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Standardization of Polymerase Chain Reaction Assay for the Authentication of *Arapaima gigas fish*

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Abstract

Pirarucu is a freshwater fish that presents a great commercial value for being well accepted by the consumers and for showing excellent meat quality. The zoological identification of fisheries during industrial processing is harmed by the removal of external morphologic characteristics, facilitating fraudulent practices in commercialization. In this context, the identification at the molecular level is an important tool in the inspection and commercialization of the fishery. The DNA resists to the processing methods, like the salting, the most common way of commercializing the pirarucu. The Polymerase chain reaction, PCR assay, were applied in samples that suffered degradation or have gone under industrialization methods. This work aimed use the PCR technique as a tool to authenticate the *Arapaima gigas* species and to avoid possible frauds in commercially available products. The obtained data showed the efficiencies of the DNA extraction, the amplification of the target sequence, and identification of the genetic material through PCR. It is possible to conclude that the PCR technique that was standardized in the present study showed high sensibility, precision, and specificity for the detection of the genetic material of *Arapaima gigas*, constituting a useful tool for the monitoring and inspection during its commercialization.

Keywords: fish, fraud, pirarucu, PCR

1. Introduction

Arapaima gigas (Schinz, 1822) is a member of the Order Osteoglossiform and family Osteoglossidae and it is popularly known in Brazil as pirarucu. In other regions, this fish is called by different names, like bodecos (high Amazonas) and paiche (Peru and Ecuador). Its distribution is referenced in South America (Amazon Basin and Guyana rivers), and it can be found in Brazil, Colombia, Guyana, Peru and Ecuador, where native species occur (Rosa *et al.*, 2020).

Pirarucu is considered the largest freshwater fish with scales in the world and it can grow up to four meters of length and 200 kg of body mass (Chu-Koo *et al.*, 2008). This fish inhabits mainly lowland lakes and flooded forests (Castello, 2008). In Amazon, its consumption is a traditional habit that has expanded to other regions and countries (Santos-Cipriano, 2015). Its collection in the Amazonian region has relevant ecological and socio-cultural functions,

representing economic and nourishing importance since the colonial times, especially due to the meat flavor, the nutritional value and the commercial value.

These characteristics increase its vulnerability in terms of conservation, leading this species to show signs of overfishing (Campos-Silva and Peres, 2016; Begossi *et al.*, 2018). This fish also presents great potential to aquiculture due to its characteristics that are favorable to the creation, like rapid growth, the capacity of aerial respiration, easy adaptation to feed, the meat of high quality, and great income (Paiva, 2015; Santos-Cipriano, 2015). The potential aquiculture can make the species get out of the overfishing situation, increasing its production and commercialization such in the internal market as in the external market.

The *Arapaima gigas* species is also known as "cod of the Amazon" and it is commercialized mainly in in salting and dry form. However, this processing of this fish is still rudimentary (Nunes *et al.*, 2012). In salting processing, there is the removal of morphological characteristics (skin and head), because it makes the species hard to be identified and increases the chances of adulteration and mistakes in the labeling (Brito *et al.*, 2015).

The frauds in the food can be in many ways: alterations, adulterations, and falsification, which are done with the goal of obtaining higher profits. This practice is generally adopted when there are accentuated differences in the price or in the availability of products (Fox *et al.*, 2018). This situation may be occurring with the pirarucu (*Arapaima gigas*) commercialization in the north region of Brazil, once its predatory fishing has decreased the natural stock.

Considering the losses that the frauds can cause, the existence of tools to detect and identify the different species of commercially available fish is necessary. Currently, the existing methods of identification are be based on the morphologic and anatomic analysis of the muscles, on the protein analysis with electrophoresis and on the analysis of the lipids. However, although these methods are cheap and fast, they use proteins and lipids for their development and these compounds can be denatured or degraded during the food processing (heating, conserving or drying), producing alterations in the obtained results (Hsieh *et al.*, 2010; Dalama *et al.*, 2015).

In this context, an alternative for food authentication that was studied by many authors is the Polymerase Chain Reaction (PCR). The PCR is a technique that amplifies a *DNA* template to produce *specific DNA fragments in vitro*, allowing the detection of genetic material, even in low quantities. This method presents advantages like speed, high sensitivity, and specificity, besides being able to detect DNA regardless of its fount, showing more efficiency than techniques like chromatography and electrophoresis (Mayer, 2005; Dalmasso *et al.*, 2011). The PCR is a useful tool for the monitoring and inspection of food destined for human consumption.

Several studies used the PCR for detection of frauds in fish and other products of animal origin (Aguilar *et al.*, 2012; Khallaf *et al.*, 2014; Ali *et al.*, 2015; Brito *et al.*, 2015; Mousavi *et al.*, 2015; Mueller *et al.*, 2015; Safdar and Junejo, 2015; Sumathi *et al.*, 2015; Tisza, *et al.*, 2016). Although this technique was applied on the identification of several species, there is

still few information on the applicability of this method in the detection of fraudulent changes in fish and fish-derived products (Mousavi *et al*., 2015).

Considering all this information, the present study aimed to standardize a PCR for the authentication of pirarucu (*Arapaima gigas*) that can be used as a tool to prevent possible frauds in commercially available products.

2. Material and Methods

2.1 Raw Material

Samples of pirarucu (*Arapaima gigas*) were acquired in Tefé, Amazonas – Brazil. Samples of black caiman (*Melanosuchus niger*), pantanal alligator (*Caiman crocodilus),* gillbacker sea catfish (*Hexanematichthys parkeri*), acoupa weakfish (*Cynoscion acoupa*), school shark (*Galeorhinus galeus*), meagre (*Argyrosomus regius*), kumakuma (*Brachyplathystoma filamentosum*), dourada (*B. rousseauxii*), *laulao catfish* (*B. vaillant*), crevalle jack (*Caranx hippos*), smooth weakfish (*Cynoscion leiarchus*) and *common stingray (Dasyatis guttata*) were collected in municipality of Castanhal (1°17'27.8"S 47°55'23.0"W), state of Pará, Brazil. The collected material was forwarded to the Laboratory (1°18'42.6"S 47°56'49.9"W), and it was stored under freezing at -16°C until the execution of the proposed methodology.

2.2 DNA Extraction

The DNA samples were extracted, following the protocol validated by Darwish *et al.* (2009), with modifications suggested by Oliveira *et al.* (2015). The changes were carried out in the initial step of the protocol, where fractions of 0.3 g of the sample was macerated and added with lysis buffer STES (0.2 M of Tris base, 0.5 M of sodium chloride, 0.1 % (w/v) of Sodium Dodecyl Sulfate and 0.01 M of Ethylenediaminetetraacetic Acid). The material was homogenized in an orbital shaker, and it was incubated in a stove at 56°C overnight with 10µL of proteinase K (20 mg mL^{-1}). The other parts of the sample processing were performed according to the original protocol (Darwish *et al.*, 2009); the DNA was extracted eluted in 25 µL of TRIS-EDTA (TE) buffer, pH 8.0.

After all the related procedures, the obtained DNA was quantified in spectrophotometer *BioTek® Gen5™* and absorbance measurements were performed at 280 nm and at 230 nm in order to verifying the purity of the samples. The 280 nm length corresponded to the absorption peak of ultraviolet (UVs) rays and the 230 nm length corresponded to the absorption peak of UVs from organic contaminants, according to Lambert-Beer law (Lambert, 1760; Beer, 1852).

Specific initiators for the *Arapaima gigas* (*Primer forward 5'* TCACACGATGGGGCAATCAA3' and *Primer reverse* 5' TGTCGTAAGCAGATCGAGCC 3') were used. The primers were designed with the PRIMER-BLAST tool, provided by the NCBI (*National Center for Biotechnology Information*) and they amplified sequences of 439 base pairs (bp). The design was made from the sequence obtained through the gene bank, which refers to the complete mitochondrial genome from the studied fish. The oligonucleotides were prepared following the instruction of the manufacture (Ludwing

Biotec®) and eluted in buffer TE pH 8.0 until the concentration of 100 pmolμL^{-1}.

Tests to establish the ideal temperature of annealing were carried out in order to to determine the efficiency of the produced primers. For that, five temperatures (52°C, 53°C, 54°C, 55°C e 56°C) were performed and the selection was carried out through visual evaluation of the band patterns regarding the intensity.

The utilized PCR solution was calculated for a final volume of 25 µL for each reaction. For that, there were utilized 50 nM of KCl and 10 mM Tris-HCl (buffer 1X), 10 nM dNTP mix, approximately 1.6 ng of DNA mold, 1U Taq DNA Polymerase, 10 ρmol of each initiator and the volume was completed with MiliQ water. The Thermocycler was programmed to 30 cycles and the temperatures and times utilized to denaturation, annealing and extension were 93°C30s^{-1}, from 52 to 56°C 30s^{-1} and 72°C30s^{-1}, respectively, increased by initial denaturation at a temperature of 93°C 3min^{-1} and a final extension at 72°C10min^{-1}.

The visualization of the amplified material was performed through electrophoresis in agarose gel at 1.5 %, utilizing 1 µL of Safer colorant (6X) for each 5µL of the sample. The electrophoresis was conducted in a horizontal tank containing TBE 0.5X (89 nM Tris-HCl, 89 nM of boric acid and 20 nM of EDTA), with amperage of 90 A. The analysis of the electrophoresis results was performed with the help of an equipment of photo documentation under ultraviolet light (*Gel Documentation System, Gel Doc™, Bio-Rad®*).

The evaluation of the PCR analytic sensitivity was performed from the serial dilution of DNA previously extracted and quantified. For that, ten microtubes were prepared, in triplicate, for serial dilution and each one contained 90 µL of sterilized Milli Q water. This way, dilutions from 10^{-1} to 10^{-10} were obtained. Besides these ones, the dilutions 1:1 and 1:4 were also tested. The DNA solutions, as well as their respective dilutions, were submitted to the proposed PCR. The target-DNA threshold detection was based on the last dilution that presented band visualization (PCR product) in electrophoresis. The technique of sensitivity threshold was calculated through the ratio between the concentration of the minor amplified dilution and the concentration of the utilized DNA; this value was expressed in percentage, according to what was previously suggested by Brodmann and Moor (2003).

The specificity of the initiators was tested concerning to the presence of genomic material from other species of fish commercialized in the Amazon region. For that, the DNAs from 12 species were utilized: *Melanosuchus niger*, *Caiman crocodilus***,** *Hexanematichthys parkeri*, *Cynoscion acoupa*, *Galeorhinus galeus*, *Argyrosomus regius*, *Brachyplathystoma filamentosum*, *B. rousseauxii*, *Caranx hippos*, *B. vaillant*, *Cynoscion leiarchus* and*Dasyatis guttata*. Five repetitions of the DNA extraction of each species and five repetitions of the amplification reactions were performed, all under the same conditions.

3. Results and Discussion

The observed results showed that the DNA was obtained with enough quality and concentrations for the PCR execution with the proposed methodology. The purity and the yield of the total extracted DNA were verified through measurement of absorbance at 230 nm, 260 nm and 280 nm, which indicated values with purity parameters and medium DNA

concentration in the samples of 1.6 ngμL^{-1}.

The protocol that was used in the present study, adapted from Darwish *et al.* (2009) proved to be effective in all the tested dilutions. This result can be related to the use of Proteinase K, which has been utilized in several extraction protocols due to its protein degradation action in different samples, even in the presence of detergents. Although the technique used in the present study has been very efficient in the DNA extraction, many authors refer to the DNA extraction as a critical step during the food analysis, mainly in the most processed products. Some factors, such as DNA degradation, matrix effect, and presence of PCR inhibitors, imply a low limit of detection or quantification.

The food analysis presents some difficulties because it can contain interferers in its composition, such as proteins, polysaccharides, fat, salt, colorants, and others. Besides that, the use of physical and chemical treatments may predispose the DNA fragmentation, which blocks its utilization in the PCR (Marcelino *et al.*, 2008; Cardoso *et al.*, 2019). The extraction methodology proposed has been utilized by several researchers for the obtainment of DNA from milk, cheese and meat samples (Colgan *et al.*, 2001; Brodmann, and Moor 2003; Kesmen *et al.*, 2007; Kesmen *et al.*, 2009; Amaral *et al.*, 2014; Karabasanavar *et al.*, 2014; Dantas *et al.*, 2019). However, until the moment of this study, this methodology were not utilized for a fishery. In this way, our results point out a viable alternative to the DNA extraction of fish, which can be tested in studies that utilize another species besides *Arapaima gigas.*

The test that evaluated the ideal annealing temperature of the initiators showed that the designed *primer* was capable of annealing the target-DNA at the five analyzed temperatures. Based on this result, the temperature of 55°C was elected the more suitable one, since it presented a clearer band with 439 base pairs in agarose gel at 1.5%, as it is seen in Figure 1B.

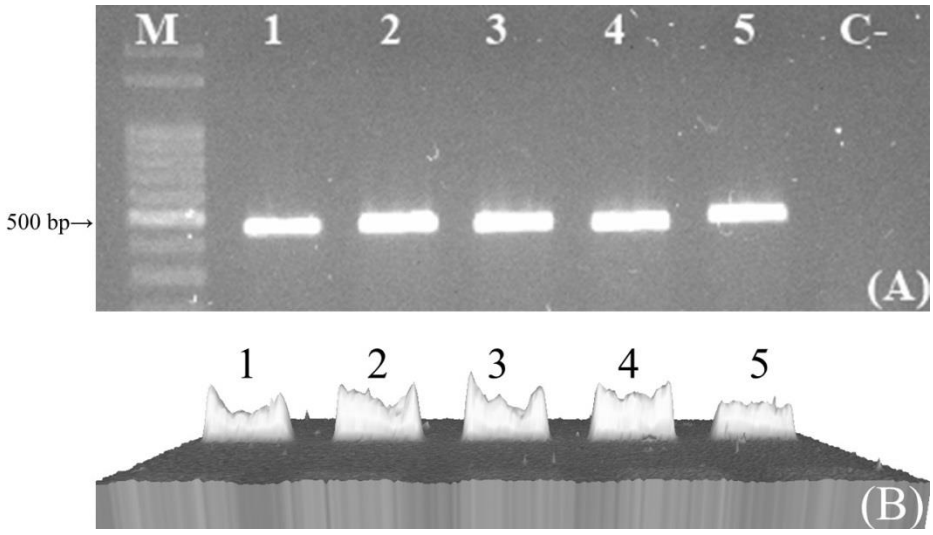

Figure 1. (A) Agarose gel 1.5%, colored with Safer, showing the presence of bands (439bp) specific to the *Arapaima gigas* species, obtained through PCRs with annealing temperatures varying from 52°C to 55°C

M: molecular marker 1kb; 1 to 5: obtained bands through PCR with annealing temperatures of 52°C, 53°C**,** 54°C, 55°C and56°C and negative C-Control. (B) 3D Demonstration of *Arapaima gigas'* DNA fragments amplification (439bp), obtained through PCRs with different annealing temperatures, generated by *Gel Doc XR+* from *Bio rad* with software *Image Lab Version 5.2.1.*; 1 to 5: bands obtained through PCR with annealing temperatures of 53°C**,** 54°C, 55°C and 56°C

Until this moment, literature does not present primers for the *Arapaima gigas* species. The primers proposed in this study are efficient and capable of annealing at a wide range of temperatures, which proves the viability of their utilization in the detection of frauds and in *multiplex* reactions in future studies. This technique increases the information content by ge1, decreases the time spent, which reduces the costs for the PCR around 50%, and the costs for the agarose gel around 85% when compared to the conventional procedure (Masi *et al.*, 2003).

The analytical sensitivity test for PCR showed that the technique used in this study was capable of amplifying DNA fragments of *Arapaima gigas* until the dilution 10^{-2} (0.016 ng/μL^{-1}), as it is shown in Figure 2. The technique presented sensitivity threshold of 1%, justifying its utilization as a method of authentication for the species. Therefore, the dilutions 1:1, 1:4, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} corresponded to the concentrations of 0.8 ngμL^{-1}, 0.4 ngμL^{-1}, 0.16 ngμL^{-1}, 0.016 ngμL^{-1}, 0.0016 ngμL^{-1}, 0.00016 ngμL^{-1}, 0.016 ρgμL^{-1}, 0.0016 ρgμL^{-1}, 0.00016 ρgμL^{-1}, 0.016 fgμL^{-1}, 0.0016 fgμL^{-1}, 0.00016 fgμL^{-1}, respectively.

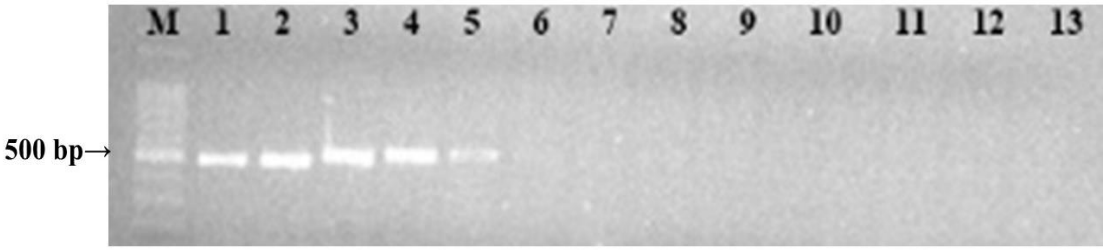

Figure 2. Agarose gel 1.5% colored with Safer demonstrated presence of DNA fragments of *Arapaima gigas* (439bp), obtained through PCR of different DNA dilutions

M: Molecular marker 1kb, 1: DNA at 1.6ngμL^{-1}, 2: Dilution 1:1**1 (0.**8ngμL^{-1}), 3: Dilution 1:4 **(**0.4ngμL^{-1}), 4: Dilution 10^{-1}, 5: Dilution 10^{-2}, 6: Dilution 10^{-3}, 7: Dilution 10^{-4}, 8: Dilution 10^{-5}, 9: Dilution 10^{-6}, 10: Dilution 10^{-7}, 11: Dilution 10^{-8}, 12: Dilution 10^{-9} and 13: Dilution 10^{-10}.

Researches that also performed authentication for other food of animal origin obtained results similar to the present study. Sultana *et al.* (2018), while evaluating the sensitivity of specific DNA initiators from bovines, porcine, and fishes, obtained a detection limit of 0.1% to 0.001% for the specific initiators.

Researches like the one of Karabasanavar *et al.* (2014), who performed a PCR assay to detect adulteration in pork, showed detection levels of 0.01% when evaluating the sensitivity of the

technique from series dilutions. Amaral (2014) performed a study to authenticate the sausage and evaluated the sensitivity of the technique. The detection levels obtained were 0.01% for the DNAs of rabbit, cow and hare and 0.1% for the DNAS of dear, pork, and red meat. Hossain (2019) researched the presence of cow, buffalo, chicken, cat, dog, swine, and fish in raw and processed foods, obtained a sensitivity threshold of 0.001% for raw food and 0.5% for Heat-Treated Food Products.

In the analytical specificity test of PCR (Figure 3), it was verified that there were no cross-reactions in the DNA amplification when comparing the 12 non-target species that were analyzed, proving the specificity of this technique for the detection of genetic material from *Arapaima gigas.* The precision of the PCR was also evaluated and after the performance of five repetitions from the referred assay utilizing DNA from five different extractions of the twelve evaluated species, it was verified that the results were identical, proving the precision of the method.

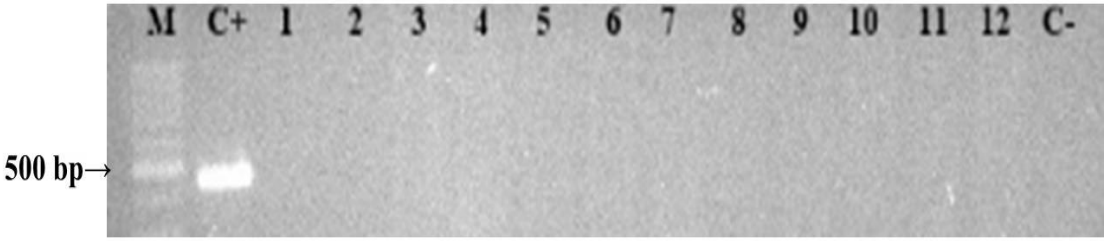

Figure 3. Agarose gel 1.5%, colored with Safer, showing one of the repetitions from the specificity test for the *primers* that weredesigned for the authentication of *Arapaima gigas*

M: molecular marker 1 kb, C+: Band of 439 bp, correspondent to *Aparaima gigas* (positive control); 1: PCR of *Melanosuchus niger* DNA; 2: PCR of *Caiman crocodilus* DNA; 3: PCR of *Hexanematichthys parkeri* DNA; 4: PCR of *Cynoscion acoupa* DNA; 5: PCR of *Galeorhinus galeus* DNA; 6: PCR of *Argyrosomus regius* DNA; 7: PCR of *Brachyplathystoma filamentosum* DNA; 8: PCR of *B. rousseauxii* DNA; 9: PCR of *Caranx hippos* DNA; 10: PCR of *B. vaillant* DNA; 11: PCR of *Cynoscion leiarchus* DNA; 12: PCR of *Dasyatis guttata* DNA and C: negative control.

The specificity obtained by PCR in this study was high and was compared to the values obtained in other studies, as the one performed by Karabasanavar *et al.* (2014), who carried out a PCR assay to detect adulterations in pork and tested their initiators in other 24 species of animals. These authors verified that the animals presented high level of specificity for the initiator with the target sequence. Colgan *et al.* (2001), besides the pork, also developed a PCR to identify beef, ovine meat and poultry meat and verified, after specificity test, that the utilized initiators were specific for each target species.

In same way, Kesmen *et al.* (2007) and Amaral *et al.* (2014) performed authentication assays in sausages. Kesmen et al. (2007) tested the specificity of each one of the species-specific initiators, confirming amplification in bovine, ovine, equine, asinine, and swine. Amaral *et al.* (2014) evaluated the specificity of their initiators testing the PCR amplification for other animal species ordinarily utilized as food, like boar, duck, pheasant, poultry, ostrich, caprine,

ovine, and confirmed the specificity of the initiators designed for the *Bostaurus* with 100% of identity.

In the same context, Colgan *et al.* (2001), while developing a PCR for the identification of bovine, ovine, swine and poultry meat, verified, after specificity test, that the utilized initiators were specific for each target species. Still, Kesmen *et al.* (2009), who performed an experiment for the identification of equine, asinine and pork meat, tested their initiators for 7 types of meat (bovine, swine, equine, donkey, ovine, chicken and turkey), and also concluded that the specific initiators for the species of donkey and swine did not show any cross-reaction, proving its specificity.

Since there are no designed *primers* commercially available for the species *Arapaima gigas,* the present study designed and tested for the first time this technique. The quality of the initiators was fundamental for the PCR success, for these were capable of identifying the target species with exactitude and presented specific bands for the analyzed species without showing cross-reactions or dimer formations, which validates its precision. The technique also proved to be highly sensitive and this result is compatible with the ones reported by several authors like Colgan *et al.* (2001), Brodmann and Moor (2003), Kesmen *et al.* (2007), Kesmen *et al.* (2009), Amaral *et al.* (2014), Karabasanavar *et al.* (2014). In this way, the results obtained in the present study prove that the proposed technique may be an important tool in the monitoring and inspection of the species *Arapaima gigas.*

Although the presented data are extremely relevant, more studies aiming the authentication of samples obtained in the commerce must be performed, in order to take the proposed methodology to a quotidian use in different conditions than the ones proposed in this experiment. The use of PCR is already widespread in studies of several food-products of animal origin. However, there are few studies regarding the authentication of fish and fish-derived products. These studies are extremely important, once the processing of the products eliminates morphological features, facilitating fraudulent practices in processed fishery, which is the case of the species *Arapaima gigas.*

Considering the wide variety of fish species and derived products, the flaws in the inspection of these products' commercialization that facilitates their fraudulence and the small number of published researches regarding the fishery authentication, this study demonstrated as a methodologic base for the performance of new researches with another species of fish.

4. Conclusion

The initiators proposed in this study present high sensitivity, precision and specificity thresholds for the detection of genetic material from pirarucu (*Arapaima gigas*), proving that the proposed PCR technique was standardized with success. The PCR represents an excellent alternative for the detection of frauds caused by the replacement of pirarucu for other species, and constitutes a useful tool for the authentication of this product by the official entities of inspection.

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