Title: Identification of novel *Cryptosporidium* species in aquarium fish.

Authors: N. Zanguee, J.A. Lymbery, J. Lau, A. Suzuki, R. Yang, J. Ng, U. Ryan

PII: S0304-4017(10)00431-0
DOI: doi:10.1016/j.vetpar.2010.08.006
Reference: VETPAR 5418

To appear in: *Veterinary Parasitology*

Received date: 1-6-2010
Revised date: 29-7-2010
Accepted date: 10-8-2010

Please cite this article as: Zanguee, N., Lymbery, J.A., Lau, J., Suzuki, A., Yang, R., Ng, J., Ryan, U., Identification of novel *Cryptosporidium* species in aquarium fish., *Veterinary Parasitology* (2010), doi:10.1016/j.vetpar.2010.08.006

Identification of novel *Cryptosporidium* species in aquarium fish.

N. Zanguee[a, b], J A. Lymbery[b], J. Lau[c], A. Suzuki[c], R. Yang[b], J. Ng, U. Ryan[c*].

[a]*Department of Fisheries, Faculty of Marine Natural Resources, Khoramshahr Marine Science and Technology, Khoramshahr, Khouzestan, Iran.*

[b]*Fish Health Unit, Centre for Fish and Fisheries Research, Murdoch University, Murdoch, Western Australia, 6150.*

[c]*Division of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Western Australia, 6150.*

**Corresponding author. Mailing address: Division of Health Sciences, School of Veterinary and Biomedical Sciences, Murdoch University, Murdoch, Western Australia, Australia 6150. Phone: 61 89360 2482. Fax: 61 89310 414. E-mail: Una.Ryan@murdoch.edu.au*

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 twenty-one 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.

Keywords: *Cryptosporidium*, ornamental fish, 18S rRNA, new 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). *Cryptosporidium 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.

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

0.5µL 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, California) according to the manufacturer's instructions with the exception that the annealing temperature was raised to 58ºC. 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, Arizona, USA). Bootstrap analyses were conducted using 1,000 replicates to assess the reliability of inferred tree topologies. Maximum Likelihood (ML) analyses were conducted using the program PhyML (Dereeper et al., 2008) and the reliability of the inferred trees was assessed by the approximate likelihood ratio test (aLRT) (Anisimova and Gascuel, 2006).

2.4. Calculation of prevalences

Prevalences were expressed as percentage of positive samples, with 95% confidence intervals calculated assuming a binomial distribution, using the software Quantitative Parasitology 3.0 (Rozsa et al., 2000).

3. Results

3.1. Prevalence of Cryptosporidium in fish hosts

Of the 200 fish sampled during this study from 39 different species, twenty-one positives were detected in 13 different species by PCR (10.5% prevalence, with 95% CI 6.3-14.7) (Table 1). Infected hosts were angelfish (n=1), butter-bream (n=2), dispar anthias (n=1), false gramma (n=1), golden algae eater (n=3), green chromis (n=2), guppy (n=1), kupang damsel (n=1), madder seaperch (1), neon tetra (n=2), oscar (n=4), upside down cat fish (n=1), a bristle tooth tang (n = 1) and a wedgetailed blue tang (n=1). No PCR inhibition was detected.

3.2. Identification of Cryptosporidium species in fish

Partial sequences were obtained for nineteen of the twenty-one positive isolates at the 18S rRNA locus. Unfortunately no isolates were successfully amplified at the actin locus despite numerous attempts. Neighbour-joining, parsimony and ML analysis of the 18S rRNA sequences from these 19 isolates and a range of *Cryptosporidium* species and genotypes

obtained from GenBank produced similar results (Figure 1-ML tree shown). Twelve of the 19 isolates were similar or identical to previously described species/genotypes of *Cryptosporidium*. Isolate 101 from a neon tetra was 100% identical to piscine genotype 1 (GenBank accession no. AY524773). Isolates 13 and 112 and 113, all from oscar fish and isolate 110 from a neon tetra were genetically identical and exhibited just one single nucleotide polymorphism (SNP) from piscine genotype 2 (GenBank accession no. FJ769050). Isolate 9 from a butter bream, 107 from an upsidedown catfish, 127 from a wedgetailed blue tang, 144 from a madder seaperch, 145 from a bristle tooth tang and 151 from a green chromas were 100% identical to each other and 99.2% identical (three SNPs) to *C. molnari* (GenBank accession number HM243547). Isolate 117 from a golden algae eater had 8 SNPs from *C. molnari* and 9 SNPs from isolates 9, 107, 127, 144, 145 and 151. The remaining seven isolates represent three novel species/genotypes; piscine genotype 4 which comprised isolate16 from a golden algae eater, 106 from a kupang damsel and 114 from an oscar (100% identical); piscine genotype 5 which comprised isolates 95 from an angelfish and 129 from a butter bream and isolate 121 from a golden algae eater. Isolates 95 and 129 were 100% identical to each other and had two SNPs from isolate 121; piscine genotype 6 which comprised isolate192 from a guppy. The genetic distance between these three novel species/genotypes and other, previously described species and genotypes of *Cryptosporidium* is shown in Table 2.

3.3. Nucleotide sequence accession numbers

The unique partial 18S rRNA sequences of the isolates 9, 16, 95, 101, 117, 121 and 192 have been deposited in the GenBank database under the accession numbers HM989832 to HM989837 and HM991857.

4. Discussion

In the present study, the overall prevalence of *Cryptosporidium* spp. in the ornamental fish was 10.5% (CI 6.3-14.7). This was considerably higher than the prevalence of 0.8% (6/709) for *Cryptosporidium* reported in 709 cultured, wild marine and wild freshwater fishes in a recent study (Reid et al., 2010). Other studies have reported prevalences of up to 100%, mostly among juvenile fish (Landsberg and Paperna, 1986; Sitja-Bobadilla et al., 2005; Alvarez-Pellitero et al., 2004; 2009; Murphy et al. 2009). The higher prevalence of *Cryptosporidium* in ornamental fish in the present study compared to the study by Reid et al., (2010) may have been due to the crowded environment of the aquarium tanks and the frequent introduction of new species; infectious diseases are often much more prevalent in ornamental fish than in wild fish or captive aquaculture fish (Burgess et al., 1999).

In the present study, a variety of new hosts were identified for established piscine species/genotypes of *Cryptosporidium.* Piscine genotype 1 (AY524773) was identified in a neon tetra and piscine genotype 2 (FJ769050) was identified in a neon tetra and oscar fish. *Cryptosporidium molnari*-like genotypes were identified in a butter bream, a golden algae eater, a green chromas, a madder sea-perch, an upsidedown catfish and a wedgetailed blue tang.

In addition to these extensions of host range for established species/genotypes of *Cryptosporidium*, three new, distinct piscine genotypes numbered 4-6, were identified in angelfish, butter bream, golden algae eater, kupang damsel, oscar and guppy (Figure 1). A previous study by Reid et al., (2010), identified piscine genotype 3 in sea mullets. In the present study, this genotype was not identified and the isolates examined exhibited 5.6-12.6% genetic distance from piscine genotype 3.

Molecular and phylogenetic analysis of the 18S rRNA locus supports the species status of these genotypes. The genetic distance between the piscine genotypes 4 to 6 and other fish-derived species of *Cryptosporidium* was 4.0-13.4% and between the novel genotypes and other *Cryptosporidium* species and genotypes was between 10.5 and 18.3%. This is considerably greater than the genetic distance between most other species of *Cryptosporidium*. For example, *C. parvum* and *C. hominis* differ by 0.6% at the 18S locus and the genetic distance between *C. parvum* and all other intestinal species is 0.6% - 2.3%. Within *Cryptosporidium* taxonomy, if the genetic distance between a new genotype and its closest relative is equal to or greater than the genetic distance between established *Cryptosporidium* species, then this is regarded as a valid criterion for claiming species status (Xiao et al., 2004). By this criterion, the novel genotypes found in ornamental fish in this study appear to be separate species. Unfortunately, we have not yet been able to examine the morphological or life history characteristics of these genotypes. The genetic distance between *C. molnari* and the *C. molnari*-like genotypes was 0.8-2.8% and therefore the *C. molnari*-like genotypes are also likely to be separate species. Genetic characterization at additional loci in the future will be important for clarification of their species status.

Histological examination of intestinal/stomach tissue of infected ornamental fish was conducted, but due to rapid autolysis of fish tissue were unable to confirm the presence of *Cryptosporidium* spp. in either intestinal or stomach tissues. Some of the fish were dead for

194 several hours prior to being collected and some were obtained frozen from suppliers, which
195 would also contribute to the substantial autolysis seen.

196 Phylogenetic analysis of 18S rRNA sequences indicated that all the piscine genotypes
197 identified in the present study and all piscine genotypes from previous studies (with the
198 exception of *C. parvum*, *C. xiaoi* and pig genotype II identified in whiting in a previous study
199 by Reid et al., 2010), formed clades that were basal to all other *Cryptosporidium* species and
200 genotypes in the tree, suggesting that piscine *Cryptosporidium* isolates may be the most
201 primitive of *Cryptosporidium* species. Piscine genotype 1 (AY524773) was previously
202 referred to as *C. molnari*-like on the basis of detection in the stomach and presence of
203 oogonial and sporogonial stages deep within the epithelium, similar to *C. molnari* (Ryan et
204 al., 2004). However, phylogenetic analysis in the present study, has revealed that piscine
205 genotype 1 (AY524773) is genetically distinct from *C. molnari* and shared only 91.2%
206 similarity with *C. molnari* at the 18S locus. The present data support suggestions from other
207 recent studies of *Cryptosporidium* in fish (Ryan et al., 2004; Reid et al., 2010) that the
208 epidemiology and evolutionary relationships of piscine-derived *Cryptosporidium* species are
209 much more complex than previously thought, and additional piscine species will undoubtedly
210 be identified as more fish are examined. Further studies are required to more fully understand
211 the phylogenetic relationships and evolutionary timelines of fish-derived *Cryptosporidium*
212 isolates.

213 The pathogenesis of the *Cryptosporidium* species identified in aquarium fish in the
214 present study is unknown. However, previous studies on piscine genotype 2 (FJ769050),
215 which was identified in a hatchery, revealed that infected fish exhibited variable levels of
216 emaciation, poor growth rates, swollen coelomic cavities, anorexia, listlessness and increased
217 mortality (Murphy et al., 2009). In affected fish, large numbers of protozoa were identified
218 both histologically and ultrastructurally associated with the gastric mucosa. Piscine genotype

1 (AY524773) was associated with high mortalities amongst guppies and was detected in the stomach, with oogonial and sporogonial stages observed deep within the epithelium, similar to *C. molnari* (Ryan et al., 2004). All of the ornamental fish examined in the present study appeared unwell and exhibited a variety of symptoms including emaciation, anorexia, listlessness, tail and/or fin rot and swollen coelomic cavities. Isolate 106 from a kupang damsel (piscine genotype 4) had skin lesions and a bacterial infection, while three of the four oscar fish-derived isolates (112, 113, 114) (piscine genotypes 2 and 4) had concomitant bacterial and gill fluke infections.

The pathogenesis of *Cryptosporidium* is an issue of potentially much wider significance than its effects on companion aquarium fish and the ornamental fish industry. A large number of freshwater ornamental fish species have been released, either accidentally or deliberately, into waterways throughout the world, where they have established self-sustaining populations (Rahel, 2002). Populations of exotic ornamental fish may adversely affect the native freshwater fish through competition, predation and the introduction of diseases (Arthington, 1991; Morgan et al., 2004; Kennard et al., 2005; Lymbery et al., 2010). The high prevalence of potentially pathogenic *Cryptosporidium* found in ornamental fish in the present study may, if these fish are released, constitute a major threat to the highly endemic and threatened freshwater fish fauna of Western Australia.

Acknowledgements

We would like to thank Dr. Alvarez-Pellitero, Dr. Sitja-Bobadilla and Dr Oswaldo Palenzuela at the Institute of Aquaculture Torre de la Sal in Spain for the provision of the *C. molnari* 18S rRNA sequence used in the present study.

245 **References**

246

247 Alvarez-Pellitero, P., Sitja-Bobadilla, A., 2002. *Cryptosporidium molnari* n. sp.
248 (Apicomplexa: Cryptosporidiidae) infecting two marine fish species, *Sparus aurata* L.
249 and *Dicentrarchus labrax* L. Int. J. Parasitol. 32, 1007 - 1021.
250 Alvarez-Pellitero, P., Quiroga, M.I., Sitjà-Bobadilla, A., Redondo, M.J., Palenzuela, O.,
251 Padrós, F., Vázquez, S., Nieto, J.M., 2004. *Cryptosporidium scophthalmi* n. sp.
252 (Apicomplexa: Cryptosporidiidae) from cultured turbot *Scophthalmus maximus*. Light
253 and electron microscope description and histopathological study. Dis. Aquat. Organ. 62,
254 133-145.
255 Alvarez-Pellitero, P., Perez, A., Quiroga, M.I., Redondo, M.J., Vázquez, S., Riaza, A.,
256 Palenzuela, O., Sitjà-Bobadilla, A., Nieto, J.M., 2009. Host and environmental risk
257 factors associated with *Cryptosporidium scophthalmi* (Apicomplexa) infection in
258 cultured turbot, *Psetta maxima* (L.) (Pisces, Teleostei). Vet. Parasitol. 165, 207-15.
259 Anisimova, M., Gascuel, O., 2006. Approximate likelihood-ratio test for branches: A fast,
260 accurate, and powerful alternative. Syst. Biol. 55, 539-552.
261 Arthington, A. H., 1991. Ecological and genetic impacts of introduced and translocated
262 freshwater fishes in Australia. Can. J. Fish. Aquat. Sci. 48 (Suppl. 1), 33-43.
263 Burgess, P.H., Exell, A., Bailey, M.T., 1999. A-Z of Tropical Fish Diseases and Health
264 Problems. Howell Books New York, pp. 12–13.
265 Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J. F.,
266 Guindon, S., Lefort, V., Lescot, M., Claverie, J. M. and Gascuel, O., 2008.
267 Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res.
268 36, W465-469.

269 Kennard, M. J., Arthington, A. H., Pusey, B. J., Harch, B. D., 2005. Are alien fish a reliable
270 indicator of river health? Fresh, Biol, 50, 174-193
271 Landsberg, J.H., Paperna, I., 1986. Ultrastructural study of the coccidian *Cryptosporidium* sp.
272 from stomachs of juvenile cichlid fish. Dis. Aquat. Organ. 2, 13-20.
273 Lymbery, A.J., Hassan, M., Morgan, D.L., Beatty, S.J., Doupé, R.G., 2010. Parasites of native
274 and exotic freshwater fishes in south-western Australia. J. Fish Biol., in press.
275 Morgan, D. L., Gill, H. S., Maddern, M. G., Beatty, S. J., 2004. Distribution and impacts of
276 introduced freshwater fishes in Western Australia. N. Z. J. Mar. Fresh. Res. 30, 511-523
277 Murphy, B.G., Bradway, D., Walsh, T., Sanders, G.E., Snekvik, K., 2009. Gastric
278 cryptosporidiosis in freshwater angelfish (*Pterophyllum scalare*). J. Vet. Diagn. Invest.
279 21, 722-727.
280 Ng, J., Pavlasek, I., Ryan, U., 2006. Identification of novel *Cryptosporidium* genotypes from
281 avian hosts. Appl. Environ. Microbiol. 72, 7548-7553.
282 Rahel, F.J. 2002. Homogenization of freshwater faunas. Ann. Rev. Ecol. Syst. 33, 291-315.
283 Reid, A., Lymbery, A., Ng, J., Tweedle, S., Ryan, U., 2010. Identification of novel and
284 zoonotic *Cryptosporidium* species in marine fish. Vet Parasitol. 168, 190-5.
285 Rozsa, L., Reiczigel, J., Majoros, G., 2000. Quantifying parasites in samples of hosts. J.
286 Parasitol. 86, 228-232.
287 Ryan, U., Xiao, L., Read, C., Zhou, L., Lal, A.A., Pavlasek, I. 2003. Identification of novel
288 *Cryptosporidium* genotypes from the Czech Republic. Appl. Environ. Microbiol. 69,
289 4302-4307.
290 Ryan, U., O'Hara, A., Xiao, L., 2004. Molecular and biological characterization of
291 *Cryptosporidium molnari*-Like isolate from a guppy (*Poecilia reticulata)*. Appl.
292 Environ. Microbiol. 70, 3761-3765.

293 Sitjà-Bobadilla, A., Padrós, F., Aguilera, C., Alvarez-Pellitero, P. 2005. Epidemiology of
294 *Cryptosporidium molnari* in Spanish Gilthead Sea Bream (*Sparus aurata* L.) and
295 European Sea Bass (*Dicentrarchus labrax* L.) cultures: from hatchery to market size.
296 Appl. Environ. Microbiol. 70, 131-139.
297 Van de Peer, Y., R. De Wachter., 1994. TREECON for Windows: a software package for the
298 construction and drawing of evolutionary trees for the Microsoft Windows environment.
299 Comput. Appl. Biosci. 10, 569–570.
300 Xiao, L., Fayer, R., Ryan, U., Upton, S.J., 2004. *Cryptosporidium* taxonomy: recent advances
301 and implications for public health. Clin. Microbiol. Rev. 17, 72-97.

302
303

304
305

306 **Fig. 1**. Evolutionary relationships of *Cryptosporidium* piscine-derived isolates inferred by ML
307 analysis of 18S rRNA sequences. Percentage support (>50%) from 1000 pseudoreplicates
308 from neighbor-joining, parsimony and ML analyses indicated at the left of the supported node
309 (ns = not supported).
310
311
312
313

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

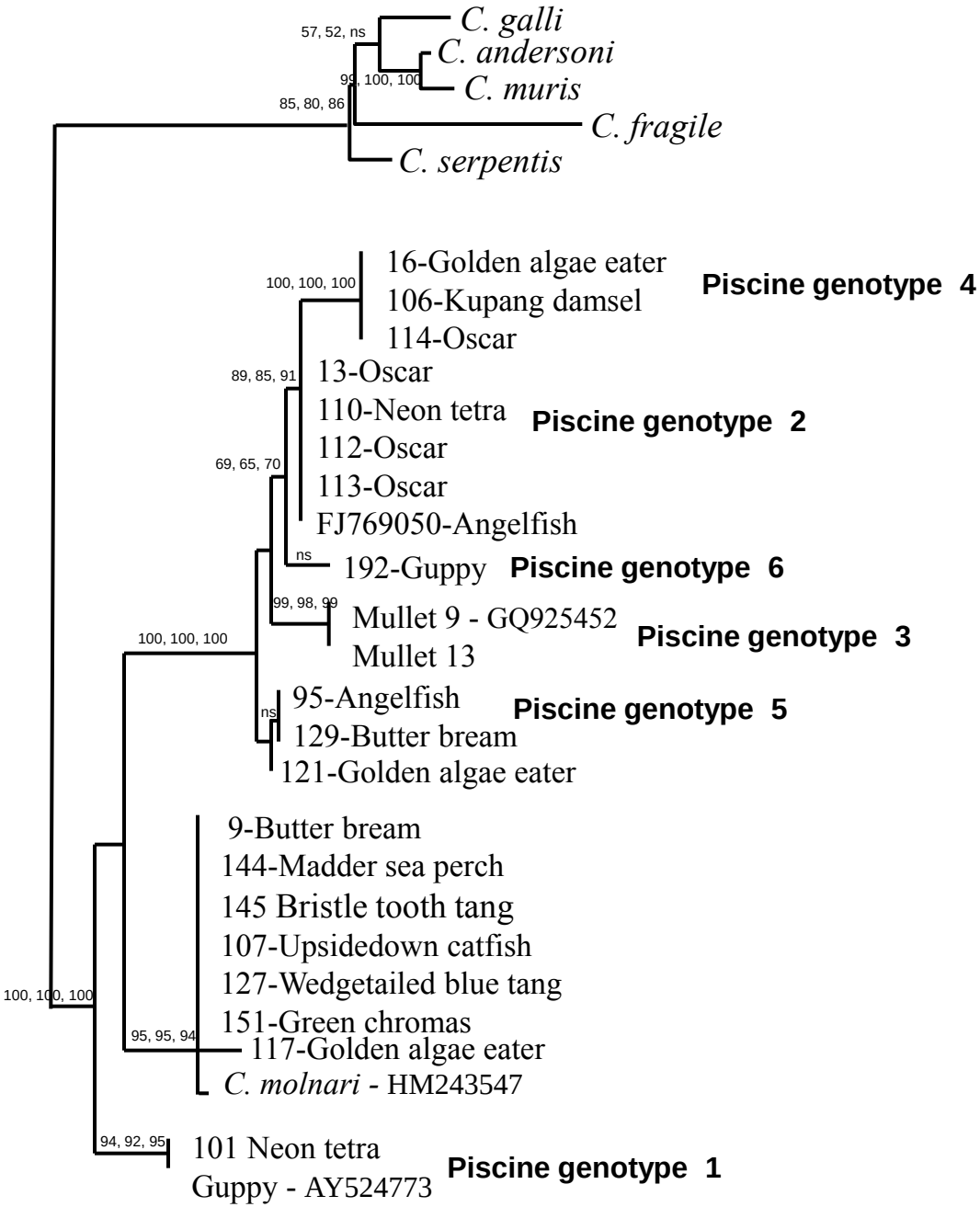
Silver gourami	*Trichogaster trichopterus*	Freshwater	2	0
Silver shark	*Balantiocheilos melanopterus*	Freshwater	9	0
Striped kuhli loach	*Pangio kuhlii*	Freshwater	1	0
True rummy nose tetra	*Hemigrammus bleheri*	Freshwater	10	0
Upside down cat fish	*Synodontis nigriventris*	Freshwater	2	1
Wedgetailed 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**

Table 2

Percentage sequence difference at the 18S rRNA locus between new genotypes of *Cryptosporidium* found in ornamental fish in this study and other *Cryptosporidium* species and genotypes calculated using Kimura's distance.

	C. molnari	**Mullet genotype GQ925452**	**Angelfish genotype FJ769050**	**Guppy genotype AY524773**	**New genotype 1 (isolates 16, 106, 114)**	**New genotype 2 (isolates 95, 129)**	**New genotype 3 (isolate 192)**	***C. muris***	***C. baileyi***	***C. parvum***
C. molnari	0									
Mullet genotype GQ925452	12.9	0								
Angelfish genotype FJ769050	10.6	5.7	0							
Guppy genotype AY524773	8.8	12.6	14.2	0						
16-Golden algae eater	12.8	5.6	4.0	13.4	0					
95-Angelfish	10.5	6.5	4.3	12.5	7.3	0				
192-Guppy	12.3	7.3	4.6	12.7	8.1	5.9	0			
C. muris	14.1	17.4	17.1	15.2	15.7	13.2	15.4	0		
C. baileyi	13.2	13.6	14.6	13.8	17.0	11.7	15.6	9.2	0	
C. parvum	16.7	18.2	16.3	15.9	18.4	16.1	16.4	12.1	7.3	0

Figure


57, 52, ns
C. galli
C. andersoni
99, 100, 100
C. muris
85, 80, 86
C. fragile
C. serpentis
100, 100, 100
16-Golden algae eater
106-Kupang damsel
114-Oscar
Piscine genotype 4
89, 85, 91
13-Oscar
110-Neon tetra
Piscine genotype 2
69, 65, 70
112-Oscar
113-Oscar
FJ769050-Angelfish
ns
192-Guppy
Piscine genotype 6
99, 98, 99
Mullet 9 - GQ925452
Mullet 13
Piscine genotype 3
100, 100, 100
95-Angelfish
ns
129-Butter bream
Piscine genotype 5
121-Golden algae eater
9-Butter bream
144-Madder sea perch
145 Bristle tooth tang
107-Upsidedown catfish
127-Wedgetailed blue tang
151-Green chromas
100, 100, 100
95, 95, 94
117-Golden algae eater
C. molnari - HM243547
94, 92, 95
101 Neon tetra
Guppy - AY524773
Piscine genotype 1