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18S ribosomal DNA-based PCR test for avian and mammalian DNA identification in meat products

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ABSTRACT

This work has aimed to create a PCR test to identify avian and mammalian DNA in meat products. The test is based on phylogenetic analysis of 18S ribosomal RNA (rRNA) of four major groups of *Tetrapod*: *Amphibia*, *Reptilia, Mammalia*, and *Aves*. 18S rDNA complete coding sequences from GenBank have been used for phylogenetic analysis by the Maximum Likelihood method. The alignment of these 18S rDNA sequences has been used for PCR primers modeling. We have received the following PCR fragment for these primers: for birds – 97 base pairs (bp), and for mammals – 134 bp. The difference between them in 37 bp is sufficient for separating these fragments in standard agarose gel. We have tested this PCR to identify avian or mammalian DNA in sausage products and confirmed the suitability of this test for avian (chicken) and mammalian (sheep, cows) meat identification and meat identification in sausage products.

Introduction

Nowadays, all over the world, meat and meat products are widely consumed in the diet among non-vegetarians. To reduce the cost of meat production, manufacturers often resort to falsification, replacing more expensive meat with cheaper ones, or even resorting to all kinds of fraudulent substitutes (Abozinadh, Yacoub, Ashmaoui & Ramadan, 2015). Replacing expensive meat with cheap one is associated with a violation of human rights in his choice to resort to dietary preferences. It is very important for many consumers to know the true content of meat products and to be sure that there are no falsifications.

It is known that a good source of protein is poultry meat, which is also more affordable in terms of price (Tan, de Kock, Dykes, Coorey & Buys, 2018). On the other hand, pork meat has the highest fat content compared to chicken meat (Pereira & Vicente, 2013). In addition, some religious restrictions (amongst communities of religious Muslims and Jews) have a problem with pork meat and its products' consumptions (Yayla & Ekinci Doğan, 2021). The dietary concern of eating red meat (beef, veal, pork, lamb, and mutton) have been clarified in studies that show that red meat consumption for a long time is associated with increased risk of several chronic diseases (total mortality, cardiovascular disease, colorectal cancer, and type 2 diabetes, in both men and women) (Battaglia Richi et al., 2015; Wolk, 2017). Furthermore, dietary preference for valuable and expensive deer meat is associated with the high temptation to falsify it (Kaltenbrunner, Hochegger & Cichna-Markl, 2018). Deer meat is considered dietary meat with a balanced ratio of omega-6 – omega-3 essential fatty acids, low total fat content, and delicious taste (Fajardo, González, Rojas, Garcia & Martín, 2010; Poławska, Cooper, Jyźwik & Pomianowski, 2013). Horse meat is considered highly valuable in terms of nutritional value, but cases of falsifications with horse meat lead this meat become a controversial product, although the central issue of consumers in this question is the knowledge of what people eat precisely and not the nutritional characteristics of horse meat (Lorenzo et al., 2017). Meat swapping has been the focus since the European horse meat scandal in 2013. And today, cases of falsification of meat are repeated worldwide (Cavin, Cottenet, Cooper & Zbinden, 2018).

Therefore, to protect consumers from meat adulteration the development of a method for meat authentification there is a big necessity. (Kumar et al., 2015).

PCR tests have been widely used for different DNA identification since developing molecular biology methods. Currently, PCR tests are used for meat DNA identification instead of numerous methods (anatomical, histological, organoleptic, chemical, biochemical,

; PCR, Polymerase chain reaction.

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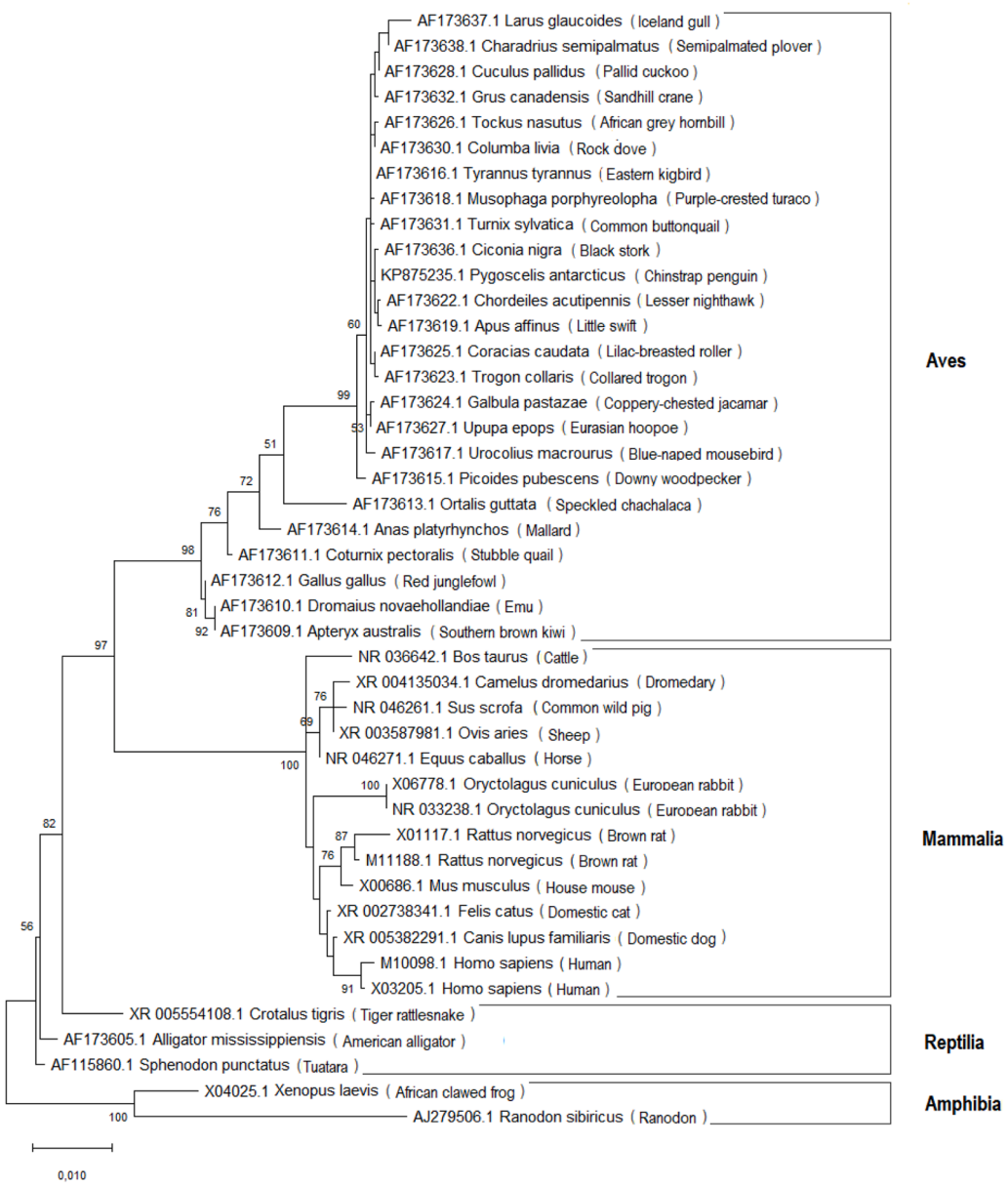

Fig. 1. Tetrapod 18S rRNA phylogenetic analysis by the maximum likelihood method. The tree with the highest log-likelihood. The percentage (50 and higher are shown) in which the associated sequences clustered together are shown next to the branches.

spectrophotometric, chromatographic, electrophoretic, immunological, and immuno-electrophoretic assay, etc.) earlier had been used for that (Kumar et al., 2015). There are several PCR (and real-time PCR) tests for different kinds of meat detection based on mitochondrial DNA: *cytochrome b* gene (Abuelnaga et al., 2021; Chen, Lu, Xiong, Xiong & Liu, 2020; Jiang et al., 2018; Kumar, Singh, Karabasanavar, Singh & Umapathi, 2014; Matsunaga et al., 1998,1999a; Meyer, Höfelein, Lüthy & Candrian, 1995; Natonek-Wiśniewska, Słota & Kalisz, 2010; Rajapaksha, Thilakaratne, Chandrasiri & Niroshan, 2002; Sahilah et al., 2012; Tanabe et al., 2007; Yacoub & Sadek, 2017; Zha, Xing & Yang, 2010; Zhang et al., 2020), and *16S* and *12S rRNA* genes (Li et al., 2019; Sahilah et al., 2012; Wang et al., 2010; Yang et al., 2014; Fajardo et al., 2009, 2008a,b,2007a,2006; Gupta et al., 2008), and mitochondrial D-loop (Fei et al., 1996; Fajardo et al., 2009,2008b,2008c,2007b); satellite DNA (Buntjer, Lenstra & Haagsma, 1995; Chikuni, Tabata, Kosugiyama, Monma & Saito, 1994), and *kappa-casein precursor* gene (Kaltenbrunner et al., 2018), and exon DNA (Druml, Grandits, Mayer, Hochegger & Cichna-Markl, 2015).

A few PCR methods for detecting domestic animal meat use nuclear rRNA genes: There is a 5S ribosomal RNA (rRNA) gene test for differentiation of goose and mule duck (Rodriguez et al., 1991), 18S rRNA gene-based PCR test for differentiation of mammalian, poultry, and fish in meat products. (Matsunaga, Shibata, Yamada, Shinmura & Chikuni, 1999b). However, the *18S rRNA* gene is frequently used to identify parasites (Khosravinia et al., 2021; Bahrami, Tabandeh & Tafreshi, 2017; Barroso et al., 2007; de Sousa et al., 2018; Ghaemi, Hoghoogh-Rad, Shayan & Eckert, 2012; Kourenti & Karanis, 2006; Mahmood & Mustafa, 2020; Wong, Carson & Elliott, 2004). rRNAs are well-known macromolecules for cells of any living organism. They serve an essential function in the ribosomes, the basic machinery of cells for protein synthesis (Noller, 1991). rRNAs are widely used to study the evolutionary history and taxonomy of living organisms since Woese (1998) reconstructs the phylogenetic history of all living organisms in the Tree of Life, widely known in all textbooks as the three-domain system in biological classification.

The 18S rRNA of a small subunit of a eukaryotic ribosome is used for

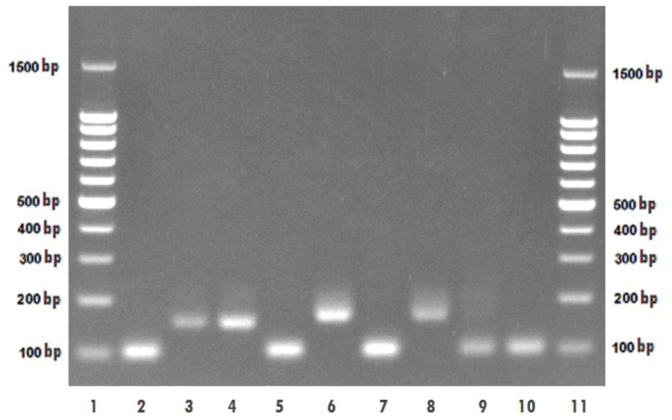

Fig. 2. Electrophoregram of the 1.7% agarose gel of PCR fragments of tested samples. PCR DNA fragments: 2 – chicken leg, 3 - sheep blood, 4 – cow blood, 5–10 – different sausage samples, and 1, 11 – DNA marker.

phylogenetic analysis of eukaryotes (Xie, Lin, Qin, Zhou & Bu, 2011), and the 18S rRNA phylogenetic analysis of major *Tetrapod* groups: Amphibia, Reptilia, Mammalia, and Aves, belong to different phylogenetic branches, although birds are closer to reptiles (Xia, Xie & Kjer, 2003). This suggests that a conservatism level of the 18S rRNA gene can be used to identify and differentiate tetrapods by PCR tests based on this gene. From this, we have been proposed that the 18S rDNA PCR test should be adequate to identify avian and mammalian DNA. 18S rRNA gene-based PCR test for differentiation of mammalian, poultry, and fish in meat products of Matsunaga et al. (1999b) had used only a few partial sequences of 18S rRNA gene, because of this, the purpose of this study is to develop the PCR test based on full phylogenetic analysis of complete coding part of 18S rRNA gene sequences to identify the poultry and mammalian DNA in meat products.

Materials and methods

The ethical statement. Experiments in this study have been carried out under guidelines for the ethical and humane use of animals for research according to the "Guidelines for accommodation and care of laboratory animals. Rules for keeping and care of farm animals" approved by The Interstate Council for Standardization, Metrology, and Certification of the Commonwealth of Independence States (GOST 34,088–2017).

Samples. Blood samples of bovine and ovine have been collected from farms (Moscow region). Chicken leg tissue samples and sausage products are from a one of the chain supermarkets of Moscow. All samples have been storage at −20 °*C* before use.

DNA extractions. DNA has been isolated using kits of SYNTOL company (Moscow, Russia): a set of "M-sorb-blood" have been used for whole blood and "DNA-EXTRAN-2″ − for mammalian tissues according to protocols.

Primers. Two primers GGRDNA-F (5′-GAGCTAATA-CATGCCGACGAG-3′) и GGRDNA-R (5′-CTAGAGTCACCAAAGCTGCC-3′) have been synthesized by CJSC Evrogen, Moscow, Russia. The primer modeling strategy is described below (see Results).

PCR. Ribosomal DNA fragment amplification from these primers has been performed on the BIOER thermocycler (Hangzhou Bioer Technology Co., Ltd., Hangzhou, China) using HS Taq DNA-polymerase (CJSC Evrogen, Moscow, Russia). 0.5 μg of total DNA for 50 μl of PCR mixture have been used. Amplification conditions have been used as follows. First, an initial denaturation for 3 min at 95 °*C* has been used. Then 40 cycles have been performed: denaturation for 15 *s* at 95 °*C*, annealing for 15 *s* at 58 °*C*, and elongation for 20 *s* at 72 °*C*. A standard 1.7% agarose gel electrophoresis has been used to analyze PCR fragments.

The tetrapod 18S rDNA phylogenetic analysis. We use 44 nucleotide sequences of complete coding parts of *18S rRNA* genes from GenBank of four major groups of tetrapods (*Amphibia*, *Reptilia*, *Mammalia*, and *Aves*). The evolutionary analysis of these 44 sequences of 18S rDNA has been performed with the MEGA X (Version 10.0.5) program (Kumar, Stecher, Li, Knyaz & Tamura, 2018), using the Maximum Likelihood method and the Tamura-Nei model (Tamura & Nei, 1993) with 500 bootstrap replications and uniform rates among sites.

Results and discussion

We have carried out a phylogenetic analysis of 44 sequences of the cording parts of *18S rRNA* genes of the four major groups of Tetrapod available from GenBank (https://www.ncbi.nlm.nih.gov), which has confirmed that the *Amphibia*, *Reptilia*, *Mammalia*, and *Aves* are in separate phylogenetic groups (Fig. 1). Based on the sequence alignment of these 44 sequences of 18S rDNA, conserved regions have been found, which have been used to design PCR primers (see Methods). PCR fragments from these primers are of different lengths to birds (chicken) and mammals (ovine and cow): 97 bp and 134 bp, respectively (Fig. 2). This difference is sufficient for the standard agarose gel electrophoretic separation of them. Because of this, it is suitable for differential PCR test identification and separation of bird and mammal DNA in meat products, from which DNA can be isolated.

PCR fragment lengths in this test for reptiles and amphibians (with some exceptions) are the same as for birds. Exceptions are for Tigris rattlesnake (the reptilia) and African clawed frog (the amphibia) with fragments length of 98 bp and 103 bp, respectively. The difference in one and six base pairs (for 98 bp and 103 bp, respectively) is not essential for the standard agarose gel electrophoretic separation of these fragments from the bird fragment (97 bp). Therefore, it is impossible to separate birds from reptiles and amphibians with the help of this PCR test. The meat of reptiles and amphibians is not widely distributed for nutrition, compared with chicken, turkey, sheep, goats, cows, and pigs. Hence this PCR test is still suitable for detecting avian and mammalian DNA in mixed meat products.

Matsunaga et al. (1999b) had used only a few partial sequences of the 18S rRNA gene in their PCR test for differentiation of mammalian, poultry, and fish in meat products, as we have said above, therefore, our PCR test based on complete 18S rDNA is more validated.

PCR methods to test meat products (such as sausages, cold cuts, and pate composed of poultry or a combination of pork and beef) are big concerns. It is known that twelve laboratories from Switzerland and Germany use such PCR tests (Eugster, Ruf, Rentsch & Köppel, 2008, 2009). In Russia, sausage products are still controlled by organoleptic, chemical, and bacteriological methods by GOST 9792–73, which leaves unscrupulous manufacturers to resort to falsifications. In this study, we have tested six sausage samples from various Russian manufacturers. Four of them have turned out to be produced from poultry (Fig. 2), although this has not always been stated.

Conclusion

We have received in this study the PCR test based on 18S rDNA phylogeny and alignment for identification avian and mammalian DNA. Furthermore, we have proved this test on samples from the chicken leg, sheep, and cow blood and six different sausage products. Because of this, it is confirmed the suitability of this test for avian (chicken) and mammalian (sheep, cows) meat identification and meat identification in sausage products.

Ethical statement

Experiments in the short communication entitled "18S ribosomal DNA-based PCR test for avian and mammalian DNA identification" by Irina M. Zyrianova, Ph.D., and Oleg G. Zaripov, Ph.D., have been carried out under guidelines for the ethical and humane use of animals for

research according to the "Guidelines for accommodation and care of laboratory animals. Rules for keeping and care of farm animals" approved by The Interstate Council for Standardization, Metrology, and Certification of the Commonwealth of Independence States (GOST 34,088–2017).

Declaration of Competing Interest

Authors Irina M. Zyrianova, Ph.D., and Oleg G. Zaripov, Ph.D., declare no conflicts of interest in the short communication entitled "18S ribosomal DNA-based PCR test for avian and mammalian DNA identification".

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