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Genetic diversity of *Cryptosporidium* in fish at the 18S and actin loci and high levels of mixed infections

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ABSTRACT

Cryptosporidium is an enteric parasite that infects humans and a wide range of animals. Relatively little is known about the epidemiology and taxonomy of *Cryptosporidium* in fish. In the present study, a total of 775 fish, belonging to 46 species and comprising ornamental fish, marine fish and freshwater fish were screened for the prevalence of *Cryptosporidium* by PCR. The overall prevalence of *Cryptosporidium* in fish was 5.3% (41/775), with prevalences ranging from 1.5 to 100% within individual host species. Phylogenetic analysis of these *Cryptosporidium* isolates as well as 14 isolates from previous studies indicated extensive genetic diversity as well as evidence for mixed infections. At the 18S locus the following species were identified; *Cryptosporidium molnari*-like genotype ($n = 14$), *Cryptosporidium huwi* ($n = 8$), piscine genotype 2 ($n = 4$), piscine genotype 3-like ($n = 1$), piscine genotype 4 ($n = 2$), piscine genotype 5 ($n = 13$), piscine genotype 5-like ($n = 1$) and five novel genotypes ($n = 5$). At the actin locus, species identification agreed with the 18S locus for only 52.3% of isolates sequenced, indicating high levels of mixed infections. Future studies will need to employ both morphological characterization and deep sequencing amplicon-based technologies to better understand the epidemiological and phylogenetic relationships of piscine-derived *Cryptosporidium* species and genotypes, particularly when mixed infections are detected.

1. Introduction

Currently three piscine *Cryptosporidium* species are recognised; (1) *Cryptosporidium molnari*, which was initially identified in gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) (Alvarez-Pellitero and Sitja-Bobadilla, 2002) and was characterized genetically in 2010 (Palenzuela et al., 2010), (2) *Cryptosporidium scophthalmi*, which was described in turbot in 2004 (*Scophthalmus maximus*) (Alvarez-Pellitero et al., 2004) and characterized genetically in 2015 (Costa et al., 2015) and (3) the recently described *Cryptosporidium huwi* (previously piscine genotype 1) (Ryan et al., 2015).

Molecular characterization based mainly on short 18S rRNA sequences (250–404 bp) has identified seven additional piscine genotypes (piscine genotypes 2–8) as well as *Cryptosporidium parvum*, *Cryptosporidium xiaoi*, *Cryptosporidium scrofarum*, *Cryptosporidium hominis* and rat genotype III in fish (Murphy et al., 2009; Reid et al., 2010; Zanguee et al., 2010; Morine et al., 2012; Koinari et al., 2013; Ryan and Xiao, 2014). Understanding the epidemiology and zoonotic potential of piscine *Cryptosporidium* is important because *Cryptosporidium* can cause a variety of clinical signs in fish and as it can cause high morbidity, it is also economically important (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Alvarez-Pellitero et al., 2004; Ryan et al., 2004, 2015; Murphy et al., 2009; Zanguee et al., 2010). A sound taxonomy of piscine-derived *Cryptosporidium* species is also important as previous research suggests that they may be the most primitive of *Cryptosporidium* species (Ryan et al., 2004, 2015; Palenzuela et al., 2010; Reid et al., 2010; Zanguee et al., 2010; Morine et al., 2012; Koinari et al., 2013) and therefore, provide important information on the evolution of the genus. The purpose of the present study was to examine the extent of genetic diversity of *Cryptosporidium* species in fish hosts using a longer region (~553 bp) of the 18S gene (Silva et al., 2013) and to construct a molecular phylogeny at the actin locus to better understand the phylogenetic relationships of piscine *Cryptosporidium* species and genotypes.

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Table 1
Fish species screened for *Cryptosporidium*. NA = no amplification. Prevalence (and 95 confidence intervals—CI) is given for each species. Unless specified, all fish were obtained from a local aquarium.

Host common name	Host scientific name	Habitat	No. sampled	No. positive	Prevalence (%) (95% CI)	Isolates sequenced	Species/genotype identified 18S locus	Species/genotype identified actin locus
Albino bristlenose	*Ancistrus* sp.	Freshwater	1	0	0		–	
Angelfish	*Pterophyllum altum*	Freshwater	5	2	40.0 (8.0–82.9)	CA70, LC15	Piscine genotype 5 (CA70), *C. molnari*-like (LC15)	Piscine genotype 5 (CA70), *C. molnari*-like (LC15)
Anthia	*Anthiinae*	Marine	1	0	0		–	
Azure damsel	*Chrysiptera hemicyanea*	Marine	3	2	66.7 (13.3–98.0)	LC47, LC51	*C. molnari*-like (LC47), Novel (LC51)	*C. molnari*-like (LC47), NA (LC51)
Balloon molly	*Poecilia latipinna*	Freshwater	5	0	0		–	
Bicolor angelfish	*Centropyge bicolor*	Marine	1	0	0		–	
Black ghost knife fish	*Apteronotus albifrons*	Freshwater	4	2	50.0 (1.0–99.0)	CA125, CA247	Piscine genotype 4 (CA125), piscine genotype 5 (CA247)	NA
Blue damsel	*Chrysiptera cyanea*	Marine	2	0	0		–	
Blue tang	*Paracanthurus hepatus*	Marine	1	1	100 (100–100)	LC09	Piscine genotype 5 (LC09)	Piscine genotype 5 (LC09)
Bolivian butterfly fish	*Mikrogeophagus altispinosus*	Freshwater	1	0	0		–	
Bristlenose catfish	*Ancistrus cirrhosus*	Freshwater	3	0	0		–	
Freshwater cobbler[a]	*Tandanus bostocki*	Freshwater	35	0	0			
Chromis	*Chromis viridis*	Marine	4	0	0		–	
Cichlid	*Cichlidae*		2	0	0		–	
Clown loach	*Botia macracantha*	Freshwater	1	0	0		–	
Corydorus catfish	*Corydorus* spp.	Freshwater	4	0	0		–	
Ebili angel fish	*Centropyge eibli*	Marine	1	0	0		–	
Flagatail catfish	*Dianema urostriatum*	Freshwater	1	0	0		–	
Frontosa	*Cyphotilapia frontosa*	Freshwater	1	0	0		–	
Golden algae eater	*Gyrinocheilos aymonieri*	Freshwater	1	0	0		–	
Goldfish	*Carassius auratus*	Freshwater	110	6	5.5 (1.2–9.7)	LC16, LC38, KS07, KS09, KS10, KS11	*C. molnari*-like (LC16a, LC38, KS09), piscine-genotype 3-like (KS11), piscine genotype 5 (LC16b, KS07, KS10)	*C. molnari*-like (LC16, LC38, KS09, KS11), piscine genotype 5, (KS07, KS10)
Gold ram	*Mikrogeophagus ramirezi*	Freshwater	4	0	0		–	
Golden tiger barb	*Puntigrus tetrazona*	Freshwater	22	1	4.5 (0–13.2)	KS52	*C. huwi* (KS52)	*C. huwi* (KS52)
Gourami	*Trichogaster trichopterus*	Freshwater	7	0	0		–	
Guppy	*Poecilia reticulata*	Freshwater	108	4	3.7 (0.1–7.3)	CA47, CA49, KS43, KS46	*C. huwi* (KS43, KS46), *C. molnari*-like (CA47), piscine genotype 5 (CA49)	*C. huwi* (KS43, KS46), *C. molnari*-like (CA47), piscine genotype 5 (CA49)