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Identification of novel *Cryptosporidium* species in aquarium fish

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ABSTRACT

Little is known about the prevalence and genotypes of *Cryptosporidium* in fish. The present study investigated the prevalence of *Cryptosporidium* species in 200 aquarium fish of 39 different species in Western Australia by PCR amplification at the 18S rRNA locus. A total of 21 positives were detected by PCR (10.5% prevalence) from 13 different species of fish. Nineteen of these isolates were successfully sequenced. Of these, 12 were similar or identical to previously described species/genotypes of *Cryptosporidium*, while the remaining seven isolates appeared to represent three novel species.

1. Introduction

Currently little is known about the epidemiology, taxonomy, pathology and host specificity of *Cryptosporidium* species infecting piscine hosts. There are two recognised species of *Cryptosporidium* in fish: *Cryptosporidium molnari* in gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) and *Cryptosporidium scophthalmi* in turbot (*Psetta maxima*, syn. *Scophthalmus maximus*) (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Alvarez-Pellitero et al., 2004). *C. molnari* primarily infects the epithelium of the stomach and seldom the intestine (Alvarez-Pellitero and Sitja-Bobadilla, 2002), whereas *C. scophthalmi* mainly infects the epithelium of the intestine and very seldom the stomach (Alvarez-Pellitero et al., 2004). Currently, genetic sequences are available in GenBank for *C. molnari* (GenBank accession number HM243547) but not *C. scophthalmi.* To date only three additional studies have generated genetic sequences from piscine-derived *Cryptosporidium* spp.; an isolate from a guppy (*Poecilia reticulate*) (hereafter referred to as piscine genotype 1) (Ryan et al., 2004), a freshwater angelfish (*Pterophyllum scalare*) (hereafter referred to piscine genotype 2) (Murphy et al., 2009) and more recently *C. parvum*, *C. xiaoi* and pig genotype II were identified in whiting (*Sillago vittata*) (Reid et al., 2010) and a novel *Cryptosporidium* spp. was identified in sea mullet (*Mugil cephalus*) (hereafter referred to as piscine genotype 3) (Reid et al., 2010).

The aim of the present study was to determine the prevalence of different species of *Cryptosporidium* in ornamental fish in Western Australia (WA).

2. Materials and methods

2.1. Sample collection

A total of 200 ornamental fish from 39 different species (see Table 1) were collected from local aquariums and pet shops in metropolitan WA. These fish included both marine and freshwater, and tropical and temperate species. On arrival in the laboratory, fish were measured for length and weight and dissected. Stomach and intestinal epithelial cells were scraped off using a scalpel blade and placed into a 1.5 mL Eppendorf tube. Remaining stomach and intestinal tissue were stored separately in 10% formalin.

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Table 1
Ornamental fish species sampled and tested for infection with *Cryptosporidium* during this study.

Common name	Scientific name	Freshwater/marine	No. collected	No. positive
Acei	*Pseudotropheus sp.*	Freshwater	3	0
Albino cory	*Corydoras paleatus*	Freshwater	4	0
Angelfish	*Pterophyllum scalare*	Freshwater	4	1
Azure damselfish	*Chrysiptera hemicyanea*	Marine	3	0
Molly	*Poecilia latipinna*	Freshwater	7	0
Balloon kissing gourami	*Helostoma temminckii*	Freshwater	4	0
Banded dwarf cichlid	*Apistogramma bitaeniata*	Freshwater	3	0
Black ghost	*Apteronotus albifrons*		5	0
Black widow tetras	*Gymnocorymbus ternetzi*	Marine	5	0
Blue star leopard wrasse	*Macropharyngodon bipartitus*	Marine	5	0
Bristlenose catfish	*Ancistrus cirrhosus*	Freshwater	8	0
Bristle tooth tang	*Ctenochaetus tominiensis*	Marine	1	1
Butter bream	*Monodactylus Argenteus*	Marine	3	2
Madder seaperch	*Pseudanthias dispar*	Marine	2	1
Electric yellow	*Labidochromis caeruleus*	Freshwater	5	0
False gramma	*Pseudochromis paccagnellae*	Marine	1	1
Golden algae eater	*Crossocheilus aymonieri*	Freshwater	5	3
Goldfish	*Carassius auratus auratus*	Freshwater	7	0
Green acara/Green terror	*Acara rivulata*	Freshwater	2	0
Green chromis	*Chromis viridis*	Marine	13	2
Guppy	*Poecilia reticulata*	Freshwater	43	1
Hornet (Bumblee) cichlid	*Maylandia crabro*	Freshwater	1	0
Humbug damsel	*Dascyllus aruanus*	Marine	5	0
Kupang damsel	*Chrysiptera hemicyanea*	Marine	1	1
Moss green tiger barb	*Puntius tetrazona*	Freshwater	3	0
Neon tetra	*Paracheirodon innesi*	Freshwater	2	2
Orange anemone (clownfish)	*Amphiprion percula*	Marine	14	0
Oscar	*Astronotus ocellatus*	Freshwater	4	4
Red hi fin platy	*Xiphophorus maculatus*	Freshwater	1	0
Red melon discus	*Symphysodon discus*	Freshwater	2	0
Schwartz cory	*Corydoras schwartzi*	Freshwater	2	0
Silver gourami	*Trichogaster trichopterus*	Freshwater	2	0
Silver shark	*Balantiocheilos melanopterus*	Freshwater	9	0
Striped kuhli loach	*Pangio kuhlii*	Freshwater	1	0
True rummy nose tetra	*Hemigrammus bleheri*	Freshwater	10	0
Upside down cat fish	*Synodontis nigriventris*	Freshwater	2	1
Wedgetailed blue tang	*Paracanthurus hepatus*	Marine	1	1
Yellow tailed damsel	*Chrysiptera parasema*	Marine	2	0
Zebra fish	*Danio rerio*	Freshwater	5	0
Total			200	21

2.2. DNA extraction and PCR amplification

DNA was extracted from ~250 mg of pooled intestinal and stomach tissue scrapings from each fish sample using a Qiagen DNeasy tissue kit (Qiagen, Germany). DNA was eluted in 50 μL of AE buffer to concentrate the DNA. All extracted DNA samples were stored at −20 °C until required for screening.

All samples were screened at the 18S rRNA locus and positives were genotyped by sequencing. A two-step nested PCR protocol was used to amplify the 18S rDNA gene of *Cryptosporidium* as previously described (Ryan et al., 2003). For all isolates that were positive at the 18S locus by PCR, attempts were also made to amplify the actin locus as previously described (Ng et al., 2006). PCR contamination controls were used including negative controls and separation of preparation and amplification areas. The amplified DNA fragments from the secondary PCR products were separated by gel electrophoresis and purified using the freeze-squeeze method (Ng et al., 2006). A spike analysis (addition of 0.5 μL of *Cryptosporidium* positive control into each sample) was conducted on randomly selected *Cryptosporidium* negative samples from each group of DNA extractions to determine if negative results were due to PCR inhibition.

2.3. Sequence and phylogenetic analysis

Purified PCR products were sequenced using an ABI Prism™ Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions with the exception that the annealing temperature was raised to 58 °C. Nucleotide sequences were analyzed using Chromas lite version 2.0 (http://www.technelysium.com.au) and aligned with reference genotypes from GenBank using Clustal W (http://www.clustalw.genome.jp).

Phylogenetic trees were constructed using additional isolates from GenBank. Distance estimation was conducted using TREECON (Van de Peer and De Wachter, 1994), based on evolutionary distances calculated with the Kimura's distance and grouped using Neighbour-Joining. Parsimony analyses were conducted using MEGA version 3.1 (MEGA3.1: Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, AZ, USA). Bootstrap analyses were conducted using 1000 replicates to