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Sciadicleithrum variabilum (Dactylogyridae: Monogenea) Infection in *Symphysodon discus*: a Case Report

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ABSTRACT—During the winter of 2009, we observed an outbreak of severe respiratory disease in *Symphysodon discus* in a fish farm. The disease began with anorexia, respiratory distress and behavioral disorders and caused the death of all affected juveniles (80%) within a few days. Gill filaments were inflamed, hyperplastic and necrotic, and were infected with *Sciadicleithrum variabilum*. We amplified the cytochrome oxidase I (COI) gene of the monogenean using PCR and sequenced the gene as a molecular marker to rapidly identify the species. We demonstrated successful sequencing of the COI gene for *S. variabilum* identification, together with the need to monitor this pathogen in fish farms.

Key words: pathology, *Sciadicleithrum variabilum*, PCR, Monogenea, cytochrome oxidase I, *Sympysodon discus*

Every year, more than one billion ornamental fish, over 4,000 freshwater and 1,400 marine species are traded worldwide, which inevitably leads to a high risk of infectious disease transmission[1)]. About 90% of the ornamental fish trade concerns freshwater species, above all Cichlids[2)]. Discus *Symphysodon discus*, an Amazonian Cichlid freshwater fish, is a very popular aquarium fish. It is 20 cm long and eats worms, crustaceans, insects and plants. The eating habits of the juveniles of the species are especially interesting as they feed on the skin mucus of their parents[3)]. Parasitic diseases are frequent in ornamental fish and are one of the most common causes of fish mortalities[4–6)]. Their diffusion has been enhanced by high breeding density, lack of effective quarantine methods and poor sanitary control[7)]. Neo-tropical species of the Cichlidae family are frequently parasitized by monogenean parasites. They inhabit the skin and gills of their hosts causing epidermal hyperplasia due to disruption by parasite attachment and feeding. Increased mucus production might also occur. The members of three monogenean genera are found in cichlid fishes: 1) *Gussevia* Kohn and Paperna, 1964; 2) *Sciadicleithrum* Kritsky, Thatcher and Boeger, 1989; 3) *Trinidactylus* Hanek, Molnar and Fernando, 1974.

Morphological identification of Monogenea at specific level is often difficult and requires good systematic competence. Moreover, the Dactylogyridae family is morphologically variant, at times making precise identification impossible[8)]. Recently, some authors suggested the cytochrome oxidase I (COI) gene, a small segment of the genome, as a marker to bar-code various organisms[9)], including monogenean parasites[10)]. It has also been proven that, in some cases, COI can prevent the misidentification of monogenean sister species[11)].

The goals of this work were: 1) to describe a severe infection with *Sciadicleithrum* sp. in juvenile *Symphysodon discus*, 2) to classify the parasite by morphological identification, 3) to sequence the cytochrome oxidase I gene (COI) as a molecular marker to quickly identify the monogenean species.

During winter 2009, we observed an outbreak of severe respiratory disease among *Symphysodon discus* kept in indoor glass tanks on an ornamental fish farm located in Northern Italy. Water in the tanks was recycled, being partially changed (about 70%) twice a week, with temperature ranging between 28–29°C. Fries and juvenile discus were often bred together with their parents for a minimum of one month to a maximum of two months. Sometimes, young fish came from different aquaria. Tanks had a capacity of 50 liters or 100 liters, and fish density complied with the capacity of each tank. After the acclimatization period, adults were separated from young fish.

Juvenile discus, aged between 2–3 days to 2–3 months, showed signs of anorexia, severe respiratory distress and behavioral disorder. Breathing was accelerated with rapid and alternate respiratory activity with movement of the operculum of one side, while that of the other side was immobile. The fish rubbed their heads against hard surfaces, lost weight rapidly and died within a few days. Eighty per cent of the two or three-day-old discus and 50% of the others died within 2–3 weeks after the onset of symptoms. Surviving fish were thin, weak and did not increase in weight. Adults were asymptomatic.

During the outbreak, we examined 15 juvenile discus ranging from 3 cm to 10 cm in length. At necropsy, gills appeared hyperemic, with diffuse hemorrhage and covered by abundant greyish mucus. The mucus

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contained many monogenean worms. Parasites were equipped with haptor, hamuli and bars, and were morphologically consistent with the Dactylogyridae family (Fig. 1A–C).

We observed approximately from 50 to more than 200 parasites for each examined fish. No lesions were observed on other organs. However, we carried out microbiological investigations on gills, liver and spleen. We incubated TSA and Blood agar at 22°C for 72 h; no bacterial pathogens were isolated.

To assess gill lesions, we fixed them in 10% buffered formalin. Subsequently, the samples were embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin-eosin. From one to eight monogenean parasites were found on each of the primary gill lamellae. The parasites were attached to epithelial cells with haptor and median hooks (anchors). Sometimes, parasites were attached to the proximal ends of gill filaments (Fig. 2A & C). The gills were infiltrated by mononuclear cells, which were mostly lymphocytes and eosinophilic granular cells (Fig. 2D & E). Epithelial cells were hyperplastic, fused together, with "clubbing" of the gill filaments (Figs. 2A–E).

Identification of monogenean parasites was carried out on individually fixed parasites. The parasites were gently detached from the gills and fixed in 65°C heated 5% formalin solution. We studied the sclerotized organs (haptoral structures and male copulatory organ) according to the protocol described by Harris *et al.*[12], and the non-sclerotized organs (ovary, testis, prostatic gland and ducts, intestinal caeca, pharynx and oral sucker) with Gomori's trichrome and acid carmine stains. Samples were mounted in Euparal[13,14].

The helminth specimens were identified using the morphological key as described in Kritsky *et al.*[15], comprising ventral bar with bilateral umbelliform membranes (haptor structure), overlapping gonads, and a cirrus (copulatory organ sclerite) with a loose coil of clockwise rings. These features are species-specific within the *Sciadicleithrum* genus. So, the helminth specimens were identified as *Sciadicleithrum variabilum* species, synonyms *Urocleidoides variabilis*[16] and *Ancyrocephalus kostomarovi*[17], belonging to the Dactylogyridae family (Fig. 1B & C).

Parasites were removed from gill arches and immediately placed in digestion solution. DNA extraction was carried out according to the protocol described by Zietara *et al.*[18]. For each specimen a portion of mitochondrial cytochrome oxidase subunit I (COI) was amplified by PCR. The amplification was carried out using

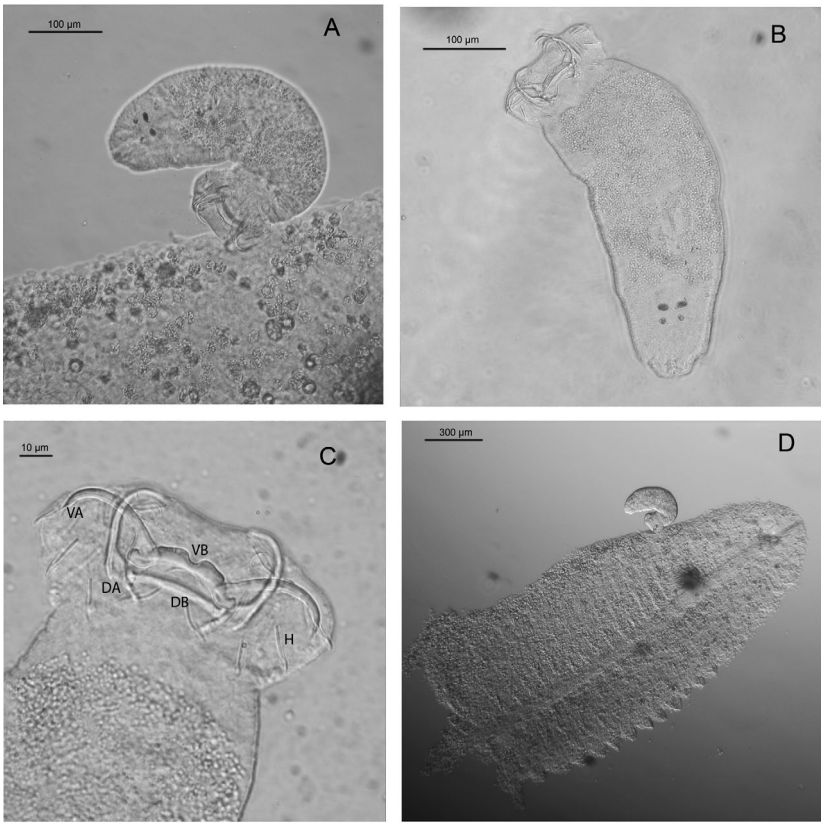

Fig. 1. *Sciadicleithrum variabilum* prepared with proteinase K and ammonium picrate, optical microscope images. A) *Sciadicleithrum variabilum* attached to gill lamella; B) Dorsal view of *Sciadicleithrum variabilum,* haptor; C) Detail of the sclerotized structures of the haptor: dorsal bar (DB), ventral bar (VB), two dorsal anchors (DA), two ventral anchors (VA) and 14 hooks (H); D) *Symphysodon discus* gill lamella with *Sciadicleithrum variabilum* attached.

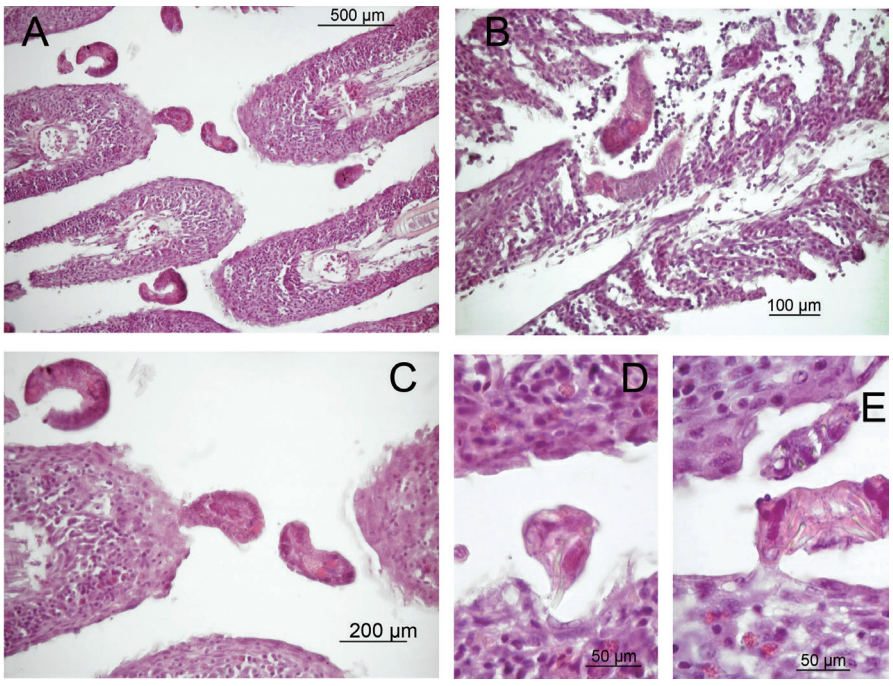

Fig. 2. Histological micrographs of gill lamellae stained with hematoxylin and eosin. The gills are inflamed, hyperplastic with "clubbing" of the gill filaments. A) Monogenean parasites attached and free on gill filaments; B) Gill filaments are inflamed and destroyed. A monogean parasite is attached to epithelial cells with haptor; C) Detail of photomicrograph A. A monogenean parasite is attached to the proximal end of gill filament with haptor; D and E) Details of the haptor fused with tissues of the host. Gill filaments are inflamed and contains eosinophilic granular cells.

the following primers COI mono 5 and COI mono int3[19]. PCR were conducted with a single extension in a 50 μL solution containing 1.5 U Taq polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 10 ρM of each primer and 2 μL of undiluted DNA solution. Thermal cycling was performed with an initial denaturation for 3 min at 94°C, followed by 40 cycles (30 s at 94°C, 30 s at 45°C and 2 min at 72°C), with a final extension of 7 min at 72°C[19]. The amplified products were run on a 1.5% agarose gel containing ethidium bromide. Sequencing was performed with COI mono int3. To test specificity we aligned the obtained sequences with those in the International Nucleotide Sequence Database (INSD) using BLAST search engine. The alignment was manually checked and adjusted with BioEdit 5.0.9[20]. Identification of polymorphic sites was conducted with DnaSP 3.52 software[21] and overall mean distance was calculated with Mega4 software[22].

The molecular characterization based on cytochrome oxidase I provides a 825 bps alignment of sequences from 5 specimens, yielding 2 haplotypes, of *S. variabilum* deposited in EMBL (EMBL code FN400763 and FN400764) with 3 polymorphic sites. The overall mean distance, between haplotypes, and the standard error (0.002 ± 0.001) were calculated in order to make a comparison with the threshold for intraspecific variation in monogenean parasites.

Several studies have been carried out on the effects of Monogenea on fish hosts. The pathogenic effect of attached worms has also been studied, although the virulence of *S. variabilum* has not yet been fully understood[23]. Some authors found *S. mexicanum*[24] and *S. variabilum*[23] damaged gill tissue almost exclusively at the attachment site; this damage was recorded only in epithelial cells with slight necrosis and a slight pathogenic effect.

In our experience, *S. variabilum* directly injured the epithelial cells of the gill lamellae, caused inflammation and increased mucus production. We assumed that these lesions correlated to the elevated number of parasites adhering to a large surface of the gills. However, as reported by Onal *et al*.[23], a small number of *S. variabilum* can be detected on the gills of discus larvae without strong evidence of lesions. In our case study, the delicate structure of the gills was unable to tolerate massive parasite damage, which provoked inflammation and respiratory failure. Furthermore, juvenile fish were unable to offset the existing gas imbalance, dying within a few days[25]. It is known that juvenile fish are more susceptible to disease than adults. As their immune system is immature environmental changes can easily cause stress; these conditions can be more harmful to juvenile discus due to the *S. variabilum* infestation[26–30]. Furthermore, management errors such as high water temperature, crowded tanks, parental care, and juvenile eating habits, all play important roles in the diffusion of infection.

In the outbreak we described, the adult discus living with the juveniles were, in our opinion, the main source of contamination. The adults probably carried a mild unapparent infection, but the high reproductive potential of *S. variabilum*, due to egg resistance to any environmental conditions and their direct and rapid life-cycle, contributed to the successful spread of the infection[31,32]. Moreover, the inefficient sanitary control carried out for adult discus, the high breeding temperature and the habit of mixing juvenile discus from different aquariums contributed to the diffusion of *S. variabilum*. We therefore speculate that 80% mortality in juvenile discus was directly related to the *S. variabilum* infection. Our experience underlines the fact that sanitary control in breeding fish stocks is fundamental, especially when fries and juveniles live together with their parents. Inefficient management can lead to the high mortality and stunned growth of surviving fish, together with serious economic damage.

Morphological evaluation of Monogenea is still the gold standard even if it requires good systematic knowledge of the Class Monogenea. In this study, DNA barcoding of the mitochondrial gene cytochrome oxidase subunit I (COI) has been associated to morphological identification in order to provide a molecular reference that could be easily and quickly used for future identification of *S. variabilum* species. Even if COI sequences of strictly related species, belonging to the same genus or family, are not yet available on GenBank (web nucleotide databases), a lot of data related to intraspecific and interspecific average variation values for monogenean parasites are available[10]. Several studies validated COI as molecular marker able to identify organisms at specific levels[9], included monogenean parasites[10,11]. Plaisance *et al.*[19] used COI for phylogeography of Dactylogyridae, proving that this marker could also be useful for intra-specific identification of haplotypes. Our data show that the overall mean distance between sequenced haplotypes (0.2%) is considerably below the 3% threshold for intra-specific variation in monogenean parasites[10], confirming that our haplotypes belong to the same genetic unit, morphologically identified as *S. variabilum*. However, the future development of COI sequences belonging to strictly related monogenean species could provide a further and exhaustive validation of COI as molecular marker able to discriminate species within *Sciadicleithrum* genus. The identified COI sequence could be also successfully applied to detect morphologically different stages of the parasite (larval stages, *oncomiracidia,* or eggs) in water, in order to prevent massive infections.

In conclusion, in the future *S. variabilum* infection on *Symphysodon discus* might easily be identified by simply comparing COI sequences of the parasites with deposited haplotypes. Moreover, this method, able to identify different stages of the parasite potentially present in water, could be useful for diagnostic purposes to quickly identify the parasite and to set up prophylaxis and therapeutic protocols focused on its life cycle.

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