Species Identification of the Wild Meat Food Products

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Abstract

Anseriformes as the large waterfowl living in the northern hemisphere, with the size and shape is between that of duck and swan. Its aquatic habit and characteristics were worse than that of duck and swan. With the continuous development of economy, the temptation of high profits and the weak awareness of environmental protection, the cases of killing the wild geese illegally have been reported in our country. However, in the process of law enforcement, the executor often lack of clear morphological evidence supporting, which makes the identification difficult. In this paper, we successfully identify several wild goose meat products by means of molecular genetics, and discuss the operation techniques and marker selection of molecular genetics in species identification based on related researches. At the same time, it also provides a streamlined and practical technical route for the determination of species in such cases.

Keywords— *geese, 12S rRNA gene, molecular genetics, species identification*

I. INTRODUCTION

The structure of mitochondrial genome is relatively simple, and can be independently copied, which has high specificity and uniqueness [1]. The high conservation of mitochondrial genes in eukaryotes makes them become the mark of evolutionary research. In modern biology, genetic analysis occupies a very important position. Among them, the polymorphism analysis of the mitochondrial DNA (mtDNA) in animals is a powerful tool to protect biology and evolutionary biology. The polymorphism analysis of mtDNA can detect the diversity of population genetic, protect and manage the gene bank. Genetic diversity is closely related to the adaptability of natural selection and the evolutionary potential of species. In the management of wildlife, genetic diversity of populations can be used to monitor and maintain the genetic diversity of populations as well as to identify divergent populations [2].

In this study, several samples of wild meat products that might be suspected of wild geese were amplified by using common primers12S rDNA, after sequencing and compared it with the homologous sequences of GenBank, that is, identify the exact source of species by molecular phylogenetic analysis clearly, the purpose is to provide a viable technical route for species identification of such samples, protect wildlife resources, standardize management and protection of law enforcement. In this research, the author participated in the whole process of sample pretreatment to data analysis, experienced the rigorous research, and realized the joy of success.

II. MATERIALS AND METHODS

A. Experimental Materials

A total of three samples were collected from three meal restaurants in Jiujiang, Jiangxi Province in the experiment (TDY, March 2010), (JDE, March 2010), and (XTE, March 2010) The samples in the study were all finished products after being braised or otherwise processed. Selected muscle tissue in the study, placed it in the centrifuge tube of 50ml, add 2 to 3 times the volume of ethanol (95%) for preservation.

B. Experimental methods

1) Extraction of DNA

Pretreatment and digestion of samples. Each sample was cut about 0.5 g of muscle tissue, and it was broken by surgical scissors, knife, etc. and then placed in the centrifuge tube of a 2ml, washed it 2 ~ 3 times with 95 % ethanol. Then washed it with 75 % ethanol twice, soaked it in double distilled water of 1ml for 2 hours, and changed the water for 1 - 2 times in the period. After the soak, the lysate of 500μl(10mmol/L Tris-Cl, 1.0 % SDS, 1mmol/L $CaCl_2$) and Proteinase K of 10μl (20 mg / ml) were added to the sample tube and digested overnight in a water bath at 55℃,or until the muscle tissue was completely digested.

DNA sample extraction. The digested sample solution was extracted twice by phenol-chloroform-isoamyl alcohol solution (25: 24: 1, pH=8.0) of equal volume and chloroform-isoamyl

alcohol solution (24: 1). Anhydrous ethanol (2.5 times of the extracted supernatant's volume) and sodium acetate (1/10 volume of the extracted supernatant's volume) were added to the extracted supernatant to precipitate DNA. The precipitate was vacuum dried and dissolved in a suitable amount of buffer solution (10 mMTris- HCl, 1mM EDTA, pH= 8.0). The extracted was electrophoreses by 1.2 % EB - sepharose and imaged by UV gel imaging system. In the study, sterile filter paper was used as a negative control from the extraction step to avoid contamination.

*2) **PCR Amplification, Product Purification and Sequencing***

We used the common primersL1091(5′-AAAAAGCTTCAAACTGGGATT AGATACCCCACTAT-3′) and H1478(5′-TGACTGCAGAGGGTGACGGGCGGTG TGT-3′)amplified the partial sequence of mitochondrial gene12S rRNA to amplify the template of extracted DNA, the amplification reaction was carried out in a standard system of 50μl,the conditions of amplification reaction are as follows: the volume of TaKaRaPreMix*Taq*(containing TaKaRa Ex *Taq*1.25U, dNTP Mixture 0.4mM, Buffer 2 ×, 4mM Mg^{2+}) was 25μl,the volume of primers(10μmol/L) were 1μlseparately,the volume of the template of extracted DNA was 3~5μl,The total volume of the reaction system was replenished to 50μlby using double distilled water, the cycle setting of the PCR reaction: pre-denatured at 95 ℃ for 5 min, then denatured at 95 ℃ for 30 s, annealed at 55 ℃ for 40 s, extended at 72 ℃ for 60s, and extended at 72 ℃ for 7 min after 35 cycles of reaction.

The amplified product was electrophoreses on 1.2% EB-Sepharoseat 120 V for 30 min and then detected by gel imager. The PCR product was purified by E.Z.N.A. Cycle-pure Kit (Omega Company) and subjected to bidirectional sequencing by using ABI 310 Genetic Analyzer of PE Company and its accompanying kit BigDyeTM.

*3) **Bioinformatics analysis of DNA sequence***

The sequenced gene fragment of mitochondrial 12srRNA was searched by blast in GenBank. After obtaining the homologous region sequence with high similarity to the sequence, the studied sequence was combined with the homologous region sequence and compared with the sequence by Clustal W software. After comparison, the genetic distance between DNA sequences was calculated by using the Kimura-2-parameter (k - 2 - p) method using MEGA software (version 4.0), and the phylogenetic tree was constructed by neighbor - joining (NJ) method based on the distance matrix. Maximum parsimony was also used to construct phylogenetic relationships between sequences. The confidence level at the branch of the phylogenetic tree is estimated by bootstrap test, and the number of repetitions is 1000.

III. RESULTS AND ANALYSIS

A. Detection of DNA samples

The results showed that the DNA of all three samples had strong degradation, but the small fragment of DNA was faintly visible. The results of electrophoresis are shown in fig. 1. The directions of electrophoresis are all from top to bottom, with DNA marker using m2000, the same below.

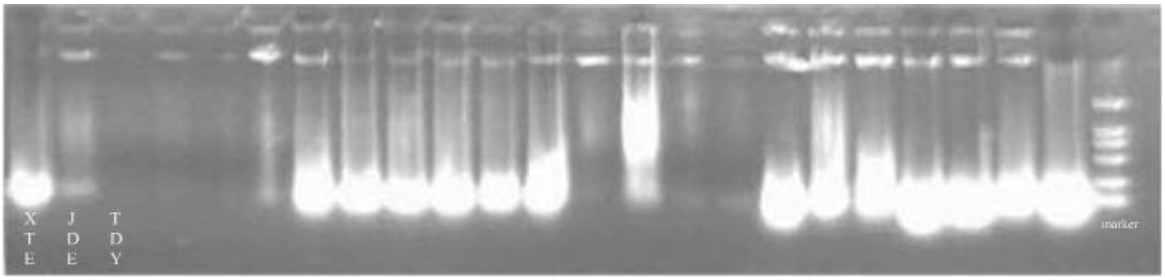

Fig. 1 Electrophoretogram of the Template of Extracted DNA

B. Detection of PCR products

Positive amplification products were obtained in all three DNA templates using primers l1091 and h1478, and compared with DNA marker, the size of the amplified product was approximately 400 bp, which was in line with the expected size of the amplified fragment in the experiment. The negative control unamplified fragment in the study indicated that the study process was not contaminated with foreign DNA, and the results are shown in fig. 2. Each sample was repeated twice in the graph, and the negative control was repeated twice too. The diffusion bands in the negative control were primer dimer, which accorded with the general rule of negative experimental results.

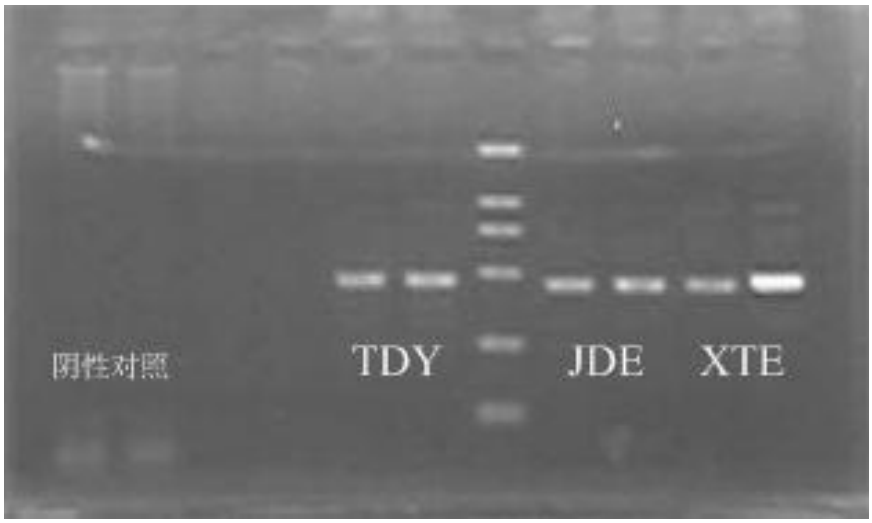

Fig. 2 Electrophoretogram of the Amplification Products of 12S DNA

C. Sequencing results of PCR products

After the comparison of Clustal, the amplification products of the partial sequence of 12S in the experimental samples were obtained as follows:

XTE12S
-CTAAATCTTGATACTTACTTTACCGAAGTAT
CCGCCAGAGAACTACGAGCACAAACGCT
JDE12S
-CCTAATCTTGATACTTACTTTACCGAAGTAT
CCGCCAGAGAACTACGAGCACAAACGCT
TDY12S
CCTAAATCTTGATACTTACTTTACCGAAGTAT
CCGCCAGAGAACTACGAGCACAAACGCT

XTE12S
TAAAACTCTAAGGACTTGGCGGTGCCCCAAA
CCCACCTAGAGGAGCCTGTTCTACAATCG
JDE12S
AAAACTCTAAGGACTTGGCGGTGCCCCAAAC
CCACCTAGAGGAGCCTGTTCTACAATCG
TDY12S
AAAACTCTAAGGACTTGGCGGTGCCCCAAAC
CCACCTAGAGGAGCCTGTTCTACAATCG

XTE12S
TAATCCACGATTAACCCAACCACCCCTTGCC
AACACAGCCTACATACCGCCGTCGCCAG
JDE12S
TAATCCACGATTAACCCAACCACCCCTTGCC
AACACAGCCTACATACCGCCGTCGCCAG
TDY12S
TAATCCCCGATTAACCCAACCACCCCTTGCC
AACACAGCCTACATACCGCCGTCGCCAG

XTE12S
CCCACCTCGAATGAGAGCACAACAGTGGAC
ACAATAGCATCCCGCTAATAAGACAGGTCA
JDE12S
CCCACCTCGAATGAGAGCACAACAGTGGAC
ACAATAGCATCCCGCTAATAAGACAGGTCA
TDY12S
CCCACCTCGAATGAGAGCACAACAGTGGAC
ACAATAGCACCCCGCTAATAAGACAGGTCA

XTE12S
AGGTATAGCCTATGGAGTGGAAGAAATGGG
CTACATTCCCTATACATAGGGCACACGGAA
JDE12S
AGGTATAGCCTATGGAGTGGAAGAAATGGG
CTACATTCCCTATACATAGGGCACACGGAA
TDY12S
AGGTATAGCCTATGGAGTGGAAGAAATGGG
CTACATTCCCTATTCATAGGGCACACGGAA

XTE12S
AGAAGTTTGAAATTGCTTCTGGAAGGAGGAT
TTAGCAGTAAAGTGGGACAATAGAGCCTA
JDE12S
AGAAGTTTGAAATTGCTTCTGGAAGGAGGAT
TTAGCAGTAAAGTGGGACAATAGAGCCTA
TDY12S

GAAGCGTGAAACCACTTCTGGAAGGCGGATT
TAGCAGTAAAGTGGGACAATAGAGCCTA
XTE12S
CTTTAAGCCGGCCCTGGGGCACGTACACACC
GCCCGTCACCCTCTGCAGTCAA
JDE12S
CTTTAAGCCGGCCCTGGGGCACGTACACACC
GCCCGTCACCCTCTGCAGTCAA
TDY12S
CTTTAAGGCGGCCCTGGGGCACGTACACACC
GCCCGTCACCCTCTGCATTCAA

From the point of sequence consistency, the amplification results are all partial fragments of 12S we need, which is also confirmed by the bioinformatics research.

D. Bioinformatics Analysis of DNA sequence

1) Homology Analysis of Sequences

In the sequencing results of three samples, the specific sequence length of 393 - 405 base was obtained after removing some ambiguous data peaks and primer part at the end. After blast comparison of 400 bp sequence in NCBI database (http: / / www.ncbi.nlm.nih.gov), 24 species with high homology in the sequences were selected:

Bucephalaclangula, login number of sequenc e:AF173712; *Pteronettahartlaubi*, AF173703; *Sark idiornismelanotos*,AF173702.*Chloephagamelanopter a*, AF173710; *Neochenjubatus*, AF173709;*Marmar onettaangustirostris*, AF173700;*Histrionicus*, AF17 3713;*Dendrocygnaautumnalis*,AF173717;*Uriaaalge*, DQ485794;*Thalassornisleuconotus*,U83739;*Aix ga lericulata*,AY164515;*Cygnus cygnus*, AY164523;*C ygnus columbianus*,DQ083161;*Anserindicus*, AY16 4532;*Brantasandvicensis*, U83735;*Anasacuta*,AY16 4525;*Mergusalbellus*, AY164516;*Cairinamoschata*, AM902523;*Tadornatadorna*,AY164521;*Mergus me rganser*,AY164529;*Somateriafischeri*,U83738;*Cyano chencyanopterus*, AF173704;*Tadornaradjah*, AF17 3708.

2) Genetic Distance and Phylogenetic Analysis Based on K-2-P

The analysis results of genetic distance showed that the genetic distance between the unknown species and the above species was in the scope of 0.0 ~ 0.206. The genetic distance between the unknown species TDY and the known species (*Anserindicus*, AY164532), the unknown species JDE, XTE and the known species (*Cygnus columbianus*, DQ083161) is zero, this result suggested that the sample TDY in the study may be from the *Anserindicus*, JDE and XTE is the same species, may be *Cygnus columbianus*, the same results were obtained by phylogenetic analysis. And high confidence was obtained: TDY was 97, JDE and XTE were 70.

IV. DISCUSSION

A. The Fragment of 12SrRNA can be Used as an Effective Molecular Marker for Identification of Wild Animal Products

Our research showed that although food has been subjected to water boiling, high temperature, high pressure, and a variety of flavoring processes, and the molecules of DNA in the tissues have a certain degree of degradation, but still can provide a considerable length of DNA fragments, making it possible to carry out the genetic analysis. In the researches of Liu and Wang also showed that the molecules of DNA in the meat food can maintain a relatively stable in the processing process of high temperature and high pressure[3]. Therefore, from the perspective of sample, the meat products after similar processing process can be able to carry out the molecular genetic analysis through DNA.

Currently, the direct sequencing method generally determines the gene fragments of Cytb,12S rRNA, D - loop in the DNA and so on, the sequence of the12SrRNA or Cytb of species is determined, and then the similar sequences are then searched for in GenBank and analyzed to determine species. By using this method, we successfully identified the species of Chinese muntjac, black muntjac, black bears, pangolin, African kudu, impala, African springbok, African elephants, peacocks, Varanus bengalensis and various livestock and poultry, and provided important help for supervision of import and export and law enforcement of key protected animals.

In this study, direct sequencing method was used to identify species (wild goose). The DNA of Mitochondrial contains 22 tRNAs, 2 rRNAs and 13 protein coding genes. It has great advantages in species identification because of the genetic characteristics of mitochondrial DNA. After years of practical experience, it has been found that not all identification results of mitochondrial genes were perfect. Such as: the amplification of 28S rRNA gene, even if a single species will produce more fragments, is not suitable for the species identification of mixed samples; The Cytb gene can't be used to distinguish the specific species of human and animal without sequencing or enzyme cutting, even if combined with the amplification of D-Loop, can only distinguish the test samples between those of human and animal. The 12s rRNA gene is a fragment of mitochondrial DNA which has about 950 - 1050 basic groups [4]. Compared with Cytb gene, the application of 12s rRNA for species identification has two obvious

advantages: first, it has high conservation, the second is that the 12s rRNA sequence has obvious identifiability among species and the statistical comparison of the existing data showed that the degree of variation of minimum sequence in the species is 0.8 % [5]. Because most of the nuclear DNA has been degraded, 12s rRNA is more suitable for species identification by primer amplification, sequencing and sequence comparison.

B. The Practical Significance of the Analysis Results to the Protection of Wild Goose and Other Birds

The wild Anatidae in China are all migratory species, the breeding of them is mainly concentrated in the northeast area, a few in the north area and the west area such as Qinghai, Xinjiang, inner Mongolia and other provinces, the overwintering of Anatidae mainly concentrated in the south area of the Yangtze river and southwest area China[6]. Nowadays the most Anatidae need to migrate from one protected area to another for the overwintering because of the serious damage to the ecological environment, the number of the Anatidae has a sharp decline in the way of migration because of illegal hunting, at the same time, the phenomenon of illegal hunting in protected areas also occurs from time to time.

Although the awareness of the protection of wild birds is more and more popular, the meat food processed by wild bird often appear in the restaurants often around the country, according to the survey, the birds accounted for 21 species in the 53 kinds of wild animals, few of them are artificial breeding, more than 20000 wild birds are eaten every day only in Guangzhou [7], wild birds are facing strong hunting pressure, species and quantity decreased sharply.

The Anserindicus involved in this study belongs to the Anatidae, those two species are similar, but the female bird is slightly smaller, the breeding of them is concentrated in Qinghai, Tibet, western Xinjiang, Gansu, inner Mongolia and the northwest area of China, they usually go into the breeding ground in late March and early April, and pass the winter in areas of Shanxi, Hunan, Sichuan, Yunnan, Guizhou and Tibet, there are more wintering population in Jiangxi province, which is the second national key protected animal. Cygnus columbianus is about 110 cm in length. Weight 4 ~ 7 kg, and the female bird is slightly smaller, and lives in the reedy lakes, reservoirs and ponds, feed the aquatic plant roots and seeds as food, distributed in the areas of northeast, inner Mongolia, northern Xinjiang and north China, the south area, occasionally seen in Taiwan, pass the winter in the protected area in Poyang lake, Jiangxi province, is the national secondary key protected animals. The emergence of these two kinds of wild bird meat dishes in our province's major restaurants shows that the phenomenon of illegal hunting in Qinghai and other places is serious, and the phenomenon of illegal hunting also occurs in our province when *Cygnus* columbianus and other bird immigrate here for passing the winter. This shows that bird protection in our country is still facing severe test. It is suggested that the relevant departments should increase publicity efforts to improve people's awareness of bird protection, and increase law enforcement efforts to crack down on illegal poachers and punish illegal sale of bird dishes restaurants, and protect the wild bird from the root.

V. CONCLUSION

In addition to being used as a tool for systematic classification, evolutionary analysis and drug quality monitoring, 12S rRNA also can also be used as an effective genetic marker for species identification. With the establishment of gene bank and the improvement of various gene comparison software, this new research method will be more convenient and faster, so as to be applied to the identification of more species.

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