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FORENSIC APPLICATIONS OF MITOCHONDRIAL CYTOCHROME *B* GENE IN THE IDENTIFICATION OF DOMESTIC AND WILD ANIMAL SPECIES

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ABSTRACT

Identification of animal species based on biological samples such as blood, hair, bone, muscle and others is an important practice in forensic analysis of DNA, but need a standard analytical method particularly when the DNA is low or mixed. The mitochondrial genome contains several forensically informative nucleotide sequences (FINS) that can be utilized for both intra-species and inter-species differentiation. Mitochondrial DNA (mtDNA) has several advantages than nuclear genomes due to greater abundance in the samples and high number of copies in each cell. Therefore, amplification and sequencing of mtDNA genes could constitute relatively sensitive procedures in species identification even if the tested samples are old or degraded. Cytochrome b (Cyt b) gene is among the most important mitochondrial genes which have been used in forensic species identification. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis and sequencing of the mitochondrial cyt b gene could be used as a robust tool in identification of different animal species including domestic, wild and fish species and for determination of meat origin in processed food products due to the presence of species specific mutation sites. The mitochondrial cytochrome-b sequence based species identification has wide forensic and judicial applications.

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1 Introduction

Forensic identification of animal species and individualization of animal samples is important for many purposes, such as investigations of alleged crimes of wildlife, poaching, illegal trading and conservation of endangered animal species, attacking of animals, robbery of livestock or the determination of meat origin in meat products or identification of animal species in foods of animal origin (for religious and health purposes) (Bottero & Dalmasso, 2011; Silva-Neto et al., 2016; Lopez-Oceja et al., 2016). Species identification using molecular-genetic or bio-molecular approaches is more reliable and specific than other methods such as immunological approaches based on antigen-antibody reactions which lack specificity and changed by environmental conditions (Tobe & Linacre, 2008). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) which involves the extraction of DNA, PCR, and the use of restriction enzymes to digest the PCR products is one of the most commonly used molecular techniques as it results in species-specific restriction pattern. However it is unable to identify unknown species in mixed samples (Fernandes et al., 2008; Spychaj et al., 2009). Amplification of specific region using universal primer and sequencing of such region to compare its sequence with the public database in GeneBank is more reliable to determine the most related sequence of unknown sample (Naidu et al., 2012; Muangkram et al., 2018). Mitochondrial DNA (mtDNA) is highly polymorphic and thousand copies of it are present inside each cell, therefore it's amplification and sequencing could be used as a powerful tool in species identification even in old or degraded samples (Bravi et al., 2004 ; Muangkram et al., 2018).

The mitochondrial cytochrome b (MT-cyt b) gene is one of the best known proteins that make up the complex III of oxidative phosphorylation system in the mitochondria and is the only one encoded by the mitochondrial genome. Cyt b genes have been utilized as powerful indicators in species identification by analysis techniques of DNA (Andrejevic et al., 2019). It is also used in studies of forensic investigations, legal medicine and molecular evolution (Bataille et al., 1999; Wolf et al., 1999; Prusak et al., 2004). Species identification can be performed efficiently by using short fragments of MT-cyt b, especially in samples that are degraded or are low in DNA quantity (Andrejevic et al., 2019). While developing species specific primers, it is important to verify the primer specificity on other animal species so that the diagnostic test will remain as highly specific for the species against which it is developed (Kim et al., 2020). Cytochrome b (cyt -b) gene has species specific mutation sites making it useful for species identification, therefore, cyt b gene is extensively employed in systematic studies to resolve divergences at several taxonomic levels. The species identification studies depending on cyt b gene are various including amplification of short (< 400 base pair) and long (>900 base pair) fragments using PCR-PFLP and sequencing in addition to variable size species-specific multiplex PCR (Matsunaga et al., 1999; Parson et al., 2000; Izeni et al., 2001; Bellis et al., 2003; Guha et al., 2006). The short fragment of cytochrome b gene can be considered as the universal DNA barcode region of individual species. It can be used as an accurate and efficient tool for discriminating different domestic and wild animal species (Yacoub et al., 2013). This review highlights the role of the mitochondrial cytochrome b gene in identification of different animal species including domestic, wild and fish species.

2 Use of cyt b gene in identification of domestic animal species

Bing et al. (1999) developed a technique that has successfully enabled the differentiation of DNA from human and non-human [*Gallus gallus* (chicken), *Sus scrofa* (pig), *Bos taurus* (cow) and *Equus caballus* (horse)] origins by amplifying the MT-cyt b and displacement loop (D-loop). Where, the amplification products were of one band for non-human DNA and as two bands for human. Such technique was also used by Bellis et al. (2003) to identify *G. gallus* (chicken), *Ovis aries* (sheep), *Capra hircus* (goat), *B. taurus* (cow), *S.scrofa* (pig), *E. caballus (*horse*), Rattus noregicus* (rat), *Felis catus* (domestic cat), *Cnis familiaris* (domestic dog *)* in addition to *Panthera tigris tigris* (Bengal tiger). The 127 bp long target fragment of MT-cyt b chosen by Andrejevic et al. (2019) for amplification using universal primers exhibited very low intraspecies genetic diversity ranging from 0 to 4.72%. Whereas, the interspecies genetic diversity of the same fragments were very high (8.36% to 42.52%), indicating great potential for species discrimination.

Nucleotide sequence analysis of cyt b gene was done by Parson et al. (2000) to identify DNA from 44 different animal species including birds, mammals, fishes, amphibians and reptiles. They stated that sequencing of cyt b was very powerful and sensitive for identification of various samples even the problematic ones like feathers, hair and bristles. Similarly, Ramatla et al. (2019) could differentiate between rodent species which in the poultry farms in Mafikeng in South Africa by amplifying and sequencing of PCR products of cyt b gene. Similarly, Awad et al. (2015) partially sequenced a cyt b segment (358pb) which helped in identification of some avian species such as chicken (*Gallus gallus*), Japanese quail (*Coturnix japonica*), muskovy duck (*Cairina moschata*), rock pigeon (*Columba livia*), and laughing dove (*Streptopelia senegalensis*). The cytochrome b gene sequence based analysis is a very efficient tool that can be used to discriminate among native chicken strains and other species of *Gallus gallus* fowl (Yacoub et al., 2013). The PCR-RFLP of cytochrome b gene was performed over 39 DNA samples collected from different meatball shops in Indonesia to confirm the contamination of sausage, nugget and meatballs with pork, and amplification of cytochrome b gene was

proved promising in detecting the meat contamination by another type of meat as 9 meat samples were found contaminated with porcine DNA (Erwanto et al., 2011; Erwanto et al., 2012; Erwanto et al., 2014). In another study, 565 blood-fed Culicoides females were collected from forest, pasture, donkey shelters and sheep shelters in France during 2012-2014. They were identified based upon morphological features and on the basis of Cyt b marker gene of mitochondrial DNA. Results showed that cyt b was suitable marker for confirmation of species (Hadj-Henni et al., 2015).

Identification of meat in foods and feedstuffs as well as processed meats is a matter of great importance due to many considerations related to health, religion and economic affairs (Farag et al., 2015a). Analyzing and sequencing the products of PCR- RFLP of cyt b gene (359 bp amplicon was exposed to digestion by restriction enzymes to give species specific patterns by electrophoresis) helped in detecting the meat meal in the animal feed stuffs and identified the species of origin (Bellagamba et al., 2001). The same technique could identify animal species in processed food with chicken, pork and bovine meat (Pancorbo et al., 2004). Aina et al. (2019) used cyt b gene to identify wild boar meat in meatball products.

PCR-RFLP of cyt be gene has been also used by Bravi et al. (2004) who amplified a fragment from cyt b gene from human, cattle, pig, sheep, donkey, horse, cat, dog, chicken and rabbit from blood and meat samples using universal primers and the restriction enzymes (Hae III , Alu I, and Hinf I). Similarly, Abdel-Rahman et al. (2009) developed a PCR and PCR-PFLP technique to identify dog's, cat's, horse's and donkey's meat. Moustafa et al. (2017) could differentiate between horse and donkey (*Equus asinus*) species using the same technique. Additionally, Farag et al. (2015b) used the PCR-RFLP analysis of cyt be gene to identify domestic species such as camel, buffalo and sheep. Donkey-specific primer pairs developed from mitochondrial cytochrome b gene was found to be effective in detecting raw donkey meat as well as different meat mixtures like grinded, boiled, fried, roasted, dried, and autoclaved meat (Kim et al., 2020). The specificity of the developed donkey-specific primers was also verified using 20 animal species.

PCR-RFLP of cyt b gene succeeded in identification of cattle (*Bos Taurus*), goat (*Capra hircus*), sheep (*Ovis aries*), red deer (*Cervus elaphus*) and roe buck (*Capreolus capreolus*) from blood traces obtained from a leaf which is considered as unknown source (Pfeiffer et al., 2004).

Species specific repeat (SSR) and PCR-RFLP analysis of cyt b gene were effective in identification of meat samples from cattle, buffalo, sheep and pig (Ahmed et al., 2007). Novianty et al. (2017) used a genetic marker of cyt b by duplex-PCR to identify pork contamination in meatballs. Jain et al. (2007) used multiplex PCR analysis of cyt b gene for identification of sheep, goat, buffalo, chicken, horse and pig meat through mixing different ratios of species specific primers .Similar results were obtained by Matsunaga et al. (1999) for all the same species except buffalo. By using the same primers in different ratios, Obrovska et al. (2002) also obtained species specific bands for cattle, horse, pig and chicken. RT-PCR that uses specific primers targeting MT-cyt b gene is considered as the standard detection tool for identifying the presence of pork in food samples intended for halal consumption (Orbayinah et al., 2019). They designed the primer 5′-ACG CGA TAT AAG CAG GTA AA-3′ (forward); and 5′-CTG CTT TCG TAG CAC GTA TT-3′ (reverse) which was found to be effective in detecting pork meatball formulations at different series of dilution.

Tobe & Linacre (2008) could design species specific primers based on single nucleotide polymorphism (SNPs) in the cyt b gene through collecting the sequences obtained from Gene bank and used them to identify various animal species including dog, cat, cow, donkey, horse, pig, house mouse (*M. musculus*), human (*H. sapien*), lamb (*O. aries*), rat, rabbit (*O. cuniculus*), guinea pig (*C. porcellus*) in addition to fox (*V. vulpes*), badger (*M. meles*) and red deer (*C. elaphus*)

3 Use of cyt b gene in identification of wild animal species

Wildlife forensics using cytochrome b is an applied aspect of new DNA based technologies for identification of wildlife species (Ogden et al., 2009). Matsunaga et al. (1998) used cyt b gene sequence to design species-specific primers to identify the meat of dear in meat products and meat and could distinguish red and sika dear from bovine, sheep and pigs. To differentiate between sika deer and red deer they exposed the PCR products to digestion by restriction enzymes (EcoRI, ScaI, and BamHI). The fragment of red deer was digested by EcoRI only into two fragments (67 + 127bp).While, the fragment of sika deer fragment was digested by ScaI and BamHI to 49+145 bp and 48+146 bp fragments. They discriminated between the two deer kinds from the digestion pattern of restriction enzymes. In Zambia, to identify the origin of meat from wild animals, among 29 animals of eleven species of bovidae mitochondrial DNA, mainly cytochrome b (Cytb) and cytochrome oxidase I (COI) were used as chief target for PCR and sequencing (Lynch & Jarrell, 1993; Syakalima et al., 2016). The presence of forensically informative nucleotide sequences (FINS) in the mitochondrial genome of Indian antelope or Blackbuck (*Antilope cervicapra*) were evaluated and found the presence of FINS in the Cytochrome Oxidase I, Cytochrome b, and 16S rRNA genes (Shukla et al., 2019). The identified FINS can be used for both intra-species and Inter-species differentiation studies in this wild animal species.

Zhang & Ryder (1998) could differentiate between old world monkeys (*Maccaca mulatta, Mandrillus leucophaeus, Mandrillus*

sphinx, Semnopithecus entellus, T. johni, T. francoisi, T. phagrei, Rhinopithecus roxellanae, Trachypithecus vetulus, Pygathrix nemaeus, R. avunculus, R. bieti, Nasalis larvatus and *Colobus polykomos*) using the partial sequence of the cyt b gene segment (393-402 bp) .

Cook et al. (1999) partially sequenced 450 bp fragment of the cyt b gene and 512 bp from mitochondrial control region and could determine the monophyletic of sika deer by studying 28 individuals of sika deer subspecies. Partial DNA sequence of cyt b (402 bp fragments) has been used by Hsieh et al. (2001) to identify endangered species in Tiwan from the remains of Formosan gem-faced civets, lion, tigers, leopard cats, clouded leopards, water buffalo, *Formosan muntjacs, Formosan sambars, Formosan sika deers, Formosan serows,, Formosan macaques* and *Formosan pangolins*. Then the resulting sequences were compared with some domestic species such as dogs, cats, sheep, pigs, cattle and humans. Endangered and domestic animals of the same species could be clustered in the neighbour-joining tree.

Hsieh et al. (2003) used a partial sequence of cyt b (402 bp) to identify and determine the phylogenetic relationship of rhinoceros horns species and found that there were 4 major branches among this species based on GeneBank sequences. On a similar ground, Hsieh et al. (2005) used a partial sequence of cyt b to identify tortoises using 100 shell samples from the species *Kachuga tecta*. They could classify them in to 4 haplotypes of DNA sequences as follows; haplotype I and II (*K. tecta*), haplotype III (*Morenia ocellata*) and haplotype IV (*Geoclemys hamiltonii*) compared to GeneBank sequences.

Nagata et al. (2005) used the PCR- RFLP of cyt b gene to develop species-specific primers for identification of the leopard *Panthera pardus* and *tiger Panthera tigris* from domestic dog, domestic cats and red fox, roe deer, red deer, sika deer, wild boar and humans. Quantitative polymerase chain reaction (q-PCR) using species specific primers (forward: 5′-CGG TTC CCT CTT AGG CAT TT-3′; Reverse: 5′-GGA TGA ACA GGC AGA TGA AGA-3′) that targets MT-cyt b gene can be used for the detection of wild boar meat. This has great application both in wildlife forensics as well as in the identification of non-halal meats in commercial meat products (Aina et al., 2019).

PCR- RFLP of cyt be gene has been also used by Abo-Hadeed et al. (2011) to identify some non-domestic animals (American black bear (*Urus americanus*), Blue Nile monkey (*Ceropithecus mitis*), Bactrian camel (*Camelus bactrianus*) Barbary sheep (*Amotracus lervia*), and Llama (*Lama glama*) from hair samples by amplifying a fragment from cyt b gene (358pb) using universal primers and the restriction enzymes (Hae III, Alu I, and Hinf I). Differentiating the wild and domestic population of animal species is necessary to resolve certain categories of judicial cases. Gonzalez et al. (2020) made an attempt to differentiate among domestic, wild and hybrid camelids of South America. MT-cyt b genetic variation was found to be an ideal tool for distinguishing between Lama and Vicugna (Gonzalez et al., 2020).

Partial sequence of cyt b succeeded in identifying the origin of unknown samples. The first one was ornithological trace “a hair from a bird nest” related by cyt b to goat (100% similarity with GneBank sequence). The second sample was forest trace “blood stain from Notecka forest in Poland and related by cyt b to elk (Alces alces) with 97.8% similarity. The third one was zoological trace “muscle fragment of a dead reptile from Zoological Garden in Warsaw and related by cyt b to the endangered species “*Python molurus*” (Prusak et al., 2005).

Yan et al. (2005) used various sequences of cyt b gene to design species specific PCR primer for identification of the protected species “Chinese alligators” and other species of crocodilians. Additionally, Cyt b gene has helped in developing of species-specific markers for identification of eight endangered Indian *Pecoran species* (Guha et al., 2006).

Lee et al. (2009) used nested PCR within cyt b for identification of endangered and protected elephant species using highly degraded DNA ivory samples (360African savanna elephants “*Loxodonta africanta*", 14 African forest sample "*Loxodonta cyclotis*" and 8 Asian elephants). The resulting sequences matched with GeneBank sequences of Loxodonta and *E. maximus* species with about 99% of similarity.

4 Use of cyt b in identification of fish species

Izeni et al. (2001) stated that cyt be could be utilized as a molecular marker in establishing of the phylogenetic tree of the fish family Cichidae. Aranishi et al. (2005) optimized a rapid PCR-PFLP technique to distinguish the occurrence of 3 closely related species of gadoid fish: Alaska Pollack "*Theragra chalcogrmma*", Atlantic cod "*Gadus morhua*" and Pacific cod "*Gadus macrcephalus*" in commercial products of sea foods. They designed a universal primer for Gadoid to amplify a cyt b gene fragment (558 bp) which was then exposed to digestion with the restriction enzymes Eco105I and Eco32I which gave 2 fragments in Pacific cod, 3 fragments in Alaska Pollack and no cleavage in Atlantic cod.

Akasaki et al. (2006) used the PCR-RFLP analysis of cyt b (385 bp) and restriction enzymes (MseI, MboI, FokI, and AluI) as a rapid screening method to identify imported products of cod fish in Japan. With the same technique, Wu et al. (2008) could identify commercial species of *Aluterus scriptus , Aluterus monoceros, , Monacanthus chinensis, , Thamnaconus modestus, Thamnaconus hypergyrus* and *Chaetodermis penicilligerus.* They designed H15149and L14735 in the cyt b gene to amplify a 465bp fragment

from processed meats of filefish. Then the resulting fragments were digested by HaeIII enzyme which gave species-specific pattern for each species;, *A. scriptus* (180+159+126 bp), *A. monoceros* (285+180bp), M. chinensis (424+41bp), *T. modestus* (229+180+56 bp), *T. hypergyrus* (180+159+94+32 bp) and *C. penicilligerus* (285+139+41bp). The mitochondrial cyt b sequence of *Catla catla* was found to be highly variable among the wild and cultured populations. It is also interesting to note that the genetic distance between the wild populations were more compared to the cultured populations. The genetic distance between the cultured populations were found to be almost absent or present at very low values indicating that cyt b sequence is highly conserved (Garg & Mishra, 2018).

5 Conclusion and Future Prospects

The use of cyt b gene for species identification has been shown to be a simple, reliable and sensitive method which allows analysis of minute amount of DNA even in old and highly degraded samples and could determine the origin of biological evidences from different domestic and wild animals, and fish species even in the absence of reference samples. Mt- cyt b gene is highly conserved among different species at the same time shows diversity among the different unrelated populations of the same species. Hence, it can be used as a marker to differentiate among different animal species and also has potential for identifying ecological habitat based on the genetic diversity among different populations. Species identification based on molecular tools that targets mitochondrial cyt b gene has wide forensic and judicial applications.

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