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Research Article

Cytochrome c Oxidase Sequences of Zambian Wildlife Helps to Identify Species of Origin of Meat

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Accurate species identification is a crucial tool in wildlife conservation. Enforcement of antipoaching law is more achievable with robust molecular identification of poached meat. Determining the region where the animal may have been taken from would also be a useful tool in suppression of cross-border trade of poached meat. We present data from a cytochrome c oxidase "barcoding" study of Zambian ruminants that adequately identifies the species of origin of meat samples. Furthermore, the method demonstrates possible improvement and application in regional variation in sequence identity that has a potential for discriminating meat samples from different subpopulations.

1. Introduction

Marginal wildlife utilization has historically been an integral part of most African cultures. Unfortunately, recent trends have shown that this has now been transformed from subsistence into an illegal multimillion dollar industry [1, 2], with often negative consequences on target animal species' populations and the environment.

In Zambia, poaching for game meat or trade in wildlife products is one of the most damaging human activities to wild animal populations. Poachers evade law enforcement by removing easily recognisable body parts when transporting game meat or by processing or semiprocessing meat to make it difficult to identify the species from which it was derived. After such treatment, commonly used identification methods such as smell or meat texture appearance lack scientific credibility and cannot be used during prosecution.

Wildlife forensics often provides scientific credibility in prosecuting wildlife crime. This study is based upon the use of DNA sequence analysis of meat or other animal tissues to ensure accurate species identification. The most common methods of species identification use mitochondrial DNA, particularly the cytochrome b (Cytb) and cytochrome oxidase I (COI) [2–5] genes. The COI gene has been identified by the Barcode of Life Project as the best gene for differentiating animal species. A broad range of primers are available for this gene, which are applicable to a wide range of taxa [6, 7]. The sequence of the gene is relatively short and changes to the sequence occur more slowly than in the Cytb gene [8]. The COI gene can be used to characterize individuals not only at higher taxonomic levels (phyla and order) but also at a species level [9, 10]. Through the Barcode of Life Project (http://www.barcodinglife.org/), the COI gene is the focus of a worldwide molecular effort to characterize all living animals using a single genetic entity [11]. The COI gene therefore potentially allows forensic scientists to identify the species of origin of biological samples, through DNA sequence analysis and comparison with the Barcode of Life Database (BOLD). This database is still being built, but it will be possible to identify the species from which a sample is originated if the species has already been deposited in the database. The presence of many sequences from different regions also has potential to help in analysing geographical clustering of barcoding sequences which can be very useful for bushmeat

Table 1: The species, number of samples, and the method of sample preservation used in this study.

Common name	Scientific name	Animals sampled	Sample preservation
Puku	*Kobus vardoni*	7	70% ETOH
Eland	*Taurotragus oryx*	3	70% ETOH
Impala	*Aepyceros melampus*	3	70% ETOH
Kafue Lechwe	*Kobus leche kafuensis*	3	70% ETOH & PBS
Bushbuck	*Tragelaphus sylvaticus*	2	PBS and frozen
Waterbuck	*Kobus ellipsiprymnus*	2	PBS and frozen
Buffalo	*Syncerus caffer*	2	PBS and frozen
Wildebeest	*Connochaetes taurinus*	2	PBS and frozen
Sable	*Hippotragus niger*	2	PBS and frozen
Reedbuck	*Redunca arundinum*	2	Formalin
Hartebeest	*Alcelaphus buselaphus*	1	Formalin

ETOH: ethanol; PBS: phosphate buffered saline.

identification and tracing. A preliminary validation study has already been conducted against forensic standards [12] but the technique still needs to be tested in different parts of the world for both identification and geographical clustering of barcoding sequences [13].

This study focused on the application of this technique to some Zambian wildlife and comparison of the sequences obtained by COI barcoding to previously characterized species from elsewhere in Africa. Several common game species for which there was no previous sequence identification available in the publicly accessible databases were also characterized and deposited.

2. Materials and Methods

2.1. Study Samples. Tissue samples were collected from 29 known individual animals representing 11 species of Bovidae in game ranches and national parks around Lusaka and south Luangwa, Zambia. The samples were collected with the help of wildlife police officers and game ranch staff to guarantee species identification. Table 1 shows the species sampled, the number of animals sampled for each species, and the methods used to preserve samples before DNA extraction.

2.2. DNA Extraction. DNA was extracted using the Qiagen Blood and Tissue Kit, according to standard protocols for all samples except those preserved in formalin where the Qiagen FFPE kit was used.

2.3. Polymerase Chain Reaction and Sequencing. The COI gene fragments were amplified using the polymerase chain reactions (PCR) in 25 μL reaction mixture. The primers used were COIbF (5′-TTTCAACCAACCACAAAGACATCGG-3′) and COIbR (5′-TATACTTCAGGGTGTCCAAAGAATCA-3′) [13]. Amplification was carried out in 10x reaction buffer containing 2.5 mM of $MgCl_2$, 0.2 mM each dNTP and 0.75 units of Taq DNA polymerase (All New England Biolabs; https://www.neb.com/), 10 mM each primer (Invitrogen, www.invitrogen.com), and 1 uL template DNA. PCR amplification was performed on Techne TC-512 Thermal Cycler (Barloworld Scientific Ltd.) with 15 min at 95°C for initial polymerase activation, followed by 35 cycles of amplification (30 s at 94°C, 30 s at 55°C, and 1 min at 72°C) and final extension for 10 min at 72°C. Products were viewed via gel electrophoresis with ethidium bromide and amplified PCR products were purified using a kit (Nucleospin® Extract II, Macherey-Nagel). Sequencing was performed using BigDye terminator v3.1 as per protocol on an ABI 3730 DNA sequencer (Applied Biosystems).

2.4. Data Analysis. Bidirectional contig assembly and editing were carried out using the CAP3 contig assembly program [14] through BIOEDIT [15]. Species identification was performed via BLASTn [16] searches against the NCBI nucleotide collection and BOLD databases.

Species identifications were verified using a phylogenetic dataset consisting of 27 sequences representing most subfamilies of Bovidae present in Africa. Where available, sequences from southern or central African isolates were used. Accession numbers and geographic origins of these sequences are shown in Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/1808912. Sequences were aligned using MUSCLE [17, 18] under the default settings. Phylogenetic trees were generated using PhyML [18, 19] under the general time reversible nucleotide substitution model with optimised tree topology and between site variations. Branch support was calculated through PhyML using the approximate likelihood ratio test [19]. A sequence from the deer *(Dama dama)* was used as an outgroup.

After species identification, phylogenetic trees were generated to establish whether barcoding information is sufficient to establish the geographic origin of samples. A dataset was created with all available COI sequences of the test species set. A phylogenetic tree for this dataset was generated as above. The sequences included in this analysis are listed in Supplementary Table 2.

3. Results

Of the 11 species examined the COI genes of 9 species were amplified successfully. The most representative sequence of

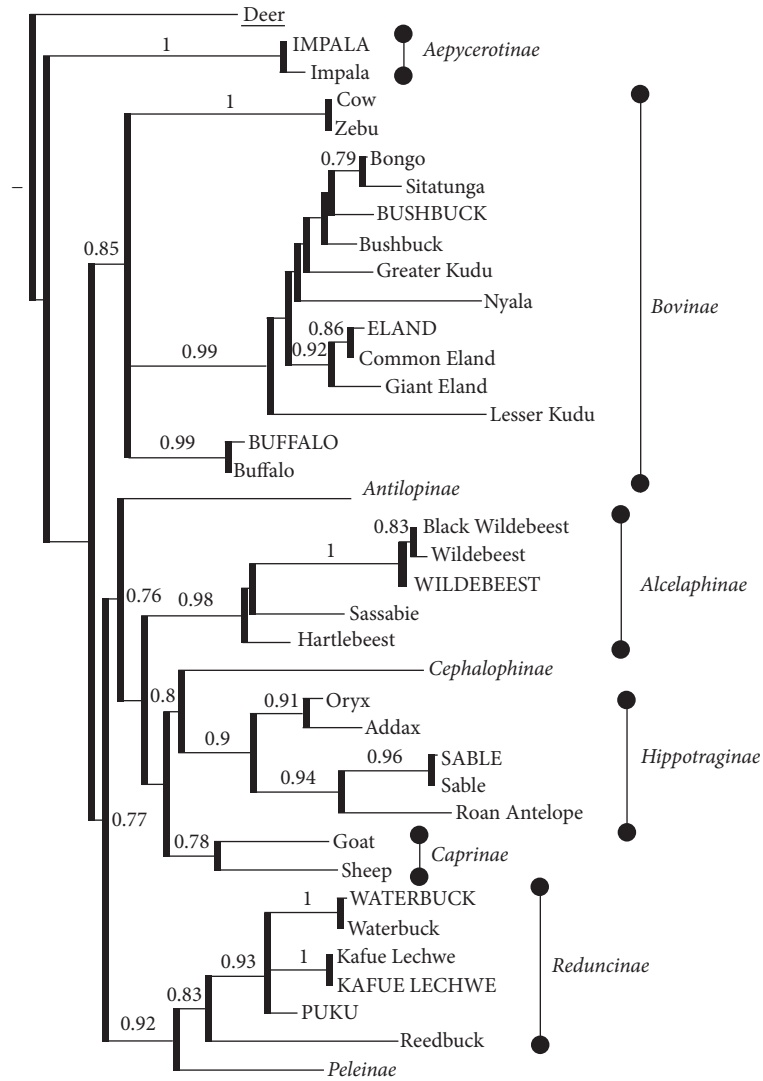

Figure 1: PhyML maximum likelihood phylogenetic tree of the cytochrome c oxidase subunit 1 gene for waterbuck, impala, reedbuck, oryx, addax, duiker, sheep, goat, nyala, sitatunga, bongo, hartebeest, sassabies, cattle, deer, kudu, wildebeest, eland, bushbuck, and buffalo sequences. Zambian test sequences are shown in capital letters (Supplementary Table 1). Bootstrap values exceeding 75% are shown on the nodes. The deer sequence was used as an outgroup to root the tree. GenBank accession numbers and geographical information are provided in Supplementary Table 1.

each of these species was deposited in the GenBank database with the following reference numbers: puku *(Kobus vardoni)* JQ690383, eland *(Taurotragus oryx)* JQ690384, impala *(Aepyceros melampus)* JQ690386, Kafue Lechwe *(Kobus leche kafuensis)* JQ690387, waterbuck *(Kobus ellipsiprymnus)* JQ690391, buffalo *(Syncerus caffer)* JQ690392, wildebeest *(Connochaetes taurinus)* JQ690393, sable *(Hippotragus niger)* JQ690394, and bushbuck *(Tragelaphus scriptus)* JQ690389. No PCR products were recovered from reedbuck *(Redunca arundinum)* or Lichtenstein's hartebeest *(Alcelaphus buselaphus lichtensteini)*.

All nine of these species could be robustly identified by similarity searching or phylogenetic analysis. The BOLD database has sequences for all the test species except puku. All other samples could be identified robustly at a species level, with the exception of the wildebeest, which was only resolved to genus level and bushbuck, which was not recognised. Using BLASTn against the nr database, which is slightly less stringent, bushbuck was identified at species level and wildebeest and puku at genus level. In phylogenetic analysis all samples with an available African barcode sequence clustered closely and robustly with their species of origin as shown by the values in Figure 1.

In the geographic analysis (Figure 2), there was no consistent relationship between country of origin and phylogeny. For impala and wildebeest, sequences tended to cluster by country. For buffalo, bushbuck, waterbuck, and eland however, several clades contained sequences of mixed origin, including some geographically distant sequences such as waterbuck sequences from Tanzania and Niger. Zambian sequences were generally fairly distinct from sequences from elsewhere in Africa. Insufficient sequences

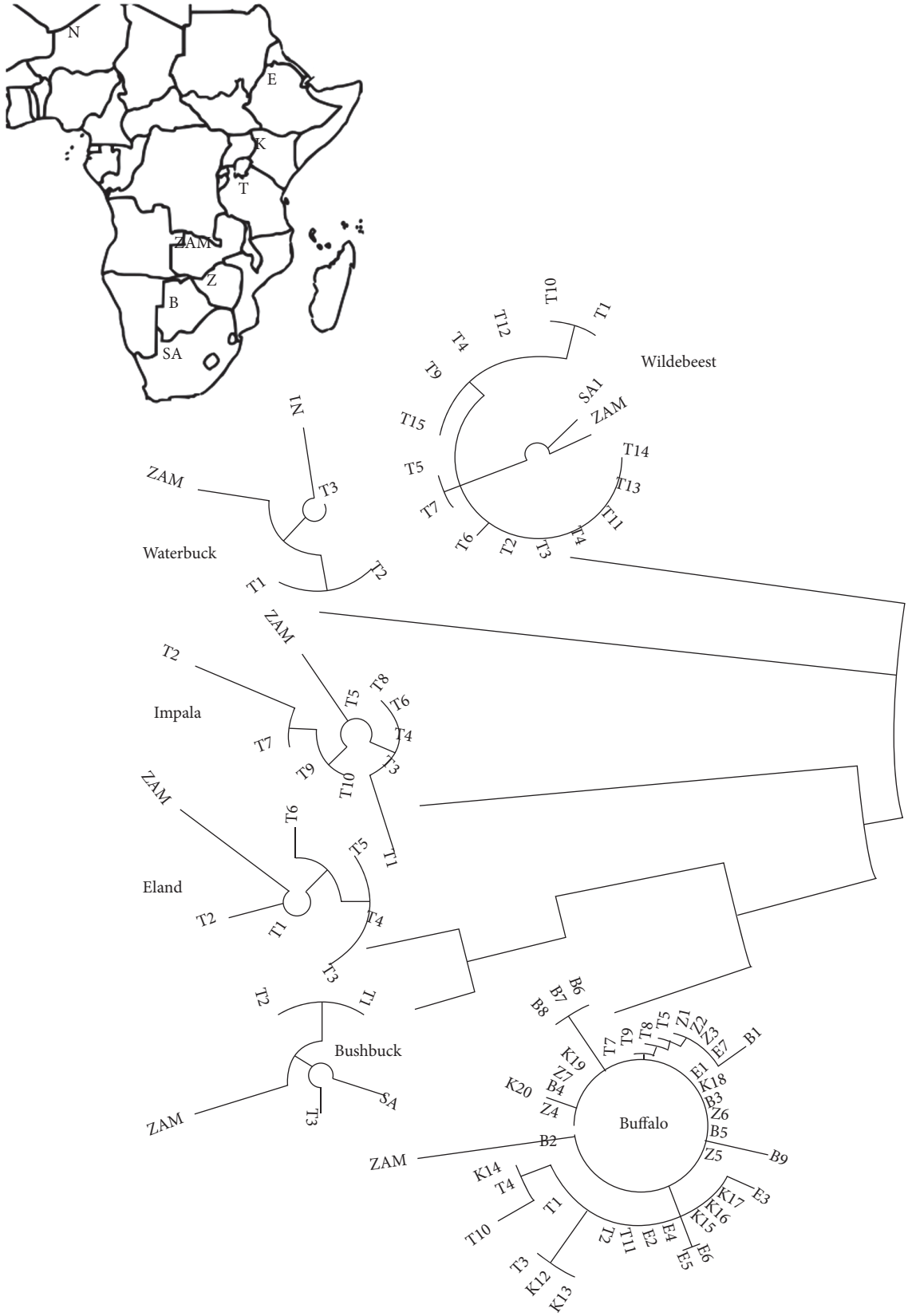

Figure 2: PhyML maximum likelihood phylogenetic tree for available COI sequences from waterbuck, impala, wildebeest, eland, bushbuck, and buffalo of known geographic origin. Sequences are labelled as follows: Zambia, ZAM; Ethiopia, E; Kenya, K; Tanzania, T; Zimbabwe, Z; Botswana, B; South Africa, SA; Niger, N. GenBank accession numbers are provided in Supplementary Table 2.

were available for geographic analysis of the remaining samples.

4. Discussion

The basic technique of COI barcoding proved a robust means of identification of meat samples from the species examined. Our findings here were in broad agreement with that of Bitanyi et al. [13] in Tanzania that covered an overlapping range of species, confirming the applicability of this methodology in forensic identification and conservation genetics of game species in general [20]. The only species for which sequence data could not be obtained were for those specimens preserved in formalin which has a known detrimental effect on DNA quality [21]. We therefore concluded that using formalin to preserve samples may not be ideal for this method, which is unfortunate because most samples collected from the Zambian wild are preserved in formalin.

Two species, wildebeest and puku, could only be identified at a genus, rather than species level. For puku, this is simply the result of a lack of data as no previous puku barcode sequences were available at the time of the study. The barcoding technique was not suitable for very detailed identification of samples if the species had not been sequenced previously. However, phylogenetic analysis showed that the sample was of a *Kobus* sequence and not *K. ellipsiprymnus* or *K. leche*, which only leaves four possible species and is likely to be adequate for most purposes. This problem will become less common as the barcoding database grows. For wildebeest, the ambiguity was between two very closely related species, the blue wildebeest *C. taurinus* and black wildebeest *C. gnou*. The COI gene may be too conserved to distinguish between very similar species, although again the technique is likely to be sufficient for most purposes when prosecuting poaching cases.

The geographic analysis showed that the COI sequence is generally not very reliable in determining the geographic origin of a sample. Some species, wildebeest, impala, and eland, did show distinct geographical clustering separating Tanzanian and Zambian sequences. For buffalo, bushbuck, and waterbuck this was not the case. Zambian sequences tended to fall outside of groups of sequences from other African countries and it would be worthwhile to sequence more samples from these populations to see if they would form unique clusters. All sequences analysed, except the waterbuck sequence from Niger, were from adjacent countries (Figure 2), so it is possible that the populations analysed from different countries are not isolated from each other. It is also likely that, given the short length and high conservation of the COI gene, there is too little genetic variation for detailed within species analysis. However, it would generally be possible to establish whether or not a sample is likely to have come from a particular well characterized population.

5. Conclusion

COI barcoding was consistently successful in easily identifying the species of origin of samples from Zambian wildlife species, providing that the species has been previously barcoded. When this was not the case, samples could be identified at genus level. Therefore, this technique would allow identification of the origin of poached meat samples. However, without significantly more data, the technique cannot reliably establish which population of animals a sample is from.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] P. Ferrier, "The economics of agricultural and wildlife smuggling," *United States Development Agency Economic Research Report*, vol. 8, pp. 1–35, 2009.

[2] Interpol, "Illegal Ivory and Rhino horn trade targets of Interpol co-ordinated operation across Southern Africa," http://www.interpol.int/News-and-media/News-media-releases/2010/PR036.

[3] C. Bellis, K. J. Ashton, L. Freney, B. Blair, and L. R. Griffiths, "A molecular genetic approach for forensic animal species identification," *Forensic Science International*, vol. 134, no. 2-3, pp. 99–108, 2003.

[4] H.-M. Hsieh, L.-H. Huang, L.-C. Tsai et al., "Species identification of rhinoceros horns using the cytochrome b gene," *Forensic Science International*, vol. 136, no. 1–3, pp. 1–11, 2003.

[5] W. Parson, K. Pegoraro, H. Niederstätter, M. Föger, and M. Steinlechner, "Species identification by means of the cytochrome b gene," *International Journal of Legal Medicine*, vol. 114, no. 1-2, pp. 23–28, 2000.

[6] O. Folmer, M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek, "DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates," *Molecular Marine Biology and Biotechnology*, vol. 3, no. 5, pp. 294–299, 1994.

[7] S. K. Verma and L. Singh, "Novel universal primers establish identity of an enormous number of animal species for forensic application," *Molecular Ecology Notes*, vol. 3, no. 1, pp. 28–31, 2003.

[8] M. Lynch and P. E. Jarrell, "A method for calibrating molecular clocks and its application to animal mitochondrial DNA," *Genetics*, vol. 135, no. 4, pp. 1197–1208, 1993.

[9] P. D. N. Hebert, A. Cywinska, S. L. Ball, and J. R. DeWaard, "Biological identifications through DNA barcodes," *Proceedings*

of the Royal Society of London B: Biological Sciences, vol. 270, no. 1512, pp. 313–321, 2003.

[10] P. D. N. Hebert, S. Ratnasingham, and J. R. DeWaard, "Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species," *Proceedings of the Royal Society B: Biological Sciences*, vol. 270, no. 1, pp. S96–S99, 2003.

[11] S. Ratnasingham and P. D. N. Hebert, "bold: the barcode of life data system (http://www.barcodinglife.org)," *Molecular Ecology Notes*, vol. 7, no. 3, pp. 355–364, 2007.

[12] N. Dawnay, R. Ogden, R. McEwing, G. R. Carvalho, and R. S. Thorpe, "Validation of the barcoding gene COI for use in forensic genetic species identification," *Forensic Science International*, vol. 173, no. 1, pp. 1–6, 2007.

[13] S. Bitanyi, G. Bjørnstad, E. M. Ernest et al., "Species identification of Tanzanian antelopes using DNA barcoding," *Molecular Ecology Resources*, vol. 11, no. 3, pp. 442–449, 2011.

[14] X. Huang and A. Madan, "CAP3: a DNA sequence assembly program," *Genome Research*, vol. 9, no. 9, pp. 868–877, 1999.

[15] T. A. Hall, "BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT," *Nucleic Acids Symposium Series*, vol. 41, pp. 95–98, 1999.

[16] S. F. Altschul, W. Gish, W. Miller, E. W. Myers, and D. J. Lipman, "Basic local alignment search tool," *Journal of Molecular Biology*, vol. 215, no. 3, pp. 403–410, 1990.

[17] R. C. Edgar, "MUSCLE: multiple sequence alignment with high accuracy and high throughput," *Nucleic Acids Research*, vol. 32, no. 5, pp. 1792–1797, 2004.

[18] M. Gouy, S. Guindon, and O. Gascuel, "SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building," *Molecular Biology and Evolution*, vol. 27, no. 2, pp. 221–224, 2010.

[19] S. Guindon and O. Gascuel, "A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood," *Systematic Biology*, vol. 52, no. 5, pp. 696–704, 2003.

[20] R. Ogden, N. Dawnay, and R. McEwing, "Wildlife DNA forensics—bridging the gap between conservation genetics and law enforcement," *Endangered Species Research*, vol. 9, no. 3, pp. 179–195, 2009.

[21] M. Srinivasan, D. Sedmak, and S. Jewell, "Effect of fixatives and tissue processing on the content and integrity of nucleic acids," *American Journal of Pathology*, vol. 161, no. 6, pp. 1961–1971, 2002.