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Non-lethal detection of DNA from *Cichlidogyrus* spp. (Monogenea, Ancyrocephalinae) in gill mucus of the Nile tilapia *Oreochromis niloticus*

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ABSTRACT: Infection of Nile tilapia *Oreochromis niloticus* by monogeneans of the genus *Cichlidogyrus* is harmful. Currently, diagnosis of this infection is based on invasive techniques and the identification of isolated parasites by their morphology. To facilitate diagnosis, we have developed a non-lethal polymerase chain reaction (PCR) test for detection of *Cichlidogyrus* spp. DNA in the gill mucus of *O. niloticus*, using 5 pairs of specific primers based on *Cichlidogyrus sclerosus* 28S rRNA (Cicly 1 to Cicly 5) which generate fragments of approximately 188, 180, 150, 159 and 189 bp, respectively. PCR specificity was tested using genomic DNA extracted individually from 175 isolated *Cichlidogyrus* spp., 75 *Gyrodactylus cichlidarum* and 75 endopararasitic *Enterogyrus* spp., as well as from 75 protozoans *Trichodina* spp. The Cicly primers were used to detect *Cichlidogyrus* spp. DNA in mucus from the gills of 23 Nile tilapia confirmed to be infected with the parasite. Negative controls consisted of 45 uninfected Nile tilapia. The limit of sensitivity of the assay was 1.2 ng of purified parasite DNA. The Cicly primers did not amplify DNA from the mucus of non-infected Nile tilapia, *G. cichlidarum*, *Trichodina* spp. or *Enterogyrus* spp. In all cases, the sensitivity and specificity of the test were 100%. The sequences of all the amplified fragments showed a high similarity to that of the 28S rRNA region of *C. sclerosus* (93 to 100% identical to GenBank Accession No. DQ157660.1). We provide evidence for a safe and non-invasive DNA-based diagnostic method for the presence of *Cichlidogyrus* in the gill mucus of *O. niloticus*.

KEY WORDS: Diagnosis · Ectoparasite · Monogenean · PCR

INTRODUCTION

Nile tilapia *Oreochromis niloticus* has a high economic demand around the world, making it attractive for culture (Fitzsimmons & Carvalho 2000). It grows rapidly, is highly adaptable to water conditions, and is very tolerant to stress caused by handling (El-Sayed 2006). Over 2 million tonnes of the fish are estimated to be produced annually worldwide (Gonçalves et al. 2009). Monogeneans of the genus *Cichlidogyrus* (order Monophisthocotylea) are among the major parasites of Nile tilapia, causing cichlidogyriasis. These ectoparasites attach themselves to their hosts' gills by a structure called a haptor located in the posterior region of the body; the haptor is armed with hooks and bars but does not

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have discrete multiple suckers and clamps. These monogeneans feed on mucus, skin cells and blood from their site of attachment in the gill lamellae, using an anterior oral sucker (Gonçalves et al. 2009). Histological sections of gill tissue from severe monogenean infestations show excessive mucus production and hyperplasia that could lead to fusion of gill lamellae. Lesions in the gills could be fatal, especially under poor environmental conditions such as low oxygen content (Kabata 1985). In natural environments, *Cichlidogyrus* spp. are commonly found in apparent equilibrium with their host fish; in free-ranging populations it is not easy to determine whether monogeneans are the direct cause of mortality in Nile tilapia. However, the situation can be very different in cultured Nile tilapia. Because of their direct life cycle, these parasites can proliferate very quickly in environments with high densities of fish, such as tanks or ponds with inadequate sanitation and poor water quality; the parasites may thus be transmitted readily from fish to fish, resulting in epidemic levels of morbidity and mortality (Buchmann & Lindenstrom 2002). *Cichlidogyrus sclerosus* has been reported as the cause of death in cultured *Oreochromis* spp. introduced to Southeast Asia (Khalil 1971). A few monogeneans on a healthy, mature fish are usually not significant; however, moderate numbers on young fish can cause significant mortalities (Kabata 1985).

Clinical signs of cichlidogyriasis are not easily noticed in the early stages of infection. Fishes may become lethargic, and swim near the surface or seek the bottom of the tank, but in heavy parasitic infestations the gills may be swollen and pale, the respiration rate may be increased, and the fish will be less tolerant of low levels of oxygen; fishes with severe respiratory distress may be observed (R. Rodríguez-Canul pers. obs.). Secondary infection by bacteria and fungus is common in tissues that have been damaged by monogeneans (Wu et al. 2006).

Currently, the identification of monogeneans is carried out, post-mortem, by examination of fish gills; identification is based on an analysis of the parasite's morphology and on the size of sclerotized parts of the haptor and the reproductive organs (Paperna & Thurston 1968, Smith et al. 1992). Even though parasitology is considered to be the 'gold standard' test, this technique is lethal for the fish and it depends on the experience of a well-trained technician. Serological tests based on enzyme-linked immunosorbent assay (ELISA) identify IgM antibodies specific for *Cichlidogyrus* spp. (Sandoval-Gío et al. 2008). A major limitation of both of these techniques is that they are invasive to the host. Moreover, the presumptive diagnosis of cichlidogyriasis in a given farm implies additional costs for definitive diagnosis, management and treatment. Currently, several assays have been developed for the detection of pathogens in other species of fish using non-lethal sampling methods (Miriam et al. 1997, López-Vázquez et al. 2006, Lindstrom et al. 2009, Sanders & Kent 2011).

A non-invasive test that could detect pre-patent or low levels of monogenean infection would be useful. In this study, we describe a test based on the identification of genomic DNA from *Cichlidogyrus* sp. in the gill mucus of Nile tilapia; the test is novel, safe, and non-invasive to the fish, and it can be useful for presumptive diagnosis of cichlidogyriasis.

MATERIALS AND METHODS

Biological material

Cichlidogyrus spp. (175 individuals) were isolated from the gills of naturally infected Nile tilapia from the CINVESTAV — IPN Unidad Mérida facilities and stored individually in Eppendorf tubes with 70% ethanol. Briefly, Nile tilapia were sacrificed by inserting a knife behind the eyes and towards the upper edge of the operculum, piercing the brain; this procedure was performed very quickly, resulting in instant death. Gills were then removed and placed individually in Petri dishes containing 0.7% saline solution. Parasites were counted and isolated at 10×, and morphological features were observed at 40× and 100×.

The heterologous group included 75 individuals of *Gyrodactilus cichlidarum* (Paperna 1968), 75 protozoans (*Trichodina* spp.) and 75 individuals of *Enterogyrus* spp.; these organisms were isolated from the stomach of Nile tilapia.

Isolation of mucus from the gills of Nile tilapia and extraction of DNA

Mucus from 23 Nile tilapia, known to be infected with *Cichlidogyrus* spp., was collected by swabbing. Briefly, a Nile tilapia was placed on a clean surface, and 1 technician restrained it firmly with his hand while another technician handled a sterile dry cotton swab. The swab was placed gently inside the operculum and rotated gently for 5 s on the external surface of the left and right sides of the fish gills, avoiding causing excessive tissue damage. The swab was then

gently removed from the gills and placed in a sterile 0.5 ml Eppendorf tube with a hole in the bottom. This tube was placed on a collector tube of 1.5 ml and centrifuged at 13 500 × *g*. About 40 to 50 µl of mucus was collected. This was separated, in equal volumes, into 2 tubes for extraction of DNA. The contents of 1 tube was fixed in ethanol (96%) and the contents of the other tube was immediately used for extracting DNA using the Wizard® genomic DNA purification kit (Promega®) according to the manufacturer's protocol. To assess the worm burden, fishes were then dissected and gills removed for isolation of the parasites at 10× and 40× (Jiménez-García et al. 2001).

For negative controls, mucus was collected from 45 uninfected Nile tilapia and used for the extraction of DNA, as described above for the positive control group.

Table 1. Primers used for detecting the DNA of *Cichlidogyrus* sp. F: forward primer; R: reverse primer; Amplicon length: length of the region amplified by a given pair of primers

Primer set	Primer sequence	Amplicon length (bp)
Cicly 1	F 5′-GCT TGT ACC TGG GAT CGT GT-3′ R 5′-GCC TTG GAT GGA GTT TAC CA-3′	188
Cicly 2	F 5′-CCG TGA GGG AAA GTT GAA AA-3′ R 5′-TTG AAG CGC ATT CAG AAC AC-3′	180
Cicly 3	F 5′-GCG AGT GAA CGG AGA TTA GC-3′ R 5′-GAC TTT CAC CCG CTA TGG AA-3′	150
Cicly 4	F 5′-GAG GGA TTG ATG CTG AGA GC-3′ R 5′-CTG TGC AGC GAG GAA TAC AA-3′	159
Cicly 5	F 5′-GAG GGA TTG ATG CTG AGA GC-3′ R 5′-GCC TTG GAT GGA GTT TAC CA-3′	189

Extraction of parasitic genomic DNA and design of primers

Genomic DNA was isolated by a modification of the method described by Mollaret et al. (2000). DNA was extracted from single specimens placed individually in Eppendorf tubes incubated at 55°C for 2 h with a mixture of 10 µl of Proteinase K (20 µg ml^{-1}) (Sigma) and 150 µl of 10% Chelex (Sigma).

The concentration of DNA was determined using a NanoDrop 2000c spectrophotometer (Thermo Scientific) by measuring absorbance values at 260 nm and 280 nm, and determining the ratio A_{260}/A_{280}. Samples with a value of ~1.8 and above were used in the subsequent polymerase chain reaction (PCR) assays.

Primers were synthesized on the basis of the region D1 to D2 of the large subunit (LSU) 28S rDNA gene of *Cichlidogyrus sclerosus* (DQ157660.1) (Wu et al. 2006) with the aid of the primer3 (v.0.40) software program (Rozen & Skaletsky 2000). The following criteria were applied to the design of the new primers: GC content from 45 to 60%; minimal secondary structure; no primer-dimers; melting temperature (T_m) between 50 and 70°C; and a length of 20 to 24 bases. Details of the primers are shown in Table 1.

In all cases, 15 serial dilutions of each primer (100 pM to 1 pM) and 15 serial dilutions of the DNA (from 64 to 0.0039 ng) were performed to assess the limitation of the PCR assays.

The sensitivity and specificity of each PCR was evaluated by χ^2 (2 × 2) (Galen & Gambino 1995).

PCR assays

The PCR reactions were carried out in a total volume of 20 µl containing 2 µl of template (~32.5 ng of DNA of single parasites), 0.30 µM of each primer, 1.5 mM of $MgCl_2$, 10× reaction buffer (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, pH 9.0), 0.3 mM dNTPs mixture (Promega®) and 3.0 U of *Taq* DNA polymerase (BioLabs®). The PCR reactions were run on a thermal cycler (TECHNE TC-312) and the cycling conditions were 95°C for 3 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2.5 min, with a final extension of 72°C for 10 min. Electrophoresis of PCR products was run in a 2% agarose gel with a 100 bp DNA ladder. Bands were visualized using 0.1% ethidium bromide stain on a UV documentation system (MiniBis Pro®). In all cases, ultra-pure water and gill tissue of *Oreochromis niloticus* were used as negative controls, whereas DNA from the parasites was used as a positive control.

All PCR analyses were done in triplicate, and positive fragments were forward- and reverse-sequenced at the CINVESTAV IPN-Unidad Irapuato. Sequences were aligned manually, and BLASTn searches were performed against public databases in GenBank for preliminary matching and comparison with original sequences from the 28S rRNA-based identification of monogeneans of the genus *Cichlidogyrus* (http://www.ncbi.nlm.nih.gov/blast/Blast).

RESULTS

PCR with *Cichlidogyrus* spp. genomic DNA

We were not able to identify, to species level, all of the *Cichlidogyrus* spp. isolated in this study. We evaluated only their main morphological characteristics, but in a qualitative way we were able to assess that *Cichlidogyrus sclerosus* occurred more frequently than other species of this genus in *Oreochromis niloticus*—followed by *Cichlidogyrus tilapiae, C. haplochromii* and *C. longicornis* (Jiménez-García et al. 2001).

DNA from single individuals was used for the PCR standardization to increase the sensitivity of the test for its subsequent use with mucus from Nile tilapia. All PCR assays were highly sensitive with the 5 sets of primers used individually. We observed amplicons of size 188 bp with primer set Cicly 1, 180 bp with primer set Cicly 2, 150 bp with primer set Cicly 3, 159 bp with primer set Cicly 4, and 189 bp with primer set Cicly 5 (Fig. 1A). We also observed some variability after titrating the primers. The lower detection limit was 4 pM for Cicly 1, 30 pM for Cicly 2, 2 pM for Cicly 3, 2 pM for Cicly 4, and 4 pM for Cicly 5. To avoid this variability, we decided to use each set of primers at 30 pM in subsequent analyses. We used these conditions to titrate DNA isolated from each parasite, and the lower detection limit was 0.03 ng for Cicly 1, 1 ng for Cicly 2, 0.006 ng for Cicly 3, 0.007 ng for Cicly 4, and 0.12 ng for Cicly 5.

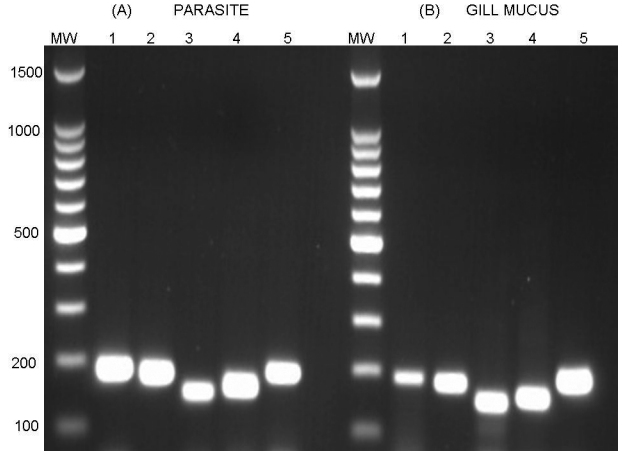

Fig. 1. PCR assays for detection of *Cichlidogyrus* spp. genomic DNA from (A) parasite tissue and (B) gill mucus from infected Nile tilapia *Oreochromis niloticus*. Lane MW: 100 bp DNA ladder; Lane 1: Cicly 1, amplicon size = 188 bp; Lane 2: Cicly 2, amplicon size = 180 bp; Lane 3: Cicly 3, amplicon size = 150 bp; Lane 4: Cicly 4, amplicon size = 159 bp; Lane 5: Cicly 5, amplicon size = 189 bp

We also tested the specificity of the *Cichlidogyrus* spp. PCR assays against DNA from *Gyrodactylus cichlidarum*, *Trichodina* spp. and *Enterogyrus* spp. None of these parasite tissues amplified in specific regions for the primer sets Cicly 1 to 5.

PCR on *Cichlidogyrus* spp. DNA in mucus from Nile tilapia

No fish died as a result of handling stress during the collection of mucus. The PCR on mucus from the 23 infected Nile tilapia amplified DNA fragments of the same size as for the *Cichlidogyrus* spp. PCR assays (Fig. 1B). In this case, we corroborated the PCR-positive reaction with the presence of monogeneans by microscopy. The number of parasites isolated ranged from 1 to 162 per fish.

We did not observe amplification of DNA from the mucus of the 45 parasite-free *Oreochromis niloticus*. In all cases, 100% sensitivity and specificity was observed.

Sequence matching of PCR products using *Cichlidogyrus* spp. DNA

Sequences of the PCR products obtained with the 5 sets of primers were submitted to GenBank to check against the genome of *Cichlidogyrus sclerosus* (D1 to D2 region of the LSU 28S rDNA gene from which the primers were designed). The PCR products obtained with all 5 sets of primers had ~95 to 100% homology with the corresponding region (DQ157660.1 GenBank) (Wu et al. 2006). The amplicons also matched the LSU rDNA region of *C. cubitus* (HQ010037.1), *C. falcifer* (HQ010024.1), *C. tilapiae* (HQ010029.1), *C. arthracanthus* (HQ010022.1), *C. ergensi* (HQ010038.1), *C. pouyaudi* (HQ010039.1), *C. digitatus* (HQ010023.1), *C. longicirrus* (HQ010026.1) *C. acerbus* (HQ010036.1), *Cichlidogyrus* sp. 2 AS-2010 (HQ010028.1), *Cichlidogyrus* sp. 1 AS-2010 (HQ010027.1), *C. aegypticus* (HQ010021.1), and *C. halli* (HQ010025.1) (Mendlová et al. 2010), and also some regions of the D1 to D2 domain of LSU rDNA and the combined LSU and partial sequence of small subunit (SSU) rDNA data sets of *Cichlidogyrus* sp. 1 XW-2006 (DQ537367.1) and *Cichlidogyrus* sp. 2 XW-2006 (DQ537368.1) (Wu et al. 2007).

A small region (25% of total coverage) showed >90% homology with the D1 to D2 regions of LSU rDNA from *Scutogyrus longicornis* (DQ157659.1) (Wu et al. 2006) and the LSU rDNA region of *S. longicornis* (HQ010035.1) (Mendlová et al. 2010). The

amplicons also matched the partial C1, full D1 and partial C2 domains of 28S rDNA from *Cichlidogyrus* sp. MLJ1 (AF218124.1) (Mollaret et al. 2000) (Table 2). Additionally, Cicly 2 recognized a small region (12% of coverage; 89% of homology) with the partial 28S rDNA of a species of Dactylogyridae, 1 YS-2008 (EU836211.1), and *Haliotrema* sp. 1 TY-2005 (DQ058213.1) (Tingbao et al. 2006).

Sequence matching of PCR products using DNA from gill mucus

As with DNA from the parasite, the amplified products using DNA from mucus were also compared and aligned first with the original sequence of *Cichlidogyrus sclerosus*. The 5 primer sets amplified sequences which had ~91 to 98% homology with the original region D1 to D2 of LSU rDNA (28S gene) (DQ157660.1) GenBank (Wu et al. 2006). The amplified sequences also matched the same set of parasites previously described: the LSU rDNA region of *C. cubitus* (HQ010037.1), *C. falcifer* (HQ010024.1), *C. tilapiae* (HQ010029.1), *C. arthracanthus* (HQ010022.1), *C. ergensi* (HQ010038.1), *C. pouyaudi* (HQ010039.1), *C. digitatus* (HQ010023.1), *C. longicirrus* (HQ010026.1), *C. acerbus* (HQ010036.1), *Cichlidogyrus* sp. 2 AS-2010 (HQ010028.1), *Cichlidogyrus* sp. 1 AS-2010 (HQ010027.1), *C. aegypticus* (HQ010021.1) and *C. halli* (HQ010025.1) (Mendlová et al. 2010). The amplicons also matched regions of the D1 to D2 domain of LSU rDNA and the combined LSU and partial sequence of small subunit (SSU) rDNA data sets of *Cichlidogyrus* sp. 1 XW-2006 (DQ537367.1) and *Cichlidogyrus* sp. 2 XW-2006 (DQ537368.1) (Wu et al. 2007).

A small region (8% of total coverage; >90% of homology) matched the D1 to D2 regions of LSU rDNA from *Scutogyrus longicornis* (DQ157659.1) (Wu et al. 2006) and (HQ010035.1) (Mendlová et al. 2010). The amplicons also matched the partial C1, full D1 and partial C2 domains of 28S rDNA of *Cichlidogyrus* sp. MLJ1 (AF218124.1) (Mollaret et al. 2000) (Table 3). Only sequences amplified by Cicly 4 and Cicly 5 had 86% homology with *C. pouyaudi* (DQ157655.1) (Wu et al. 2006).

Additionally, Cicly 2 recognized a small region (8% of coverage; 88% of homology) with the partial 28S rDNA of a species of Dactylogyridae, 1 YS-2008 (EU836211.1) and *Haliotrema* sp. 1 TY-2005 (DQ058213.1) (Tingbao et al. 2006).

Table 2. Percentage (%) similarity of the PCR products obtained by using primers Cicly 1 to Cicly 5 (see Table 1), with DNA from *Cichlidogyrus sclerosus* tissue, to various partial sequences of the 28S rDNA of monogeneans described in GenBank. **Bold**: incomplete coverage (96%); *italic*: minimal coverage (15%); –: no matching

Accession number	Parasite (partial sequence of 28S rDNA)	%Similarity (Cicly primer set) 1	2	3	4	5	Source
DQ157660.1	*C. sclerosus*	99	100	100	100	95	Wu et al. (2006)[a]
HQ010037.1	*C. cubitus*	97	96	98	90	95	Mendlová et al. (2010)[b]
HQ010024.1	*C. falcifer*	97	95	97	90	95	Mendlová et al. (2010)
HQ010029.1	*C. tilapiae*	97	96	97	89	96	Mendlová et al. (2010)
HQ010036.1	*C. acerbus*	96	96	97	90	95	Mendlová et al. (2010)
HQ010028.1	*Cichlidogyrus* sp. 2 AS-2010	96	96	97	90	95	Mendlová et al. (2010)
HQ010027.1	*Cichlidogyrus* sp. 1 AS-2010	96	97	96	91	94	Mendlová et al. (2010)
HQ010021.1	*C. aegypticus*	95	95	98	90	94	Mendlová et al. (2010)
HQ010025.1	*C. halli*	94	96	98	90	93	Mendlová et al. (2010)
HQ010022.1	*C. arthracanthus*	95	95	96	90	94	Mendlová et al. (2010)
HQ010035.1	*Scutogyrus longicornis*	**95**	**94**	**95**	**89**	**92**	Mendlová et al. (2010)
DQ157659.1	*S. longicornis*	**95**	**94**	**95**	**89**	**92**	Wu et al. (2006)
HQ010038.1	*C. ergensi*	95	95	*98*	89	94	Mendlová et al. (2010)
HQ010039.1	*C. pouyaudi*	88	94	97	*86*	*86*	Mendlová et al. (2010)
HQ010023.1	*C. digitatus*	97	95	96	*88*	95	Mendlová et al. (2010)
HQ010026.1	*C. longicirrus*	96	96	96	*87*	94	Mendlová et al. (2010)
DQ537367.1	*Cichlidogyrus* sp. 1 XW-2006	96	93	97	–	95	Wu et al. (2007)
DQ537368.1	*Cichlidogyrus* sp. 2 XW-2006	97	96	97	89	96	Wu et al. (2007)[c]
AF218124.1	*Cichlidogyrus* sp. MLJ1	95	–	96	–	94	Mollaret et al. (2000)[d]

[a]Partial C1, full D1 and partial C2 domains of 28S rDNA data
[b]LSU rDNA data
[c]D1 to D2 domain of LSU rDNA and the combined LSU and partial sequence of SSU rDNA data sets
[d]Partial C1, full D1 and partial C2 domains of 28S rDNA data

Table 3. Percentage (%) similarity of the PCR products obtained by using primers Cicly 1 to Cicly 5 (see Table 1), with DNA from mucus of Nile tilapia gills, to various partial sequences of the 28S rDNA of monogeneans described in GenBank. **Bold**: incomplete coverage (96%); *italic*: limited coverage (8%); –: no matching

Accession number	Parasite (partial sequence of 28S rDNA)	%Similarity (Cicly primer set) 1	2	3	4	5	Source
DQ157660.1	*C. sclerosus*	96	95	97	91	98	Wu et al. (2006)[a]
HQ010037.1	*C. cubitus*	96	98	96	95	98	Mendlová et al. (2010)[b]
HQ010029.1	*C. tilapiae*	97	97	97	98	99	Mendlová et al. (2010)
HQ010024.1	*C. falcifer*	96	98	98	91	97	Mendlová et al. (2010)
DQ537368.1	*Cichlidogyrus* sp. 2 XW-2006	97	97	97	98	99	Wu et al. (2007)[c]
HQ010023.1	*C. digitatus*	96	97	98	96	98	Mendlová et al. (2010)
HQ010036.1	*C. acerbus*	96	98	98	96	97	Mendlová et al. (2010)
HQ010028.1	*Cichlidogyrus* sp. 2 AS-2010	96	98	98	96	97	Mendlová et al. (2010)
HQ010026.1	*C. longicirrus*	96	97	98	**89**	97	Mendlová et al. (2010)
DQ537367.1	*Cichlidogyrus* sp. 1 XW-2006	96	97	98	94	97	Wu et al. (2007)
HQ010027.1	*Cichlidogyrus* sp. 1 AS-2010	95	96	97	92	97	Mendlová et al. (2010)
HQ010021.1	*C. aegypticus*	95	98	97	93	96	Mendlová et al. (2010)
HQ010025.1	*Cichlidogyrus halli*	95	97	97	92	96	Mendlová et al. (2010)
HQ010022.1	*C. arthracanthus*	95	98	95	93	95	Mendlová et al. (2010)
HQ010038.1	*C. ergensi*	96	98	**98**	93	96	Mendlová et al. (2010)
HQ010035.1	*Scutogyrus longicornis*	*95*	*95*	*96*	*92*	*96*	Mendlová et al. (2010)
DQ157659.1	*S. longicornis*	*95*	*95*	*96*	*92*	*96*	Wu et al. (2006)
HQ010039.1	*C. pouyaudi*	–	96	94	93	87	Mendlová et al. (2010)
AF218124.1	*Cichlidogyrus* sp. MLJ1	95	–	96	–	97	Mollaret et al. (2000)[d]
DQ157660.1	*C. sclerosus*	96	95	97	91	98	Wu et al. (2006)[a]
HQ010037.1	*C. cubitus*	96	98	96	95	98	Mendlová et al. (2010)[b]
HQ010029.1	*C. tilapiae*	97	97	97	98	99	Mendlová et al. (2010)
HQ010024.1	*C. falcifer*	96	98	98	91	97	Mendlová et al. (2010)
DQ537368.1	*Cichlidogyrus* sp. 2 XW-2006	97	97	97	98	99	Wu et al. (2007)[c]
HQ010023.1	*C. digitatus*	96	97	98	96	98	Mendlová et al. (2010)
HQ010036.1	*C. acerbus*	96	98	98	96	97	Mendlová et al. (2010)
HQ010028.1	*Cichlidogyrus* sp. 2 AS-2010	96	98	98	96	97	Mendlová et al. (2010)
HQ010026.1	*C. longicirrus*	96	97	98	**89**	97	Mendlová et al. (2010)
DQ537367.1	*Cichlidogyrus* sp. 1 XW-2006	96	97	98	94	97	Wu et al. (2007)
HQ010027.1	*Cichlidogyrus* sp. 1 AS-2010	95	96	97	92	97	Mendlová et al. (2010)
HQ010021.1	*C. aegypticus*	95	98	97	93	96	Mendlová et al. (2010)
HQ010025.1	*Cichlidogyrus halli*	95	97	97	92	96	Mendlová et al. (2010)
HQ010022.1	*C. arthracanthus*	95	98	95	93	95	Mendlová et al. (2010)
HQ010038.1	*C. ergensi*	96	98	**98**	93	96	Mendlová et al. (2010)
HQ010035.1	*Scutogyrus longicornis*	*95*	*95*	*96*	*92*	*96*	Mendlová et al. (2010)
DQ157659.1	*S. longicornis*	*95*	*95*	*96*	*92*	*96*	Wu et al. (2006)
HQ010039.1	*C. pouyaudi*	–	96	94	93	87	Mendlová et al. (2010)
AF218124.1	*Cichlidogyrus* sp. MLJ1	95	–	96	–	97	Mollaret et al. (2000)[d]

[a]Partial C1, full D1 and partial C2 domains of 28S rDNA data
[b]LSU rDNA data
[c]D1–D2 domain of LSU rDNA and the combined LSU and partial sequence of SSU rDNA data sets
[d]Partial C1, full D1 and partial C2 domains of 28S rDNA data

DISCUSSION

For many years the presence of monogeneans, such as *Cichlidogyrus* spp., has been a recurrent problem for Nile tilapia farms. Control of cichlidogyriasis is based on chemical substances that are inefficient and which could have potential toxicity and/or involve additional costs. Current methods for detecting this parasite require lethal sampling of fish, which increases costs in local farms. In this study, we have standardized PCR-based testing that can detect DNA isolated directly from *Cichlidogyrus* spp. as well as the parasite's DNA in mucus from Nile tilapia *Oreochromis niloticus*.

The PCR standardization reported herein has several advantages. The 5 pairs of primers were able to amplify their target sequences under the same PCR conditions (Coleman & Tsongalis 2006). All the PCR-positive results from gill mucus were confirmed by the presence of the parasite at necropsy. One of the

major findings is that the PCR carried out on gill mucus is able to detect a single parasite; this is an advantage because, with this test, we can detect low levels of parasites (i.e. early infection) without killing the fishes.

All the isolated parasites resembled *Cichlidogyrus* spp. in that they had sclerotized structures in the attachment organ (the haptor) and characteristic morphology of the reproductive organs (Mollaret et al. 2000). In a previous study, 5 species of *Cichlidogyrus* (*C. tilapiae, C. sclerosus, C. dossui*, *C. haplochromii* and *C. longicornis*) were reported from *Oreochromis aureus* and *O. niloticus* (Jiménez-García et al. 2001). We used Nile tilapia from the same facilities; we found that *C. sclerosus* was more prevalent, but we also found fewer of the other 4 species described previously. Thus, we decided to standardize the PCR tests at 'genus' level to avoid any misleading information. All PCR standardizations were performed using DNA from single individuals, and no false-positive and/or false-negative results were found. This PCR approach, which uses a highly conserved gene, has another advantage: it could be used in Nile tilapia farms around the world because, in both natural and culture conditions, several species of *Cichlidogyrus* are always present. Some of these species are morphologically highly variable, so it can be difficult to distinguish species on a morphological basis (Pouyad et al. 2006, Boungou et al. 2008, Mendlová et al. 2010); but what is important is that all of these species can give rise to cichlidogyriasis, and hence are potential causes of economic loss.

During the process of primer standardization, we observed some variability in primer sensitivity, ranging from 2 pM to 30 pM. The minimum concentration of DNA detected ranged from 0.007 ng to 0.12 ng of DNA. During the alignment analyses of products obtained by amplification of DNA from the parasite we found ~100% homology between the PCR products of all 5 pairs of primers and DNA from the original sequence of *Cichlidogyrus sclerosus* (Wu et al. 2006). Likewise, the sequences in the DNA amplified from mucus were specific to the original sequence of the genus *Cichlidogyrus* (DQ537367.1) (Wu et al. 2007). Alignment and matching were similar to those found in previous studies with sequences from the parasite (93 to 99%) (Mollaret et al. 2000, Wu et al. 2006, 2007).

Special caution has to be taken with the primer pair Cicly 2, as a small region of the amplified sequence matches the original sequences of *Haliotrema* sp. and *Scutogyrus longicornis*. In this case, it is necessary to validate the PCR tests with DNA of these monogeneans to assess the primers' specificity because, after all, in this study, we are reporting only matches from a very conserved gene. According to some molecular phylogenetic studies, *S. longicornis* could be included within the genus *Cichlidogyrus*, suggesting the non-monophyly of *Cichlidogyrus* (Pouyad et al. 2006, Wu et al. 2007, Mendlová et al. 2010).

In conclusion, we report the first use of a specific sequence from the 28S rDNA gene of *Cichlidogyrus* spp. for molecular diagnosis. Our PCR assays detected DNA obtained directly from the parasite and also the parasite's DNA present in the gill mucus of Nile tilapia, so that this approach could indicate a putative infestation. This presumptive test could be used in conjunction with parasitological and/or serological tests for early detection of the presence of the parasite in order to avoid the possibility of epizootic outbreaks of cichlidogyriasis in culture systems. The detection of DNA from gill mucus of Nile tilapia is a novel approach which is not invasive to the host, so this approach might facilitate the use of testing in order to help avoid economic losses on Nile tilapia farms.

As parasites feed on gill mucus and debris, it is possible that the PCR test is detecting DNA from excretory/secretory products of the parasite. This is a hypothesis that we are currently evaluating, as the PCR can detect DNA from a single egg (data not shown). In further studies it will be necessary to include a panel of DNA from *Scutogyrus* sp. and *Haliotrema* sp. to evaluate the specificity of the PCR assays.

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