OPTIMIZATION OF RANDOM AMPLIFIED POLYMORPHIC DNA PROTOCOL FOR MOLECULAR IDENTIFICATION OF *Lophius gastrophysus*[1]

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SUMMARY

Lophius gastrophysus has important commercial value in Brazil particularly for foreign trade. In this study, we described the optimization of Random Amplified Polymorphic DNA (RAPD) protocol for identification of *L. gastrophysus*. Different conditions (annealing temperatures, $MgCl_2$ concentrations, DNA quantity) were tested to find reproducible and adequate profiles. Amplifications performed with primers A01, A02 and A03 generate the best RAPD profiles when the conditions were annealing temperature of 36ºC, 25 ng of DNA quantity and 2.5 mM $MgCl_2$. Exact identification of the species and origin of marine products is necessary and RAPD could be used as an accurate, rapid tool to expose commercial fraud.
Keywords: fraud, fish identification, blackfin goosefish, RAPD-PCR.

RESUMO

OTIMIZAÇÃO DO PROTOCOLO DE AMPLIFICAÇÃO RANDÔMICA DE DNA POLIMÓRFICO PARA IDENTIFICAÇÃO MOLECULAR DE *Lophius gastrophysus*. *Lophius gastrophysus* apresenta importante valor comercial no Brasil, principalmente para a exportação. Neste estudo, descrevemos uma otimização do protocolo de amplificação aleatória de DNA polimórfico (RAPD) para identificação de *L. gastrophysus*. Diferentes condições (temperatura de recozimento, quantidade de DNA e concentração de $MgCl_2$) foram testadas para obter perfis reprodutíveis. Os iniciadores A01, A02 e A03 geraram os melhores resultados de amplificação quando utilizados temperatura de recozimento de 36ºC, 25 ng de DNA e 2,5 mM de $MgCl_2$. A identificação exata de espécies e da origem dos produtos marinhos faz-se necessária e a RAPD é uma ferramenta rápida e precisa para expor fraudes comerciais.
Palavras-chave: fraude, identificação de peixes, peixe sapo, RAPD-PCR.

1 - INTRODUCTION

Due to the increase of the international trade in seafood and seafood products, there is a need for suitable methods for the identification of fish species or part of it to ensure compliance with the labeling regulations, quality and pricing policies imposed by various countries and, thereby, to prevent the substitution of fish species [2, 8]. Identification might be required to support enforcement of regulations, for example restrictions on fishing for a particular species or enforcement of regulations relating to naming of fish offered for sale, or in testing for compliance with specifications. The use of such techniques will help to protect consumers' rights by enabling the enforcement of labeling regulations. It will also help to regulate imports from producer countries and it will assist in the protection of endangered species [11].

Molecular biological methods represent the future in food control laboratories [8]. Methods based on comparison of DNA fragments have been developed. The polymerase chain reaction (PCR) method has been used successfully to identify fish species and to avoid fraudulent label [2, 4, 5, 11, 12]. The random amplified polymorphic DNA (RAPD) [13, 14] technique consists in the amplification, by PCR, of random segments of genomic DNA using a single short primer with arbitrary sequences, thus, one can expect to scan the genome more randomly than using conventional techniques [3] to produce a fingerprint of the DNA contained in the sample [7]. The relatively low cost of the technique and requirement of only nanograms of template DNA provide advantages in the use of RAPD in fish and fish products identification [3]. This technique can be used to differentiate individuals or breeding stocks with a given species but also to differentiate among different species. The conditions of the analysis can be optimized to reveal only species-specific differences, since all individuals belonging to a given species have more of their genetic material in common than with individuals from other species [7].

The Lophiidae family comprises four genus and twenty-five species, where the *Lophius* is the most important one, due to its economical potential. In Brazil, it is possible to find the *L. gastrophysus* species, which lives in depth between 100 and 500 meters, main target of the fishing fleet that operates above the isobath of 200 meters in the south and southeast region [9]. The blackfin goosefish has been detached by the high availability and very good acceptance in the international market, becoming one of the most valuable fisheries in Brazil.

Several authors have demonstrated the discriminatory power of PCR analysis for animal species identification [2, 4, 5, 11, 12]. *L. gastrophysus* can be easily distinguished if it is sold as a whole fish by means of traditional taxonomic classification methods. In the case of transformed products, another technique for identifying fish species is needed to avoid fraud through species substitution.

In this study, we described the optimization of RAPD protocol for identification of *L. gastrophysus*. Different conditions (annealing temperatures, $MgCl_2$ concentrations, DNA quantity) were tested in order to find reproducible and adequate profiles.

2 - MATERIAL AND METHODS

2.1 - Sample and extraction of DNA

Fresh fish of fourteen specimens from *L. gastrophysus*

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were collected from Brazilian coast. Genomic DNA was isolated from fresh muscle according to DOYLE & DOYLE [6] (method 1) and from frozen (- 20ºC) muscle of each individual according to ASAHIDA *et al.* [1] (method 2), after minor modifications described below. About 300-500 mg of tissue was ground in 500 μL homogenization buffer (10 mM Tris-HCl pH 8.0; 125 mM NaCl; 10 mM EDTA pH 8.0; 0.5% SDS; 4 M urea). Then 3.5 μl proteinase K (10 mg/ml) were added and the mixture was incubated at 37ºC overnight. DNA was purified with standard phenol:chloroform: isoamyl alcohol (25:24:1) extractions, treated with RNAse, precipitated with sodium acetate (3 M pH 5.3) and ice cold absolute ethanol, wash with 70% ethanol and resuspended in 100 μl TE (Tris-EDTA pH 8.0) buffer. DNA presence was verified by agarose gel electrophoresis and its concentration and purity were determined with a spectrophotometer set at 260 and 280 nm absorbances.

2.2 - PCR conditions

PCR reactions were performed with five commercial 10-mer primers (Invitrogen) with the following 5'-3' sequences: (A01) CAGGCCCTTC, (A02) TGCCGAGCTG, (A03) AGTCAGCCAC, (A11) CAATCGCCGT and (A16) AGCCAGCGAA. Optimal conditions for RAPD analysis were determined by the highest number of fragments and accurate bands.

DNA amplifications were performed in a final volume of 25 μl containing 1 unit of *Taq* DNA polymerase (Invitrogen), 20 mM Tris-HCl pH 8.4, 50 mM KCl, 0.2 mM of each dNTP, 2.5 mM or 2.0 mM $MgCl_2$, 1 μM primer and 12.5 or 25.0 ng of DNA template. The amplifications were carried out in a thermal cycler (MJ Research Inc., MiniCycler™, Watertown, MA, USA) programmed for initial heat denaturation in one step of 2 minutes at 94ºC. Subsequent 44 cycles of 1 minute at 94ºC, 30 seconds at 36ºC or 40ºC and 2 minutes at 72ºC; followed by one final step of primer extension at 72ºC for 7 minutes. One negative control (absence of template DNA) was performed for each set of amplifications. Amplification products were resolved by gel electrophoresis on 1.5% agarose gels stained with ethidium bromide in Tris Borate EDTA (TBE) buffer for 2 hours at 80 volts. Subsequently, gels were visualized on UV *light* and photodocumentation was performed. DNA fragments sizes were estimated by comparison with the standard marker 1 kb ladder (λ DNA/ Hind III, Invitrogen and DRIgestTM III, Amersham).

3 - RESULTS AND DISCUSSION

It is well known that problems related to the standardization of the amplification conditions with thermal profiles have been observed. The laboratorial practice showed that other variables, like final reagent concentration and template DNA quality, influence the attainment of reproducible results.

DNA extracted from frozen muscle by method 2, that is based in a high concentration of urea to modify the cell lysis, showed large amounts of high-quality DNA than DNA isolated from fresh muscle by method 1 fundamented on the use of the detergent CTAB (cetyltrimethylammonium bromide) that solubilizes the membranes, forming with DNA a complex that facilitates a posterior precipitation. For this reason, fish DNA extracted by method 2 was used for PCR amplification.

Firstly, tests with annealing temperature (36ºC and 40ºC), DNA (12.5 ng and 25.0 ng) and $MgCl_2$ (2.0 mM and 2.5 mM) concentrations were performed with eight samples with four commercial 10-mer primers (A01, A02, A03 and A16).

For RAPD analysis, the employed annealing temperature is lower than the used PCR reaction average, since lower temperatures slow the reaction specificity so that the highest reproducible fragments number may be amplified. In our test, the annealing temperature of 36ºC provided a higher number of amplified fragments than the temperature of 40ºC (results not shown).

Regarding the DNA concentration, the used extracting process (phenol:chloroform:isoamyl alcohol), might leave impurities (proteins, phenol, NaCl) that jeopardize the DNA amplification through PCR. In general, 1 ng/μl concentration in the reaction is adequate for a vast variety of eucariotic organisms with nuclear genomes varying between 10^8 and 10^{10} base pairs [10]. It was observed that the use of 25 ng of DNA provided the best results, probably because in this concentration (1 ng/μl) the contaminant quantity is low while the haploid genomes number (for teleostean fish range of 0.4 to 4.4 pg) is in accordance with the recommended quantity.

Since the $MgCl_2$ is co-factor of the *Taq* polymerase enzyme, it influences the DNA amplification process. Lower quantities than the necessary ones lead to the amplification failure or to deficient amplification or still, to low experiments reproducibility. In our experiment the 2.5 mM $MgCl_2$ was ideal to amplify the *L. gastrophysus* DNA.

Amplifications performed with primers A01, A02 and A03 generate the best RAPD profiles when the conditions were annealing temperature of 36ºC, 25 ng of DNA quantity and 2.5 mM $MgCl_2$, but primer A16 generate few bands (*Figure 1*).

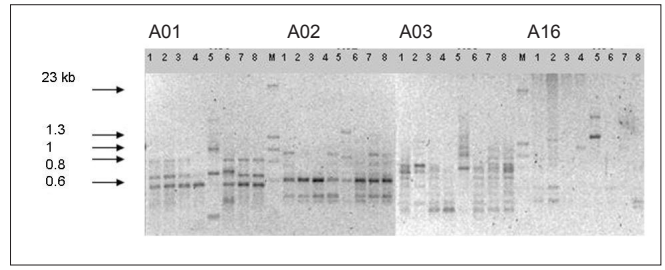

FIGURE 1 – Amplifications performed with primers A01, A02, A03 and A16 when the conditions were annealing temperature of 36 ºC, 25.0 ng of DNA quantity and 2.5 mM $MgCl_2$. Lanes 1-8: specimens of *L. gastrophysus*; M: standard marker 1 kb ladder (DRIgest™ III, Amersham)

To conduce RAPD experiments for the *L. gastrophysus* specie it was established the following reaction conditions for the final volume of 25 μl: 1 unit of *Taq* DNA polymerase, 20 mM Tris-HCl pH 8.4, 50 mM KCl, 0.2 mM of each dNTP, 2.5 mM MgCl2, 1 μM primer and 25.0 ng of DNA template.

After amplification conditions were established, fourteen samples were performed with the primers A01, A02, A03 and A11. The results from this study present the first data obtained applying the RAPD-PCR method to *L. gastrophysus* identification. All the primers cited above produced different RAPD fragment patterns. The number of fragments generated per primer varied between four and eight, with a mean of 6.5 bands per primer and 1.9 per individual. From a total of twenty-six scorable bands, two monomorphic bands with size of approximately 600 bp and 400 bp were obtained for A01 and A03 primers respectively (*Figure 2*). In the future, RAPD-PCR technique can be used for unambiguous identification of *Lophius* at the species level and for investigating their genetic relationships.

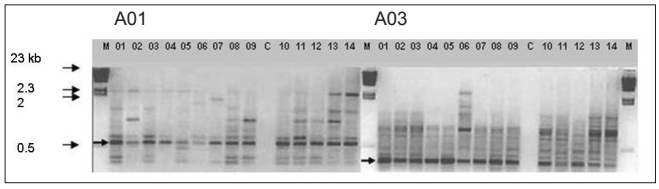

FIGURE 2 – RAPD profiles to *L. gastrophysus* obtained with primers A01 and A03. Lanes 1-14 specimens of *L. gastrophysus* amplified with primer A01 and A03. M: standard marker 1 kb ladder (λ DNA/ Hind III, Invitrogen). C: amplification without DNA. Arrows show monomorphic bands

4 - CONCLUSIONS

The RAPD-PCR method can be very valuable in studies involving molecular identification, particularly in species such as *Lophius* spp. that are difficult to identify using morphological characteristics when only fillets are available, once the RAPD protocol has been optimized.

Further developments are likely in the search for specific DNA fragments in order to construct species-specific probes. These would be used for hybridization with a target sequence and to identify the species in order to be a useful tool to reveal frauds in the substitution of *Lophius* spp. The authors intend to apply the methodology for the identification of those species, which are subjected to commercial frauds causing detriment to the consumer.

5 - LIBRARY REFERENCE

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