From fish-markets to restaurants: Substitution prevalence along the flatfish commercialization chain in Brazil

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1. Introduction

Seafood has played a prominent role in human diet, particularly in the last decades, exceeding the consumption of all terrestrial animal foods combined, with world per capita fish consumption estimates rising above 20.5 kg a year in 2018 (FAO, 2020). Seafood products are commercialised in several forms and the complexity of their supply chain makes their trade especially vulnerable to mislabelling (Kroetz et al., 2020). Several factors contribute to the prevalence of mislabelling, including deliberate fraud (Fraser, 2018) and non-intentional substitution, which may result from the mislabelling of morphologically-similar species or the use of ambiguous common names in labelling legislation (Crego-Prieto et al., 2012; Muñoz-Colmenero et al., 2015). Despite advances in regulations fuelled by the application of molecular species identification, seafood mislabelling is widespread (Kroetz et al., 2020).

Groups such as flatfish, which are prized by consumers, are a main target for economic gains through deliberate mislabelling, which reaches 37% in the Americas, 22% in Europe and 15% in Asia (Kappe1 and Schröder, 2016; Naaum and Hanner, 2016; Pardo et al., 2018). Mislabelling is detrimental not only economically (i.e., leads to product devaluation and the decrease in fish stocks), but also to the health of consumers due to the possible presence of allergenic or contaminant agents in substitute species (Kulawik et al., 2016). Additionally, mislabelling can mask the illegal trade of endangered species and hamper stock management of overexploited fish species (Donlan and Luque, 2019; Kroetz et al., 2020).

Official lists correlating common and scientific names of fisheries species and guidelines for the inspection of seafood products are maintained by different regulatory bodies and differ across geopolitical regions. For instance, the European Union Parliament has determined that labels should contain the scientific name of the species, its geographic origin, and production method (Carvalho et al., 2011; Kroetz et al., 2020). In the United States, the Food and Drug Administration (FDA) publishes an online regulatory fish encyclopaedia that lists the
acceptable market names for each seafood species together with photographs of whole fish and their marketed product forms such as fillets (Yancy et al., 2008; FDA, 2018). Similarly, normative instruction number 29 (IN 29) determines which common names can be used for the 454 main commercial fishes sold in Brazil (MAPA, 2015). Under the IN 29, the only species of fish that can be sold as flounder or sole (“linguado” in Portuguese) are those belonging to the genera *Syacium* and *Paralichthys* (family Paralichthyidae, order Pleuronectiformes). However, species of lower commercial value such as the panga (*Pangasianodon hypophthalmus*) have been illegally used as substitutes for flounders in Brazil (Carvalho et al., 2017; Staffen et al., 2017). Contrary to some parts of the world, pleuronectiforms like the arrow-tooth flounder (*Atheresthes stomias*), plaice (*Pleuronectes platessa*), and Pacific halibut (*Hippoglossus stenolepis*) are not legally considered flounders in Brazil (Kappel and Schröder, 2016; Naum and Hanner, 2016; Pardo et al., 2018).

The incidence of mislabelling can vary along the seafood supply chain, making regulation logistically complex and vulnerable to fraud (Crego-Prieto et al., 2012). Studies comparing substitution rates among different sectors are still rare, but most indicate that the mislabelling rate increases along the supply chain (Kappel and Schröder, 2016; Pardo et al., 2018; Shehata et al., 2019). For example, a study in Canada found mislabelling rates of 17.6 %, 27.3 %, and 38.1 % in importers, registered processors, and final retailers, respectively (Shehata et al., 2019). Substitution must be adequately detected and estimated to control seafood mislabelling. Morphological identification is the most traditional and simple method of determining the species of origin in fisheries products, but it is usually unsuitable after products undergo filleting, canning, and other processing procedures (Catanese et al., 2010). Molecular approaches circumvent those limitations, enabling identification even for high levels of processing (Christiansen et al., 2018; De Brito et al., 2015). The most commonly used genetic markers are mitochondrial sequences from the cytochrome b (*Cytb*) and cytochrome oxidase subunit I (*COI*) genes. Their use in fish species identification is widespread and well-established (Delpiani et al., 2020; Pappalardo and Ferrito, 2015; Pardo et al., 2018; Shehata et al., 2019).

Despite the increasing application of molecular techniques in recent years, fisheries mislabelling reports in Brazil have been largely limited to a few groups such as elasmobranchs and sciaenids (Barbosa et al., 2020; Bunholi et al., 2018), while many other important groups are still poorly characterized. Mislabelling in Brazilian flounders has only been investigated in two studies with markedly different estimates (13 % vs. 75 %) owing to limited samples sizes (n = 4 and 23, respectively) (Carvalho et al., 2017; Staffen et al., 2017). We use mitochondrial sequencing data from a larger sample (N = 260) to estimate the prevalence of substitution in seafood labelled as “flounder” at multiple points in the commercialization chain, from street markets to restaurants, in Rio de Janeiro. We also compared the prevalence of mislabelling in samples collected before and after the fish labelling regulations came into force in Brazil. Our findings reveal a high prevalence of substitution and a stark variation in mislabelling rates across the supply chain.

2. Material and methods

2.1. Sample collection and DNA extraction

Fish samples (n = 260) were collected from street markets, fishmongers, supermarkets, and restaurants in Rio de Janeiro, Brazil between 2012 (pre-IN29 regulation, n = 77) and 2020 (post-IN29 regulation, n = 183) (Supplementary Appendix A, Tables A1 and A2). Tissues were kept in 96 % ethanol and stored at −20 °C. DNA was purified from 20 to 40 mg of tissue using a salting-out protocol (Miller et al., 1988) and assessed using 1% agarose gel electrophoresis in 0.089 M Tris, 0.089 M Borate, 0.002 M EDTA buffer, pH 8.0.

2.2. Polymerase chain reaction (PCR) and sequencing

Each PCR reaction consisted of 5–10 ng of sample DNA, 2 μl of 5 X FIREPol® Master mix containing 12.5 mM MgCl₂ (final concentration of 2.5 mM) (Solis BioDyne, Tartu, Estonia), 0.25 μM of each primer, 200 μM dNTPs, 0.5 mg BSA, and nuclease-free water for a final volume of 10 μl. The primer pair L14735 (F) and H15149AD (R) (Sotelo et al., 2001) was used for *Cytb* amplifications, whereas primers FishF2-tf-F forward (F) and FishR2-tf reverse (R) (Ivanova et al., 2007) were used for amplifying *COI*. The few samples that were ambiguously identified by COI and *Cytb* were additionally analysed using the ribosomal RNA 16S locus (16S rRNA) using the 16Sall_F (3′ ACCAAACAYCGGC 5′) and 16Sall_R (3′ TGTCTTGATCCAAATCG 5′) primers. PCR was performed with an initial denaturing step of 4 min at 94 °C, followed by 35 cycles at 93 °C for 30 s, 50 °C for 40 s, and 72 °C for 1 min and a final extension step of 7 min at 72 °C. PCR products were inspected using 2% agarose gel electrophoresis and purified enzymatically by incubating 5 μl of PCR product for 15 min at 37 °C in 6 μl of an aqueous solution containing 0.08 units of alkaline phosphatase (FastAP, Fermentas, Waltham, MA, USA) and 0.83 units of exonuclease 1 (Fermentas). Sequencing was performed using BigDye Terminator v3.1 Ready Mix kit (Applied Biosystems, Foster City, CA, USA) on a 3500 Genetic Analyzer (Applied Biosystems).

2.3. Sequence analysis and molecular identification

Forward and reverse DNA sequences were assembled using Geneious Prime software version 2019.1.2 (Biomatters Ltd., Auckland, New Zealand). The sequences were used for species identification using two approaches: similarity searches and phylogenetic analyses. In the similarity-based approach, a threshold of 98 % similarity was used for species identification. Custom local BLASTn searches were conducted against a database containing all fish *COI* and *Cytb* sequences available at NCBI supplemented by an in-house sequence database. Phylogenetic identification was performed using an alignment approach including the sample sequences, representatives of possible species according to BLAST search, and the outgroup *Scleropages formosus*. Sequences were aligned using Clustal Omega (Sievers and Higgins, 2018) and manually curated using the Geneious prime software. Phylogenetic reconstruction was performed using maximum likelihood as implemented in the IQ-Tree package (Minh et al., 2020) with the Kimura two-parameter (K2P) substitution model (Kimura, 1980) defined *a priori* as the most used and well-established model for forensic analysis of seafood products (Delpiani et al., 2020; Christiansen et al., 2018).

2.4. Mislabelling statistics

To test and quantify possible financial implications of mislabelling cases, the normalized Δmislabel measure (Nₐmislabel) was estimated for fish samples where individual price was recorded (n = 97). The Nₐmislabel was calculated by $\frac{P_{label} - P_{substitute}}{P_{label}}$ (Donlan and Luque, 2019), where $P_{label}$ is the price of the putative fish and $P_{substitute}$ is the average price of the actual fish species identified genetically. Because the distributions of $P_{label}$ and $P_{substitute}$ were not homoscedastic, their means were compared using an unequal variance paired Student’s t test. The differences in mislabelling rates before and after implementation of labelling regulation IN29 and between supply chain stages were tested using the chi-square ($X^2$) test.

3. Results and discussion

3.1. Species identification

In total, 260 samples were analysed, all of which were successfully
sequenced for at least one gene while 166 samples were sequenced for both genes, resulting in 189 COI and 237 Cytb sequences. Aligned sequences were 553 bp- and 335 bp-long for COI and Cytb, respectively (Fig. 1). All samples were at least 98% similar to reference sequences in the database comprising NCBI, Fish-BOL and in-house sequences, and were assigned to species using a 2% cut-off. Species assignments were further corroborated by phylogenetic analysis (Fig. 1, Supplementary Tables A1 and A2). A third gene (16S) was included to unambiguously assign samples to species when Cytb and COI sequences gave conflicting results (n = 14, Supplementary Appendix A, Table A2). In total, 15 species were found being sold as flounder: nine pleuronectiform and six non-pleuronectiform species (Fig. 2 and Supplementary Tables A1 and A2).

3.2. Incidence of mislabelling

Roughly half of all flounder samples (130 of 260, 50%) were mislabelled, a substantially higher prevalence of mislabelling than the global rate of 27% for seafood in general, especially so when compared to the global mislabelling rate of 18% reported for flounders (Supplementary Table C1). Previous international seafood mislabelling studies reported similar (Willette et al., 2017) and contrasting (ChristianSEN et al., 2018; Delpiani et al., 2020) mislabelling rates depending on country and species analysed. Mislabelling is less common in Europe (22%), some regions of Oceania (15%), and South Africa (20%). In contrast, North and South America exhibit high mislabelling incidence, especially Brazil, where substitution rates can be as high as 46% for finfish products such as cod, croaker, tuna, and salmon (Supplementary Table C3). Similarly, high rates for flounders were also found in Spain and Malaysia, but the small sample sizes of those studies make them difficult to compare with ours (Supplementary Table C1).

The striped catfish (Pangasianodon hypophthalmus), also known as panga or Asian catfish, accounted for over 75% of mislabelling cases in our data. The comparison of our estimates to those from the literature indicates that despite the variety of local substitutes, panga is the main substitute species also globally (Fig. 3). The striped catfish is, indeed, one of the most common substitutes for more desirable, higher-priced seafood products worldwide (Calosso et al., 2020; Luque and Donlan, 2019; Minoudi et al., 2020), probably because of its low price and widespread availability from freshwater aquaculture (Calegari et al., 2019). Substitution of panga for valuable flatfish species causes not only economic losses but poses health concerns because of contamination by heavy metals and antibiotics (Luque and Donlan, 2019; Kulawik et al., 2016).

The flatfish Xysteurus rasile (South American flounder) was the second most common fish used as a flounder substitute (17%). This is likely to represent a case of inadvertent mislabelling. There is no report in the literature of Xysteurus as a substitute, because this genus is legally labelled as “fantail flounder” in all countries where reports are available (Astarloa, 2002; Díaz de Astarloa and Fabré, 2001). X. rasile is a local unhithen stuted puneonectiform whose ommision form the Brazilian labelling regulation is unjustified.

This is the first report of blackfin goosefish (Lophius gastrophus), the unicorn leatherjacket filefish (Aluterus monocercus), and the Nile tilapia (Oreochromis niloticus) being sold as flounder substitutes, albeit at low frequencies. The use of blackfin goosefish as a substitute for flounder in Brazil may result from the recent export ban of this species to the European Union (EU), as reported in local news media.1 Other three substitutes recorded at low frequencies were the Argentine hake (Merluccius hubbsi), the Acoupa weakfish (Gymnocton acoupa), and the arrow-tooth flounder (Atheresthes stomias), all of which have been previously reported in flounder mislabelling cases in other countries (Pardo et al., 2018; Pardo and Jimenez, 2020; Staffen et al., 2017). The arrow-tooth flounder was widely traded in Brazil, where it was largely commercialised as flounder until 2015, but its use as a substitute has been drastically reduced since the introduction of the fish labelling legislation in late 2015 (Barbosa, 2016; Carvalho et al., 2015).

3.3. Mislabelling along the commercialisation chain

Mislabelling varied significantly along the supply chain and rates of mislabelling were higher in street markets and restaurants (Fig. 2). Here, the term “commercialisation chain” is used for increasing levels of processing, as commonly done in the literature (e.g., Deconick et al., 2020; Helgoe et al., 2020; Shehata et al., 2019) and does not imply a strict sequence between sectors. The complexity of seafood supply chains is often regarded as the main contributing factor to mislabelling, which appears to be cumulative throughout the supply chain (Grego-Prieto et al., 2012; Muñoz-Colmenero et al., 2017; Shehata et al., 2019).

High mislabelling rates are often reported from restaurants, retailers, canteens, and sushi bars, which are the points where seafood products are more heavily processed (ChristianSEN et al., 2018; Helgoe et al., 2020; Kappel and Schroder, 2016; Shehata et al., 2019). The high substitution rate found in street markets in the current study would at first glance seem to be at odds with the assumed relationship between mislabelling and seafood processing level, since fish are usually sold in street markets at the lowest level of processing. However, unlike in European countries, for example, flounder is sold already filleted in the fish stalls of street markets in Brazil. That, combined with insufficient on-site labelling inspection, explains the high mislabelling rates observed in street markets. A similar pattern was observed in Spain for the trade of tuna, megrim, and hake, which are sold in a more processed form (Cawthorn et al., 2015).

Flounder substitutions varied across the supply chain not only in quantity but also in taxonomic diversity (Fig. 2). The highest diversity of substitute species was observed in restaurants, where six of the seven substitute species were detected (Fig. 2), likely reflecting fish species availability in the restaurant’s kitchen at the time of sale. The low prevalence of substitute species in supermarkets and fishmongers is likely the result of more stringent federal inspections in these sectors, as recently shown for other fish groups (Alvarenga et al., 2021).

3.4. Effects of changes in labelling regulation

The mislabelling rate was not reduced by the introduction of labelling regulation IN29 in late 2015 (Fig. 4) and might have even increased in some sectors due to the omission of several pleuronectiforms. A suggestive decrease of 10% in mislabelling involving panga was observed in supermarkets, which might indicate progress in the enforcement in this sector. In Spain and Greece, it took approximately the same time for labelling regulation to produce a 10% decrease in substitution cases (Minoudi et al., 2020; Muñoz-Colmenero et al., 2015), in line with the overall seafood mislabelling rates in the EU, which declined from 23% to 7% between 2011 and 2015 (Warner et al., 2016). In fact, only samples identified as non-pleuronectiforms (e.g., panga or filefish) would have been considered mislabelled before the introduction of regulation IN29 in 2015. A large proportion of samples (40%) collected before 2015 consisted of flatfish species (X. rasile, A. stomias, Limanda aspera, Lepidopsetta polyxystra, and Pleuronectes sp.) that would have been considered mislabelled after the revisions introduced in 2015 (Fig. 4). The omission of Pleuronectiform species, notably X. rasile, should be reviewed since it leads to confusion and to underestimates of mislabelling. Other Pleuronectiform species (A. stomias, L. aspera, L. polyxystra, and Pleuronectes sp.) are also omitted from current legislation, but these are imported into Brazil and are found at a low frequency.
We observed a sharp (50%) decline in the numbers of *Paralichthys patagonicus* (Patagonian flounder) sold in Brazil (Fig. 4B), likely resulting from overfishing and depletion of natural stocks, which has gone undetected because all *Paralichthys* species landed are treated as "lin- guado" (i.e., flounder), a general name that includes all species of the genus, for fishery statistics purposes. Moreover, the high prevalence of substitutes featuring on the market likely contributes to the overestimation of current stocks (Mariani et al., 2017), which makes it harder to identify overfishing of one or other species, directly affecting fisheries management as observed in the past for species like the red snapper (*Lutjanus campechanus*) and hake (*Merluccius* spp.) and leading to catastrophic consequences over time (Cawthorn et al., 2018; Garcia-Vazquez et al., 2012; Marko et al., 2004).

*P. patagonicus* is ranked as vulnerable by the IUCN and as threatened by the Brazilian environmental agency. Fisheries overexploitation in Brazil, whose coastal waters host half of the global population of this species has been pointed out. The identity of samples in each group is listed in Appendix B, Tables B1 and B2 in the Supplementary material. Bootstrap support values retrieved from 1000 replicates and values < 98% are indicated at the nodes. The outgroup was *Scleropages formosus* (EU594454 for Cyb and JF946625 for COI).

**Fig. 1.** Maximum-likelihood trees for market samples of flounder collected in this study and reference sequences from GenBank and in-house sequence database based on sequence alignments of COI and Cytb genes. (A) Maximum-likelihood tree based on a 335-bp alignment of the Cytb gene with 237 Cytb sequences from market samples of flounder and 14 sequences from reference samples. (B) Maximum-likelihood tree based on a 553-bp alignment of the COI gene with 189 COI sequences from market samples of flounder and 22 reference sequences. Samples were collapsed to improve visualization, the number of samples in each tip is shown in brackets. The identity of samples in each group is listed in Appendix B, Tables B1 and B2 in the Supplementary material. Bootstrap support values retrieved from 1000 replicates and values < 98% are indicated at the nodes. The outgroup was *Scleropages formosus* (EU594454 for Cyb and JF946625 for COI).
the likely cause (Riestra et al., 2020).

3.5. Price and flounder mislabelling

Prices of substitute species were significantly lower than those of the species they were substituted for (the expected product, i.e., the species that appeared on the label) (N = 97; Student’s t = 10.79; p < 0.001). This result suggests that flounder substitution was intentional and motivated by economic gain and, therefore, can be classified as seafood fraud. Normalized Δmislabel ratios indicate that consumer loss increases proportionally to processing level across the supply chain (street markets < fishmongers < restaurants, Supplementary Table D1). In the very few mislabelling cases recorded in supermarkets, however, no significant difference was found between the average price of the species on the label (the expected product) and the actual species in the package, suggesting substitution was unintentional. The greatest economic losses (up to 80%) occurred when the lesser valued species P. hypophthalmus and C. acoupa were used as flounder substitutes (Supplementary Table D2). Sample prices are seldom reported in seafood substitution studies, hampering comparisons of mislabelling for profit (Luque and Donlan, 2019). For flatfishes, studies have mostly addressed the later stages of the supply chains. In European restaurants, for example, the mislabelled species sold as flounder fillets cost between 50% and 70% less than the prices charged on the menu (Deconinck et al., 2020; Kappel and Schröder, 2016).

Fig. 2. Distribution of mislabelled flounder samples by pleuronectiform and non-pleuronectiform substitute species. Mislabelling prevalence and species proportions for (A) the entire supply chain and (B) each stage in the supply chain. Total mislabel ratios for both the entire supply chain and for each supply chain stage are shown alongside their respective labels (Total, Restaurant, Fishmonger, Street-market and Supermarket).
4. Conclusions

Roughly half of all samples analysed were mislabelled, with over 75% of substitutions involving *Pangasianodon hypophthalmus*. Mislabelling was correlated directly with the level of processing and varied along the commercialization chain, reaching 82% in restaurants. The relatively low rates observed in supermarkets are likely the result of federal inspections, which are more frequent and stringent than local inspections and have significantly improved compliance by large fish processing companies. The 2015 revisions were ineffective in controlling mislabelling and led to an increase of non-conformity to legislation by inexplicably omitting local non-threatened pleuronectiforms (e.g., *X. rasile*). This oversight should be solved in updated versions of the list. Our results indicate that financial gain is the key driver and that consumer loss is greater in more processed food items. The causes of mislabelling in the Brazilian flounder trade are complex and multifactorial but there is clearly the need for updating labelling regulations and improving inspections, particularly in street markets and restaurants.
CRediT authorship contribution statement

Daniela Santos Souza: experimental design, data collection and analysis, original draft preparation; Weidy Rozendo Clemente: experimental design and data analysis; Frederico Henning: data analysis, manuscript editing and review; Antonio Mateo Sol-Cava: experimental design, data analysis, manuscript editing and review.

Funding

This work was supported by a National Council for Scientific and Technological Development (CNPq) productivity grant to AMSC and by Carlos Chagas Filho Foundation (FAPERJ) grants to AMSC (CNE and GARPA–RIO Programs) and to FH (202.459/2017). DSS was funded by fellowship from the Coordination for the Improvement of Higher Education Personnel (CAPES) through the Post-Graduate Program in Biodiversity and Evolutionary Biology (PPGBBE) of the Federal University of Rio de Janeiro (UFRJ).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Rafaela dos Santos Soares, Jessica Faria, Marcela Alvarenga Simões, Carine Belau, Gabriela Dias, Paulo Vianna and Isabel Willmer helped during sampling and provided comments that significantly improved the manuscript. This work benefited from invaluable discussions during a REGeneC (Latin American network for conservation genetics) workshop.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.fishres.2021.106095.
