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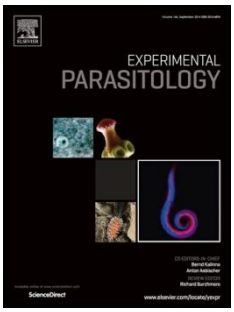

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Cryptosporidium huwi n. sp. (Apicomplexa:Eimeriidae) from the guppy (*Poecilia reticulata)*

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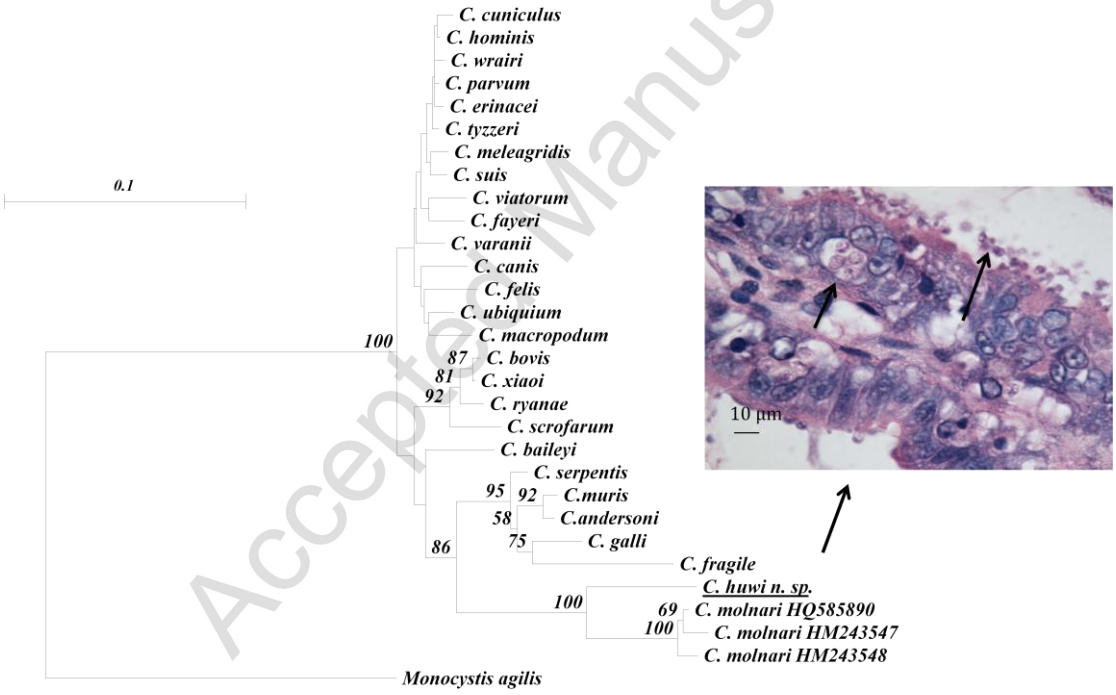
Highlights

- Description of a new *Cryptosporidium* species
- *Cryptosporidium huwi n. sp.*
- Formerly piscine genotype 1
- Molecular characterisation at two loci
- Morphological characterisation

Graphical Abstract

Cryptosporidium huwi* n. sp. (Apicomplexa:Eimeriidae) from the guppy (*Poecilia reticulata)



Abstract

The morphological, biological, and molecular characteristics of *Cryptosporidium* piscine genotype 1 from the guppy *(Poecilia reticulata)* are described, and the species name *Cryptosporidium huwi* n. sp. is proposed to reflect its genetic and biological differences from gastric and intestinal *Cryptosporidium* species. Oocysts of *C. huwi* n.

sp. over-lap in size with *Cryptosporidium molnari*, measuring approximately 4.4-4.9 µm (mean 4.6) by 4.0-4.8 µm (mean 4.4 µm) with a length to width ratio of 1.04 (0.92-1.35) (n = 50). Similar to *C. molnari*, *C. huwi* n. sp. was identified in the stomach only and clusters of oogonial and sporogonial stages were identified deep within the epithelium. However, phylogenetic analysis of 18S rRNA sequences indicated that *C. huwi* n. sp. exhibited 8.5-9.2% and 3.5% genetic distance from *C. molnari* isolates and piscine genotype 7 respectively. At the actin locus, the genetic distance between *C. huwi* n. sp. and *C. molnari* was 16.6%. The genetic distance between *C. huwi* n. sp. and other *Cryptosporidium* species at the 18S locus was 13.2%-17% and at the actin locus was 18.9%-26.3%. Therefore *C. huwi* n. sp. is genetically distinct from previously described *Cryptosporidium* species.

Keywords: Cryptosporidium huwi n. sp.; morphology, genetic characterization; 18S rRNA; actin gene; phylogeny.

1. Introduction

Until recently, little was known about the epidemiology, taxonomy, pathology and host specificity of *Cryptosporidium* species infecting piscine hosts. The parasite has been described in both fresh water and marine piscine species with parasitic stages located either on the gastric or intestinal surface, or at both sites (Ryan, 2010; Ryan and Xiao, 2014).

Currently the only recognised species infecting fish is *Cryptosporidium molnari,* which was initially identified in gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labarx*) (Alvarez-Pellitero and Sitja-Bobadilla, 2002) and was characterised genetically in 2010 (Palenzuela et al., 2010). *Cryptosporidium molnari* primarily infects the gastric but seldom the intestinal epithelium (Alvarez-Pellitero and Sitja-Bobadilla 2002). In 2004, *C. scophthalmi* was described in turbot (*Psetta maxima*. sny. *Scopthalmus maximus*) (Alvarez-Pellitero et al., 2004). However this species is considered invalid until genetic data are acquired because of the likely existence of multiple morphologically similar intestinal species in fish (Ryan et al., 2014).

In 2004, a novel piscine-derived *Cryptosporidium* spp. (piscine genotype 1) was described in a guppy (*Poecilia reticulate*), using histopathological and molecular data (Ryan et al., 2004). Subsequent molecular characterization has identified seven additional piscine genotypes (piscine genotypes 2-8) as well as *C. parvum, C. xiaoi, C. scrofarum, C. hominis* and rat genotype III (Murphy et al., 2009; Reid et al., 2010; Zanguee et al., 2010; Morine et al., 2012; Koinari et al., 2013; Ryan and Xiao, 2014).

The purpose of the present study was to determine the prevalence of piscine genotype 1 in ornamental fish and to provide the necessary comparative genetic characterization of piscine genotype 1 at the 18S and actin loci with all available

piscine-derived *Cryptosporidium* genotypes. Based on these data and results of previous histological analysis, we have concluded that piscine genotype 1 is genetically and biologically distinct and propose to name it *Cryptosporidium huwi* n. sp.

2. Materials and methods

2.1 Sampling

A total of 155 ornamental fishes, belonging to 6 species, were collected from a commercial aquarium in Perth, Western Australia (Table 1). All fishes were collected alive specimens for harvesting fresh tissues. All fish were euthanized using an ice slurry upon arrival at the laboratory under animal ethics permit no W2325/10. They were then weighed and measured (length and width) and dissected using a fresh scalpel blade for each fish. The intestine and stomach of each fish were dissected out using a fresh scalpel blade, and stored at -20 C for further analysis.

2.2 Genomic DNA extraction and PCR amplification

DNA was extracted from ~25 mg of intestinal and stomach tissues using the PowerSoil DNA Isolation Kit (Mo Bio, California, USA). All samples were screened at the 18S rRNA locus as previously described (Ryan et al., 2003). Positive isolates were also analysed at the actin locus using PCR primers optimized for amplification of piscine-derived *Cryptosporidium* species (which produce a ~ 392 bp product), as

previously described (Koinari et al., 2013). No template controls consisting of DNA-free molecular grade water were used during each PCR run. Physical separation of sample preparation and amplification areas was practiced to prevent contamination of test samples by PCR products. The amplified DNA fragments from the secondary PCR products were separated by gel electrophoresis and purified for sequencing using an in-house filter tip-based method without any further purification as previously described (Yang et al., 2013).

2.3 Sequence and phylogenetic analysis

Positives were sequenced using an ABI Prism™ Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions. Nucleotide sequences were analyzed using Finch TV Version 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com) and aligned with reference *C. huwi* n. sp. 18S (AY524773) and actin (AY524772) sequences from GenBank using Clustal W (http://www.clustalw.genome.jp). Multiple-sequence alignments were constructed using additional isolates from GenBank. Distance, parsimony and maximum likelihood trees were constructed using MEGA version 5 (Tamura et al., 2011). Prevalences were expressed as the percentage of samples positive by PCR, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rózsa et al., 2000).

3. Results

3.1 Prevalence of C. huwi n. sp. in ornamental fish hosts

121

122 At the 18S locus, a total of 11 positives were detected by PCR and sequence
123 analysis, an estimated prevalence of 7.1% (11/155)(3.1-11.1 CI) in ornamental fish
124 (Table 1). Of these positives, 10 were identified as *C. huwi* n. sp. at the 18S locus and
125 of these, 5 were successfully amplified and sequenced at the actin locus. All 5 isolates
126 sequenced at the actin locus were identified as *C. huwi* n. sp. The prevalence of *C.*
127 *huwi* in neon tetras was 7.8% (7/90), in guppies was 20% (2/10) and in tiger barbs
128 was 10% (1/10). One positive in oscar fish (5% - 1/20) was identified as piscine
129 genotype 2 (Table 1).

130

131 *3.2 Phylogenetic analysis of C. huwi* n. sp. *at the 18S locus*

132

133 Phylogenetic analysis at the 18S locus based on 485 bp of sequence data
134 (AY524773), using distance, parsimony and maximum likelihood produced similar
135 trees (Fig. 1A, distance tree shown). In this analysis, *C. huwi* n. sp. and *C. molnari*
136 were most closely related and exhibited 8.5-9.2% genetic distance from each other.
137 The genetic distance between *C. huwi* n. sp. and all other *Cryptosporidium* species
138 ranged from 13.2% (*C. andersoni*) to 17% (*C. fragile*). The genetic distance between
139 *C. huwi* n. sp. and *C. parvum* was 14.6%. Phylogenetic analysis based on shorter 18S
140 sequences (288 bp), which included piscine genotypes 1-8 was also conducted (Fig.
141 1B, distance tree shown). In that analysis, *C. huwi* n. sp. was most closely related
142 (3.5% difference) to piscine genotype 7 (JQ995775) previously identified in neon
143 tetra's (Morine et al., 2012). The 10 *C. huwi* n. sp. specimens sequenced as part of the
144 present study were 100% identical to the reference *C. huwi* n. sp. sequence
145 (AY524773).

146

147 *3.3 Phylogenetic analysis of C. huwi. n. sp. at the actin locus*

148 Phylogenetic analysis at the actin locus based on 618 bp of sequence
149 (AY524772), using distance, parsimony and maximum likelihood produced similar
150 trees (Fig. 2, distance tree shown). At the actin locus, the genetic distance between *C.*
151 *huwi* n. sp. and *C. molnari* was 16.6% and between *C. huwi* n. sp. and all other
152 *Cryptosporidium* species ranged from 18.9% (*C. baileyi*) to 26.3% (*C. canis*). Despite
153 numerous attempts, we were unable to amplify and sequence piscine genotype 7 at the

actin locus and therefore the phylogenetic relationship between *C. huwi* and piscine genotype could not be determined at this locus. Alignment of the five shorter *C. huwi* n. sp. actin sequences (~390 bp) generated as part of the present study, with the reference *C. huwi* n. sp. isolate (AY524772) showed that all five isolates exhibited 1 single nucleotide polymorphism (SNP) compared to AY524772.

3.4. Histological analysis of C. huwi n. sp.

Previous histological analysis of *C. huwi* n. sp. based on 5 µm hematoxylin and eosin stained sections from a guppy (*Poecilia reticulata*) (Ryan et al., 2004) identified the parasite multifocally on apical surfaces as well as deep within the epithelium of the gastric mucosa, whereas adjacent areas were largely not infected (Fig. 3A and B). Oocysts were not identified in the intestine. Clusters of oogonial and sporogonial stages were present deep within the epithelium (Fig. 3C and D). Oocysts of *C. huwi* n. sp. measured approximately 4.4-4.9 µm (mean 4.6) by 4.0-4.8 µm (mean 4.4 µm) with a length to width ratio of 1.04 (0.92-1.35) (n = 50). 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. PCR analysis of DNA extracted from these sections, confirmed the presence of *C. huwi* n. sp. (Ryan et al., 2004). *Cryptosporidium* was not observed on histological examination of gastrointestinal tissues taken from the ornamental fish tested by PCR in the present study.

3.5 Species description

Species name: *Cryptosporidium huwi n. sp.* (Fig 3).

Type hosts: *Poecilia reticulata* (guppy).

Type locality: *Jandakot, Perth, Western Australia.*

Prevalence: *C. huwi sp. was detected in 10/155 samples screened, an estimated prevalence of 6.4% (2.6-10.3 CI) in ornamental fish.*

Other hosts: *Neon tetra (Paracheirodon innesi)* and Tiger barb (*Puntius tetrazona*).

Prepatent period: *Unknown.*

Patent period: *Unknown.*

Site of infection*:* Stomach.

Material deposited*: DNA sequences have been deposited in GenBank under accession numbers AY524773 for the 18S locus and* AY524772 *for the actin locus.*

Etymology: This species is named *Cryptosporidium huwi* n. sp. in honor of the late Prof. Huw Smith who has contributed greatly to the biology and epidemiology of *Cryptosporidium* species.

4. Discussion

In the present study *C. huwi* n. sp. was detected in 6.4% (10/155) of ornamental fish samples screened. The most common fish host species was neon tetra with a prevalence of 7.8% (7/90) in this host species. A previous study which examined two neon tetra isolates identified *C. huwi* n. sp. in one and piscine genotype 2 in the second isolate (Zanguee et al., 2010). Another study examined 4 neon tetra isolates but did not detect *C. huwi* n. sp., however piscine genotype 4 was detected in one of these isolates (Morine et al., 2012).

Cryptosporidium huwi n. sp. oocysts measured approximately 4.6 by 4.4 µm and overlap in size with many intestinal *Cryptosporidium* species and are very similar to the dimensions described for *C. molnari* (4.72 by 4.47 µm) (Alvarez-Pellitero and Sitja-Bobadilla 2002) and for *C. scophthalmi* (4.44 x 3.91 µm) (Alvarez-Pellitero et al., 2004). However, morphological overlap in oocyst size is common amongst *Cryptosporidium* species and size measurement is not a useful criterion for delimiting species in this genus (Fall et al., 2003).

208 At the 18S locus *C. huwi* n. sp. exhibited a 3.5% genetic distance from piscine
209 genotype 7 and 8.5-9.2% genetic distance from *C. molnari*. At the actin locus the
210 genetic distance between *C. huwi* n. sp. and *C. molnari* was 16.6%. This clearly
211 supports the species status of *C. huwi* n. sp., as these differences are greater than
212 many currently accepted species. For example, the genetic distance at both the 18S
213 and actin loci between *C. parvum* and the recently described *C. erinacei* is 0.5%
214 (Kváč et al., 2014) and the genetic distance between *C. muris* and *C. andersoni* at the
215 18S and actin loci is 0.9% and 3.5% respectively.
216 Earlier phylogenetic analyses identified two main branches in the genetic
217 structure of *Cryptosporidium*; gastric and intestinal (Xiao et al., 2004). However, the
218 present study and more recent phylogenetic analysis supports the existence of a
219 piscine clade that includes *C. molnari*, *C. huwi* n. sp. and piscine genotypes 2-8
220 (Palenzuela et al., 2010; Reid et al., 2010; Zanguee et al., 2010; Morine et al., 2012;
221 Koinari et al., 2013), which branches off at a basal position relative to all other
222 *Cryptosporidium* species and is supported by high bootstrap values (99-100%). An
223 unusual feature of the piscine clade is that sporulation takes place deep within the
224 epithelium (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Ryan et al., 2004; Palenzuela
225 et al., 2010). This is in contrast with the epicellular location of *Cryptosporidium*
226 species from other vertebrates. In addition, both *C. molnari* and *C. huwi n. sp*. have
227 been associated with necrosis and sloughing of epithelial cells (Alvarez-Pellitero and
228 Sitja-Bobadilla, 2002; Ryan et al., 2004; Palenzuela et al., 2010), compared to the less
229 invasive mucosal pathogenesis of *Cryptosporidium* species from other vertebrates.
230 This data combined with the considerable genetic distance between the piscine clade
231 and gastric and intestinal clades at the 18S (13.2%-17%) and actin loci (18.9%-
232 26.3%), supports the original assertion by Paperna and Vilenkin, (1996), that

Cryptosporidium species infecting piscine hosts, probably should be classified as a separate genus, designated *Piscicryptosporidium.* Evidence to date suggests that considerable genetic diversity exists within the piscine clade (Murphy et al., 2009; Reid et al., 2010; Zanguee et al., 2010; Morine et al., 2012; Koinari et al., 2013). Further morphological and molecular characterization of these novel piscine genotypes will help to clarify the validity of *Piscicryptosporidium* as a genus.

In the present study, *Cryptosporidium* was not observed on histological examination of gastrointestinal tissues taken from the ornamental fish tested by PCR. It is possible that *Cryptosporidium* was not observed in these fish due to low number of parasites and the multifocal nature of infection in gastrointestinal tissues. In addition, due to the small size of fish species tested in this study, it was not always possible to sample tissues for both PCR and histology from the same fish and of the 155 samples screened by PCR, only 41 had sufficient tissue for histological analysis.

In conclusion, morphological, genetic, and biological data support the establishment of *Cryptosporidium* piscine genotype 1 as a new species and we propose the name *C. huwi.*

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Fig. 1a. Evolutionary relationships of *C. huwi* n. sp. and other *Cryptosporidium* spp. inferred by distance analysis of 18S rRNA sequences. Percentage support (>50%) from 1000 pseudoreplicates from neighbor-joining analyses is indicated at the left of the supported node. b) Phylogenetic relationship of *C. huwi* n. sp., with other piscine *Cryptosporidium* genotypes.

Fig. 2. Evolutionary relationships of *C. huwi* n. sp. and other *Cryptosporidium* spp. inferred by distance analysis of actin sequences. Percentage support (>50%) from 1000 pseudoreplicates from neighbor-joining analyses is indicated at the left of the supported node.

Fig. 3. Hematoxylin and eosin-stained sections of a guppy stomach showing large numbers of *C. huwi* n. sp. organisms along the epithelial lining of the stomach (A) with adjacent areas not infected (B). Clusters of oogonial and sporogonial stages are located deep within the epithelium (C and D).

Table 1. Prevalence of *C. huwi n. sp*. in ornamental fish in the present study (95% confidence intervals are given in parenthesis).

Host common name	**Host species name**	**No sampled**	**No positive**	**Prevalence**	***Cryptosporidium* species/genotype**
Neon Tetra	*Paracheirodon innesi*	90	7	7.8 (2.2-13.3)	*C. huwi*
Guppy	*Poecilia reticulata*	10	2	20 (0.0-44.8)	*C. huwi*
Tiger Barb	*Puntius tetrazona*	10	1	10 (0.0-28.6)	*C. huwi*
Ruby Barb	*Puntius nigrofasciatus*	5	0	0 (0.0-0.0)	-
Oscar	*Astronotus ocellatus*	20	1	5 (0.0-14.6)	Piscine genotype 2
Gold Gourami	*Trichogaster trichopterus*	5	0	0 (0.0-0.0)	-
Goldfish	*Carassius auratus*	15	0	0 (0.0-0.0)	-

auratus			
	155	11	7.1 (3.1-11.1)

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