Molecular characterization of Cryptosporidium molnari reveals a distinct piscine clade

Running title: Molecular characterization of C. molnari

Oswaldo Palenzuela*, Pilar Alvarez-Pellitero, and Ariadna Sitjà-Bobadilla

Instituto de Acuicultura de Torre de la Sal. Consejo Superior de Investigaciones Científicas (CSIC). 12595 Ribera de Cabanes, Castellón, Spain

*Corresponding author. Phone: +34 964319500; Fax: +34 964319509.

E-mail: oswaldo@iats.csic.es
ABSTRACT

Multilocus phylogenetic analysis of SSU rRNA and actin from Cryptosporidium molnari clustered this species with a C. molnari-like genotype from guppy, although both fish isolates seem distinct species. The analysis of available piscine genotypes provides some support for cladistic congruence of the genus Piscicryptosporidium, but additional piscine genotypes are needed.
Recent reviews accept over 20 valid cryptosporidium species (7, 20), and characterization of additional isolates is expanding this list rapidly (21). In addition, numerous morphotypes or genotypes have been proposed whose taxonomic affiliation is unsettled due to incomplete characterization, according to minimum consensus standards (5, 7, 25). Five species have been proposed for fish isolates (15), but only C. molnari and C. scophthalmi (2, 4) stand as valid species (20), although not without discussion (7). Fish cryptosporidia present some unique features, which even led to propose the genus *Piscicytosporidium* (13). However, lack of genetic support keeps this genus and several fish morphotypes as *incertae sedis* (12, 15, 25). Detailed biological data of *C. molnari* and *C. scophthalmi* have been previously presented (3, 18, 19) but no molecular characterization has been yet conducted, thus hampering species identification of other fish isolates (7, 25) and evaluation of their relationships within the genus (15). Ribosomal and actin gene data of an isolate from guppy fish (*Poecilia reticulata*) have been obtained and preliminary analyses of these sequences indicated a basal position in the cryptosporidia tree (17). Although regarded as *C. molnari*-like, biological characterization of this isolate was limited. The purpose of this work was to provide the necessary *C. molnari* comparative genetic data, and to clarify the relationship of available fish isolates in a phylogenetic context.

**Fish and parasite material.** Juvenile *S. aurata* were obtained from a local fish nursery facility. Small pieces of stomach were fixed in 10 % buffered formalin and embedded in Technovit-7100 resin (Heraeus Kulzer, Werheim, Germany). The gastric mucosa was gently scrapped and a small drop was examined fresh or after Kinyoun acid-
fast staining, at the microscope. The remaining scraping material was fixed in absolute ethanol and kept at 4°C until use.

**DNA extraction, gene amplification, cloning and sequencing.** Samples containing *C. molnari* oocysts were briefly hydrated in TE buffer (10mMTris, 1mM EDTA, pH8) and genomic DNA was purified using a silica-based kit (Roche Applied Science, Barcelona, Spain). Two SSU rDNA fragments (823 bps and 572 bps) were amplified by two different nested PCR approaches (26, 8, 16). In addition, a 1,103 bps fragment of the actin gene was amplified as described previously (23). Amplified products were cloned into pCR4-TOPO plasmid vector (Invitrogen, Paisley, UK). Both strands of at least two cloned products per target gene fragment were sequenced using M13 forward and reverse primers.

**Phylogenetic analyses.** Homologous positions presenting differences between contigs were verified by eye inspection of the electropherograms. Sequences were used as queries to search for homologous regions using Blastn (1). Positions and determination of actin coding frame were inferred in reference to *C. parvum* (GenBank #M86241), which comprises the annotated entire coding sequence (10). Alignments with related sequences retrieved from Genbank were refined by hand, and Mega 4.1 software (24) was used for phylogenetic inference.

**Nucleotide sequence accession numbers.** The nucleotide sequences of *Cryptosporidium molnari* from gilthead sea bream (*Sparus aurata*) have been deposited in Genbank under accession numbers HM243547-HM243550 (SSU rRNA gene) and HM365219-HM365220 (actin gene).

**Diagnosis and histopathological analysis.** Samples infected by *C. molnari* were selected from the preliminary study of tissue smears (Fig. 1A, C and D). Histopathological examination of resin sections from this material revealed numerous oocysts and epicellular
merogonial and gamogonial stages in the gastric mucosa (Fig. 1A, B, E and F). The morphometry and histopathological evaluation were identical to previous studies (2) and correspond to the type material deposited at MNCN – CSIC (Madrid, Spain): holotype and paratype acquisition numbers MNCN 35.02/19 and 35.02/20, respectively.

**Genetic data and phylogeny.** The obtained SSU rDNA sequences presented high identity with *Cryptosporidium* spp. genotypes in Blast searches (89-91%), and they matched positions 193-1,029 and 291-878 relative to *C. parvum* SSU rDNA (GenBank #AF108865) (annotated sequence alignments are available from the authors upon request). Variations at 21 sites identified 4 coexisting haplotypes from only two infected fish. Variability of any given haplotype from the consensus was detected at 2-5 positions (0.35-0.87 %). Similar variability has been reported at this locus for other *Cryptosporidium* genotypes (14) and it is probably explained by the particular organization of this gene in cryptosporidia (11). The actin product aligned well with the reference *C. parvum*, matching positions 19-1,122 of its coding region (aminoacids 7-373 of the translated peptide). Confirmed variations identified two *C. molnari* actin haplotypes differing at 9 nucleotide positions (0.82 % variability). Of these, most were synonymous substitutions but translated peptide sequences differed in aminoacids 48, 120 and 348. This variability is within what has been found among bovine and non-bovine “parvum” genotypes (23), but it is higher than expected from two individual fish of the same population. In *C. parvum*, actin is encoded by a single copy gene (10) and intraspecific variations in cryptosporidia have been reported as very low or absent (23). Compared with *C. parvum* protein, the total number of aminoacids changed in *C. molnari* haplotypes was 14-15, which is less than the average divergence between bovine *C. parvum* and the gastric genotypes (23).
All phylogenetic trees generated using the actin or the rDNA data sets supported the existence of a piscine clade clustering *C. molnari* haplotypes and *C. molnari*-like from guppy (Figs. 2 and 3). This clade branched off at a basal position relative to all other *Cryptosporidium* genotypes included in the datasets and this pattern was robust and stable to different inference methods and substitution models tested (data not shown). General topology of the *Cryptosporidium* spp. cladograms using both datasets was similar, and consistent with the expected branching of the main species and genotypes (25). Although clustered together, *C. molnari* appeared rather distant to the guppy genotype, which clearly seems a distinct species.

Our results further complete the description of *C. molnari*, which now stands as the only piscine *Cryptosporidium* species with comprehensive biological and molecular characterization. Guppy *C. molnari*-like actin gene sequence presented some dubious variations, like indels resulting in frameshift mutations at conserved regions of the protein, possibly caused by formalin fixation and embedding of the material (22). The shorter length of this sequence relative to the dataset (651 bps covering roughly 57% of the deduced peptide sequence), and the presence of some sequence inaccuracies could partly explain the long branch of the guppy genotype in actin trees, and a lower support for the piscine clade inferred from this locus.

Previous phylogenetic analyses resulted in a general genetic structure of *Cryptosporidium* with two main, gastric and intestinal branches (25). Our analysis including piscine species conflicts with this pattern. The *Cryptosporidium* species thus far reported from piscine hosts share most of the morphological and ultrastructural features of other species from terrestrial hosts (2, 4). However, sporulation in piscine *cryptosporidia* takes place deeply within the epithelial cell, and oocysts accumulation in the gastric or
intestinal mucosal often results in necrosis, vacuolation, and sloughing of epithelial cells. This contrasts with the persistent epitelullar disposition of species from other vertebrates, which are usually regarded as minimally invasive mucosal pathogens. The considerable genetic distance between piscine Cryptosporidium and the remaining genotypes, together with the distinctive morpho-pathological features, stir the long-standing debate on the possible ascription of piscine species to a differentiate genus within the family Cryptosporidiidae, and the validity of the genus Piscicryptosporidium (9, 7). The limited genetic data available so far suggests the existence of a basal fish clade, sister to all the other genotypes, and thus provides some support for the cladistic congruence of a distinct piscine taxon. However, its formal endorsement seems premature. Fish genotypes still remain largely unexplored and an important diversity, consistent with the overwhelming fish taxonomical and ecophysiological variations, might be expected among them.

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