A case study on DNA barcoding for pet food mislabeling in South Korea

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Abstract

Due to the close relationship between pets and humans, pet owners are highly invested in proper diets for their pets. Even though pet food mislabeling is concerning, there are few studies on this topic. This study investigated pet food mislabeling in South Korea's market based on DNA barcoding. In total, 10 pet food products were purchased, and 200 sequences of the partial Cytochrome c oxidase subunit 1 (COI) gene were generated from clones of the samples. The obtained sequences were compared to available public databases to identify species present in the ingredients. The data analyses showed that the labeled species were consistent with species detected by COI sequences in 6 of the products. However, the expected species were not detected in 4 products, revealing possible mislabeling in these samples. Our findings indicated that DNA barcoding might represent a promising tool to detect pet food mislabeling.

Introduction

Pets are now considered companions that live and share emotions with people. Therefore, securing nutrients and health for pets is essential. Unsurprisingly, the pet food market is growing, and diverse pet food products have been produced to meet the demand. In 2019, pet food value in the USA reached $36.9 billion (American Pet Products Association, 2019). In the same year, the value of the pet food industry in the European market reached approximately 21 billion euros (European Pet Food Industry Federation, 2019). Similar to the USA and European countries, pet food markets in South Korea also showed an upward trend. The market grew 2.5-fold between 2012 and 2016, from $800 million to $2 billion (Son *et al.,* 2017). The fast market growth requires strengthening legal regulation in pet food labeling to protect the interest of pets and their owners. Labeling of pet food is considered the most important communication tool between manufacturers and purchasers (Okuma *et al.,* 2015). Accordingly, labeling is required to provide pet owners with necessary, transparent, and understandable information. However, similar to the human food supply, the fast growth in the pet food industry has been associated with the potential for intentional and accidental product mislabeling. Consequently, pet food mislabeling may affect purchasers' interest as they may pay more and receive a lower-end product. Additionally, mislabeling can possibly impact pet health if the substituents are harmful. To meet the requirements of legal regulation, it is imperative to apply a useful tool to detect pet food mislabeling.

Since its introduction, DNA barcoding has gained popularity as a powerful method for species identification. DNA barcoding is commonly used to investigate mislabeling in the human food sector (Carvalho *et al.,* 2015; Pardo *et al.,* 2018; Shehata *et al.,* 2018; Do *et al.,* 2019). In contrast, only a few studies were performed to test pet food authentication. Previous applications of DNA barcoding demonstrated a relatively high rate of mislabeling in pet food (Armani *et al.,* 2015). Although South Korea is a fast-growing pet food market, there is little known about pet food mislabeling in the country. Therefore, pet food mislabeling should be investigated to protect purchasers and their pets.

The present study applied DNA barcoding to detect species contents of pet foods in South Korea's market. Pet food samples used for cat and dog were collected and the partial Cytochrome c oxidase subunit 1 (COI) sequences were generated by Polymerase chain reaction (PCR), cloning and Sanger sequencing. The obtained sequences were compared with available public databases to determine the species present in the samples. Based on the analyses, potential mislabeling of the products was verified.

Materials and methods

Sample collection and DNA extraction

Ten different dog and cat food products were purchased from retail outlets in South Korea for this study. Collected pet food samples included both single and mixed-species labels, and different types of pet foods such as canned meats, jerky, particle, and snacks were selected. The ingredients of the collected samples are listed in Supplementary Table S1. Following collection, the products were labeled with internal codes. For DNA extraction, jerky, particle, and snack samples were pulverized with autoclaved mortars and pestles. Total DNA was extracted from 25 mg of samples using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) with modifications described by Handy *et al.* (Handy *et al.*,2011). The concentration and purity of extracted DNA were measured with a MaestroNano spectrophotometer (Maestrogen, Hsinchu, Taiwan).

Polymerase chain reaction (PCR)

PCR analysis was performed for the

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mitochondrial COI gene with the LCO1490/HCO2198 primer set (Folmer *et al.,* 1994). When species were labeled in the product but were not detected by the LCO1490/HCO2198 primer set, more specific primers were used to amplify the samples (Table 1). This step reduces the possibility that undetected species were caused by primer matching. PCR reaction was performed containing 10 µl of 2×TOPsimple™ DyeMIX-Tenuto (Enzynomics, Daejeon, South Korea), 1 µl of each primer (10 µM stock), 100 ng of template DNA, and distilled water to bring the volume to a total of 20 µl. Thermocycler protocol for gradient PCR conditions consisted of an initial denaturation for 5 minutes at 94°C and 35 cycles of 94°C for 30 seconds, 42–55°C for 1 minute, and 72°C for 1 minute, followed by a final extension for 5 minutes at 72°C. The amplified products were analyzed by electrophoresis in 1% (w/v) agarose gels in a tris-acetate buffer. Subsequently, PCR products from each sample were mixed and used for cloning. The cloning procedure was conducted with the TA Cloning® Kit following the manufacturer's instructions (Invitrogen, Carlsbad, USA). Obtained colonies were randomly selected for PCR with the M13 primer set. Finally, the M13 primer set was used to sequence the insert using an ABI 3730 DNA Analyzer (Applied Biosystems, Waltham, USA).

Sequence analyses

All sequences were assembled and edited using Geneious software version 9.1 (Kearse *et al.,* 2012). The obtained sequences were blasted in GenBank with the Basic Local Alignment Search Tool (blast.ncbi.nlm.nih.gov/Blast.cgi) and Barcode of Life Database (BOLD) Identification Engine (www.boldsystems.org). The findings of the scientific names based on COI sequences were recorded through this procedure (Armani *et al.,* 2015; Do et al., 2019). The common names of species were identified with FishBase (www.fishbase. org), Encyclopedia of Life (eol.org), and NCBI taxonomy (www.ncbi.nlm.nih.gov/t axonomy).

Results and discussion

Detection of species in pet foods by DNA barcoding

For each sample, 20 COI sequences were generated through this study. Blast with GenBank and BOLD databases showed consistent results with similarity in the range of 99.85–100% (Table 2). Based on the similarity analyses, all obtained sequences were identified as a specific species (Supplementary Table S2). The most common species detected in the pet food was chicken (*Gallus gallus*) which appeared in 7 of the 10 examined samples (Table 2). Of 7 products with chicken detected, 6 included chicken labeling, and 1 (DF4) was labeled as duck; however, chick-

Table 1. Primer sequences used to amplify COI sequences of pet food products in the present study.

Primer sequences (5'–3')	Amplification size	References
LCO1490: GGTCAACAAATCATAAAGATATTGG	658 bp	Folmer *et al.*, (1994)
HCO2198:TAAACTTCAGGGTGACCAAAAAATCA		Folmer *et al.*, (1994)
BirdF1: TTCTCCAACCACAAAGACATTGGCAC	749 bp	Hebert *et al.*, (2004)
BirdR1: ACGTGGGAGATAATTCCAAATCCTG		Hebert *et al.*, (2004)
FishF1: TCAACCAACCACAAAGACATTGGCAC	655 bp	Ward *et al.*, (2005)
FishR1: TAGACTTCTGGGTGGCCAAAGAATCA		Ward *et al.*, (2005)
FishF2: TCGACTAATCATAAAGATATCGGCAC	655 bp	Ward *et al.*, (2005)
FishR2: ACTTCAGGGTGACCGAAGAATCAGAA		Ward *et al.*, (2005)

Table 2. Summary of species identification in pet food products by DNA barcoding.

Brand*	Label	BLAST analysis	Similarity (%)	Number of sequences	Common name of species	Potentially mislabeled
DF1	Whitefish*	*Gadus chalcogrammus*	99.85	20	Alaska pollock	No
DF2	Lamp Salmon	*Ovis aries* *Oncorhynchus gorbuscha*	100 99.85	16 4	Sheep Pink salmon	No
DF3	Chicken	*Gallus gallus*	99.85-100	20	Chicken	No
DF4	Duck	*Gallus gallus*	100	20	Chicken	Yes
DF5	Salmon	*Oncorhynchus gorbuscha* *Oncorhynchus keta*	100 99.24	18 2	Pink salmon Chum salmon	No
DF6	Chicken Sheep Beef	*Gallus gallus* *Ovis aries* *Not detected*	100 99.85-100	12 8	Chicken Sheep	Yes
DF7	Chicken Beef	*Gallus gallus* *Bos taurus*	100 100	18 2	Chicken Cow	No
CF1	Chicken	*Gallus gallus*	99.85-100	20	Chicken	No
CF2	Chicken Turkey	*Gallus gallus* *Not detected*	100	20	Chicken	Yes
CF3	Chicken Tuna	*Gallus gallus* *Not detected*	100	20	Chicken	Yes

DF, Dog food; CF, Cat food. *Whitefish is a term used for several bottom species of white-fleshed fishes, including Alaska pollock (Alaska Seafood Marketing Institute, 2016).

en was found. In mixed products such as DF6 and DF7, the number of chicken sequences was also higher than other species, indicating a large amount of chicken used for production. The commonality of chicken in the pet food products may be because of the low cost of chicken compared to other meats (Okuma and Hellberg, 2015; National Chicken Council, 2022). In addition to chicken, other species were detected in the tested samples, including sheep (*Ovis aries*), cow (*Bos taurus*), Alaska pollock (*Gadus chalcogrammus*), Pink salmon (*Oncorhynchus gorbuscha*) and Chum salmon (*Oncorhynchus keta*).

Pet food mislabeling

Six of the 10 pet food products examined in this study were labeled correctly. All species that appeared on the product label were found in these samples, and undeclared species were not found throughout the study (Table 2). Of the 6 correctly labeled products, 5 products had labels similar to the common names of the species. For the DF1 sample, the label is whitefish, and the common name of *Gadus chalcogrammus* is Alaska pollock. However, Alaska pollock is one of the whitefish species (Alaska Seafood Marketing Institute, 2016), and pictures of this fish were shown on the product bag. Therefore, DF1 was considered to be correctly labeled. In contrast, 4 pet food products (DF4, DF6, CF2, and CF3) were considered potentially mislabeled. In addition to the LCO1490/HCO2198 primer set, mislabeling of tested samples was confirmed with the BirdF1/R1 primer set (DF4 and CF2) and FishF1/R1 and FishF2/R2 primer sets (CF3). Products were considered mislabeled if they contained species that were not present on the product label or did not contain species that were present on the product label (Okuma and Hellberg, 2015). In the DF6, CF2, and CF3 samples, species were labeled on the products, but they were not detected in the analysis. According to the label, sample DF6 included chicken, sheep, and cow; however, cow was not detected. Similarly, in the CF2 sample, chicken and turkey were included in the label, but only chicken was found. Also, the CF3 sample was labeled chicken and tuna, but only chicken was discovered. Moreover, the material for producing the DF4 sample was potentially substituted. Even though DF4 was labeled as duck, all of the sequences obtained did not belong to this species. Instead, the blast result indicated that all of the sequences were chicken. This suggests that chicken might substitute duck to produce the product CF4.

Pet food labels are essential for owners to understand and select suitable products for their pets. In the present study, DNA barcoding was applied to investigate mislabeling in different pet food products, including canned meats, jerky, particle, and snacks. The available reports demonstrated that DNA barcoding is a promising approach for detecting fish, meat and poultry species in food products (Handy *et al.,* 2011; Armani *et al.,* 2015; Silva *et al.,* 2020). Moreover, combining PCRs and cloning targeting a barcode region may be an effective method to determine species from mixed-species products (Silva *et al.,* 2020). However, DNA degradation during food processing may restrict the recovery of barcode sequences from examined effects. Therefore, the application of macro and minibarcoding should be considered, depending on tested products and the quality of extracted DNA. Compared to minibarcoding, full length DNA barcoding was more effective for species identification in meat and poultry products, except for canned samples (Silva *et al.,* 2020). With the increasing number of sequences deposited in the public databases for references such as GenBank and BOLD, DNA barcoding is still a promising method for pet food authentication, especially for single-species products with high-quality extracted DNA.

This study detected 4 potential mislabeled products, including 3 products (DF6, CF2 and CF3) in which labeled ingredients were not found and 1 product (DF4) in which an unlabeled ingredient was possibly substituted. Similar to our findings, previous investigations have also discovered mislabeling in several meat products from tested samples. Examining pet food products in the USA, Okuma and Hellberg (Okuma and Hellberg, 2015) revealed that 20 of the 52 tested products were potentially mislabeled. In addition to meat ingredients, pet food mislabeling was detected in fish products. Analysis of canned minnow products, Armani *et al.* (Armani *et al.*, 2015) detected that all samples labeled as minnow were mislabeled. Moreover, 40% of tuna, bonito, and mackerel fillets were also mislabeled (Armani *et al.,* 2015). The results here and previous studies suggested a relatively high rate of pet food mislabeling. Despite that high rate, the cause of pet food mislabeling is unclear (Okuma and Hellberg, 2015). Pet food mislabeling may come from intentional or unintentional substitution by manufacturers. Additionally, contamination in production plants may be possible because one plant usually produces various products, and equipment may not be thoroughly cleaned (Premanandh, 2013). Moreover, a lack of traceability from the farm to the final food product may generate an opportunity for mislabeling (Shackell, 2008; Okuma and Hellberg, 2015). Finally, even though PCR, cloning and different primer sets were applied, undetected species in mixed samples due to PCR bias could not be excluded. Bias in the PCR procedure for a specific species can be derived from factors such as primer bias and differences in mitochondrial copy number (Silva *et al.,* 2020). Therefore, further studies should apply macrobarcoding to authenticate mixed pet food products in South Korea.

Conclusions

In the present study, DNA barcoding based on PCR and cloning was applied to detect pet food mislabeling in South Korea's market. The results showed that 4 out of 10 products were potentially mislabeled. Our study and previous investigations reveal that pet food mislabeling is a growing problem. Thus, investigations on pet food authentication are necessary. Also, the findings of this study indicate that DNA barcoding is a potential method for the investigation of mislabeling in single-species product. In future studies, expansion of sample collection together with minibarcoding and macrobarcoding should be used to test pet food authentication in South Korea.

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