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Authentication of Species in Bison Products Using Molecular Methods

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Authentication of Species in Bison Products using Molecular Methods

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ABSTRACT

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American bison (*Bison bison*) meat is vulnerable to species substitution due to its high value and similar appearance to less expensive meats, such as beef from domestic cattle (*Bos taurus*). DNA barcoding of the cytochrome *c* oxidase subunit 1 (CO1) gene is a commonly used method to test for meat species mislabeling. However, due to historical hybridization between the American bison and domestic cattle, additional testing is required to confirm species. The objective of this study was to perform a market survey of products sold as bison meat and verify the species of each product using a combination of DNA barcoding and polymerase chain reaction-satellite fragment length polymorphism (PCR-SFLP). A total of 45 bison products were purchased from online retailers, national grocery chains, butchers, and restaurants. All samples underwent DNA barcoding and those that tested positive for cattle were further tested with PCR-SFLP. Of the 45 samples tested using DNA barcoding, 41 were identified as bison, 1 was identified as red deer (*Cervus elaphus*) and 3 were positive for cattle. The results of PCR-SFLP confirmed the presence of cattle for 2 of the 3 samples identified as cattle with DNA barcoding. Overall, 3 of the 45 samples (6.7%) were determined to be mislabeled. This study revealed that additional testing of species with historical hybridization provides improved species identification results as compared to testing with DNA barcoding alone. However, further research is needed in identifying the effectiveness of PCR-SFLP for other hybrid species.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CO1- cytochrome c oxidase subunit I

F_1- first filial generation of offspring

FSIS- Food Safety and Inspection Service

LOD- limit of detection

mtDNA- mitochondrial deoxyribonucleic acid

NBA- National Bison Association

NTC- non-template control

PCR- polymerase chain reaction

PCR-RFLP- polymerase chain reaction-restriction fragment length polymorphism

SFLP- satellite fragment length polymorphism

SRY- sex-determining region Y-chromosome

TSPY- testis-specific protein Y-chromosome

USDA- United States Department of Agriculture

1. Introduction

American bison (*Bison bison*) once flourished in North America, numbering in the tens of millions (Derr et al., 2012; Shaw, 1995). However, great numbers of bison were slaughtered during the peak of the hide trade in the late 1800s and the species was driven to near extinction. By the early 1900s, the remaining bison survived as small herds on 5 private ranches and within a small wild herd in Yellowstone National Park, which had less than 25 animals in 1902 (Derr et al., 2012; Meagher, 1973). Bison on the remaining ranches were crossbred with cattle in an attempt to improve the traits of cattle, including meat quality, quantity, hardiness, feed efficiency, and disease resistance (Boyd, 1914; Goodnight, 1914; Hedrick, 2010). Although the crossbreeding events were discontinued, they resulted in the incorporation of cattle DNA into American bison populations. In 1905, the American Bison Society was formed and its lobbying efforts led to the creation of several public conservation herds within the United States (Freese et al., 2007; Isenberg, 2000). Currently, there are approximately 500,000 bison living in North America (DOW, 2020): about 31,000 bison are managed within conservation herds and the remaining bison are in commercial private herds (Gates et al., 2010; NPS, 2020). The International Union for Conservation of Nature (IUCN) Red List considers American bison to be Near Threatened; however, the populations are stable due to a harvest management plan (Jørgensen et al., 2017).

According to the National Bison Association (NBA), the demand for bison food products is exceeding the supply, including for use as pet food ingredients (NBA, 2020a). Nearly 4,500 bison are slaughtered monthly for food production and the wholesale price of bison carcasses increased 40% from 2016 to 2020, indicating strong growth in the bison industry (USDA, 2020c). Bison, which is sometimes mistakenly referred to as “buffalo,” is low in fat, calories, and

cholesterol and contains high amounts of protein, iron, and vitamin B-12 (NBA, 2020a). Bison spend the majority of their lives on grasslands, with little or no time in the feedlot, and they are generally not given antibiotics or growth hormones. As the demand for bison products continues to increase, there may be increased economic incentives for intentional mislabeling of cattle products as bison for economic gain. For example, the average price for ground bison in April 2020 was $26.43/kg compared to $13.21/kg for ground beef, and bison ribeye steaks averaged $61.62/kg, which is about twice that of beef steaks at $30.86/kg (USDA, 2020a; b).

Species mislabeling of meat products is commonly detected using analytical methods, such as DNA or protein-based techniques (Quinto et al., 2016). DNA barcoding is a widely used method that utilizes universal primers targeting a short, standardized genetic region for the identification of species (HebertCywinska et al., 2003). In animals, the most commonly used region is a ~650 base-pair (bp) fragment of the mitochondrial gene coding for cytochrome c oxidase subunit I (COI) (HebertCywinska et al., 2003). Previous U.S. market surveys using DNA-based techniques such as DNA barcoding have reported the identification of cattle in commercial bison or buffalo products (Hellberg et al., 2017; Kane et al., 2016; Quinto et al., 2016). For example, a market survey conducted on game meats sold in the U.S. reported that two products labeled as “stewed bison meat” and “rib eye bison steak” were identified as domestic cattle with DNA barcoding (Quinto et al., 2016). In comparison, another U.S. market survey detected a mixture of beef, pork and horse in a product labeled as “ground bison” using real-time PCR (Kane & Hellberg, 2016).

A shortcoming of previous market surveys involving bison products was that the analytical methods used for the detection of bison were based on mitochondrial DNA (mtDNA), which is inherited through the maternal line (Quinto et al., 2016). This is problematic when testing for the

presence of bison because historical crossbreeding of the two species was reliant on breeding male bison with female cattle, as well as backcrosses of male bison with female offspring (Hedrick, 2010). Although the cross-breeding programs were halted over a century ago, some American bison populations still carry ancestral cattle DNA, with an average of 13.9% mtDNA cattle ancestry and 0.6% autosomal cattle ancestry across 22 herds studied (Hedrick, 2010). Additionally, according to U.S. regulations, the term bison may refer to American bison or the hybrid species cattalo, which is a result of direct crossbreeding between American bison and cattle (Exotic Animals and Horses, 9 C. F.R. § 352). As a result, DNA-based testing of bison products has the potential to give a false positive result for cattle due to the presence of ancestral cattle DNA in bison or the use of cattalo in a bison product. However, previous studies reporting the detection of cattle DNA in bison products did not perform additional testing to verify the identity of the product. A method that could be used to confirm the species in these situations is polymerase chain reaction (PCR)-satellite fragment length polymorphism (SFLP), which is a variation of PCR-restriction fragment length polymorphism (RFLP) that targets centromeric satellite DNA. A PCR-SFLP method was previously developed for the differentiation of bovine species, including animals of hybrid origin such as bison and cattle (Verkaar et al., 2002); however, it has not been used to verify species labeling of bison products.

The objective of this study was to perform a market survey of products sold as bison meat and verify the species in each product using a combination of molecular methods. DNA barcoding was used as an initial test for species and any bison products that tested positive for domestic cattle were further tested using PCR-SFLP to verify the species.

2. Review of Literature

2.1 Bison History

2.1.1 Bison (*Bison bison*) vs Cattle (*Bos taurus*)

American bison (*Bison bison*) and cattle (*Bos taurus*) are both within the family *Bovidae*, subfamily *Bovinae*. Bison are endemic to North America as they first entered through the Bering land bridge 500,000-250,000 BP (Burton, 1982), while the first domestic cattle arrived in the early 1500s to North America (Rouse, 1973). The two species share the same number of chromosomes (n=30) and similar autosomal gene content and order (Ying et al., 1977).

2.1.2 Bison populations in the United States

Plains bison are thought to have existed in the tens of millions in western North America (Hedrick, 2010). Over a 10-year span (1870-1880), the large numbers of plains bison were slaughtered to near extinction. Bison populations were stabilized by a group of ranchers who wanted to improve their cattle by crossbreeding with bison to introduce favorable traits to improve meat quality, quantity, hardiness, feed efficiency, and disease resistance (Boyd, 1914). Even though the attempt in obtaining upgraded beef was not achieved, bison populations were able to recover to over 500,000 by the early 2000s (Freese et al., 2007). Currently, there are about 31,000 plains bison in the United States in conservation herds and the remaining bison are managed under commercial private herds (Gates et al., 2010; NPS, 2020). As a result of hybridization between bison and cattle in North America, some bison carry ancestral cattle DNA (Halbert et al., 2005; Polziehn et al., 1995). This genetic introgression can be found in both private and conservation herds.

2.1.3 Bison Market

The approximate number of bison slaughtered under U.S. federal and state inspection in 2018 was 50,900, compared to 125,000/day for beef slaughters (USDA, 2019). For the month of April 2020, the average price for ground bison was $26.43/kg compared to $13.21/kg for ground beef (USDA, 2020a; c). The demand for bison currently exceeds the number of bison that are slaughtered to meet this demand. This increase in demand opens the door to fraudulent activity, particularly for economic profit.

2.2 Bison-cattle hybridization

American bison and domestic cattle do not readily produce offspring and would prefer to mate with their own species (Goodnight, 1914). Although this cross is not naturally occurring, bison numbers were restored to sustainable numbers through ranchers forcing the species cross in the early 1900s. Ranchers were able to cross bison males (bulls) with domestic cattle females (cows), but the reciprocal cross was not possible because bison females were reluctant to mate with domestic cattle males (Hedrick, 2010). The interspecific cross between bison bulls and domestic cows resulted in all offspring that were female and no reproductively viable male offspring. The female offspring were then backcrossed to bison bulls, resulting in nearly all female progeny with 25% cattle ancestry and 75% bison ancestry. Occasionally, bulls with 75% bison ancestry were produced, but they were generally not fertile (Boyd, 1914). For crosses between bison bulls and domestic cows, the offspring had 100% cattle mtDNA ancestry and 50% autosomal cattle ancestry. For the backcross progeny of a bison bull to F_1 cow cross, there is again 100% mtDNA cattle ancestry and 25% autosomal cattle ancestry. This assumes that there is no paternal leakage of bison mtDNA from bison male parents to the offspring (Sutovsky et al., 2004). It is predicted that paternally inherited cattle Y chromosomes are 0% from these crosses.

Therefore, no cattle Y chromosomes have been detected in herds with either cattle mtDNA or microsatellite markers (Ward et al., 2001). After 5 or 6 generations of backcrossing, the autosomal cattle ancestry is reduced to 1.56%. It also is noted that if bison cows were used, then mtDNA of bison would be introduced. However, there are some populations, such as the Williams Ranch and Houserock Ranch herds, which demonstrate nearly 100% mtDNA of cattle ancestry and low autosomal cattle ancestry, indicating that bison cows were not successfully introduced (Hedrick, 2010).

Modern crossbreeding of bison with any other species is prohibited among members of the U.S. National Bison Association. However, hybridized versions of bison are commercially available (NBA, 2020b). For example, beefalo is a cross between bison and cattle that contains 37.5% American bison and 62.5% domestic cattle. Beefalo is considered a breed of cattle and is regulated by the USDA (ABA, 2019). Bison-cattle crossbreeds that contain more than 37.5% bison are considered cattalo, which is regulated as an exotic species in the U.S., with meat of different quality and appearance of the meat from beefalo (ABA, 2019).

2.3 Pathogens associated with bison meat

2.3.1 Salmonella, *Listeria*, and *E. coli*

American bison is growing in popularity in the U.S. and European meat markets. Bison meat is not subjected to growth promoting hormones or antimicrobials that are often used in the cattle industry; however, little is known about the effects on the natural microbiological flora of these animals and the microbiological safety of bison meat for human consumption. As little is known about the microbiological safety of bison meat production, mislabeling of this product as beef could lead to potential food safety issues. A study was conducted by researchers at North Dakota State University examining the microbiological safety of bison meat and its risk of

foodborne illness for humans (Li et al., 2004). This study focused on *Salmonella spp., Listeria monocytogenes* and *Escherichia coli* O157:H7. These pathogens have been linked to a number of cases of human illness associated with meat and meat products (Hurd et al., 2000). *Salmonella* can cause gastroenteritis issues associated with nausea, vomiting and diarrhea. *L. monocytogenes* is associated with flu-like symptoms that can affect high risk groups such as immune-compromised or pregnant women. *E. coli* O157:H7 can be life-threatening and has been found primarily in cattle resulting in a large number of outbreaks of human illness. (Li et al., 2004) studied 355 bison carcasses over a 12-month time; 116 from the slaughter line and 239 from chilled carcasses. Swabs were taken from four points on the slaughter line (pre-dehiding, post-evisceration, post-USDA inspection and post-washing) for a total 703 swabs collected. Of the 355 carcasses sampled, 65 were positive for *Listeria* spp from the slaughter line and 9/239 were positive for *Listeria* spp from chilled carcasses. Positives for *Salmonella* were found in 14/355 samples and 7/239 chilled carcasses. The highest positive detection was found for *E. coli* in 136/355 samples on the slaughter line with no *E. coli* detected of any of the chilled carcasses. *E. coli* O157:H7 was detected in 5 of the 355 bison carcasses sampled (1.41%).

Previous studies have reported a range of pathogen levels in beef carcasses. For example, one study conducted by the USDA Food Safety and Inspection Service (FSIS) reported that no *E.coli* O157:H7 was found in 2112 cow and bull carcasses (USDA, 1996). In contrast, a survey conducted by (Elder et al., 2000) indicated O157:H7 was found in 43% of pre-evisceration, 18% of post-evisceration and 2.0% of post-processing beef carcasses. There are many factors thought to contribute to these variations in numbers, including improved isolation procedures, differences in the samples collected, and the type of sample. Additionally, the time of year that the samples were collected may influence the results, as *E. coli* is most prevalent in late summer and early

fall (Li et al., 2004). *L. monocytogenes* was found on chilled bison carcasses at a rate of 0.42%, which is lower that the levels of 4.1-11.3% reported previously for beef carcasses tested in the US (USDA, 1994; 1996). Li et al. (2004) reported the detection of *Salmonella* in chilled bison carcasses at a rate of 2.79%, which is very similar to 2.7% for cattle (USDA, 1996). According to Li et al. (2004), the results from their study vary amongst similar studies due to differences in the sample sizes, the type of sample collected, when the sample was collect (seasons) and improved enrichment and isolation procedures (such as immunomagnetic separation).

2.3.2 *Mycoplasma bovis*: an emerging disease in commercial bison populations

Mycoplasma bovis is a bacterial pathogen that has recently raised concern in North America for the bison industry (USDA, 2013). This pathogen is causing disease in feedlots and breeding-age cows with resulting morbidity and mortality in herds. Mortality rates have been as high as 25% which causes significant economic loss to the producers (Woodbury, 2020). This disease appears as pneumonia or pharyngitis with lesions to the organ systems throughout the body (Woodbury, 2020). The rancher can notice behavior of trailing behind the herd as this disease leads to emaciation and weakness. Bison will often be euthanized to eliminate suffering as recovery is unlikely.

Beta-lactam antimicrobials are ineffective against *Mycoplasma* because they lack a cell wall. *Mycoplasma* also doesn't synthesize folic acid making it resistant to sulfonamides. *Mycoplasma* is susceptible to drugs that interfere with protein or DNA synthesis; however, it is resistant to erythromycin (Maunsell et al., 2011). The best way to prevent this infection from spreading is to separate new animals before introducing them to the herd therefore, quarantine is encouraged.

As this disease is new to bison, it is poorly understood so continued research is needed to develop practical, bison-specific diagnostic tests and effective treatment and prevention strategies to deal with the effects of this pathogen to the bison food industry.

2.4 Meat mislabeling

Food fraud can occur by ingredient substitution and mislabeling of food products which has been found globally within the meat industry. Ingredient substitution can be intentionally carried out for economic gain by completely or partially replacing an ingredient with a lower quality alternative. However, partial replacement of meat species can also occur due to improper cleaning of the equipment when moving from one grind of meat to another, resulting in more than one species of meat being packaged together (Chung et al., 2020). Previous studies have revealed instances of mislabeling in a variety of meat products (Cawthorn et al., 2013; de Oliveira et al., 2018; Di Pinto et al., 2015; Kane & Hellberg, 2016; Naaum et al., 2018; Quinto et al., 2016). For example, one study focused on whole cuts of game meats sold online in the United States reported that 10 of the 54 samples were potentially mislabeled based on the results of DNA barcoding (Quinto et al., 2016). For example, 2 products labeled as bison and 1 labeled as yak were identified as cattle; a product labeled as black bear was revealed to contain American beaver; and pheasant was identified as helmeted guineafowl. However, the bison and yak samples could not be definitely determined to be mislabeled due to the possible detection of ancestral mitochondrial cattle DNA in these animals. Six of the samples were associated with economic incentives, specifically the products labeled bison, pheasant and yak, while four were priced lower than the listed price for the species identified.

Another U.S. study on mislabeling of meat products reported that 10 of the 48 samples tested contained undeclared species based on the results of DNA barcoding and real-time PCR (Kane &

Hellberg, 2016). Samples were purchased from 5 online specialty meat distributors and 4 outlets in Orange County, CA (supermarkets and butchers). The samples containing undeclared species included a bison sample found to contain beef, pork and horse, as well as a yak burger that was identified as beef. Similar to the results of Quinto et al. (2016), the bison and yak samples could not be definitively determined to be mislabeled due to the possible detection of ancestral mitochondrial cattle DNA in these species. The results of Kane and Hellberg (2016) indicated that mislabeling was prevalent in ground meat products especially among online specialty meat distributors (35%), followed by local butchers (18%) and then supermarkets (5.8%).

Studies have also been conducted outside of the United States which have indicated instances of mislabeling in processed meats (Cawthorn et al., 2013; de Oliveira et al., 2018; Di Pinto et al., 2015; Naaum et al., 2018; Shehata et al., 2019). For example, de Oliveira et al. (2018) investigated 91 ground meat samples sold in Brazil as ground beef and detected water buffalo (*Bubalus bubalis*) DNA in 17.5% (16/91) of the samples collected. Of those adulterated samples, 56.25% (9/16) were completely substituted as only water buffalo DNA was detected and 43.75% (7/16) contained a mixture of cattle and water buffalo DNA (de Oliveira et al., 2018).

In a study conducted in South Africa, 139 samples of minced meats, burger patties, deli meats, raw sausages and dried meats were tested for authentication using DNA barcoding (Cawthorn et al., 2013). A total of 20 burgers were tested, including 14 beef burgers, 3 mutton/lamb burgers, 1 ostrich burger, and 2 burgers with no declared species. The results revealed the presence of chicken in 8 of the 20 patties, sheep and pork in 6 samples, beef in an ostrich burger and water buffalo in a beef patty. In this study, burger patties were the second - highest category behind sausages with an incidence of substitution or contamination.

A study conducted in Valenzano, Italy, focused on 72 processed sausages and patties (chicken, pork or pate-a mixture of beef and pork) purchased from dealers, markets and supermarkets (Di Pinto et al., 2015). This study revealed 41/72 mislabeled products, of which 20/36 chicken sausages contained pork and beef, 9/12 pork sausages were positive for beef, 5/12 pate samples labeled as pork and beef mixtures contained chicken and 7/12 meat patties labeled as pork were positive for beef.

A more recent study investigated mislabeling of sausage products purchased in the Canadian market using DNA barcoding, digital PCR and real-time PCR (Naaum et al., 2018). Samples were collected from three major cities from grocery stores and specialty stores to total 100 samples. Labels stated that the sausages contained either pork (n=38), beef (n=27), chicken (n=20) or turkey (n=15). It was found that 95% of samples contained the species that matched the label on the package. The sausages labeled beef, chicken, or pork contained the predominate species matching the label on the package. Of the 15 turkey sausages, 10 contained turkey while 5 samples contained chicken. As for the sausages that contained mixed species; 7 of the 27 beef sausages also contained pork; 4 of the 20 chicken sausages also contained turkey; and 2 of the 38 pork sausages were mixed with beef. Overall, this study found a 20% mislabeling rate among ground meat products in the Canadian market, which is similar to studies conducted in the United States (Kane & Hellberg, 2016). While most of the sausages did contain the majority of the predominate labeled species, even small amounts of undeclared species can have potential health issues or raise religious concerns for consumers.

This Canadian meat mislabeling study had a follow-up study that was conducted by Shehata et al. (2019) a year later on an additional 100 sausage products purchased in Canada. The samples collected included beef, pork, chicken, and turkey sausages. The difference with this

follow-up study from the original study was the additional target testing for sheep and goat along with the previously tested species of pork, beef, chicken, turkey, and horse. The authors reported species mixtures in 17% of products; specifically, 10/30 beef sausages contained a mixture of either chicken, pork, or sheep; 5/20 chicken sausages contained a mix of beef, pork and turkey; 2/20 turkey sausages were mixed with chicken and pork; and none of the pork sausages contained species mixtures. The overall mislabeling rate of 17% for this study was similar to previous studies conducted on meat mislabeling in North America (Kane & Hellberg, 2016; Naaum et al., 2018; Quinto et al., 2016).

2.5 Species authentication testing in meat products

There are many analytical methods used for the authentication of meat products, including protein-based methods and molecular methods. Some common protein-based methods include enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC), and polyacrylamide gel electrophoresis (Lago et al., 2011). However, these methods have limited use with highly processed foods as they are less sensitive for meats that have undergone high temperatures during processing (Haunshi et al., 2009). Molecular methods include real-time polymerase chain reaction (PCR), PCR-restriction fragment length polymorphism (RFLP), and DNA sequencing. Numerous molecular methods have been developed that successfully identify processed meats and are reliable, sensitive, and fast (Di Pinto et al., 2007; Lago et al., 2011). However, many of these methods target mtDNA, which makes it difficult to authenticate hybrid species. As bison has the possibility of containing ancestral cattle DNA and may test positive for cattle with mtDNA methods, additional methods, such as PCR-SFLP, are needed to differentiate hybrid species.

2.5.1 DNA extraction

DNA extraction is the first step in DNA-based testing of meat products. This step is critical for isolating the DNA and removing food residue, additives, and preservatives (Di Pinto et al., 2007). One example of a DNA extraction method used in previous meat mislabeling studies is the DNeasy Blood and Tissue Kit (Qiagen) (Hellberg et al., 2017; Kane & Hellberg, 2016). This kit is efficient with complex and processed matrices. Furthermore, the buffering conditions and silica-column-based system allow for better DNA binding and inhibitor removal as compared to other DNA extraction processes such as cetyl trimethylammonium bromide (CTAB)-based extractions (Di Pinto et al., 2007). The DNeasy Kit is feasible and ideal for various meat products because its high sensitivity, specificity and reproducibility, making this a cost-effective method.

2.5.2 DNA barcoding for species authentication

DNA barcoding is a sequencing-based method that uses a standardized genetic region to identify biological specimens (HebertRatnasingham et al., 2003). This is an effective method in identifying multiple animal species because it shows relatively low genetic divergence within a species and high divergence between a species (HebertCywinska et al., 2003). As such, DNA barcoding has successfully been used to identify species in a variety of meat products (Ahmed et al., 2018; Cawthorn et al., 2013; Hellberg et al., 2017; Kane & Hellberg, 2016; Naaum et al., 2018; Quinto et al., 2016; Shehata et al., 2019). The DNA barcoding method used in these experiments amplifies a ~658-bp region of the gene coding for cytochrome *c* oxidase subunit 1 (COI) (Ahmed et al., 2018; Quinto et al., 2016). This method is supported by the Barcode of Life Data System (BOLD), which contains DNA barcode sequences for over 220,000 animal species.

The downside of this testing method is that it is not capable of identifying multiple species in the same product and it is unable to differentiate hybrid species as it relies on mitochondrial DNA.

2.5.3 Real-time PCR for species identification in mixtures

Real-time PCR is commonly used to test for the presence of species in ground meats because it can detect multiple species within the same product (Bottero et al., 2011). Numerous studies have used real-time PCR for this purpose (Cawthorn et al., 2013; Kane & Hellberg, 2016; Thanakiatkrai et al., 2017). One study conducted in Southeast Asia collected 93 meat samples of raw frozen meat, instant frozen, street food and Halal food (Thanakiatkrai & Kitpipit, 2017). This study found that of the raw frozen meat, 1 sample of ostrich contained pork; in instant frozen food, 2 chicken products contained pork and beef; in street food, 2 beef and 1 chicken products contained pork while 3 pork products contained chicken; and no fraud was detected in halal food. Overall, studies have found that real-time PCR is reliable to identify meat species in both raw and processed forms. Real-time PCR methods are fast, highly sensitive, and can be used for simultaneous detection of multiple targets (Hossain et al., 2017). Furthermore, several multiplex real-time PCR assays have been developed for identification of various animal species (Zhang, 2013). However, real-time PCR methods have not been developed for the identification of bison species.

2.5.4 PCR-SFLP for detection of bison with cattle ancestry

PCR-satellite fragment length polymorphism (SFLP) is a variation of PCR-RFLP that targets satellite repeats. Like PCR-RFLP, PCR-SFLP combines amplification of a DNA region with digestion of PCR products by one or more restriction endonucleases (Bielikova et al., 2010). This results in unique profiles for each meat species and is beneficial in discriminating closely related species (Burger et al., 2002). In the case of PCR-SFLP, restriction enzymes are used to

differentiate DNA from hybrid species based on the analysis of species-specific satellite repeats of related species such as sheep versus cattle and chicken versus turkey (Buntjer et al., 1995). In a study by Verkaar et al. (2002), a combination of PCR-RFLP and PCR-SFLP were performed for bovine species identification, including the differentiation of hybrids. The PCR-RFLP method was based on species-specific mutations of mtDNA of cytochrome b and cytochrome oxidase II, which is not ideal for species with historical hybridization events, such as bison. However, the use of PCR-SFLP enabled the verification of bison because the variants in the sequences of similar satellites are detected by SFLP assays (Nijman et al., 1999). PCR-SFLP specifically can differentiate between bison and cattle as the identification of bison with ancestral cattle DNA will be indicated. PCR-SFLP will be used in the current study to clarify any positive identification of bison as cattle by mitochondrial DNA by indicating the species based on the nuclear genome.

2.6 Objective

Food fraud is an area of concern in the food industry, especially as the interest in game meat is increasing in the U.S. To date, bison has been tested for species authentication in whole cuts and ground meats; however, previous studies were based on mtDNA. Due to historical hybridization events between bison and cattle, there is a possibility of bison meat testing positive for cattle when using mtDNA-based methods.

The objective of this study is to identify species in a variety of bison products, including raw, cooked, ground and whole cuts using DNA barcoding. Products identified as cattle will be further tested with PCR-SFLP to verify the species.

3. Materials and Methods

3.1 Sample collection

A total of 45 unique products advertised as containing bison or buffalo were purchased for use in this study. The selected products included uncooked burger patties/ground meat (n = 13), cooked burgers (n = 21), uncooked whole cuts (n = 10), and hot dogs (n = 1). Seventeen products were purchased from grocery stores, 21 products were purchased from restaurants, 4 from local butchers, and 3 from an online vendor. The grocery stores (n = 9), restaurants (n = 21), and butchers (n = 4) visited were located in Orange County and Los Angeles County, CA. The following information was recorded for each product: brand name, collection location, conditions of collection, product type, country of origin, packaging and distribution information, ingredient list, product description, and any additional claims made on the label. Photos were taken of the price as advertised in the venue, front and back of the label on the package, and the product outside of the package. All products were stored in a freezer at -20 °C. Prior to testing, samples were thawed overnight at 4 °C. Ground samples were placed in a 7-oz Whirl-pak bag (Nasco, Salida, CA) and the bag was massaged by hand for 60 s to obtain a homogenous mixture (Okuma et al., 2015). A tissue sample of ~25 mg was excised with a sterile scalpel and forceps from the interior portion of each meat product and placed directly into a 1.5 ml microcentrifuge tube for DNA extraction.

3.2 DNA extraction

DNA extraction was carried out using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA), Spin-Column protocol. After the tissue sample was collected, it was lysed with 180 µl Buffer ATL and 20 µl Proteinase K over a period of 3 h at 56 °C at 300 rpm in a Thermomixer C (Eppendorf, Hauppauge, NY). Each sample was immediately vortexed after lysis. Next, 200 µl

Buffer AL and 200 µl of 95% ethanol was added to each sample tube and the tube was vortexed. The samples were then transferred to spin columns and centrifuged for 1 min at 8000 rpm. The column membrane was washed with 500 µl of AW1 buffer and centrifuged for 1 min at 8000 rpm followed by a second wash of 500 µl of AW2 buffer and centrifuged for 3 min at 14,000 rpm. The columns were transferred to a sterile 1.5 mL microcentrifuge tube and 100 µl of preheated (37°C) AE buffer was added to the column. The samples were then centrifuged for 1 min at 8000 rpm to collect the eluted DNA. A reagent blank with no tissue added was included alongside each set of extracted samples.

3.3 DNA barcoding

Mammalian primer cocktails described in Ivanova et al. (2012) were used to amplify a 658-bp region of the gene coding for COI. Each PCR sample tube contain the following: 12.5 µl HotStar Taq (Qiagen), 10 µl molecular grade, 0.25 µl forward primer cocktail (10 µM) (Table 1), 0.25 µl reverse primer cocktail (10 µM), and 2 µl of template DNA. A non-template control (NTC) with added sterile water in place of DNA was included in the PCR run. Thermal cycling was carried out using a Mastercycler nexus gradient thermal cycler (Eppendorf) with the following cycling conditions: 95 ℃ for 15 min; 5 cycles of 94 ℃ for 30 s, 50 ℃ for 40 s, and 72 ℃ for 1 min; 35 cycles of 94 ℃ for 30 s, 55 ℃ for 40 s, and 72 ℃ for 1 min; and a final extension step at 72 ℃ for 10 min (Ivanova et al., 2012).

PCR products were confirmed with gel electrophoresis by loading 4 µl of PCR product along with sterile water (16 µl) and 10 µl E-Gel™ 1 Kb Plus DNA ladder (Invitrogen) on 2.0% E-gels (Life Technologies, Carlsbad, CA). The gel was run for 15 min using an E-Gel iBase Power System (Life Technologies) (Hellberg et al., 2014). The results were visualized using Foto/Analyst Express (Fotodyne, Hartland, WI) in combination with Transilluminator FBDLR-

88 (Fisher Scientific, Waltham, MA) and PCIMAGE (version 5.0.0.0 Fotodyne, Hartland, WI). The amplified products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions. Samples were sent to GenScript (Piscataway, NJ) for bi-directional sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and a 3730xl Genetic Analyzer (Life Technologies).

Table 1. Mammalian primer sets used in this study.

Primer set	Primer name	Primer sequence (3′-5′)[a]	Ratio in Cocktail	Product length[b]	Reference
Mammalian primer cocktail for COI full barcode	LepF1_t1	TGTAAAACGACGGCCAGTATTCAACCAATCATAAAGATATTGG	1	658 bp	(Ivanova et al., 2012)
	VF1_t1	TGTAAAACGACGGCCAGTTCTCAACCAACCACAAAGACATTGG	1		
	VF1d_t1	TGTAAAACGACGGCCAGTTCTCAACCAACCACAARGAYATYGG	1		
	VF1i_t1	TGTAAAACGACGGCCAGTTCTCAACCAACCAIAAIGAIATIGG	3		
	LepR1_t1	CAGGAAACAGCTATGACTAAACTTCTGGATGTCCAAAAAATCA			
	VR1d_t1	CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCRAARAAYCA	1		
	VR1_t1	CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCAAAGAATCA	1		
	VR1i_t1	CAGGAAACAGCTATGACTAGACTTCTGGGTGICCIAAIAAICA	3		

[a]Underlined segment indicates M13 tails.
[b] Product lengths are given without primers

3.4 Sequence analysis

Raw sequence data was assembled and edited using Geneious R7 (Biomatters Ltd, Auckland, New Zealand). The consensus sequences were aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) with the default settings in Geneious R7 and then trimmed to the COI DNA barcoding region (658 bp). The length, number of ambiguities and % high quality bases (HQ%) were recorded for each consensus sequence. According to quality guidelines set by Handy et al. (2011), only samples with assembled bi-directional sequences that were at least 500 bp and had <2% ambiguities or single reads with at least 500 bp and ≥98% HQ were further analyzed. Nucleotide sequences meeting these requirements were searched against the public barcode records in the Barcode of Life Data Systems (BOLD) Identification System for COI. The top species matches showing genetic similarity to query sequence were recorded. Sequences which did not yield a species match in BOLD were queried in GenBank using the Basic Local Alignment Search Tool (BLAST) and the top species matches were recorded.

3.5 PCR-SFLP

Samples that were identified as cattle after DNA barcoding underwent further testing with PCR-SFLP (Verkaar et al., 2002). Each PCR tube contained 21 µl molecular grade water, 0.5 OmniMix Bead, 1 µl forward primer (50 ng), 1 µl reverse primer (50 ng) and 2 µl template DNA, for a total volume of 25 µl. Thermal cycling was carried out using a Mastercycler nexus gradient thermal cycler with the following cycling conditions: predenaturation for 2 min at 95 ℃; 25 cycles of 15 s 92 ℃, 30 s at 38 ℃ and 45 s at 72 ℃; followed by a final extension for 5 min at 72 ℃. DNA template was amplified with the Satellite IV and the Satellite 1.711b primer sets (Table 2). PCR products amplified with the Satellite IV primer set underwent a restriction digest with *Taq*I (Fisher Scientific, Hanover Park, IL), and PCR products amplified with the

Satellite 1.711b primer set were digested with *Tru1*I [(*Mse*I) Fisher Scientific, Hanover Park, IL)]. Each restriction digest contained 10 µl of the PCR product, 2 µl of 10X buffer R, 18 µl nuclease-free water, and 1 µl (10 U) of restriction endonuclease (*Taq*I or *Tru1*I). Restriction digests were carried out for 3 h at 65 ℃ using a Mastercycler nexus gradient thermal cycler. The PCR-SFLP products were separated with gel electrophoresis using the settings described above for DNA barcoding, with the exception that the gels were run for 20 min.

Table 2. PCR primers used for PCR-SFLP.

Target	Primer direction	Primer sequence 5'-3'	Amplicon length (bp)
Satellite IV	Forward	AAGCTTGTGACAGATAGAACGAT	604
	Reverse	CAAGCTGTCTAGAATTCAGGGA	
Satellite 1.711b	Forward	CTGGGTGTGACAGTGTTTAAC	822
	Reverse	TGATCCAGGGTATTCGAAGGA	

4. Results and Discussion

4.1 Summary of sample collection

Among the 45 products collected, the price ranges of the raw ground samples (n = 13) were $5.89 to $16.99; raw whole cuts (n = 10) were $8.99 to $30.00; cooked burgers (n = 21) were $9.58 to $23.00; and the hot dog package (n = 1) was $20.00. Eight of the products were labeled as buffalo and 37 were labeled as bison. Of the 8 "buffalo" products, 6 were cooked burger patties, 1 was a frozen burger patty and 1 was a raw ground pre-packaged product. The majority of the bison/buffalo products did not declare a country of origin. Ten of the products declared USA and 1 sample was labeled with "Product of USA and Canada." Of the 10 items with country of origin labeling, 8 were purchased from grocery stores, 1 from a butcher and 1 was an online purchase.

4.2 DNA barcoding and PCR-SFLP

All 45 samples were successfully amplified and sequenced with the COI DNA barcode. Bi-directional reads were obtained for all sequences, with an average length of 655 ± 6 bp. The sequences were high quality, with an average % high quality (HQ) bases of 96.2 ± 7.5% and average percent ambiguities of 0.05 ± 0.16%. The samples were all identified at the species level using BOLD with ≥99.7% genetic similarity to the top species match. The majority of samples (n = 41) showed a top species match to both American bison (*Bison bison*) and steppe bison (*Bison priscus*). However, steppe bison became extinct about 10,000 years ago, at the end of the last Ice Age (Marsolier-Kergoat et al., 2015). Therefore, this species identification was ruled out and the samples were determined to be American bison. The remaining 4 samples were identified as a species other than bison: 1 sample was identified as red deer (*Cervus elaphus*) with 100% genetic similarity and 3 samples were identified as domestic cattle (*Bos taurus*) with 99.7-100% genetic similarity.

The three samples that tested positive for domestic cattle with DNA barcoding were subjected to confirmatory testing with PCR-SFLP (Figure 1). The results of testing with the satellite 1.711b genetic marker combined with *Taq*I restriction enzyme showed three bands around 800 bp, 550 bp, and 250 bp for samples Z003 and Z014, which is consistent with the expected result for domestic cattle/zebu/banteng/and gayal gaur. On the other hand, sample Z011 showed a single band around 800 bp, which indicates the presence of bison, yak, or wisent. The results of testing with the satellite IV genetic marker combined with *Tru1*I restriction enzyme showed bands around 600 bp for all three samples, as well as a secondary band around 500 bp that varied intensity from barely visible (sample Z003) to clearly visible (sample Z011). These results were determined to be inconclusive, as the expected result for domestic cattle was a single

band at 604 bp and the expected result for American bison was six bands with lengths of 604, 529, 467, 137, 75, and 63 bp. While it is possible that bands <150 bp may not have been observed in the E-Gel, at least three bands at 467-604 bp should have been visible for samples containing American bison. The inconclusive results associated with the satellite IV genetic marker may have been due to genetic variation in the target region for *Tru1*I, leading to alternate fragmentation patterns in the agarose gel. Overall, the combined results of DNA barcoding and PCR-SFLP indicated that among the three samples that tested positive for domestic cattle with DNA barcoding, two were domestic cattle and one sample was likely American bison with ancestral cattle DNA.

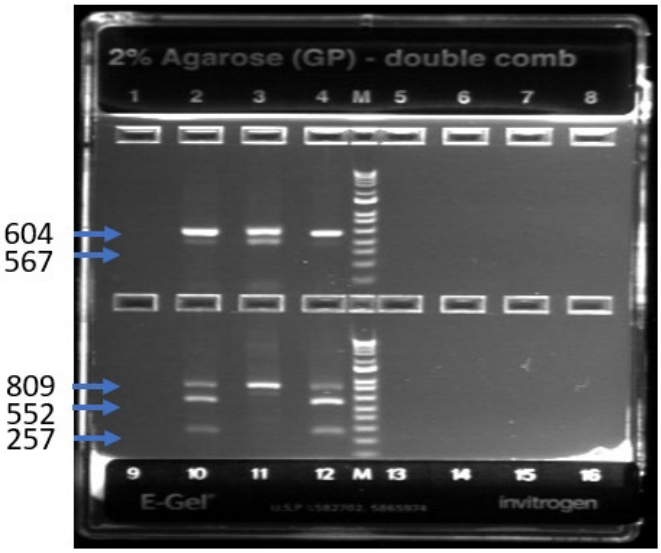

Figure 1. Gel electrophoresis results of PCR-SFLP for bison products that were positive for domestic cattle with DNA barcoding. Top row: Satellite IV primers with MseI digest; Bottom row: Satellite 1.711 primers with TaqI digest. Wells contain the NTC (wells 1 and 9), Z003 (wells 2 and 10), Z011 (wells 3 and 11), Z014 (wells 4 and 12), and 100 bp molecular ladder ("M" wells).

4.3 Mislabeled products

Based on the combined results of DNA barcoding and PCR-SFLP, 3 of the 45 samples (6.7%) tested in this study were determined to be mislabeled (Table 3). One sample (Z003) was

labeled as raw ground bison and was purchased at a grocery chain store, while the other 2 products (Z014 and Z021) were cooked bison/buffalo burgers purchased at chain restaurants. Sample Z021 was labeled as a buffalo burger but was identified with DNA barcoding as red deer. This sample was sold for $11.84; however, the price per kg could not be calculated because the weight of the burger was not included in the product description. Therefore, it is difficult to determine whether any economic profit was made based on this mislabeling event. While previous studies have not reported the identification of red deer in a bison/buffalo product, a game meats distributor in the US previously received a warning letter from the FDA for selling "Black Bear Burgers" that instead contained elk/red deer (*Cervus* sp.) (FDA, 2011).

Two samples (Z003 and Z014) were labeled as bison but confirmed to contain domestic cattle based on the combined results of DNA barcoding and PCR-SFLP (Table 3). Sample Z003 was a 0.45-kg packaged ground sample purchased from a grocery store for US $9.99 (i.e., $22.20/kg). The average cost of ground beef during the period this sample was purchased (July, 2019) was US $14.31/kg (USDA, 2020b). Therefore, sample Z003 was associated with an economic profit of US $7.89/kg. Sample Z014 was a 7-oz burger purchased from a restaurant for US $17.64. Comparatively, the price of a beef burger purchased from the same establishment was $15.00, resulting in an economic profit of $2.64 for this sample.

When products were separated based on purchasing locations, mislabeling was detected in 2 of 21 samples purchased at restaurants and 1 of the 17 samples purchased at grocery stores. None of the 4 samples purchased from butchers or the 3 samples purchased online were mislabeled. In terms of product type, all three samples determined to be mislabeled were ground bison: two were cooked burgers and one was a raw ground product. In comparison, previous studies reporting bison mislabeling purchased the mislabeled products from online vendors and grocery

stores in Orange County (Kane & Hellberg, 2016; Quinto et al., 2016). A previous study investigating whole cuts of bison with DNA barcoding identified domestic cattle in one sample of bison stew meat and one sample of bison rib eye steak purchased online (Quinto et al., 2016). Another study identified domestic cattle alone or as a mixture with other species in two yak burgers and one sample of ground bison, all of which were purchased from online vendors (Kane & Hellberg, 2016).

Table 3. Summary of mislabeled bison products identified in this study. Prices are given in US dollars.

Sample ID	Product description on label/menu	Purchase place	Paid price	Amount purchased	Product type	Identified species
Z021	Buffalo burger	Restaurant	$11.84	1 burger (no weight given)	Cooked burger	Red deer (*Cervus elaphus*)
Z003	Fresh 90/10 ground bison	Grocery store	$9.99	1 lb pre-packaged	Ground	Domestic cattle (*Bos taurus*)
Z014	Bison burger	Restaurant	$17.64	1 burger (no weight given)	Cooked burger	Domestic cattle (*Bos taurus*)

The overall rate of mislabeling in the current study (6.7%) was relatively low compared to previous studies in North America that investigated a wider scope of meat products, with reported mislabeling rates of 14-21% (Kane & Hellberg, 2016; Naaum et al., 2018; Quinto et al., 2016; Shehata et al., 2019). The differences in these results may be due to this study being conducted on one single species compared to previous studies targeting multiple species. Additionally, the sample sizes of previous studies were higher than the current study, ranging from 48 to 100 samples.

5. Conclusions

The overall results of this study indicate that the samples tested had a relatively low level of mislabeling when compared to previous meat mislabeling studies. The greatest rate of mislabeling was found in samples purchased from restaurants (9.5%), followed by grocery stores (5.9%). No mislabeling was detected in samples purchased from butchers or online sources. The common trend of lower-cost species being substituted for higher-cost species for economic gain remains evident. This study demonstrated the importance of confirmation testing for bison products that test positive for domestic cattle with DNA barcoding. While three bison products in this study initially tested positive for domestic cattle with DNA barcoding, follow-up testing with PCR-SFLP indicated that one of the products was likely bison with ancestral cattle DNA. However, additional research is needed to optimize the method and resolve the inconclusive results obtained with the *TaqI* restriction enzyme. Further research should also be conducted to examine the effectiveness of PCR-SFLP for differentiation of other species with historical hybridization events.

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