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## ARTIGO

## Molecular identification of *Pseudoplatystoma* sp. fish fillets by Multiplex PCR

### Identificação molecular de filés de peixe *Pseudoplatystoma* sp. por PCR-Multiplex

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**ABSTRACT**

Nuclear and mitochondrial genes were used as molecular markers for verifying the identity of fish fillets marketed as pintado (*Pseudoplatystoma corruscans*). Based on the polymorphisms of nuclear DNA (RAG2, globin, and EF1 genes) and mitochondrial regions (16S), we examined whether the fillets originated from inbred species of pintado or from hybrids derived from crosses between cachara (*P. reticulatum*) and pintado. Nuclear genes from both species were detected in the analyzed fillets (n = 29). This clearly identified these fish as interspecific hybrids (or F1/first filial generation) of the type “cachapinta,” resulting from a cross between female cachara and male pintado. These results demonstrate that monitoring fish fillet trading is crucial for detecting discrepancies between the marketed species and related information declared on the label. Species that are frequently hybridized, such as pintado and cachara, require special attention.

**KEYWORDS:** Cachapinta; Cachara; Genetic Identification; Hybridization; Pintado

**RESUMO**

Marcadores moleculares (PCR-Multiplex de genes nucleares e mitocondriais) foram utilizados para verificar a identidade molecular de filés de peixe comercializados como pintado (*Pseudoplatystoma corruscans*) com base em polimorfismos de regiões do DNA nuclear (genes RAG2, globina e EF1) e mitocondriais (16S) para verificar se os filés pertenciam a espécie pura de pintado ou se eram híbridos derivados do cruzamento entre cachara (*Pseudoplatystoma reticulatum*) e pintado (*Pseudoplatystoma corruscans*). Os filés analisados (n = 29) apresentaram genes nucleares de ambas espécies *P. corruscans* e *P. reticulatum*, e desta forma, foram identificados como híbridos interespecíficos ou F1 (primeira geração filial) do tipo “cachapinta” resultante do cruzamento entre uma fêmea de cachara e um macho de pintado. Estes resultados mostram que o monitoramento da comercialização de filés de peixe é fundamental para identificar situações onde existem diferenças entre as espécies comercializadas e as informações declaradas no rótulo. Espécies em que a hibridação tem sido frequentemente realizada como é o caso do pintado e da cachara, merecem atenção especial.

**PALAVRAS-CHAVE:** Cachapinta; Cachara; Hibridação; Identificação Genética; Pintado



## Introduction

The order siluriformes consists of 34 families. Pimelodidae is one of these families, which contains the genus *Pseudoplatystoma*. Some of these species exclusively live in freshwater regions and are widely distributed throughout the river basins of South America<sup>1</sup>. The siluriformes (common name: surubins<sup>2,3</sup>) are also known as leather fish because of their thick skin and lack of scales.

The common names for *Pseudoplatystoma corruscans* and *P. reticulatum* are pintado and cachara, respectively. Both are freshwater species of high commercial value because of their size, tasty meat, low fat content, and the absence of intramuscular bones<sup>4</sup>. Crosses between pintado and cachara have been conducted frequently in Brazilian aquaculture to obtain hybrids with faster growth rates and that are easier to handle than the parental species<sup>5</sup>.

The popular name of the hybrids is derived from the first half of the female's name and the second half of the male's name. Thus, "cachapinta" results from crossing a female cachara with a male pintado and "pintachara" from crossing a female pintado with a male cachara<sup>6</sup>. Hybridizations involving pintado, cachara, and other neotropical fish species are also possible<sup>7</sup>.

In the juvenile stage, morphological identification of fish is difficult and often inaccurate. Morphological identification of *Pseudoplatystoma* sp. by visual assessments of staining and the spot patterns on the body are not reliable methods, even with adult animals<sup>9</sup>. In contrast, the use of genetics can provide an accurate diagnosis of hybrid and parental species<sup>8</sup>.

Food product labels are a communication tool between merchandisers and consumers. For this reason, the information provided must be explicit to help consumers make informed food choices. In accordance with Brazilian laws, labels should not submit false or inaccurate information or cause confusion about the origin or quality of the food<sup>10</sup>.

Species of a lesser commercial value have been falsely identified as surubims and are used for the preparation of steaks and other processed products, which is a problem faced by Brazilian producers<sup>11</sup>. The identification of fish fillets usually requires the application of molecular tools, since most morphological features used for this purpose are removed during the filleting process<sup>12</sup>. Genetic fingerprinting of fish and fish products is an important tool in fraud detection and identification of interspecific crosses of hybrid catfish<sup>11</sup>.

Molecular techniques have been applied in aquaculture, allowing adequate management of several cultivated species<sup>13</sup>. Compared to other molecular techniques, multiplex polymerase chain reaction (PCR) has advantages in that it: (1) has a relatively low-cost, (2) is processed rapidly, and (3) allows for the simultaneous analysis of several species<sup>14</sup>. Other studies have confirmed the efficiency of multiplex PCR in the identification of fish species and hybrids<sup>9,15,16,17</sup>. In this context, the purpose of this study was to verify the authenticity of fish species in fillets marketed as pintado.

## Material and Methods

### Samples and DNA extraction

Samples were purchased from a company in Mato Grosso do Sul, Central-Western, Brazil (21°50'58.1"S 54°55'41.1"W) that industrializes farming of freshwater fish. We used twenty-nine frozen fish fillets marketed as pintado (*P. corruscans*) and all experiments included one DNA sample from each pure parental species of pintado and cachara as control reactions. DNA extraction was conducted using the Wizard Genomic DNA Purification Kit (Promega, WI) according to the manufacturer's protocol. DNA quantity was determined against a molecular marker standard (Low DNA Mass Ladder-Invitrogen™, Life Technologies, USA) by electrophoresis in a 1% agarose gel.

For DNA extraction, 10 mg of thawed fillet were added to 600 µl of chilled Nuclei Lysis Solution and homogenized for 10 s. Proteinase K (5 µl) was added, followed by vortex homogenization and incubation at 60°C for 2 h. RNase solution (2.5 µl) was added to the nuclei lysate and mixed, followed by incubation at 37°C for 30 min and subsequent cooling to room temperature. Protein Precipitation Solution (200 µl) was added and the sample was vortexed, chilled on ice for 5 min, and centrifuged at 13,000 rpm for 4 min. The supernatant was transferred to a fresh tube containing 600 µl of isopropanol (room temperature), mixed gently by inversion, and thereafter centrifuged at 13,000 rpm for 4 min. The supernatant was removed and mixed with 600 µl of 70% ethanol (room temperature), followed by centrifugation at 13,000 rpm for 4 min. The ethanol was aspirated and the DNA pellet allowed to dry at room temperature for 1 h. In the final step, the DNA was rehydrated overnight at 4°C in 100 µl DNA Rehydration Solution.

### Molecular markers and Multiplex PCR

To identify the species of the processed samples, PCR techniques were applied to examine the regions of nuclear β-globin (GLOB), nuclear recombination-activating gene 2 (RAG2), elongation factor 1-alpha (EF1α), and mitochondrial 16S ribosomal DNA (rDNA) genes, generating diagnostic electrophoretic fragments for the species and their hybrids being studied. Multiplex PCR was performed for each of the genes listed. DNA sequencing and sequence alignment were done as described in previous studies<sup>9,17</sup>.

The use of nuclear genes allows for the identification of pure and hybrid animals. Pure animals have nuclear genes of only one species while hybrids have nuclear genes of two species. Mitochondrial genes are maternally inherited and thus allow for the identification of the hybrids' female parent species (cachapinta or pintachara).

Amplifications were performed using PCR in a total volume of 25 µL with 200 µM of each dNTP (dATP, dTTP, dGTP, and dCTP), 1.5 mM MgCl<sub>2</sub>, 1X Taq DNA buffer (20 mM Tris-HCl,



pH 8.4, and 50 mM KCl), 0.5 units of Taq Polymerase (Invitrogen, Life Technologies, USA), 0.1  $\mu$ M of each universal primer, and 10-50 ng of genomic DNA. The reactions were performed under the following conditions: (1) for GLOB and EF1 $\alpha$ : 95°C, 5 min; 95°C, 30 s, 59°C, 30 s, 72°C, 20 s (35 times); 72°C, 5 min; (2) for RAG2 and 16S: 95°C, 5 min; 95°C, 30 s, 57°C, 30 s, 72°C, 15 s (35 times); 72°C, 5 min. DNA fragment sizes were determined by electrophoresis on 2% agarose gels stained with ethidium bromide (1 ng mL<sup>-1</sup>) and visualized by UV illumination. The images were captured with a digital camera (Olympus CAMEDIA c-5060, 5.1 Megapixel).

Specific primers developed in previous studies were used for the PCR reaction<sup>9,17</sup>. Primers for the GLOB nuclear gene were designed in a reverse order. The GLOB PcR primer (5'-CAGCCACCTTGGGGTTTCCT-3') is specific to *P. corruscans* and GLOB PrR primer (5'-GGTACGTCTAATCTCAGTAATTGAAA-3') is specific to *P. reticulatum*. The multiplex amplification of the GLOB gene (using the primer pairs GLOB SiluF/GLOB SiluR, GLOB PcR, and GLOB PrF) allows for the visualization of two fragments. One is approximately 569 bp in length (reaction control) for cachara, pintado, and hybrids resulting from the amplification with the primer pair GLOB SiluF/GLOB SiluR. The other is obtained using the species-specific primers GLOB PcR and GLOB PrF respectively and allows for the amplification of a 304 bp fragment from *P. corruscans* and a 137 bp fragment from *P. reticulatum*, respectively. Hybrid animals show a heterozygous pattern expressing 2 fragments (137 bp and 304 bp) inherited from the parents.

Primers for the EF1 $\alpha$  nuclear gene were designed in reverse order. The primer EF1 $\alpha$  PcR (5'-CAACAATGGCAGCATCTCCT-3') is specific to *P. corruscans* and the primer EF1 $\alpha$  PrR (5'-ATAAAGGACAAGACAAGATCG-3') is specific to *P. reticulatum*. The multiplex amplification of this gene allows for the visualization of an ~800 bp fragment (reaction control) for cachara, pintado, and their hybrids (EF1 $\alpha$ F/EF1 $\alpha$ R). Additionally, a 520 bp fragment of *P. corruscans* and a 630 bp fragment in *P. reticulatum* (EF1 $\alpha$ PrF/ EF1 $\alpha$ PrR) will be amplified. Hybrid animals show a heterozygous pattern expressing two fragments (520 bp and 630 bp) inherited from the parents.

Primers for the RAG2 nuclear gene were designed in reverse order. The primer RAG2 PcR (5'-AACTCCAGTCAATGAGATAAATG-3') is specific to *P. corruscans* and the primer RAG2 PrR (5'-CAGTTCCAGGTCTCTGTGGTT-3') is specific to *P. reticulatum*. The multiplex amplification of this gene (using primers RAG2 PcR, RAG2 PrR, RAG2 SiluF, and RAG2 SiluR) allows for the visualization of a ~550 bp fragment (reaction control) for cachara, pintado, and their hybrids resulting from the amplification primers RAG2 SiluF and RAG2 SiluR. The use of species-specific primers RAG2 PcR and RAG2 PrR allows for the visualization of a 330 bp fragment in *P. corruscans* and a 290 bp fragment in *P. reticulatum*. Hybrids show a heterozygous pattern expressing two fragments (290 bp and 330 bp) inherited from the parents.

Primers for the 16S mitochondrial gene were designed in reverse order. The primer 16S PcF (5'-TGACCATAAAGATCCGGCTAT-3')

is specific to *P. corruscans* and the primer 16S PrR (5'-TCTTG-GTTTTGGGGTTGTTA-3') is specific to *P. reticulatum*. The multiplex amplification of this gene (using primers 16S F, 16S R, 16S PcF, and 16S PrR) allows for the visualization of a ~650 bp fragment (reaction control) for cachara, pintado, and their hybrids resulting from the amplification primers 16S F and 16S R. The use of species-specific primers 16S PcF and 16S PrR allows for the visualization of a 200 bp fragment in *P. corruscans* and a 400 bp fragment in *P. reticulatum*. Hybrid animals show a heterozygous pattern expressing two fragments (200 bp and 400 bp) inherited from the parents.

## Results and Discussion

Multiplex PCR of the GLOB gene showed one band of about 569 bp, corresponding to the reaction control. Pure parental pintado (*P. corruscans*) DNA showed a band of ~304 bp and pure parental cachara showed a band of ~137 bp, confirming that these bands are characteristic for each species. One band of the reaction control (569 bp) and one inherited from each parental species were observed in all tested fillets confirming a hybrid origin of the samples.

In the multiplex PCR of the EF1 $\alpha$  gene, one band of ~800 bp was visualized, corresponding to the reaction control. In addition to this band, all samples of tested fillets showed two other bands: one of ~520 bp, characteristic of *P. corruscans*, and another of ~630 bp, characteristic of *P. reticulatum*. As with the GLOB PCR results, this also demonstrates hybridization.

Multiple PCR amplifications of the RAG2 gene revealed a reaction control band of ~550 bp in all samples. All analyzed fillets showed a heterozygous pattern with two bands, one of ~330 bp as in the DNA control sample of *P. corruscans* and another of ~290 bp as in the DNA control sample of *P. reticulatum*.

Multiplex PCR of the mitochondrial 16S gene revealed fragments of ~650 bp, which was the reaction control for all samples. The tested fillets showed bands of ~400 bp, as in the maternal species *P. reticulatum*, and therefore corresponded to the profile of the hybrid 'cachapinta' (Figures 1 and 2).

The catfish species *P. corruscans* (pintado) and *P. reticulatum* (cachara) have long been used to produce hybrids known by fish farmers as "cachapinta" by crossing a cachara female and pintado male and "pintachara" by crossing a pintado female and a cachara male<sup>18</sup>.

Production of hybrids is economically very important in several countries, including Brazil, mainly because they are easier to breed and offer improved productivity over their parental species<sup>19,20,21</sup>. In addition to productivity advantages, the production of hybrids like "cachapinta" also makes spawning cachara females (*P. reticulatum*) available for a longer period during the year<sup>22</sup>.

However, consumers need clear and accurate information to make informed food choices. The food content must be exactly what is declared on the label, i.e., the food must be authentic and not misdescribed<sup>23</sup>. Unambiguous identification



of fish and fish products has importance in various areas. For example, it can help to detect fraud or replacement of species in commercial transactions<sup>12</sup>. Genetic identification of fish and fish products is an important tool in both fraud detection and the identification of hybrid catfish<sup>11</sup>. When morphological characteristics are preserved, species identification is possible. However, these characteristics are lost during processing and the species are no longer recognizable<sup>24</sup>, thus underscoring the importance of molecular techniques for species identification.

According to the Regulation of Industrial and Sanitary Inspection of Animal Products (RIISPOA)<sup>25</sup>, Brazilian law stipulates that labels of animal products must not convey a false impression or falsely indicate the origin or quality of a product. The use of a specific name that indicates the true nature of the product is required<sup>10</sup>.

Molecular markers based on polymorphisms of DNA regions of nuclear and mitochondrial DNA have long been used for studying fish species<sup>14,21,26</sup>.

Previously, the morphologically similar sharks *Carcharhinus obscurus* and *C. plumbeus* were identified by multiplex PCR utilizing species-specific primers for the nuclear ribosomal ITS2 region. This method was determined to be rapid and reliable for the distinction of two globally widespread, intensively harvested species<sup>27</sup>. Species identification by PCR can also prevent fraud (use of unauthorized species) in codfish production<sup>28</sup>. Nuclear markers can be used for the discrimination of fish species when intron size differs between species and, therefore, may allow for the amplification of species-specific DNA fragments<sup>29</sup>. Furthermore, the 5S ribosomal DNA gene proved to be appro-

prate for the identification and differentiation of commercially valuable cephalopod species of the families Loliginidae and Ommastrephidae<sup>30</sup>. Another freshwater fish species cultivated in Brazil is Tambaqui (*Colossoma macropomum*). Its hybrids, derived from crosses with Pacu *Piaractus mesopotamicus* or Pirapitinga *Piaractus brachypomus*, can be identified by multiplex PCR of the nuclear gene atropomyosin<sup>31</sup>.

Here, we showed that multiplex PCR can effectively identify hybrids between the studied catfish species and can be performed in minimal time and with readily available reagents. The results for the 16S mitochondrial gene allowed us to determine that the hybrid origin of the tested fillets were of the type “cachapinta”, since this mitochondrial gene is inherited maternally. This discrimination is necessary because hybrids may have different biological, zootechnical, and nutritional characteristics<sup>18,32,33</sup>.

## Conclusion

The results of this study indicate the need for monitoring of marketed fish products in order to ensure agreement between the actual species processed and the label information. This is especially important for species where hybridization is frequently performed, as with the catfish species examined here. We conclude that multiplex PCR can be applied as an auxiliary tool for the accurate identification of hybrids from *P. corruscans* and *P. reticulatum*, especially when dealing with products where morphological features are not preserved as a result of processing.

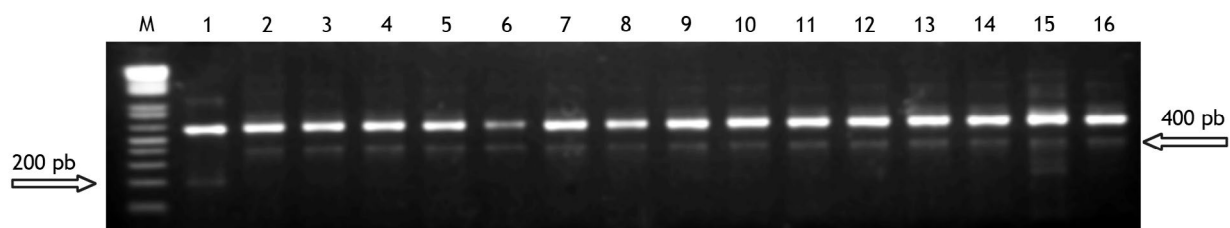


Figure 1. Electrophoresis analysis of Multiplex-PCR of 14 samples of the 16S genes. Lanes: 1 – *P. corruscans*; 2 – *P. reticulatum*; 3-16 – samples hybrid; M – molecular weight marker (1Kb).

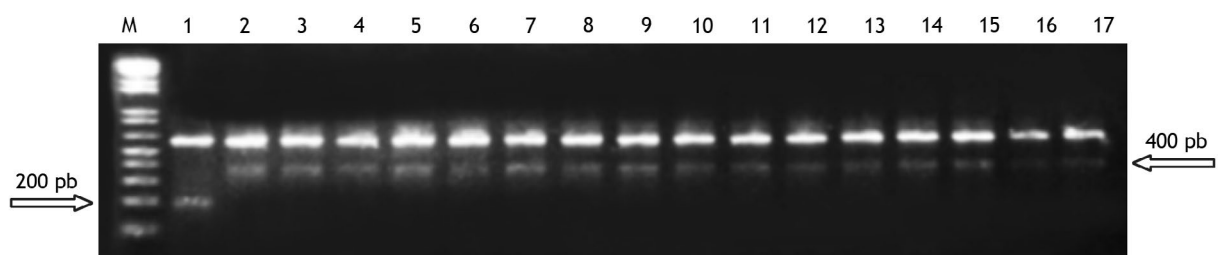
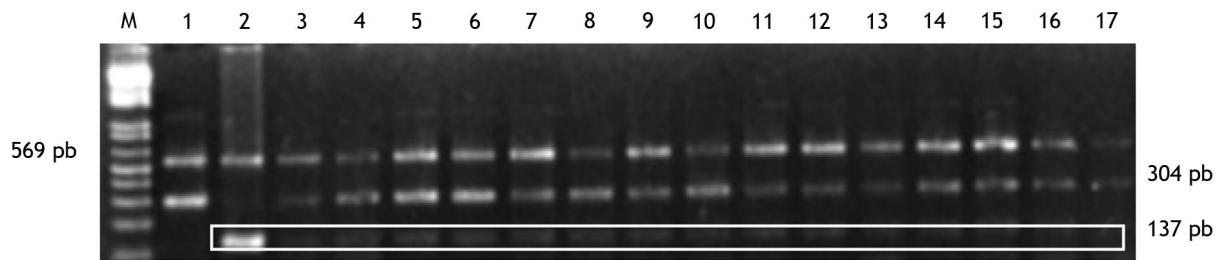
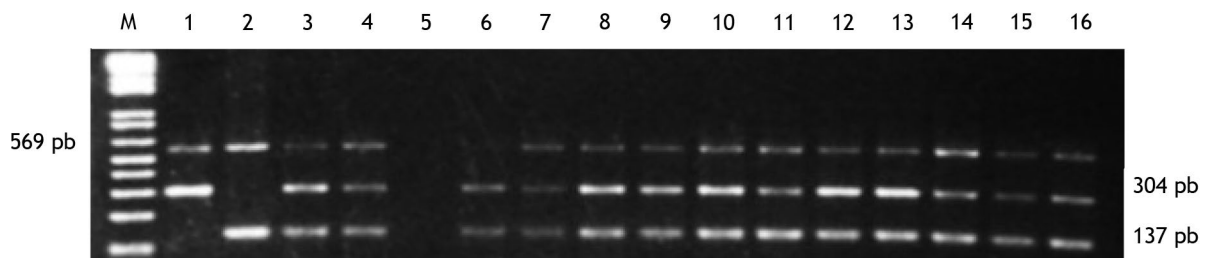


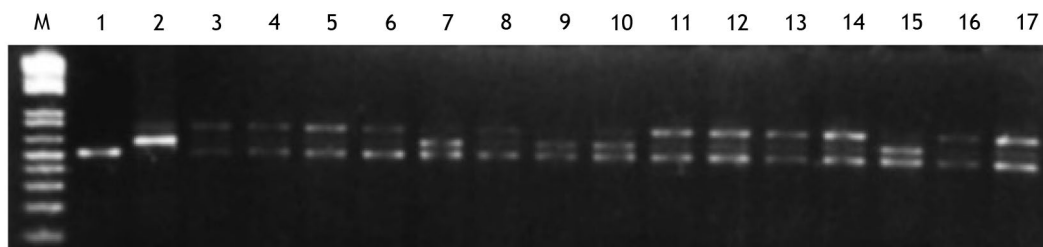
Figure 2. Electrophoresis analysis of Multiplex-PCR of 15 samples of the 16S genes. Lanes: 1 – *P. corruscans*; 2 – *P. reticulatum*; 3-17 – samples hybrid; M – molecular weight marker (1Kb).



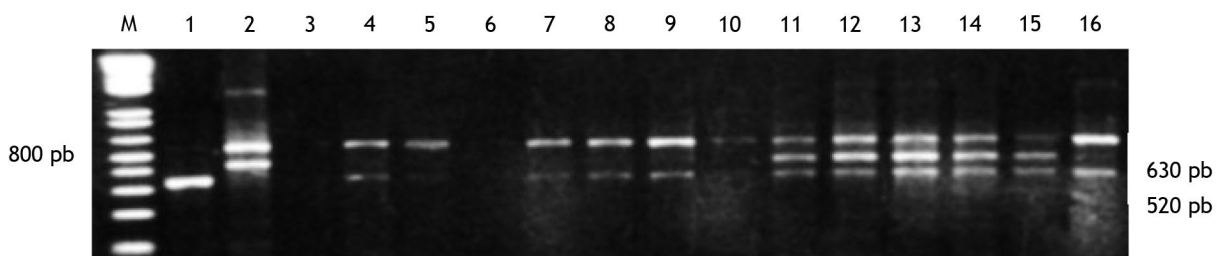
**Figure 3.** Eletrophoresis analysis of Multiplex-PCR of 15 samples of the GLOB genes. Lanes: 1 – *P. corruscans*; 2 – *P. reticulatum*; 3-17 – samples hybrid; M – molecular weight marker (1Kb).



**Figure 4.** Eletrophoresis analysis of Multiplex-PCR of 14 samples of the GLOB genes. Lanes: 1 – *P. corruscans*; 2 – *P. reticulatum*; 3-16 – samples hybrid; M – molecular weight marker (1Kb).



**Figure 5.** Eletrophoresis analysis of Multiplex-PCR of 15 samples of the EF1 $\alpha$  genes. Lanes: 1 – *P. corruscans*; 2 – *P. reticulatum*; 3-17 – samples hybrid; M – molecular weight marker (1Kb).



**Figure 6.** Eletrophoresis analysis of Multiplex-PCR of 14 samples of the EF1 $\alpha$  genes. Lanes: 1 – *P. corruscans*; 2 – *P. reticulatum*; 3-16 – samples hybrid; M – molecular weight marker (1Kb).

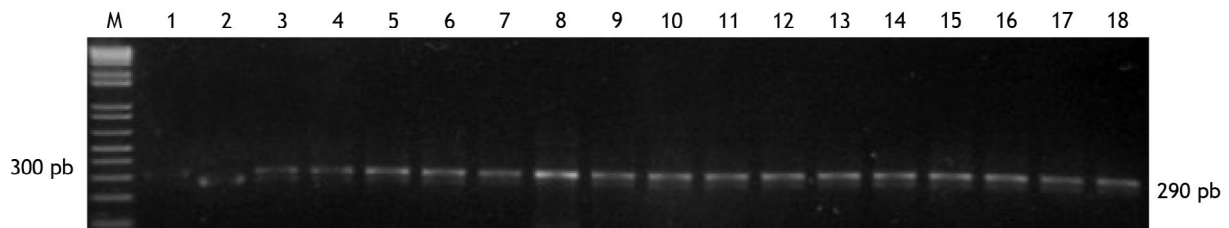


Figure 7. Eletrophoresis analysis of Multiplex-PCR of 16 samples of the RAG genes. Lanes: 1 – *P. corruscans*; 2 – *P. reticulatum*; 3-18 – samples hybrid; M – molecular weight marker (1Kb).

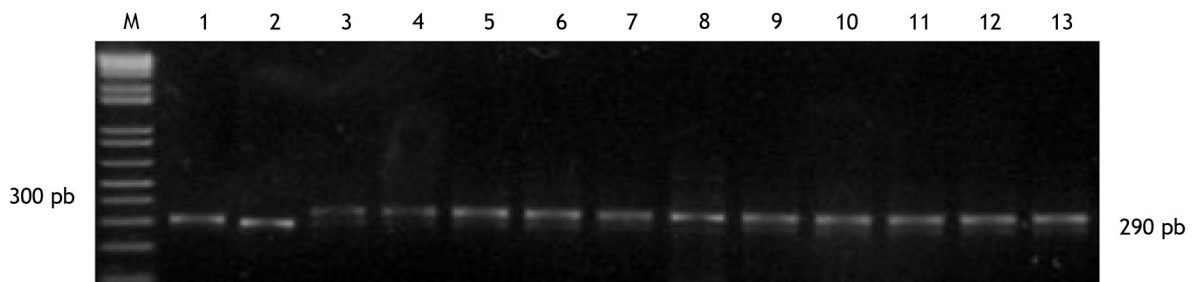


Figure 8. Eletrophoresis analysis of Multiplex-PCR of 13 samples of the RAG genes. Lanes: 1 – *P. corruscans*; 2 – *P. reticulatum*; 3-13 – samples hybrid; M – molecular weight marker (1Kb).

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