Meat Science 85 (2010) 265–269

Contents lists available at ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci

Identification of 12 animal species meat by T-RFLP on the 12S rRNA gene

Qi Wang [a], Xin Zhang [a], Hui-Yuan Zhang [a], Jie Zhang [a], Guang-Quan Chen [a], Da-He Zhao [b], Hai-Ping Ma [b], Wan-Jin Liao [b,*]

[a] Beijing Entry-Exit Inspection and Quarantine Bureau, No. 6 Tianshuiyuan Street, Chaoyang District, Beijing 100026, China
[b] State Key Laboratory of Earth Surface Processes and Resource Ecology and MOE Key Laboratory for Biodiversity Science and Ecological Engineering, Beijing Normal University, Beijing 100875, China

ARTICLE INFO

Article history:
Received 16 August 2009
Received in revised form 17 December 2009
Accepted 10 January 2010

Keywords:
Species identification
T-RFLP
Mitochondrial 12S rRNA gene

ABSTRACT

The verification of authenticity of meat products is relevant for economical, religious or public health concerning reasons. A molecular approach using terminal restriction fragment length polymorphism (T-RFLP) was developed to distinguish 12 common economically important meat species. The partial 12S rRNA gene was amplified with double-fluorescently labeled primers. The amplified fragments were digested with two endonucleases and only the terminal restriction fragment containing labeled primer was detected on capillary electrophoresis system ABI3100. *Alu*I and *Tru*9I generated differently-sized terminal fragments in different species. Pig and buffalo can be separated by 3′-terminal fragment of *Alu*I digestion. Horse, turkey, goat, sheep, deer, and cattle can be further separated by 5′-terminal fragment of *Tru*9I digestion. Dog and chicken, sturgeon and salmon can finally be separated by 5′-terminal fragment of *Alu*I digestion and 3′-terminal fragment of *Tru*9I digestion. Our results demonstrated the potential feasibility and applicability of T-RFLP method for rapid and accurate identification of animal species.

1. Introduction

Food adulterated with less costly and less desirable or even objectionable species has been around for a long time (Arvanitoyannis, Tsitsika, & Panagiotaki, 2005). With the increasing awareness of food safety and quality, species identification in food products has become of crucial importance for health, economic, religious, esthetic or legal reasons (Arvanitoyannis et al., 2005; Lees, 2003; Sun, 2008).

Rapid scientific and technological advances have shed light on the determination of food authenticity (Arvanitoyannis & van Houwelingen-Koukaliaroglou, 2003; Arvanitoyannis et al., 2005; Lees, 2003; Sun, 2008). Most of the analytical methods for meat species identification include electrophoretic techniques, antibody techniques, DNA techniques (Lees & Popping, 2003). Among them, PCR–RFLP is widely used to identify meat species in the past years (Fajardo et al., 2006, 2007, 2009; Girish et al., 2005, 2007; Hsieh, Chai, & Hwang, 2007; Meyer, Hoffelein, & Candrian, 1995; Partis et al., 2000; Sharma, Thind, Girish, & Sharma, 2008). Several mitochondrial genes have been chosen as targets for this method because of the inherent multicopy presence and great sequence diversity of mitochondrial genes (Brown, Gadaleta, Pepe, & Saccone, 1986). The mitochondrial 12S rRNA gene is one of the most widely used target genes for animal species identification (Fajardo et al., 2009; Girish et al., 2007; Park, Shin, Shin, Chung, & Chung, 2007; Sharma et al., 2008; Teletchea, Maudet, & Hanni, 2005). Fajardo et al. (2009) identified red deer, fallow deer, roe deer, chamois, mouflon, pyrenean ibex, goat, sheep, cattle, and swine using the 12S rRNA gene and PCR–RFLP.

RCR–RFLP is highly repeatable, cheap and quick (Meyer et al., 1995; Partis et al., 2000). In PCR–RFLP analysis, the digested PCR products are often separated by agarose gel electrophoresis. However, agarose gel has a low resolution, and the estimation of fragment size is subjective. Occasionally, it is not possible to detect small DNA fragments by the conventional PCR–RFLP gel-based method. Terminal restriction fragment length polymorphism (T-RFLP) is a recent molecular approach that has been used to assess subtle genetic differences between strains as well as to obtain an insight into the structure and function of microbial communities (Marsh, 1999). T-RFLP uses primers labeled with a fluorescent dye, and the digested fragments are separated by capillary electrophoresis (Layer, Ghebremedhin, König, & König, 2007). The advantage of T-RFLP is that the fragment size is estimated by an automated method, which allows accurate estimation. Moreover, the genetic patterns are easier to analyze than in PCR–RFLP since only the terminal fragment is labeled with the fluorescent dye and then detected.

To the best of our knowledge, T-RFLP has not been used to differentiate meat species despite its obvious advantages. In the present study, the mitochondrial 12S rRNA gene was PCR amplified with fluorescent-labeled primers in 12 common economically

* Corresponding author. Tel.: +86 10 58805081; fax: +86 10 58807721.
E-mail address: liaowj@bnu.edu.cn (W.-J. Liao).

0309-1740/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.meatsci.2010.01.010

important meat species, followed by digestion with restriction enzymes. The aim was to evaluate the suitability of T-RFLP for identifying animal species.

2. Materials and methods

2.1. Samples

We collected samples from 12 economically important animal species: pig (*Sus scrofa*), dog (*Canis familiaris*), cattle (*Bos taurus*), goat (*Capra hircus*), sheep (*Ovis aries*), horse (*Equus caballus*), chicken (*Gallus gallus*), salmon (*Salmo salar*), deer (*Cervus nippon*), buffalo (*Bubalus bubalis*), sturgeon (*Acipenser oxyrhynchus*), and turkey (*Meleagris gallopavo*). All these animals are commonly used in meat production.

2.2. DNA extraction

Total DNA was extracted from 20 mg of tissue using the TIANamp Genomic DNA Kit (TIANGEN Inc., China), according to the manufacturer's protocol. The DNA was resuspended in 100 μL TE buffer and stored at −20 °C.

2.3. PCR amplification of 12S rRNA

A pair of universal primers was used to amplify the mitochondrial 12S rRNA gene. The sequence of the forward primer was 5′-AAA CTG GGA TTA GAT ACC CCA CTA T-3′ while that of the reverse primer was 5′-GAG GGT GAC GGG CGG TGT GT-3′. These primers have earlier been described by Kocher et al. (1989) and were used with some modifications (Girish et al., 2005). For T-RFLP, the forward primer was labeled at the 5′-end with phosphoramidite fluorochrome 6′-carboxyfluorescein (FAM), and the reverse primer was labeled with 6-carboxy-2″,4,4″,5″,7,7″-hexachlorofluorescein (HEX). The samples were amplified in 0.2-mL PCR tubes in a final volume of 40 μL. The reaction mixture contained 4 μL of 10 × PCR buffer (200 mM Tris–HCl (pH 8.4) with 500 mM KCl), 0.8 μL of 10 mM dNTP mixture, 1.2 μL of 50 mM $MgCl_2$, 1.6 μL each of the forward and reverse primer (10 μM), 0.8 U of Platinum® *Taq* DNA Polymerase (Invitrogen), 100 ng of purified DNA, and autoclaved Milli-Q water. PCR was performed in an ABI9700 thermocycler (Applied Biosystems, USA). The amplification conditions included initial denaturation at 94 °C for 4 min, followed by 35 cycles at 94 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s. The final extension was performed at 72 °C for 7 min.

2.4. T-RFLP analysis

The fluorescent-labeled PCR products of the mitochondrial 12S rRNA gene were separated by electrophoresis on a 1.5% agarose gel and purified using the TIANgel Midi Purification Kit (TIANGEN Inc., China). The purified PCR products were resuspended and digested with the *Alu*I and *Tru*9I restriction endonucleases (Promega, USA). Digestions were performed in a volume of 10 μL containing approximately 50–100 ng of PCR amplification products and 2.5 U of enzyme. The digestion mixture was incubated for 2 h at 37 °C for *Alu*I and at 65 °C for *Tru*9I, as recommended by the manufacturer.

The digested PCR products (0.5 μL) were incubated with 9.2 μL of deionized formamide and 0.3 μL of GeneScan-500 ROX Size Standard (Applied Biosystems, USA) at 95 °C for 5 min and then separated on an ABI 3100 automated sequencer using a 50-cm capillary and the POP6™ polymer system (Applied Biosystems, USA). The sizes of the fluorescent-labeled terminal fragments were assessed using the Peak Scanner Software 1.0 (Applied Biosystems) and GeneScan-500 ROX Size Standard.

2.5. DNA sequencing and restriction enzyme analysis

For each species, the amplicons of the mitochondrial 12S rRNA gene were sequenced at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd., China. The sequences were generated using Sequence Analysis (Version 5.1, Applied Biosystems), and any discrepancies were manually corrected based on the graphical sequence file. The restriction sites of each species were analyzed using the DNASTAR MapDraw software.

3. Results

3.1. DNA sequences and theoretical restriction digestion analysis

A fragment of the mitochondrial 12S rRNA gene of approximate size 450 bp was amplified from the samples of all 12 species, i.e., pig, dog, cattle, goat, sheep, horse, chicken, salmon, deer, buffalo, sturgeon, and turkey. At least 3 samples from each species were PCR amplified and sequenced for subsequent T-RFLP studies.

Based on the restriction map of the sequences, the *Alu*I and *Tru*9I restriction enzymes were chosen to differentiate the meat species by T-RFLP. The sizes of the terminal fragments cleaved by each restriction enzyme are listed in Table 1. As shown in Fig. 1, *Alu*I generated HEX-labeled terminal fragments of length 163 bp in pig; 440 bp in buffalo; 48 bp in chicken, dog, and horse;

Table 1
Predicted and observed lengths of the FAM-labeled and HEX-labeled terminal restriction fragments of the 12S rRNA gene from *Canis familiaris*, *Gallus gallus*, *Equus caballus*, *Sus scrofa*, *Meleagris gallopavo*, *Capra hircus*, *Ovis aries*, *Salmo salar*, *Acipenser oxyrhynchus*, *Cervus nippon*, *Bos taurus*, and *Bubalus bubalis*.

Species	Sample size	Amplicon length (bp)	Terminal restriction fragment length (bp)							
			HEX-labeled *Alu*I		FAM-labeled *Tru*9I		FAM-labeled *Alu*I		HEX-labeled *Tru*9I	
			Predicted	Observed	Predicted	Observed	Predicted	Observed	Predicted	Observed
Dog	4	435	48	46.6 ± 0.02	90	84.3 ± 0.07	88	82.8 ± 0.02		
Chicken	5	443	48	46.9 ± 0.10	93	87.7 ± 0.02	242	240.6 ± 0.54		
Horse	4	450	48	46.8 ± 0.04	332	330.5 ± 0.01				
Pig	3	439	163	163.1 ± 0.01	439	435.6 ± 0.06				
Turkey	4	441	201	202.0 ± 0.29	91	86.2 ± 0.08				
Goat	5	438	200	200.7 ± 0.06	315	312.7 ± 0.03				
Sheep	4	439	200	200.6 ± 0.02	325	323.4 ± 0.02				
Salmon	5	433	223	224.2 ± 0.02	90	83.0 ± 0.04			343	342.7 ± 0.03
Sturgeon	3	434	224	225.5 ± 0.08	90	83.1 ± 0.09			273	273.4 ± 0.09
Deer	3	439	350	351.7 ± 0.04	34	26.0 ± 0.03				
Cattle	5	439	350	351.0 ± 0.03	91	85.2 ± 0.07				
Buffalo	4	440	440	440.3 ± 0.38	53	47.9 ± 0.03				