DNA barcoding revealing the mislabeling of fish in a highly tourist capital in Brazil

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The consumption of raw fish has been increasing considerably in the West, since it has an appeal to be potentially healthier (omega 3 and 6, essential amino acids and vitamins). However this potential benefit, as well as the taste, value and even the risk of extinction is different between species of fish, constituting grounds for fraud. Through the Project "Cat by Hare", using the principles of the DNA barcode, we revealed mislabelling of fish in japanese restaurants and fishmarkets in Florianópolis, a highly tourist capital in Brazil. We sequenced the COI gene of 65 samples from fishmongers and 80 from restaurants and we diagnosed 34\% of fraud in fishmongers and 17\% in restaurants. This different percentage is related to the restaurants selling only two species (Tuna and Salmon) and one category of fish called "white fish" that can be any species that has whitish musculature. We discussed that frauds may have occurred for different reasons: to circumvent surveillance on threatened species; to sell fish with sizes smaller than allowed or species that are being highly captured as being a low captured one at any time (law of supply); to induce product consumption using species with better taste. It should be noted that some substitutions are derived from incorrect identification and are not a fraud *per se*, due to confusion of popular names or misunderstanding of sellers. Therefore, we suggest the implementation of a systematic regulatory program conducted by governmental agencies to reduce mislabelling to avoid further damage to the community (in health and financial issues) and fish stocks

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ABSTRACT

The consumption of raw fish has been increasing considerably in the West, since it has an appeal to be potentially healthier (omega 3 and 6, essential amino acids and vitamins). However this potential benefit, as well as the taste, value and even the risk of extinction is different between species of fish, constituting grounds for fraud. Through the Project "Cat by Hare", using the principles of the DNA barcode, we revealed mislabelling of fish in japanese restaurants and fishmarkets in Florianópolis, a highly tourist capital in Brazil. We sequenced the COI gene of 65 samples from fishmongers and 80 from restaurants and we diagnosed 34% of fraud in fishmongers and 17% in restaurants. This different percentage is related to the restaurants selling only two species (Tuna and Salmon) and one category of fish called "white fish" that can be any species that has whitish musculature. We discussed that frauds may have occurred for different reasons: to circumvent surveillance on threatened species; to sell fish with sizes smaller than allowed or species that are being highly captured as being a low captured one at any time (law of supply); to induce product consumption using species with better taste. It should be noted that some substitutions are derived from incorrect identification and are not a fraud *per se*, due to confusion of popular names or misunderstanding of sellers. Therefore, we suggest the implementation of a systematic regulatory program conducted by governmental agencies to reduce mislabelling to avoid further damage to the community (in health and financial issues) and fish stocks.

INTRODUCTION

The "Cat by Hare Project" was named after a popular saying that emerged in the old days in Portugal where the hare meat was much appreciated. Due the high cost, traders added many condiments to cat meat making the difference almost imperceptible. This expression, quoted even in Luis de Camões poems, is used nowadays to identify a situation of taking something worthless believing it to be a more expensive product, that is fraud (Saxon-speaking usually use the expression *a pig in a poke*).

Food fraud is an intentional adulteration to mask product conditions, or allocate requirements that it does not have, such as nutritional characteristics or price Spink and Moyer (2011). Since fish is a quickly decaying product, the main strategy to extend shelf life is to process the meat, the most common is filleting. The fillet is produced cutting or slicing the flesh from the bone lengthwise, parallel to the backbone. This way many morphological structures are removed, making it difficult to recognize the species used, allowing accidental or intentional substitutions (Cawthorn et al. (2012); Galimberti et al. (2013); Galal-Khallaf et al. (2014)).

Accidental substitutions usually happen when species have similar morphological characteristics, species with the same vernacular name, or different names for the same species (Buck (2007); Ardura et al. (2010); Barbuto et al. (2010); Cawthorn et al. (2012)). On the other hand, intentional substitutions occur for the purpose of increasing profits by replacing species of high commercial value by species of low value or little market acceptance, as well as, for the marketing of vulnerable or overexploited species (Logan et al. (2008); Cawthorn et al. (2012); Huxley-Jones et al. (2012); Maralit et al. (2013); Cutarelli et al. (2014)). There is a *strong evidence that intentional mislabeling of cheaper fish products is a more frequent phenomenon mainly within processed fish*(Carvalho et al. (2015)).

Such substitutions lead to problems associated with food security where substituted species pose a potential risk to human health (Handy et al. (2011); Galimberti et al. (2013)), economic losses for both the end consumer and the fisherman who were not intentionally involved in the fraud (Ardura et al. (2010); Galimberti et al. (2013)) and ecological implications, affecting the conservation status of endangered, vulnerable species, leading to declining fish populations (Logan et al. (2008); Barbuto et al. (2010); Ardura et al. (2013)). Therefore, the aim here was to evaluate the authenticity of the identification of the fish commercialized in popular fishermarkets and japanese restaurants in Florianópolis (Santa Catarina) through an efficient molecular tool such as DNA Barcode.

MATERIAL AND METHODS

Samples

A total of 145 fish samples were collected in 12 Japanese Food Restaurants (JFR) and 09 fishmongers in Florianópolis (southern Brazil), between July 2015 and November 2015. A piece of $1cm^3$ was fragmented in triplicates and stored in 96% ethanol at -20º until DNA extraction. Data were recorded, including date, location, type of fish product and common name of the species offered. The name of the establishments has been omitted to ensure confidentiality.

This study is a continuation of a molecular surveillance program implemented by the Municipality of Florianópolis, previously described by Carvalho et al. (2015). The samples were taken in three campaigns, one of which was accompanied by officials from the PROCON - The Consumer Protection Program -, a governmental regulatory agency. The other samples were taken blindly without prior notice to the establishment, with the sampler acting as a regular consumer.

DNA extraction and sequencing

Genomic DNA was extracted from muscle tissue following the salting out protocol of K. and Nurnberger (1991) with minor modification to reduce the final volume. The Cytochrome c oxidase subunit I (COI) was amplified using the primers L5698 and H7271 (Melo et al. (2011)). PCR reaction mixtures consisted of 0.2μL of Platinum Taq DNA polymerase (5U/μL, Invitrogen, Carlsbad, CA, USA), 0.2μL of each primer (10pmol), 2.5μL of 10x buffer, 1.5μL of $MgCl_2$ (50mM), 1.0μL of genomic DNA (20ng/μL) and purified water to complete the final reaction volume (25μL). PCR conditions entailed 3 minutes at 94°C, following 35 cycles of 30s at 94°C, 80s at 56°C, 160s at 72°C, finalized by 5 minutes at 72°C, after PCR was maintained at 4°C. PCR products were visualized in 1% agarose gel for amplification check.

Positive reactions were purified with ExoSAP-IT (Exonuclease I: Recombinant; SAP: *Pandalus borealis* - USB Corporation) and sequenced using the dideoxy-terminal method with Big Dye kit reagents (ABI PrismTM Dye Terminator Cycle Sequencing Reading Reaction - PE Applied Biosystems) using an automatic capillary sequencer, model ABI3130 (Life Technologies).

Data analysis

For the main species of commercial interest, the correlation between their common names and their scientific names adopted was based on Brazilian Normative Instruction n^o 29 (Brasil and MAPA (2015)) and vernacular names were compared from FishBase (www.fishbase.org). The electropherograms were manually analyzed using the Chromas Lite 2.1.1 (www.technelysium.com.au) and sequences were checked and edited in BioEdit (Hall, 1999).

The sequences were double compared both to GenBank (www.ncbi.nlm.nih.gov/genbank/) and the BOLD (www.boldsystems.org) databases employing BOLD identification tools and Blastn Search Tool, respectively. In all cases, BOLD was the criteria for species identification adopted, considering as valid those with similarity index equal to or greater than 98% in both databases.

The sample was declared mislabeled if the species name determined through molecular identification did not match the commercially accepted name in this list. Additionally, we included the International Union for Conservation of Nature situation (IUCN Red List of Threatened Species status) obtained from http://www.iucnredlist.org and standardized from the Barcode Index Number (Accession number: AAA4896 *Carcharhinus plumbeus*, AAA5277 *Coryphaena hippurus*, AAC9439 *Cynoscion guatucupa*, AAK3830 *Isopisthus parvipinnis*, AAB9115 *Lepidocybium flavobrunneum*, AAB8513 *Micropogonias furnieri*, AAA2371 *Oncorhynchus kisutch*, AAA6537 *Oreochromis niloticus*, AAB7719 *Orthopristis* *ruber*, AAC0146 *Paralichthys orbignyanus*, AAC5845 *Peprilus paru*, AAA9142 *Pomatomus saltatrix*, AAA7096 *Prionace glauca*, AAA7096 *Prionace glauca*, AAC4059 *Rhizoprionodon lalandii*, AAB1796 *Ruvettus pretiosus*, AAA3435 *Salmo salar*, AAB7268 *Sardinella aurita*, AAC0327 *Seriola lalandi*, AAC0327 *Seriola zonata*, AAA2402 *Sphyrna lewini*, AAA7352 *Thunnus alalunga*, AAA7352 *Thunnus obesus*, AAB0166 *Trichiurus lepturus*, AAA6300 *Xiphias gladius*).

RESULTS AND DISCUSSION

We note that when samples were separated by collect origin the percentage of fraud among fishmongers (34%) is almost double than that observed in restaurants (17%) is not unexpected since Brazilian JRF (targets of this research) traditionally offers only two species (Tuna and Salmon) and a third category ("white fish") that is not related to any particular species.

In the total analized sample, the general fraud rate was 28% and the results are presented in Tables 1 and 2, one of which shows the frauds identified and the other one shows the samples in which there was no exchange of species, respectively. It is remarkable that the major motivation for these changes is apparently the cost, although some changes serve to cover up other interests, such as the sale of Croaker in place American Harvestfish. Both have similar economic value (around 3 dollars/kilo), but when Croaker are caught below average size, a common practice is filleting it and selling it as fish known to be smaller.

As mentioned, Brazilian JFR base their menu on three options: Salmon (popularly identified by the rosaceous hue), Tuna (dark meat) and "white fish", which may vary in species. So, the color (and not the texture and flavor) seem to be the most frequent criteria, making fraudulent exchanges easier. In most restaurants offering "white fish" species identified by traders were Yellow Amberjack, Dolphinfish, Weakfish or Tilapia. In nine establishments we could not retrieve the product popular name information. Identification by barcode revealed that two of them were Yellow Amberjack, two were Dolphinfish and Tilapia. Since the expression "white fish" is broad, it is not appropriate to determine whether there was some kind of fraud because the color of the meat of these identified species correspond to this category. However, two cases aroused attention where samples of "white fish" were identified as Salmon (twice) and Tuna (twice).

It is known that Salmon coloration is influenced by diet. Moreover the distribution of salmon coloration through the meat is not uniform, where the musculature whitish close to the horizontal septum, especially when there is much fat infiltrations around the miosepta (Brasil and MAPA (2016)). This substitution is suggested to be motivated not by price differences but to take advantage of all parts of the carcass, even if it is necessary to sell it as if a cheaper species. In other words, it is better to sell Tuna or Salmon as if a cheaper fish, like Yellow Amberjack, than to offer a product (part of the fish) that because of its color would not be recognized by the buyer as Salmon, and therefore be rejected.

Of all the collected samples, the most frequent is also the one of greater commercial value - Tuna, which is not a species, but a set of several of the same genre (this study identified two species: *T. obesus* and *T. alalunga*). The six Tuna fraud were identified in restaurants (and none in fishmongers) and exchanges took place with three species twice each: Salmon (*S. salar*), Yellowtail Amberjack (*S. lalandi*) and Escolar (*L. flavobrunneum*).

Some replacements can not be regarded as fraud, but as a result of confusion between vernacular names. As an example it is worth mentioning the relationship between *T. lepturus* and *X. gladius*, two very different kind of fish, not similar in shape, texture and even flavor, but both called "swordfish" in different regions of Brazil. The same is true for the fish known as Escolar, which may be the popular name for *L. flavobrunneum* or *R. pretiosus* depending on the region of Brazil. These two species cause a gastrointestinal disease and are banned in some countries, like Japan, Italy and Republic of Korea, while other countries like Canada, Denmark and Sweden require health advisories and the European Union requires fishes to be appropriately labeled to provide information to the consumers on adverse gastrointestinal effects (Dalama et al. (2015), Commission Regulation EC (2005), Giusti et al. (2016)).This draws attention to the importance of regulating the relationship between common names and their scientific names, especially for species of commercial interest.

The Atlantic salmon (*S. salar*) is a fish of high commercial value and target of a very emphatic advertising campaign to increase it's consumption. Of the 51 samples termed as Salmon, six of them were identified as fraud (once with Blue Shark, Croaker, Yellow Amberjack, Tuna and twice with Weakfish). Such frauds are possible only with addition of dyes in the feed of farmed fish. In addition to the fraud related to market value, a more dangerous fraud is also present at these cases: it is known that cold-water

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Table 1. Summary of the samples in which **mislabeling** was identified by DNA Barcoding. Parentheses show the lowest match comparisons values from BOLD and BLAST, respectively, highlighting that the highest value is 100. Red List IUCN code: NE - Not Evaluated; DD - Data Deficient; LC - Least Concern; NT - Near Threatened; VU - Vulnerable; EN - Endangered.

N	Sold as (Fishbase)	Identified as (BOLD and BLAST, respectively)		IUCN
1	American harvestfish	Croaker	*M. furnieri* (99,98)	LC
1	Conger	Croaker	*M. furnieri* (99,98)	LC
1	Croaker	Blue Shark	*P. glauca* (100,99)	NT
1	Croaker	Weakfish	*C. guatucupa* (100,99)	NE
1	Escolar	Oilfish	*R. pretiosus* (100,100)	LC
1	Flounder	Bigtooth corvina	*I. parvipinnis* (100,100)	LC
1	Flounder	Croaker	*M. furnieri* (98,98)	LC
2	Flounder	Patagonian flounder	*P. patagonicus*(100,100)	NE
2	Flounder	Weakfish	*C. guatucupa* (100,99)	NE
1	Flounder	Weakfish	*M. furnieri* (99,98)	LC
1	Grouper	Croaker	*M. furnieri* (99,99)	LC
1	Grouper	Weakfish	*C. guatucupa* (99,99)	NE
1	Ling	Croaker	*M.furnieri* (100,100)	LC
1	Panga catfish	Croaker	*M. furnieri* (100,100)	LC
1	Salmon	Banded rudderfish	*S. zonata* (100,100)	LC
1	Salmon	Blue Shark	*P. glauca* (100,100)	NT
1	Salmon	Croaker	*M. furnieri* (99,99)	LC
1	Salmon	Tuna	*T. alalunga* (100,100)	NT
2	Salmon	Weakfish	*C. guatucupa* (100,99)	NE
3	Sand tiger shark	Blue Shark	*P. glauca* (100,98)	VU/NT
1	Shark	Weakfish	*C. guatucupa* (100,99)	NE
2	Swordfish	Largehead hairtail	*T.* (100,100)	LC
1	Tuna	Banded rudderfish	*S. zonata* (100,100)	LC
2	Tuna	Escolar	*L. flavobrunneum* (100,100)	LC
2	Tuna	Salmon	*S. salar* (100,100)	LC
1	Tuna	Yellowtail amberjack	*S. lalandi* (100,100)	LC
2	Weakfish	Bigtooth corvina	*I. parvipinnis* (99,98)	LC
3	“White fish”	Salmon	*S. salar* (100,100)	LC
1	“White fish”	Tuna	*T. obesus* (100,100)	VU

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Table 2. Summary of the samples in which sold species correspond to molecular identification species using DNA Barcoding. Parentheses show the lowest match comparisons values from BOLD and BLAST, respectively, highlighting that the highest value is 100. Red List IUCN code: NE - Not Evaluated; DD - Data Deficient; LC - Least Concern; NT - Near Threatened; VU - Vulnerable; EN - Endangered.

N	Sold as (FishBase)	Identified as (BOLD and BLAST, respectively)		IUCN
4	American harvestfish	American harvestfish	*P. paru* (100,100)	LC
2	Blue Shark	Blue Shark	*P. glauca* (100,100)	NT
2	Bluefish	Bluefish	*P. saltatrix* (100,100)	VU
1	Corocoro grunt	Corocoro grunt	*O. ruber* (100,100)	LC
3	Croaker	Croaker	*M. furnieri* (100,99)	LC
1	Dolphinfish	Dolphinfish	*C. hippurus* (99,98)	LC
2	Escolar	Escolar	*L. flavobrunneum* (100,100)	LC
5	Flounder	Flounder	*P. orbignyanus* (100,100)	NE
1	Salmon	Coho salmon	*O. kisutch* (100,100)	NE
46	Salmon	Salmon	*S. salar* (100,100)	LC
1	Sardine	Sardine	*S. aurita* (100,99)	LC
3	Shark	Blue Shark	*P. glauca* (100,100)	LC
1	Shark	Brazilian sharpnose shark	*R. lalandii* (100,100)	DD
1	Shark	Sandbar shark	*C. plumbeus* (100,99)	VU
1	Shark	Scalloped hammerhead	*S. lewini* (100,100)	EN
1	Swordfish	Swordfish	*X. gladius* (100,100)	LC
1	Tilapia	Tilapia	*O. niloticus* (100,100)	NE
15	Tuna	Tuna	*T. obesus* (100,100)	VU
10	Weakfish	Weakfish	*C. guatucupa* (100,99)	NE
1	"White fish"	Banded rudderfish	*S. zonata* (100,100)	LC
2	"White fish"	Dolphinfish	*C. hippurus* (100,100)	LC
1	"White fish"	Tilapia	*O. nilouticus* (100,100)	NE

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fish like Tuna and Salmon are the best sources of polyunsaturated omega 3 fatty acids (Behs (2011)) which means that the consumer is not only deceived but their right to quality food access is denied.

There is no information of direct damages to consumers' health because the frauds identified in this study, but there are a several reports of substitutions that caused damage to human health. An example published in 2002, where at least 63 people consumed herbal tea inadvertently mixed with neurotoxic Japanese star anise (*I. anisatum*) (Vermaak et al. (2013)). Likewise, conditions like Hypercarotenemia (OMIM # 115300), an autossomal dominant disease, in which the main treatment consists of restrictive diet (Gangakhedkar et al. (2015)) can cause several heath damage to the individual who inadvertently consumes dyes used in feed farmed fish.

One sample was molecularly identified as Coho Salmon (*O. kisuth*), a fish of the Salmonidae family with meat color very similar to *S. salar*, but myoseptum not so well highlighted. This substitution was not considered as a result of intentional fraud but as misidentification.

In 13 sorts of substitutions, the most used species was the Croaker (*M. furnieri*) which was involved in eight types of fraud (62%). Since Croaker is a very common fish in the coastal area of southern Brazil, it participates significantly in the list of traded fishery species. Fillets coloration varies from white to pinkish, with predominantly white muscles, but with red muscles distributed along the horizontal caudal fin and septum. This tone variation from reddish to brown gives Croaker multiple exchange possibilities, as can be seen in Table 1 in which Croaker fillet are identified being sold as Ling, Salmon, Panga Catfish, Conger, Grouper, flounder (3 times), American Harvestfish (twice) and shark. Although it is a very common specie, the high replacement rate raises questions about the exploitation of fish stocks, since Croaker itself has a great demand and even fraudulently abastance demand on other species.

In the opposite direction than expected, we also identified two frauds where Croaker iself was replaced by Weakfish and by Blue Shark, probably as a result of occasional increased fishing of these two species, since there is little difference in the amount price by kilo (around 3 dollars).

The two samples collected as Grouper are from different establishments and both were mislabelled (by Weakfish and Croaker). Known for being a fish much appreciated in cooking, several species of Grouper are classified in the IUCN Red List of Threatened Species varying from NE (Near Threatened) to CE (Critically Endangered) due to destruction of their habitats and overfishing (Rosa and Lima (2008). Due to the low fish stocks and great appreciation, Grouper is replaced by species with greater abundance and similar white meat when filleted.

Santa Catarina is the leader Brazilian state in Flounder (*P. orbignyanus*) capture being one of the main targets of the fleet and has one of the largest fish searches (Sampaio and Bianchini (2002)). In the present study, of the 12 samples sold as Flounder 3 were rigged by Croacker (*M. furnieri*) and two by Weaker (*C. guatupuca*) certainly aiming higher profitability.

The name Shark in Brazil has a stigmatized meaning, directly associated to an animal that eats people and garbage in the sea, with a stinking meat (high concentration of ammonia). Therefore, few people ask to buy Shark meat. Instead, Brazilian people prefer the name "Cação" for the fished and processed sharks. Even though the species are the same, the former does not have the stigmatized name and is more widely accepted as food (Bornatowski et al. (2013)). What is puzzling is that more than half of the population believes they are different animals (Bornatowski et al. (2015)). Moreover, in Brazil there is no surveillance that requires the identification of shark species by commercial establishments and it is usually sold only with the generic term of "Cação" as we shown in the present study. What is even worse in this picture is the sale of endangered species, as seen here by the presence of *S. lewini* and *C. plumbeus* (that are regarded as *critically endangered* species in the Brazilian coast following IUCN criteria – (Brasil (2014))) as simple "Cação". We also verified another fact that commonly happens in mislabeling: the sale of a species for other species for financial gain and with potential to sell species at risk of extinction (Barbuto et al. (2010), Filonzi et al. (2010), Muñoz Colmenero et al. (2016)).

The fact that the fish sellers label the "Cação" sold as sandtiger shark (*C. taurus*) is based on the fact that, historically, this species was often caught by spearfishing in the surrounding area of Santa Catarina State (Souza (1994)) and their meat was sold in the fish market. However, there is already clear evidence that the population of this species is in serious decline in Brazil nowadays, also regarded as "critically endangered" (Brasil (2014)). Another interesting fact in our study is the substitution of bone fish for shark and vice versa which is probably based on on the law of supply and demand and possible financial gain. This was detected in a few cases in the literature, in which protected shark species were also sold as bony fish of high commercial value (Filonzi et al. (2010)).

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It is noteworthy that the fillet solded as Panga Catfish was identified as *M. furnieri* in both databases. To rule out the possibility of sampling error, we provided a new DNA extraction and sequencing the exchange characterizing as a curious example of a species of higher commercial value being sold as a lower commercial value. While the kilo of Corvina costs more than 3 dollars, the kilo of Panga is below one dollar.

In 2013 Neto (2013) reported a similar substitution (Tilapia being sold as Panga Catfish) and suggested that this swap is a marketing strategy to induce product consumption. Panga Catfish is an imported species form Asia and is considered not to taste as good as Tilapia or Croaker, beside being fattier. Eventual substitutions with more palatable fish may mislead the consumer to consider it not so well cooked or seasoned instead of being a fraud.

This type of studies works with a direct application of knowledge, creating benefits for environmental issues by identifying environmental crimes and restraining them, like the restriction of species in illegal times or areas or even species at risk of extinction. Moreover, it allows the society to be safer concerning health issues related to seafood consumption. Projects as "Cat by Hare Project" bring forth frauds and risks that consumers face, allowing the community to become well informed and able to make better choices, besides directly reducing mislabelling levels in a long term.

CONCLUSION

The study of mislabelling incidents in seafood markets brings forth a higher security to consumers and also increases competitivity among fishermen, sellers and restaurants who act within the norms. Comparing to the first step of the project described by Carvalho et al. (2015) where 24% of seafood mislabelling were found, the general fraud level found in this work was only 16%. It is likely that the reduction on the fraud levels in the city of Florianópolis happened because of the inspection efforts and public disclosure of the found results. Therefore, we believe that the implementation of a systematic regulatory program conducted by governmental agencies has a true impact in reducing market substitutions, bringing a direct benefit to society.

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