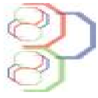

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Sequence of specific mitochondrial 12S rRNA fragment of Egyptian buffalo as a reference for discrimination between buffalo, cattle, sheep and goat

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ABSTRACT

Objective: Polymerase Chain Reaction (PCR) was conducted to amplify segment of specific mitochondrial 12S rRNA gene fragment in Egyptian water buffalo towards developing a tool for discriminating buffalo from cattle, sheep and goat.

Methodology and results: Amplification was performed using purified total genomic DNA of nine Egyptian buffalo blood samples. Direct sequence analysis showed complete conservation among all tested samples. The investigated buffalo sequences were deposited in GenBank database with the accession numbers (FJ828575-FJ828583). Multiple sequence alignment between the sequenced Egyptian buffalo and the homologous segments of buffalo, cattle, sheep and goat sequences available on GenBank database showed that the Egyptian buffaloes belong to one of two haplotypes, while the other one includes swamp buffalo. On the other hand definite single nucleotide polymorphisms (SNPs) were detected and were found to be specific for each of the four studied *Bovidae* species. In addition, other specific SNPs were detected that allow the discrimination between subfamily *Bovinae*, represented by water buffalo and cattle, and subfamily *Caprinae* represented by sheep and goat.

Conclusion and application of findings: Sequence examination and detection of specific SNPs referring to the specific Egyptian buffalo 12S rRNA gene fragment (accession numbers FJ828575-FJ828583) is an easy, accurate and highly specific method for the discrimination and identification of unknown sequences belonging to one of the four studied *Bovidae* species.

Key words: Sequence analysis, *Bovidae,* species identification, mitochondrial DNA, 12S rRNA gene.

Accession number: The nucleotide sequence data were submitted to nucleotide sequences database NCBI/ Bankit/ GenBank with the accession numbers: FJ828575-FJ828583.

INTRODUCTION

Species identification is an important aspect of forensic sciences, legal culling of animal populations, food quality control procedures or for the detection and identification of animal material in food samples. Traditional tests for species identification are based on immunological methods with antibodies against soluble protein that is specific for various species. These methods are

limited to stain material containing extractable proteins and only species for which antibodies are available can be identified. An immunological method has been developed based on monoclonal antibodies (MAbs) that can be used in an enzyme-linked immunosorbent assay (ELISA) for rapid detection of any cooked mammalian meats in cooked poultry products (Hsieh *et al.*, 1998). Other techniques which depend on protein analysis such as electrophoresis (Kim & Shelef, 1986; Skarpeid *et al.*, 1998) and liquid chromatography (Ashmoor *et al.*, 1998) have also been used. However, these methods are of limited use in species identification because proteins are heat labile and their presence and characteristics depend on special cell types. Presently, DNA based techniques are extensively used in species identification.

The specificity of genomic DNA probes for species differentiation by slot blot hybridization has been investigated. The polymerase chain reaction (PCR) was applied to identify six meats (cattle, pig, chicken, sheep, goat and horse) as raw materials by multiplex PCR (Matsunaga *et al.*, 1999). Also, a PCR-SSCP method has been developed to verify the authenticity of labeled raw material of canned fish or in products made from closely related fish species. Experiments have been performed investigating species differentiation between monkey and human and between cattle, goat and sheep. These experiments have been done using DNA hybridization method (Kirsten *et al.*, 1991).

The extensive use of cytochrome b (cyt b) gene for species identification supports the high potential of cyt b region as a tool for this purpose. Short segments of the mitochondrial cyt b gene were amplified by PCR and analyzed by single strand conformation polymorphism (SSCP) to get species-specific patterns of single- stranded DNA (ssDNA). These DNA strands were separated by polyacrylamide gel electrophoresis and visualized by silver staining (Rehbein *et al.*, 1997). On the other hand, a method has been developed to identify samples of selected animal species and man by analysis of the cyt b gene using species-specific RFLP patterns. These patterns were generated by co-restriction of PCR products with restriction endonucleases (Zehner *et al.*, 1998).

The recent technique for species identification of vertebrate animals was carried out by nucleotide sequence analysis of the cyt b gene. The sequences derived were used to identify the biological origin of the samples by aligning to cyt b gene sequence entries in nucleotide databases using the BLAST program (Parson *et al.*, 2000).

Single nucleotide polymorphisms (SNPs) variants in DNA sequences provide a shortcut to comparing genes and genomes within and among species. They occur frequently throughout the genome and tend to be relatively stable genetically, and therefore they serve as excellent biological markers in addition to their use as valuable genetic markers for revealing the evolutionary history of populations.

On the other hand, mitochondrial rRNA genes are also widely studied in evolutionary biology (Allard & Honeycutt, 1992; Whitfield & Cameron, 1998; Arctander *et al.*, 1999) and some recent studies have also proven the usefulness of unique polymorphisms present in mitochondrial rRNA genes in species identification (Carrera *et al.*, 1999; Chapela *et al.*, 2002; Vences *et al.*, 2005).

This study aimed to develop an easy method for species identification and differentiation based on sequence analysis of any unknown *Bovidae* samples (buffalo, cattle, sheep and goat) followed by alignment with homologous sequences available on GenBank database.

MATERIALS AND METHODS

DNA purification: Genomic DNA was extracted from the peripheral blood of nine Egyptian buffalo samples, using standard commercial Kit (Pure-gene Genomic DNA purification Kit) as recommended by the manufacturer.

PCR amplification and direct sequencing: Primers specific for mitochondrial 12S rRNA gene were designed: 5′-CAAACTGGGATTAGATACCCCACTAT-3′; 5′-AGGGTGACGGGCGGTGTGT-3′ (Kocher *et al.*, 1989) and directed towards the two conserved regions of the gene. The primers were synthesized by Amersham Pharmacia Biotech (U.K.). Amplification reactions were carried out using 25 µl reaction mixture consisting of 1.25 unit *Taq* polymerase (DyNAzyme), 1X enzyme buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl and 0.1% Triton X-100), 1 µM forward and reverse primers, 0.2 mM dNTPs and 100 ng of DNA. The reaction mixture was overlaid with sterile mineral oil and was run in an MJ research PTC-100 Thermocycler (MJ Research Inc., Watertown, MA, USA). The cycling conditions were: 3 min. at 95°C, followed by 30 cycles of 45 seconds at 94°C, 1 min. at 55°C and 1 min. at 71°C. Amplification products were electrophoresed with size marker (*Hae* III digest of Φ X174 DNA) on 1.5% agarose in 1X- Tris acetate buffer (TAE) containing 0.8 µl of 10 mg/ml ethidium bromide (Ausubel *et al.*, 1990). Before sequencing, PCR products were purified using QIAquick PCR purification kit (Qiagen, Inc. Valencia, CA, USA).

Sequencing was performed on an ABI Prism 310 genetic analyzer (Applied Biosystem, CA, USA) using the BigDye™ terminator cycle sequencing ready reaction mixture according to the manufacturer's instructions (Applied Biosystems). Direct submissions of the nine tested sequences were made to NCBI GenBank database with the accession numbers (FJ828575-FJ828583).

Pairwise sequence alignments were carried out using NCBI-BLASTN version 2.2.5 and PSI BLAST (Altschul *et al.*, 1997). Multiple sequence alignments and variable sites extraction of 12S rRNA sequences from Egyptian buffaloes and GenBank database samples were done using the MUSCLE 3.6 software (Edgar, 2004) and CLUSTALW 1.82 (Thomson *et al.*, 1994).

RESULTS

This study aimed to develop a highly specific and straightforward tool to identify the origin of any unknown DNA sample that may belong to one of the four studied *Bovidae* species, i.e. buffalo, cattle, sheep and goat. The 12S rRNA sequences of nine tested Egyptian buffalo samples were identical and the total length of each sequenced segment was 365 nucleotides. Only nucleotides between No. 26 to No. 349 contained variable sites while the remaining was conserved. Forty eight variable sites (single nucleotide polymorphisms, SNPs) were detected in-between the two conserved regions of the gene. The bases flanking this variable region are completely conserved in the four *Bovidae* species.

Two haplotypes of 12S rRNA sequences were identified from the multiple alignment results between the nine tested Egyptian buffalo sequences and other examples of homologous buffalo sequences selected from GenBank database Two buffalo haplotypes were revealed, of which haplotype 1 which include Egyptian buffaloes and haplotype 2 which include Chinese swamp buffalo; breed: Haikou (accession AY702618), Mediterranean (accession AY488491) and Bubalus bubalis (accession AF231028). The detected SNPs can be classified as shown in table 1.

Eleven SNPs were detected which can be used to discriminate between subfamily *Bovinae*, represented by buffalo and cattle and the subfamily *Caprinae* represented by sheep and goat. The first group is identified by guanine at position 185; adenine nucleotide at positions 38, 203 and 229; thymine at positions 37, 68, 122, 282 & 283 and cytosine at positions 270 and 274. The second group is distinguished by another set of SNPs, represented by guanine at positions 38, 203 and 229; cytosine nucleotide at positions 37, 68, 122, 282 & 283 and thymine nucleotide at positions 185, 270 and 274.

Although all the previously presented SNPs are specific for species identification, the remaining unmentioned ones (presented in Fig. 1) can also be used for the determination of the origin of an unknown sample.

DISCUSSION

The DNA molecule makes it an extremely useful tool for molecular species identification. Species identification using DNA analysis has already been described by many authors (Chikune *et al.*, 1994; Forrest &

Carnegie, 1994; Zehner *et al.*, 1998) and has replaced or at least supplemented species determination by biochemical tests in many laboratories. DNA is an extremely stable and long-lived biological molecule that can be recovered from biological material that has been under stress conditions (e.g. processed food products, blood stains, among others). It is found in all biological tissues or fluids and can provide more information than proteins.

Mitochondrial DNA (mt DNA) has become a very powerful tool in species identification and forensic sciences because of the high number of copies in each cell and the lack of recombination with paternal mtDNA. The high copy number results in increased sensitivity of mtDNA testing as compared to nuclear DNA. For species identification, a number of studies have adopted the universal primers introduced by Kocher *et al.* (1989) targeting the cyt b locus (Parson *et al.*, 2000; Hsieh *et al.* 2001; Branicki *et al.*, 2003; Bellis *et al.*, 2003). However, it is evident that regions residing in the 12S rRNA locus in the mitochondrial genome among mammals are more strictly conserved than the region in the cyt b locus. These regions are located in both the loop and stem portions in the secondary structure (Springer & Douzery, 1996). It should be noted that no such highly conserved region has been found in the cyt b region, which is commonly used as a means for species identification (Bataille *et al.*, 1999; Parson *et al.*, 2000; Bellagamba *et al.*, 2001; Hsieh *et al.*, 2001; Bellis *et al.*, 2003; Wan & Fang, 2003).

Table 1: Nucleotide variation in a specific 12S rRNA gene fragment of four studied Bovidae species. Nucleotide positions correspond to Egyptian buffaloes GenBank accession numbers (FJ828575-FJ828583).

No. of SNPs	Specificity	Representing nucleotide	Base position
8	all buffaloes (haplotypes 1 & 2)	Guanine	at positions 110, 132 and 196
		Thymine	position 172
		Cytosine	positions 71, 269, 271 and 348.
3	buffalo haplotype 1 only	Guanine	position 158
		Cytosine	position 267
		Adenine	position 293
4	buffalo haplotype 2 only	Thymine	positions 32 and 267
		Guanine	position 293
		Cytosine	position 72
12	cattle (*Bos taurus*)	Guanine	positions 193, 266 and 273
		Adenine	27 and 174
		Thymine	positions 26, 36, 186 & 190
		Cytosine	positions 253, 294 and 295.
6 + 2 indels	sheep (*Ovis aries*)	Guanine	positions 172, 231 and 300
		Adenine	position 190
		Thymine	position 271
		Cytosine	position 349
		insertion of Adenine	position 164-165
		deletion of Adenine	position 259
6 + 1 deletion	goat (*Capra hircus*)	Guanine	position 299
		Thymine	positions 260, 261 and 317
		Cytosine	positions 225 and 259
		deletion of Adenine	deletion at position 273

The present study offers a specific, reliable and straightforward way for identification of bovine and caprine species based on the sequence analysis of an unknown sample and its alignment with homologous sequences available on GenBank. Any of the nine tested 12S rRNA sequences of Egyptian water buffalo can serve as a reference since they were all found to be completely conserved. These DNA samples from Egyptian buffaloes were previously studied for phylogenetic analysis and comparison between cow and buffalo mitochondrial displacement-loop regions (Ramadan & El Hefnawi, 2008), where four haplotypes and nine polymorphic sites were identified in the entire D-loop of the Egyptian buffaloes. There were similarities with some Indian haplotypes, but the Egyptian buffaloes were shown to be closest to their Italian (Mediterranean) counterparts (Parma *et al.*, 2004; Kierstein *et al.*, 2004).

Four *Bovidae* species were compared in this study, where subfamily *Bovinae* is represented by buffalo and cattle, while subfamily *Caprinae* is represented by sheep and goat. The variable site number and the nucleotide number can indicate the origin of any unknown sequence that belongs to any of the four studied *Bovidae* species. This can be achieved by alignment of the unknown sequence with a reference (reference can be any of the accession numbers FJ828575-FJ828583), and identification of the variable sites and their positions which leads to species identification after comparison with the positions and types of the corresponding specific SNPs.

Sequences homologous to the specific 12S rRNA Egyptian water buffalo fragment were selected and aligned (Fig. 1). The multiple sequence alignment results showed 48 SNPs. Two buffalo haplotypes were revealed, of which haplotype 1 which include Egyptian buffaloes and haplotype 2 which include Chinese swamp buffalo; breed: Haikou (accession AY702618), Mediterranean (accession AY488491)and Bubalus bubalis (accession AF231028). Eight SNPs were found to be specific for the identification of the two buffalo haplotypes, three SNPs were specific for haplotype 1 only and 4 SNPs for haplotype 2. On the other hand, 12 SNPs were found to be specific for the identification of cattle (*Bos taurus*), 6 SNPs and 2 indels for sheep (*Ovis aries*) and 6 SNPs and one deletion for goat (*Capra hircus*), in addition to eleven SNPs which allowed discrimination between *Bovinae* and *Caprinae*.

In conclusion, the presented straightforward sequence examination and detection of specific SNPs (located inside the conserved region of the species) between Egyptian buffalo 12S rRNA gene fragment (accession numbers FJ828575-FJ828583) and the other three studied *Bovidae* species, is of valuable importance for species identification, an important and essential issue for food quality control-procedures, legal culling of animal populations and in forensic sciences.

Figure 1: Multiple sequence alignment results showing the detected variable sites between the specific Egyptian buffalo 12S rRNA fragment [GenBank: FJ828575-FJ828583] and examples of homologous sequences from water buffaloes (BBU), Cattle (*Bos taurus* & JBC), Sheep (*Ovis aries*) and goat (*Capra hircus*). View and download figure on a separate (adjacent) link.

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