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# Identification of novel and zoonotic *Cryptosporidium* species in fish from Papua New Guinea

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**Abstract**

There is still limited information on the distribution of *Cryptosporidium* species and genotypes in fish. The present study investigated the prevalence of *Cryptosporidium* species in cultured freshwater (*n*=132), wild freshwater (*n*=206) and wild marine (*n*=276) fish in Papua New Guinea (PNG) by PCR screening at the 18S rRNA locus. A total of seven fish (2 cultured freshwater, 1 wild freshwater and 4 wild marine fish) were identified as positive for *Cryptosporidium*. Specifically, *Cryptosporidium* was found in four different host species (Nile tilapia, *Oreochromis niloticus*; silver barb, *Puntius gonionotus*; mackerel scad, *Decapterus maracellus* and oblong silver biddy, *Gerres oblongus*), giving an overall prevalence of 1.14 % (95 % CI: 0.3 % - 2 %, *n*=7/614). Of the seven positive isolates, five were identified as *C. parvum* and two were a novel piscine genotype, which we have named piscine genotype 8. Piscine genotype 8 was identified in two marine oblong silver biddies and exhibited 4.3 % genetic distance from piscine genotype 3 at the 18S locus. Further subtyping of *C. parvum* isolates at the 60 kDa glycoprotein (*gp60*) locus identified 3 *C. parvum* subtypes (IIaA14G2R1, IIaA15G2R1 and IIaA19G4R1) all of which are zoonotic and a *C. hominis* subtype (IdA15G1). The zoonotic *Cryptosporidium* were identified in fish samples from all three groups; cultured and wild freshwater and wild marine fish. Detection of *Cryptosporidium* among aquaculture fingerlings warrants further research to gain a better understanding of the epidemiology of *Cryptosporidium* infection in cultured fish. The identification of zoonotic *Cryptosporidium* genotypes in fish from PNG has important public health implications and should be investigated further.

**Keywords:** *Cryptosporidium*, fish, 18S rRNA, *gp60*, novel genotype, zoonotic.

## 1 Introduction

The apicomplexan protozoan parasite *Cryptosporidium* infects a wide range of mammals, birds, reptiles and fish, primarily causing diarrhoea in mammals, diarrhoea and/or catarrhal respiratory signs in birds and gastritis in reptiles and possibly fish (O'Donoghue, 1995; Ryan, 2010). *Cryptosporidium* has been described in more than 17 species of both fresh and salt water fish with parasitic stages located deep within and on the surface of the stomach or intestinal epithelium (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Alvarez-Pellitero et al., 2004; Ryan et al., 2004; Murphy et al., 2009; Reid et al., 2010; Zanguee et al., 2010; Morine et al., 2012). In fish, *Cryptosporidium* can cause high morbidity with clinical signs including variable levels of emaciation, poor growth rates, swollen coelomic cavities, anorexia, listlessness and increased mortality (Murphy et al., 2009).

Currently the only recognised species infecting fish is *Cryptosporidium molnari*, which was 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 epithelium of the stomach and seldom the intestine (Alvarez-Pellitero and Sitja-Bobadilla 2002). In 2004, *C. scophthalmi* was described in turbot (*Psetta maxima*. syn. *Scopthalmus maximus*) (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Alvarez-Pellitero et al., 2004). However, no genetic sequences are available for *C. scophthalmi* and it can thus not be considered a valid species due to the high genetic heterogeneity and morphological similarity among *Cryptosporidium* species in fish. In addition to *C. molnari*, a total of 3 species and 10 genotypes have been characterised genetically in fish (Table 1).

Fish are an important part of the diet and a source of income especially for people living on the coast and along the rivers in Papua New Guinea (PNG). Freshwater fish farming began in the 1960s with the introduction of carp and trout species; however, difficulties due to lack of knowledge among farmers impeded farming progress and the spread of its nutritional and financial benefits to rural communities (Smith, 2007). Since 1995, the number of inland aquaculture operations has increased in PNG due to international programs that involve the expansion of hatcheries, training of farmers and the introduction of new fish species (Smith, 2007). To date, very little is known about the prevalence and genotypes of *Cryptosporidium* in fish or other animals in PNG (Owen, 2005; Koinari et al., 2012; Koinari et al., 2013). The present study represents a detailed investigation of the prevalence and genetic characterisation of *Cryptosporidium* in cultured freshwater, wild freshwater and wild marine fish sampled from a number of different locations throughout PNG. It is therefore the first comprehensive study describing the distribution of *Cryptosporidium* species in PNG.

## 2 Materials and Methods

### *2.1 Sample collection*

A total of 614 fish from cultured freshwater, wild freshwater and wild marine environments were collected in PNG between February and August 2011 (Fig. 1). Cultured fish ($n$=133) included three species, which were collected from four smallholder fish ponds in Kundiawa, Asaro, Mumeng and Bathem (Table 2). Wild freshwater fish ($n$=205) included six species and were collected from the Ramu and Sepik Rivers, while 276 wild marine fish consisting of 16 species were bought from local fishermen in Bilbil, Madang, Tavana and Pilapila (Table 2). On average, time lag between collection or purchasing and processing of the fish was up to 4 h. All sampling was conducted under Murdoch University Animal Ethics permit R2369/10.

The fish were weighed, measured (length and weight) and dissected. Sections of intestine and stomach were cut using a sterile scalpel blade for each fish, placed in 2 mL Eppendorf tubes and preserved in 70 % ethanol for molecular screening. The remaining stomach and intestine were fixed in 10 % buffered formalin for histological analysis. All samples were stored at 4 °C in

PNG until sample collection was completed. The samples were then transported to Murdoch University, Perth, Australia and stored at 4 °C until analysis.

*2.2 DNA isolation*

The preserved intestines and stomachs were washed 5 times with water to remove ethanol and the epithelial layers were scraped using a sterile scalpel blade for each fish. DNA was extracted from 25 mg of intestinal and stomach scrapings using a PowerSoil® DNA Isolation Kit (MO BIO laboratories, Carlsbad, California, USA) according to the manufacturer's instructions and incorporating five freeze-thaw cycles as described previously (Ng et al., 2006) to break open the *Cryptosporidium* oocysts. DNA was eluted in 50 µL of elution buffer. All extracted samples were stored at -20 °C until required for screening.

*2.3 Cryptosporidium genotyping and subtyping*

All 614 samples from fish were screened for the presence of *Cryptosporidium* at the 18S rRNA locus as previously described (Morgan et al., 1999). Prevalences were expressed as a percentage of positive samples; with 95 % confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rozsa et al., 2000). Isolates that were positive at the 18S locus were subtyped at the 60 kDa glycoprotein (*gp60*) locus using primers which produce an 850 bp product (Alves et al., 2003)., however, amplification was unsuccessful and a shorter (400 bp) product was amplified as described by Sulaiman et al. (2005).

Positive isolates were also amplified at the actin locus. New primers were designed specifically based on actin gene sequences of piscine-derived *Cryptosporidium* and a semi-nested PCR protocol was used. For the primary PCR, a PCR product of ~392 bp was amplified using the forward primer ActinallF1 (5'-GTAAATATACAGGCAGTT-3') and reverse primer ActinallR1 (5'-GGTTGGAACAATGCTTC-3'). Each PCR was performed in a reaction volume of 25 µL using 1 µL of DNA, 1 x PCR buffer (Kapa Biosystems, Cape Town, South Africa), 2.0 mM $MgCl_2$, 200 µM (each) dNTP (Fisher Biotech, Perth, Australia), 12.5 pmol of forward and reverse primers and 0.5 U of *kapa* Taq DNA polymerase (Kapa Biosystems, Cape Town, South Africa). Forty-five PCR cycles (95 ℃ for 30 s, 46 ℃ for 30 s, 72 ℃ for 30 s) were performed using a Perkin Elmer Gene Amp PCR 2400 thermocycler with an initial hot start (95 °C for 4 min) and a final extension (72 ℃ for 7 min). For the secondary PCR, a fragment of ~278 bp was amplified using 1 µL of primary PCR product with forward primer ActinallF2 (5'-CCTCATGCTATAATGAG- 3') and reverse primer ActinallR1. The conditions used for the secondary PCR were identical to those for the primary PCR.

Secondary PCR products were separated by gel electrophoresis and purified using a simple tip elution method. Briefly, the PCR product was excised from the gel using a scalpel blade and purified using an in house filter tip method and used for sequencing without any further purification as previously described (Yang et al., 2013). DNA sequencing was performed using the ABI Prism BigDye® terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 3730 DNA Analyser instrument. Nucleotide sequences were analysed using FinchTV 1.4.0 (Geospiza, Inc., Seattle, WA, USA; http://www.geospiza.com) and aligned with reference genotypes retrieved from GenBank using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Phylogenetic trees were constructed using additional sequences retrieved from GenBank. Distance estimation was conducted using MEGA5 (Tamura et al., 2011) based on evolutionary distances calculated using the *p* distance model (Nei and Zhang, 2006) and grouped using neighbour-joining. Parsimony and maximum likelihood analyses were also conducted using the MEGA5 software. Reliabilities for the trees were tested using 1000 bootstrap replications (Felsenstein, 1985) and bootstrap values exceeding 70 were considered well supported (Hills and Bull, 1993).

*2.4 Microscopy*

Sections of intestinal and stomach tissues fixed in 10 % formalin were embedded in paraffin. Histological sections were cut at 5 µm thicknesses, stained with hematoxylin and eosin and examined with an Olympus BX50 light microscope at 400 and 1000 fold magnification.

## 3 Results

*3.1 Prevalence of Cryptosporidium in fish hosts*

Of the 614 fish sampled, seven (1.14 %, 95 % CI 0.3 % - 2 %) were positive by PCR, of which, two (0.33 %, 95 % CI 0.09 % - 1.2 %) were cultured fingerlings, one (0.16 %, 95 % CI 0.03 % - 0.9 %) was a wild freshwater species and four (0.65 %, 95 % CI 0.25 % - 1.66 %) were wild marine species. Among the fish hosts infected were: two Nile tilapias (*Oreochromis niloticus*) with a prevalence of 2.4 % (95 % CI = 0 % - 5.7 %; 2/83) from fish ponds in Kundiawa and Mumeng; one silver barb (*Puntius gonionotus*) from Sepik River with a prevalence of 1.9 % (95 % CI = 0 % - 5.7 %; 1/52); two oblong silver biddies (*Gerres oblongus*) from Bilbil and Tavana with a prevalence of 3.6 % (95 % CI = 0 % - 8.5 %; 2/55) and two mackerel scads (*Decapterus macarellus*) from Pilapila with a prevalence of 6.9 % (95 % CI = 0 % - 16.1 %; 2/29) (Fig.1).

*3.2 Sequence and phylogenetic analysis of the 18S rDNA, gp60 and actin genes*

Sequence analysis identified *C. parvum* (5 isolates: Nile tilapia ON36 and ON68, mackerel scad DM17 and DM18, and silver barb PG37) and a novel piscine genotype (2 isolates: silver biddy GO18 and GO55), hereafter referred to as piscine genotype 8 at the 18S rRNA locus (Table 3). The two piscine genotype 8 isolates from silver biddies were genetically identical to each other, but distinct from all isolates previously characterised at the 18S rRNA locus (Table 4). Neighbour-joining, parsimony and maximum likelihood analysis produced similar results and indicated that piscine 8 genotype clustered most closely with the piscine 3 genotype from a sea mullet, while the other five isolates regularly clustered with *C. parvum* (85 % bootstrap support) (Fig. 2). Sequences were also obtained for the two piscine genotype 8 isolates at the actin locus. At this locus, sequence information was only available for *C. molnari* and piscine genotype 1 and piscine genotype 8 grouped more closely with *C. molnari* genotypes (7.3 % - 8.5 % genetic differences) (Fig. 3 and Table 4).

At the *gp60* locus, three subtypes belonging to *C. parvum* (family IIa: IIaA14G2R1, IIaA15G2R1, IIaA19G4R1) and a *C. hominis* subtype (family Id: IdA15G1) were identified (Table 3).

*3.3 Microscopy*

No parasites were observed during microscopic examination of the intestinal or stomach tissues due to substantial autolysis of tissues.

*3.4 Nucleotide sequence accession numbers*

The unique partial 18S rRNA and actin sequences of piscine genotype 8 from silver biddies were deposited in the GenBank database under the accession numbers KC807985 to KC807988.

## 4. Discussion

In the present study, the overall prevalence of *Cryptosporidium* sp. was low (1.14 %, 7/614). Previous studies have also reported low prevalences in similar groups of fish (0.8 %, 6/709) (Reid et al., 2010) and in ornamental fish (3.5 %, 6/171) (Morine et al., 2012) while others have reported higher prevalences (10 - 100 %) mostly among juvenile fish (Alvarez-Pellitero et al., 2004; Sitja-Bobadilla et al., 2005; Murphy et al., 2009; Zanguee et al., 2010).

This study identified three new fish hosts for *Cryptosporidium*; silver barb (*P. gonionotus*), mackerel scad (*D. maracellus*) and oblong silver biddy (*G. oblongus*). The fourth host Nile tilapia *(O. niloticus)* could also be a new host since previous studies have detected *Cryptosporidium* in the same genus but did not identified the species (Landsberg and Paperna, 1986; Paperna and Vilenkin, 1996).

No oocysts or life cycle stages were observed in the infected fish hosts in the present study due to substantial autolysis of tissues, which has been reported as an issue for *Cryptosporidium* detection in piscine hosts (Zanguee et al., 2010). Fish are known to have a very rapid rate of tissue autolysis compared to homeotherms (Roberts, 2012) and many of the fish were dead for up to 4 hours prior to being processed which contributed to the problem. Previous studies have provided histological and electron microscopic evidence of considerable cellular damage associated with several *Cryptosporidium* species/genotypes that infect fish; *C. molnari* in the stomach of fingerlings and juveniles of gilt-head sea bream (Alvarez-Pellitero and Sitja-Bobadilla, 2002), piscine genotype 1 in the stomach of a guppy (Ryan et al., 2004) and piscine genotype 3 in the intestine of a mullet (Reid et al., 2010). Piscine genotype 2 was associated with gastric infections in angelfish, with the greatest morbidity and mortality seen in larval and juvenile fish (Murphy et al., 2009). Whether the *C. parvum* and *C. hominis* identified in the present study represents actual or mechanical infections remains to be determined as the oocysts may have been passing through rather than infecting these fish and further research using histological analysis of rapidly preserved tissue specimen is required to confirm this.

The zoonotic *Cryptosporidium* genotypes identified in this study are of significance to public health. *Cryptosporidium parvum* subtypes IIaA14G2R1, IIaA15G2R1 and IIaA19G4R1 were found in cultured freshwater (Nile tilapia), wild freshwater (silver barb) and a marine (mackerel scad) fish. The zoonotic *C. parvum* IIa subtype family has predominantly been found in calves and in humans in North America, Europe and Australia (Xiao, 2010). Only one study has previously detected zoonotic *C. parvum* (subtype IIaA18G3R1) in a marine fish (Reid et al, 2010). The presence of IIa subtypes in the fish samples from the present study could be due to waterborne contamination with human and animal waste. Human sewage management systems in PNG villages typically involve dugout toilets near the homes and toilets built over the rivers or seas. No cattle farms were seen in close vicinity to where the samples were collected, whereas dugout toilets, companion animals and/or domesticated poultry and pigs were seen in the environment. Fish ponds, rivers and seas could be contaminated from rainwater runoff and from humans and/or animals bathing in them. A previous study has detected antibodies against *Cryptosporidium* among children from PNG (Groves et al., 1994), however, no molecular work has been done to confirm the species or genotypes present.

One marine fish (mackerel scad DM18) was identified as *C. parvum* at the 18S locus while at the *gp60* locus it was subtyped as *C. hominis* IdA15G1R1, indicating that a mixed *C. parvum/C. hominis* infection was present. This is the first report of *C. hominis* in fish. The only other marine organism in which *C. hominis* has been reported was a dugong (*Dugong dugon*) (Morgan et al., 2000), and its presence probably reflects human sewage contamination of the water.

The novel piscine genotype 8 was identified in two marine silver biddies. At the 18S locus, piscine genotype 8 exhibited 4.3 % genetic difference with piscine genotype 3 and 13.8 % - 17.7 % genetic difference with other *Cryptosporidium* spp. (Table 4). At the actin locus, piscine genotype 8 exhibited 7.3 % genetic difference with *C. molnari 2* and 19.0 % - 21.4 % with other *Cryptosporidium* spp. (Table 4). Based on the differences in the genetic sequences, piscine genotype 8 is unique and may represent a new species; however, further research is required to confirm this.

The present study identified zoonotic *C. parvum* subtypes in fish species, which are frequently eaten in PNG. Previous studies have not identified conclusive evidence for transmission of *Cryptosporidium* from fish to humans but one study reported that urban anglers are at a risk for

contracting cryptosporidiosis from exposures received while fishing and consuming caught fish (mean probability of infection was nearly one) (Roberts et al., 2007). It is therefore essential that fish for human consumption are handled appropriately to avoid contamination. In addition, a novel *Cryptosporidium* sp. (piscine genotype 8) was identified based on molecular data but lacks histological details of infection or morphological features of the oocysts, thus future work is required to establish this genotype as a species.

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334

**Table captions**

Table 1: Species/genotypes of *Cryptosporidium* reported in fish in previous studies.

Table 2: Cultured freshwater, wild freshwater and marine fish species collected in the present study.

Table 3**:** Species, genotypes and subtypes of *Cryptosporidium* identified in fish in the present study. Sample DM18 was typed as *C. parvum* at the 18S locus but at *gp60* locus it was typed as *C. hominis* (IdA15G1) indicating the presence of mixed *C. parvum/C. hominis* in this sample.

Table 4: Percentage of genetic differences between piscine genotype 8 and other *Cryptosporidium* species/genotypes at the 18S rRNA and actin loci.

343 **Figure Legends**

344 **Figure 1. Map of the study sites in Papua New Guinea.** Cultured freshwater fish were
345 obtained from Bathem, Kundiawa, Asaro and Mumeng; wild freshwater fish were collected in
346 the Ramu River near Sausi and the Sepik River near Pagwi and marine fish were collected from
347 Bilbil, Madang, Pilapila and Tavana.

348 **Figure 2. Evolutionary relationships of *Cryptosporidium* piscine-derived isolates inferred**
349 **by neighbour-joining analysis of *p* distances calculated from pairwise comparison of 18S**
350 **rRNA sequences.** The percentage of replicate trees in which associated taxa clustered together
351 in the bootstrap test (1000 replicates), are shown at the internal nodes (>50% only) for distance,
352 ML and parsimony (n.s. = not supported). Accession numbers are given in parentheses. Isolates
353 from the present study are marked with asterisk (*).

354 **Figure 3. Phylogenetic relationships of *Cryptosporidium* isolates inferred by neighbour-**
355 **joining analysis of the actin gene based on genetic distances calculated by the *p* distance**
356 **model.** The percentage of replicate trees in which associated taxa clustered together in the
357 bootstrap test (1000 replicates), are shown at the internal nodes (>50% only) for distance, ML
358 and parsimony (n.s. = not supported). Accession numbers are given in parentheses. Isolates from
359 the present study are marked with asterisk (*).

360

360 **Table 1:** Species/genotypes of *Cryptosporidium* reported in fish in previous studies (N = the
361 number of specimens in which each species/genotype of *Cryptosporidium* was identified.

| Species/genotype | N | Fish Host | Reference |
|---|---|---|---|
| *C. molnari* | >100 | Gilthead sea bream and European seabass | Alvarez-Pellitero and Sitja-Bobadilla, 2002; Palenzuela et al., 2010 |
| *C. scophthalmi* | 49 | Turbot | Alvarez-Pellitero and Sitja-Bobadilla, 2002; Alvarez-Pellitero et al., 2004 |
| *C. molnari -like* | 7 | Butter bream, madder seaperch, bristle tooth tang, upsidedown catfish, wedgetailed blue tang and green chromas, golden algae eater | Zanguee et al., 2010 |
| Piscine genotype 1 | 2 | Guppy and neon tetra | Ryan et al., 2004; Zanguee et al., 2010 |
| Piscine genotype 2 | >5 | Angelfish, neon tetra and Oscar fish | Murphy et al., 2009; Zanguee et al., 2010 |
| Piscine genotype 3 | 2 | Sea mullet | Reid et al., 2010 |
| Piscine genotype 4 | 4 | Golden algae eater, kupang damsel, Oscar fish and neon tetra | Zanguee et al., 2010; Morine et al., 2012 |
| Piscine genotype 5 | 3 | Angelfish, butter bream and golden algae eater | Zanguee et al., 2010 |
| Piscine genotype 6 | 1 | Guppy | Zanguee et al., 2010 |
| Piscine genotype 6-like | 1 | Gold gourami | Morine et al., 2012 |
| Piscine genotype 7 | 3 | Red-eye tetra | Morine et al., 2012 |
| Rat genotype 3-like | | Goldfish | Morine et al., 2012 |
| *C. scrofarum* | 2 | School whiting | Reid et al., 2010 |
| *C. parvum* | 1 | School whiting | Reid et al., 2010 |
| *C. xiaoi* | 1 | School whiting | Reid et al., 2010 |

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**Table 2**: Cultured freshwater, wild freshwater and marine fish species collected in the present study.

| Cultured freshwater fish | Locations | | | | |
|---|---|---|---|---|---|
| | Kundiawa | Asaro | Mumeng | Bathem | Total |
| Nile tilapia (*Oreochromis niloticus*) | 36 | 20 | 27 | - | 83 |
| Common carp (*Cyprinus carpia*) | - | 11 | 32 | - | 43 |
| Mozambique tilapia (*Oreochromis massambic*) | - | - | - | 7 | 7 |
| **Total** | **36** | **31** | **59** | **7** | **133** |
| Wild freshwater fish | | Ramu River | Sepik River | | Total |
| Silver barb (*Puntius gonionotus*) | | 13 | 39 | | 52 |
| Highfin catfish (*Neoarius berneyi*) | | - | 20 | | 20 |
| Mozambique tilapia (*Oreochromis massambic*) | | - | 15 | | 15 |
| Pacu (*Colossoma bidens*) | | - | 34 | | 34 |
| Indo-Pacific trapon (*Megalops cyprinoides*) | | - | 70 | | 70 |
| Redtail catfish (*Phractocephalus hemioliopterus*) | | - | 14 | | 14 |
| **Total** | | **12** | **192** | | **205** |
| Wild marine fish | Bilbil | Madang | Pilapila | Tavana | Total |
| Bigeye scad (*Selar crumenopthalmus*) | 46 | - | 60 | - | 106 |
| Bigeye trevally (*Caranx sexfasciatus*) | 4 | - | - | - | 4 |
| Blackfin barracuda (*Sphyraena qenie*) | 9 | - | - | - | 9 |
| Coachwhip trevally (*Carangoides oblongus*) | - | - | - | 1 | 1 |
| Indo-Pacific trapon (*Megalops cyprinoides*) | 4 | - | - | - | 4 |
| Mackerel scad (*Decapterus macarellus*) | 5 | - | 24 | - | 29 |
| Oblong silver biddy (*Gerres oblongus*) | 1 | - | - | 54 | 55 |
| Oriental bonito (*Sarda orientalis*) | - | 4 | - | - | 4 |
| Pinjalo snapper (*Pinjalo pinjalo*) | - | - | 1 | - | 1 |
| Rainbow runner (*Elagatis bipinnulatus*) | - | 5 | - | - | 5 |
| Reef needlefish (*Strongylura incisa*) | - | 4 | - | - | 4 |
| Slender pinjalo (*Pinjalo lewisi*) | - | - | - | 14 | 14 |
| Spanish mackerel (*Scomberomous maculatus*) | - | 3 | - | - | 3 |
| Talang queenfish (*Scomberoides commersonnianus*) | - | - | 2 | - | 2 |
| Wahoo (*Acanthocybium solandri*) | - | 1 | - | - | 1 |
| Yellow fin tuna (*Thunnus albacares*) | - | 34 | - | - | 34 |
| **Total** | **69** | **51** | **87** | **69** | **276** |

**Table 3**: Species, genotypes and subtypes of *Cryptosporidium* identified in fish in the present study. Sample DM18 was typed as *C. parvum* at the 18S locus but at *gp60* locus it was typed as *C. hominis* (IdA15G1) indicating the presence of mixed *C. parvum/C. hominis* in this sample.

| Sample | Host species | Group | 18S | *gp60* | Actin |
|---|---|---|---|---|---|
| ON36 | Nile tilapia | Cultured | *C. parvum* | IIaA19G4R1 | - |
| ON68 | Nile tilapia | Cultured | *C. parvum* | IIaA14G2R1 | - |
| PG37 | Silver barb | Wild freshwater | *C. parvum* | IIaA19G4R1 | - |
| DM17 | Mackerel scad | Wild marine | *C. parvum* | IIaA15G2R1 | - |
| DM18 | Mackerel scad | Wild marine | *C. parvum*, | IdA15G1 | - |
| GO18 | Silver biddy | Wild marine | Piscine genotype 8 | - | Piscine genotype 8 |
| GO55 | Silver biddy | Wild marine | Piscine genotype 8 | - | Piscine genotype 8 |

372 **Table 4**: Percentage of genetic differences between piscine genotype 8 and other
373 *Cryptosporidium* species/genotypes at the 18S rRNA and actin loci.

| | 18S locus | Actin locus |
|---|---|---|
| Piscine genotype 1 | 13.0 | 17.4 |
| Piscine genotype 2 | 5.1 | Not analysed* |
| Piscine genotype 3 | 4.3 | Not analysed* |
| Piscine genotype 4 | 6.3 | Not analysed* |
| Piscine genotype 5 | 5.5 | Not analysed* |
| Piscine genotype 6 | 13.0 | Not analysed* |
| Piscine genotype 7 | 11.8 | Not analysed* |
| *C. molnari* | 10.8 | 7.3-8.5 |
| *C. parvum* | 15.4 | 21.4 |
| *C. hominis* | 15.4 | 21.4 |
| *C. bovis* | 17.7 | 19.0 |
| *C. baileyi* | 14.7 | 19.4 |
| *C. muris* | 15.0 | 19.4 |
| *C. andersoni* | 13.8 | 20.1 |

374 *Actin sequences were not available for these genotypes.
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