Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 5, Issue, 09, pp.2430-2433, September, 2013

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

PCR-RFLP ANALYSIS OF MITOCHONDRIAL 12S R RNA GENE FOR IDENTIFICATION OF MEAT AND MEAT PRODUCTS IN SOME AVIAN SPECIES

***[1]Ismail A. Abdul-Hassan and [2]Ghufran J. Al-Aqeeli**

[1]Genetic Engineering and Biotechnology Institute, University of Baghdad, Iraq
[2]University of Wasit

ARTICLE INFO

Article History:
Received 26th June, 2013
Received in revised form
15th July, 2013
Accepted 27th August, 2013
Published online 14th September, 2013

Key words:
PCR-RFLP, 12S r RNA gene,
Avian meat species.

ABSTRACT

A molecular approach using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to distinguish four avian meat species in addition to pig. The partial 12S rRNA gene was amplified with universal primers. The amplified fragments (456 bp for all species studied) were digested with Acil restriction enzyme.The results showed that each of chicken, turkey, quail, duck and pig species exhibited a unique specific RFLP pattern that allowed its identification and discrimination from others. Meat products studied were confirmed through PCR-RFLP of 12S r RNA gene and showed conformity with the declared label.PCR-RFLP analysis of mitochondrial 12S r RNA gene was found to be an accurate and authentic method for meat species identification.

INTRODUCTION

Meat serves as the principal source of animal origin protein for mankind and it plays an important role in social, cultural andreligious life style of every community throughout theworld.There is a variation in the taste, nutritive and aesthetic value ofmeat from different food animal species (Mahajan *et al.*, 2011). Therefore, the demand and cost of themeat varies according to the animal species. Currently, various types of frozen poultry and meat products such as nuggets, sausages, balls, burgers, andother cooled poultry meat are well accepted in Iraq. Adulteration of meat with cheaper ambiguous meats of differentorigin during preparation of meat products is a common practice inmany countries. Because meat adulteration and mislabeling are illegaland raise many health, religious and economic issues (Wang *et al.*, 2010), the detection of adulterations and identification of adulterantsin meat products is crucial for the enforcement of labeling legislationand prevention of unfair competition (Kesmen *et al.*, 2010). This is also important for the implementation of national standards aswell as to protect the consumer preference (Singh *et al.*, 2007). Recent food scares and inappropriate risk management practices have increased consumer's awareness regarding the origin and composition of the food products they purchase. The identification of animal species in meat products has been gaining in practical importance as a tool to avoid unfair competition and to assure accurate and truthful labeling. The manufacturers of food products need to find efficient methods to confirm the validity of their products components in harmony with government legislations (Calvo *et al.*, 2001; Sun, 2008). There are several protein-based methods for meat identification, but the negative aspects of these methods are insensitive and do not have the ability to detect highly heat-treated meat due to denaturation of protein during processing ; while DNA-based methods are more specificity and sensitivity for identification of food components (Mackie *et al.,* 2000; Montowska and Pospiech, 2007). DNA molecule is more stable than protein, so it is the best for use in species identification techniques of meat subjected to high temperature and pressure during processing (Lanzilao *et al.,* 2005). Recently, many studies applied various methods to identify meat species, including PCR amplification (Tanabe *et al.,* 2007), PCR-based amplified fragment length polymorphism (AFLP) (Sasazaki *et al.,* 2004), random amplification of polymorphic DNA (RAPD) (Calvo*et al.,* 2001), multiplex PCR (Asensio *et al.,* 2008), nested primer PCR (Miguel and Begona, 2004), microsatellite (Rikimaru and Takahashi, 2007; Tajima *et al.,* 2002) and real-time PCR (Fajardo *et al.,* 2008). In addition to the above techniques, a PCR-RFLP analysis has been widely developed for the typing and the differentiation of animal and fish species (Meyer *et al.,* 1995; Murray *et al.,* 1995; Partis *et al.,* 2000; Perez and Presa, 2008). This method has been used to analyze various genes, especially those from mtDNA. Tartaglia *et al.* (1998) designed a dedicated PCR-RFLP assay exclusively for the detection and distinction of bovine mitochondrial DNA in ruminant feeds.Using similar assay, Aida *et al.* (2005) developed method involving cyt b gene of pig mitochondrial DNA in fats of pigs. Also, based on 12S rRNA gene and PCR-RFLP assay, Che Man *et al.* (2007) studied the traceability of pork in food products, where the PCR amplification of 12S rRNA yielded a band of 387 bp for the pork sausages. In the this work, species differentiation of rawmeat and meat productswere investigated by the use of PCR-RFLPanalysis of mitochondrial 12S rRNA gene.

Corresponding author: Ismail A. Abdul-Hassan
Genetic Engineering and Biotechnology Institute, University of Baghdad, Iraq

MATERIALS AND METHODS

Meat samples

Muscle tissue samples from chicken (*Gallus gallus*), turkey (*Meleagrisgallipavo*), quail (*Coturnixcoturnix*), duck (*Anasplatyrhynchos*) and pig (*Susscrofadomestica*) were collected

from Baghdad and Irbil markets and stored at – 20 C till further treatment. In addition, some meat products were analyzed. These include different products of chicken sausage, roasted chicken burgers, roasted roll of turkey, pork sausage and pork hamburger. Also, samples of pig meat were subjected to different temperatures up to 135 C. Each sample was prepared and analyzed in five replicates.

DNA Extraction

DNA was extracted from the samples using the DNeasy® blood and tissue kit (QIAGEN, Germany) according to the manufacturer's instructions. Subsequently, the quality of DNA was assessed by agarose gel electrophoresis using 1% agarose gel stained with ethidium bromide. The purity and concentration of DNA was estimated using Nanodrop at 260 and 280 nm. The DNA sample showing the OD 260:280 nm value of 1.70 to 1.90 was considered as good quality.

Primers

Universal primers from mitochondrial 12S r RNA gene were used for PCR amplification as described by Kocher *et al.* (1989).
Forward 5'- CAAACTGGGATTAGATACCCCACTAT- 3'
Reverse 5'- GAGGGTGACGGGCGGTGTGT-3'

PCR amplification

Polymerase chain reaction (PCR) was performed in 25 μl of reaction mixture containing 2 μl of genomic DNA, 12.5 μlof master mix, 1 μlof each primer and 8.5 μlRNase-free water to make a final volume. Amplification was performed on thermal cyclerusing 0.2 ml reaction tubes. The PCR program consisted of 4 min denaturation at 96°C, followed by 35 cycles of denaturation (94°C, 30 s), annealing (63°C, 30s and primer extension (72°C, 30 s). The final cycle was followed by extension at 72°C for 7 min.

Restriction fragment length polymorphism

PCR amplicons of the mitochondrial 12S rRNA genewere subjected to restriction enzyme digestion with Acil restriction enzyme according to the suppliersinstructions. Briefly, enzyme-buffer mix was preparedby mixing 2 μl of restriction enzyme with 8 μl of therespective buffer.Reaction mix was prepared by mixing 10 μl PCRproduct with 2 μl of enzyme buffer mix. Volume wasmade up to 20 μl with autoclaved MilliQ water and incubatedovernight at 37C. Digested product was visualizedby electrophoresis in agarose gel along with100 bp ladder.

Gel electrophoresis

The horizontal agarose gel electrophoresis was used for analysis of PCR products. Agarose (1.5%) was used for preparation of gel. For that 0.3 g of agarose was put in 20 ml of 1x TBE solution and heated to completely dissolve the agarose. Then 1 µl (5%) ethidium bromide solution was added as gel visualizing agent and mixed thoroughly. The electrophoresis was done for 15 min at 100 V then 40 min at 50 V. The PCR product was finally analyzed using UV transilluminator. The ready to use 100 bp ladders was used for the present work.

RESULTS AND DISCUSSION

This study was used universal primers to amplified common fragment (456 bp in size) of mitochondrial 12S r RNA gene from chicken, turkey, quail, duck and pig (Figure 1). Six DNA samples from each species were extracted and subjected to PCR amplification for use in the RFLP trials. PCR products were successfully amplified to the expected 456 bp fragment within 12S r RNA gene. It is more efficient to detect species-specific DNA using mitochondrial DNA than genomic DNA (Cheng *et al.,* 2003), because there are approximately 104 copies of mitochondrial DNA available per cell compared to only one copy of genomic DNA.

Fig. 1. Polymerase chain reaction (PCR) amplification of mitochondrial 12S r RNA gene from pig and some avian species.Amplicons were analyzed by 1% agarose gel electrophoresis

Restriction map of sequenced amplicons of chicken, turkey, quail, duck and pig mitochondrial 12S r RNA partial sequences along with Acil restriction site are given in Table 1. Acil enzyme was chosen for PCR-RFLP studies, so as to detect and differentiate meat species.Acil enzyme generated fragments of 150, 95, 90, 80, 45 and 20 bp in chicken; 260, 95, 75, 45 and 20 bp in turkey; 210, 95, 80, 50, 45 and 20 bp in quail; 130, 75, 65, 60, 45, 43, 30 and 20 bp in duck; and 250, 135, 95 and 20 bp in pig (Figure 2). These results show that each animal species exhibited a unique specific RFLP pattern that allowed its identification and discrimination from others. Chicken, turkey, quail and duck which belongs to the class Aves produced different genetic profiles based on 12S r RNA gene and Acil restriction enzyme used in this study, enabling easy and unambiguous interpretation of restriction results. PCR-RFLP of 12S r RNA gene in this study could differentiate closely related meat species such as chicken-turkey. As noted in the Table 1 and Figure 2, it is not possible to detect fragments (< 50 bp in size) by the conventional

Table 1. PCR-RFLP analysis using Acil restriction enzyme of 12S r RNA gene from pig and some avian species

S.N	Species	Samples	456 bp	Fragment size in bp							
1	Chicken	5	+	150	95	90	80	45	20	-	-
2	Turkey	5	+	260	95	75	45	20	-	-	-
3	Quail	5	+	210	95	80	50	45	20	-	-
4	Duck	5	+	130	75	65	60	45	43	30	20
5	Pig	5	+	250	135	95	20	-	-	-	-

-No fragment.

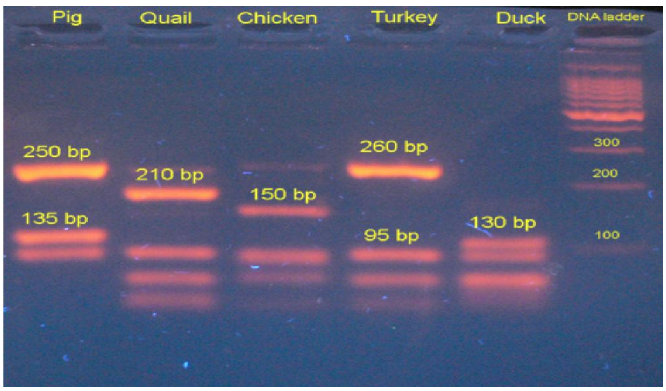

Fig.2. Restriction fragment length polymorphism (RFLP) of mitochondrial12S rRNA gene from pig, quail, chicken, turkey and duck. PCR amplicons were subjected to restrictionanalysis with Acilresulting in the fragments that presented in Table 1. 100 bpDNA ladder.

Table 2. Application of PCR-RFLP analysis of mt 12S rRNA gene using AciI restriction enzyme in meat identification of pig, chicken and turkey species from field samples

S.N.	Sample code	456 bp product	AciI restriction enzyme digests			(fragment size in bp)			Species identity	Conformity
1	C-H1	+	150	95	90	80	45	20	chicken	+
2	C-H2	+	150	95	90	80	45	20	chicken	+
3	C-H3	+	150	95	90	80	45	20	chicken	+
4	C-H4	+	150	95	90	80	45	20	chicken	+
5	C-S1	+	150	95	90	80	45	20	chicken	+
6	C-S2	+	150	95	90	80	45	20	chicken	+
7	C-S3	+	150	95	90	80	45	20	chicken	+
8	C-S4	+	150	95	90	80	45	20	chicken	+
9	T-M1	+	260	95	75	45	20	-	turkey	+
10	T2	+	260	95	75	45	20	-	turkey	+
11	P-D	+	250	135	95	20	-	-	pig	+
12	P-S1	+	250	135	95	20	-	-	pig	+
13	P-S2	+	250	135	95	20	-	-	pig	+
14	P-S3	+	250	135	95	20	-	-	pig	+

-No fragment.

PCR-RFLP gel-based method due to the low resolution of the agarose gel. Therefore, as related with the analysis of restriction profiles generated, only the typical major bands were taken into account. Different heat treatments did not affect the RFLP pattern and results were similar in raw meat sample and heat-treated samples at 120, 125, 130 and 135 C for 30 minutes (Figure 3). These results indicate that the specificity did not vary with heating up to 135 C. Successful PCR amplification was done from DNA extracted from various meat samples that heat-treated at different temperature viz., 120, 125, 130 and 135 C for 30 minutes. Since, mitochondria are present in large numbers in each cell; even less amount of sample is sufficient to give detectable amplification. Fairborther*et al.* (1998) were reported the capacity of PCR to amplify relatively short sequences in highly degraded DNA, thus, it was not surprising to see 456 bp fragment of mitochondrial 12S r RNA gene amplification in highly heat-treated meats. In addition, similar signals were seen at all temperatures of heating (Figure 3). This result is disagree with the results of Matsunaga*et al.* (1998), who observed weak amplification of mt DNA in meat samples processed at 120 C for 30 minutes. High copy number of mt DNA in a cell may contribute to the survival of at least a few copies, when the tissue is subjected to extreme processing conditions.

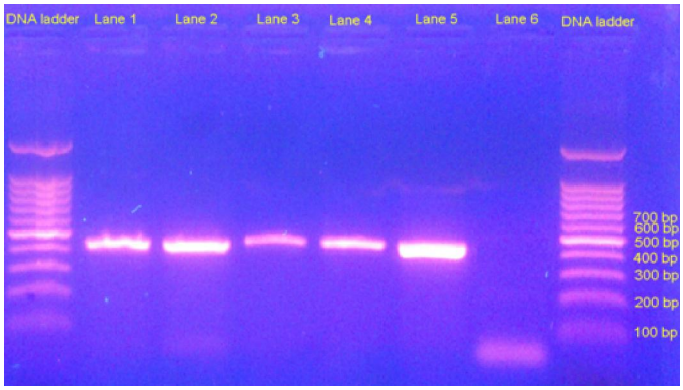

Fig. 3. Electrophoretic analysis of 12S rRNA gene in pig. 100 bp DNA ladder; Lane 1: raw muscle; Lanes 2-5: heat-treated muscles; 120, 125, 130 and 135 C for 30 minutes, respectively; Lane 6: control; 100 bp DNA ladder.

The identification of the species origin ofmeat is a valuable tool for the assessment of risk associated withintroduction of animal material that might be harmful to human health (Corona *et al.,* 2007). With the increasing awarenessof food safety and quality, species identification in foodproducts has become of crucial importance for health, economic, religious, esthetic or legal reasons (Arvanitoyannis *et al.,* 2005; Lees, 2003; Sun, 2008). For the evaluation of the accuracy of meat product labeling, the species identifications confirmed through PCR-RFLP of 12S r RNA gene were compared with species declared on the product packaging. Meat products that selected in this study (Table 2) showed conformity with the label of these products. Depending on the results that obtained in this study, this technique can be adopted as an accurate analytical technique for pig, chicken, turkey, quail and duck meat identification targeting 12S r RNA gene of mitochondrial DNA for enforcement of labeling regulations.

REFERENCES

Aida, A. A. Che Man, Y. B., Wong, C. M. V. L., Raha, A. R. and Son, R. (2005). Analysis of raw meats andfats of pigs using polymerase chain reaction for Halalauthentication. Meat Science 69: 47-52.

Arvanitoyannis, I. S., Tsitsika, E. V., and Panagiotaki, P. (2005). Implementation ofquality control methods (physico-chemical, microbiological, and sensory) inconjunction with multivariate analysis towards fish authenticity. InternationalJournal of Food Science and Technology, 40, 237–263.

Asensio, L., Gonzalez, I., Pavon, M. A., Garcia, T., and Martin, R. (2008).An indirectELISA and a PCR technique for the detection of grouper (Epinephelusmarginatus) mislabeling. Food Additives andContaminants: Part A Chemistry, Analysis, Control, Exposureand Risk Assessment, 25(6), 677–683.

Calvo, J. H., Zaragoza, P., and Osta, R. (2001). Random amplified polymorphic DNAfingerprints for identification of special in poultry pate. Poultry Science, 80,522–524.

Che Man, Y. B., Aida, A. A., Raha, A. R., and Son, R. (2007). Identification of porkderivatives in food products by species-specific polymerase chain reaction (PCR) for Halal verification. Food Control, 18, 885–889.

Cheng, Y. H., Wen, C. H., Ding, S. T., Kao, C. C. andKuo, T. Y. (2003). Detecting meat and bone meal inruminant's feeds by species-specific PCR. Journal ofAnimal Feed Science12:851-860.

Corona, B., Lleonard, R., Carpio, Y., Uffo, O., and Martínez, S. (2007). PCR detection ofDNA of bovine, ovine-caprine and porcine origin in feed as part of a bovinespongiform encephalopathy control program. Spanish Journal of AgriculturalResearch, 5, 312-317.

Fairborther, K. S., Hopwood, A. J., Lockley, A. K., andBardsley, R. G.(1998). The actin multigene family and livestock speciation using thepolymerase chain reaction. Animal Biotechnology, 9(2), 89–100.

Fajardo, V., Gonzalez, I., Martin, I., Rojas, M., Hernandez, P. E., Garcia, T., et al. (2008).Real-time PCR for quantitative detection of chamois (Rupicaprarupicapra) andpyrenean ibex (Capra pyrenaica) in meat mixtures. Journal of Association ofOfficial Analytical Chemists International, 91(1), 103–111.

Kesmen, Z., Yetim, H., and Şahin, F. (2010). Identification of different meat species used insucuk production by PCR assay. GIDA, 35(2), 81–87.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Paabo, S., Villablanca, F.X., Wilson, A.C. (1989). Dynamics of mitochondrial DNA evolution in animals: Amplifications andSequencing with conserved primer. *Proc. Natl. Acad.Sci., USA. 86*: 6196-6200.

Lanzilao, I., Burgalassi, F., Fancelli, S., Settimelli, M., and Fani, R. (2005). Polymerasechain reaction-restriction fragment length polymorphism analysis ofmitochondrial cytb gene from species of dairy interest. Journal of Associationof Official Analytical Chemists International, 88(1), 128–135.

Lees, M. (2003). Food authenticity and traceability. Cambridge England: WoodheadPublishing Ltd.

Mackie, I. M., Craig, A., Etienne, M., Je´ roˆme, M., Fleurence, J., Jessen, F., et al. (2000).Species identification of smoked and gravad fish products by sodiumdodecylsulphate polyacrylamide gel electrophoresis, urea isoelectric focusingand native isoelectric focusing: A collaborative study. Food Chemistry, 71, 1–7.

Mahajan, M.V.; Gadekar, Y.P.; Dighe, V.D. ; Kokane, R.D. and Bannalikar, A.S. (2011). Molecular detection of meat animal species targeting MT 12S r RNA gene. Meat Science, 88:23-27.

Matsunga, T., Chikuni, K., Tanabe, R., Muroya, H., Shibata, K., Yamada, J., andShinmura, Y. (1998). Determination of mitochondrialcytochrome b gene sequence for red deer (Cervuselaphus) and the differentiationof closely related deer meats. Meat Science, 49(4), 379–385.

Meyer, R., Hoefelin, C., Luethy, J., and Candrian, U. (1995). Polymerasechain reaction-restriction fragment length polymorphismanalysis: a simple method for species identification in food. Journalof AOAC International, 78(6), 1542–1551.

Miguel, A. P., andBegona, P.-V. (2004). Identification of commercial canned tunaspecies by restriction site analysis of mitochondrial DNA products obtained bynested primer PCR. Food Chemistry, 86, 143–150.

Montowska, M., and Pospiech, E. (2007). Species identification of meat by Electrophoretic Methods. Acta Sci. Pol., Technol. Aliment. 6 (1), 5-16

Murray, B. W., McClymont, R. A., and Strobeck, C. (1995). Forensic identification ofungulate species using restriction digests of PCR-amplified mitochondrial DNA. Journal of Forensic Sciences, 40, 943–951.

Partis, l., Croan, D., Guo, Z., Clark, R., Coldham, T. and Murby, J. (2000). Evaluation of a DNA fingerprinting method determining the species origin of meats. *Meat Science* 54: 369-376.

Perez, M., and Presa, P. (2008). Validation of a tRNA-Glu-cytochrome b Key for themolecular identification of 12 Hake Species (Merluccius spp.) and Atlantic Cod(Gadusmorhua) Using PCR-RFLPs, FINS, and BLAST. Journal of Agricultural andFood Chemistry, 56(22), 10865–10871.

Rikimaru, K., and Takahashi, H. (2007). A method for discriminating a Japanese brandof chicken, the Hinai-jidori, using microsatellite markers. Poultry Science, 86,1881–1886.

Sasazaki, S., Itoh, K., Arimitsu, S., Imada, T., Takasuga, A., Nagaishi, H., et al. (2004).Development of breed identification markers derived from AFLP in beef cattle.Meat Science, 67, 275–280.

Singh, Y., Brahmbhatt, M. N., Bhong, C. D., Jain, S., and Joshp, C. G. (2007). Detection ofmeat species by polymerase chain reaction of actin gene family. Haryana Veterinary,46, 25–27.

Sun, D. W. (2008). Modern techniques for food authentication. Academic Press.

Tajima, K., Enishi, O., Amari, M., Mitsumori, M., Kajikawa, H., Kurihara, M., *et al*.(2002). PCR detection of DNAs of animal origin in feed by primers based onsequences of short and long interspersed repetitive elements. Bioscience,Biotechnology, and Biochemistry, 66, 2247–2250.

Tanabe, S., Miyauchi, E., Muneshige, A., Mio, K., Sato, C., and Sato, M. (2007). PCRmethod of detecting pork in foods for verifying allergen labeling and foridentifying hidden pork ingredients in processed foods. Bioscience,Biotechnology, and Biochemistry, 71(7), 1663–1667.

Tartaglia, M., Saulle, E., Pestalozza, S., Morelli, L., Antonucci, G., andBattaglia, P. A.(1998). Detection of bovine mitochondrial DNA in ruminant feeds: A molecularapproach to test for the presence of bovine-derived materials. Journal of FoodProtection, 61, 513–518.

Wang, Q., Zhang, X., Zhang, H. Y., Zhang, J., Chen, G. Q., Zhao, D. H., et al. (2010).`Identification of 12 animal species meat by T-RFLP on the 12S rRNA gene. MeatScience, 85(2), 265–269.

****