

## Research Article

# Application of Next-Generation Sequencing Technology Based on Single Gene Locus in Species Identification of Mixed Meat Products

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Polymerase chain reaction (PCR) detection is a commonly used method for species identification of meat products. However, this method is not suitable for the analysis of meat products containing multiple mixtures. This study aimed to test whether next-generation sequencing (NGS) technology could be used as a method for the certification of mixed meat products. In this study, five kinds of common meat (pigs, cattle, sheep, chickens, and ducks) were mixed as samples with different proportions. The primers designed from mitochondrial 16S rRNA and nuclear genome gene (growth hormone receptor, GHR), respectively, were used to detect these meats. The sequencing results of NGS were analyzed using a self-designed bioinformatics program. The fragments with similar sequences were classified and compared with the database to determine their species. The results showed that all five kinds of meat components could be correctly identified using these two primers. The meat composition could be detected as low as 0.5% in the mixed samples using the NGS technology targeting GHR gene fragments, which was superior to those targeting mitochondrial 16S rRNA. However, the quantitative detection of species in the mixture was not likely to be quite accurate due to the amplification bias of PCR amplification. These results showed that the NGS technology could be applied to identify meat species in mixtures.

## 1. Introduction

Low-cost meat is often used in the consumer market as high-quality meat or high value-added meat [1], which damages the economic interests of consumers and leads to social problems such as food safety and religious ethics [2]. DNA in meat contains species-specific genetic information; it is stable in terms of chemical properties and is not easily destroyed in food processing. It is especially suitable for identifying different species in meat [3, 4]. In the last two decades, many DNA-based methods have been developed to identify meat species. Most of them rely on PCR to amplify specific DNA fragments and analyze them by different methods [5]. Previously, indirect methods, such as restriction fragment length polymorphism or single-strand conformation polymorphism, were mainly used to

distinguish the specific DNA sequences of different species [6]. Nowadays, the most commonly used technology is to amplify the complete specific gene sequence by PCR and then use the conventional Sanger sequencing method for sequencing analysis. The target genes usually detected are mitochondrial cytochrome b (cytb) or cytochrome oxidase I (cox1) genes [7]. However, these methods usually detect only one species at a time; also, the species in the sample needs to be known in advance so as to identify them using specific analysis steps. To overcome these limitations, the dot blot and gene chip methods have been developed in recent years, which can detect mixed meat samples [8] and analyze more than one species simultaneously. However, these methods rely on the species-specific gene probes prepared in advance, which makes it impossible to detect unknown species in mixed meat.

The emergence of next-generation sequencing (NGS) technology has greatly improved the speed and accuracy of DNA sequencing [9], overcoming the limitation that the conventional Sanger sequencing method can sequence only a single DNA fragment [10]; NGS can sequence different DNA fragments in parallel. It has become a standard DNA sequencing analysis method for whole-genome sequencing, metagenomics research, transcriptomics research, environmental microbial polymorphism research, and other applications requiring sequence data [11, 12]. NGS technology can be used to analyze meat products containing different species mixtures by parallel sequencing of different template molecules from a sample.

Recently, the application of high-throughput sequencing technology in food species identification is still in its infancy. In the species identification of tuna, two short cytochrome b gene (cytb) fragments were sequenced on Illumina MiSeq platform, which could identify the species whose content was less than 1% but not marked on the biological commodity label [6]. Besides, according to the genes of expanded 12S and 16S rRNA mitochondrial DNA, the library was constructed for NGS sequencing. The mixture of 13 kinds of common meat, such as pigs, cattle, and sheep, was detected, which proved that NGS sequencing could be applied to identifying meat species in the mixture [13].

The aforementioned studies were based on the sequencing analysis of mitochondrial DNA genes in meat, but the content of mitochondrial DNA in different species was very different. Therefore, the sequencing analysis could only be used for qualitative detection and not for the quantitative analysis of species in mixed meat. In this study, we used the single-copy gene GHR in the nuclear genome as the target gene site, designed primers to construct the library, compared the results of NGS sequencing with GHR gene and 16S rRNA gene as the target site, and verified the potential of high-throughput sequencing technology in the quantitative detection of different species in mixed meat.

## 2. Materials and Methods

**2.1. Primers and Conventional Sanger Sequencing.** The GHR gene was used as the target gene because of the great difference in the mitochondrial DNA content in muscles of different species. GHR genes of pigs, cattle, sheep, chickens, and ducks were downloaded from the GeneBank database, and primers were designed according to the common conservative region; the mitochondrial DNA primer 16S\_ki was used as control (Table 1) [14]. PCR reactions were performed in volumes of 20  $\mu$ L containing about 20 ng extracted DNA, 10 pmol of forward and reverse primers, and 10  $\mu$ L of PCR super mix (Bio-Rad, CA, USA). Temperature profiles were as follows: 10 min at 95°C followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C. Reactions were completed with a final extension step of 7 min at 72°C. The DNA of pigs, cattle, sheep, chickens, and ducks were used as templates. The fragments were amplified using the primers GHR\_1 and 16S\_ki. The size of the fragments was verified by electrophoresis, followed by confirmation using conventional Sanger sequencing.

**2.2. Sample Preparation.** Five samples containing muscle tissues were prepared. The samples were frozen at  $-70^{\circ}\text{C}$  for 24 h and then lyophilized with a lyophilizer to remove moisture from the samples. The freeze-dried samples were ground into minced meat with a tissue grinder and then mixed according to different mass ratios to obtain artificially mixed samples, 10 g for each sample. Sample S1 was an equal mixture of pigs, cattle, sheep, chickens, and ducks. The pork content in sample S2a–S2h was 80%, 50%, 20%, 10%, 5%, 1%, 0.5%, and 0.1%, respectively; the remaining was equally mixed with four other kinds of muscle tissues. Samples S3a–S3h, S4a–S4h, S5a–S5h, and S6a–S6h contained tissues from cattle, sheep, chickens, and ducks with different mass ratios, respectively; the preparation method was the same as that for pork samples (S2A–S2h).

**2.3. DNA Isolation and NGS on the Illumina MiSeq Platform.** DNA of samples was extracted using a Qiagen's meat DNA extraction kit (Qiagen, Germany). A microspectrophotometer was used to determine DNA concentration to ensure that the A260/A280 value was between 1.8 and 2.0. Fragments were amplified in separate PCRs with the primers GHR\_1 and 16S\_ki. The primers featured additional Illumina adapter sequences at the 5'-end, including binding sites for the hybridization of PCR products, binding sites for sequencing primers, and poly(N)-regions for unique sample identification. The quality of PCR amplicons was analyzed with agarose gel electrophoresis and/or spectrophotometric analysis (NanoDrop spectrophotometer, Thermo Scientific, MA, USA). Amplicons were applied in equimolar ratios to bridge amplification and sequencing by synthesis on the Illumina MiSeq platform (Illumina, CA, USA). The samples were submitted to two independent NGS runs to assess the reaction's repeatability.

**2.4. Data Analysis.** The reads were obtained by NGS sequencing. Then, the same reads were given the same order number and collected in a FastA document. A basic local alignment search tool (BLAST) was used in the mentioned FastA document to remove the primer sequences in the reads. The reads were collected later in another FastA document, which were then taken to BLAST in the document containing the sequences from tissues of pigs, cattle, sheep, chickens, and ducks. The reads matching the different kinds of meats were counted.

## 3. Results

**3.1. Mixed Samples and Sequence Variability.** All the sequences could be amplified by the primers GHR\_1 and 16S\_ki. The two primers amplified fragments of the expected size for pigs, cattle, sheep, chickens, and ducks under the same PCR conditions. No nonspecific products were detected by agarose gel electrophoresis. The amplified fragments were confirmed by Sanger sequencing. The results showed that all the sequences were correct as expected.

TABLE 1: Primer pairs used for DNA amplification.

Name	Primers (5' to 3'): forward and reverse	Amplified fragment (bp)
16S_ki	GCCTGTTTACCAAAAACATCACCTCCATAGGGTCTTCTCGTCTT	243
GHR_1	CACCACAGAAAGCCTTACCACTACTGTGCTCACATAGCCAC	191

**3.2. Analysis of NGS Sequencing Results of Mixed Samples.** Sample S1 was a mixture of pigs, cattle, sheep, chickens, and ducks, and each meat accounted for 20% of the total mass. Sample S1 was sequenced using the primer 16S\_ki, and 14,977 reads were obtained. The results of BLAST showed 60,008 reads (40.21%) in sheep, 59,762 reads (40.05%) in pigs, 27,217 reads (18.24%) in cattle, 1735 reads (1.16%) in ducks, and 478 reads (0.32%) in chickens. Using the primer GHR\_1, the results of NGS sequencing showed 105,942 reads in total. These included 59,639 reads (56.65%) in ducks, 17,839 reads (16.94%) in sheep, 15,434 reads (14.66%) in cattle, 7525 reads (7.94%) in pigs, and 4804 reads (4.65%) in chickens (Figure 1). A small number of reads for geese and horses were also identified, which might be due to the contamination of the sample or the mismatch in the sequencing.

The results showed a significant difference between the number ratio and the mass ratio of reads obtained by sequencing with these two primers, which could not be directly used for quantitative analysis. The reason might be the difference in DNA content or PCR amplification efficiency in different kinds of meat. The amplification efficiency of the 16S\_Ki primer was lower than that of the GHR\_1 primer in birds such as chickens and ducks, but had high amplification efficiency in mammals (pigs, cattle, and sheep). The amplification efficiency of the GHR\_1 primer was the highest in ducks, but not different in mammals, and the lowest in chickens.

**3.3. NGS Sequencing Results of Mixed Samples with Different Proportions.** NGS sequencing was carried out on five common meat samples with different mass ratios (80%–0.1%), and the results of the sequencing of two primers were compared (Tables 2 and 3). The results showed that the number of reads obtained by NGS sequencing changed with the change in the content in the mixed samples of five kinds of common meat with different proportions. The change trend was consistent, but the absolute number was not proportional; therefore, it could not be used for quantitative detection. The amplification efficiency of the 16S\_Ki primer was higher in mammals (pigs, cattle, and sheep), and the number of reads was significantly more than that of the GHR\_1 primer (more than 100 reads could be detected with 0.1% content). However, the amplification efficiency of birds (chickens and ducks) was low in the 16S\_Ki primer. When the content of chickens and ducks was less than 10%, only a small number of reads could be obtained, which was easily confused with foreign pollutants and could not be detected effectively. The GHR\_1 primer could get higher reads in mammals and birds. When the content was more than 0.5%, more than 1000 reads could be obtained, which could be used for the qualitative detection of these 5 kinds of common

meat. To distinguish false positives such as sample contamination or mismatching during sequencing, we regarded the results with >100 reads as positive and those with <100 reads as false positive. The 16S\_Ki primer could detect mammalian components as low as 0.1%, but could only detect more than 10% of poultry components. The GHR\_1 primer could detect 0.1% of the four components except chickens, and the detection rate of chicken components was 0.5%. The results indicated that GHR\_1 primer was more suitable for the detection in these five kinds of common meat.

#### 4. Discussion

NGS sequencing has great application potential in species identification. The mitochondrial gene 16S rRNA was used as the target gene, and the NGS method was used to identify mammalian samples in mixed samples, with a detection limit of less than 1% [15]. Illumina sequencing was used to detect species DNA from mammals (pigs, cattle, horses, and sheep) and birds (chickens and turkeys) in sausages, which could distinguish species at the 1% level [16].

In this study, we tested the possibility of using Illumina second-generation sequencing technology to identify common meats (pigs, cattle, sheep, chickens, and ducks) in meat products. Adulteration identification of meat products is a hot topic in society. The low value meat, such as chickens, ducks, and pigs, is often impersonated as high value meat such as cattle and sheep, leading to a high concern worldwide [17]. Besides, many kinds of meat may produce different kinds of harmful substances in the process of deep processing [18]. At the same time, the sales scale of artificial plant meat products is growing rapidly in the global food market, and the taste and color are more and more close to the traditional meat products [1, 19]. All these put forward new demands for species identification of meat products. The advantage of NGS in species identification is that it can identify multiple species simultaneously from mixed samples without any prior information [20]. Species information can be obtained by matching the sequencing results with the existing sequence data in the database using bioinformatics methods. In this study, we did not consider the sequence differences among individuals because the sequence differences within species were less than that among species. Using the program algorithm, we can combine the sequences with individual differences into one class for analysis. According to the literature review [13], the target genes used in the existing NGS identification methods are mostly concentrated in mitochondrial DNA. Its main advantage is that mtDNA is rich in animal cells, is easy to extract, and contains multiple DNA sequences with both highly variable regions (differences between species) and conserved regions (highly similar between species), which

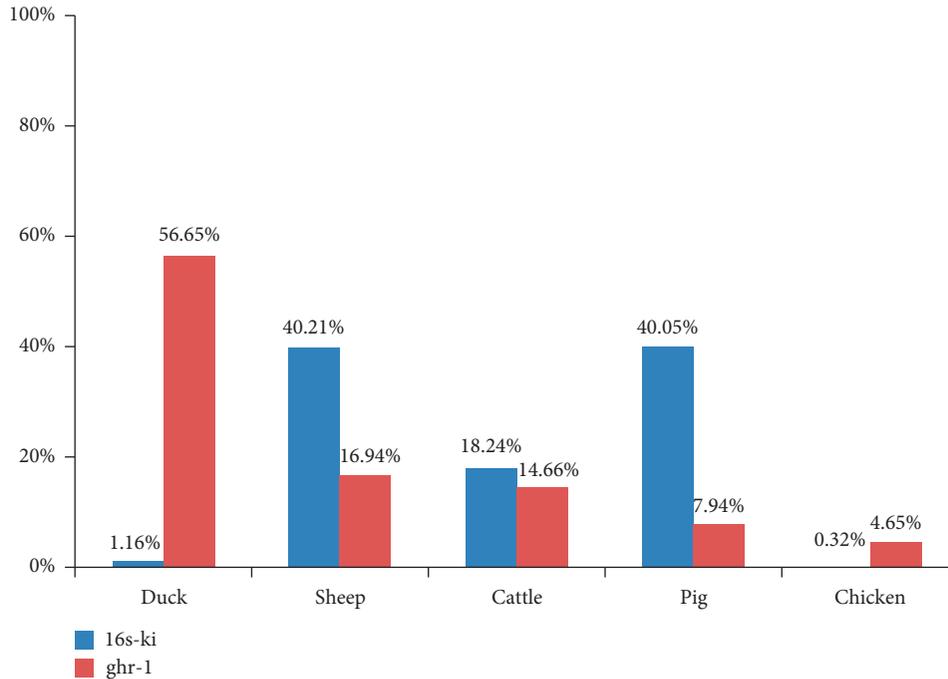


FIGURE 1: NGS sequencing results of mixed samples with an equal mass ratio.

TABLE 2: NGS sequencing results by 16S\_Ki primer.

Content (%)	Number of reads for each species				
	Pigs	Cattle	Sheep	Chickens	Ducks
80	127913	104762	189945	5143	17271
50	100808	40736	102967	2013	8933
20	59762	27217	60008	213	1735
10	31030	22441	30514	128	459
5	8925	4931	12858	4	21
1	1918	2587	4366	4	8
0.5	440	1443	3832	7	6
0.1	279	396	1957	1	4

TABLE 3: NGS sequencing results by GHR\_1 primer.

Content (%)	Number of reads for each species				
	Pigs	Cattle	Sheep	Chickens	Ducks
80	82930	96455	113943	80336	135320
50	30518	50870	76538	53362	81978
20	7551	15537	17831	4819	59849
10	5425	8989	13558	4217	37926
5	3387	5386	8555	1397	19547
1	2041	2485	3876	1863	2141
0.5	1740	1073	1399	1012	5841
0.1	162	1287	854	8	1253

are suitable for species identification, such as 16S RNA gene. However, the mtDNA content in mammals is higher than that in birds due to the significant difference in the mtDNA content in different species, which makes the detection efficiency of the original detection method for birds low. False negatives may be detected for a low content of bird components in mixed samples. The content of nuclear genome monoclonal genes is the same in different species of cells, and their number can directly reflect the number of cells. In recent years, it has been widely used in droplet digital PCR (DDPCR) quantitative detection of species [21–23] and has strong application potential in NGS.

We designed the primer GHR\_1 based on the auxin receptor gene GHR; the mitochondrial DNA primer 16S\_Ki was also used in NGS. The results showed that it was feasible to use the Illumina MiSeq NGS method to identify two short fragments of mitochondrial 16S RNA gene and nuclear genome GHR gene. The 16S\_Ki primer can help detect as low as 0.1% of mammalian components (pigs, cattle, and sheep) in the samples, but the detection effect on poultry was not good. However, GHR\_1 primer could detect as low as

0.5% of the components in all the samples. The limit of detection depends largely on species composition. Due to the amplification bias of PCR amplification, it is unlikely that the quantification of species in the mixture will have considerable accuracy. The target site of the primer GHR\_1 is the nuclear genome monoclonal gene, which has a certain quantitative detection potential. However, the present study found that the number of reads detected by different species had a certain correlation with the species content, but it was not linear. Therefore, an accurate quantitative analysis could not be carried out, and hence further research is needed.

A small number of other species are detected, probably due to experimental pollution. Therefore, it is necessary to distinguish the positive results from possible pollution. The 16S\_Ki primer had poor detection effect on poultry samples, and the number of reads obtained was very low, which might be confused with the background pollution. The GHR\_1 primer was better, and thousands of reads could be obtained for 0.5% species, which was significantly different from the background pollution. For NGS technology, DNA residues detected in the sequencer may pollute the next detection;

therefore, appropriate monitoring methods should be used. In addition, DNA extraction, PCR, and NGS sequencing should be carried out in different rooms to avoid accidental contamination between samples.

In conclusion, NGS sequencing is a promising tool for detecting possible species mixing in meat products. With the decreasing cost of NGS detection equipment and services, the advantages of NGS sequencing, such as high throughput and nontargeting, have become more prominent. It may become a routine method for species identification in food.

## Data Availability

The data are available from the corresponding author upon request.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Authors' Contributions

Xinmei Liu and Zhiyang Liu contributed equally to this study.

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