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Validation of the barcoding gene COI for use in forensic genetic species identification

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Abstract

The application of forensics to wildlife crime investigation routinely involves genetic species identification based on DNA sequence similarity. This work can be hindered by a lack of authenticated reference DNA sequence data resulting in weak matches between evidence and reference samples. The introduction of DNA barcoding has highlighted the expanding use of the mtDNA gene, cytochrome *c* oxidase I (COI), as a genetic marker for species identification. Here, we assess the COI gene for use in forensic analysis following published human validation guidelines. Validation experiments investigated reproducibility, heteroplasmy, mixed DNA, DNA template concentration, chemical treatments, substrate variation, environmental conditions and thermocycling parameters. Sequence similarity searches using both GenBank BLASTn and BOLD search engines indicated that the COI gene consistently identifies species where authenticated reference sequence data exists. Where misidentification occurred the cause was attributable to either erroneous reference sequences from published data, or lack of primer specificity. Although amplification failure was observed under certain sample treatments, there was no evidence of environmentally induced sequence mutation in those sequences that were generated. A simulated case study compared the performance of COI and cytochrome *b* mtDNA genes. Findings are discussed in relation to the utility of the COI gene in forensic species identification.

Keywords: DNA barcoding; Cytochrome *c* oxidase I; COI; Wildlife forensics; Validation; Cytochrome *b*

1. Introduction

Genetic species identification has been used to investigate the illegal hunting of animals [1,2], trade in protected species and species' derivatives [3,4] and animal tissues in human murder cases [5]. When morphology is compromised, genetic species identification attempts to match an unknown evidence sample to a known reference sample by comparing sequences of genes, usually mitochondrial DNA (mtDNA) loci that are known to vary between species. The genes most commonly used in the forensic identification of species are cytochrome *b* (cyt *b*) [6,7] and the hyper-variable displacement loop (D-Loop) [2], although other genes have proven useful [8,9]. Mitochondrial genes have a high copy number allowing a greater yield of mtDNA to be recovered from trace samples compared to nuclear DNA [10]. In addition, mtDNA genes typically lack recombination promoting the loss or fixation of mtDNA haplotypes, reducing within species diversity and thus enabling species identification [11,12].

The recent introduction of DNA barcoding has led to the suggestion that the mtDNA gene cytochrome *c* oxidase I (COI) be used as a 'barcode' for most animal life [13,14]. This is a rapidly expanding area of research and is supported by an international consortium of major natural history museums, herbaria and other organisations [15]. The marker itself has already received some attention in forensic entomology [16,17], and in a non-forensic context has been used to identify species of fish [18], birds [19], insects [20] and primates [21]. The anticipated growth in COI data has recently led one leading journal to form a dedicated barcoding section for COI sequence publication, paving the way for the COI gene to become a key taxonomic identification tool.

The central concept in species identification is to match the sequence of the evidence item to a reference sequence, either through DNA sequence similarity searches [22] or by

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phylogenetic reconstruction [3]. The major limitation to this process is the lack of authenticated reference DNA sequence data, with high sequence matches often limited to comprehensively studied species for which multiple haplotypes are documented. Where cyt *b* and D-Loop sequences do exist, very few of these have been generated as part of forensic studies [23], with the vast majority submitted to databases as part of phylogenetic, molecular ecological and conservation studies [e.g. 24–26]. Such sequence data can be generated and accepted into databases without any need for standard protocols or quality control, raising doubts over their suitability for forensic application. A key aim of barcoding is the production of COI reference sequences recovered from multiple voucher specimens using standard protocols, resulting in numerous authenticated haplotypes. Given the development of the barcoding initiative and associated protocols, the future application of COI as a forensic marker, alongside existing genes, appears inevitable.

Prior to its use in forensic casework any novel marker or tool needs to be validated. Validation of a genetic marker is designed to determine its reproducibility and limitations by testing its ability to provide accurate results under a variety of conditions. There are currently no published validation guidelines for non-human forensic work, so the methodology adopted here followed human forensic guidelines provided by the Scientific Working Group on DNA Analysis Methods (SWGDAM) (http://www.cstl.nist.gov/div831/strbase/validation/SWGDAM_Validation.doc). The validation studies examine reproducibility and heteroplasmy, mixed DNA, DNA template concentration, chemical treatments, substrate variation, environmental conditions and thermocycling parameters.

2. Materials and methods

2.1. Study species

Validation studies were performed on authenticated specimens of mammal (cow, *Bos taurus)*, bird (chicken, *Gallus gallus*) and fish (cod, *Gadus morhua*), and were chosen as well-studied commercial species each represented by multiple reference sequences held on the GenBank and the Barcode Of Life (BOLD) databases. Sample types were blood (chicken and cow) and muscle tissue (cod). Where required, human DNA was recovered from a variety of different tissues from a single individual.

2.2. DNA extraction and polymerase chain reaction (PCR) amplification

DNA from all samples was recovered using the QIAGEN DNeasy tissue kit and extracts were quantified using the fluorescent dye PicoGreen (Molecular Probes Inc.), and Galaxy Fluostar apparatus (BMG Labtechnologies Ltd.). DNA was amplified using COI universal primers HC02198: 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′and LC01490: 5′-GGTCAACAAATCATAAAGATATTGG-3′ [27]. Before the validation studies commenced, PCR reaction conditions and thermocycling parameters were first defined: a 20 μl reaction containing 0.36 units of ABgene Thermo-Start DNA Polymerase, 2.5 mM $MgCl_2$, 0.14 mM each dNTP, 0.72 × reaction buffer, 20 pmol each primer and 2 μl of template DNA (2–10 ng/μl). PCR [28] was performed on a PTC-200 MJ Research thermocycler using the following cycling parameters: 15 min denaturation step at 95 °C; 40 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1, 5 min elongation step at 72 °C. A high number of amplification cycles were used to account for reduced primer specificity in some species. PCR product was visualised under UV light following electrophoresis on an ethidium bromide stained 2% agarose gel. Positive and negative controls were used throughout.

2.3. Validation studies

To test the reproducibility of the COI region, three independent PCR amplifications were performed on a single individual from each species and the DNA sequences compared. To test for heteroplasmy, DNA was recovered from a single individual and COI sequences produced and compared from human blood, buccal swab, nail and hair root [29].

To examine the effect that human contamination has on species identification DNA from each of the representative species was mixed with human DNA at 2 ng/μl in the following ratios: 20:1, 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10, 1:20. Effects of DNA template concentration were assessed by amplifying DNA from cow, chicken and cod at concentrations of 10, 5, 2.5, 1.25, 0.62, 0.31 and 0.15 ng/μl.

The effect of chemical treatments on DNA recovery and amplification was investigated by treating samples of cow and chicken with bleach, soap, gasoline, and 0.1M sodium hydroxide. Ten replicates of each species were treated with each chemical (50 μl:50 μl chemical to blood mix). DNA was recovered from five replicates one hour after treatment and from the remaining five replicates after five days' storage in ambient indoor conditions. The effect of denim, suede, wood, and metal on DNA recovery and amplification for subsequent species identification was investigated in cow and chicken. Ten replicates of each species were used (50 μl blood deposited on 5 cm^2 of substrate). DNA was recovered as for the chemical treatments. In addition, five replicates of cow, chicken and cod were subjected to environmental conditions typical of those experienced by forensic samples. DNA from cow and chicken was recovered from swabs treated with 50 μl unpreserved blood subjected to outdoor ambient conditions sheltered from rain during January to March (winter). DNA from cod was recovered from 0.1 g muscle tissue exposed to identical outdoor ambient conditions and sea water (room temperature). To investigate temporal DNA degradation and its effect on amplification efficiency, extractions were performed at weeks zero, two, four, and six and amplified via PCR.

PCR reaction components and thermocycling parameters were varied from those defined above to determine windows of acceptable performance. The following parameters were tested: (i) annealing temperature, testing ± 2 and ± 4 °C, (ii) cycle number, testing ± 1 and ± 2, and (iii) $MgCl_2$ concentration (1.5, 2.0, 2.5 and 3.0 mM). Positive results were defined as PCR product occurring at the correct fragment size of ∼658 base pairs (bp) in the representative species and the positive control.

2.4. DNA sequencing

All amplification products occurred at the correct fragment size and were cleaned using exonuclease I (New England Biolabs) and shrimp-alkaline phosphotase (Invitrogen) [30]. Product was sequenced unidirectionally using the HC02198 primer, on an Applied Biosystems, Inc (ABI) 3730xl or Beckman Coulter CEQ8000. Sequences were visualised and edited using Chromas 1.6 (Technelysium Pty Ltd.). Sequence similarity searches were performed using GenBank BLASTn (http://www.ncbi.nlm.nih.gov/BLAST/) and BOLD Identification System (http://www.boldsystems.org). Sequences were examined for stop codons using MEGA Version 2.1 [31]. ClustalX [32] was used to compare DNA sequence data.

3. Results and discussion

3.1. Reproducibility and heteroplasmy

No DNA sequence variation was observed among the independent replicates of each individual. All sequences provided correct species identification results when using the GenBank BLASTn and BOLD search engines. In chicken, both search engines provided joint highest matches for *G. gallus* and *G. sonneratii*. The inability to differentiate between these

species due to a lack of variation at this locus is not surprising given that species in the *Gallus* genus including the grey jungle fowl, *G. sonneratii*, and chicken, *G. gallus*, are known to hybridise [33]. A lack of differentiation between closely related species has been reported in other studies for the COI gene [20] and also the cyt *b* gene [7]. No heteroplasmy was observed with different human sample types.

The human sequences generated in these experiments were consistently identified in GenBank as 100% *Homo sapiens*, however in the BOLD search engine in addition to *Homo sapiens*, joint sequence matches were also returned for five invertebrate species: four echinoderms, *Macrophiothrix* spp and a caddisfly, *Parapsyche elsis* (Table 1). The five matching invertebrate DNA sequences were obtained from published sequence data [34,35], but were not generated from barcoding studies. The observed results could be explained by either the COI region being unable to differentiate between these species over the region amplified, or that contamination of DNA sequences has occurred. Although COI has been reported to be unable to differentiate between some closely related species (see above), the existence of highly conserved regions of the gene over hundreds of base pairs is considered unlikely given its phylogenetic utility demonstrated by barcoding studies. We also consider it unlikely that the human fragment amplified in this study belonged to an invertebrate species given the contamination controls in place [23], and the fact that no such species have previously been analysed in the laboratory. We suggest that contamination occurred during original sequence submission.

To explore this explanation, a complete human COI sequence was obtained from GenBank and aligned with the COI sequences of the five invertebrate species using the bl2seq program on NCBI (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) (Table 1). The results suggest that the high percentage match to the four echinoderm species is due to human contamination, as the sequences match over the majority of the sequence length. In contrast, the *H. sapiens* and *P. elsis* sequences match over only 479 bp of the 881 bp *P. elsis* sequence, indicating that the original *P. elsis* sequence was chimeric with a human sequence. This is supported by the discrepancy in published sequence length (441 bp) [34] and submitted sequence length (881 bp, GenBank Accession AF436568).

Much of the initial sequence data held on BOLD is unauthenticated reference data transferred from GenBank that is currently included to populate the database. Correspondence with BOLD has facilitated the inclusion of an option to search under authenticated sequence data only; in time such data should dominate the database. While search engine mismatches undoubtedly have an impact on the interpretation of the result, they do not invalidate COI as a forensic species identification marker; the same result may be obtained from any marker using unauthenticated reference sequence data.

3.2. Mixture analysis and DNA template concentration

The addition of human DNA prevented identification of cow and chicken when mixed with human at a ratio of 10:1 or lower (non-human:human) and of cod when mixed at 20:1 or lower. The preferential amplification of human DNA over the target species may be explained by differences in primer affinity between species. Aligning the primer sequences against the entire COI region from each of the target species shows that point mutations promote preferential primer annealing to human DNA (Table 2). This has been previously observed when using forensic cyt *b* primers [7] and while useful in analysis of human DNA, may be a limitation in wildlife species identification.

Variation in primer specificity also affected sequence recovery at low DNA concentrations. While DNA from cow and chicken yielded sequences at the lowest DNA concentration (0.15 ng/μl), cod DNA concentrations of 0.31 ng/μl and below failed to return species-informative sequences.

3.3. Chemical, substrate and environmental exposure

DNA was successfully recovered for all chemical and substrate treatments in cow and chicken at day 0 and day 5, although DNA yield from the chemical treatments was generally reduced (data not shown). Cow DNA successfully amplified in all five replicates regardless of treatment, and sequence data returned a 100% match to the target species over ~480 bp from both search engines. Chicken DNA treated with bleach did not amplify while gasoline, sodium hydroxide, soap, wood and leather reduced amplification success. Reasons for the observed difference between cow and chicken are unclear. Previous studies have shown that inhibitors in denim, suede and wood can have an effect on PCR [36,37], although previous studies on cyt *b* using cat DNA show no effect of sodium hydroxide, soap and gasoline on PCR [7].

In the environmental exposure experiments DNA was recovered from cow, chicken and cod tissue throughout the

Table 1
Results of GenBank bl2seq alignment of a *Homo sapiens* complete COI sequence (AY963575) against five invertebrate species and chimpanzee (*Pan troglodytes*)

Species	GenBank Accession number	Sequence length	GenBank identity score	Percentage similarity
Parapsyche elsis	AF436568	881	479/485	98
Ophiothrix ciliaris	AY365181	604	601/607	99
Macrophiothrix spongicola	AY365178	603	599/606	98
Macrophiothrix paucispina	AY365171	613	613/616	99
Macrophiothrix nereidina	AY365168	607	607/607	100
Pan troglodytes	AY544154	603	545/603	90

Unusually high sequence similarity was observed between the human and invertebrate COI regions, relative to more closely related species.

Table 2
Results of primer alignment against primer annealing sites in amplified species

Primers/species	Primer sequence and species primer annealing sites																											Matching bases
LC01490	5′	G	G	T	C	A	A	C	A	A	A	T	C	A	T	A	A	A	G	A	T	A	T	T	G	G		3′	
H. sapiens	5′	T	C	.	.	T	.	.	.	.	.	C	.	.	C	.	.	.	.	.	C	.	.	.	.	.		3′	19/25
G. gallus	5′	T	C	.	.	.	.	.	C	.	.	C	.	.	C	.	.	.	.	.	C	.	.	.	.	.		3′	19/25
G. morhua	5′	T	C	.	.	G	.	.	C	.	.	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.		3′	19/25
B. taurus	5′	T	C	.	.	.	.	.	C	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.		3′	21/25
HC02198	5′	T	A	A	A	C	T	T	C	A	G	G	G	T	G	A	C	C	A	A	A	A	A	A	T	C	A	3′	
H. sapiens	5′	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	3′	25/26
G. gallus	5′	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	G	.	.	G	.	.	.	.	.	3′	23/26
G. morhua	5′	.	.	.	.	.	.	.	.	G	.	.	A	.	.	C	.	.	G	.	.	G	.	.	.	.	.	3′	21/26
B. taurus	5′	.	.	G	.	.	.	.	.	G	.	.	.	.	.	T	.	.	.	.	.	G	.	.	.	.	.	3′	22/26

Whole COI sequences downloaded from GenBank: *H. sapiens* (AY963575); *G. gallus* (AP003322); *G. morhua* (X99772); *B. taurus* (AY676873). Primer LC01490 shows highest similarity to *B. taurus*, while Primer HC02198 shows highest similarity to *H. sapiens*. Variation in primer specificity corresponds to differential amplification success among species.

six week period when stored in outdoor ambient conditions. No DNA was recovered from the cod stored in sea water after week two due to tissue breakdown. Cow DNA was successfully amplified and sequenced at week six, while Chicken DNA failed to amplify after week four. DNA recovered from cod muscle could be amplified at all weeks, however sequence results revealed mixed species profiles that could not be easily identified. Despite the effect of environmental exposure, no evidence of environmentally induced mutation was witnessed, a finding consistent with previous data on the stability of other genetic markers in post-mortem tissues [38].

Non-human forensic samples typically consist of body tissues, or whole organisms belonging to obscure species. As such, DNA degradation is generally not observed at the same level as in human forensic mtDNA analysis. Although a relatively long DNA fragment was amplified here, the results showed that PCR product was still obtained after four weeks in all species in the outdoor ambient test, indicating that this size of the COI fragment is suitable for wildlife forensic samples of this type. Nevertheless, it is sometimes desirable that forensic genetic markers be diagnostic over shorter sequence lengths in cases of extreme DNA degradation. For this purpose, COI has recently been shown to be diagnostic over ~100 bp, allowing for the amplification of small DNA fragments if necessary [39].

3.4. Thermocycling parameters

The COI region of cow and cod amplified at ± 2 °C and ± 4 °C of the annealing temperature of 50 °C. The COI region of chicken amplified at −4 °C and ± 2 °C, but failed to amplify at +4 °C. The COI region in cow and chicken amplified at 40 cycles ± 1 and ± 2. The COI region of cod amplified at ± 1 and +2 cycles but showed no visible product at −2 cycles. The COI region of cow amplified at all $MgCl_2$ concentrations tested. Chicken failed to amplify at 1.5 mM $MgCl_2$, and cod failed to amplify at 1.5 mM and 3.0 mM $MgCl_2$. These results provide windows of optimal performance allowing the COI region to be amplified in other laboratories.

4. Simulated case report

The Eurasian badger, *Meles meles*, is a protected species in the United Kingdom (UK) due to persecution. Samples from badger persecution cases typically consist of blood or hairs recovered from the suspect's clothing or the suspect's dog. In this simulated case study a sample of unidentified species blood was provided by the Royal Society for the Prevention of Cruelty to Animals (RSPCA). DNA was recovered, amplified and sequenced following the standard protocols for COI described above, and for cyt *b* following existing procedures [6]. A 509 bp sequence of COI was obtained and a sequence similarity search performed using the GenBank and BOLD search engines. GenBank provided the highest matches of 85% over 427 bp to *Taxidea taxus*, the American badger, and 86% over 396 bp to *Gulo gulo*, wolverine. The highest match using the BOLD engine was 84.1% to *Gulo gulo*. From these results it was not possible to confidently identify the species from which the sample originated. For cyt *b* a 520 bp sequence gave a 99% GenBank match to the Eurasian badger, *Meles meles*, allowing confident identification of the sample. Subsequent consultation with the RSPCA revealed the sample originated in the UK from a Eurasian badger. The COI sequence recovered here for *Meles meles* was submitted to GenBank (Accession DQ487091).

The difference between COI and cyt *b* results in GenBank is due to the larger number of cyt *b* sequences held in the database. In this example, no COI reference sequence data exists for the Eurasian badger. This case study highlights the current situation associated with COI for forensic species identification.

5. Summary

This study demonstrates that the cytochrome *c* oxidase I gene enables accurate animal species identification where adequate reference sequence data exists. Species-diagnostic COI sequences were obtained in all validation experiments. Any misidentification that occurred was due to errors in the reference databases and although some effects of sample treatment were observed, the results do not compromise the use of the COI gene in forensic species determination.

The use of universal primers is necessary for retrospective species identification as they allow amplification across a wide taxonomic range. However this property complicates correct species identification where samples may be mixed or contaminated. The results of the mixture, DNA template concentration and environmental experiments highlight the possible need for alternative primers to be used in conjunction with those used here, whether for the amplification of smaller fragments, or where information allows more specific primers to be employed.

The issue that may slow the acceptance of COI as a forensic genetic species identification marker is that currently more data from a larger number of species exist for cyt *b* than for COI, generally allowing a higher percentage match to be obtained. This does not prevent the use of COI as a marker for well-studied species, indeed the availability of two corroborative markers will be of benefit to forensic zoologists. Although it is expected that current forensic cases requiring species identification will rely predominantly on cyt *b* and GenBank BLASTn tools at present, the continued submission of 'barcode' data should eventually lead to COI becoming a more powerful tool than existing markers in terms of data quality and quantity.

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