MULTIPLEX PCR-GENETIC DIFFERENTIATION OF INTER-SPECIES MEAT MIXTURES BASED ON ORTHOLOGOUS GENE, CYTOCHROME-b.

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Identification of the purity of meat samples offered for sale and in eating joints is necessary for consumer preference and regulatory surveillance. In this regard, the discrimination of species specific meat in food and raw materials assures the quality standards and in monitoring of commercial frauds in meat-containing products. Determination of the species from traces of source material is sometimes difficult and critical task in forensic DNA analysis. In several countries, becomes of particular importance due to socio-religious issues associated with the preference. The Egyptian ministry of health frequently reported cases of meat adulteration with meats strictly forbidden to be used for human consumption for medical and religious reasons.

Unfortunately, inter-species meat adulteration is quite often difficult to distinguish visually. The conventionally available methods include various forms of electrophoresis (Skarpeid *et al*., 1998), liquid chromatography (Ashoor *et al*., 1998) and use of immune sera in agar gel diffusion (Hsieh *et al*., 1998). However, these methods depend on protein expression pattern which is heat-labile and get denatured after processing and cooking. This results in the alteration of electrophoresis pattern and loss of biological activity. Moreover, immune sera often show cross species reactivity (Rastogi *et al*., 2004). In comparison with proteins, DNA is stable against technological treatments and independent of the considered tissue. With the help of PCR the identification can be realized in a very fast way (just from very small quantities), cooked samples and prolonged storage products as well. In addition even after subjecting to heat during cooking, the DNA would still be amenable to PCR amplification. Moreover, even if there is partial degradation after prolonged storage, minute fragments could still be amplified. For these reasons, nucleic acid-based analyses are now the preferred techniques for species identification in processed food (for comprehensive review sees Lenstra *et al*., 2001).

The first genetic approach for determination of species identity was the dot-blot technique, (Wintero *et al*., 1991). Some approaches based on RAPD-PCR (Lee and Chang, 1994) and others are

focused on RFLP analysis, Calvo *et al*. (2001). Existing techniques consist of laborious and costly DNA sequencing procedures. Recently, PCR amplification of species-specific gene fragment is adopted. Bottero *et al*. (2003) developed a method which involved the ability of primers to amplify wider target sequences. This PCR based assay demonstrated high sensitively and useful in routine feedstuff analysis.

Although different genes might serve as target for PCR approach, mitochondrial genes represent a good source to ortholog variable samples. Mitochondrial 12S rRNA gene sequence analysis has been extensively used in molecular taxonomy and phylogeny. Also, 12S rRNA is used for species identification and detection of cross-contamination in cell lines and also for identification of a disputed skin sample received in a forensic laboratory, Rastogi *et al*. (2004). The mitochondrial cytochrome b gene has been used in phylogenetic as well as in forensic investigations (Kocher *et al*., 1989; Zehner *et al*., 1998; Bataille *et al*., 1999) and has been shown in a variety of studies to be a very useful DNA-region for species characterization (Wolf *et al*., 1999; Yan *et al*., 2005).

The present work aimed to develop degenerate primer for simultaneous identification of six commonly used species for meat production in Egypt viz., cattle, buffaloes, camels, pigs, goats and sheep. To ortholog these species mitochondrial cytochrome-b gene was employed for multiplex-PCR approach.

MATERIALS AND METHODS

1. Meat samples preparation

Meat samples of the six species used herein; (cattle, C; buffaloes, B; camels, Ca; Pigs, P; goats, G; and sheep, S); were collected from Al-Basateen governmental slaughterhouse, Cairo, Egypt. At least, five animals were randomly sampled and specimens were taken from longitudinal dorsal muscle of each animal. Then, the samples were kept in plastic bags, tagged and transported in icebox to the meat lab. Each sample was visually examined for general physical appearance, where it rinsed and the extraneous fat was removed. The samples were individually minced and kept frozen until DNA extraction.

2. DNA extraction

Total cellular DNA from the meat samples was isolated following the method of Krieg *et al*. (1983) with some modifications. Briefly, 1.0 g of frozen minced tissue was extracted in 3 ml of SSE buffer (0.3 M Sodium acetate, 0.5% SDS, 5 mM EDTA, pH 8.3). Then, the supernatants were organic-extracted in 3 ml phenol chloroform (1:1). After well mixing the samples were subjected to centrifugation (3000 g for 15 min). The aqueous phase was extracted twice with chloroform. Finally, the aqueous phase was transferred and the DNA was recovered by alcoholic precipitation (2

volumes of cold absolute ethanol). The DNA was collected by centrifugation, washed (in 70% ethanol), air dried and dissolved in TE buffer. The extracted DNA was visualized on agarose and its quantity was adjusted to a final concentration of 50 ŋg/µl with spectrophotometer (Beckman, DU600 series).

3. Retrieving sequences, alignment and primers designing

To ortholog the selected six species presented herein, mitochondrial cytochrome-b (*Cyt-b*) genes sequences embedded in Gene Bank database were retrieved and analyzed. Their accessions are; *AY95295* for *Bos taurus* (Cattle), *D88637* for *Bubalus bubalus* (Buffaloes), *U06426* for *Camules dromedarius* (Camels), *AB015079* for *Sus scrofa domesticus* (*Pigs, AB004075* for *Capra hircus* (Goats) and *DQ097430 for Ovis aries* (Sheep). Oligonucleotide primers were designed from different regions of mitochondrial DNA (cytochrome-b gene) after alignment of the downloaded sequences. Six regions covered different sites specific to the six species were used in the present work producing varied amplicons.

All of sequence handling, alignments, distances and phylogeny tree reconstruction were done by the aid of DNA star software package version 4.

4. Simplex PCR

In a preliminary phase of this investigation, primers specificity was individually assessed with DNA extracted from each of the target species. In this regard, samples homogeneity was evaluated with applying simplex PCR approach for at least five individuals of each species. PCR amplification was performed in a final volume of 50 µl containing 75 mM Tris–HCl (pH 8.8), 1 unit of Platinum *Taq* DNA Polymerase (Invitrogen, USA), 0.1 mg/ml BSA (Sigma), 0.2 mM each of dATP, dCTP, dGTP, dTTP (Pharmacia), 2 mM MgCl2, 25 pmol of primers and 250 ŋg of DNA template. Amplification was performed in a Thermal Cycler 2400 (Perkin Elmer) with the following main cycling parameters were: 94°C for 8 min, followed by 35 cycles of 94°C/30s, 60°C/40s and 72°C/1 min with a final extension at 72°C/0 min. Annealing temperature ranged from 58°C to 62°C and optimized to 60°C for all species.

5. Multiplex PCR

For the simultaneous detection of each species in mixes, a one step multiplex PCR was developed using primer cocktail of the previously designed individual primers. Amplification was performed in a final volume of 25 µl containing 75 mM Tris–HCl (pH 8.8), 1.5 unit of Platinum *Taq* DNA Polymerase (Invitrogen, USA), 0.1 mg/ml BSA (Sigma), 0.25 mM each of dATP, dCTP, dGTP, dTTP (Pharmacia), 2.5 mM $MgCl_2$, 150 ŋg of DNA template and primers were mixed as F Cytb: reverse ones in the ratio of 1F: different reverses ratio (0.6 (S) : 0.2 (G) : 3 (P) : 0.6 (Ca) : 2

(B) :3 (C). Thermal cycling was programmed following the same procedure as in simplex PCR. All PCR products were detected by Agarose gels electrophoresis (1% in TAE buffer).

RESULTS AND DISCUSSION

1. Retrieving sequences, alignment and primers designing

Primer design was carried on the basis of sequences identities and divergences to cover varied amplicons. The forward primer was chosen according to a highly identical region (8-36) with some degenerate bases. Furthermore, reverse primers were chosen according to divergent regions and different amplicons of sizes. The analysis of used sequences presented in the current study revealed six different regions specific to each species. As predicted, the resulted amplicons were 134 bp for sheep, 215 bp for goats, 271 bp for pigs, 361 bp for camel, 476 bp for buffalo and 562 bp for cattle. Athor factors affecting primer design belong to its entire sequence characteristics; e.g., primer-primer dimmers (involved three combinations; F:F, F:R & R:R), annealing temperatures, etc.).

Table (1) summarizes sequence accessions numbers, primer sequences (both forward and reverses), primer lengths and sites and predicted amplicons of different species. The degenerate forward sequence is given as international nucleotides codes (M= A or C, P= purines, Y= perymidines and W= A or T). Partial sequences multiple-alignment (590 bp) covering the amplified region is shown in Table (2). Priming-sites are presented in italic bold face letters and the amplified segments belonging to different species are presented as shaded characters. As shown in the table, forward primer matched degenerate conserved region, whilst reverse ones matched different divergent regions as markers to different species. All of sequences, handling, alignments, distances and phylogeny tree reconstruction were done by the aid of DNA* software package version 4.

Table (3) shows produced pairwise identities and divergence among the six species used herein using weighed residue. The upper diagonal presents pairwise identities, whilst the lower one indicates sample divergences. The sequences analysis revealed entire differences among species aiding in distinct genetic differentiation in correspondence to each meat origin. It is worthy to indicate that the higher identity value was between Goats and Sheep, while, the lowest one was between Cattle and Camel. This observation reveals sufficient divergences among *Cyt-b* sequences (11.7:24.6) to design universal primers amplifying corresponding amplicons.

Phylogenetic tree reconstruction using CLUSTALW method with weighed residue is presented in Fig. (1). A dendrogram revealed one main group of four species including two subgroups. Subgroup 1 included both C and B and subgroup included G and S. The remaining

two species P and Ca represented distant sequences.

2. Simplex PCR specificity and homogeneity Test

To confirm samples homogeneity within each species at least five individuals were evaluated. The same simplex PCR conditions was applied and revealed samples identities within each species (data not shown). This results support the usefulness of proposed technique presented herein as detection method to differentiate between detection species. To verify the specificity of the primers and optimize PCR conditions, simplex PCR were carried out on bulked DNA extracted of each species. The PCR products are shown in Fig. (2-a). The primers generated specific amplicons of approximately 562, 476, 361, 271, 215 and 134 bp in length for cattle (C), buffaloes (B), camels (Ca), pigs (P), goats (G) and sheep (S), respectively. These different amplicons sizes serve as Potential genetic markers to differentiate the six meat species in their different mixture. In addition, it might be concluded these fragments could be used as specific probes in further work. Cytochrome-b gene has been shown in a variety of investigations to be a very useful DNA-region for species characterization, Wolf *et al*. (1999), Partis *et al*. (2000) and Yan *et al*. (2005). Among different genomic background (Partis *et al*., 2000) applied *Cyt-B* gene as genetic tool to distinguish 22 meat origins belong to different taxa (animal livestock, avian, fish, human and others).

3. Multiplex PCR specificity

The multiplex PCR was carried out on analogous samples and the results are given in Fig. (2-b). It appeared that the multiplex PCR produced a single band of target size corresponding to its species origin. As shown in Fig. (2-b), no non-specific fragment was produced supporting the consistency of the proposed methods described herein. in the case of 12S rRNA mitochondrial gene. It might be due to the method of sample preparation, primer design, PCR conditions and other technical PCR points affecting success of amplification. Primer specificity and Tm are more critical for multiplex-PCR than conventional approaches, (Matsunaga *et al*., 1999). The PCR products from sheep, goat, pig, camel buffalo and cattle were unambiguous and giving corresponding distinct single DNA amplicons of ≈134, ≈215, ≈271, ≈361, ≈476 and ≈562 bp, respectively. These results confirmed the ability of PCR approach presented herein to discriminate the six meats species. The present study showed lower mismatching between such species-specific primer and the other sequences (1% vs. 15%). Different meat mixes combinations were adopted to circumvent the discrepancy of the method. Meat mixing falls into three categories, all meat mix, big animal meat mix (C, B & Ca) and small animal meat mix (P, G, & S). With referring to Table (3), divergences average among three large animals

is (23.266), whilst it is (17.3) among small ones. However, distinct bands were obtained and fit corresponding meat origin.

In addition, to test the potentiality of designed primers and illustrated PCR approach presented herein in meat industry, different mixtures ratios between cattle and pig meats were obtained. Figure (3) shows distinct produced amplicons fitting meat mixture ratios. These mixs were 100% C (lane 1), 75% C+ 25% P (lane 2), 1:1 C:P mix (lane 3), 25% C + 75% P (lane 4) and 100% P (lane 5) The results obtained revealed the sensitivity of proposed approach to detect meat adulteration even at low percentage.

4. Conclusion

Mitochondrial DNA sequence is highly conserved in different species of animals (Antoinette *et al*., 1995). T he Mitochondrial *Cyt-b* gene has been well characterized among different vertebrate groups and has been sequenced, Chikuni *et al*. (1994). This has enabled designing of universal primers for the *Cyt-b* gene, which can amplify corresponding fragments in a wide variety of organisms. General 5 differences between mitochondrial *Cyt-b* gene sequences are sufficient for species identification of different biological samples (Prakash *et al*., 2000). Molecular techniques have been tried elsewhere for meat identification. Although, DNA sequencing and analysis is accurate and authentic, it is costly, time consuming and not suitable for routine species identification studies. In the present study, a partial sequence of mitochondrial *Cyt-b* gene was targeted for the PCR study, so as to identify the meat species, generate molecular probes for further work and fit meat adulteration even at low quantities. As each cell contains more than one thousand copies of mitochondrial DNA, PCR assays based on its amplification were shown to be more sensitive as compared to single or low copy nuclear DNA targets, (Partis *et al*., 2000). Since, the quantity of PCR products generated corresponded to the copy number of the target DNA sequence (Partis *et al*., 2000), a higher copy number of mitochondrial DNA ensures a sufficiently high quantity of PCR product, even when small amounts of fresh/or processed meat samples are used. Because of the high copy number of small, circular mitochondrial DNA in cells, the chances of their survival under different processing conditions are higher, making it ideal for processed meat species identification. However, mitochondrial genes are monophyletic (Palumbi & Cipriano, 1998) and highly conserved (Anderson *et al*., 1982; Partis *et al*., 2000). The method described in the present investigation proved to be highly sensitive and has the potentiality to distinguish between different meat origins. In addition, it might be served as forensic analysis and meat industry application.

SUMMARY

In this study, sequence analysis of mitochondrial cytochrome-b gene was

applied for meat species identification. Six species meat origins which are commonly used in meat production and industry were sampled, viz, cattle, buffaloes, camels, pigs, goats and sheep. Five individuals from each species were tested for homogeneity. Based on conserved and divergent gene region, universal primers were designed and successfully produced species-specific amplicons which ranged from 134 bp to 562 bp. In this regard, two PCR approach were carried out, simplex and multiplex PCR. On the other hand, different combinations of meat mixtures were prepared and, also, different ratios of meat sources were obtained. A multiplex PCR was performed by mixing the six primers in appropriate ratios. The primers generated specific amplicons of ≈562, ≈476, ≈361, ≈271, ≈215 and ≈134 bp in length for cattle, buffaloes, camels, pigs, goats and sheep, respectively. The multiplex PCR proposed in this study might be considered as potential methods for identification of meat samples originated from different sources. In addition, the proposed method described herein supports its potentiality to detect meat contamination at low quantities. The results obtained confirm the power of mitochondrial *Cyt-b* gene to ortholog different species resources as well conspecific populations and thus, might be applied as a detection method in forensic applications in meat industry.

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Table (1): The accession numbers, the used forward and reverse primers (5'-3') of each six species, priming sites and different produced amplicons.

Accession No.*	Primer type	Species (symble)	Primers sequences (5' – 3')	Primer length	Priming site	Amplicons
	Forward	Degenerate	ACATCCGAAAAPWCMACCCAYTWMTAAAA	29	8-36	------
AY952958		Cattle, (C)	GCGATGATGAATGGGAAAATAAAGTGA	27	544-569	562
D88637		Buffaloes, (B)	AACCAGACTTGTACCAATGTATGGGATTG	29	456-486	476
U06426		Camel, (Ca)	ACTGTGAACAATAAAACAATTCCAACGT	28	342-368	361
AB015079	Reverse	Pigs, (P)	CAAATAAAGAACATGGATGCTCCGTTTG	28	251-278	271
AB004075		Goats, (G)	ATTTACATCTCGACAAATGTGAGTT	25	198-222	215
DQ097430		Sheep, (S)	GTTAGAATCTGTAAAATTAAGCAA	24	117-140	134

* GB, Gene Bank database ** Sequences are presented as international code

Table (2): Partial nucleotides sequences (1-590 bp) multiple alignment of the six cytochrome b indicating conserved segments (degenerate forward primer, 8-36), divergent regions (6 reverse primers) and 6 produced amplicons (grey-shaded letters). Priming sites are presented as italic bolded face characters.

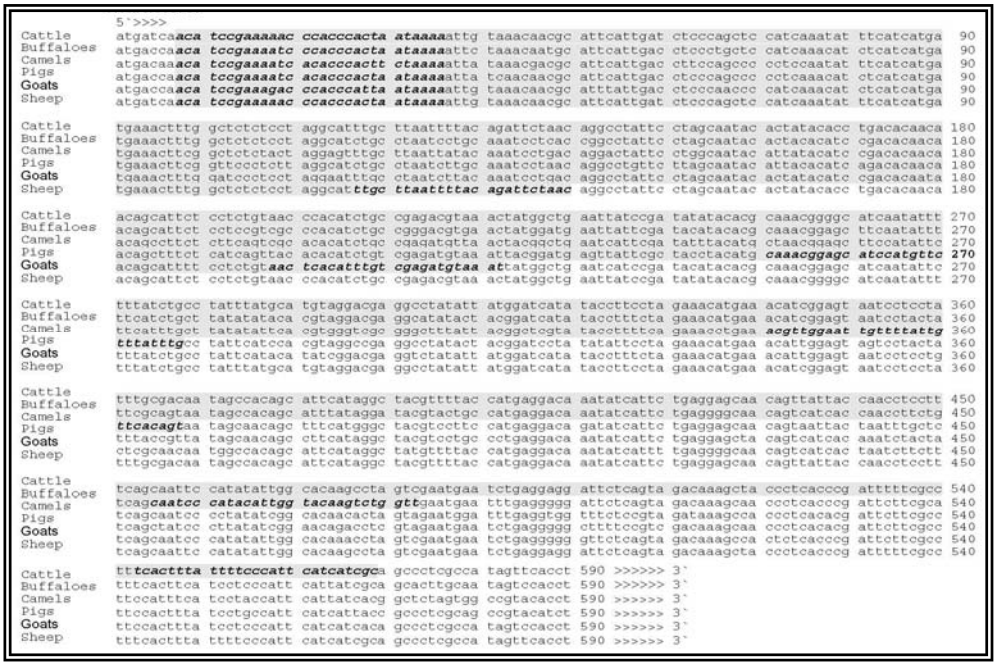

Table (3): Sequence pair-wise distances produced using CLUSTALW method with weighed residue. Identities are given in upper-diagonal and divergences are given in lower-diagonal. The highest identity value (bolded) resulted between Goats (G) and Sheep (S), while the lowest one (bolded) was between Cattle (C) and Camel (Ca).

		Identities					
		C	B	Ca	P	G	S
Divergences	C		87.6	77.6	79.8	85.6	84.4
	B	13.2		78.8	82.5	85.4	87.1
	Ca	24.1	22.5		80.0	78.0	76.9
	P	21.5	19.1	21.8		82.0	80.7
	G	15.9	15.5	24.1	19.1		**88.8**
	S	16.8	13.8	24.6	21.1	11.7	

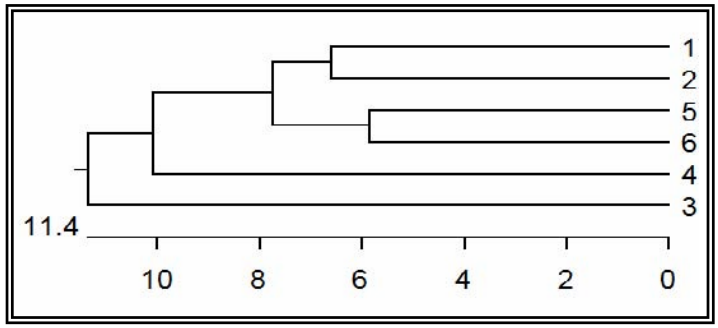

Fig. (1): Phylogenetic tree of Cyt-B using clustalW method with weighed residue indicating main group of 4 species (includes two sub-groups, (C, B) cluster1 and (G, S) cluster 2) and out-groups of two species (P & Ca).

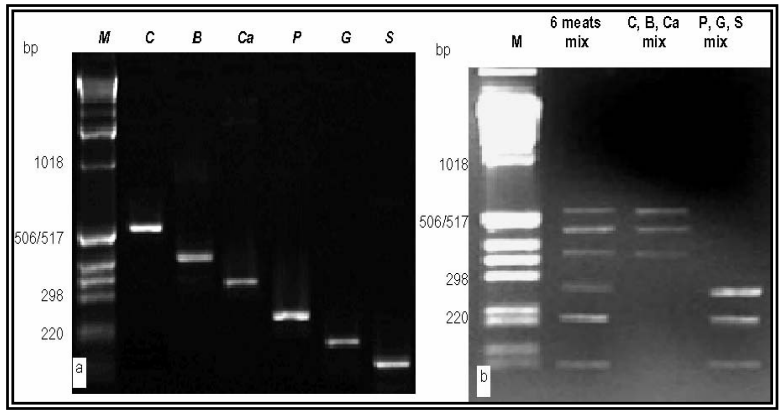

Fig. (2): Agarose gel (1% in TAE buffer pH 8.3) electrophoretic patterns of both a: simplex PCR amplicons of single species meats and b: multiplex PCR amplicons of different meats combinations including 6meats mix (lane 1), 3 meats (big animals; C, B and Ca) mix (lane 2) and 3 meats (small animals; P, G and S) mix (lane 3). The designed primers here in produce distinct bands belong to their different species origins both in simplex and multiplex PCR. *C*, cattle; *B*, buffalo; *Ca*, camel; *P*, pig; *G*, goat; *S*, sheep and *M*, DNA sizing marker.

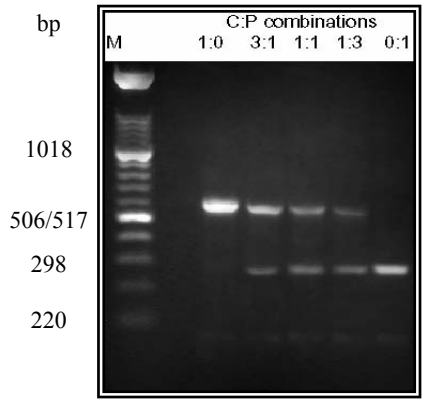

Fig. (3): Agarose gel electrophoresis of PCR-amplified *Cyt-B* gene segments targeting different combinations of Cattle (C) and Pigs (P) meat mixture confirming method sensitivity of disigned primers to species-specific adulteration. 100% C (lane 1), 57% C+ 25% P (lane 2), 1:1 C:P mix (lane 3), 25% C + 75% P (lane 4) and 100% P (lane 5).