Conservation Genetics Resources (2021) 13:127–130
https://doi.org/10.1007/s12686-021-01195-7

TECHNICAL NOTE

Simple and safe approach for molecular identification of the endangered species *Epinephelus itajara*

Yrlano Oliveira[1] · Roberto Alencar[1] · Yrlan Oliveira[1] · Rodrigo A. Torres[2] · Iracilda Sampaio[1] · Marcelo Vallinoto[1] · Gláucia C. Silva-Oliveira[1]

Received: 6 August 2020 / Accepted: 9 February 2021 / Published online: 20 February 2021

Abstract

The Atlantic goliath grouper, *Epinephelus itajara* (Lichtenstein, 1822), is considered a critically endangered species. In Brazil, the harvesting, transportation, and sale of *E. itajara* or it's by products have been prohibited, but illegal harvesting still occurs. This study developed a simple, sensitive and accurate procedure for the safe identification of *E. itajara* by polymerase chain reaction (PCR). A specific primer (16S-RYOP4) was designed, synthesized and grouped with universal primers. The efficiency of specific amplification of the new set of primers was proven using samples of *E. itajara*, four species of epinephelids and five other species of fish marketed in northern Brazil. This procedure can be used to inspect products sold in stores, supermarkets and fairs, especially in places of reduced scientific, technological and economic development, where there is a record of illegal fishing and selling of *E. itajara*.

Keywords Atlantic goliath grouper · Endangered species · Molecular identification · Illegal sale

Technical note

The Atlantic goliath grouper, *Epinephelus itajara* (Lichtenstein, 1822), is the largest grouper species found in the Atlantic Ocean and is listed by the International Union for Conservation of Nature as "critically endangered", due to population decline resulting from overfishing and habitat degradation (Espinosa-Perez et al. 2015). In Brazil, the harvesting, transportation, and sale of *E. itajara* or it's by products have been prohibited by law since 2002. However, illegal harvesting occurs, especially in northern Brazil (Silva-Oliveira et al. 2008). The large coastal extension and the practices of mischaracterization of the specimens (with the removal of the head and fins) make it difficult to inspect and identify the illegal sale of *E. itajara* (ICMBio/MMA 2018). This means that the development of low-cost and rapid procedures for the reliable identification of this species, based on samples of the typical by products sold in shops and markets, will provide an important tool for the suppression of the illegal trade in this critically endangered species (Horreo et al. 2013; Melo-Palmeira et al. 2013).

Modern methods, such as real-time polymerase chain reaction (PCR), multiplex PCR and PCR–RFLP, for the identification of epinephelid species are available, but they are complex and/or require expensive equipment and laboratory resources (Damasceno et al. 2016; Torres et al. 2013; Chen et al. 2012). In regions with low human development index and reduced scientific and technological development, as the coast of the north and northeast regions of Brazil, the diagnosis of the product sold illegally has also been a major challenge. Considering the urgent need to conserve Atlantic goliath grouper stocks and the importance of identifying this species where illegal harvesting is disguised by falsifying fishery products, the present study developed a simple, sensitive and accurate procedure for the safe identification of *E. itajara* by PCR.

A specific primer (16S-RYOP4, 5′-AGTTCTGTTAATTAGAGTTGTCA) was designed for *E. itajara*, using the FastPCR program (Kalendar et al. 2014), based on comparisons with the epinephelid sequences available in GenBank (Craig and Hastings 2007) and the use of the universal primers 16S-L1987 (5′-GCCTCGCCTGTTTACCAAAAAC-3′)

✉ Gláucia C. Silva-Oliveira
gcoliveira@ufpa.br

[1] Institute of Coastal Studies, Federal University of Pará, Alameda Leandro Ribeiro s/n, Bragança, PA 68600-000, Brazil

[2] Center of Biological Science, Federal University of Pernambuco, Recife, PE, Brazil

and 16S-H2609 (5′-CCGGTCTGAACTCAGATCACGT-3′) (Palumbi et al. 1991) (Fig. 1). The efficiency of specific amplification of the new set of primers (16S-L1987/16S-RYOP4) was assessed using grouper samples identified in previous studies (Silva-Oliveira et al. 2008, 2013), as well as samples of other epinephelids obtained from fishery catches (SISBIO license number 19289), which were identified using Cervigón et al. (1993). Four epinephelid species were included here—the coney (*Cephalopholis fulva*, Linnaeus, 1758), the dusky grouper (*Mycteroperca marginata*, Lowe, 1834), the rock grouper (*Epinephelus adcensionis*, Osbeck, 1765), and the red grouper (*Epinephelus morio*, Valenciennes, 1828). Finally, samples of other fish species that are marketed in northern Brazil were also evaluated, such as acoupa weakfish (*Cynoscion acoupa*, Lacepède, 1801), the giant sea catfish (*Arius luniscutis*, Valenciennes, 1840), the Spanish mackerel (*Scomberomorus brasiliensis*, Collette, Russo & Zavala-Camin, 1978), the King mackerel (*Scomberomorus cavalla*, Cuvier, 1829), and the white mouth croaker (*Micropogonias furnieri*, Desmarest, 1823) in order to assist in the assessment of the specificity and sensitivity of the approach proposed by PCR.

A total of 125 samples were used in the present study: five samples representing each of the fish species traded in northern Brazil (*C. acoupa, A. luniscutis, S. brasiliensis*, *S. cavalla, and M. furnieri; n = 25*), five samples representing each of the five epinephelid species (*C. fulva, M. marginata, E. adcensionis, E. morio, and E. itajara; n = 25*), and an additional 75 samples of *E. itajara*. The DNA was isolated using a Dneasy kit (Qiagen), and the samples were amplified by PCR. The basic reaction protocol was done with a total volume of 12.5 µl containing 2 µl of the dNTPs mix (1.25 mM), 1.25 µl of buffer (Invitrogen—Tris–HCl, pH 7.8), 0.5 µl of $MgCl_2$ (50 mM), 0.3 from each primer, approximately 1 µl of isolated DNA, 0.1 µl of Taq (5U/µl) polymerase (Invitrogen) and sterile distilled water to complete the final reaction volume. The amplification conditions were 94 °C for 2.5 min, followed by 40 cycles of 35 s at 94 °C, 1 min at 60 °C and 1 min at 72 °C, with a final extension of 5 min at 72 °C. The PCR products were electrophoresed in 1% agarose gel at 120 volts for 45 min. Finally, the two fragments of the *E. itajara* mitochondrial genome amplified by PCR and visualized after electrophoresis were isolated using the QIAquick PCR Purification Kit (Qiagen), subjected to nucleotide sequencing and then deposited on GenBank (MT538327 and MT538328).

The amplification obtained by the 16S-L1987/16S-H2609/16S-RYOP4 primers was evaluated in samples of Epinephelidae fishes. The combination of the universal primers 16S-L1987/16S-H2609 and 16S-RYOP4 enabled the identification of two different patterns. In samples of *E. itajara*, two fragments were amplified and, consequently, two bands were visualized after electrophoresis: one band of 621 base pairs (bp) and the other of 423 bp. In contrast, the other samples of Epinephelidae fishes have only one band (~ 621 bp) (Fig. 2). Thereafter, the remaining 75 samples of *E. itajara* were tested using the 16S-L1987/16S-H2609/16S-RYOP4 primers. The same two-band pattern was observed in all *E. itajara* samples. Regarding the samples belonging to the fish families marketed in northern Brazil, all samples showed only a single band (~ 621 bp) after the amplification procedures (data not shown).

In this approach, the visualization of a 621 bp fragment will indicate that DNA isolation and amplification of genetic material have been successful. It is an indication of the quality of the test performed an internal control. The specificity of this experiment is clearly identified by the visualization of a 423 bp fragment, amplified only in *E. itajara* samples. As a limitation, there is a risk of contamination during the execution of the experiment. However, this risk can be minimized. The appropriate physical structure, the type of storage, the handling of the sample and reagents, the workflow, etc., all steps applied to perform the PCR must be controlled and turned to minimize the chances of contamination. For example, a commercial kit for DNA isolation was used in this study in order to guarantee the quality and avoid contamination of the samples. This step can be adjusted by a DNA isolation protocol that maintains the quality and safety of the procedure, but at a lower cost. Good practices and strategies aimed at developing quality and safety PCR assays are recommended.

The characteristics of the procedure developed are very interesting and can be used to inspect products sold in stores, supermarkets and fairs, mainly where there is a record of

Fig. 1 Location and length of the two fragments of the 16S rRNA gene contained in the mitochondrial DNA (mtDNA) of *Epinephelus itajara*, whose amplification by PCR was described in the present study

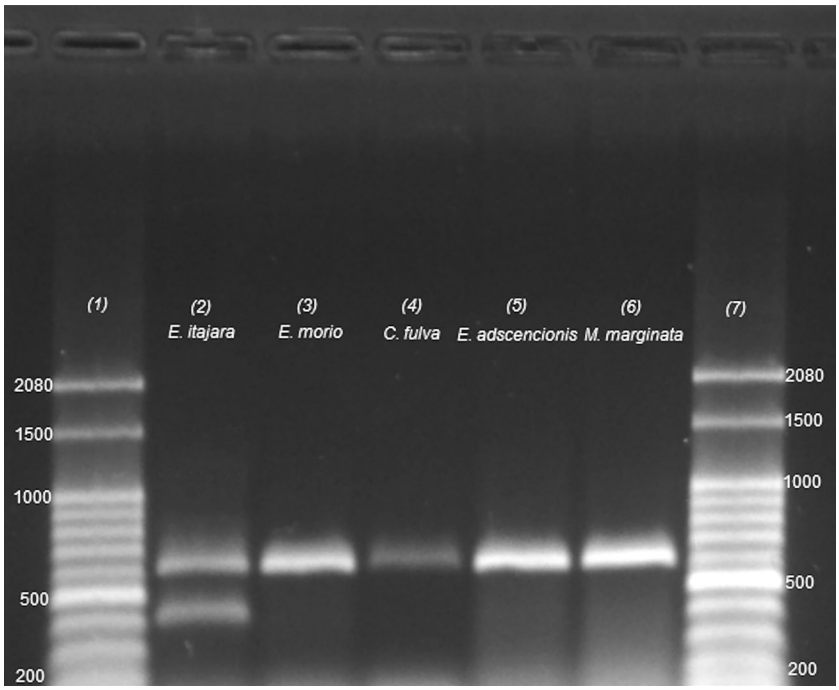

Fig. 2 Agarose gel showing positive amplification of the two fragments of the 16S mtDNA gene of *Epinephelus itajara* using the protocol developed in the present study. (1) and (7) DNA ladder: the numbers refer to the size (200 to 2000 base pairs) of the DNA markers; (2) Sample of *Epinephelus itajara*; (3) Sample of *Epinephelus morio*; (4) Sample of *Cephalopholis fulva*; (5) Sample of *Epinephelus adcensionis*; (6) Sample of *Mycteroperca marginata*

illegal fishing and selling of *E. itajara*. For example, Brazilian agencies for inspection, protection, preservation and conservation of biodiversity such as the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA), and the Chico Mendes Institute for Biodiversity Conservation (ICMBIO) can use the procedure described here or establish partnerships with universities or other public institutions to evaluate suspicious samples whenever necessary.

Acknowledgements The authors are grateful for the instructions, collaboration, support and coexistence with Horacio Schneider (*in memoriam*), an inspiring scientist. This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Number 306233/2009-6) and Fundação Amazônia de Amparo a Estudos e Pesquisas (FAPESPA – PRONEX 2007). YO, RA, IS, MV and GCSO received fellowships from CNPq or Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

References

Cervigón F, Cipriani R, Fischer W, Garibaldi L, Hendrickx M, Lemus AJ, Márquez R, Poutiers JM, Robaina G, Rodriquez B (1993) FAO species identification sheets for fishery purposes: Field guide to the commercial marine and brackish-water resources of the northern coast of South America. FAO species identification sheets for fishery purposes. http://www.fao.org/3/t0544e/t0544e00.htm. Accessed 11 Mar 2014

Chen S, Zhang J, Chen W, Zhang Y, Wang J, Xu D, Zhou Y (2012) Quick method for grouper species identification using real-time PCR. Food Control 27:108–112. https://doi.org/10.1016/j.foodcont.2012.03.011

Craig MT, Hastings PA (2007) A molecular phylogeny of the groupers of the subfamily Epinephelinae (Serranidae) with a revised classification of the Epinephelini. Ichthyol Res 54:1–17. https://doi.org/10.1007/s10228-006-0367-x

Damasceno JS, Siccha-Ramirez R, Oliveira C, Mendonça FF, Lima AC, Machado LF, Tosta VC, Farro APC, Hostim-Silva M (2016) Molecular identification of Atlantic goliath grouper *Epinephelus itajara* (Lichtenstein, 1822) (Perciformes: Epinephelidae) and related commercial species applying multiplex PCR. Neotrop Ichthyol 14:e150128. https://doi.org/10.1590/1982-0224-20150128

Espinosa-Perez H, Carpenter KE, Sedberry G, Zapp-Sluis M, Claro R (2015) *Epinephelus itajara*: The IUCN Red List of Threatened Species 2015: e.T195409A70323667. https://www.iucnredlist.org/species/195409/70323667. Accessed 11 Apr 2020

Horreo JL, Ardura A, Pola IG, Martinez JL, Garcia-Vazquez E (2013) Universal primers for species authentication of animal foodstuff in a single polymerase chain reaction. J Sci Food Agric 93:354–361. https://doi.org/10.1002/jsfa.5766

Instituto Chico Mendes de Conservação da Biodiversidade, Ministry of the Environment (ICMBio/MMA), Brazil (2018) Livro Vermelho da Fauna Brasileira Ameaçada de Extinção: Vol. VI - Peixes. https://www.icmbio.gov.br/portal/images/stories/comunicacao/publicacoes/publicacoes-diversas/livro_vermelho_2018_vol6.pdf. Accessed 20 Apr 2020

Kalendar R, Lee D, Schulman AH (2014) FastPCR software for PCR, in silico PCR, and oligonucleotide assembly and analysis. Methods Mol Biol 1116:271–302. https://doi.org/10.1007/978-1-62703-764-8_18

Melo-Palmeira CA, Rodrigues-Filho LFDS, Sales JBL, Vallinoto M, Schneider H, Sampaio I (2013) Commercialization of a critically endangered species (largetooth sawfish, *Pristis perotteti*) in fish markets of northern Brazil: authenticity by DNA analysis. Food Control 34:249–252. https://doi.org/10.1016/j.foodcont.2013.04.017

Palumbi SR, Martin A, Romano S, Mcmillan WO, Stice L, Grabowski G (1991) The Simple Fool's Guide to PCR. Vers 2.0. University of Hawaii, Honolulu

Sadovy Y, Eklund AM (1999) Synopsis of biological data on the Nassau grouper, *Epinephelus striatus* (Bloch, 1792), and the jewfish, *E. itajara* (Lichenstein, 1822). NOAA Technical Report NMFS, 146. http://spo.nwr.noaa.gov/tr146.pdf. Accessed 10 Apr 2020

Silva-Oliveira GC, Rêgo PS, Schneider H, Sampaio I, Vallinoto M (2008) Genetic characterisation of populations of the critically endangered Goliath grouper (*Epinephelus itajara*, Serranidae) from the Northern Brazilian coast through analyses of mtDNA. Genet Mol Biol 31:988–995. https://doi.org/10.1590/S1415-47572008005000016

Silva-Oliveira GC, Silva ABC, Oliveira Y, Nunes ZP, Torres RA, Sampaio I, Vallinoto M (2013) New nuclear primers for molecular studies of Epinephelidae fishes. Conserv Genet Resour 5:165–168. https://doi.org/10.1007/s12686-012-9759-6

Torres RA, Feitosa RB, Carvalho DC, Freitas MO, Hostim-Silva M, Ferreira BP (2013) DNA barcoding approaches for fishing authentication of exploited grouper species including the endangered and legally protected goliath grouper *Epinephelus itajara*. Scientia Marina 77:409–418. https://doi.org/10.3989/scimar.03805.29A