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Cardicola aurata sp. n. (Digenea: Sanguinicolidae) from Mediterranean *Sparus aurata* L. (Teleostei: Sparidae) and its unexpected phylogenetic relationship with *Paradeontacylix* McIntosh, 1934

A.S. Holzer [a,*], F.E. Montero [b], A. Repullés [a], M.J. Nolan [c], A. Sitja-Bobadilla [d], P. Alvarez-Pellitero [d], C. Zarza [e], J.A. Raga [a]

[a] Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia, P.O. Box 22085, 46071 Valencia, Spain
[b] Department of Animal Biology, Plant Biology and Ecology, Autonomous University of Barcelona, 08193 Bellaterra, Barcelona, Spain
[c] Department of Veterinary Science, Parasitology Section, University of Melbourne, 250 Princes Highway, Werribee, Victoria 3030, Australia
[d] Instituto de Acuicultura de Torre de la Sal (IATS) (CSIC), 12595 Ribera de Cabanes, Castellón, Spain
[e] Fish Health Service, Skretting, Carretera de la Estación s/n, 09620 Cojóbar (Burgos), Spain

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ABSTRACT

A new sanguinicolid trematode, *Cardicola aurata* sp. n., is described from gilthead seabream *Sparus aurata* L., from off the Spanish Mediterranean coast. The morphology of *C. aurata* sp. n. generally agrees with the diagnosis of the genus, however, in contrast to all other reported *Cardicola* spp. the male pore is located submedially at the posterior end of the body instead of sinistrally before the posterior end of the body. Based on a comparison of the morphology as well as partial 28S and ITS2 rDNA sequence data from the present species with that from closely related species, it was decided to emend the diagnosis of *Cardicola* rather than create a new genus, as the aberrant position of the male pore is likely to be an autapomorphy. The phylogenetic analyses revealed a close relationship between *Cardicola* and *Paradeontacylix*, two genera with considerable morphological differences; *C. aurata* sp. n. occupies a position intermediate to these genera. Thus, a morphological comparison of *Cardicola*, *Paradeontacylix* and *Braya*, a genus which is morphologically similar to *Cardicola* but clusters basal to the *Cardicola*/*Paradeontacylix* clade, was conducted. The results of this comparison showed that despite large differences with regard to body shape, the organisation of the internal organs is very similar in species of *Cardicola* and *Paradeontacylix*. The synopsis of morphological data and molecular phylogeny allows for interpretations regarding the importance of different morphological features for the phylogenetic inference of the Sanguinicolidae.

1. Introduction

The Sanguinicolidae von Graff, 1907, trematodes inhabiting the blood system of marine and freshwater fish, currently consists of 25 recognized genera (April 2008; [1–6]). Whereas most genera can be distinguished by combinations of morphological characters, morphology sometimes fails to distinguish closely related congeners [5,6]. However, molecular variation can be more pronounced between such species and has been used to support the proposal of new sanguinicolid species and genera where morphological differences are few (for example [5,6]). As a result, molecular studies have become important when examining the inter-relationships of the Sanguinicolidae and resolving the family's taxonomy.

In the Mediterranean, cage-cultured *Sparus aurata* L. (Perciformes: Sparidae) suffer inflammatory reactions and necrosis of the gill tissues due to the presence of sanguinicolid eggs in the primary and secondary gill filaments [7]. Unfortunately, these authors did not provide a detailed description of the parasite in their publication. The aim of the current study was to conduct an epidemiological survey of several *S. aurata* culture sites along the Spanish Mediterranean coast and investigate the prevalence and seasonality of this sanguinicolid species, to describe it morphologically and molecularly and estimate its phylogenetic position within the Sanguinicolidae. During this study, a close molecular relationship of the investigated sanguinicolid with species of *Cardicola* Short, 1953 and the morphologically distinct genus *Paradeontacylix* McIntosh, 1934 was determined. This led to a re-evaluation of morphological features of these two genera and *Braya* Nolan & Cribb, 2006, which was supposed to be the closest relative of *Cardicola* [5].

* Corresponding author. Marine Zoology Unit, Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia, P.O. Box 22085, 46071 Valencia, Spain. Tel.: +34 963543685; fax: +34 963543733.
E-mail address: astrid.holzer@uv.es (A.S. Holzer).

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2. Materials and methods

2.1. Collection sites and methods

S. aurata were collected from off-shore sea cages at six sites in the Western Mediterranean, with a longitudinal distribution over approximately 450 km, from the Delta of the Ebro (40° 13′ N, 0° 17′ E) in the north to Carboneras (36° 58′ N, 1° 53′ W) in the south (Fig. 1). From sites 1 and 2 (Fig. 1), 0+ *S. aurata* of 9–16 cm total length and 15–65 g body weight were obtained every one to two months between January 2006 and May 2007. Fifteen to 30 fish were examined for the presence of blood flukes at each sampling date. Live fish were transported to the Marine Zoology Unit at the University of Valencia in aerated sea water or were killed and placed on ice. All fish were examined within 24 h of being euthanized by cranial concussion and neural pithing. The heart, gills, kidney, spleen and liver were removed from the fish and examined in 0.85% saline using a dissection microscope. The muscles and blood vessels associated with the pectoral girdle were also screened under a dissection microscope. Adult flukes were removed from host tissues with a pipette and transferred to a 1.5 ml Eppendorf tube containing saline where they were shaken vigorously to remove host tissue from their surfaces. Sites 3–6 (Fig. 1) were sampled on four dates between April 1999 and May 2000 and on five dates between May 2003 and March 2005. Each sample consisted of eight to 20 fishes which were screened for eggs by examining the various gill arches histologically, without screening for adults. Gills were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 24 h in the fridge. They were then washed in phosphate buffer, dehydrated in a graded series of ethanols, transferred to resin and semithin sections were cut, stained with methylene blue and mounted with Entellan® (Merck).

2.2. Morphological data

Digital images of sanguinicolids were taken of three live specimens. Thereafter, two specimens were fixed in 70% ethanol and stained in iron acetocarmine according to Ref. [8]. Specimens were differentiated in 1% HCl and 70% ethanol and dehydrated in an ethanol series (70% to 100%), transferred into dimethyl phthalate (98%) for clearing and mounted in Canada balsam. Measurements, in micrometres, were taken on digital images of live individuals, using the computer program Image Tool 3.00 (developed at the University of Texas Health Science Center at San Antonio and available at http://ddsdx.uthscsa.edu/dig/itdesc.html). Measurements are given as a range followed by the mean in parentheses. Worms were drawn using a drawing tube.

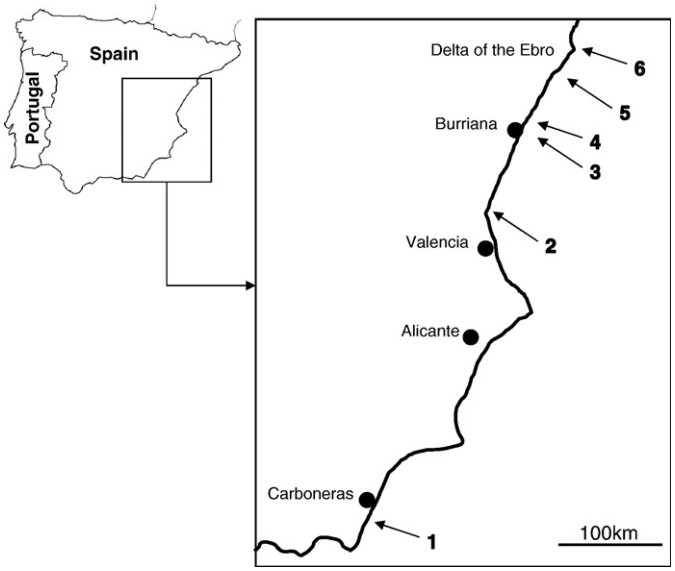

Fig. 1. Culture sites of *Sparus aurata* off the Spanish Mediterranean coast (Sites 1–6) which were checked for *Cardicola aurata* sp. n. infection.

Due to the unexpected findings of the present study morphological voucher specimens of *Cardicola milleri* Nolan & Cribb, 2006 (QM G22513, QM G22514), *Cardicola lafii* Nolan & Cribb, 2006 (QM G225230, QM G225231), *Cardicola parilus* Nolan & Cribb, 2006 (QM G225233, QM G225234), *Cardicola palmeri* Bullard & Overstreet, 2003 (USNPC 93199.00/MT32-2P), *Cardicola forsteri* Cribb, Daintith & Munday, 2000 (USNPC 94131.00/133A-23), *Cardicola lauruei* Short, 1953 (USNPC 37378.00/395), *Braya psittacus* Nolan & Cribb, 2006 (QM G225249, QM G225250), *Braya yantschi* Nolan & Cribb, 2006 (QM G225253) and *Braya jexi* Nolan & Cribb, 2006 (QM G225257, QM G225258) were obtained from the Queensland Museum (QM) and from the US National Parasite Collection (USNPC), for comparison with the present material in addition to published drawings and descriptions.

2.3. Molecular data and phylogeny

One worm was dissolved in 300 µl of TNES urea (10 mM Tris–HCl (pH 8), 125 mM NaCl, 10 mM EDTA, 0.5% SDS, 4 M urea) and genomic DNA extracted using a phenol-chloroform protocol described previously [9].

Using PCR, we amplified two DNA fragments from the genomic ribosomal DNA (rDNA) tandem units. Partial 28S ribosomal DNA (D1–D3) was amplified using the forward primer 'U178' (5′-GCACCCGCTAAYTTAAG-3′) [10] and the reverse primer 'L1642' (5′-CCAGCGCCATCCATTTTCA-3′) [10]) and the internal transcribed spacer 2 (ITS2) was amplified using '3S' (5′-GGTACCGGT GGATCACGTGGCT AGTG-3′,) [11] and 'ITS2.2' (5′-CCTG GTTAGTTTCTTTTCCTCCGC-3′) [11]. PCR cycle sequencing reactions were performed in 30 µl reactions containing 1.5 units of THERMOPRIME PLUS DNA polymerase and the related 10× buffer containing 15 mM $MgCl_2$ (ABgene, UK), 0.2 mM of each dNTP, 0.5 mM of each primer and approximately 80 ng of template DNA. For both PCRs, initial denaturation of DNA (95 °C for 2 min) was followed by 35 cycles of amplification (95 °C for 50 s, 55 °C for 50 s, and 72 °C for 1 min 20 s) and ended with a 4 min extension (72 °C). PCR products were excised from a 1.0% agarose gel in sodium acetate buffer [12] and purified for sequencing using a High Pure PCR Product Purification Kit (Roche Diagnostics, Germany). The same primers used for PCR were used for cycle sequencing of the fragments in a 48 capillary ABI 3730 sequencer (Applied Biosystems) using the BIG Dye Terminator v3.1 Ready Sequencing Kit (Applied Biosystems) according to the manufacturers protocol.

Consensus sequences were constructed using Vector NTI Suite 9 (Invitrogen). Sequences were submitted to the BLAST on GenBank™. Using Clustal X [13], the partial 28S rDNA sequence and the ITS2 rDNA sequence of the sanguinicolid from *S. aurata* was aligned with various sanguinicolid taxa. Information on these taxa, their hosts, geographic origins and GenBank™ accession numbers are summarized in Table 1.

28S rDNA sequence alignment and phylogenetic analyses were conducted in order to determine the position of the sanguinicolid from *S. aurata* in relation to other taxa of the family Sanguinicolidae and included two isolates of *Cardicola coeptus* Nolan & Cribb, 2006a (sequences unpublished) and 18 other taxa. This represents all 28S rDNA sequences of sanguinicolids published to date with the exception of *Paracardicoloides yamagutii* Manter, 1974 (U42562), which was excluded due to its shortness (131 bp, only domain D1) and subsequent potential misplacement in phylogenetic analyses.

ITS rDNA sequences generally show a higher variability between taxa than 28S rDNA sequences [19] and thus ITS2 rDNA data were used for determining the position of the present sanguinicolid species amongst closely related taxa, i.e. 14 *Cardicola* spp., five *Paradeontacylix* spp. and five *Braya* spp. available on GenBank™. Secondary structure information was retrieved for the ITS2 region of all *Cardicola* spp. using the ITS2 Database [20] and secondary structure alignment was