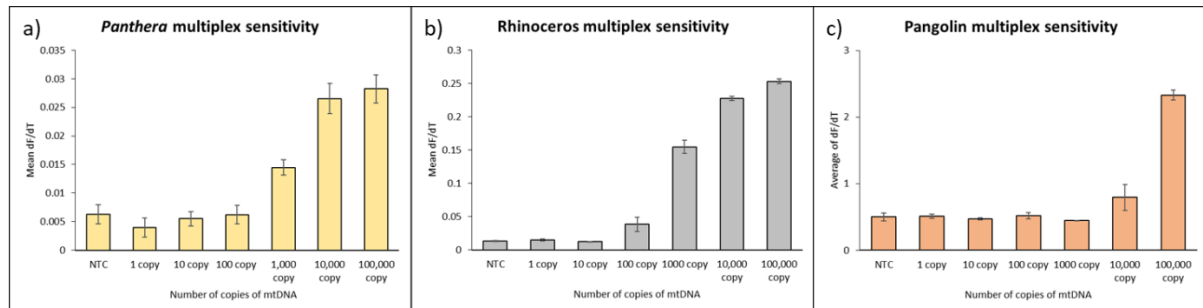
74 When assessing the utility of probes for wildlife forensic species detection it is important to
75 consider the protective legislation for the study species. Is species level identification required
76 or is genus level or family level identification sufficient? The latter example allows some
77 flexibility in probe design while the former example makes probe design more complicated.
78 The melt derivative peaks obtained from the *Panthera* probe (Figure 1b) show a clear
79 differentiation between the two tested tiger subspecies species with the tiger (*P. tigris tigris*)
80 showing a distinct peak at 59.8°C with the Sumatran tiger (*P. tigris sondaica*) showing a
81 distinct peak at 52.8°C. There were no temperature differences observed between the leopard
82 (*P. pardus*) and the jaguar (*P. onca*) with both species displaying a peak at 42.8°C. The inability
83 to differentiate between the African leopard and the South America jaguar is unexpected given
84 that there is an additional A/G transition observed in the leopard DNA sequence (Table 1).
85 Despite this, the probe shows utility for the investigation of IWT as all the target species are
86 CITES Appendix I listed and therefore illegal to trade [24]. The melt derivative peaks obtained
87 from the rhinoceros probe (Figure 1d) show a clear differentiation between the three Asian
88 species with Indian rhinoceros (*Rhinoceros unicornis*) showing a distinct peak at 46.2°C, the
89 Javan rhinoceros (*Rhinoceros sondaicus*) showing a distinct peak at 55.5°C and the Sumatran
90 rhinoceros (*Dicerorhinus sumatrensis*) showing a distinct peak at 57.5°C. There was no
91 difference in melt temperature between the two African species with the black rhinoceros
92 (*Diceros bicornis*) and the white rhinoceros (*Ceratotherium simum*) both showing a peak at
93 49.2°C. The lack of differentiation between the African species is due to them both sharing a
94 T/C transition albeit in a different place meaning there is no relative difference in melt
95 temperature. However, given all the species tested are CITES Appendix I listed and therefore
96 illegal to trade, the probe remains useful for the detection of the Rhinocerotidae family. The
97 rhinoceros data also highlights the potential to differentiate between the two geographic regions

98 (Asia and Africa) which, while not needed to level a criminal charge, may still provide useful
99 forensic intelligence to investigators attempting to understand IWT routes and the composition
100 of seizures [25]. Indeed, such work has been performed previously using microsatellite markers
101 to determine the geographic origin of large shipments of ivory [26,27]. While a tantalising
102 possibility, it is considered unlikely that there is sufficient variation in the mtDNA genome to
103 allow a finer 'population specific' origin to be determined using the approach described. The
104 melt derivative peaks obtained from the pangolin probe (Figure 1f) show a clear differentiation
105 between the two Asian species with Chinese pangolin (*Manis pentadactyla*) showing a distinct
106 peak at 52.5°C, and the Phillipine pangolin (*Manis culionensis*) showing a distinct peak at
107 43.5°C. Of the African species, only the ground pangolin (*Smutsia temminckii*) also showed a
108 species specific melt peak at 53.2°C, with the remaining species, tree pangolin (*Phataginus*
109 *tricuspis*), long-tailed pangolin (*Phataginus tetradactyla*) and the giant pangolin (*Smutsia*
110 *gigantean*) showing some shared melt peak temperatures at 44.2°C and 48.2°C. The inability
111 to differentiate between these remaining species does not invalidate the use of the probe for
112 supporting the IWT investigations as all the species are CITES Appendix I listed [28] so the
113 Manidae family detection remains useful.

114 The hypothetical Limit Of Detection (LOD) of the multiplex assay showed the LOD is 1000
115 copies of mtDNA for *Panthera* species (Figure 2a), 100 copies of mtDNA for rhinoceros
116 species (Figure 2b), and 10,000 copies of mtDNA for pangolin species (Figure 2c). It is
117 possible that the lack of sensitivity displayed by the pangolin species is due to the use of two
118 forward and two reverse primers, which were necessary given the diversity observed in the
119 aligned pangolin sequences. The use of multiple primer sets, designed in the same region,
120 increases the possibility that they may form hairpins effectively preventing them from
121 amplifying DNA. Using a conservative estimate of 1000 copies of mtDNA in each cell [29,30]

122 the level of sensitivity for the multiplex test is calculated at approximately 10 cells which
123 matches the sensitivity requirements of a rapid wildlife forensic field-test [31].

124



125

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

128

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

144 *tigris jacksoni*), Siberian tiger (*Panthera tigris altaica*), the Indochinese tiger (*Panthera tigris*
145 *corbetti*) and the South China tiger (*Panthera tigris amoyensis*). This is a problem that the
146 wildlife forensic community is attempting to solve through the development of a dedicated
147 database [32] which houses DNA sequence data from these forensically important species.
148 Once completed, it is likely that more researchers will be able to use the resource to help
149 develop molecular approaches to support wildlife forensic investigations leading to the
150 development of field-based assays for rapid sample screening at points of seizure [31]. Finally,
151 the development of wildlife forensic tests require extensive validation before application
152 [12,33,34] and further development and optimisation is necessary before the approach
153 described can be used in casework. Such work needs to increase the number of replicates used
154 across validation studies, transition from using synthetic DNA to extracted species DNA,
155 attempt detection of ‘real world’ samples, closely related ‘non-target’ species and mock
156 casework samples to ensure PCR amplification and HyBeacon detection is consistent and
157 reproducible. Future work will require collaboration with wildlife forensic laboratories to
158 ensure accurate and reproducible results are obtained before the probes are used in criminal
159 casework.

160

161 **Acknowledgments**

162 This work was carried out Alice Masters, who was funded by the Peoples Trust in Endangered
163 Species, and Kimberley George, in partial fulfilment of her MSc in Forensic Bioscience.

164

165 **References**

166 [1] World Wildlife Fund Report. Fighting Illicit Wildlife Trafficking. A Consultation with
167 Governments. Published 2012. Available at http://wwf.panda.org/our_work/wildlife/

168 problems/illegal_trade/wildlife_trade_campaign/wildlife_trafficking_report/. Accessed on
169 7.12.2019

170 [2] Nijman V, Shepherd CR. Trade in tigers and other wild cats in Mong La and Tachilek,
171 Myanmar—A tale of two border towns. *Biological Conservation* (2015) **182**:1-7.

172 [3] Wyler LS, Sheikh PA. International illegal trade in wildlife: Threats and US policy.
173 Published 2013. Library of Congress Washington DC Congressional Research Service.

174 [4] Ayling J. What sustains wildlife crime? Rhino horn trading and the resilience of criminal
175 networks. *Journal of International Wildlife Law & Policy* (2013) **16**:57-80.

176 [5] Shepherd CR. Overview of pangolin trade in Southeast Asia. *Proceedings of the Workshop*
177 *on Trade and Conservation of pangolins Native to South and Southeast Asia* (2009) **30**:6-9.

178 [6] Wolf C, Rentsch J, Hübner P. PCR– RFLP analysis of mitochondrial DNA: A reliable
179 method for species identification. *Journal of Agricultural and Food Chemistry* (1999) **47**:1350-
180 5.

181 [7] Meganathan PR, Dubey B, Haque I. Molecular identification of Indian crocodile species:
182 PCR-RFLP method for forensic authentication. *Journal of Forensic Sciences* (2009) **54**:1042-
183 5.

184 [8] Lee JC, Chang JG. Random amplified polymorphic DNA polymerase chain reaction
185 (RAPD PCR) fingerprints in forensic species identification. *Forensic Science International*
186 (1994) **67**:103-7.

187 [9] Congiu L, Chicca M, Cella R, Rossi R, Bernacchia G. The use of random amplified
188 polymorphic DNA (RAPD) markers to identify strawberry varieties: a forensic application.
189 *Molecular Ecology* (2000) **9**:229-32.

190 [10] Berry O, Sarre SD. Gel-free species identification using melt-curve analysis. *Molecular*
191 *Ecology Notes* (2007) **7**:1-4.

- 192 [11] Malewski T, Draber-Mońko A, Pomorski J, Łoś M, Bogdanowicz W. Identification of
193 forensically important blowfly species (Diptera: Calliphoridae) by high-resolution melting
194 PCR analysis. *International Journal of Legal Medicine* (2010) **124**:277-85.
- 195 [12] Dawnay N, Ogden R, McEwing R, Carvalho GR, Thorpe RS. Validation of the barcoding
196 gene COI for use in forensic genetic species identification. *Forensic Science International*
197 (2007) **173**:1-6.
- 198 [13] Branicki W, Kupiec T, Pawlowski R. Validation of cytochrome b sequence analysis as a
199 method of species identification. *Journal of Forensic Science* (2003) **48**:1-5.
- 200 [14] Dawnay N, Stafford-Allen B, Moore D, Blackman S, Rendell P, Hanson EK, Ballantyne
201 J, Kallifatidis B, Mendel J, Mills DK, Nagy R. Developmental Validation of the ParaDNA®
202 Screening System-A presumptive test for the detection of DNA on forensic evidence items.
203 *Forensic Science International: Genetics* (2014) **11**:73-9.
- 204 [15] Blackman S, Dawnay N, Ball G, Stafford-Allen B, Tribble N, Rendell P, Neary K, Hanson
205 EK, Ballantyne J, Kallifatidis B, Mendel J, Mills D, Wells S. Developmental validation of the
206 ParaDNA® Intelligence System—A novel approach to DNA profiling. *Forensic Science*
207 *International: Genetics* (2015) **17**:137-48.
- 208 [16] Gaied NB, Richardson JA, Singleton DG, Zhao Z, French D, Brown T. End-capped
209 HyBeacon probes for the analysis of human genetic polymorphisms related to warfarin
210 metabolism. *Organic & Biomolecular Chemistry* (2010) **8**:2728-34.
- 211 [17] Dawnay N, Hughes R, Court DS, Duxbury N. Species detection using HyBeacon® probe
212 technology: Working towards rapid onsite testing in non-human forensic and food
213 authentication applications. *Forensic Science International: Genetics* (2016) **20**:103-11.
- 214 [18] Richardson JA, Gerowska M, Shelbourne M, French D, Brown T. Six-colour HyBeacon
215 probes for multiplex genetic analysis. *ChemBioChem* (2010) **11**:2530-3.

216 [19] Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Sayers EW. GenBank. *Nucleic Acids*
217 *Research* (2009) **37**:D26-31.

218 [20] Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H,
219 Remmert M, Söding J, Thompson JD. Fast, scalable generation of high-quality protein multiple
220 sequence alignments using Clustal Omega. *Molecular Systems Biology* (2011) **7**: 539.

221 [21] Kumar S, Nei M, Dudley J, Tamura K. MEGA: a biologist-centric software for
222 evolutionary analysis of DNA and protein sequences. *Briefings in Bioinformatics* (2008)
223 **9**:299-306.

224 [22] Stafford-Allen B, Dawnay N, Hanson EK, Ball G, Gupta A, Blackman S, French DJ,
225 Duxbury N, Ballantyne J, Wells S. Development of HyBeacon® probes for specific mRNA
226 detection using body fluids as a model system. *Molecular and Cellular Probes* (2018) **38**:51-
227 9.

228 [23] Sanchez JA, Pierce KE, Rice JE, Wangh LJ. Linear-After-The-Exponential (LATE)-PCR:
229 An advanced method of asymmetric PCR and its uses in quantitative real-time analysis.
230 *Proceedings of the National Academy of Sciences* (2004) **101**:1933-8.

231 [24] The CITES Appendices. Available at <https://www.cites.org/eng/app/index.php>. Accession
232 on 12.7.2019.

233 [25] Ewart KM, Frankham GJ, McEwing R, Hogg CJ, Wade C, Lo N, Johnson RN. A rapid
234 multiplex PCR assay for presumptive species identification of rhinoceros horns and its
235 implementation in Vietnam. *PloS one* (2018) **13**:e0198565:1-10

236 [26] Ogden R, Linacre A. Wildlife forensic science: a review of genetic geographic origin
237 assignment. *Forensic Science International: Genetics* (2015) **18**:152-9.

238 [27] Wasser SK, Brown L, Mailand C, Mondol S, Clark W, Laurie C, Weir BS. Genetic
239 assignment of large seizures of elephant ivory reveals Africa's major poaching hotspots.
240 *Science* (2015) **349**:84-7.

241 [28] Kumar VP, Rajpoot A, Shukla M, Nigam P, Goyal SP. Inferring the molecular affinity of
242 Indian pangolin with extant Manidae species based on mitochondrial genes: a wildlife forensic
243 perspective. *Mitochondrial DNA Part B* (2018) **3**:640-4.

244 [29] Zhang P, Lehmann BD, Samuels DC, Zhao S, Zhao YY, Shyr Y, Guo Y. Estimating
245 relative mitochondrial DNA copy number using high throughput sequencing data. *Genomics*
246 (2017) **109**:457-62.

247 [30] Tobe SS, Linacre AM. A technique for the quantification of human and non-human
248 mammalian mitochondrial DNA copy number in forensic and other mixtures. *Forensic Science*
249 *International: Genetics* (2008) **2**:249-56.

250 [31] Masters A, Ogden R, Wetton JH, Dawnay N. Defining end user requirements for a field-
251 based molecular detection system for wildlife forensic investigations. *Forensic Science*
252 *International* (2019) **301**:231-239.

253 [32] Ahlers N, Creecy J, Frankham G, Johnson RN, Kotze A, Linacre A, McEwing R, Mwale
254 M, Rovie-Ryan JJ, Sitam F, Webster LM. 'ForCyt' DNA database of wildlife species. *Forensic*
255 *Science International: Genetics Supplement Series* (2017) 6:e466-8.

256 [33] SWGDAM Validation Guidelines for DNA Analysis Methods. [https://docs.wixstatic.](https://docs.wixstatic.com/ugd/4344b0_813b241e8944497e99b9c45b163b76bd.pdf)
257 [com/ugd/4344b0_813b241e8944497e99b9c45b163b76bd.pdf](https://docs.wixstatic.com/ugd/4344b0_813b241e8944497e99b9c45b163b76bd.pdf). Accessed on 19.2.2019.

258 [34] Dawnay N, Ogden R, Thorpe RS, Pope LC, Dawson DA, McEwing R. A forensic STR
259 profiling system for the Eurasian badger: a framework for developing profiling systems for
260 wildlife species. *Forensic Science International: Genetics* (2008) **2**:47-53.