ISSN : 0974 - 7427 Volume 6 Issue 3

Trade Science Inc.

BIOCHEMISTRY
An Indian Journal

Regular Paper

BCAIJ, 6(3), 2012 [71-77]

Forensic biological identifications of mammal species through DNA barcodes

Magdy M.Youssef[1,2]*, Yehia A.Hussein[3,4], Abd Rahman Heraba[3], Saad S.Al-Shokair[3]

[1]Biochemistry Division, Chemistry Department, Faculty of Science, Mansoura University, (EGYPT)

[2]Chemistry Department, College of Science, King Faisal University, (KSA)

[3]Clinical Studies Department, College of Vet. Med. & Animal Resources, (KSA)

[4]Forensic Medicine and Toxicology Dept.,Faculty of Vet.Med., Alexandria University, (EGYPT)

Received: 19th February, 2012 ; Accepted: 19th March, 2012

ABSTRACT

One of the grand challenges of modern biology is to develop accurate and reliable technologies for a rapid screening of DNA variations. This topic of research is of prime importance for the detection and identification of species in numerous fields of investigation. Variety of DNA based approaches have been developed for the identification of individuals in a myriad of taxonomic groups. In the present work, We extracted genomic DNA from whole blood samples obtained from variant eukaryotic species and compared the results of the product obtained in terms of quantity (concentration of DNA extracted and DNA obtained per ml of blood used) and quality (260/280 ratio of the obtained yield. A series of random oligonucleotide primers were designed and used it with the genome DNA of individuals' species of Human, Horse, Camel, Rabbit, Cow, Chicken, Sheep and goats to identify and evaluate these species by utilizing polymerase chain reaction (PCR) based assays. The DNA was amplified and the migration pattern of the amplified specific short and long amplified DNA elements was quantified by the aid of agarose gel electrophoresis. The species specificity of the PCR amplicons was demonstrated by the ability of the assays to accurately detect and identify of species specific DNA from mixed sources. The PCR assay reported here will help facilitate the sensitive detection of common domestic animal and bird species DNA from complex biomaterials. The dendogram of the studied individuals' species was demonstrated. A critical evaluation of all methods is presented focusing on their discriminatory power, reproducibility and user friendliness. The current trend was used to develop small scale devices with a high throughput capacity.

INTRODUCTION

The diversity of life underprops all biological studies, but it is also a punitive load. Whereas physicists agreement with a cosmos accumulated a fundamental subdivisions, biologists confront a planet populated by millions of species. Their discrimination is no informal assignment. In fact, subsequently insufficient taxonomists can censoriously identify more than 0.01% of the predictable ten to fifteen million species[10], a community of fifteen thousand taxonomists will be required, in perpetuity, to identify life if our reliance on morphological analysis is to be continuous. Genomic attitudes to taxon analysis exploit variety among DNA sequences to classify creatures[15,24]. The application of forensics to wildlife crime investigation routinely includes genetic species identification based on DNA sequence similarity. A lack of authenticated reference DNA sequence data can be resulting in weak matches between evidence and reference samples[4]. The goal of DNA barcoding is to develop a species specific sequence library for eukaryotes. A 650 bp fragment of the cyto-

Regular Paper

chrome c oxidase 1 gene has been used fruitfully for species level identification in several animal groups[19]. It may be difficult in practice, however, to retrieve a 650 bp fragment from archival specimens or from environmental samples. The DNA analysis established the potential of much smaller fragments, mini barcodes, for identifying unknown specimens. Kitano *et al*.[14] established a simple method using universal primers for species identification based on direct PCR sequencing. Two primer sets were designed based on the conserved regions of the 12S and 16S rRNA loci detected by the comprehensive sequence comparison among 30 mammalian whole mitochondrial genomes. In humans, the expected sizes of PCR products of the 12S and 16S rRNAs were 215 and 244 bp, respectively. Both primer sets successfully amplified the expected PCR products from various kinds of vertebrates including mammals, birds, reptiles, amphibians, fish, and the sequenced segments contained sufficient nucleotide differences to identify each animal species[2]. A case example of the identification of a piece of buried bone of unknown species is presented, and the species was identified as a pig by this technique. Bellis *et al*.[3] investigated potential markers within chromosomal, mitochondrial DNA and ribosomal RNA with the aim of developing a DNA based method to allow differentiation between animal species. Such discrimination tests may have important applications in the forensic science, agriculture, quarantine and customs fields.

The difficulties encountered in the segregation and purification of DNA specially from eukaryotic species include degradation of DNA due to endonucleases, extremely viscous polysaccharides and other secondary metabolites which straight or circuitously interfere with the enzymatic reactions. Furthermore, the contaminating RNA that precipitates along with DNA reasons many difficulties including suppression of PCR amplification[22], intrusion with DNA amplification involving random primers, e.g. Random Amplification Polymorphic DNA (RAPD) analysis[18], and indecorous priming of DNA templates during thermal cycle sequencing. Different eukaryotic species often may not permit best DNA yields from one isolation procedure. For example, some closely related species of the same genus require different isolation protocols. Thus, an effective procedure for isolation of DNA as well as the optimization of the PCR circumstances is required. Various procedures for DNA extraction have been successfully applied to many eukaryotic species[7,26], which were further modified to provide DNA suitable for several kinds of analyses[23,25].

The integrated primate biomaterials and information resource provides essential research reagents to the scientific community by establishing, confirming, maintaining, and distributing DNA and RNA derived from primate cell cultures[16]. Characterization of species specific molecular markers and development of a method for identification of many species is necessary to monitor illegal trade of parts and products for better conservation and management of the endangered species. Universal primers were used for the amplification of the mitochondrial 12S rRNA gene from genomic DNA variant species[8].

Karlsson and Holmlund[13] identified mammal species by using species specific DNA pyrosequencing. In forensic casework it is highly relevant to be able to deduce the species origin of an unknown biological sample. For such a purpose they designed and developed an assay for species identification based on DNA sequencing of two short mitochondrial DNA amplicons. In short, partial 12S rRNA and partial 16S rRNA fragments are amplified by PCR followed by direct sequencing using pyrosequencing technique[11]. With millions of species and their life stage transformations, the animal kingdom provides a challenging target for taxonomy. Recent work has suggested that a DNA based identification system, founded on the mitochondrial gene, cytochrome c oxidase subunit 1, can aid the resolution of this diversity[12].

In this study we address the important issue of minimum amount of sequence information required for identifying species in DNA barcoding. The DNA samples from eight eukaryotic different species male and female individuals (including human) were analyzed. DNA extraction and quantitation followed by RAPD- PCR amplification and visualization formed the basis of the experimental analysis. The RAPD PCR method can be used an a novel approach based on a much shorter barcode sequence and demonstrate its effectiveness in archival specimens. This approach will significantly broaden the application of DNA barcoding in biodiversity studies.

MATERIALS AND METHODS

Blood collection

The whole bloods were collected from the male and female of the following eukaryotes rabbit, cow, camel, chicken, horse, sheep, goats, and human. Blood samples of cows, camels, horses, sheep, and goats were taken through venipuncture of jugular veins. The samples of rabbit and chickens were collected from ear and wing veins, respectively. Blood sample of human was taken from the vein in the antecubital fossa. Bloods were collected in a heparin containing Vacutainer tubes and stored at 4 °C and extracted within the same working day.

Extraction of DNA from whole blood

The DNA was extracted from the whole blood, according to the method described by Sambrook *et al.*[20].

In a 15-mL falcon tube 3 ml of the whole blood were added to 11 mL of reagent A (10mM Tris- HCl pH 7.4, 320m*M* sucrose, 5mM $MgCl_2$, 1% Triton X 100) and mixed on a rolling for 4 min at room temperature. Then, centrifuged at 3000 g for 5 min at room temperature. Without disturbing cell pellet the supernatant was discarded and the remaining moisture were removed by inverting the tube and blotting onto tissue paper. One ml of reagent B (40mM Tris-HCl, 150 mM NaCl, 60mM EDTA, 1% sodium dodecyl sulphate, pH 8.0) were added and mixed briefly to resuspend the cell pellet. Then, 250 µl of 5M sodium perchlorate were add and mixed by inverting tubes several times. The extract incubated in a water bath for 20 min at 65°C and allowed to cool to room temperature. 2 ml of ice cold chloroform were added to the extract and mixed on a rotating mixer for 60 min. The tubes were centrifuged at 2400g for 2 min and upper phase layer were transferred into a clean falcon tube using a sterile pipet. 3 mL of ice cold ethanol were added and the DNA allowed to precipitate by inverting the tubes gently. Using a freshly prepared flamed Pasteur pipet the DNA were spooled onto the hooked. The DNA samples were transferred into a 1.5-mL Eppendorf tube,allowed to air dry and resuspended in 200 µL of TE buffer (10 mM Tris-HCl pH 7.6, 1mM EDTA).

Amount and purity of DNA

The yield of DNA per ml of blood extracted was measured utilizing a UV Spectrophotometer at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. Any protein contamination of the DNA samples causes great problems during the subsequent handling. The DNA concentration and purity was also determined by running the samples on 0.7 % agarose gel electrophoresis. The nucleic acid concentration was calculated following Sambrook *et al.*[20].

Optimization of RAPD reaction

The RAPD-PCR was carried out using three universal oligonucleotide primers; the first was BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG-'3), the second was ERIC1R primer (5'-ATGTAAGCTCCTGGGGATTCAC-3') and the third was ERIC2 primer (5'-AAGTAAGTGACTGGGGTGAGCG-3') (metabion international AG, Lena-Christ-Strassa 44/I, D-82152 Martinsried/ Deutschland). The reactions were carried out in a DNA Thermocycler (9700 thermal cycler PCR, Eppendorf, Germany). Reactions without DNA were used as negative controls. The PCR reaction mixtures were prepared with 1µl of eukaryotic genomic DNA, 5 µl of Taq buffer 10x, 200 µmol of each deoxynucleoside triphosphate, 20 pmol each primer, 2.5 U Taq DNA polymerase (Promega, Germany) and sterile filtered mille water to a final volume of 50 µl. The PCR program was as follows: Primary denaturation at 95ºC for 4min, 38 cycles was applied as follow: 95ºC for one min., 45 ºC for one min. and 72ºC for 2min in Final extension step at 72ºC for 10 min and PCR reaction kept on 4ºC until removing the PCR tubes.

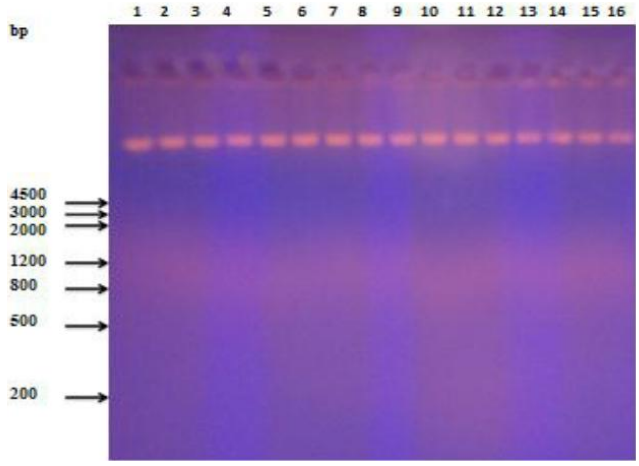
Agarose gels

DNA was analyzed by using horizontal agarose gel electrophoresis. The DNA mixed with 1/6 volume of loading dye (10% w/v ficol 400, 0.06% w/v bromophenol blue and 0.5% w/v SDS) and loaded onto the 0.8% agarose gel in TAE buffer (40mM Tris-HCl pH 7.9, 5mM sodium acetate, 1mM EDTA). The electrophoresis was performed in TAE buffer and stained with ethidium bromide (0.5 µg/ml).

Regular Paper

RESULTS AND DISCUSSION

Genomic DNA is a significant component in order to achieve molecular applications involving genomic studies. Whole blood is the primary source for DNA isolation[1]. The strategy of RAPD PCR is started with isolation the total chromosomal DNA from the variant eukaryotic species. Therefore, the chromosomal DNA of the male and female of rabbit, cow, camel, chicken, horse, sheep, goats and human were isolated and purified. It is problematic to extract high superiority DNA from eukaryotic species as rabbit, cow, camel, chicken, horse, sheep, goats and human because of the high substances of polysaccharides[17]. Moreover, it has been reported that the reproducibility of RAPD band patterns in eukaryotic species was affected by the method of DNA extraction and purification[18]. The quality of the isolated chromosomal DNA of variant eukaryotic species was evaluated by migrating the DNA in 0.7% agarose gel electrophoresis as represented in Figure 1. The procedure utilized in the DNA extraction gave a good yield of DNA ranging from 5.9 to 8.7 μg ml^{-1} blood (TABLE 1) across all eight eukaryotic species (rabbit, cow, camel, chicken, horse, sheep, goats and human). This yield was comparatively high to that of Pereira *et al*.[21] where the DNA yield ranged from 2.3 to 5.0 μg ml^{-1}. The procedure utilized in the DNA extraction revealed A260/280 ratio ranging from 1.5 to 1.9 (TABLE 1) indicating the absence of proteins and polyphenol contaminants and proved that the DNA extracted has a good quality and pure enough to be utilized with RAPD PCR technique. The purity of DNA was further confirmed by restriction digestion analysis using the restriction endonucleases *Eco* RI and *Hind* III (data not shown).



Lane 1 : Chromosomal DNA of male rabbit
Lane 2 : Chromosomal DNA of female rabbit
Lane 3 : Chromosomal DNA of male cow
Lane 4 : Chromosomal DNA of female cow.
Lane 5 : Chromosomal DNA of male camel.
Lane 6 : Chromosomal DNA of female camel.
Lane 7 : Chromosomal DNA of male chicken.
Lane 8 : Chromosomal DNA of female chicken.
Lane 9 : Chromosomal DNA of male horse.
Lane 10 : Chromosomal DNA of female horse.
Lane 11 : Chromosomal DNA of male sheep.
Lane 12 : Chromosomal DNA of female sheep.
Lane 13 : Chromosomal DNA of male goats.
Lane 14 : Chromosomal DNA of female goats.
Lane 15 : Chromosomal DNA of male human.
Lane 16 : Chromosomal DNA of female human

Figure 1 : 0.7% agarose gel electrophoresis showing the chromosomal DNA isolated from male and female of rabbit, cow, camel, chicken, horse, sheep, goats and human as an eukaryotic species.

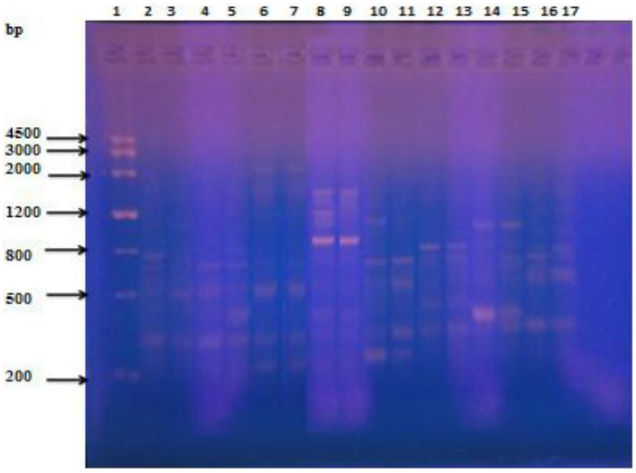
TABLE 1 : DNA yield and purity, isolated from different male and female of eukaryotic species (rabbit, cow, camel, chicken, horse, sheep, goats and human) using protocol of Sambrook *et al.*,[20].

Eukaryotic Species	A260	A280	A260/ A280	DNA Concentration (μg/ml^{-1}) blood
Rabbit (M)	0.148	0.093	1. 591	7.4
Rabbit (F)	0.132	0.089	1.483	6.6
Cow (M)	0.127	0.074	1.716	6.4
Cow (F)	0.134	0.068	1.970	6.7
Camel (M)	0.151	0.082	1.841	7.6
Camel (F)	0.133	0.091	1.681	6.6
Chicken (M)	0.141	0.079	1.784	7.1
Chicken (F)	0.126	0.083	1.518	6.3
Horse (M)	0.119	0.065	1.830	5.9
Horse (F)	0.127	0.078	1.628	6.4
Sheep (M)	0.144	0.083	1.734	7.2
Sheep (F)	0.174	0.094	1.851	8.7
Goats (M)	0.166	0.098	1.693	8.3
Goats (F)	0.172	0.097	1.773	8.6
Human (M)	0.148	0.089	1.662	7.4
Human (F)	0.136	0.096	1.416	6.8

Of 12 primers tested in the present work, only 3 were finally selected for analysis; these yielded strong and reproducible bands with all studied eukaryotic species. A total of 276 bands, ranging from 200 to 2500 base pair (bp). The representative RAPD PCR of the rabbit, cow, camel, chicken, horse, sheep, goats and

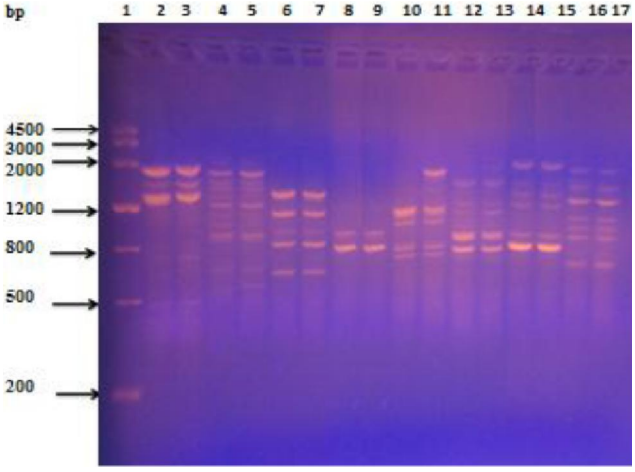
Regular Paper

human male and female DNA electrophoresis patterns with BOXA1R, ERIC1R and ERIC2 primers are shown in Figures 2, 3 and 4, respectively. The sequences and G+C contents of the primers, together with the number of bands they produced, are listed in TABLE 2. The DNA of all eukaryotic species in the present study were subjected to RAPD PCR using three universal primers, BOXA1R, ERIC-1R and ERIC-2. The RAPD band patterns resulting from the three primers were analyzed using UPGMA method to construct a similarity matrix and to generate a dendrogram indicating the relationship between the eight tested eukaryotic species of male and female of rabbit, cow, camel, chicken, horse, sheep, goats and human. The genetic relationships among rabbit, cow, camel, chicken, horse, sheep, goats and human are illustrated in the UPGMA dendrogram (Figure 5). The presence or absence of any particular DNA bands was the only parameter considered in the computer analysis. The dendrogram generated to show linkage distance (Figure 5) indicated that the eukaryotic species were classified into two main clusters (cluster I and cluster II) from one ancestor at 75% linkage distance. Cluster I was human, sheep and goats. Cluster I was subdivide at 64% linkage distance into cluster Ia of human and cluster Ib of sheep and goats. Also, the cluster Ib was subdivided at 55% linkage distance into cluster Ic of sheep and Id of goats. All the eukaryotic species at cluster II were divided at linkage distance 70% into two clusters, cluster IIa was of horse and cluster IIb was of rabbit, cow, camel and chicken. At linkage distance 67% cluster IIb was subdivided into cluster IIc of camel, chicken and cluster IId was of rabbit and cow. The cluster IIc at linkage distance 55% was subdivided into cluster IIf of chicken



Lane 1 : Gelpiolt DNA molecular weight marker (QIAGEN) Wide Range Ladder (100)
Lane 2 : Chromosomal DNA of male rabbit
Lane 3 : Chromosomal DNA of female rabbit
Lane 4 : Chromosomal DNA of male cow
Lane 5 : Chromosomal DNA of female cow.
Lane 6 : Chromosomal DNA of male camel.
Lane 7 : Chromosomal DNA of female camel.
Lane 8 : Chromosomal DNA of male chicken.
Lane 9 : Chromosomal DNA of female chicken.
Lane 10 : Chromosomal DNA of male horse
Lane 11 : Chromosomal DNA of female horse.
Lane 12 : Chromosomal DNA of male sheep.
Lane 13 : Chromosomal DNA of female sheep.
Lane 14 : Chromosomal DNA of male goats.
Lane 15 : Chromosomal DNA of female goats.
Lane 16 : Chromosomal DNA of male human.
Lane 17 : Chromosomal DNA of female human.

Figure 2 : Showing 2% agarose gel electrophoresis containing the RAPD PCR product of the BOXA1R nucleotide primere with the genomic DNA of variant eukaryotic species.



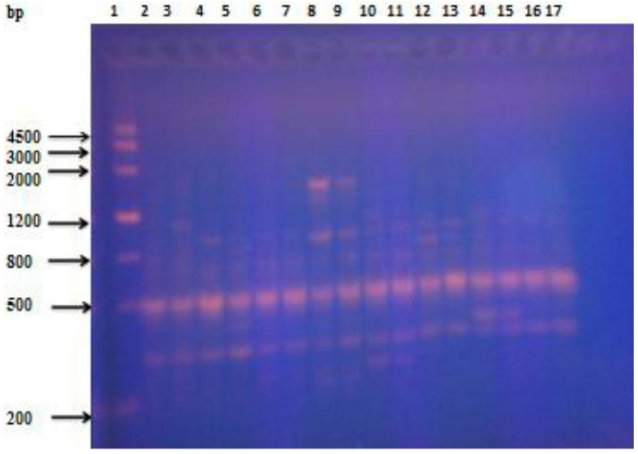
Lane 1 : Gelpiolt DNA molecular weight marker (QIAGEN) Wide Range Ladder (100)
Lane 2 : Chromosomal DNA of male rabbit
Lane 3 : Chromosomal DNA of female rabbit
Lane 4 : Chromosomal DNA of male cow
Lane 5 : Chromosomal DNA of female cow.
Lane 6 : Chromosomal DNA of male camel.
Lane 7 : Chromosomal DNA of female camel.
Lane 8 : Chromosomal DNA of male chicken.
Lane 9 : Chromosomal DNA of female chicken.
Lane 10 : Chromosomal DNA of male horse
Lane 11 : Chromosomal DNA of female horse.
Lane 12 : Chromosomal DNA of male sheep.
Lane 13 : Chromosomal DNA of female sheep.
Lane 14 : Chromosomal DNA of male goats.
Lane 15 : Chromosomal DNA of female goats.
Lane 16 : Chromosomal DNA of male human.
Lane 17 : Chromosomal DNA of female human.

Figure 3 : Showing 2% agarose gel electrophoresis containing the RAPD PCR product of the ERIC IR nucleotide primere with the genomic DNA of variant eukaryotic species.

Regular Paper

TABLE 2 : DNA sequences of the primers used for random amplified polymorphic DNA (RAPD) analysis

Primer	DNA sequence (5' → 3')	G+C content %	No. bands Products
BOXA1R	(5´-CTACGGCAAGGCGACGCTGACG-`3)	68.2	86
ERIC1R	(5´-ATGTAAGCTCCTGGGGATTCAC-`3)	50	98
ERICII	(5´-AAGTAAGTGACTGGGGTGAGCG-`3)	54.6	92



Lane 1 : Gelpiolt DNA molecular weight marker (QIAGEN) Wide Range Ladder (100)
Lane 2 : Chromosomal DNA of male rabbit
Lane 3 : Chromosomal DNA of female rabbit
Lane 4 : Chromosomal DNA of male cow
Lane 5 : Chromosomal DNA of female cow.
Lane 6 : Chromosomal DNA of male camel.
Lane 7 : Chromosomal DNA of female camel.
Lane 8 : Chromosomal DNA of male chicken.
Lane 9 : Chromosomal DNA of female chicken.
Lane 10 : Chromosomal DNA of male horse
Lane 11 : Chromosomal DNA of female horse.
Lane 12 : Chromosomal DNA of male sheep.
Lane 13 : Chromosomal DNA of female sheep.
Lane 14 : Chromosomal DNA of male goats.
Lane 15 : Chromosomal DNA of female goats.
Lane 16 : Chromosomal DNA of male human.
Lane 17 : Chromosomal DNA of female human.

Figure 4 : Showing 2% agarose gel electrophoresis containing the RAPD PCR product of the ERIC II nucleotide primere with the genomic DNA of variant eukaryotic species.

and cluster IIe of camel. While, the cluster IId at linkage distance 57% was subdivided into cluster IIh of rabbit and cluster IIg cow. Whenever, at distance linkage 25 % there is no similarity had been observed. The RAPD procedure has the benefits of simplicity, quickness, and requiring no specific sequence information of the target genome[20]. Thus, it is possible for molecular marker supported breeding, population inheritances, and biodiversity assessments. Based on the great level of polymorphisms in eukaryotic species, it was proven that the RAPD marker was reliable in revealing the relationships among variant eukaryotic species. It has been

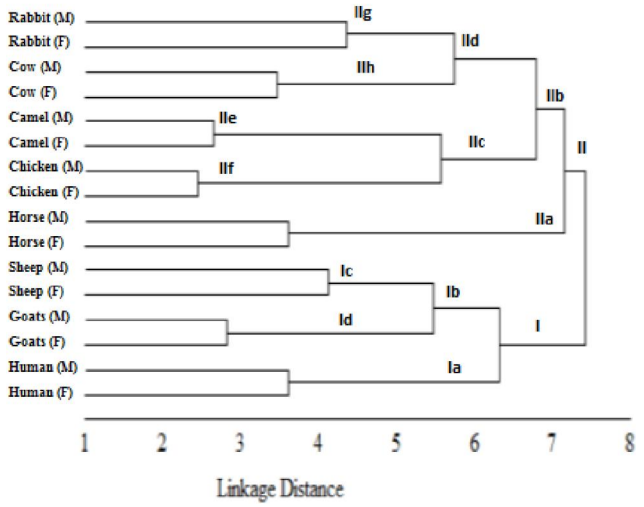


Figure 5 : Unweighted pair group method with arithmetic mean (UPGMA) dendrogram of the studied eukaryotic species based on the simple matching coefficient for of random amplified polymorphic DNA analysis using BOXA1R, ERIC1R and ERIC2 primers. (NB: M=Male and F=Female).

reported that the G+C content of the primer would affect the amplification results. We did not find any difference between primers with different G+C contents.

In conclusion, the present study suggests that RAPD analyses could be used to identify, to assess and to distinguish between Rabbit, Cow, Camel, Chicken, Horse, Sheep, Goats, Human eukaryotic species.

ACKNOWLEDGEMENTS

The financial support by the Deanship of Scientific Research (Project Number 120056) King Faisal University, Saudi Arabia is gratefully acknowledged.

REFERENCES

[1] A.A.Abd El-Aal, N.A.Abd Elghany, A.M.Mohamadin, A.A.El-Badry; Int.J.Health Sci., **3(1)**, 285-287 **(2010)**.
[2] J.An, M.Y.Lee, M.S.Min, M.H.Lee, H.Lee; Forensic Sci.Int., **167(1)**, 59-61 **(2007)**.
[3] C.Bellis, K.J.Ashton, L.Freney, B.Blair, L.R.Griffiths; Forensic Sci.Int., **134(2-3)**, 99-108 **(2003)**.

[4] M.Blaxter, J.Mann, T.Chapman, F.Thomas, C.Whitton, R.Floyd, E.Abebe; Philos.Trans.R Soc.Lond.B Biol.Sci., **360(1462)**, 1935-1943 **(2005)**.

[5] N.Dawnay, R.Ogden, R.McEwing, G.R.Carvalho, R.S.Thorpe; Forensic Sci.Int., **173(1)**, 1-6 **(2007)**.

[6] F.Di Pietro, F.Ortenzi, M.Tilio, F.Concetti, V.Napolioni; Mol.Cell Probes., **25(1)**, 44-48 **(2011)**.

[7] J.J.Doyle, J.L.Doyle; Phytochem.Bull., **19**, 11-15 **(1987)**.

[8] A.R.Gupta, R.C.Patra, D.K.Das, P.K.Gupta, D.Swarup, M.Saini; Mitochondrial DNA. **4**, 394-400 **(2008)**.

[9] M.Hajibabaei, G.A.Singer, E.L.Clare, P.D.Hebert; BMC Biol., **5**, 24 **(2007)**.

[10] D.L.Hawksworth, M.T.Kalin-Arroyo; Magnitude and Distribution of Biodiversity. In Global Biodiversity Assessment, V.H.Heywood, (Ed); Cambridge University Press, 107-191 **(1995)**.

[11] P.D.Hebert, A.Cywinska, S.L.Ball, J.R.de Waard; Proc.Biol.Sci., **270(1512)**, 313-321 **(2003a)**.

[12] P.D.Hebert, S.Ratnasingham, J.R.de Waard; Proc. Biol.Sci., **270(Suppl 1)**, S96-S99 **(2003b)**.

[13] A.O.Karlsson, G.Holmlund; Forensic Sci.Int., **173(1)**, 16-20 **(2007)**.

[14] T.Kitano, K.Umetsu, W.Tian, M.Osawa; Int.J.Legal Med., **121(5)**, 423-427 **(2007)**.

[15] C.P.Kurtzman; Yeast, **10**, 1727-1740 **(1994)**.

[16] J.G.Lorenz, W.E.Jackson, J.C.Beck, R.Hanner; Philos.Trans.R Soc.Lond.B Biol.Sci., **360(1462)**, 1869-1877 **(2005)**.

[17] C.Mayes, G.W.Saunders, I.H.Tan, L.D.Druehl; J.Phycol., **28**, 712-716 **(1992)**.

[18] M.Mejjad, F.Vedel, G.Ducreux; Plant Mol.Biol.Rep., **12**, 101-105 **(1994)**.

[19] I.Meusnier, G.A.Singer, J.F.Landry, D.A.Hickey, P.D.Hebert, M.Hajibabaei; BMC Genomics, **9**, 214, 12 May **(2008)**.

[20] M.U.Patway, J.P.van der Meer; J.Phycol., **30**, 91-97 **(1994)**.

[21] J.C.Pereira, R.Chaves, E.Bastos, A.Leitão, H.Guedes-Pinto; Int.J.Mol.Sci., **12(11)**, 8086-8095 **(2011)**.

[22] M.J.Pikkart, B.Villeponteau; Biotechniques, **14**, 24-25 **(1993)**.

[23] Y.Wang, D.E.Taylor; Biotechniques, **14**, 748-750 **(1993)**.

[24] K.H.Wilson; Clin.Infect.Dis., **20(Suppl.)**, 192-208 **(1995)**.

[25] B.Ziegenhagen, F.Scholz; Methods for Difficult Plant Species. In: Molecular Tools for Screening Biodiversity, A.Karp, P.G.Issac, D.S.Ingram, (Eds); Kluwer Academic Publishers, Belgium, 32-35 **(1998)**.

[26] B.Ziegenhagen, P.Guillemaut, F.Scholz; Plant Mol.Biol.Rep., **11**, 117-121 **(1993)**.

[27] J.Sambrook, E.Fritsch, T.Maniatis; A laboratory manual, Cold Sprint Harbor Laboratory Press, Cold Spring Harbor , NY **(1989)**.