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Rapid polymerase chain reaction–restriction fragment length polymorphism method for discrimination of the two Atlantic cryptic deep-sea species of scabbardfish

SERGIO STEFANNI,* RAUL BETTENCOURT,* HALVOR KNUTSEN† and GUI MENEZES*

*IMAR/DOP, University of the Azores, Cais Sta Cruz, 9901-862 Horta, Azores, Portugal, †Institute of Marine Research, Flødevigen, N-4817 His, Norway

Abstract

The present investigation provides an efficient diagnostic method based on polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis to discriminate between two cryptic species of scabbardfish, *Aphanopus carbo* and *A. intermedius*, with commercial relevance in several European fish markets. Two DNA fragments from the mtDNA, including control region and partial cytochrome oxidase subunit I genes of about 1100 bp and 700 bp, respectively, were isolated by PCR amplification. Digestion of the amplicon including the control region with *Hae*II and the amplicon including the COI gene with *Sau*3AI restriction enzymes allowed an unequivocal discrimination between the two scabbardfish species. This PCR–RFLP method allowed a clear and rapid discrimination of the trichiurid species studied.

Keywords: Atlantic, control region, cytochrome oxidase subunit I, PCR-RFLP, species discrimination

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Scabbardfish belong to the family Trichiuridae and are included in the genus *Aphanopus* that comprises six species distributed worldwide (Parin 1986, 1995). In the Atlantic, only two species are known to science, *Aphanopus carbo* (Lowe 1839) and *Aphanopus intermedius* (Nakamura & Parin 1993; Parin ~~1993~~). These species are indistinguishable in morphology but, as at first it was thought that different latitudinal distribution ranges separated them, the problem of identification was assumed to be solved. Only recently, Stefanni & Knutsen (2007) demonstrated that these two species live in sympatry in some areas: Azores, Madeira, Canaries and the northwestern coast of Africa.

Scabbardfish is a valuable marketed fish in several European countries, in particular the Portuguese island of Madeira, where there is a specialized fishery (Maul 1950) responsible for 55% of local catches (FAO 2002), and in Sesimbra (mainland Portugal) with an established fishery targeting this fish since the 1980s. Nevertheless, when marketed, these two species are considered as single unit sold as black scabbardfish (*A. carbo*), providing incomplete information on species captures and potential consequences to fisheries management.

As mentioned above, the specific morphological characters (size, shape, appearance and basic taxonomic characters) of these two scabbardfish species overlap (~~Carvalho *et al.*, in preparation~~): only genetic markers secure a reliable way of distinguishing the two species. However, as the use of mitochondrial DNA (mtDNA) sequences (Stefanni & Knutsen 2007) is a costly and time-consuming method, we here provide a novel and efficient diagnostic method for discriminating between two cryptic species of scabbardfish. This method relies on polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) analysis using two independent fragments encoding the mitochondrial control region (CR) and cytochrome oxidase subunit I (COI).

A total of 145 scabbardfish were collected from five different locations (30 from the Azores, 30 from Madeira, 30 from the Canaries, 30 from Morocco and 25 from mainland Portugal) and were sequenced for two mtDNA fragments: control region + tRNAPhe + partial 12S rRNA (GenBank Accession nos. EU853865–EU854002) and partial COI

Correspondence: Sergio Stefanni, Fax: +351 2922 00411;
E-mail: sstefanni@uac.pt

(GenBank Accession nos. EU854003–EU854146). The same individuals were also analysed for a detailed morphological investigation (Carvalho *et al.* in prep.).

Small portions of white muscle were fixed in 95% ethanol before being stored at –20 °C. Mitochondrial DNA was extracted following the procedure of Sambrook *et al*. (1989), with slight modifications (Stefanni 2000). Fragments of the mtDNA including CR were amplified following the protocol and PCR profile reported in Stefanni & Knutsen (2007). The same pairs of primers were also used for sequencing. Fragments including partial COI were amplified using the FishF1 and FishR1 primers (Ward *et al*. 2005). The thermal cycling profile first cycle started with 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 1.5 min with a final extension at 72 °C for 7 min. PCR products of both mtDNA regions were visualized on 1.2% agarose gels and the most intense products were selected for sequencing.

From close inspection of these PCR sequences, we selected suitable restriction endonucleases that target polymorphic regions producing a species-specific pattern of hydrolysed fragments that can be easily identified based on their size. PCR products derived from amplification of both mtDNA regions were subjected to restriction digestion without previous purification. All restriction enzymes employed, *Eco*RI and *Hae*II for CR + 12S rRNA amplicon, and *Sau*3AI for partial COI amplicon, were purchased from Promega. These restriction nucleases were selected directly from sequences because they target polymorphic regions within the amplicon producing a species-specific pattern of hydrolysed fragments. Reaction mixtures were slightly modified from the protocol proposed by the manufacturer and included: 2 µL of PCR product, 2 µL of buffer (RE10× for *Eco*RI; RE for *Hae*II and *Sau*3AI), 0.5 µL (for *Eco*RI) or 0.2 µL (for *Hae*II and *Sau*3AI) of BSA, 0.5 µL (for *Eco*RI) or 1 µL (for *Hae*II and *Sau*3AI) of enzyme solution and, finally, purified water to a final volume of 20 µL. All mixtures were incubated at 37 °C for 4 h.

DNA fragments were separated by size using high-resolution pre-casted PhastGels and the PhastSystem electrophoresis apparatus (GE Healthcare Life Sciences), while visualization of the bands was based on silver staining technique following the manufacturer's protocol (Bio-Rad). After testing different polyacrylamide homogeneous and gradient gels, we considered that the PhastGel with gradient 4–15 was the most efficient for our purpose.

The two mtDNA fragments resulted in different length, approximately 1100 bp (including complete control region + tRNAPhe + partial 12S rRNA) and about 700 bp (including partial COI gene starting from its origin). Aligned sequences of shorter portions containing polymorphic sites of these two amplicons for the two species are shown in Tables S1 and S2, Supporting information.

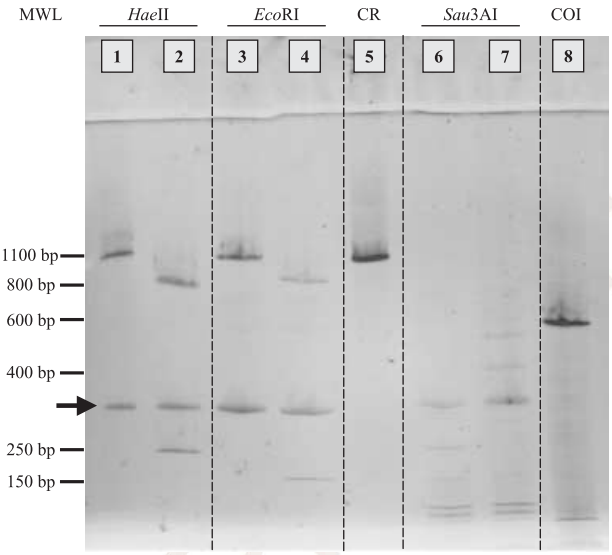

Fig. 1 Polyacrylamide PhastGel with gradient 4–15 used for PCR-RFLP analysis of control region + tRNAPhe + partial 12S rRNA genes (1–4) and partial COI (6–7). PCR amplicons previous to digestions are in wells 5 and 8, respectively. PCR products after digestions of *Aphanopus carbo* are in slots 1, 3 and 6; while *Aphanopus intermedius* are in 2, 4 and 7. MWL reproduces the molecular weight ladder. The arrow points at a band that corresponds to the electrophoretic mobility of the restriction enzyme (here visualized using the silver-staining protocol).

Direct digestion of the PCR products separately with *Eco*RI and *Hae*II (Table S1) and *Sau*3AI (Table S2) resulted in species-specific restriction pattern.

For the amplicon that included the CR, the corresponding expected profiles were as follows: an uncut band for *A. carbo* and double bands for *A. intermdius*: about 200-bp and 900-bp fragments using *Eco*RI; or about 300-bp and 800-bp fragments using *Hae*II (Fig. 1). On the other hand, for the amplicon that included partial COI gene, the corresponding expected profiles using *Sau*3AI were three bands for *A. carbo*; about 100-bp, 200-bp and 400-bp fragments; and two bands for *A. intermedius*: about 100-bp and 600-bp fragments (Fig. 1).

An additional 35 scabbardfish, collected on seamounts south of the Azores (Atlantis, Plato, Irving and Great Meteor) during the DeeCon cruise 2007, were screened for species identification by this technique to verify the efficiency of the protocol. All 35 specimens were unambiguously assigned to either *A. carbo* (34 samples) or *A. intermedius* (one sample) using the restriction enzymes *Hae*II with the amplicon containing the CR and *Sau*3AI with the COI fragment. The RFLP results were further verified by direct sequencing of the PCR products from representative specimens. However, two individuals assigned to *A. carbo* based on *Hae*II digest of CR and *Sau*3AI digest of COI, showed the restriction profile of *A. intermedius* when the CR region was cut with

*Eco*RI. Sequencing of these samples revealed that they possessed a single nucleotide substitution (C to T) at position 178, which is shared by *A. intermedius* samples. This finding underlies the importance of assaying multiple polymorphic sites in making species determinations.

This simple, robust and reliable protocol involving PCR amplification of either the CR or COI mtDNA regions followed by RFLP analyses with *Hae*II and *Sau*3AI (respectively) can be routinely performed in differentiating the two commercially important species of scabbardfish. The PCR–RFLP analysis presented here allows an unequivocal discrimination between the two cryptic species of scabbardfish, *A. carbo* and *A. intermedius*, using two mtDNA markers. This technique appears fast and inexpensive compared to DNA sequencing; therefore, it has been largely applied in the field of fish authentication (es: Cespedes *et al*. 1998, 2000; Aranishi 2005a, b; Chakraborty *et al*. 2005).

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 Portion of aligned sequences for the first 600 bp of the CR of the two scabbardfish species. Polymorphic sites are in bold and the binding sites for the endonucleases EcoRI (in light grey) and HaeII (in dark grey) are shaded

Table S2 Portion of aligned sequences for the first 600 bp of the COI gene of the two scabbardfish species. Polymorphic sites are in bold and the binding sites for the endonucleases Sau3AI are shaded

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