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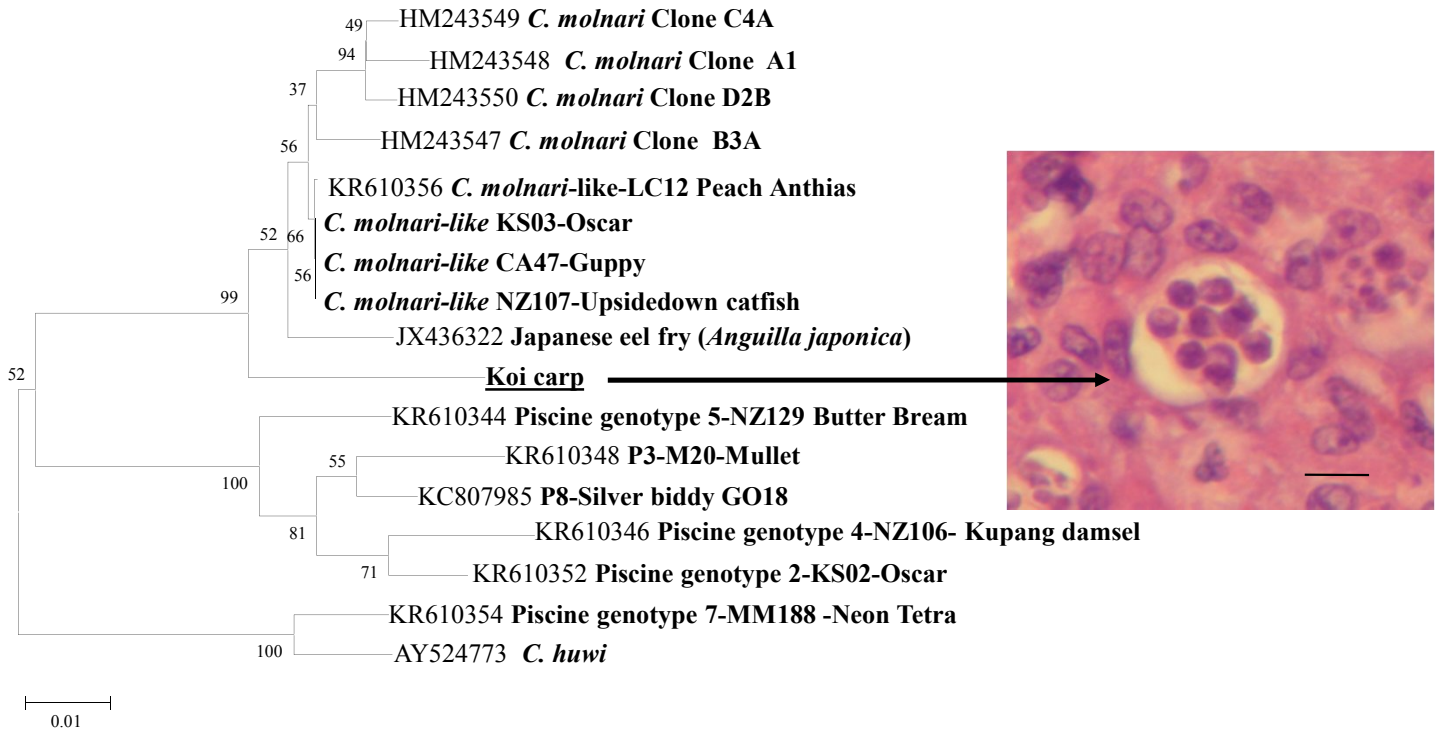

**Short Communication**

# Molecular characterisation of a disseminated *Cryptosporidium* infection in a Koi carp (*Cyprinus carpio*)

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**Highlights**

- First report of a disseminated cryptosporidiosis infection in a fish
- Parasite stages identified in the intestine, kidneys, spleen, liver and gills
- Molecular characterization at two loci; 18S ribosomal RNA (rRNA) and actin
- Novel *Cryptosporidium* genotype, most closely related to *C. molnari*.

**Abstract**

*Cryptosporidium* is a protozoan parasite that infects a wide range of hosts, yet relatively little is known about the epidemiology of cryptosporidiosis in fish. Here we report a disseminated *Cryptosporidium* infection in a male Koi carp (*Cyprinus carpio*), with parasite stages identified deep within the epithelium of the intestine, kidneys, spleen, liver and gills causing severe granulomatous inflammatory lesions. Molecular characterization at two loci; 18S ribosomal RNA (rRNA) and actin, revealed this to be a novel *Cryptosporidium* genotype, most closely related to *C. molnari*.

*Keywords: Cryptosporidium molnari;* fish; Koi carp; histology, genetic characterization; 18S rRNA; pathology; intestine; kidneys; spleen; liver; gills.

## 1. Introduction

*Cryptosporidium* is an important protozoan parasite that can cause severe diarrhea in a wide range of vertebrates. Relatively little is known about *Cryptosporidium* species infecting piscine hosts but cryptosporidiosis in fish can cause high morbidity and a variety of clinical signs (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Alvarez-Pellitero et al., 2004; Ryan et al., 2004; Murphy et al., 2009; Zanguee et al., 2010; Ryan and Xiao, 2014; Ryan et al., 2015; Yang et al., 2015). Currently three species are recognized in fish hosts; *Cryptosporidium molnari* which was originally described 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), (2) *Cryptosporidium scophthalmi* was described in turbot in 2004 (*Psetta maxima*. sny. *Scopthalmus maximus*) (Alvarez-Pellitero et al., 2004) and characterized genetically in 2016 (Costa et al., 2016), and (3) *Cryptosporidium huwi* (previously piscine genotype 1) (Ryan et al., 2015).

The prevalence of *Cryptosporidium* in fish is highly variable with prevalences ranging 0.8% to 100% mostly among juvenile fish (Landsberg and Paperna, 1986; Sitjà-Bobadilla et al., 2005; Alvarez-Pellitero et al., 2004, Murphy et al., 2009; Reid et al., 2010; Zanguee et al., 2010; Morine et al., 2012; Koinari et al., 2013; Certad et al., 2015; Yang et al., 2015).

Molecular characterization has identified additional piscine genotypes (piscine genotypes 2-8, *C. molnari*-like and 5 un-named novel genotypes) as well as *C. parvum, C. xiaoi, C. scrofarum, C. hominis* and rat genotype III in adult and juvenile fish (Murphy et al., 2009; Reid et al., 2010; Zanguee et al., 2010; Barugahare et al., 2011; Morine et al., 2012; Koinari et al., 2013; Ryan and Xiao, 2014; Certad et al., 2015; Yang et al., 2015). In the present study, we present evidence of a disseminated case of cryptosporidiosis in the gills, all parenchymous organs and intestines of a Koi carp (*Cyprinus carpio*) and molecular identification as a novel isolate, that was most closely related to *C. molnari*.

## 2. Materials and methods

An adult male Koi carp (*Cyprinus carpio*) was presented alive for necropsy at the NOIVBD pathology laboratory in The Netherlands. This Koi carp was one of a group that had been imported a few years previously from Japan and were housed in a pond containing ~20,000 liters of water with a biological and mechanical filter. The fish was 38 cm long and weighed 787 grams. This fish previously had skin infections over a two-year period, for which anti-bacterial therapy was not successful. Some fish in the same pond died over this period. Upon inspection, the skin showed irregular hemorrhages, swelling of the scales and necrosis at the fins (Fig. 1). The carp was euthanized with T61 0.5 ml intra-muscular.

During necropsy, impression smears were taken from the skin, tail-fin, gills, liver, spleen and intestinal mucosa for cytological evaluation. These impressions were air-dried, fixed in ethanol 70% and stained with Hemacolor® (Hemacolor® Rapid staining #111661, Merck Nederland Amsterdam) and examined with the light microscope with immersion oil at 1000 x magnification. Sections of skin, gill, liver, spleen, kidney and intestinal tissues were fixed in 10% buffered formalin and embedded in paraffin. Histological sections were cut at 5 µm thicknesses, stained with haematoxylin and eosin (H & E) and Giemsa and examined with a light microscope at 100 and 400 x magnification. A sample of the liver was fixed in ethanol 70% and tested for Koi Herpes Virus (KHV) using a KHV PCR test kit (Tauros Diagnostik, Bielefeld, Germany). Material from the cutis and spleen were cultured aerobically at 20 $^{o}$C and 37 $^{o}$C on Columbia Agar 5-7% blood and Brilliant Green Agar modified pH 7.0 (Tritium Microbiology, Eindhoven).

DNA was extracted from ~25 mg of paraffin-embedded intestinal, spleen and skin tissues using a Qiagen Tissue extraction kit (Qiagen, USA). All samples were screened at the 18S rRNA locus using previously described primers and conditions (Silva et al., 2013). Isolates positive at the 18S locus were also analysed at the actin locus using a hemi-nested PCR optimized for

amplification of piscine-derived *Cryptosporidium* species, as previously described (Koinari et al., 2013). The isolate was also also screened using a *C. parvum* and *C. hominis* specific qPCR at a unique *Cryptosporidium* specific gene (Clec) coding for a novel mucin-like glycoprotein that contains a C-type lectin domain (CTLD) previously described (Yang et al., 2013). This was done to determine if there was a mixed infection with *C. parvum* and/or *C. hominis* in the fish. Negative 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).

Amplicons were sequenced in both directions using an ABI Prism$^{TM}$ Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, California) according to the manufacturer's instructions. Nucleotide sequences were analysed using Finch TV Version 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com) and aligned with reference *C. huwi* 18S (AY524773) and actin (AY524772) sequences and reference *C. molnari* 18S (HM243547, HM243548 and JX436322) and actin (HM365219 and HM365220) 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 (www.megasoftware.net/). Genetic distances were calculated in MEGA using the Kimura 2 parameter model.

**3. Results**

At a macroscopical level, superficial haemorrhages (Fig.1a) were present in the skin, hyperaemia of the gills was noted, almost no fat-tissue was present, the liver was pale brown and the spleen was swollen and irregularly granular (Fig. 1b). The intestines were empty and pale with a

thickened mucosa (Fig.1c). Superficial haemorrhages were also present at a macroscopical level on the intestinal epithelium. In the stained impressions of all locations, *Cryptosporidium*-like organisms were seen. Extensive inflammation was evident on histological analysis, with many granulomas present in the intestine (tip of the villi in the lamina propria and near the crypts), liver, kidneys (interstitium) spleen, gills (base of the secondary lamellae and the cartilage), subcutaneous layer and in the fatty-tissue between the skeletal muscles. In the stained sections, many *Cryptosporidium*-like organisms were present free and intracellular (associated with granulomas) in cyst-like structures in the kidneys (Fig. 2a and 2b), spleen (2c), liver (2d), gills (2e) and intestine (not shown). The PCR for KHV was negative. The bacterial culture from cutis and spleen showed no growth.

18S sequences were obtained from intestine and spleen. All sequences obtained were identical. Phylogenetic analysis at the 18S locus based on 547 bp of sequence, using distance, parsimony and maximum likelihood produced similar trees (Fig. 3, distance tree shown). The carp isolate grouped separately and exhibited 3.8% genetic distance from *C. molnari* isolate, clone B3A (HM243547), 4.4% genetic distance from *C. molnari* isolates clone A1 (HM243548C), clone C4A (HM243549) and clone D2B (HM243550) and 5.1% genetic distance from *C. molnari*-like isolate LC12 (KR610356).

At the actin locus, a 278 bp sequence was obtained from the spleen sample. Phylogenetic analysis at the actin locus also revealed that the carp isolate to be genetically distinct and most closely related to *C. molnari* (3.0% and 4.0%, genetic distance from *C. molnari* isolates HM365219 and HM365220 respectively). It exhibited 7.8% and 8.3% genetic distance from *C. molnari*-like isolates LC12 (KR610337) and KS09 **(**KR610338). The isolate was negative for *C. parvum* and *C. hominis* using the species-specific qPCR.

Partial 18S and actin sequences for the carp isolate were deposited in GenBank under the following accession numbers; KX033348 and KX033349.

## 4. Discussion

In the present study, histological analysis of a Koi carp identified that many *Cryptosporidium*-like organisms were present free and intracellular (associated with granulomas) in cyst-like structures in the intestine, kidneys, spleen, liver and gills causing severe granulomatous inflammatory lesions. At both the 18S and actin loci, the carp isolate was genetically distinct but was most closely related to *C. molnari* (3.9-4.5% and 3.0-4.0% genetic distance respectively).

Relatively little is known about the clinical significance and histopathology of *Cryptosporidium* in fish. *Cryptosporidium molnari* was first reported mainly in the stomach epithelium of gilthead sea bream and European sea bass (Alvarez-Pellitero and Sitja-Bobadilla, 2002). Clinical signs included whitish faeces, abdominal swelling and ascites and *C. molnari* was associated with fish mortalities (Alvarez-Pellitero and Sitja-Bobadilla, 2002). The accumulation of *C. molnari* oocysts within the mucosal tissue resulted in cellular injury including consecutive necrosis, vacuolation, and sloughing of epithelial cells, but in contrast to the present study, no inflammation reaction was observed (Alvarez-Pellitero and Sitja-Bobadilla, 2002). However, leucocyte inflammatory infiltration has been reported for *C. scolpthalmi* infections (Alvarez-Pellitero et al., 2004) and in gastric cryptosporidiosis in juvenile red drum (*Sciaenops ocellatus*) (Camus and López, 1996).

Other studies of *Cryptosporidium*-infected fish have reported anorexia, regurgitation of food, emaciation, poor growth rates, swollen coelomic cavities, atrophy of skeletal muscle, tucked abdomen, listlessness and increased mortality, particularly in larval and juvenile fish (Camus and López, 1996; Ryan et al., 2004; Murphy et al., 2009; Gabor et al., 2011) and that *Cryptosporidium* distorted the normal mucosal architecture (Landsberg and Paperna, 1986; Paperna, 1987; Camus and López, 1996; Alvarez-Pellitero et al., 2004; Ryan et al., 2004; Gabor et al., 2011). In the

present study, the presence of granulomas was suggestive of a bacterial infection but no bacteria were detected.

*Cryptosporidium* normally infects the intestine and stomach of fish (Ryan, 2010). To the best of our knowledge, this is the first report of a disseminated *Cryptosporidium* infection in fish, with parasite stages identified in several tissues. Disseminated cases of cryptosporidiosis have been previously reported in other hosts, particularly immunosuppressed hosts, and in addition to the gastrointestinal and respiratory tracts, *Cryptosporidium* has been identified in the hepatobiliary system, pancreas and urinary bladder (cf. Sponseller et al., 2014). In the present study, the immune status of the host is unknown but was likely to be dysfunctional. Similar to previous studies, parasite stages were observed deep within the epithelium of carp tissues, which appears to be a key feature of piscine cryptosporidiosis (Alvarez-Pellitero and Sitja-Bobadilla, 2002; Murphy et al., 2009; Ryan et al., 2004).

In conclusion, in the present study a disseminated cryptosporidiosis infection was identified in a Koi carp and molecular analysis identified a novel genotype of *Cryptosporidium* most closely related to *C. molnari*. Further studies on additional isolates are required to better understand if this novel genotype represents a new species of *Cryptosporidium*.

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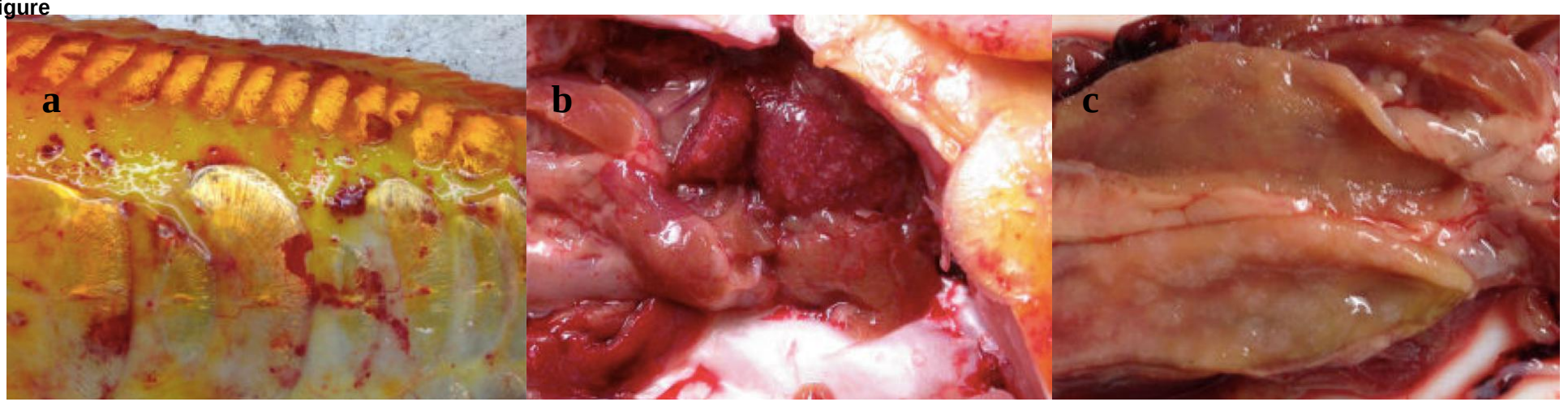
Fig 1. Macroscopic findings at necropsy of the Koi carp. (1a) Skin with hemorrhages, (1b) liver and spleen, (1c) intestines.

Fig. 2. Histological analysis of sections of carp tissue. Sections were stained with hematoxylin and eosin (H & E) and Giemsa. *Cryptosporidium* organisms (arrows) in granulomatous inflammatory lesion in the kidney (2a and 2b H & E - scale bar 50µm and 10µm respectively), spleen (2c H & E), liver (2d Giemsa) and gills (2e Giemsa), all with scale bar = 10 µm. * = the presence of inflammation.

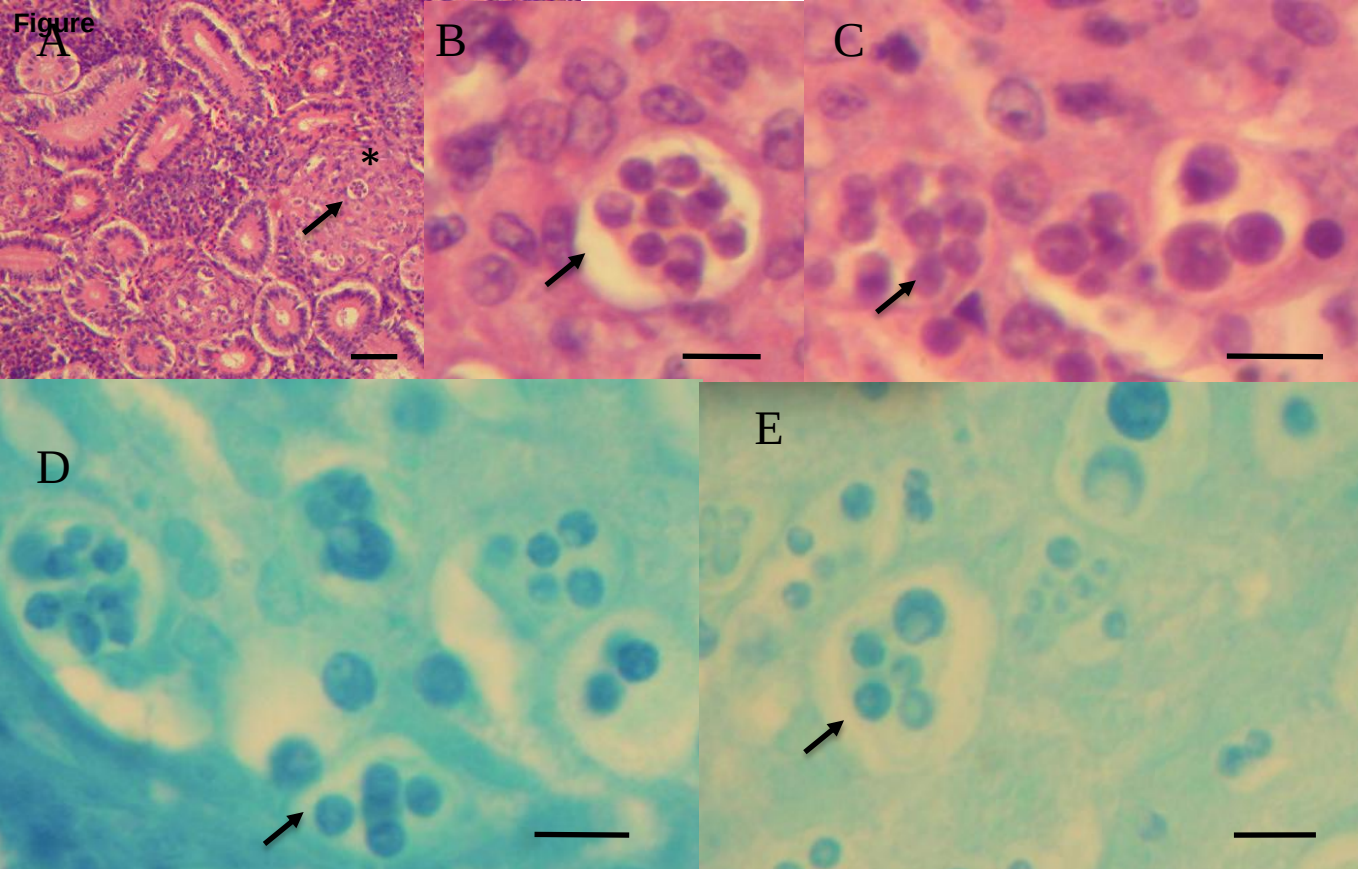
Fig. 3. Evolutionary relationships of the novel *Cryptosporidium* species from Koi carp (underlined) with other piscine-derived *Cryptosporidium* and genotypes inferred by distance analysis of 18S rRNA sequences. Percentage support (>50%) from 5000 pseudoreplicates from neighbor-joining analyses is indicated at the left of the supported node.

Fig. 4. Evolutionary relationships of the novel *Cryptosporidium* species from Koi carp (underlined) with other piscine-derived *Cryptosporidium* and genotypes inferred by distance analysis of actin sequences. Percentage support (>50%) from 5000 pseudoreplicates from neighbor-joining analyses is indicated at the left of the supported node.

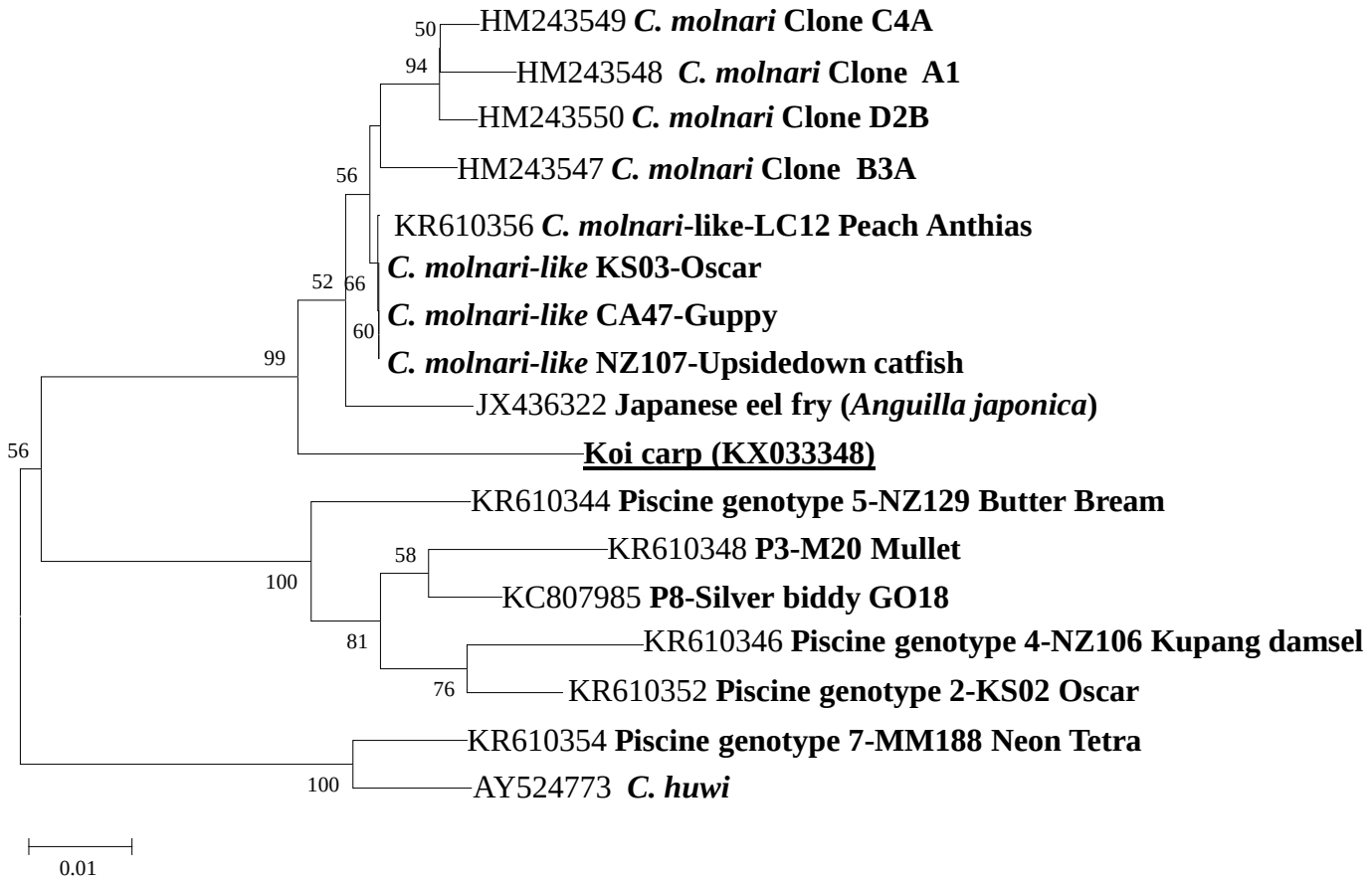
Figure



Figure



**Figure**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HM243548 C. molnari Clone A1
HM243550 C. molnari Clone D2B
HM243547 C. molnari Clone B3A
KR610356 C. molnari-like-LC12 Peach Anthias
C. molnari-like KS03-Oscar
C. molnari-like CA47-Guppy
C. molnari-like NZ107-Upsidedown catfish
JX436322 Japanese eel fry (Anguilla japonica)
Koi carp (KX033348)
KR610344 Piscine genotype 5-NZ129 Butter Bream
KR610348 P3-M20 Mullet
KC807985 P8-Silver biddy GO18
KR610346 Piscine genotype 4-NZ106 Kupang damsel
KR610352 Piscine genotype 2-KS02 Oscar
KR610354 Piscine genotype 7-MM188 Neon Tetra
AY524773 C. huwi
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94
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52
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58
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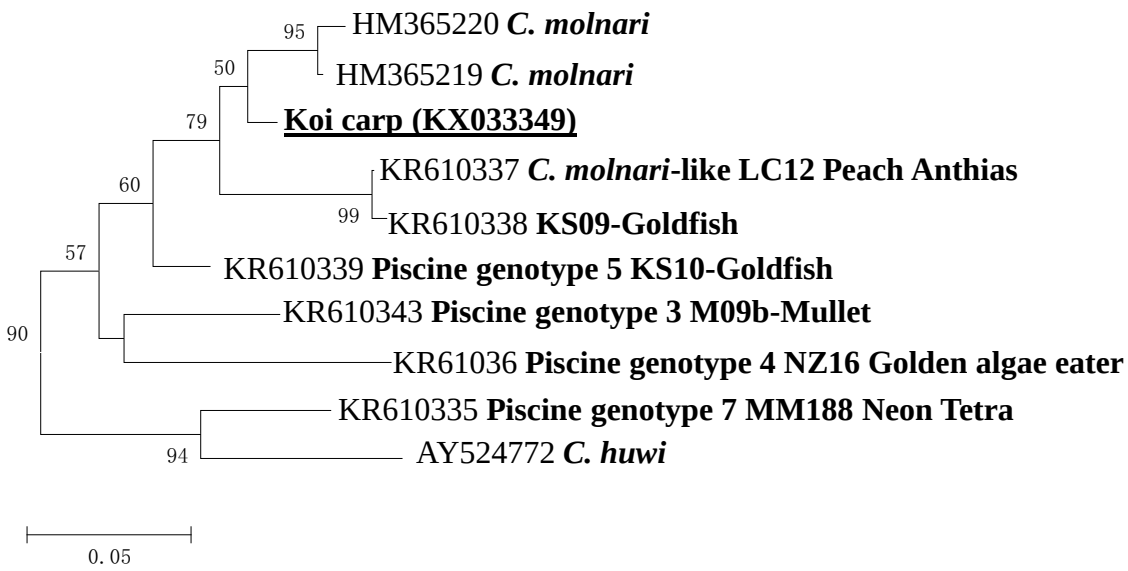
100
0.01

**Figure**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HM365220 C. molnari
50
HM365219 C. molnari
79
Koi carp (KX033349)
60
KR610337 C. molnari-like LC12 Peach Anthias
99
KR610338 KS09-Goldfish
57
KR610339 Piscine genotype 5 KS10-Goldfish
KR610343 Piscine genotype 3 M09b-Mullet
90
KR61036 Piscine genotype 4 NZ16 Golden algae eater
KR610335 Piscine genotype 7 MM188 Neon Tetra
94
AY524772 C. huwi
0.05