

1 Empowering conservation practice with efficient and economical genotyping from poor quality
2 samples

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19 Running title: Genetic data to empower conservation

20 Abstract:

- 21 1. Moderate to high density genotyping (100+ SNPs) is widely used to determine and
22 measure individual identity, relatedness, fitness, population structure and migration in
23 wild populations.
- 24 2. However, these important tools are difficult to apply when high-quality genetic material
25 is unavailable. Most genomic tools are developed for high quality DNA sources from lab
26 or medical settings. As a result, most genetic data from market or field settings is limited
27 to easily amplified mitochondrial DNA or a few microsatellites.
- 28 3. To enable genotyping in conservation contexts, we used next-generation sequencing of
29 multiplex PCR products from very low-quality DNA extracted from feces, hair, and
30 cooked samples. We demonstrated utility and wide-ranging potential application in
31 endangered wild tigers and tracking commercial trade in Caribbean queen conch.
- 32 4. We genotyped 100 SNPs from degraded tiger samples to identify individuals, discern
33 close relatives, and detect population differentiation. Co-occurring carnivores do not
34 amplify (e.g. Indian wild dog/Dhole) or are monomorphic (e.g. leopard). 62 SNPs from
35 conch fritters and field-collected samples were used to test relatedness and detect
36 population structure.
- 37 5. We provide proof-of-concept for a rapid, simple, cost-effective, and scalable method (for
38 both samples and number of loci), a framework that can be applied to other conservation
39 scenarios previously limited by low quality DNA samples. These approaches provide a
40 critical advance for wildlife monitoring and forensics, open the door to field-ready testing,
41 and will strengthen the use of science in policy decisions and wildlife trade.

42

- 43 Keywords: conch, conservation genetics, endangered species monitoring, genotyping, multiplex
- 44 PCR, non-invasive samples, SNPs, tigers

45 **Introduction**

46 Stemming the tide of global species decline requires continuous monitoring and nimble,
47 adaptive management to promote species recovery. Effective monitoring relies on identifying
48 species presence and the ability to track specific individuals and their familial relationships.
49 While species recovery is critically dependent on tracking individuals and their dynamics locally,
50 integrating data across the species range allows monitoring of global large-scale threats including
51 population range reduction and illegal wildlife trade.

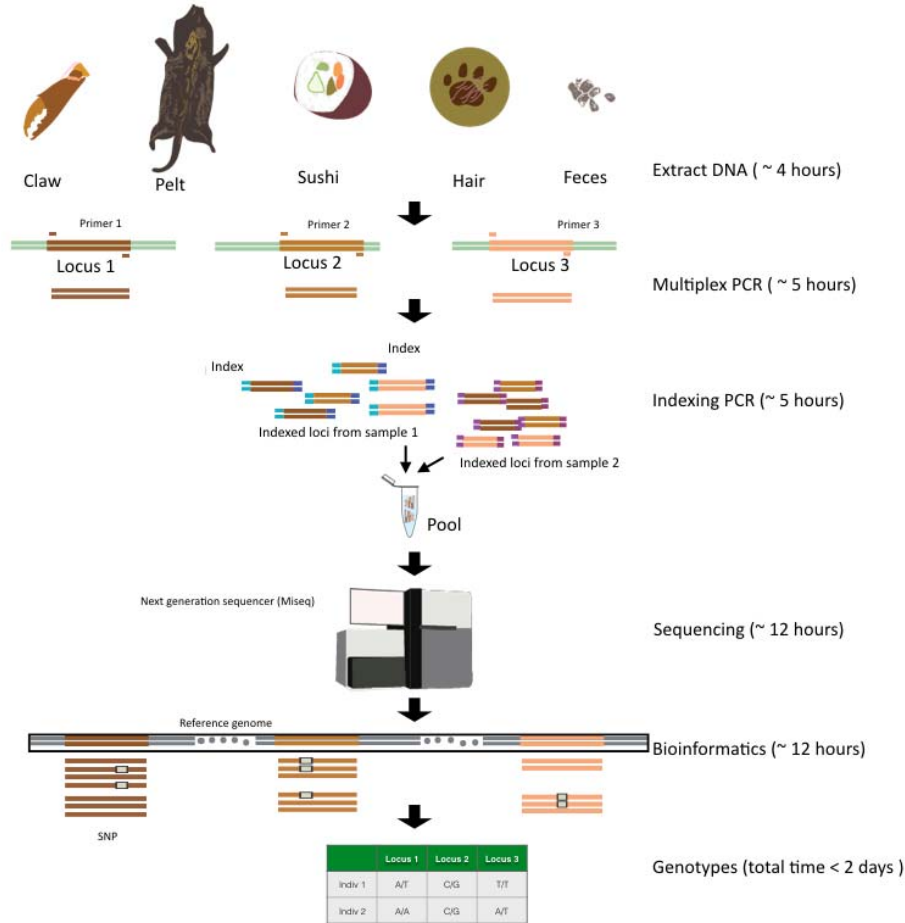
52 In principle, all of these goals can be achieved via genotyping a modest number of loci
53 such as microsatellites or single nucleotide polymorphisms (SNPs). To study endangered species
54 that are rare and elusive, approaches must be able to accommodate non-invasive sources of DNA
55 such as feces, shell, feathers, hair, and saliva, which yield impure, mixed, and/or extremely small
56 amounts of degraded DNA. Moreover, market samples generated by wildlife trade may be
57 processed, cooked, dried, or mixed with other species, again providing low quality and often
58 mixed DNA. Current approaches tend to require relatively large amounts of DNA (Carroll et al.
59 2018, nanograms of DNA) from the target species, or demand expensive and generally
60 inefficient enrichment strategies (Chiou & Bergey, 2018). Approaches designed for lower
61 concentration DNA samples (Kraus et al. 2015) require expensive and specialized equipment.

62 Here we demonstrate that a multiplex PCR approach followed by next-generation
63 sequencing satisfies all the requirements necessary for inexpensive, fast, and easy genotyping of
64 low-quality samples. Our approach is similar to GT-seq (Campbell, Harmon, & Narum, 2014)
65 but can use publicly available software for designing primers and calling SNPs, and targets only
66 very short fragments in order to succeed with degraded DNA. We illustrate the power of this
67 method for two endangered species in very divergent conservation contexts and real-life settings:

68 genotypes from feces, shed hair, and saliva found on killed prey from wild Indian tigers and from
69 CITES-regulated Caribbean queen conch imported to the US and sold in fried fritters (method
70 schematic Fig. 1a). Methods include DNA extraction, a multiplex PCR, a second barcoding PCR,
71 Illumina miseq sequencing and bioinformatics for SNP genotyping.

72 The tiger (*Panthera tigris*), a charismatic carnivore classified as endangered by the IUCN
73 red list. Their distribution across 14 countries (Goodrich et al. 2015) makes it critical that locally
74 collected data be comparable across their range. Genotyping of scat or hair, along with rapid
75 forensic testing of confiscated skins or other traded parts can verify species, individual identity,
76 and source populations. We developed a multiplex primer set for 192 SNP loci and tested them
77 on fecal, tissue, saliva, and hair samples from captive and wild tigers. We also genotyped two
78 sympatric carnivores, the Dhole (*Cuon alpinus*), and the leopard (*Panthera pardus*), that may be
79 confused with tigers when targeting noninvasive samples.

80 Our second example illustrates the use of this approach even when reference genomes are
81 unavailable, and again highlights use in difficult samples: in this case fried conch fritters.
82 Although formerly abundant, the queen conch (*Strombus gigas*) was listed in CITES Appendix II
83 in 1990, which allows for control of trade to reduce over-exploitation. Identifying geographic
84 ancestry of illegally traded queen conch products in Florida markets will aid conservation action
85 that will allow recovery of this formerly lucrative fishery. Because the most direct access to
86 imported conch is from the hundreds of restaurants in Florida, we sought techniques that would
87 allow genotyping from the most abundant menu item, fried conch fritters.



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Figure 1a A schematic for protocol and approximate time taken. Available samples include a variety of sources. Details in the main and supplementary text.

93 Methods

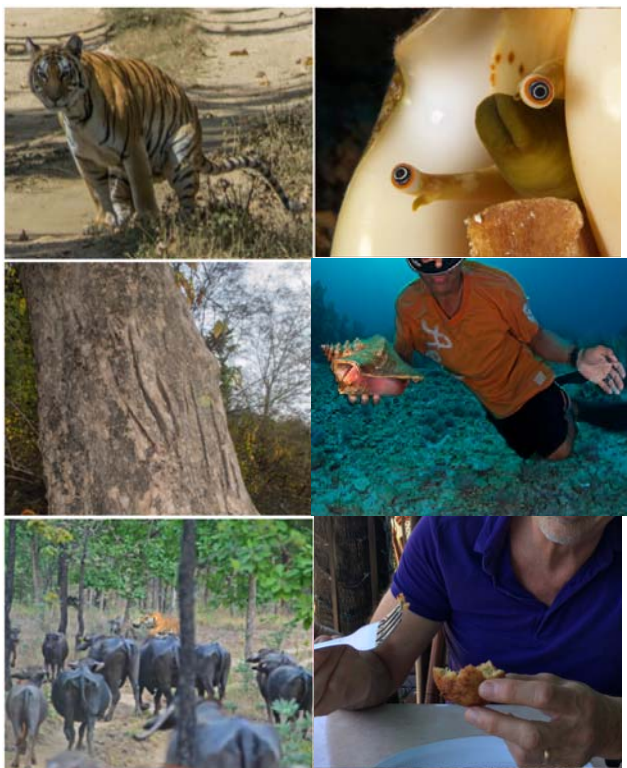
94 *Sample Collection*

95 For tigers, multiple samples from 13 captive tigers (USA zoos) representing different
96 scenarios were used for standardization (details in Table S1). Blood and corresponding fecal
97 swabs from captive tigers, (including one parent-offspring and one sibling pair) were collected in
98 India. Wild tigers were sampled noninvasively from multiple protected areas across India (Table
99 S2).

100 Scat and saliva (from predator bites on the prey) were sampled by swabbing the surface of the
101 sample with moistened synthetic swabs (Ramón-Lacaet al. 2015); tips were stored in lysis buffer
102 in 2ml microcentrifuge. Hair was sampled using forceps and stored in ziplock bags. DNA from
103 legacy fecal samples (collected in alcohol in 2014) were also tested. DNA extraction (first step in
104 Figure 1a) and quantification are described in SM1. Two leopards and one Dhole were included.
105 Most samples were genotyped in triplicate.

106 Tissue samples from live caught Queen Conch mantle were collected using a sterilized
107 biopsy forceps were preserved in RNALater. Samples from conch fritters purchased from Miami
108 restaurants were frozen until dissected to isolate animal tissue fragments (SM 3a).

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114 **Figure 1b** On the left: a tiger defecating (photo: Himanushu Chhattani), a scrape mark (and
115 associated hair samples, photo: Kaushal Patel), and a tiger hunting (photo: Shantanu Prasad,

116 associated lick marks). On the right, a conch emerging from its shell, a fisherman holding his
117 catch and a fritter.

118

119 *SNP Identification and filtering*

120 Tigers

121 We identified SNPs from whole genome sequencing of 75 tigers of wild and captive origin from
122 *P. t. tigris*, *P. t. jacksoni*, *P. t. altaica*, and *P. t. sumatrae* subspecies (SM 1a). Alignment,
123 filtering and pruning were used to identify SNPs (SM 1a). We calculated minor allele
124 frequencies (MAF) for each SNP within each subspecies, and retained SNPs with MAF 10, 15,
125 20, 25, and 30 percent. We identified fixed SNPs that differentiated populations. We prioritized
126 SNPs within contigs greater than 10, 5, and 1MB respectively. We selected 10 differentiating
127 SNPs from each subpopulation, and 9992 polymorphic SNPs from each of the aforementioned
128 MAF cutoffs, for a total of 50,000 SNPs.

129 Conch

130 We extracted RNA from 96 *L. gigas* individuals (SM 3a, table S3). Four queen conch individuals
131 (one each from Aruba, Belize, Florida, and St. Eustatius) were imported into TRINITY v2.2.0
132 (Grabherr et al. 2013) to assemble a *de novo* transcriptome (SM 3a).

133 480,962 SNPs were discovered by aligning 96 conch sequences from 6 populations in the
134 Caribbean to the assembled transcriptome(SFG pipeline -
135 <https://github.com/bethsheets/Population-Genomics-via-RNAseq>, SM 3a).

136

137 *Primer Design*

138 Publically available Primer3(amplicon size 50-90 bp, primer size 17-25 bp, and T_m of 60-61°C)
139 was used to design primers for tiger SNPs. Conch primers were designed using the G4C

140 (Genotyping for Conservation) script library and the same criteria. No attempt was made to
141 identify incompatibilities between primer-pairs and 192 primer pairs were shortlisted for both
142 species (SM 1b, SM 3b).GT-seq indexes and adapters were used (Campbell, Harmon, & Narum,
143 2014).

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145 *PCR amplification and sequencing*

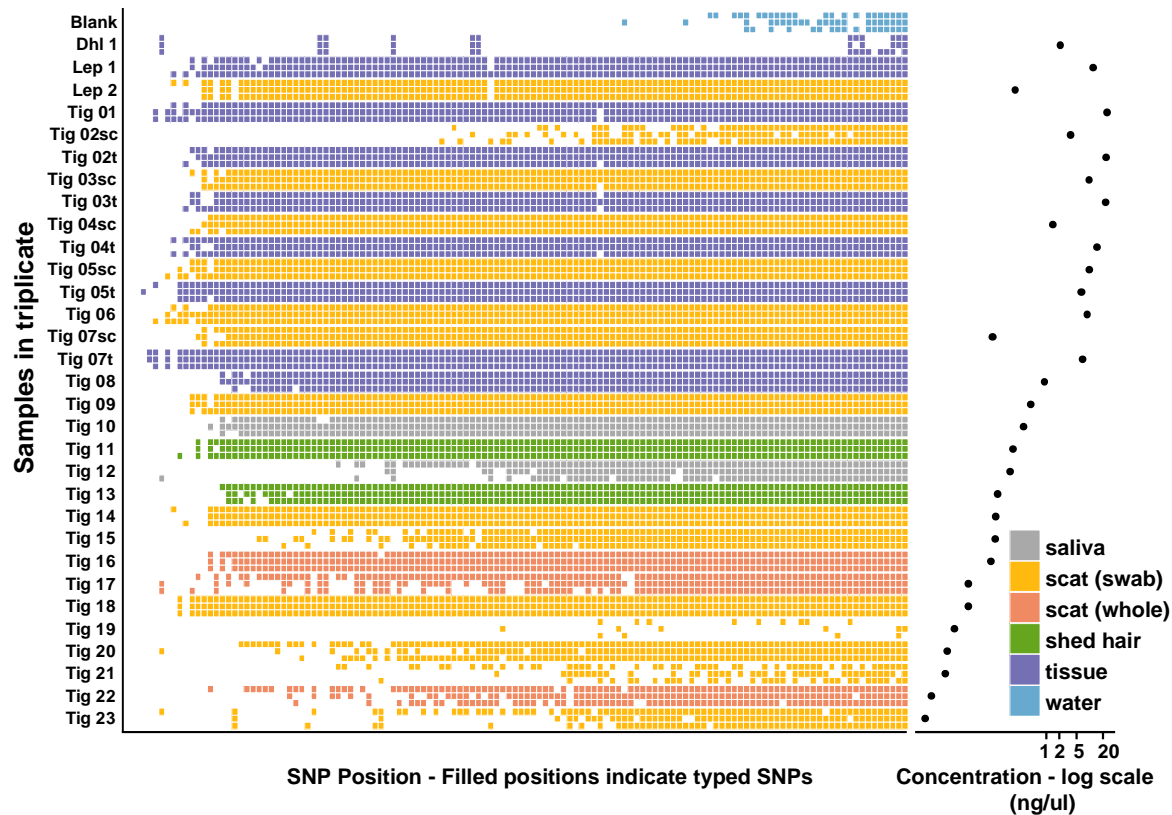
146 Library preparation consisted of an initial multiplex PCR reaction, a second PCR reaction
147 to add sequencing adapters and indexes, and sample pooling. Sample input DNA volume was
148 adjusted to a maximum of 1ng per reaction. The multiplex PCR simultaneously amplified all
149 target regions for each sample separately (in a 96 well plate). The second PCR reaction added a
150 combination of forward (i5) and reverse (i7) Illumina indexes to uniquely identify each sample.
151 The sequencing library contained equal volumes of each sample's barcoded product and was
152 cleaned with Ampure beads. Sequencing of single 50bp reads was performed on Illumina MiSeq.
153 A detailed protocol is in Supplement 1f. Alignment and genotype calling followed GT-Seq for
154 the conch study (SM3b; Campbell, Harmon, & Narum, 2014) and used standard open source
155 tools for the tiger study (SM1h). Figure 1a illustrates the steps described above.

156 For tigers, genotyping success, genotype concordance across replicates, relatedness
157 between pairs of individuals of known and unknown relationship, probability of identity of the
158 SNP panel and population structure were assessed (details in SM1h). For conch, genotyping
159 success and genotype concordance were tested by comparing SNPs across replicate conches.
160 Genetic distance and ability to assign the conch samples to the correct population was estimated
161 by comparison of the 96 transcriptome samples (details in SM 4).

162 **Results**

163 *Tiger*

164 126 targets (66% of 192 attempted) produced the most consistent results (SM2b, Figure
165 S1, Table S4), and were then tested on non-invasive samples from wild tigers across India and
166 zoo individuals (Fig. 1b, Table S2).

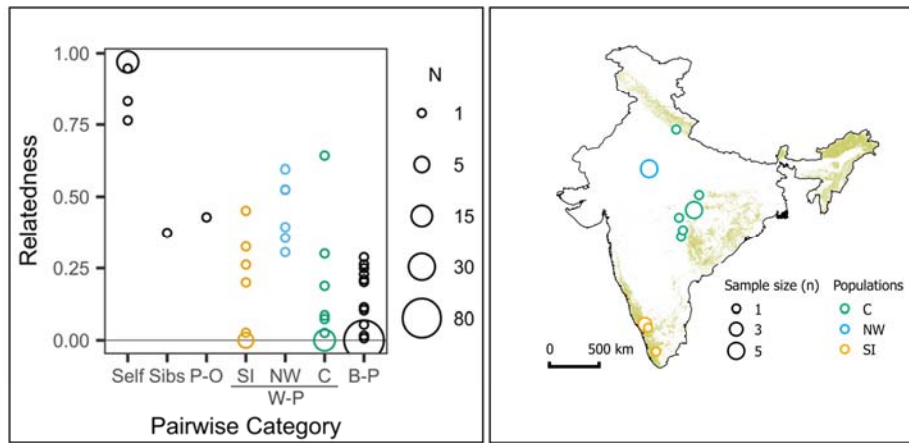


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169 **Figure 2** SNP typing success (in triplicate) for various tiger samples and controls. Filled cells
170 indicate successfully typed SNPs; color indicates sample type. DNA concentrations are on the
171 right.

172
173 The 126 SNP panel for wild tigers had a high overall genotyping success rate (Fig. 2, SM
174 2c). An average of 95 SNPs (75%, range: 4 - 114) were successfully typed across all samples.
175 When tiger DNA was > 0.01ng/ul (all sample types) an average of 105 SNPs (83%, range: 48 -
176 114) were typed.

177 Sample replicates were highly concordant with a high proportion of genotype matches
178 (n=28 triplicates, genotype concordance, mean: 0.957, range: 0.757 - 1.0). Different sample
179 types from the same individual also had highly concordant genotypes (n=5, concordance, mean
180 0.97; range 0.91 - 0.99). Our error rates were comparable to low microsatellite genotyping error
181 rates in some studies (Thaden et al. 2017) or lower other non-invasive studies (Mondol et al.
182 2014). Our probability of misidentification was vanishingly low ($p_{ID_{sibs}} = 1.6E-22$).

183 The co-occurring carnivores, leopard and dhole, could be distinguished from tiger
184 genotypes. The dhole tissue sample had very poor amplification success (mean across replicates
185 <10 SNPs) as expected. Leopards had high amplification success (mean: 110 SNPs), but SNPs
186 were monomorphic and nearly identical across two individuals (mean: 0.03% mismatches).



187 Figure 3: Pairwise relatedness (PI_HAT): Self: three replicates of each sample; same individual
188 but different sample, Sibs: siblings, P-O: parent-offspring pair, W-P: within population, B-P:
189 between populations. True relatedness unknown for W-P and B-P. Sampling locations and
190 corresponding colours represented in the India map on right.
191

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193 The known parent-offspring and sibling pairs (captive individuals, India) had relatedness
194 values close to expected values (0.5, Figure 3). Observed pair-wise relatedness was higher within
195 than between known genetic clusters or populations (see Natesh et al. 2017). As expected,
196 relatedness among individuals from a small, isolated population (NW, Figure 3) was high. Wild
197 individuals fell into three genetic clusters/populations as expected (Figure S2).

198

199 *Queen Conch*

200 We tested a 192 primer-pair panel on 279 conch samples from 14 populations, including
201 48 fried conch fritters from Miami, Florida restaurants. Our SNP success was lower for conch
202 than tigers (SM 4). Approximately half the 192 conch primer-pairs failed to provide data, but 62
203 targets reached an 86% success rate similar to the 126 good tiger targets (Fig. S3). Replicate
204 samples shared 99%-100% of their alleles, and different processed conch samples were
205 genetically identical, suggesting recapture. There were no obvious close relatives among the
206 samples (Fig. S4 and S5). Outlying islands (Aruba, St. Eustatius) were genetically differentiated
207 from the central Caribbean and Florida ($F_{ST} = 0.037, 0.048$ respectively, Table S5). Samples
208 from the same island group (e.g. Florida, Bahamas) or the same coast were not differentiated.
209 These patterns parallel a recent survey of queen conch with microsatellites (Truelove et al. 2017).

210 Conch DNA from deep-fried fritters had lower success rates than from biopsies. However,
211 success was high enough for individual identification and initial population comparison. Twenty-
212 three of the fritters were probably not Queen conch (fewer than 18 SNPs amplified), 8 fritters
213 revealed poor SNP amplification (average 41% success), and 17 fritters were comparable to fresh
214 samples (77% vs 86% success, Fig. S3). These samples revealed lower (average) genetic identity
215 to Florida populations (average 78.6 – 80.5 alleles shared, Fig. S6) compared to Puerto Rico, or

216 Andros Island (81.2-82.3, Fig. S6). While positive population identification may require greater
217 geographic sampling and more SNPs (from several whole genomes), our pilot data suggest that
218 the fritters we genotyped are less likely to be from the Florida Keys and Nassau. Importantly,
219 high resolution nuclear data was readily obtained from processed commercial samples to address
220 key conservation challenges.

221

222 **Discussion**

223 Our pilot datasets provide proof of concept for multiplex SNP genotyping of non-
224 invasive and processed market samples from two species with vastly different physiologies,
225 ecologies, and conservation challenges. The approach is successful for degraded, cooked, mixed,
226 or small and low-concentration samples (down to 10^{-3} ng DNA/ul), making it an ideal tool for
227 monitoring individuals under field conditions or from commercial markets.

228 Conservation practitioners assume that genetics is expensive. However, our method is
229 cheap, while providing rich information important for conservation. Designing a similar protocol
230 for a new species of interest would include costs for method development and implementation.
231 Development costs include polymorphic SNP ascertainment, primer design and synthesis.
232 However, note that SNPs have already been identified for many endangered and fisheries species
233 (e.g. Steiner et al. 2013). If no SNPs have been identified, practitioners could ascertain SNPs
234 using whole genome sequencing (e.g. genome assembly of reasonable quality ~\$2,000,
235 Armstrong et al., 2017) and pooled sequencing of 10 individuals at approximately 50X total
236 coverage, ~ \$1000). The upfront cost of primer design and synthesis is between \$1,000 to
237 \$10,000 (for 100 to 1,000 primer pairs, \$10 per primer pair). Once synthesized, primers can be
238 used for 38,000 reactions (~ 400 plates). The continual advance in sequencing and oligo synthesis

239 will drive down these initial development costs. Most important and attractive to conservationists,
240 we estimate implementation costs (for 1000 SNPs) can be as low as \$5 per sample (when
241 processing several hundred samples).

242 Increasing the number of SNPs beyond a few hundred can provide additional information.
243 For this pilot, we constrained the number of targeted SNPs, but it should be possible to target
244 many more. Primers chosen to amplify clusters of closely located SNPs should allow detection
245 of very recent inbreeding using long contiguous runs of homozygosity (Kirin et al. 2010). Linked
246 SNPs could generate microhaplotypes, particularly useful in pedigree reconstruction (Baetscher
247 et al. 2018). SNP panels could allow simultaneous species, individual, and diet identification for
248 sets of species, e.g. large carnivores and common prey species in India or sub-Saharan Africa.

249 Effective monitoring of individuals, populations and species is critical to designing rapid
250 conservation action and management of endangered species like tigers. Small, isolated
251 populations will require inbreeding management, genetics-based population assessment, and
252 genetically-informed introduction strategies. Likewise, identification of commercial products
253 from illegal fishing, bush meat hunting or highly processed market samples provides important
254 management information. Ability to assay such samples could provide a powerful incentive to
255 enforce local conservation laws. Rapid genetic monitoring of endangered species from
256 commonly occurring non-invasive samples will provide a data-pathway to species recovery. We
257 believe that multiplex PCR presents an example of such rapid, accessible, cheap and efficient
258 technology that will make this possible.

259

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330 analyses. MN, RWT, DP, UR, EAH, SP wrote the paper. **Competing interests:** RWT and

331 End2End Genomics LLC received NIH small business funding (R43HG009482) to develop tools
332 to study non-model species. **Data Availability:** Raw sequences are uploaded to NCBI
333 (PRJNA516037) and primer sequences for both tigers and conch are in Supplementary File
334 (Primer_Sequences).