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Molecular and Biological Characterization of a *Cryptosporidium molnari*-Like Isolate from a Guppy (*Poecilia reticulata*)

Una Ryan,[1]* Amanda O'Hara,[1] and Lihua Xiao[2]

Division of Health Sciences, School of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Western Australia 6150, Australia,[1] and Division of Parasitic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, U.S. Public Health Service, U.S. Department of Health and Human Services, Atlanta, Georgia 30341[2]

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Histological, morphological, genetic, and phylogenetic analyses of a *Cryptosporidium molnari*-like isolate from a guppy (*Poecilia reticulata*) identified stages consistent with those of *C. molnari* and revealed that *C. molnari* is genetically very distinct from all other species of *Cryptosporidium*. This study represents the first genetic characterization of *C. molnari*.

Little is known about the prevalence or geographic distribution of *Cryptosporidium* isolates that infect fish. The first report of a *Cryptosporidium* species in fish was in 1981, in a tropical marine fish (*Naso lituratus*) (3), and *Cryptosporidium nasorum* was subsequently proposed as a species in 1984 by Levine (4). However, only developmental stages of the parasite on the microvillous surface of intestinal epithelial cells were observed by light and electron microscopy. No measurements of viable oocysts were provided, and no taxonomically useful diagnostic features were presented. The species was named solely on the basis of the presumed host specificity of *Cryptosporidium* spp. and is now considered a nomen nudem (10).

Recently, *Cryptosporidium molnari* was isolated from two teleost fish, the gilthead sea bream (*Sparus aurata* L.) and the European sea bass (*Dicentrarchus labrax* L.) (1). The parasite was found mainly in the stomach epithelium and infrequently in the intestine. The oocysts were nearly spherical (shape index, 1.05). There was a great range in the sizes of the oocysts, but the average was 4.72 by 4.47 μm. Merogonial and gamogonial stages appeared in the typical extracytoplasmic position, whereas oogonial and sporogonial stages were located deeply within the epithelium (1).

Unfortunately, no molecular characterization of *C. molnari* has been conducted thus far, and its relationship to other species of *Cryptosporidium* remains unknown. This may cause problems in the identification and naming of other *Cryptosporidium* spp. in fish. In the present study, we present histological, genetic, and phylogenetic analyses of a *C. molnari*-like isolate from a guppy (*Poecilia reticulata*).

Fish. Over a period of 3 to 4 days, a guppy breeder experienced a loss of approximately 40 guppies. Two live guppies (approximately 5 cm long) were sent for examination; the fish were euthanized by cervical separation and immediately necropsied. After the body cavity of each fish was opened and samples were collected for microbiology, the fish were immersed and fixed in 10% buffered formalin for a minimum of 24 h.

Histology. Once the tissues were fixed, transverse sections (3 to 5 mm thick) were made along the length of each fish before routine processing and embedding in paraffin. Histological sections were cut at 5 μm and stained with hematoxylin and eosin. Oocysts were measured with the aid of an ocular micrometer in a Zeiss Axioskop microscope at a magnification of ×1,000.

18S rDNA gene amplification and sequencing. DNA was purified from formalin-fixed stomach sections using a QiAmp DNA extraction kit (Qiagen, Hilden, Germany). A two-step nested PCR protocol was used to amplify the 18S ribosomal DNA (rDNA) as previously described (6). Secondary PCR products were sequenced directly in both directions. Each isolate was sequenced at least twice.

PCR products were purified using Qiagen spin columns and sequenced using an ABI Prism Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, Calif.) according to the manufacturer's instructions. Sequences were analyzed using SeqEd version 1.0.3. (Applied Biosystems). Additional *Cryptosporidium* 18S rDNA sequences were obtained from GenBank.

Actin gene amplification and sequencing. A ~1,066-bp fragment of the actin gene was amplified by nested PCR and sequenced as previously described (7).

Phylogenetic analyses. Nucleotide sequences were aligned using Clustal X (8). (Sequence alignments can be obtained from the authors upon request.) A neighbor-joining tree was constructed from aligned sequences using the program Treecon (9), and genetic distance was calculated using the Kimura two-parameter model. In the construction of neighbor-joining trees, a sequence of *Eimeria bovis* (U77084) was used as an outgroup for 18S rDNA gene analysis, and a sequence of *Plasmodium falciparum* (M19146) was used for actin gene analysis. Bootstrap analyses were conducted using 1,000 replicates.

Histological analysis. Large numbers of *Cryptosporidium* organisms were detected along the epithelial lining of some areas of the stomach, whereas adjacent areas were largely not infected (Fig. 1A and B). Clusters of what appeared to be oogonial and sporogonial stages were present deep within the

* Corresponding author. Mailing address: Division of Health Sciences, School of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Western Australia 6150, Australia. Phone: 61 8 9360 2482. Fax: 61 8 9310 4144. E-mail: unaryan@central.murdoch.edu.au.

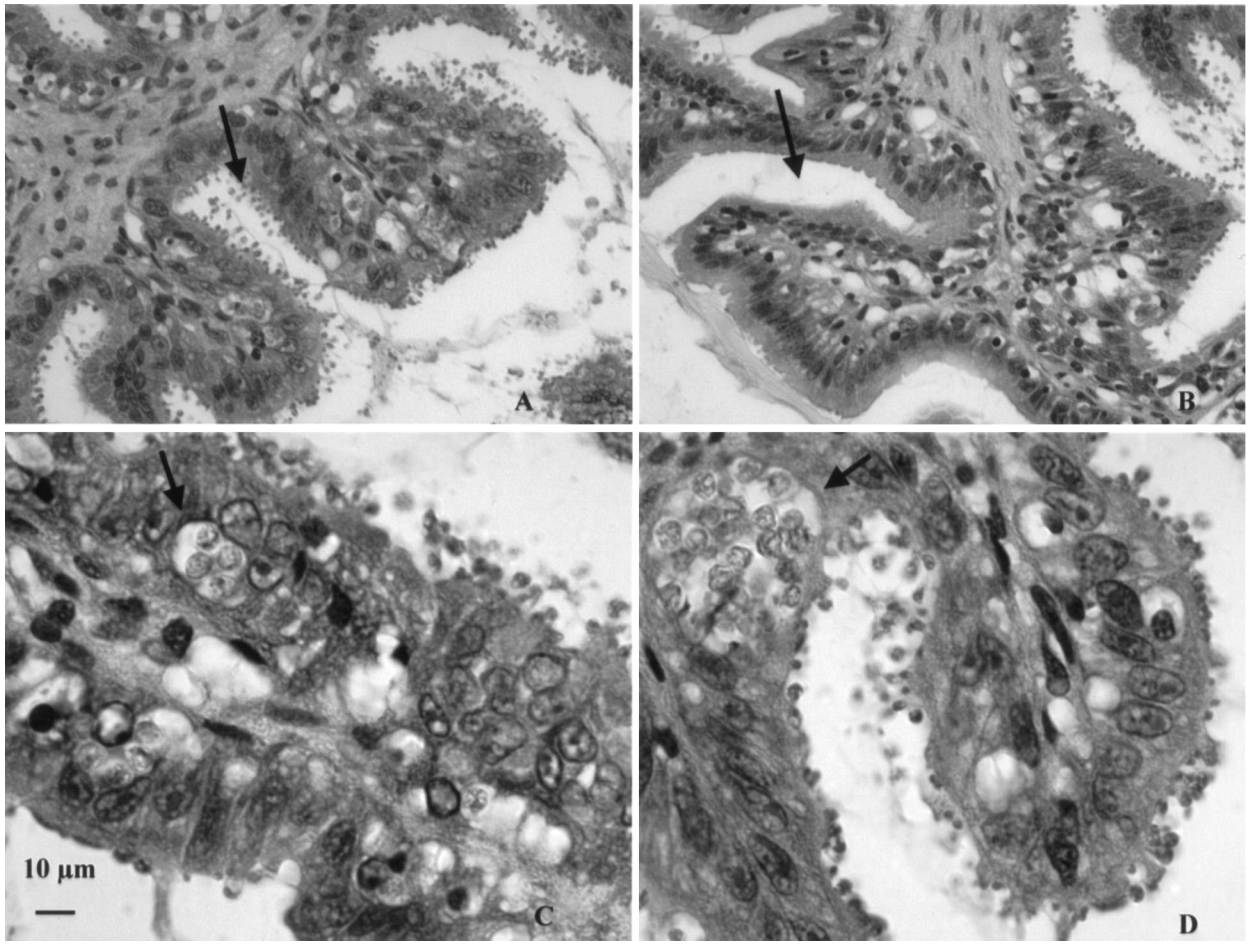

FIG. 1. Histological analysis of sections of the guppy stomach. Sections of the guppy stomach were stained with hematoxylin and eosin. (A and B) Large numbers of *Cryptosporidium* organisms along the epithelial lining of the stomach (A) with adjacent areas not infected (B). (C and D) Clusters of what appear to be oogonial and sporogonial stages located deep within the epithelium.

epithelium (Fig. 1C and D). Accompanying the parasites was a mild to moderate, multifocal infiltrate of granulocytes beneath the mucosa and within the muscular tunic and serosa. The thickness of the mucosa was variable, and there was irregular loss of mucosal glands. *Cryptosporidium* organisms were not detected within the intestinal tract. Oocysts were approximately 4.6 by 4.4 μm ($n = 50$), which is similar to the dimensions described by Alvarez-Pellitero and Sitjà-Bobadilla (4.72 by 4.47 μm) (1). However, the oocyst measurements in the present study may not be accurate due to the effects of fixation.

Sequence and phylogenetic analyses of the 18S rDNA and actin genes. BLASTn GenBank searches were performed on the rDNA, actin DNA, and translated actin sequence obtained from the *C. molnari*-like isolate. In all cases, at least the first 30 matches were *Cryptosporidium* with high homologies. Pairwise comparison of the *C. molnari*-like isolate 18S rDNA sequence with a range of *Cryptosporidium* species and genotypes revealed a unique sequence (Fig. 2). Phylogenetic analysis produced three major groups with all the intestinal *Cryptosporidium* spp. forming one broad group, the gastric *Cryptosporidium* spp. forming a second group, and the *C. molnari*-like isolate forming a distinct group at the base of the neighbor-joining tree This was supported by bootstrap analysis (89%) (Fig. 3). A neighbor-joining tree was also constructed with partial sequences from the actin gene. Analysis of the actin gene produced a tree with a topography similar to that of the 18S rDNA gene, with the *C. molnari*-like isolate again forming a separate group at the base of the tree (Fig. 4).

In the present study, a *C. molnari*-like isolate was analyzed by microscopy and genetically characterized at both the 18S rDNA and actin gene loci. Genetic analysis was not used in the original species description of *C. molnari* (1), and it has not been possible to subsequently obtain isolates of *C. molnari* for genetic analysis. Therefore, it is not certain that the *C. molnari*-like isolate that we describe in this study is in fact *C. molnari*. However, the size of the oocysts, the location of the oocysts in the stomach, and the presence of oogonial and sporogonial stages deep within the epithelium are consistent with *C. molnari*.

In the previous study by Alvarez-Pellitero and Sitjà-Bobadilla (1), pathological effects, mostly in fingerlings and juvenile fish, were seen in more than 24% of gilthead sea bream versus 4.6% of sea bass. In addition, considerable histopathological damage was evident. The wide zones of epithelium invaded by oogonial and sporogonial stages appeared necrotic, with abundant cell debris and sloughing of epithelial cells, which de-

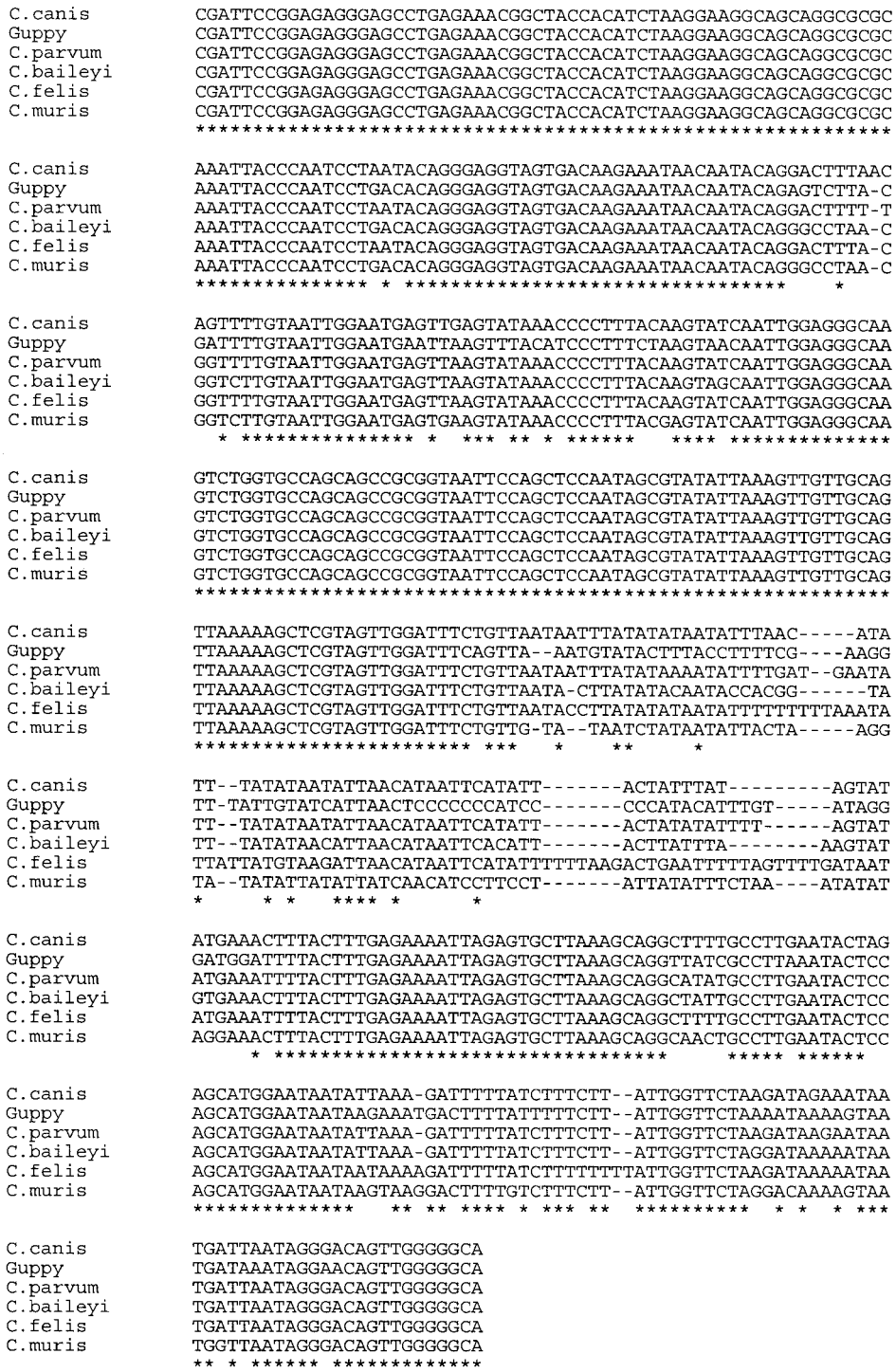

FIG. 2. Clustal X alignment of 18S rDNA sequences from selected *Cryprosporidium* spp. and guppies (ca. positions 340 to 828). Residues that were conserved in all species shown (asterisks) and gaps introduced to maximize alignment (dashes) are indicated.

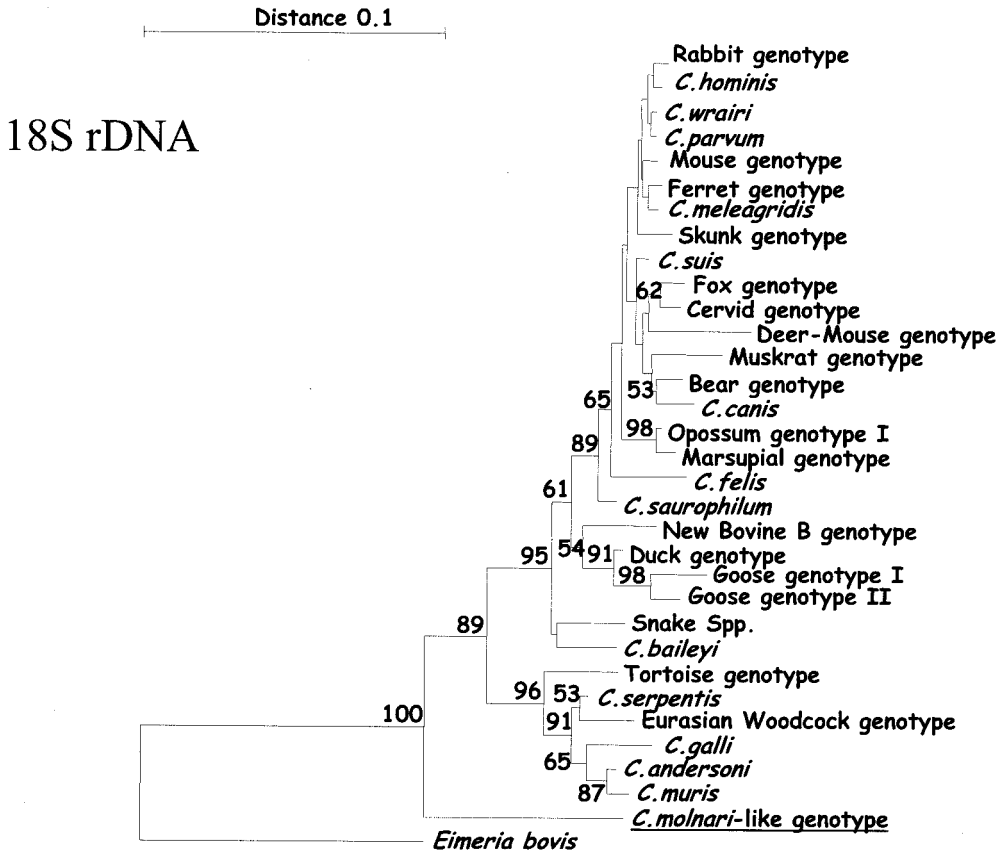

FIG. 3. Phylogenetic relationships of *Cryptosporidium* parasites inferred by the neighbor-joining analysis of the 18S rDNA gene based on genetic distances calculated by the Kimura two-parameter model. The tree was rooted with an 18S rDNA sequence from *Eimeria bovis* (U77084). Bootstrap values (as percentages) above 50 from 1,000 pseudoreplicates are shown at the nodes. The scale bar indicates the genetic distance of 0.1 substitution/site.

tached to the lumen. No inflammatory reaction was observed, and the cellular reaction was limited to the cells involved in the engulfment of intraepithelial stages and debris, probably macrophages. In this study, the large numbers of *Cryptosporidium* in the stomach were associated with variable disruption of the gastric mucosa and loss of mucosal glands (mucosal glandular atrophy). The changes described by Alvarez-Pellitero and Sitjà-Bobadilla (1) are relatively acute, whereas the changes in the stomach of the fish in the present report are subacute to chronic. The differences in the histological descriptions of the two reports may reflect differences in host susceptibility and host-parasite adaptation. Few studies have been conducted on piscine cryptosporidiosis. *Cryptosporidium* sp. was found in Australian barramundi in association with intestinal cells in the lamina propria (2) and intracellularly in necrotic epithelial cells in the stomachs of hatchery-reared fry and fingerling cichlids from a lake in Israel (5).

Phylogenetic analysis of both the 18S rDNA and actin loci placed the *C. molnari*-like isolate in a separate group that was basal to all other *Cryptosporidium* spp., indicating that the *C. molnari*-like isolate may be the most primitive of *Cryptosporidium* species. On the basis of the parasite's location in the stomach, it would be logical to assume that the *C. molnari*-like isolate may be genetically related to other gastric *Cryptosporidium* spp., such as *C. muris*, *C. andersoni*, and *C. galli*. However, phylogenetic analysis revealed that the *C. molnari*-like isolate was genetically distant from all other *Cryptosporidium* spp. The genetic similarity between the *C. molnari*-like isolate and the intestinal parasites ranged from 73 to 80%, and the genetic similarity between the *C. molnari*-like isolate and the gastric parasites ranged from 78 to 80%. Thus, the *C. molnari*-like parasite is likely the most primitive *Cryptosporidium* sp. and may represent a member of the genus prior to the split of the gastric and intestinal *Cryptosporidium* spp.

Previously *C. molnari* has been reported only in two teleost fish, the gilthead sea bream (*Sparus aurata* L.) and the European sea bass (*Dicentrarchus labrax* L.) (1). The detection of a *C. molnari*-like isolate in a guppy (*Poecilia reticulata*) in the present study potentially broadens the host range for this species. This study represents the first genetic characterization of a *C. molnari*-like isolate and will facilitate not only the screening and detection of *C. molnari* in fish but will also assist in the identification and naming of other *Cryptosporidium* spp. in fish. Future studies are required to determine the host range for *C. molnari* in fish and the extent of *Cryptosporidium* species diversity in fish.

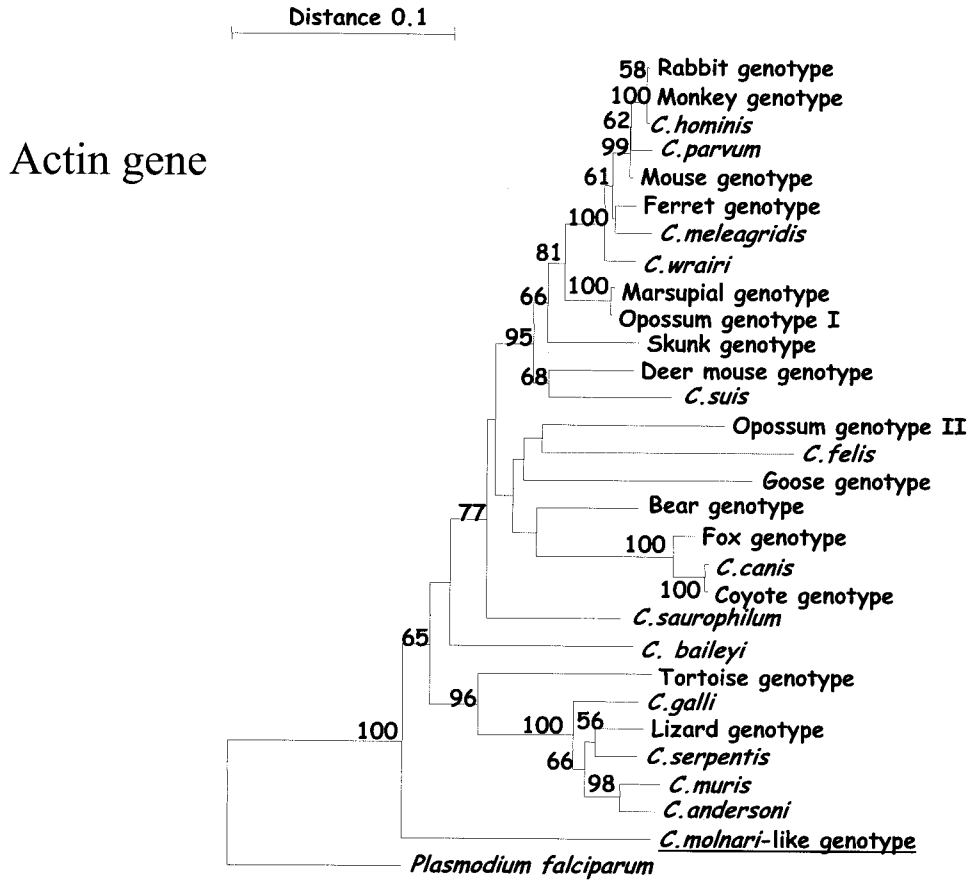

FIG. 4. Phylogenetic relationships of *Cryptosporidium* parasites inferred by the neighbor-joining analysis of the actin gene based on genetic distances calculated by the Kimura two-parameter model. The tree was rooted with an actin sequence from *P. falciparum* (M19146). Bootstrap values (as percentages) above 50 from 1,000 pseudoreplicates are shown at the nodes. The scale bar indicates the genetic distance of 0.1 substitution/site.

Nucleotide sequence accession numbers. The nucleotide sequences of the 18S rRNA and actin sequences of the *C. molnari*-like *Cryptosporidium* isolate have been deposited in GenBank under accession numbers AY524772 and AY524773.

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