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Short communication

Differentiation of two South African otter species (*Aonyx capensis* and *Lutra maculicollis*) from spraint based on partial *CytB* primer sets

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ABSTRACT

Accurate species identification based on visual cues can be challenging due to morphological similarities and the cryptic nature of certain species. Thus a more conclusive method of identification is required, namely DNA barcoding. This is the case regarding two South African otter species, Cape Clawless otter (*Aonyx capensis)* and the spotted necked otter (*Lutra maculicollis*). Due to the cryptic nature of these animals faecal samples, known as spraints, are the easiest way of confirming the presence of the animal in an area. In this study, we compared results obtained for universal and partial *CytB* primer sets on collected spraint and tissue control samples. Universal *CytB* primers revealed a low percentage of amplified otter species from faecal samples (species specific amplification success of 10.9%) whereas, the partial *CytB* primer set resulted in successful amplification of 45 out of 55 (82%) samples. We were thus able to positively differentiate between the two otter species using the partial *CytB* primer set developed in this study. The ability to accurately identify species using partial DNA will be beneficial in understanding numerous aspects of the behaviour and ecological importance of animals in their environment.

1. Introduction

Accurate species identification based on visual cues can often be difficult due to cryptic morphological features of species. In addition, morphological identification keys often rely on a certain life stage or gender of an animal to be used correctly (Hebert et al., 2003; Bonito, 2009; Chaves et al., 2012). In the case of two cryptic, co-occurring South African otter species, namely the Cape Clawless otter (*Aonyx capensis)* and the spotted necked otter (*Lutra maculicollis*), faeces (spraint) is used as a means of determining presence, space use and diet (Arden-Clarke, 1986; Van Niekerk et al., 1998; Perrin and Carugati, 2000; Angelici et al., 2005). However, due to similar diets the spraint can most often be visually similar, making it difficult to accurately identify the species occurring in a given area. In addition, erosion of spraint through exposure to environmental

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conditions leads to the physical breakdown of the spraint changing its appearance making it even more difficult to visually differentiate between the two species of otters based solely on the spraint (Rowe-Rowe, 1992). This can be problematic when trying to understand the movement patterns of the species, which is important for conservation purposes. This would, for example, influence the design of green corridors in urban environments, as the two species have different habitat requirements.

The South African Red Data Book (Friedmann and Daly, 2004) has listed *A. capensis* as 'least concern' due to habitat loss because of infrastructure development as a main threat. *L. maculicollis* is listed as 'near threatened' with indication of human induced habitat loss through agriculture, infrastructure development, exploitation/direct loss (persecution trapping as fish competitors and livestock predators) and accidental mortality caused by fish traps (Friedmann and Daly, 2004). *L. maculicollis* is listed as a protected species in National Environmental Management: Biodiversity Act (NEMBA, 2004), suggesting it is an indigenous species of high conservation value or national importance that requires national protection.

The development of a reliable method to differentiate these two otter species makes it easier to conduct a rough estimation of the occurrence rate of each species in an area, as well as help to better define their preferred habitats. Recent studies have shown how the use of genetic barcoding can be effectively used as a means of identifying species based on mitochondrial DNA (mtDNA) sequences (Hebert et al., 2003; Flynn et al., 2005; Sass et al., 2007; Corse et al., 2010; Chaves et al., 2012). Although this is a relatively easy and informative technique and; mtDNA is abundant in cells, most studies use non-degraded DNA such as that from tissue and blood samples. In the case of elusive animals it is often difficult to obtain tissue and blood samples, and alternative non-invasive samples can be used by collecting material left behind by the animal including hair, shed skin, egg shells and even faeces (Rowe-Rowe, 1992; Prigioni et al., 2005; Waits and Paetkau, 2005). The use of these non-invasive samples has furthered research on a variety of elusive, rare and endangered species which previously could not be studied due to various reasons such as ethics, safety concerns and even unavailability of good samples (Taberlet et al., 1997; Palomares et al., 2002; Medina-Vogel and Gonzalez-Lagos, 2008). Considering the obvious advantage of these non-invasive samples, there are also several drawbacks. Otter faecal samples do contain epithelial cells from the intestinal mucosa as excreta are pushed through the intestines the quality of the sample is low as there is a combination of the animal's DNA and DNA from prey eaten by the animal such as fish, small rodents, crabs, birds, and frogs (Dallas et al., 2000). This may be problematic as general barcoding of faecal samples used to identify unknown species tend to amplify the DNA of prey more than the target species as the primers used are usually universal and non-specific (Dallas et al., 2000). Another problem that arises from using faecal samples is the manner in which weather conditions can lead to degradation of genetic material (Hung et al., 2004). It requires technique optimisation to be able to use small amounts of DNA and target the amplification of short fragments. Despite this, degraded faecal samples have been used in previous studies to acquire genetic material (Meusnier et al., 2008). Considering this, the aim of this study is to describe the amplification of a short fragment ($<$150 bp) of the mitochondrial Cytochrome b (*CytB*) gene, isolated and amplified from faecal samples, and discuss its usefulness in genetically differentiating between the Cape clawless otter (*A. capensis*) and the Spotted necked Otter (*L. maculicollis*) found in South Africa. We have selected *CytB* as it is commonly used as a species-level marker and particularly so in mammalian biosystematics (Bradley and Baker, 2001; Pfunder et al., 2004).

2. Materials and methods

This study forms part of a larger project on the ecology of otters along river systems in the Gauteng province of South Africa, which required surveying of rivers banks for collecting otter spraints to conduct a detailed dietary analysis. The Jukskei River, Klip River, Hennops River, Crocodile River, Mooi River and Pienaars River along with their associated tributaries were the focus rivers of this study (Fig. 1). During the survey of the riverbank we aimed to collect fresh spraint with jelly (gelatinous secretion of anal scent glands), however the age of the sample and amount of jelly varied. Spraint found was collected with an inverted plastic zip-lock bag so as to prevent contamination of the sample. The bag was then reverted, sealed and allocated a unique identification number, and samples were immediately stored at −10 °C for further processing. The QIAGEN® QIAamp® DNA Stool Mini Kit (QIAGEN, 2010) was used for DNA extraction from otter spraint following the manufactures protocol for isolation of DNA from stool for human DNA analysis. A total of 55 samples were collected from 10 river systems.

2.1. Universal CytB primers

Initially, universal primers; L14724: CGAAGCTTGATATGAAAAACCATCGTTG and H15149: AAACTGCAGCCCCTCAGAAT-GATATTTGTCCTCA (Hsieh et al., 2003) were used to amplify approximately 400 bp of the *CytB* gene region, thereafter sequencing of this fragment was used to genetically assign collected samples to either of the two otter species found in South Africa. A total of two reference tissue samples, one each from the otter species; a road killed Cape clawless otter (*A. capensis*) from Knysna and spotted necked otter (*L. macullicollis*) found drowned from Grahamstown were used as controls.

Amplicons for sequencing were generated using the Dreamtaq PCR mastermix (Thermo Scientific, USA) at a final reaction volume of 25 μL. The reaction consisted of 12.5 μL of Dreamtaq mastermix (2x), 9.5 μL double distilled water (ddH_20), 10 pmol forward and reverse primers (Integrated DNA technologies, Coralville, IA, USA) and 1 μL (20 ng) of template DNA. The PCR conditions for the universal primers were as follows: one cycle of denaturation at 94 °C for 5 min (min) followed by

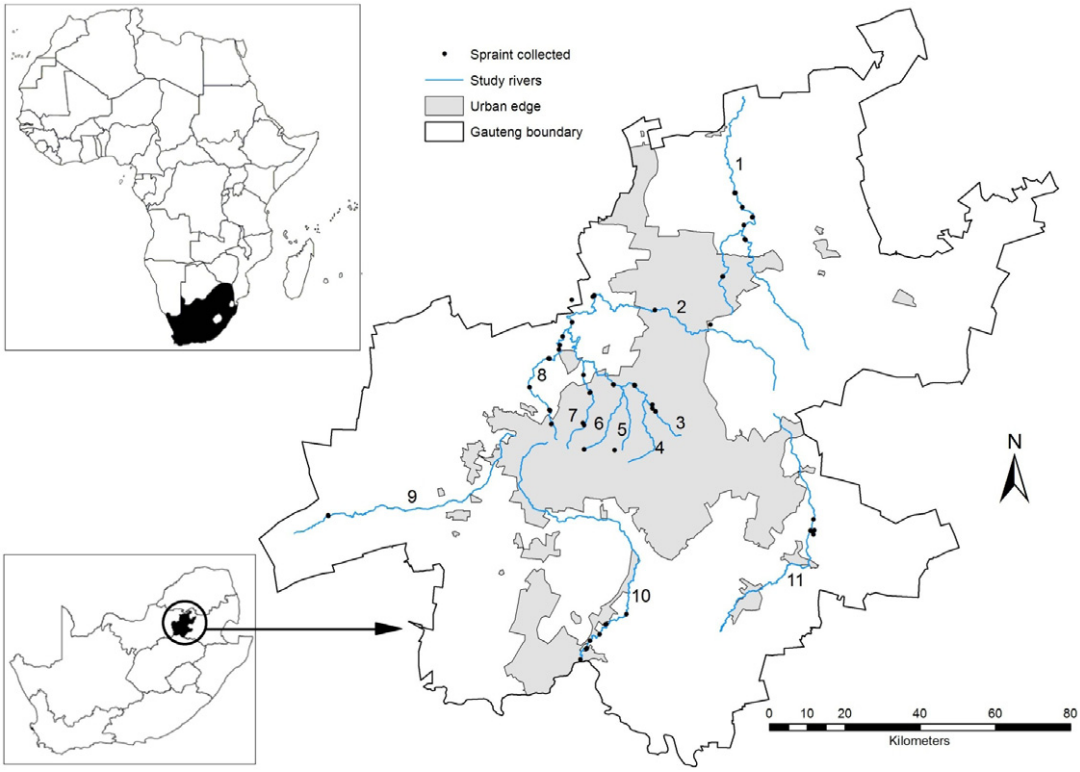

Fig. 1. Map of Gauteng province (South Africa) indicating the rivers surveyed, (1) Pienaars River; (2) Hennops River; (3) Modderfontein Spruit; (4) Jukskei River; (5) Sand Spruit; (6) Braamfontein Spruit; (7) Klein Jukskei River; (8) Crocodile River; (9) Mooi River; (10) Klip River; (11) Blesbok Spruit and locations at which spraint was located and collected. Gauteng is located in the northern section of South Africa as indicated by the map insert.

30 cycles of denaturation at 94 °C for 30 s (s); annealing at 50 °C for 30 s and extension at 72 °C for 1 min and then a final extension at 72 °C for 10 min. The PCR was conducted in a T100^{TM} Thermal Cycler (Bio-Rad Laboratories, Inc., CA, USA). Amplicons generated during PCR were purified using FastAP alkaline phosphatase and exonuclease I and the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) was used for cycle sequencing. Sequences were resolved on an ABI3130 automated sequencer (Applied Biosystems). Sequence Navigator 1.01 (Applied Biosystems) was used for initial sequence analysis of each individual specimen while the resulting sequence chromatograms were viewed and edited in the Chromas programme which is embedded in MEGA 5 (Tamura et al., 2011). Comparison to reference sequences generated from tissue samples enabled individual species identification.

2.2. Partial CytB primers

Partial *CytB* primers; CytB.S.A3 F: TGCCTGTTCCTACACGT and CytB.S.A3 R: AGTTAGTGATTACGGTTG, were developed based on aligned sequences of the cape clawless and spotted necked otter tissue samples generated above. Primers were positioned in areas flanking inter-specific sequence differences and were designed using OligoAnalyzer 3.1 (Integrated DNA technologies) with the aim of amplifying a 130 bp fragment of the *CytB* gene. Additionally, a tissue sample of Eurasian otter (*Lutra lutra*) from Hungary was analysed to determine the amplification success of these primers on related otter species. Amplicons for sequencing were generated using the above mentioned Dreamtaq PCR mastermix protocol. The PCR conditions for the partial *CytB* primers were as follows: one cycle of 94 for 5 min then 10 cycles of 94 °C for 30 s, 55 °C for 50 s and 72 °C for 1 min; 15 cycles of 94 °C for 30 s, 50 °C for 50 s and 72 °C for 1 min; and lastly 20 cycles of 94 °C for 30 s, 45 °C for 50 s and 72 °C for 1 min and finally one cycle at 72 °C for 10 min. Purification, sequencing and sequence analysis was performed as discussed above.

2.3. Phylogenetic analysis

Following sequence alignment using the ClustalW function in MEGA5, the National Center for Biotechnology Information (NCBI) BLAST function was used to identify all possible GenBank reference samples (Eurasian otter, *Lutra lutra* EF689067; Cape clawless otter, *A. capensis* AF057118 and the Spotted necked otter, *L. maculicollis* AF057125), as well as a suitable outgroup (Stoat, *Mustela erminea*_JX130899) for phylogenetic analyses. Distance based analyses (Neighbour-joining, NJ) of

Table 1
Alignment of the partial CytB gene. Sequences were aligned in Mega. Each dot "." in the sequence indicates a nucleotide (A, G, C or T) that is identical to the one at the corresponding position in the sequence of *Lutra lutra*. The "A", "G", "C" or "T" indicates sequence differences between the samples and hyphens "-" indicate gaps present in the sequence. All sequences analysed were approximately 100 bp in size.

	10	20	30	40	50	60	70	80	90
EF689067.1_Lutra lutra	ATTCTACTAT	TCGCAACCAT	AGCAACAGCA	TTCATAGGTT	ACGTATTACC	ATGAGGACAA	ATATCCTTTT	GAGGCGCAAC	CGTAATCACC AA
AF057125.1_Lutra maculicollis	..CT......	.T..GGT...	G.........	....	....-.....	....	....	....T.....	.........T ..
AF057118.1_Aonyx capemsis	..CTA-T...	..A....T..	....	....	....C.....	......G...	........C.	....	.........T ..
JX130899.1_Mustela erminea	..CT..T...	.....GTT..	......T...	....	....T.....	....	.....A..C.	....	......T..T ..
Lutra lutra ref	....	....	....	....	....	....	....	....	.........T ..
Lutra maculicollis ref	..CT......	.T..GGT...	G.........	....	....T.-...	....	....	....T.....	.........T ..
Aonyx capensis ref	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	....-....T ..
Unknown1	..CT..T...	..A....T..	.........-	.....-....	....C.....	......G...	........C.	....	....-....T ..
Unknown2	..CT..T...	..A....T..	.........-	....	....C.....	......G...	........C.	....	.........T ..
Unknown3	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	.........T ..
Unknown4	.ACT..T...	..A-C..T..	....	.-..A.....	....C.....	......G...	........C.	....	..-......T ..
Unknown5	..CTA-T...	..A....T..	....	....	....C.....	......G...	........C.	....	.........T ..
Unknown6	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	..-......T ..
Unknown7	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	..-......T ..
Unknown8	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	.........T ..
Unknown9	..CT..T...	..A-...T..	....	....	....C.....	......G...	........C.	....	..-......T ..
Unknown10	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	..-......T ..
Unknown11	..CTA.T...	..A....T..	....	....	....C.....	......G...	........C.	....	.........T ..
Unknown12	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	..-......T ..
Unknown13	..CT......	.T..GGT...	G.........	....	....T.....	....	....	....T.....	.........T ..
Unknown14	..CT......	.T.AGGT...	G.........	....	....T.....	....	....	....T.....	.........T ..
Unknown15	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	..-......T ..
Unknown16	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	.TA......T ..
Unknown17	..CT......	.T..GGT.T.	G...C....T	...T......	....T.....	....	....	....T.....	.........T ..
Unknown18	..CT......	.T..GGT...	G...C....T	....	....T.....	....	....	....T.....	.........T ..
Unknown19	..CT......	.T..GGT...	G.........	....	....T.....	....	....	....T.....	.........T ..
Unknown20	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	.........T ..
Unknown21	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	.........T ..
Unknown22	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	.........T ..
Unknown23	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	.........T ..
Unknown24	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	..-......T ..
Unknown25	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	..-......T ..
Unknown26	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	.........T ..
Unknown27	..CT.-A.T.	..A....T..	....	....	....C.....	......G...	........C.	....	..A......T ..
Unknown28	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	..A......T ..
Unknown29	..CT..T.-.	..A....T..	....	....	....C.....	......G...	........C.	....	.........T ..
Unknown30	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	.........T ..
Unknown31	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	..-......T ..
	10	20	30	40	50	60	70	80	90
EF689067.1_Lutra lutra	ATTCTACTAT	TCGCAACCAT	AGCAACAGCA	TTCATAGGTT	ACGTATTACC	ATGAGGACAA	ATATCCTTTT	GAGGCGCAAC	CGTAATCACC AA
Unknown32	..CT.-A.T.	..A....T..	....	....	....C.....	......G...	........C.	....	..A......T ..
Unknown33	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	.........T ..
Unknown34	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	.........T ..
Unknown35	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	.........T ..
Unknown36	..CT.-A.T.	..A....T..	....	....	....C.....	......G...	........C.	....	..A......T ..
Unknown37	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	..-......T ..
Unknown38	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	..A......T ..
Unknown39	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	.........T ..
Unknown40	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	.........T ..
Unknown41	..CT..T...	..A....T..	....	....	....C.....	......G...	........C.	....	....G....T ..
Unknown42	..CT......	.T..GGT...	G.........	....	....T.....	....	....	....T.....	.........T ..
Unknown43	..CT......	.T.AGGT...	G.........	....	....T.....	....	....	....T.....	.........T ..
Unknown44	..CT......	.T..GGT...	G...C....T	....	....T.....	....	....	....T.....	.........T ..
Unknown45	..CT......	.T..GGT.T.	G...C....T	...T......	....T.....	....	....	....T.....	.........T ..

the final dataset was conducted in MEGA5 using p-distance estimates with nodal support being assessed through 10 000 non-parametric bootstrap replications.

3. Results and discussion

Universal *CytB* primers revealed a low percentage of amplified otter species from faecal samples. Amplification was achieved in 11 out of 55 samples. However, BLAST search of sequences generated using universal CytB sequences revealed prey species (45%) and just six (55%) samples were identified as otter with 97%–100% similarities to the NCBI database sequences. The overall species specific amplification success rate was 10.9% (6/55), thus this primer set presented low amplification efficiency, as well as low specificity to otter DNA.

Thus partial primers were developed that sequenced a 130 bp fragment of the gene. Sequence chromatograms generated from the partial primer set were well defined with no background interference at the baseline. The partial *CytB* primer set resulted in successful amplification of 45 out of 55 (82%) samples. Lack of amplification in the remaining ten samples could be due to (i) type of sample, spraint with no or little jelly, and (ii) temperature, samples exposed to increased temperature

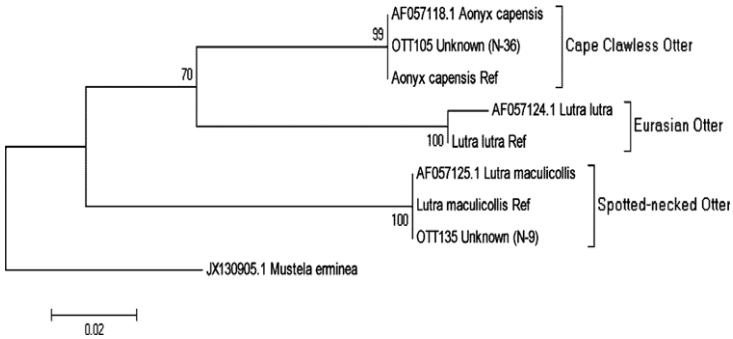

Fig. 2. Evolutionary relationships of taxa as inferred by Neighbour-Joining analyses of partial *CytB* fragment (CC-Cape clawless otter, EO-European otter, SN-Spotted necked otter, N-total number of identical samples/sequences). Accession numbers precede all reference samples downloaded from GenBank.

at time of collection. Amplification of prey remains was not observed. Subsequent BLAST results identified 36 samples as Cape clawless Otter and 9 as spotted necked otter with sequence similarities varying from 90% to 98% for both otter species. The alignment for this section is presented in Table 1.

Distance based analyses of the complete 52 sample dataset (45 faecal samples that were analysed during this study, two reference tissue samples and one sample from Eurasian otter sequenced during this study and four reference samples acquired from GenBank) revealed a clear genetic separation between the Cape clawless otter and the spotted necked otter based on this 130 bp fragment (Fig. 2). Bootstrap support for individual species clusters was high (98%–99%), although support for the phylogenetic relationship between the three otter species was not well supported. The bootstrap value for the relationship among the three species is expected to not be robust due to the size of the fragment (120–130 bp). Phylogenetic relationships among the Mustelidae family have been resolved in various studies such as that by Koepfli et al. (2008) which show that these three otter species represent different phylogenetic lineages. The grouping observed in the study presented here is supported by the phylogram published by Koepfli et al. (2008). Separate clustering of the European otter suggests that this protocol will also be able to identify samples from this species. Thus, due to the inter-specific differences observed, the partial protocol presented here can be used to accurately differentiate this additional species and does not result in the amplification of prey remains. This method is a relatively easy and informative technique to be used on degraded DNA samples.

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