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SPECIES IDENTIFICATION OF TEN COMMON FARM ANIMALS BASED ON MITOCHONDRIAL 12S rRNA GENE POLYMORPHISMS

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Bio-techniques such as genetic manipulation, marker-assisted selection, and identity test have largely facilitated the modern animal production practices. In the present study, we established a reliable and cost-effective molecular method of species identification for common farm animals. We first (re-)analyzed 179 mitochondrial 12S rRNA gene sequences of ten farm animal species to determine the intra-species and species-specific variations. The PCR-RFLP method was subsequently designed to identify these species by using endonucleases* BshNI, ScaI, AluI, *and* BfaI. *The poultry and livestock species were first discriminated by one double-digestion of both* BshNI *and* ScaI, *which generated different fragment patterns (325 bp and 115 bp for poultry vs. 364 bp and 76 bp for livestock). The ten species could be further discerned according to species-specific restriction pattern by subjecting to digestion of* AluI *and* BfaI, *respectively. Our approach would be more reliable by taking the intra-species variations into consideration and could be applied to species identity test, commercial fraud, and wildlife crime.

Keywords: 12S rRNA gene; Farm animals; Mitochondrial DNA; Restriction fragment length polymorphism; Species identification

Animal identity tests could almost be classified into species identification, breed/population assignment, and individual parentage analysis according to the specific genetic markers at different levels (1–3). The species identification of farm animals via DNA typing has been wildly applied to the fields of commercial fraud in food industry (4), genetic traceability (5), wildlife crime (6), and crime scene investigation 7.

The genetic markers used for species identification are always referred to as mitochondrial DNA (mtDNA) genes (8, 9). Due to the relatively high copy number of mtDNA genomes per cell, mtDNA is more suitable than the nuclear genome for

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studies on degraded, or otherwise compromised, materials (10). The amplified fragments of mtDNA usually included D-loop region (11), Cytochrome *b* gene (7, 12), 16S rRNA gene (8), and 12S rRNA gene (4, 13). Methodologically, PCR-restriction fragment length polymorphism (RFLP) has been proposed to be one of the most efficient methods in terms of cost, detection power, and applicability to large scale screening as reviewed by Pereira and colleagues (14).

PCR-RFLP approach based on mitochondrial DNA polymorphism has already been established for species identification of common farm animals; however, their target species were separately restricted to different animal types, namely the poultry species (15, 16) and livestock species (17, 18). When the poultry species and livestock species were combined together, at least five endonucleases were needed for RFLP analysis according to previously published reports (5, 19). In this study, we aimed to simultaneously identify ten common farm animal species (five poultry species and five livestock species) based on sequence variations of mitochondrial 12S rRNA gene by using a minimum number of endonucleases. We first obtained intra-species sequence variations for each species by analyzing the available 12S rRNA sequences (including the newly obtained sequences in this study and those retrieved from GenBank), then we selected endonucleases to recognize species-specific variations. Our PCR-RFLP method was confirmed to be easy and reliable for identifying the panel species.

MATERIALS AND METHODS

Sample Collection and Genomic DNA Extraction

Twenty-four tissue samples were collected from five poultry species, including chicken (*Gallus gallus*, N = 5), duck (*Anas platyrhynchos*, N = 5), goose (*Anser anser*, N = 5), muscovy duck (*Cairina moschata*, N = 4), and quail (*Coturnix coturnix*, N = 5). These individuals were morphologically confirmed in advance. Genomic DNA was extracted by using the standard phenol/chloroform method (20). Together with these newly collected samples, we re-analyzed the sequence data of 17 samples belonging to five livestock species (cattle, yak, buffalo, goat, and pig), which were reported in our recent study for meat authentication of commercial beef jerky (4).

PCR Amplification and Sequencing

A universal primer pair (Forward: 5′-CAA ACT GGG ATT AGA TAC CCC ACT AT-3′; Reverse: 5′-GAG GGT GAC GGG CGG TGT GT-3′) was used to amplify a fragment of mitochondrial 12S rRNA gene with a size of ~440 bp (17). The detailed conditions of PCR amplification and sequencing were described in our recent study (4). Briefly, PCR amplification was performed in a 50 μL reaction mixture including 100 ng of DNA, 10 mmol/L Tris-HCl (pH 8.3), 2.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 10 pmol/L of each primer, and 1 unit of Taq DNA polymerase (Takara, Dalian, China). PCR product was purified on spin columns (Watson BioTechnologies, Shanghai, China) and was sequenced in both directions using the BigDye Terminator sequencing kit (Applied Biosystems, Foster City,

California, USA). The 24 newly obtained sequences have been deposited in GenBank under accession numbers JN695748-JN695771.

Restriction Fragment Length Polymorphism

The 24 newly obtained mitochondrial 12S rRNA gene sequences were edited by using DNAstar program (DNAS Inc, Madison, WI, USA). We aligned these sequences with 154 reported sequences available in GenBank (Table 1 and Supplementary Table 1) to analyze intra-species and species-specific variations.

Four restriction enzymes, *Bsh*NI, *Sca*I, *Alu*I, and *Bfa*I (Fermentas, Shanghai, China), were selected for RFLP analysis based on the species-specific sequence variations. Among them, endonucleases *Bsh*NI and *Sca*I were combined together for one double-digestion. The predicted restriction sites and fragment length patterns were listed in Table 1 and Fig. 1. PCR products were digested at 37°C for 6 hours in a total volume of 10 μL, which contained 1 μL of template, 3 U of restriction enzymes, and 1 μL of digestion buffer. The digested products were visualized by electrophoresis on a 10% polyacrylamide gel and stained with ethidium bromide.

RESULTS

By using this universal primer pair, we successfully amplified the mitochondrial 12S rRNA gene fragment (~440 bp) for all panel species. We subsequently validated sequence authenticity by direct sequencing the PCR products. Together with these retrieved sequences, a total of 179 mitochondrial 12S rRNA gene sequences from the ten farm animal species were aligned to discern intra-species and species-specific

Table 1 RFLP analysis of mitochondrial 12S rRNA gene of ten farm animals

		Restriction enzymes and fragments (bp)[b]			
Types/Species	N[a]	*Bsh*NI	*Sca*I	*Alu*I	*Bfa*I
Poultry	72 (24)	—	—	—	—
Goose	9 (5)	325, 115	nc	437	307, 130
Muscovy duck	9 (4)	325, 115	nc	441	191, 131, 90, 29
Chicken	34 (5)	325, 115	nc	244, 154, 47	313, 90, 30, 12
Duck	13 (5)	325, 115	nc	392, 47	224, 131, 55, 29
Quail	7 (5)	325, 115	nc	241, 194	305, 88, 30, 12
Livestock	107	—	—	—	—
Cattle	50	nc	364, 76	91, 349	48, 82, 310
Yak	8	nc	364, 76	91, 349	130, 310
Buffalo	14	nc	364, 76	440	35, 130, 275
Goat	10	nc	364, 76	200, 240	130, 310
Pig	25	nc	364, 76	162, 278	30, 100, 310
Total	179	—	—	—	—

[a]The 24 newly sequenced poultry samples were denoted in brackets. Detailed information for these retrieved or reanalyzed sequences was listed in the supplementary Table 1.

[b]"nc" means the species could not be digested by this endonuclease.

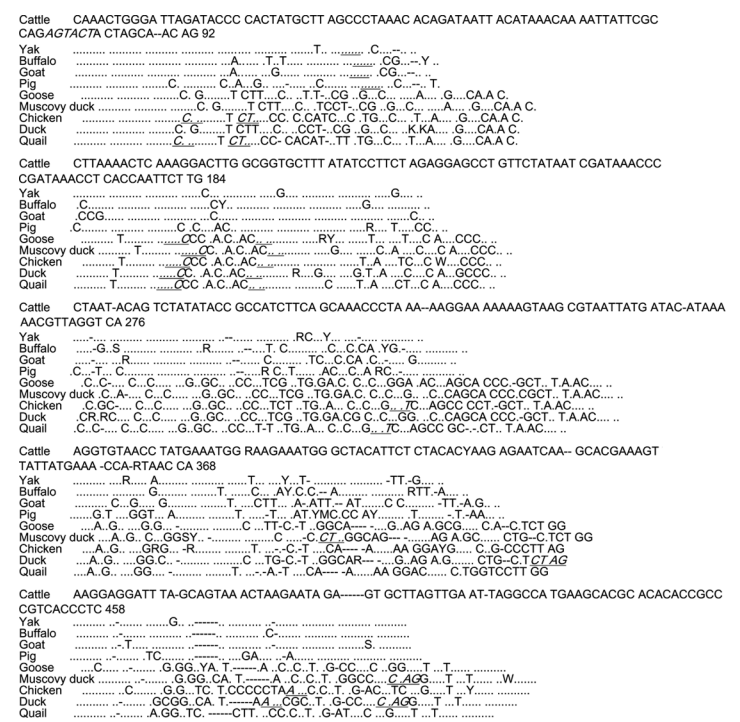

Figure 1 Alignment of modified consensus sequences of mitochondrial 12S rRNA gene fragments of ten poultry and livestock species. The intra-species variations identified in each species are presented by IUPAC codes (e.g., "Y" means the nucleotides C and T at same site in different individuals). The restriction sites of endonucleases *Bsh*NI (5′-G∧GYRCC-3′), *Sca*I (5′-AGT∧ACT-3′), *Alu*I (5′-AG∧CT-3′), and *Bfa*I (5′-C∧TAG-3′) are underlined. Dots (·) denote the identity with the reference sequence (cattle). Short lines (-) represent the base pair deletion.

sequence variations (Fig. 1). A total of 175 polymorphic sites including inserts/deletions were recognized in this data set of mtDNA sequences. Among them, 37 variable sites had intra-species polymorphisms and were subsequently excluded in the process of endonucleases selection.

Based on the species-specific variations, the poultry species and livestock species were first discriminated when they were subjected to a double-digestion of *Bsh*NI and *Sca*I, which generated 325 bp and 115 bp fragment pattern for poultry and 364 bp and 76 bp fragment pattern for livestock, respectively (Table 1 and Fig. 2). Different poultry species and livestock species could be further recognized by individual digestion with *Alu*I and *Bfa*I, respectively (Table 1 and Fig. 3). Among the five poultry species, *Alu*I digestion of quail PCR product resulted in two fragments with a size of 241 bp and 194 bp, which were distinguishable from the two fragments (392 bp and 47 bp) that were observed for duck; in contrast, three fragments (244 bp, 154 bp, and 47 bp) were observed for chicken after the *Alu*I digestion. PCR products of goose and muscovy duck could not be digested by *Alu*I, but they could be obviously distinguished using *Bfa*I digestion, in which two fragments were discerned for goose (307 bp and 130 bp) and four fragments for muscovy duck (191 bp, 131 bp,

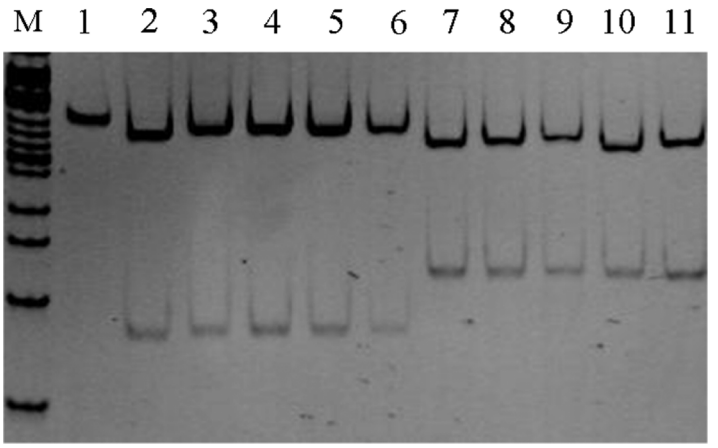

Figure 2 Gel electrophoresis of mitochondrial 12S rRNA gene fragments of ten poultry and livestock species that were submitted to a double-digestion of *Bsh*NI and *Sca*I. Lane M, 50 bp DNA ladder; Lane 1, undigested PCR product of cattle; Lanes 2–6 refer to digested PCR products of cattle, yak, buffalo, goat, and pig; Lanes 7–11 refer to digested PCR products of goose, muscovy duck, chicken, duck, quail.

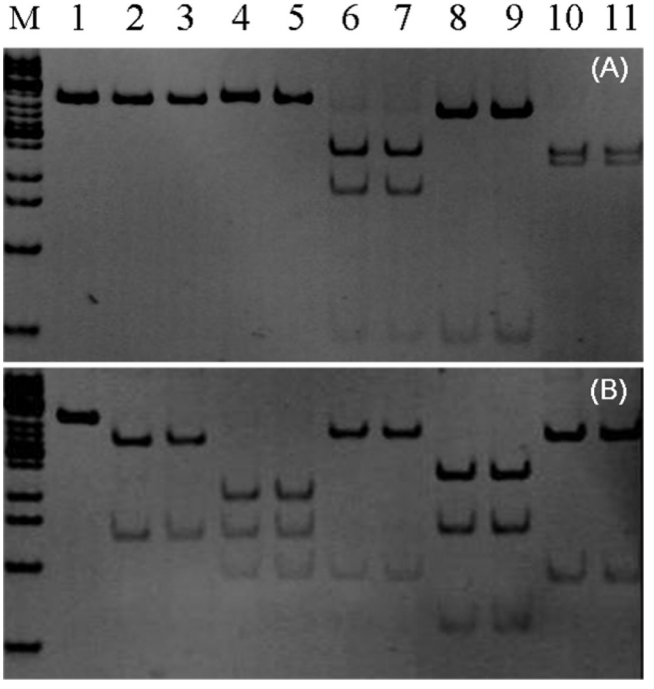

Figure 3 Gel electrophoresis of mitochondrial 12S rRNA gene fragments of the five poultry species digested by *Alu*I (A) and *Bfa*I (B). Lane M, 50 bp DNA ladder; Lane 1, undigested PCR product of cattle; Lanes 2–3, digested PCR products of geese; Lanes 4–5, digested PCR products of muscovy ducks; Lanes 6–7, digested PCR products of chicken; Lanes 8–9, digested PCR products of ducks; Lanes 1–-11, digested PCR products of quail.

90 bp, and 29 bp). The *Bfa*I restriction enzyme generated a similar four-fragment digestion pattern for chicken (313 bp, 90 bp, 30 bp, and 12 bp) and quail (305 bp, 88 bp, 30 bp, and 12 bp) that could not be effectively distinguished, but duck had a species-specific *Bfa*I digestion profile (224 bp, 131 bp, 55 bp, and 29 bp). Note that the deduced small bands (12 bp, 29 bp, and 30 bp) in corresponding species using *Bfa*I digestion were almost unobservable in the 10% polyacrylamide gel (Fig. 3).

The five livestock species could be distinguished by fragment patterns digested by *Alu*I and *Bfa*I, respectively (Table 1), and these results had been described in our former report for species authentication of commercial beef jerky (4). Briefly, the species of buffalo, goat, and pig could be discerned according to digestion profile of endonucleases *Alu*I. Cattle and yak species showed the three fragments profile (48 bp, 82 bp, and 310 bp) and two fragments profile (130 bp and 310 bp) when they were subjected to *Bfa*I digestion, respectively.

DISCUSSION

Molecular methods have largely revolutionized the biological species identification during the last decade. The most important advance in this field was the international cooperation of Barcode of Life Data System (BOLD) launched in May 2004, which has aimed at species identification and discovery of new animals, fungi, plants, and protists through analysis of a 648 bp fragment of cytochrome *c* oxidase I (COI) gene (1). However, Wilson-Wilde and colleagues recently tested the identification power of BOLD for forensic purpose and proposed that further exploration will be needed, especially when the more closely related species were considered (9).

In addition to such universal species classification of BOLD, the reliable and cost-effective molecular method would also be necessary to identify the small-scale species. By designing the species-specific primers to amplify mitochondrial cytochrome *b* gene, Tobe and colleagues designed a multiplex assay for species identification of 18 European mammals (7). For common farm animals, different PCR-based methods have been proposed for species identification as reviewed by Bottero and colleagues (21). However, poultry and livestock animals, two main types widely distributed throughout the world, have rarely been distinguished especially by using the easy and cost-effective methods. In the present study, we successfully discriminated the five poultry species from five livestock species using a simple double-digestion of *Bsh*NI and *Sca*I based on the newly designed PCR-RFLP method. Meanwhile, this is also the main advancement in this study compared with our former report, which aimed to authenticate commercial beef jerky products of the five livestock species (4).

Together with the subsequent *Alu*I and *Bfa*I digestion, we only employed four endonucleases for RFLP analysis to identify ten common poultry and livestock animals. Compared with other molecular methods, such as terminal restriction fragment length polymorphism (22) and DNA sequencing (8), the PCR-RFLP procedure established in this study would be more cost-effective and could be easily conducted in a laboratory without requirement for high-standard equipments. One limitation to practical application of our approach is that the turkey and sheep species, as two important farm animals, were not included in the present study, which could not be differentiated from these ten animals by using this panel of endonucleases (data

not shown). Furthermore, we could not absolutely exclude the potential false positives when this approach is applied to animals beyond the scope of our panel species. In addition, our approach was also successfully applied to commercial beef jerky products with a sensitivity threshold of approximately 20% in binary mixture sample (4), which therefore was not specially tested in the present study. However, the materials of milk and egg products were not confirmed for genomic DNA extraction and PCR-RFLP analysis.

One merit of our PCR-RFLP method that distinguished ours from other similar ones is that we had considered the intra-species variations, which could lead to a gain or loss of restriction sites and result in a false identification. This problem would become more obvious when only a few sequences were analyzed to deduce the so-called species-specific DNA sequence variation (23, 24). Together with our newly generated sequences, we analyzed the available mitochondrial 12S rRNA gene sequences from published sources as much as possible to discern the intra-species variations. Indeed, we observed a relatively high proportion of intra-species variations in the 458 bp fragment. With the exclusion of these intra-species variations, we thought that our approach will be more robust and consistent to provide the reliable species identification. Of course, more sequence data for each species and more knowledge about intra-species variation will be further needed to justify our method.

CONCLUSIONS

In summary, a reliable and cost-effective PCR-RFLP method was successfully established to identify ten common poultry and livestock animals, which could be effectively applied to species identity test, commercial fraud, and wildlife crime. The false identification potentially resulted from the interruption of intra-species variation could be largely avoided as we had analyzed a relatively large number of sequences per species.

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Supplementary Table 1 Mitochondrial 12S rRNA sequences of ten poultry and livestock species analyzed in this study

Species	Origins (N)	GenBank accession numbers
Goose	Sequenced (5)	JN695748 - JN695752
	Retrieved (4)	AY164530; AJ583550; EU932689; NC_011196
Muscovy duck	Sequenced (4)	JN695753 - JN695756
	Retrieved (5)	EU755254; NC_010965; AM902523; L16769; AF173705
Chicken	Sequenced (5)	JN695757 - JN695761
	Retrieved (29)	AB086102; AJ490505; AP003317 - AP003319; AP003321; AP003323; AP003580; AY235570; AY235571; DQ648776; DQ885561; EF362685 - EF362688; EF362698; EF362699; EF362701; EF362713; EF362714; EF362716; EF362717; EF362720; EF362721; FJ610338 - FJ610340; X52392
Duck	Sequenced (5)	JN695762 - JN695766
	Retrieved (8)	AF173685; APU59666; EU009397; EU755252; EU755253; FJ167857 ; L16770; NC_009684
Quail	Sequenced (5)	JN695767 - JN695771
	Retrieved (2)	AM902516; X57245
Cattle	Re-analyzed (7)	GU066734 - GU066738; GQ926965; GQ926966
	Retrieved (43)	AB074962; AB074964; AB074967; AF492350; AJ490501; AY126697; AY676855 - AY676857; AY676859; AY676861; AY676862; AY676865; AY676866; AY676871; AY676873; DQ124371; DQ124374; DQ124375; DQ124382; DQ124385; DQ124396; DQ124397; DQ124404; DQ124406; DQ124407; DQ124409; DQ124418; DQ867006; EU177816; EU177819; EU177828; EU177841; EU177853; EU177860; EU177861; EU177868 - EU177870; EU851893; FJ971088; GQ129208; NC_005971
Yak	Re-analyzed (2)	GQ926973; GQ926974
	Retrieved (6)	AF091686; AY684273; EF494177; EF494178; EF494179; EU521723
Buffalo	Re-analyzed (4)	GU119953 - GU119956
	Retrieved (10)	AF231028; AF547270; AJ457159; AJ490502; AJ846850; AY488491; AY702618; DQ867005; EU760478; EU908276
Goat	Re-analyzed (2)	GQ926969; GQ926970
	Retrieved (8)	AJ490504; AJ630113; AJ885199; DQ867007; EU851902 ; EU851903; M55541; NC_005044
Pig	Re-analyzed (2)	GQ926971; GQ926972
	Retrieved (23)	AB298688; AF304200; AF304202; AF486857 - AF486860; AF486863; AF486866; AF486867; AF486873; AF486874; AJ002189; AP003428; AY334492; AY574046; EF545567; EF545578; FJ236991 - FJ236993; FJ236996; FJ236997
Total	138	—

The 138 retrieved mitochondrial 12S rRNA gene sequences of ten farm animal species were only denoted by GenBank accession numbers.

Erratum

During the production of our paper, there was an unexpected error that caused confusion for Figure 1. Here is the correct version.

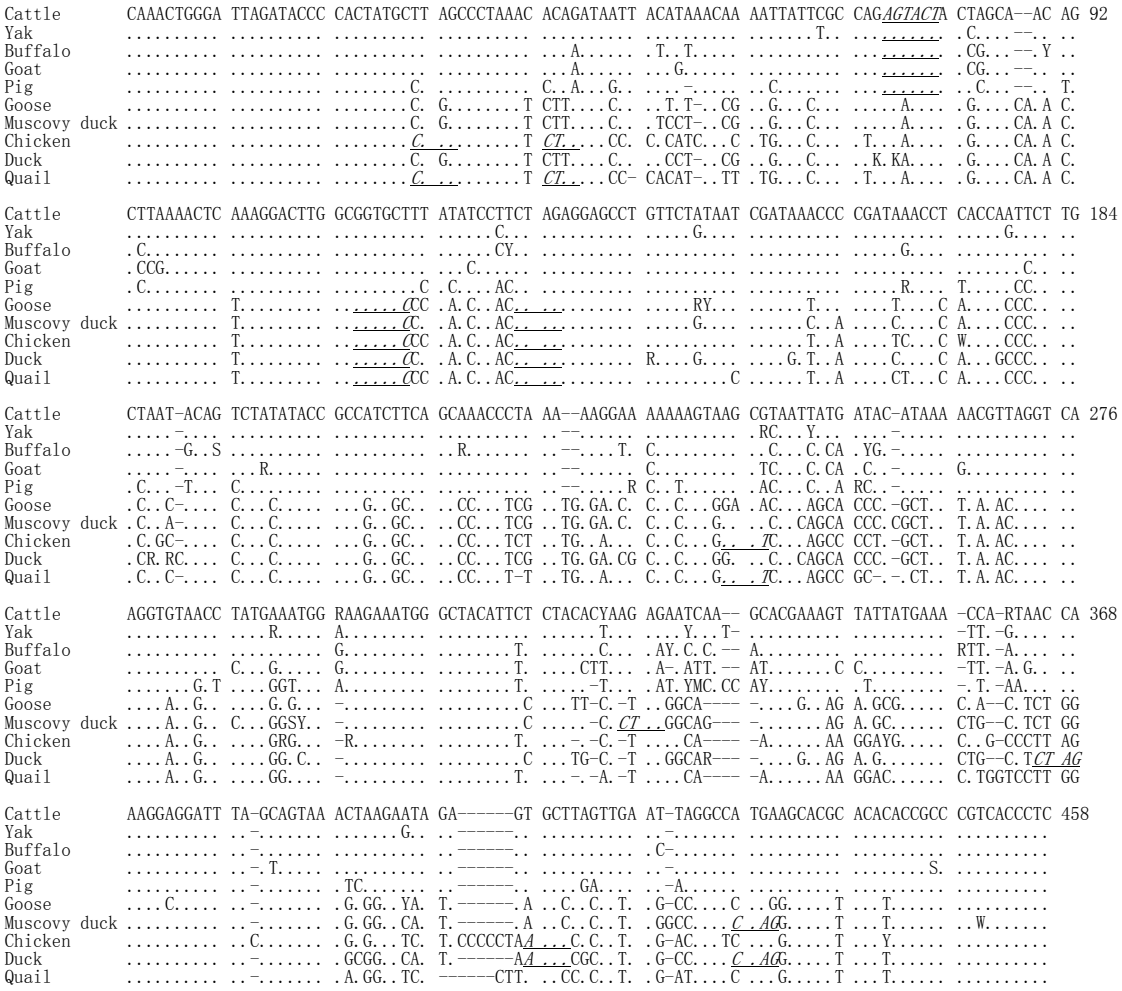

Figure 1 Alignment of modified consensus sequences of mitochondrial 12S rRNA gene fragments of ten poultry and livestock species. The intra-species variations identified in each species are presented by IUPAC codes (e.g., "Y" means the nucleotides C and T at same site in different individuals). The restriction sites of endonucleases *Bsh*NI (5'-G^GYRCC-3'), *Sca*I (5'-AGT^ACT-3'), *Alu*I (5'-AG^CT-3'), and *Bfa*I (5'-C^TAG-3') are underlined. Dots (·) denote the identity with the reference sequence (cattle). Short lines (-) represent the base pair deletion.