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Development of a PCR-RFLP marker to genetically distinguish *Prosorhynchus crucibulum* and *Prosorhynchus aculeatus*

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ABSTRACT

The cercariae stages of *Prosorhynchus crucibulum* and *Prosorhynchus aculeatus* are morphologically indistinguishable. However, the differentiation of these two species is crucial to understand the transmission dynamics between these primary hosts (mussels) and the secondary hosts (fish). In this way, the objective of this study is to develop an accurate molecular identification tool to differentiate the cercariae stage of *P. crucibulum* and *P. aculeatus*. We targeted the 18S nuclear ribosomal DNA region by PCR amplification and sequenced this amplicon. By generating these sequences, we developed a RFLP tool with the use of the enzymes HincII and FokI that produced different restriction profiles between *P. crucibulum* and *P. aculeatus*. Each enzyme generated different-sized fragments specific to the species examined and no cross-reaction between the species was detected in their restriction pattern. By sequencing, no intraspecific-polymorphism was detected since there is 100% homology among *P. aculeatus* or *P. crucibulum*. These results indicate that PCR-linked restriction analysis of the 18S rDNA region provided us with rapid and reliable molecular tools for distinction of the cercariae of these species. The sequences generated were deposited in GenBank accession numbers for *P. crucibulum* cercariae (FJ463407, FJ463408 and FJ463409) and adult worm (FJ429096, FJ429097), and for *P. aculeatus* adult (FJ429094 and FJ429095).

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1. Introduction

Prosorhynchus crucibulum (Rudolphi, 1819; Odhner, 1905) and *P. aculeatus* (Odhner, 1905) are parasites of the Bucephalidae family, with a complex life cycle, infecting successive hosts in the marine environment: the adult stage development occurs inside the digestive tract of *Conger conger*, where they reproduce asexually and produce eggs that will be released with the host faeces; inside the egg the development of the miracidium takes place , the first larval phase in the worm life cycle; this miracidium infects the mussels, the first intermediate host, where it develops into a sporocist, which reproduces asexually and generates innumerable cercariae. It was reported that the larval form of *Prosorhynchus* spp. infects the fishes (*Sole sole*, *Scophitalmus maximus*, *Limanda limanda*, *Onus mustelus*, *Pomatoshistus minutus*, *Pomatomus saltatrix*, *Liparis liparis*, *Boops boops* and *Pleuronectes flesus*) and encysts into a metacercariae [1–8]; those infected hosts will be eaten by conger eels developing the adult form and thus closing the life cycle.

In southern Europe, *Mytilus* spp. is a highly appreciated mollusk and therefore an important commercial species; it is the first intermediate host of the bucephalid digenean *Prosorhynchus* sp. [3,9,10]. The bucephalid of the genus *Prosorhynchus* had been described as causing serious problems, like castration and weakening of the adductor muscle [11–17]. Furthermore, molecular data from these worms are limited, since in the GenBank there is one sequence deposited referring to the genus of *Prosorhynchus* sp. and not specifying the species.

Taking into account the fact that *P. crucibulum* and *P. aculeatus* are morphologically undistinguishable at cercariae stage, but not in adult stage, the molecular analysis could be a useful tool to identify the species of these parasites at early stages. In this way, the main aim of this study is to develop molecular markers, that would allow us to rapidly distinguish the cercariae of the *P. crucibulum* and *P. aculeatus* using PCR-RFLP analysis of the 18S nuclear ribosomal DNA partial region, and thus, collaborate in the development of tools for controlling this parasite.

2. Materials and methods

2.1. Species identification and sequences examined

The adult worms of *P. crucibulum* and *P. aculeatus* were isolated from the digestive tract of *Conger conger* fish, and the main differences

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observed were the size and shape of the rhynchus at the anterior end, which in *P. crucibulum* is larger and triangular and in *P. aculeatus* is small and oval [3,18]. The cercariae were collected in the mantle from *Mytilus galloprovincialis*. For identification of the genus of the cercariae, their morphology was compared with that of *P. squamatus* and *P. crucibulum* [3,19]. In order to identify the species, molecular analysis was performed. For that and since there are no molecular data for these two species deposited in the GenBank , we obtained nucleotide sequences from several genera including *Prosorhynchus sp*., as suggested in the literature [20], and after alignment of these homologous sequences, primers targeting a fragment of the 18S rDNA were designed.

2.2. DNA preparation, amplification and sequencing

Adult worms of *P. crucibulum* ($n=1$, 5, 10, 15, and 22) and *Prosorhynchus aculeatus* ($n=1$, 5, 8, 13 and 22) were collected from the digestive tract of 14 conger eels (*Conger conger*). The worms were washed in saline solution and stored in 70% ethanol. A commercial kit (QIAGEN) was used to extract DNA from worms according to the protocol of the manufacturer. Three samples of cercariae from *Mytilus galloprovincialis* ($n=3$), its intermediate host, were collected from Aveiro estuary (40° 38. 620′N 8° 44. 802′W), for genus identification. In order to obtain molecular data that enable us to distinguish the two worm species by sequencing or by the use of restriction sites, a partial region of the 18S rDNA was amplified by PCR using the primers obtained by us: SSU_Fwd (5′TCTGGGTCGCATC3′) and SSU_Rev (5′ CCATTACTTCGGATC 3′). Sensitivity of the PCR method was accessed by using different numbers of the adult worms.

PCR was performed using the described primers in a total volume of 50 µl. Each reaction contained 1 standard unit Taq polymerase (Thermo Scientific), 5 µl PCR buffer 10×, 4 mM $MgCl_2$, 0.2 mM µl dNTP (Roche), 200 nM of each primer, 5 and 10 µl template DNA of *P. crucibulum* and *P. aculeatus*, respectively, made up to 50 µl with water. This mix was placed in a ThermoCycler Primus 25/96 Hain Lifescience with the following conditions: initial hot start of 94 °C during 4 min followed by 40 cycles of 95 °C for 30 s, 40 °C for 30 s and 72 °C for 30 s. Final extension of the product was at 72 °C for 5 min.

After amplification, the PCR products were checked by electrophoresis in a 1% agarose gel (BioRad) and fragment sizes were estimated using a λ DNA-HindIII digest ladder (New England BioLabs). For PCR product purification, a low melting 1% agarose gel was made (Promega) and a commercial kit (GFX PCR DNA and Gel band purification kit, GE Healthcare) was used according to the manufacturer's instructions. DNA fragments were sequenced in both strands in an external laboratory (EUROFINS MWG OPERON, Germany). The sequences obtained were compared with the sequences in the Genbank by using the Blastn tool (www.ncbi.nlm.nih.gov). These sequences were aligned with reference to the 18S rDNA region from *Prosorhynchus sp.* (AJ224458) within PROSEQ® version 2.91 (ProSeq, Dimitri Filatov). Sequence data from the two species were compared and restriction analysis simulated in the software MB® DNA Analysis 6.82 (Molbiosoft, Oleg Simakov).

2.3. PCR-RFLP analysis

The PCR-amplified products were then subjected to digestion with two restriction enzymes within the 18S rDNA partial region: FokI and HincII. Digestion was performed in a total volume of 20 µl containing 17.3 µl of PCR product, 5 units of restriction enzyme (10 units/µl) (Promega), 2 µl of 10× reaction buffer, and 0.2 µl of BSA (10 µg/µl) at 37 °C for 1 h 30 min. The resulting restriction fragments were separated by electrophoresis in 2% agarose gel. Restriction profiles of digested PCR products were checked, using UV illumination in Gel Documentation System (BioRad), by comparison with molecular markers of 50 bp (50 bp DNA ladder, New England BioLabs).

3. Results

Concerning the sensitivity, the PCR was positive when applied to the DNA extracted from 1, 5, 10, 15 and 22 adult worm of *P. crucibulum* and 1, 5, 8, 13 and 22 adult worms of *P. aculeatus* (Fig. 1).

The PCR products of the adult worms sequenced in both directions showed high similarity to *Prosorhynchus* sp. but also presented some differences. This molecular analysis of the sequences from the 18S rDNA partial region (Fig. 2) of adult worms of *P. crucibulum* and *P. aculeatus* revealed fixed nucleotide differences that allow us to distinguish these two species. These sequences were deposited in the GenBank under the accession nos. FJ429096 and FJ429097 for *P. crucibulum* and FJ429094 and FJ429095 for *P. aculeatus*. The sequences showed no intragenic variation once no differences were found inside the sequences of *P. crucibulum* or *P. aculeatus*. The identification of cercariae was made by PCR and sequencing taking into account the differences in DNA sequence previously observed in adult worms. These data were deposited in the GenBank under the accession nos. FJ463407, FJ463408 and FJ463409. We observe that all cercariae were identified as *P. crucibulum* with 100% homology, and no differences were observed between the sequences of cercariae and adult worms of *P. crucibulum* (Figs. 2 and 3).

The PCR-RFLP of the 18S rDNA partial region using restriction enzymes HincII and FokI generated different-sized fragments specific to the species examined (the adult worms) confirmed by sequencing. These results were compared with the digestion of the 18S rDNA partial region of cercariae. The HincII enzyme cuts twice the 18S rDNA *P. crucibulum* sequence (at 118 and 151 bp) creating five possible bands in the agarose gel (33, 118, 151, 166, and 199 bp), although only 118 bp, 166 bp and 199 bp bands are observed. In *P. aculeatus* 18S rDNA sequence, the same enzyme cuts only in one position (151 bp) creating two bands of 151 and 166 bp in the agarose gel (Fig. 4). For the same PCR, the FokI enzyme cuts the 18S rDNA sequence in only one site, at 176 bp, creating two bands (176 and 141) and did not digest the PCR fragment of *P. aculeatus* (Fig. 5). The use of HincII and FokI enzymes in the 18S rDNA region produced the same restriction pattern in the cercariae as in the adult.

4. Discussion

The knowledge of the genotype from the two species is relevant to our understanding of these species life cycle, as well as the development of reliable molecular tools for the distinction between cercariae of the *P. crucibulum* and *P. aculeatus*. In 1973, the morphology and life cycle of *P. crucibulum* was reported [3] and the life cycle of the *P. squamatus* was also confirmed [19]. Matthews [3] verified that both cercariae developed in branching sporocysts in *Mytilus edulis* and the metacercariae encysted in eight fishes that served as second intermediate hosts. However, the cercaria stage of *P. crucibulum* and *P. squamatus* is easily distinguished, by showing

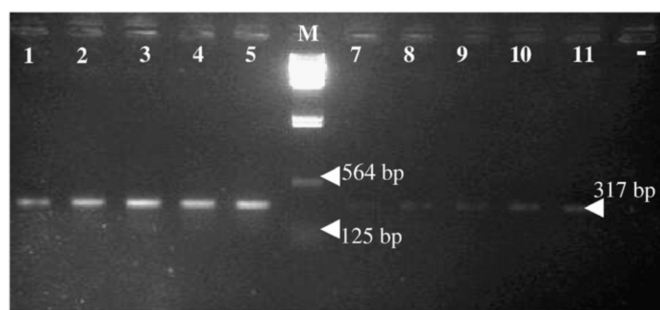

Fig. 1. Sensitivity of PCR applied to the extracted DNA. Lanes 1–5 *Prosorhynchus aculeatus;* Lane 6 (M): DNA/HindIII-23130pb marker; Lanes 7–11 *P. crucibulum;* Lane 12 (−): negative.

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P. aculeatus    ttctgggtcg  catgactgct  tgccgttgct  cagcatccgg  tctgatcgtg  attggactgt   60
P. crucibulum   ..........  ..........  ..........  ..........  ..........  ..........   60
cercariae       ..........  ..........  ..........  ..........  ..........  ..........   60

                                                                                 HincII
P. aculeatus    ttgttgggtc  ggcgtagtgg  ttgtgcagcc  tttctgccgt  gtctgtttcg  acaggtgttg  120
P. crucibulum   ..........  ..........  ..........  ..........  ..........  ..........  120
cercariae       ..........  ..........  ..........  ..........  ..........  ..........  120

                                                    HincII                  FokI
                *                                               *
P. aculeatus    gctggctgat  gggttcgtcc  tgttggcttg  ttgacatgct  tccagatgcc  tttaaacggg  180
P. crucibulum   a.........  ..........  ..........  ..........  ...g......  ..........  180
cercariae       a.........  ..........  ..........  ..........  ...g......  ..........  180

P. aculeatus    tgtctggggc  ggacggcatg  tttactttga  acaaatttga  gtgctcaaag  caggcctgtg  240
P. crucibulum   ..........  ..........  ..........  ..........  ..........  ..........  240
cercariae       ..........  ..........  ..........  ..........  ..........  ..........  240

P. aculeatus    tgcctgaaaa  gtcttgcatg  gaataatgg   ataggacttc  ggttctattt  tgttggtttt  300
P. crucibulum   ..........  ..........  ..........  ..........  ..........  ..........  300
cercariae       ..........  ..........  ..........  ..........  ..........  ..........  300

P. aculeatus    cggatccgaa  gtaatgg                                                     317
P. crucibulum   ..........  .......                                                     317
cercariae       ..........  .......                                                     317
```

Fig. 2. Sequence alignment of 18S rDNA partial region from *Prosorhynchus aculeatus* (adult worm), and *P. crucibulum* (cercariae and adult). (asterisks): polymorphic sites; (arrows): cut sites by the HincII or FokI restriction enzymes.

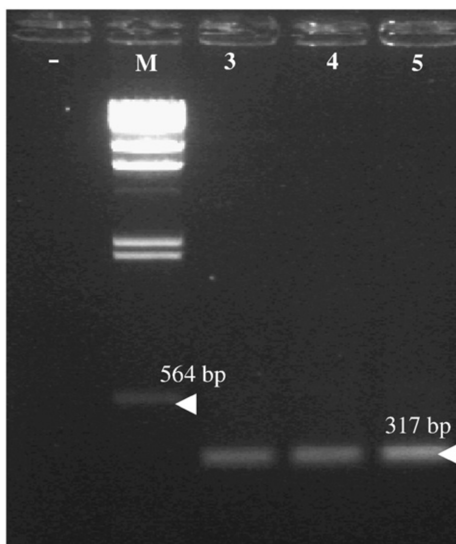

Fig. 3. Electroforesis of the PCR over the DNA of the cercariae isolated from *Mytilus galloprovincialis*. Lane 1 (−): negative; Lane 2 (M): lambda DNA/HindIII-23130pb marker; Lanes 3–5, PCR products of DNA of cercariae.

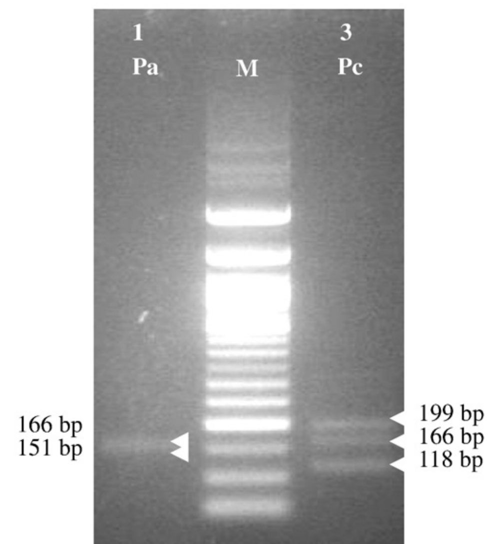

Fig. 4. HincII-digested PCR products display different profiles corresponding to the expectation of restriction maps. Lane 1 (Pa): *Prosorhynchus aculeatus*; Lane 2 (M): Marker 50 bp; Lane 3 (Pc): *P. crucibulum*.

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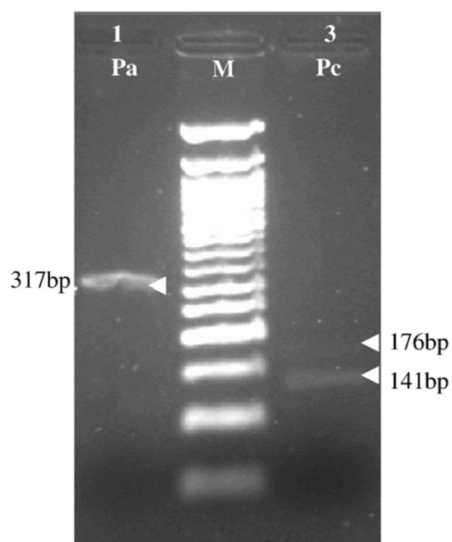

Fig. 5. FokI-digested PCR products, displaying different profiles corresponding to the expectation of restriction maps. Lane 1 (Pa): Undigested PCR products of *Prosorhynchus aculeatus;* Lane 2 (M): Marker 50 bp; Lane 3 (Pc): *P. crucibulum*.

differences in the morphology of the base of the tail. The anatomy of the adult worm *Skrjabiniella aculeatus* (Odhner), later renamed *P. aculeatus* [18] is well known, however, the life cycle of this parasite is yet little understood. It is known that a bivalve is the first intermediate host in the marine environment, and the definitive host of *P. crucibulum* and *P. aculeatus* parasites is *Conger conger* [8,21,22].

Few studies are available about the polymorphism between the species *P. crucibulum* and *P. aculeatus*. This information is essential for the development and refinement of systems for pathogen identification using molecular tools. In 1999, the phylogenetic relationship within the 18S rDNA partial region between 13 species of Fellodistomidae from four subfamilies and eight species from seven other digenean families was reported. In this research, the 18S rDNA partial region of *Prosorhynchus* sp. from family Bucephalidea was obtained, and it was possible to compare our results within the Genbank database with the results of *Prosorhycnhus* sp. [20], from the same V4 partial region. The cercariae from *P. crucibulum* and *P. aculeatus* are morphologically undistinguishable owing to the life cycle of this parasite being as yet poorly understood. The differentiation of these two species is crucial to understand the transmission dynamics between the primary and secondary host, the fish. In this way, the development of a molecular tool able to differentiate the cercariae stage of both species is crucial. We targeted the 18S nuclear ribosomal DNA partial region and generated data concerning the DNA sequence of the species *P. aculeatus* and *P. crucibulum* previously characterized according to morphological aspects. We observed fixed differences in the DNA sequence between the two species of parasites, *P. aculeatus* and *P. crucibulum*: two polymorphic sites within the 18S partial region (121 pb and 164 pb of the amplified product) which enable us to differentiate the two species. A 100% homology was detected between all the *P. crucibulum* sequences or *P. aculeatus* sequences. Additionally, we verified that only one adult worm DNA sample can yield enough DNA for detection by PCR method. The PCR-RFLP is a rapid, easy and cheap technique used in species identification. Based on these DNA differences between the two species, restriction enzymes HincII and FokI enabled us to easily distinguish the cercariae of species *P. crucibulum* and *P. aculeatus*.

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