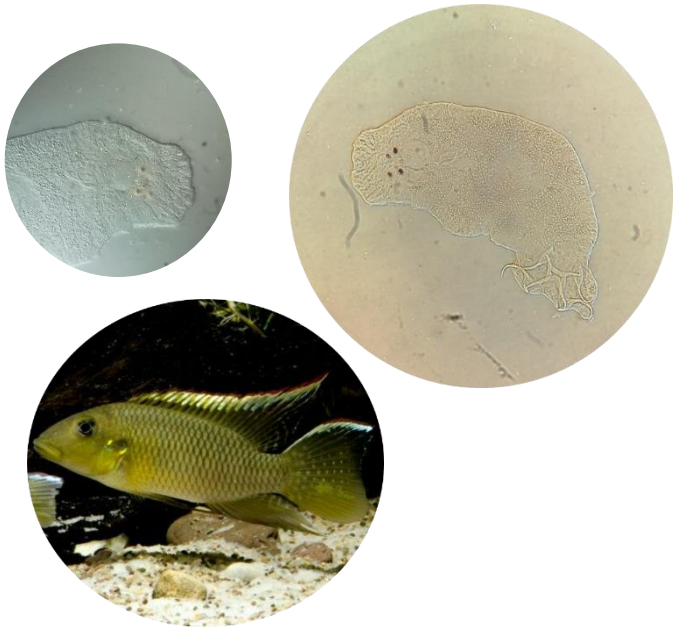

Intraspecific variability in two new species of *Cichlidogyrus* Paperna, 1960 (Platyhelminthes: Monogenea) infecting the gills of species of *Chromidotilapia* Boulenger, 1898 (Teleostei: Cichlidae) from Gabon and the Republic of Congo.

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Table of contents

Abstract

Cichlid fishes and their gill parasites belonging to *Cichlidogyrus* Paperna, 1960 (Platyhelminthes: Monogenea) are one of the most extensively studied host-parasite species networks in Africa. This network has been proposed as a model system for the macroevolutionary processes shaping host-parasite interactions. Yet only a small proportion of the parasite species is known. To close this knowledge gap, we investigate the parasite fauna of cichlid species belonging to *Chromidotilapia* Boulenger, 1898. To date, only one of these species has been examined for parasites before. The gills of seven fishes belonging to *C. kingsleyae* Boulenger, 1898 and one fish belonging to *C. elongata* Lamboj, 1999 were examined for monogeneans. Overall, 108 parasites were cut in three parts for morphological and genetic studies. The hard parts of the attachment and reproductive organs were measured and drawn using interference microscopy. Genetic studies were executed following previously established DNA extraction and amplification protocols, and next generation sequencing. Two new species of *Cichlidogyrus* were found all new to science: *C*. sp. 'diglossiae' on *C. kingsleyae* and *C. elongata*, and *C*. sp. 'gnomon on *C. elongata*. The first species is considered to be genus specific whereas the second is species specific. Species recorded from the Ogooué basin and Congo basin show morphological similarities, which could be explained by convergent evolution or the morphological similarities between the host species. *Chromidotilapia kingsleyae* harbours two species and *C. elongata*, both described in this study. This new information provides new information on the diversity of *Cichlidogyrus* in the Nyanga and Congo basin.

Keywords: Africa, Cichlidae, Monogenea, *Cichlidogyrus*, *Chromidotilapia*, Gabon, Congo

Introduction

The majority of species on Earth lead a parasitic lifestyle (Windsor, 1998). Parasites have some of the most spectacular radiation events (Price, 1980; Huyse et al., 2005) such as anaskid nematodes (Kuhn et al., 2011) and endoparasitic snails infecting corals (Gittenberger & Gittenberger, 2011). Parasites can provide information on their hosts ecology and distribution (Manter, 1966; Nieberding & Olivieri, 2007). For instance, they can provide new insights to understand distribution patterns, ecological interactions and the history of geographic regions and biota; and can be used as keystones in biogeographical research (Hoberg 1997; Nieberding and Olivieri 2007). Phylogenetic data of parasites can provide supporting and complementary information on their host phylogenies (Hennig, 1966; Page & Holmes, 1998). Despite their high diversity and abundance, only the most remarkable parasite faunas have received attention (Vanhove et al., 2015) ignoring the vast biomass including small organisms such as helminths (Fonseca et al., 2010; Lafferty et al., 2008). A large potential for speciation research based on parasites is still unexploited (Huyse et al., 2005), which is even more noticeable in host species that underwent an explosive speciation event (Pariselle et al., 2003).

In both the New World and the Old World, cichlid fishes (Teleostei: Cichlidae) have diversified extensively (Farias et al., 1998; Keenlyside, 1991) but African cichlids provide arguably the most spectacular examples for explosive speciation events, adaptive radiations (Kornfield & Smith, 2000). A considerable number of species with a great diversity of behaviours (Barlow, 2000; Grosenick et al., 2007), mouth morphology (Streelman & Albertson, 2006), body morphology (Fryer & Iles, 1972; Barlow, 2000) and coloration emerged as a result from this adaptive radiation (Seehausen , 2000; Schluter, 2000). Much of this diversity has been associated with specialisation in habitat use and diet (Salzburger, 2009). Therefore, African cichlids are an excellent model in evolutionary research (Koblmuller et al., 2015). African cichlids are infected by monogenean flatworms belonging to the genus *Cichlidogyrus* Paperna, 1960 (Platyhelminthes: Monogenea, Dactylogyridae). *Cichlidogyrus* is the most species-rich monogenean genus infecting cichlids (Vanhove et al. 2015; le Roux & Avenant-Oldewage, 2010). These flatworms have been proposed as markers for biodiversity and speciation studies (Pariselle et al., 2003) because monogenean parasites have a direct life cycle, i.e. there are no intermediate hosts (Pariselle et al., 2011), and have a relatively high host specificity (Mendlová & Šimková, 2014). Hence, many studies on cichlid monogenean interactions have provided insights on ecology, diversity, epidemiology and evolution of these interactions (Vanhove et al., 2016).

As a consequence, the cichlid–*Cichlidogyrus* system was proposed as a macroevolutionary study system for host-parasite interactions. (Pariselle et al., 2003; Pouyaud et al., 2006; Vanhove et al., 2016; Cruz-Laufer et al., 2020). The system is the most extensively described host-parasite network from a species-rich host radiation. Yet only a low proportion of the parasite species is likely known (Vanhove et al., 2016). In terms of their parasitology, economically relevant hosts are often more thoroughly investigated due to limited human and financial resources. This bias creates a knowledge gap with regard to the diversity of *Cichlidogyrus* (Cruz-Laufer et al., 2020).

Closing this knowledge gap is important to gain more information to perform a genus-wide study without the chances of inconclusive results (Kmentová et al., 2016). A series of recent studies have already expanded the knowledge and taxonomic coverage of the hosts of species of *Cichlidogyrus* (see Cruz-Laufer et al., 2020). These studies have looked at underrepresented host species of cichlid tribes including species belonging to the tribes Eretmodini Poll, 1986; Haplochromini Poll, 1986; and Tylochromini Hoedeman, 1947 (Rahmouni et al., 2017; Van Steenberge et al., 2015; Muterezi Bukinga et al., 2012; Rahmouni et al., 2018).

Nevertheless, many cichlid species have still not been examined. The tribe Chromidotilapiini is one of these understudied groups. Chromidotilapiini comprises the most species-rich group of African cichlids in West and Central Africa followed by Oreochromini and Coptodonini. Most chromidotilapiines are riverine (Schwarzer et al., 2015). Chromidotilapiini is also one of the most basal African cichlid lineages together with Tylochromini, Pelmatochromini, Hemichromini and Heterochromidini, with

Heterochromis multidens (Pellegrin 1900) as the most basal species of Cichlidae (Schwarzer et al., 2009). A strong allopatric pattern shows that species divergence within the chromidotilapiines, has been driven by ancient geographic patterns rather than ecology (Schwarzer et al., 2015). Of all 78 described species belonging to Chromidotilapiini, parasites of only three species (*Chromidotilapia guntheri* (Sauvage, 1882), *Parananochromis caudifasciatus* (Boulenger, 1913) and *Benitochromis batesii* (Boulenger, 1901)) have been reported (Pariselle & Euzet 2009).

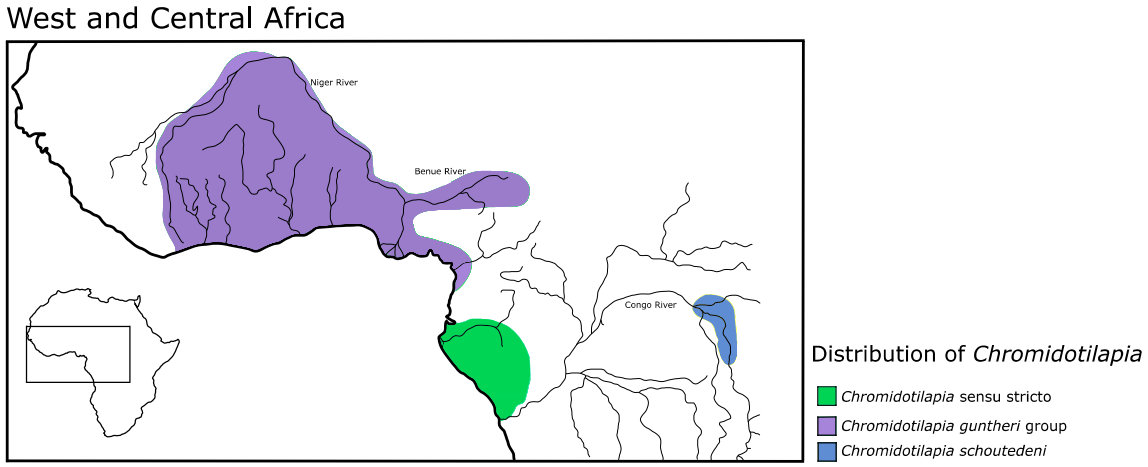

Figure 1: Distribution of the genus *Chromidotilapia*. *Chromidotilapia* sensu strictu restricted to Gabon and the Republic of Congo. *Chromidotilapia schoutedeni* restricted to Upper Congo and '*C. guntheri* group' occurring from Lower Guinea to West Africa. Figure based on Fig1 of Schwarzer et al. (2015)

Objectives

To adress the knowledge gap concerning the gill parasites of chromidotilapiine cichlids, this study will investigate species belonging to the genus *Chromidotilapia* Boulenger, 1898, in particular *Chromidotilapia* sensu strictu (Schwarzer et al. 2015). Schwarzer et al. (2015) discovered the paraphyly of the genus *Chromidotilapia* and suggested to refer to the group comprising the type species as '*Chromidotilapia* sensu stricto', due to the lack of a formal revision. *Chromidotilapia kingsleyae* Boulenger, 1898; *Chromidotilapia melaniae* Lamboj, 2003; *Chromidotilapia elongata* Lamboj, 1999 belong to this group, as do possibly *Chromidotilapia nana* Lamboj, 2003 and *Chromidotilapia mrac* Lamboj, 2002 (Schwarzer et al., 2015). These species are all confined to coastal drainages in Gabon and the Republic of Congo (Schwarzer et al., 2015) (see Fig.1). The region comprises the Lower Guinea coastal forest and savannah block, meaning that the fishes inhabit clear rainforest streams with a mixture of rock and sand (Lamboj, 2004). The two additional *Chromidotilapia* clades, '*Chromidotilapia guntheri* group' and *Chromidotilapia schoutedeni* (Poll & Thys van den Audenaerde 1967), are more closley related to other genera. The '*Chromidotilapia guntheri* group*'* comprises of *C. guntheri*, which lives predominantly in West Africa, *C. linkei* and *C. regani* from northern Lower Guinea. *Chromidotilapia schoutedeni* occurs in the Upper Congo (Schwarzer et al., 2015)(see fig.1). Little is known about ecology and parasitology of these groups, except for *C. guntheri* of which the parasites are

known. By looking at their parasites we will get new insights on both host and parasites and their coevolution.

We will focus on *Chromidotilapia kingsleyae* Boulenger, 1898 and *Chromidotilapia elongata* Lamboj, 1999. Since these species have never been examined on parasites, we expect to find new species as monogeneans express a high level of host specificity (Vanhove et al., 2011). These results could be a step closer to developing a cichlid–*Cichlidogyrus* model system.

Material and methods

Parasite collection and morphological examination

Fish specimens were obtained from the ichthyological collection of the Royal Museum for Central Africa (RMCA). We dissected the gills of the fish and stored the gills in 100% ethanol for preservation. Gills were then screened under a stereomicroscope for the presence of monogenean infections. Parasite specimens were cut in three parts. The anterior and posterior part were mounted on slides with a drop of Hoyer's medium for morphological identification. The rest was used for genetic identification and transferred to Eppendorf tubes with 99% ethanol. Parasite identification and description were conducted using the Leica DM 2500 LED microscope (Leica Microsystems, Wetzlar, Germany) on 400x and 1000x. High resolution pictures were taken through the software LasX v3.6.0 (Leica Microsystems, Wetzlar, Germany).

Morphometrics

Species delineation of dactylogyrid monogenean species is mainly based on the morphology of the sclerotised structures of the attachment and reproductive organs (Pugachev et al., 2009; García-Varela et al., 2016). Therefore, an analysis was conducted to check for interspecific phenotypic variability in haptor morphology. Measurements of the hard parts were taken at a magnification of 1000x through a Leica DM 2500 LED microscope. In total, 19 different parameters of the hard parts and male copulatory organs (MCOs) were measured (see Fig.2). Terminology was based on Pariselle et al. (2014). Measurements were analysed through a principal component analysis (PCA) in the *R* package *ggbiplot* (Vu, 2011; R Core Team, 2013) to detect potential clusters of specimens.

Molecular data generation

Whole genomic DNA was extracted following Laumer's protocol. TNES buffer contained 400mM NaCl, 20mM EDTA, 50mM Tris pH 8 and 0.5% SDS. First, TNES buffer and proteinase K (20mg/ml) were added to the samples, to break down the tissue of the specimen. After incubation for 30-60 minutes to dissolve the specimen, tYeast RNA (10mg/ml) (Invitrogen, USA), 5M NaCl and 96% EtOH are added to separate the DNA from the cellular debris. Samples are stored in the freezer. Afterwards 3 washing steps are performed with 70% chilled EtOH for purifying the DNA. Finally, 0.1X TE with 0.02% Tween 20 (Surfact-Amps Detergent Solution) for storaging. Individual samples were pooled per species to

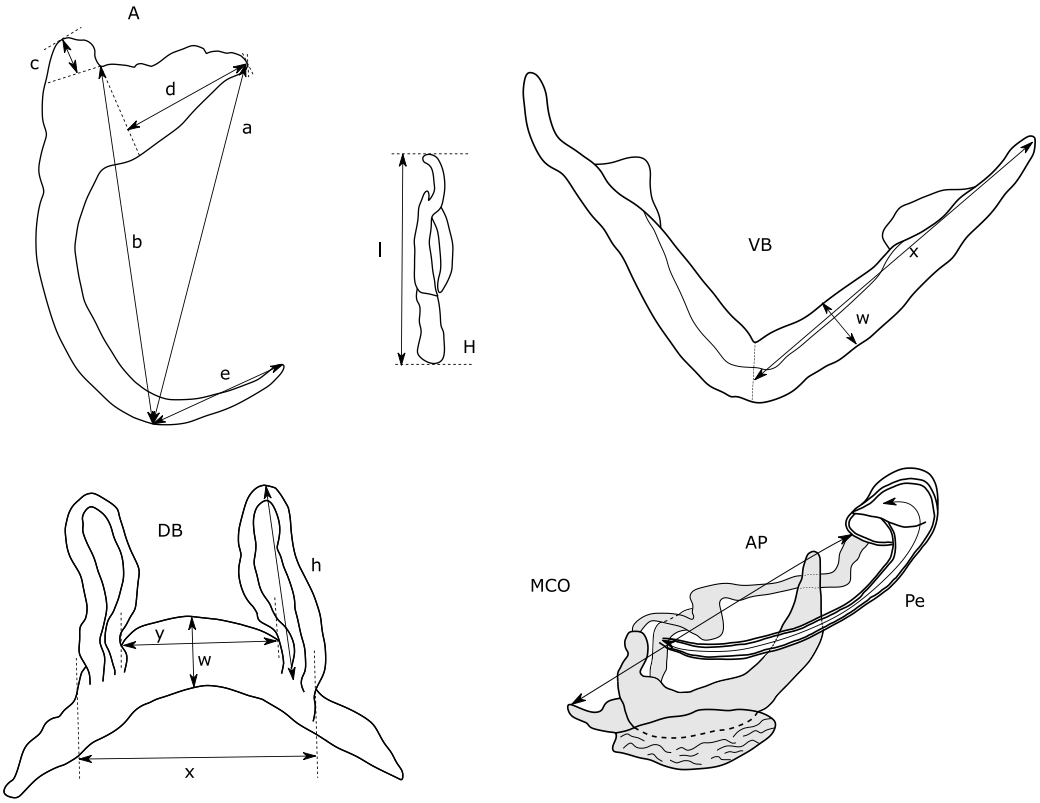

Figure 2: Measurements used for sclerotised structures of haptor and reproductive organs of *Cichlidogyrus* sp. A Anchor: a- Total length, b-Length to notch, c-Outer root length, d- Inner root length, e- Point length; H - Hook length; VB Ventral bar: x- Branch length , w- Branch width; DB Dorsal bar: h-auricle length, w- maximum straight width, x- total length, y- distance between auricles; MCO Male copulatory organ: AP- accessory piece straight length, Pe- copulatory tube curved length.

increase the chance of succeeding in amplifying the DNA. As our samples were preserved in formaldehyde during collection, which causes DNA fragmentation (Heindler et al., 2008), DNA concentration were measured with a Qubit Fluorometer using the Qubit dsDNA HS Assay kit (Life Technologies, USA). Each sample contained DNA samples from a different amount of individuals. We measured samples with 1, 3, 13 and 30 individuals originating from museum samples and a sample with a single non-formaldehyde exposed parasite specimen as control. The samples contained only partial specimens as distal and proximal parts of the worms were used for morphological examination.

We used three different nuclear sequences to verify species delineation. The small and large nuclear ribosomal subunit genes (18S and 28S rDNA), the internal transcribed spacer 1 (ITS-1) and the mitochondrial cytochrome oxidase subunit 1 gene (COI mtDNA). The S1 (5´-ATTCCGATAACGAACGAGACT-3´) (Sinnappah, et al., 2001) and Lig5.8R (5´-GATACTCGAGCCGAGTGATCC-3´) (Blasco-Costa et al., 2012) primers were used to amplify partial 18S rDNA together with ITS-1. Each PCR reaction mix contained 5 units per µl *Taq* polymerase (Thermo Fisher Scientific, USA), 5X PCR buffer, 20mg/ml bovine serum albumin (BSA), 10mM dNTPs, 10µM of each primer and 2µl of isolated DNA (concentration varied from sample to sample). The total reaction volume of 25 µl was amplified using the Bio-Rad Thermal cycler (Applied

Biosystems, USA), under the following conditions: 2 min at 95°C,39 cycles of 1 min at 94°C, 1 min at 64°C and 1 minute and 30 seconds at 72°C, and lastly 10 minutes at 72°C. To amplify the partial 28SrDNA gene, the primers C1 (5´-ACCCGCTGAATTTAAGCAT-3´) and D2 (5´-TGGTCCGTGTTTCAAGAC-3) (Hassouna et al., 1984), were used. Each reaction mix contained 5 units per µl *Taq* Polymerase, 5X PCR buffer, 20 mg/ml BSA, 10mM dNTPs, 10µM of each primer and 2µl of isolated DNA in a total reaction volume of 25µl under the following conditions: 2 min at 95°C, 39 cycles of 20 seconds at 94°C, 30 seconds at 65°C and 1min 30 seconds at 72°C, and finally 10 min at 72°C. The primers ASmit1(5'-TTTTTTGGGCATCCTGAGGTTTAT-3') in combination with Schisto3 (5'-TAATGCATMGGAAAAAAACA-3'), and with ASmit2 (5'-TAAAGAAAGAACATAATGAAAATG -3'), due to nested PCR, were used to amplify a part of the mitochondrial COI gene (Littlewood et al., 1997). The total volume of the amplification reaction contained 25µl: 24µl of PCR mix, which contained 5 units per µl *Taq* polymerase, 5X PCR buffer, 20 mg/ml BSA, 10µM of each primer, and 1 µl of genomic DNA. For primers COI-ASmit1 and Schisto3, the total reaction volume was put under the following conditions, 5min at 95°C, 40 cycles of 1 min at 94°C, 1min at 55°C and 1 min at 72°C, and lastly 7 minutes at 72°C; for primers COI-ASmit1 and COI-ASmit2, 5 min at 95°C, 40 cycles of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C, and finally 7 min at 72°C. The primers ASmit1 and Asmit2 where used separately as well, with the same reaction volume as for the nested PCR. Reaction volume was put under the following conditions: 5 minutes at 95°C, 40 cycles of 1 minute at 94°C, 1 minute at 55°C and 1 min at 72°C, and finally 7 minutes at 72°C. Another set of COI primers were used to increase the amplification success: Mono5 (5'-TAATWGGTGGKTTTGGTAA-3') in combination with Mono3 (5'-TAATGCATMGGAAAAAAACA-3') and Mono3-int (5'-ACA TAA TGA AAR TGA GC-3') (Plaisance et al., 2008). Total volume of reaction mix contained 25µl: 24µl PCR mix, which contained 5 units per µl *Taq* polymerase, 5X PCR buffer, 20mg/ml BSA, 10mM dNTPs, 10µM of each primer and 1µl of isolated DNA. Both combinations of primers are put under the same conditions: 5 minutes at 97°C, 40 cycles of 1 minute at 97°C, 1 minute at 55°C and 1 minute at 72°C, and lastly 7 minutes at 72°C.Amplification results were checked through agarose gel electrophoresis.

Due to the fragmentation of the DNA, we prepared DNA libraries for next generation sequencing. Library preparation was performed with the Nextera DNA Flex library prep kit (Illumina, USA) following manufacturer guidelines. Dependent on the sample, different volumes of DNA were used as input DNA. Volumes were put under following conditions to conduct the PCR: 3 minutes at 68°C, 3 minutes at 98°C, 12 cycles of 45 seconds at 98°C, 30 seconds at 62°C and 2 minutes at 68°C, and lastly 1 minute at 68°C. The parameters were set according to the protocol. The quality of the prepared libraries was checked using Agilent DNA 1000 kit (Agilent Technologies, USA) to infer PCR fragment size and DNA concentration using the 2100 Bioanalyzer (Agilent Technologies, USA), following manufacturer guidelines. Sanger sequencing of the three pools was done by MACROGEN, INC (South Korea).

Sequencing of the libraries was also done by through the ILLUMINA Next Generation Sequencing (NGS) platform using the HiSeq 2500 Sequencer and the TruSeq DNA PCR-Free Library Prep Kit.

Results

Morphological examination

Seven specimens of *Chromidotilapia kingsleyae* and one specimen of *Chromidotilapia elongata* were examined. We found a total of 135 and 6 monogenean parasites on the gills respectively, of which 79% and 100% were identified respectively. Two species of *Cichlidogyrus* were found, which are all new to science. Descriptions are presented below.

Family Dactylogyridae Bychowski, 1933

Genus *Cichlidogyrus* Paperna, 1960

Name: *Cichlidogyrus* sp. 'diglossiae'

Type-host: *Chromidotilapia kingsleyae* Boulenger, 1898 (Perciformes: Cichlidae)

Additional host : *Chromidotilapia elongata* Lamboj, 1999 (Perciformes: Cichlidae)

Locality: small stream, affluent of Moukalaba, Nyanga basin, Gabon; 02°47'S-10°46'E; 19/09/2001

Additional locality: Congo Republic ; 04°17'S-12°27'E on *Chromidotilapia elongata*

Site in host: Gills

Infection parameters: 3 of 7 fish of *C. kingsleyae* infected with 133 and one fish of *C. elongata* infected with 2 specimens.

Description [Based on 76 specimens; Fig. 3]. Anchors 2 pairs. Ventral anchors with a reduced outer root. Inner root more developed. Dorsal anchors about the same size as the ventral anchors. Outer root well-developed and inner root reduced, not as distinct as in the ventral anchor. Between the inner and outer root, the anchor shows a little bulge. Ventral transverse bar V-shaped with triangular membranous attachments at distal half. Dorsal transverse bar thick midsection with 2 pronounced auricles. Hooks 7 pairs; pairs 1,3,5,6 and 7 about the same length, slightly longer than pair 4. Pair 2 smaller than pair 4. Secondary shaft shorter in pair 1 and 4. Male copulatory organ (MCO) consists of a copulatory tube and an accessory piece. Copulatory tube long and slightly curved with distal opening, narrowing at the distal extremity. Base broad with heel attached. Accessory piece connecting at the base of the copulatory tube, bifurcating at the level of the end of the copulatory tube, connecting with the next part at the level of the bulbous end; slightly curved, broad piece partly laying under the copulatory tube and partly laying over the connection. Distal end of accessory piece bifurcating, one end protrudes in a bulbous end, the other end forms a hook with a wing shaped, serrated structure. A considerable variation in the MCO morphology is observed. (see Fig. 4). A string-like structure attaches to the end of the base of the copulatory tube. This attachment point is similar in all individuals. The attachment points to the rest of the accessory piece are also similar but the shape differs in some individuals (see Fig. 4 A, B). In some

individuals, the string-like structure draws a loop and or is curved. In other individuals, this structure is not visible or broken. Hence, the shape of the structure was not always observed. Similar to Fig 4 C, the accessory piece attaches to the copulatory tube where the string-like structure would attach. Furthermore, the wing-shaped structure is larger and more open in these individuals (Fig. 4 C), in other individuals this is mostly (partially) overlaps the big part of the accessory piece. Vagina not observed.

Discussion: The specimens show typical features of species of *Cichlidogyrus*: (i) two pairs of anchors (one ventral and one dorsal), two transverse bars (V-shaped ventral bar, doral bar with two auricles); (ii) hooks 7 pairs; (iii) male copulatory organ consisting of a copulatory tube and generally an accessory piece; and (iv) a vagina which can be sclerotised (Paperna, 1960; Pariselle & Euzet, 2009). *Cichlidogyrus* sp. 'diglossiae' resembles *Cichlidogyrus sclerosus* Paperna & Thurston,1969; *C. amphoratus* Pariselle & Euzet, 1995; *C. fontanai* Pariselle & Euzet, 199; *C. buescheri* Pariselle et al., 2015 and *C. frankwillemsi* Pariselle & Vanhove, 2015. *Cichlidogyrus sclerosus*, infecting fishes from numerous cichlid tribes (Cruz-Laufer et al., 2021), shows resemblances in the morphology of the ventral anchors. The roots are barely seperated (Douëllou, 1993). For the dorsal anchors, the roots are seperated. The inner root is more developed than the outer root, which is more pronounced than in the ventral anchor. Both species show a small hook at the end of the accessory piece but in *Cichlidogyrus* sp 'diglossiae' this hook appears to belongs to a separate plate. No wing-like serrated structure has been reported for *C. sclerosus*. Furthermore, the accessory piece of *C. sclerosus* consists of a single piece that

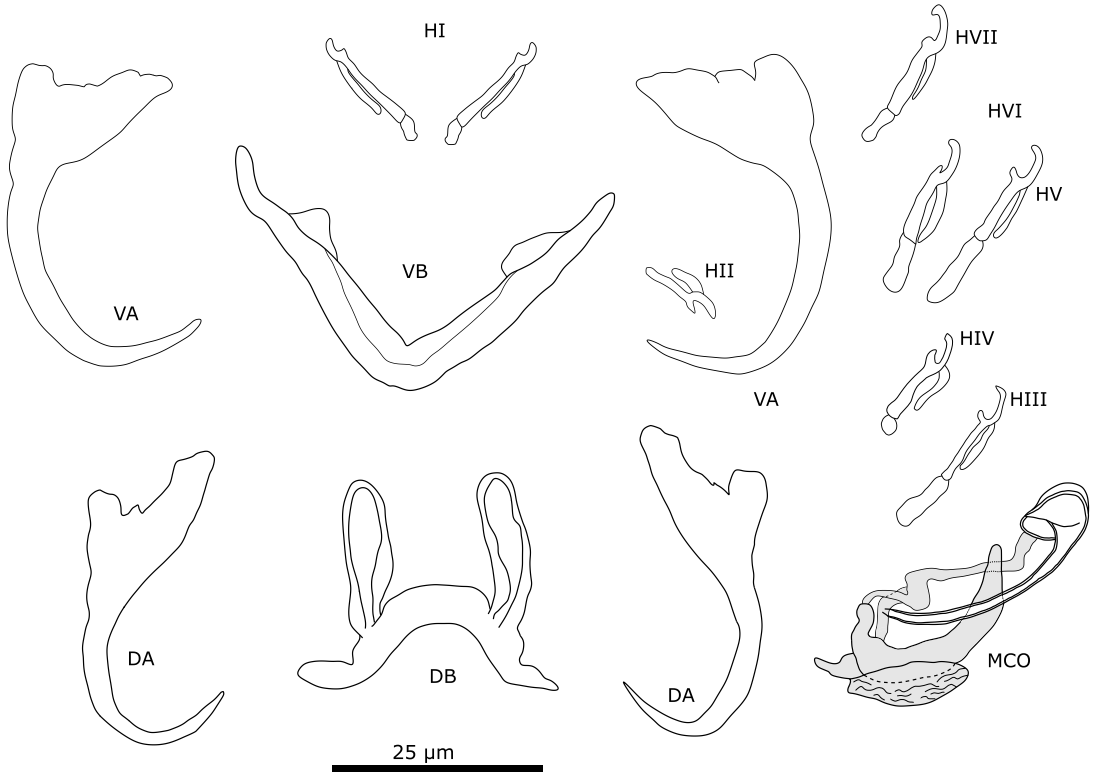

Figure 3: Sclerotised structures of *Cichlidogyrus* sp. 'diglossiae'. Abbreviations: HI-HVII, hooks; VA, ventral anchor; VB, ventral transverse bar; DA, dorsal anchor; DB, dorsal transverse bar; MCO, male copulatory organ.

is connected to the base of the copulatory tube. *Cichlidogyrus amphoratus* Pariselle & Euzet, 1995, sister species to *C. sclerosus*, also resembles *C.* sp. 'diglossiae' regarding the morphology of the haptor. The size of the hooks are similar, but secondary shafts generally longer in *C.* sp. 'diglossiae'. The indentation between inner and outer root of the ventral anchors is deeper in *C. amphoratus*. In the dorsal anchor, the outer root is larger in *C.* sp. 'diglossiae' than in *C. amphoratus*. The MCO of *C. amphoratus* shows a hook at the distal end of the accessory piece, which resembles *C.* sp. 'diglossiae'. However, a swelling in the distal opening of the copulatory tube is not seen in *C.* sp. 'diglossiae'. *Cichlidogyrus fontanai* Pariselle, & Euzet, 1998 differs from *C.* sp. 'diglossiae' in the anchors. Ventral anchor shows distinct roots, where the inner root is more developed than the outer root. In *C.* sp. 'diglossiae' there are no distinct roots. Roots of dorsal anchor in *C.* sp. 'diglossiae' more fused. Dorsal bar with longer auricles in *C.* sp. 'diglossiae' and a slender midsection. Accessory piece of both show bifurcating at the distal

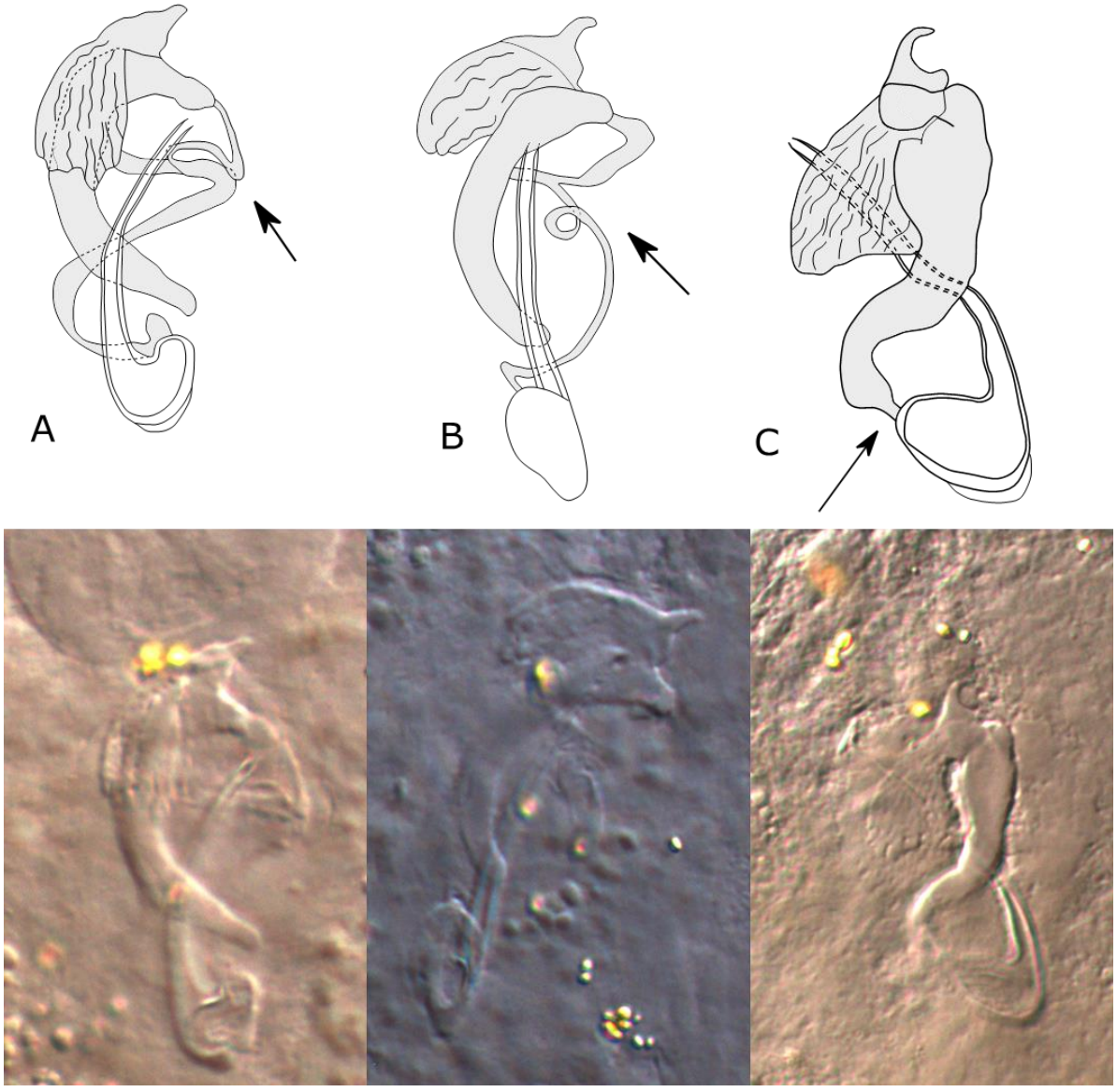

Figure 4: Drawings and microscopic pictures of the male copulatory organs of multiple individuals of *C.* sp. 'diglossiae'. Arrows indicating the variation seen in different specimens

end. The dorsal bar and anchors of *C. frankwillemsi* Pariselle & Vanhove, 2015 and *C. buescheri* Pariselle et al., 2015 resemble those of *C.* sp. 'diglossiae'.

Family Dactylogyridae Bychowski, 1933

Genus *Cichlidogyrus* Paperna, 1960

Name: *Cichlidogyrus* sp. 'gnomon'

Type-host*: Chromidotilapia elongata* Lamboj, 1999 (Perciformes: Cichlidae)

Locality: Mavemba river, tributary of Loukoula on the right bank, 2 km downstream from Mpounga, Congo Republic; 04°17'S-12°27'E; 30/07/1991

Site in host: Gills

Infection parameters: one fish examined, infected with 4 specimens.

Description: [based on 4 specimens, Fig. 5] Two pairs of anchors. Ventral anchor with reduced outer root, inner root more developed. Dorsal anchor about the same size as the ventral anchor. Outer root of dorsal anchor reduced, slightly larger than the outer root of the ventral anchor. Inner root more developed and larger than the inner root of the ventral anchor. Ventral transverse bar V-shaped with triangular membranous attachments along distal half. Dorsal transverse bar, thick midsection with auricles. Auricles are drop-shaped. Hooks 6 pairs observed (1, 3-7). Pair 1, 3-7 about the same length. Secondary shaft of pair 1 and 4 shorter. Male copulatory organ consists of a copulatory tube and an accessory piece. Distal opening of the copulatory tube slightly curved and narrows towards the distal end, with a broad base where a small heel attaches. At two points, accessory piece connects to the base of the copulatory tube. Distal end of the accessory piece split, forms a long and short projection, which two bulbous portions. Accessory piece bent in the middle. Shorter protrusion connected to a plate. This plate has a hook-like projection and a little bulge at the distal end and a drop-like projection at the proximal end.

Discussion: All specimens all show diagnostic features of species of *Cichlidogyrus* (see "Discussion" *C.* sp. 'diglossiae'). *Cichlidogyrus* sp. 'gnomon' resembles *C. fontanai* Pariselle & Euzet, 1997 (infecting *Sarotherodon occidentalis* (Daget, 1962) in River Bourouma (Guinea) (Pariselle, 1997)). The species can be distinguished based on the morphological characteristics of the haptor and the MCO. Protrusions at the distal end of the accessory piece hook-like in *C. fontanai* (Pariselle & Euzet, 1997), but bulbous in *C.* sp. 'gnomon'. The outer roots of the dorsal anchor are larger in *C.* sp. 'gnomon' and the inner roots of ventral anchor slightly larger than the inner root of the dorsal anchor. In *C. fontanai*, the inner roots of dorsal and ventral anchors are approximately the same size. The shape of dorsal transverse bar is similar, but the dorsal bar is generally larger in *C. fontanai*. The midsection of the dorsal bar is also larger. The dorsal bar and dorsal anchors of *C. muterezii* Pariselle & Vanhove, 2015 resembles *C.* sp. 'gnomon'. In *C.* sp. 'diglossiae', the accessory piece attaches to the base of the copulatory tube with a small string like extension, whereas in *C.* sp. 'gnomon' the accessory piece

attaches with the base to the base of the copulatory tube. The accessory piece bifurcates at the distal end for both species but in *C.* sp. 'gnomon' this results in two bulbous protuberances. To one of these protuberances an extra structure is connected, which is not serrated.

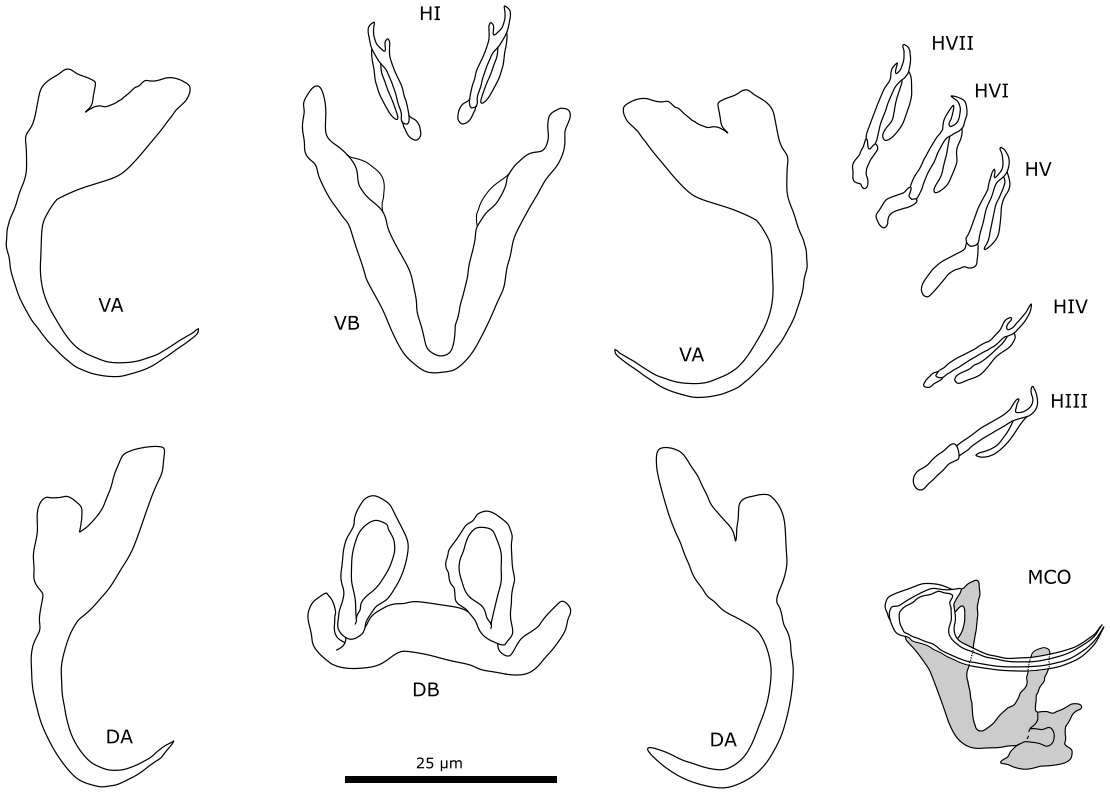

Figure 5: Sclerotised structures of *Cichlidogyrus* sp. 'gnomon'. Abbreviations: HI-HVII, hooks; VA, ventral anchor; VB, ventral transverse bar; DA, dorsal anchor; DB, dorsal transverse bar; MCO, male copulatory organ.

Morphometrics

To examine and visualise the morphometric differences between the species, a principal component analysis was conducted. The analysis was done using haptoral morphometric parameters of 33 individuals of *C.* sp. 'diglossiae' and 2 individuals of *C.* sp. 'gnomon'. The first PC explained 27.3% and the second 19.1% of the variation in the dataset (Fig. 6). Observation of two species clustered in different areas of the plot (Fig. 6). For the PCA, we used the ventral anchor, ventral bar, HI and width of the dorsal bar. The highest contributing variables are the width of the ventral bar, outer root length and point length of the ventral anchor.

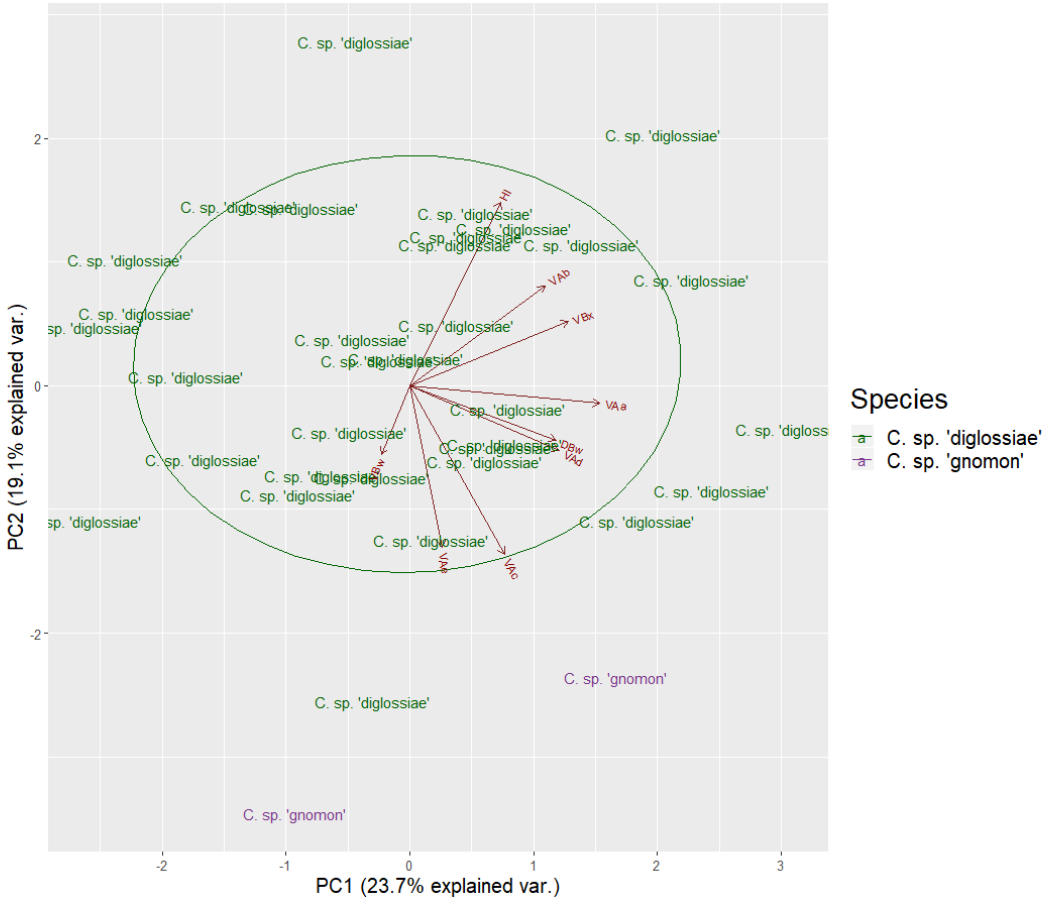

Figure 6: Biplot of PCA (first two axis) based on the measurements of haptoral structures of *C.* sp. 'diglossiae' ad *C.* sp. 'gnomon'.

Molecular data generation

The concentration increases with the amount of individuals, except that the sample with three individuals exhibits a lower concentration than the sample with a single individual. Comparing the samples of specimens with no prior formaldehyde exposure and sample with the formaldehyde exposure, the non-formaldehyde exposed samples show a higher concentration. The non-formaldehyde exposed sample has a concentration of 2.14 ng DNA/µl, while the formaldehyde exposed sample has a concentration of 1.01 ng DNA/µl, approximately double the concentration.

Sample quality control suggest DNA fragments in two of the museum samples: The pool with 30 individuals and the one with 13 individuals. The sample with 30 individuals contained DNA fragments between 300 and 1000 bp, for the one with 13 individuals this was between 300 and 700 bp.

Different PCR protocols were performed to obtain the nuclear sequences of four gene regions. Yet only a fragment of the COI mtDNA was amplified successfully using the primers ASmit1 and ASmit2, under the conditions with 97°C initial denaturation temperature and denaturation temperature. Sequencing of this fragment using sanger sequencing showed that there were no matches with monogenean sequences. This was also the case with next generation sequencing.

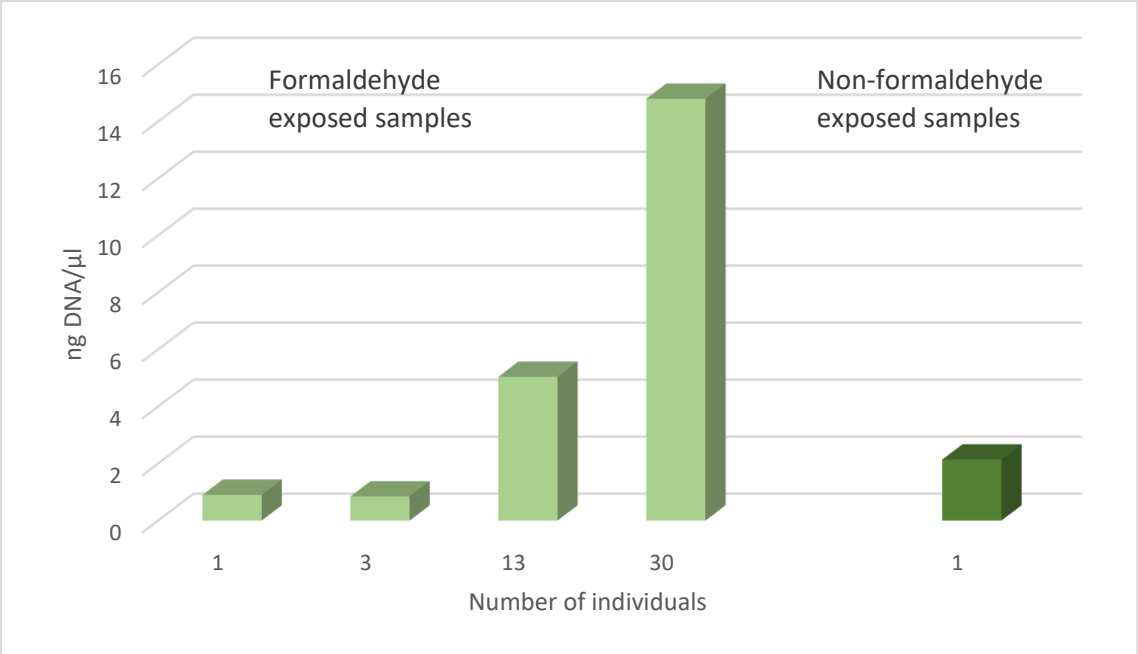

Figure 7: Concentrations of DNA in samples with different amount of individuals. Light green: formaldehyde exposed samples. Dark green: non-formaldehyde exposed samples.

Discussion

Monogenean species richness and comparison with other species of Cichlidogyrus

Based on the hard-part morphology, two new species of *Cichlidogyrus* were discovered: *Cichlidogyrus* sp. 'diglossiae' found on *Chromidotilapia kingsleyae* and *Chromidotilapia elongata;* and *Cichlidogyrus* sp. 'gnomon' found on *Chromidotilapia elongata*. No studies have investigated the parasite fauna on these fishes to date. Therefore, these are the first parasites described infecting these cichlid species. Previously, parasites have been described from *Chromidotilapia guntheri* (Pariselle & Euzet, 2009). *Chromidotilapia guntheri* belongs to a different clade, the '*Chromidotilapia guntheri* group' (Schwarzer et al., 2015), than the species examined in this study, which belong to *Chromidotilapia* sensu stricto. Due to the fact that the level of host specificity is relatively high in species of *Cichlidogyrus* (Mendlová & Šimková, 2014), the species infecting *C. guntheri* will differ from the species infecting *Chromidotilapia* sensu stricto. Previous reports show infections of *C. guntheri* with *Cichlidogyrus dionchus* Paperna, 1968, *C. longicirrus* Paperna, 1965, *C. tilapiae* Paperna, 1960, and *Onchobdella krachii* Paperna, 1968 (Pariselle & Euzet, 2009). Most of these records are relatively old, the last one for *Cichlidogyrus* being from 1968 (Paperna, 1969; Pariselle, 1995) and have not been verified since. Also, there may be a possibility that these species were misidentified, especially the ones from earlier records. Since then many new species have been described and species complexes from then described species have been discovered, i.e. the species complex of *C. tilapiae* (Pouyaud et al., 2006).

The haptor morphology of the species of *Cichlidogyrus* reported from *C. guntheri* resemble in the anchors, dorsal and ventral bar of the new species infecting *C. kingsleyae* and *C. elongata*. This resemblance is, however, not reflected in the morphology of the male copulatory organ (MCO). The species infecting *C. guntheri* also infect species of other tribes as of *Oreochromis, Sarotherodon, Hemichromis, Tilapia* and *Haplochromis* belonging to Oreochromini, Hemichromini, Tilapiini and Haplochromini respectively. Except for Haplochromini, these are basal lineages similar to Chromidotilapiini. These reports mean that the species infecting *C. guntheri* are not specific to chromidotilapiines but have a relatively wide host range. This host range contradicts the notion that monogeneans are usually highly host specific (Mendlová & Šimková, 2014; Pariselle et al., 2003). Unlike species infecting *C. guntheri*, the species described in this study are specific to chromidotilapiine cichlids. Event though that the new species differ in haptor morphology, *C.* sp. 'gnomon' having more robust anchors and dorsal bar than *C.* sp. 'diglossiae', both morphologies are found in other species infecting the basal tribes.

The haptors of species infecting *Chromidotilapia* sensu stricto are generally slender, but larger than the haptor infecting tropheines. The newly described species resemble some species in the other parts of the haptor, which are *C. muterezii* Pariselle & Vanhove, 2015, *C. frankwillemsi* Pariselle & Vanhove 2015 and *C. buescheri* Pariselle et al., 2015. Although the haptor likely reflects the phylogenetic relationships of the parasites (Vignon et al., 2011; Cruz-Laufer et al., 2021), rather than species identity, we cannot unambiguously assign the species to any previously proposed clades (see Cruz-Laufer et al., 2021). Genetic data will be needed to infer the phylogenetic position of the new species.

The male copulatory organ (MCO) morphology of the species infecting hosts belonging to *Chromidotilapia* sensu stricto is unique among the species of *Cichlidogyrus* described to date. Therefore, it is highly likely that these species form a separate group. The uniqueness lies in the structure of the extra plate in *C.* sp. 'gnomon' and the hook attached to the wing-like structure in *C.* sp. 'diglossiae', which looks similar to each other. This structure is a possible common trait for this group. There are some species that show also a bifurcation in the accessory part of their MCO, which are *C. cubitus* Dossou, 1982; *C. flexicolops* Pariselle & Euzet, 1995; *C. fontanai; C. lemoallei* Pariselle & Euzet, 2003; *C. rognoni* Pariselle, Bilong Bilong & Euzet, 2003. Apart from this bifurcation, they have no further resemblances with the new species. Generally, it remains difficult to assign the new species group anywhere due to the high morphological variability of both haptor and MCO within groups of *Cichlidogyrus* and in the genus itself (Cruz-Laufer et al., 2021; Vignon & Sasal, 2010).

We observed an overlap in the distribution of the different fish genera and species infecting these genera show similarities in their morphology. Some species are reported from the same geographical areas as *C.* sp. 'diglossiae' and *C.* sp. 'gnomon' as their host species share the same ecosystem. For instance, species of *Sarotherodon* and *Hemichromis* occur in West Africa (Bitja-Nyom et al., 2021), species

belonging to *Oreochromis* in Central and East Africa and species belonging to *Tilapia* in West and Central Africa (El-Sayed, 2019). Furthermore, species belonging to *Haplochromis* occur in Central and East Africa with one lineage in the Congo basin (Schwarzer et al., 2012), and the tropheines are endemic to Lake Tanganyika (Vanhove et al, 2015). According to Vignon et al. (2011), fish from different genera with a similar morphology will result in a non distinct selection pressure on the haptor morphology of the parasites and, hence, similarly shaped haptors. This process might have shaped the monogenean parasites infecting species belonging to the tribes Oreochromini and Tilapiini (Vignon et al., 2011; Pouyaud et al., 2006). The similarities with species infecting tropheines could be a result of convergent evolution. However, since the morphology of the haptor of species infecting tropheines is generally variable (Cruz-Laufer et al., 2021), these similarities could result from genetic drift. Genetic material and additional sampling is necessary to validate these hypotheses.

Variation in Cichlidogyrus sp. 'diglossiae'

The string-like structure in the MCO of *C.* sp. 'diglossiae' shows a considerable amount of structural variation (see Fig. 4). A similar case has been reported for *C. cirratus* Paperna, 1964. In this species, variability was also detected in a thin transparent structure when looking at it from different perspectives (Zhang et al., 2019). Future studies should use scanning electron microscopy or scanning confocal fluorescence microscopy to obtain three-dimensional images of the MCO. In complex structures, such as the MCO, three-dimensional imaging can give us a better insight of the morphology and the chances of sample destruction decreases (Zhang et al., 2019; Rossin et al., 2017; Galli et al., 2006).

Host specificity

Considering the limited number of hosts and parasites examined and adopting host specificity delimitation in Mendlová & Šimková (2014). *Cichlidogyrus* sp. 'diglossiae' appears to be an intermediate specialist, infecting congeneric host species and *Cichlidogyrus*. sp. 'gnomon' a strict specialist, infecting only one host species. Records show that species from *Oreochromis*, *Sarotherodon*, *Tilapia* and *Hemichromis* also occur in the Ogooué basin, which is in close proximity to the Nyanga basin, and in Gabon and the Congo basin. Monogenean species that infect these hosts are species specific or generalists. Species sampled from *C. guntheri* are intermediate generalists (*C. longicirrus*) or generalists (*C. dionchus*, *C. tilapiae*). Considering that *C. guntheri* is placed in a different clade and has a different distribution range (Schwarzer, 2015), this may suggest that the host specificity of the new species may change as more fish are examined on parasites. To validate the level of host specificity of the new species, additional sampling of potential hosts, such as other chromidotilapiine cichlids, is needed. Furthermore, host ranges of a parasite species may differ considering different scales, local or global, or different regions (Mendlová & Šimková, 2014; Poulin et al, 2011; Krasnov & Poulin, 2010).

Conclusion

Examining *Chromidotilapia kingsleyae* and *Chromidotilapia elongata* revealed two new species of *Cichlidogyrus*. These species are *C.* sp. 'diglossiae', which is genus-specific as it infects both host species studied; and *C.* sp. 'gnomon', which is species-specific as it only infects one host species. These conclusions are made considering the low numbers of hosts examined. These are the first species that are being described from these hosts and hence, this study provides new information on the parasite diversity of cichlid species occurring in the Nyanga and Congo basin. The morphology of the attachment organ, i.e. the haptor, reflects the phylogeny of these parasites. Looking at the new describes species, similarities with species reported from Tilapiini, Oreochromini, Haplochromini and Tropheini are reported. The similarities in morphology in the haptor can be due to the similar bauplan of the basal lineages in cichlids. The similarities with species infecting more derived lineages of cichlids, can be explained by convergent evolution or due to genetic drift. Additional sampling and DNA is needed in future research to accept or reject these hypotheses

Summary

The majority of species on Earth lead a parasitic lifestyle (Windsor 1998). Parasites can provide information on their hosts ecology and distribution (Manter, 1966; Nieberding & Olivieri, 2007), and their phylogenetic data can also provide supporting and complementary information on the evolutionary history of their hosts (Hennig, 1966; Page & Holmes, 1998). Despite their high diversity and abundance, only the most remarkable parasite faunas have received attention (Vanhove et al., 2015). Cichlid fishes and their gill parasites belonging to *Cichlidogyrus* (Platyhelminthes: Monogenea) are one of the most extensively studied host-parasite species networks in Africa. Therefore, the cichlid-*Cichlidogyrus* model system has been proposed as a macroevolutionary study system for host-parasite interactions (Pariselle et al., 2003; Pouyaud et al., 2006; Vanhove et al., 2016; Cruz-Laufer et al., 2020). Yet, only a low proportion of the parasite species is likely known (Vanhove et al., 2016). The current knowledge is insufficient for conclusive genus-wide evolutionary analyses (Kmentová et al, 2016). To close this knowledge gap this, study focuses on parasite infecting cichlid fishes belonging to the tribe Chromidotilapiini, in particular *Chromidotilapia kingsleyae* Boulenger, 1898 and *C. elongate* Lamboj, 1999, which is one of the understudied groups in terms of their parasite diversity.

Monogenean individuals were isolated from the gills of their hosts. Specimens were cut in three, the parts containing the systematically informative attachment organ, i.e. haptor, and male copulatory organ were mounted on a glass slide with a drop of Hoyer's medium for morphological examination. The remaining part was collected for genetic studies. The hard parts of the attachment and male copulatory organ were measured using interference microscopy. The data was used afterwards for a principal component analysis to detect potential clusters of specimens. Following DNA extraction, individuals were pooled per species to increase the chance of amplifying the DNA. Three nuclear DNA sequences

were amplified to assess species delineation, the small and large ribosomal subunit genes (18S and 28S rDNA), the internal transcribed spacer 1 (ITS-1) and cytochrome oxidase subunit 1 gene (COI). Using different PCR protocols. As formaldehyde-exposed samples lead to DNA fragmentation (Heindler et al., 2008), we also conducted a DNA library preparation protocol for next generation sequencing using the Nextera DNA Flex library prep kit.

Two new species of *Cichlidogyrus* were found on *C. kingsleyae* and *C. elongata*. *Cichlidogyrus* sp. 'diglossiae' occurs on both hosts, as *C.* sp. 'gnomon' only occurs on *C. elongata.* The two new species differ in the morphology of the anchors, and dorsal bar. *Cichlidogyrus sclerosus* Paperna & Thurston, 1969 and *C. amphoratus* Pariselle & Euzet, 1995 resemble *C.* sp. 'diglossiae' in multiple haptoral parts and *C. fontanai* Pariselle & Euzet, 1997 resembles *C.* sp. 'gnomon'. These species infect respectively *Oreochromis mossambicus* (Peters, 1852), *Tilapia louka* (Thys van den Audenaerde, 1969) and *Sarotherodon occidentalis* (Daget, 1962). Results from the principal component analyses, on variables of the haptor, show a distinction between the two species.

The morphology of the haptor the new species differs in the ventral anchor and dorsal bar. Both the morphologies are seen in species infecting basal lineages such as Oreochromini, Hemichromini and Tilapiini. Species infecting host belonging to Tropheini, which is a more derived clade, show also similarities in the haptor morphology to the new species. The MCO of both species is unique in having a wing-shaped structure. The structures differ slightly between the two species. In *C.* sp. 'diglossiae' the wing-shaped structure is serrated which is not the case in *C.* sp. 'gnomon'. No described species of *Cichlidogyrus* shows a strong resemblance this morphology of the MCO. We also observed considerable variation in the MCO of *C.* sp. 'diglossiae'. Considering the low number of hosts examined, the host specificity of *C.* sp. 'diglossiae' is genus-specific and *C.* sp. 'gnomon' is species-specific. The similarities in the haptor morphology with species infecting cichlids belonging to Tilapiini, Oreochromini, Haplochromini and Tropheini might be explained by the similar bauplan of the basal lineages of cichlids (Vignon et al., 2011) or convergent evolution with species infecting more derived lineages.

Cichlidogyrus sp. 'diglossiae' and *C.* sp. 'gnomon' are the first parasites to be described from *C. kingsleyae* and *C. elongata*. Here, new information on the parasite diversity is provided in cichlid species occurring in the Nyanga and Congo basin. The morphology of the attachment organs is important in reflecting the phylogeny of these parasites. Additional sampling and genetic material are needed in future research to validate the hypotheses made in this study.

Samenvatting

De meerderheid van de soorten op aarde leiden een parasitaire levensstijl (Windsor, 1998). Parasieten kunnen informatie voorzien over de ecologie en distributie van hun gastheer (Manter, 1966; Nieberding & Olivieri, 2007), en hun fylogenetische data kan ook aanvullende informatie bieden over de evolutionaire geschiedenis van hun gastheren (Hennig, 1966; Page & Holmes, 1998). Ondanks hun grote diversiteit en abundantie, hebben alleen de meest opvallende parasiet faunas aandacht gekregen (Vanhove et al., 2015). Cichliden en hun kieuwparasieten die behoren tot *Cichlidogyrus* (Platyhelminthes: Monogenea), is een van de meest onderzochte gastheer-parasiet netwerken in Afrika. Hierdoor, het cichliden-*Cichlidogyrus* modelsysteem is voorgesteld als een macro evolutionair studiesysteem voor gastheer-parasiet interacties (Pariselle et al., 2003; Pouyaud et al., 2006; Vanhove et al., 2016; Cruz-Laufer et al., 2020). Tot de dag van vandaag is er maar een kleine proportie van de parasieten soorten gekend (Vanhove et al., 2016). De huidige kennis is niet voldoende voor besluitmakende genus overkoepelende analyses, omdat deze tot misleidende conclusies kunnen leiden (Kmentová et al, 2016). Om het kennistekort aan te vullen, focust deze studie zich op soorten die behooren tot Chromidotilapiini, in het bijzonder *Chromidotilapia kingsleyae* Boulenger, 1898 en *C. elongata* Lamboj, 1999. Deze tribus is een van de weinig bestudeerde groepen op het vlak van parasitologie.

Hiervoor zijn individuen van Monogenea geïsoleerd van de kieuwen van hun gastheer. Specimens werden in drie gesneden, de delen die het aanhechtingsorgaan, haptor, en het mannelijk copulatieorgaan bevatte werden gefixeerd op een preparaat glaasje met een druppel Hoyer's medium voor morfologische studies. Het resterende deel werd bewaard voor genetische studies. De harde delen werden gemeten met het gebruik van interferentie microscopie. De data werd achteraf gebruikt voor een principal component analyses in R om mogelijke clusters van specimens te detecteren. DNA werd geïsoleerd volgens Laumer's extractie protocol. Individuen werden gepoold per soort om de kansen te vergroten om het DNA te amplificeren. DNA-concentraties werden gemeten met Qubit Fluorometer gebruik makend van Qubit dsDNA HS Assay kit (Life Technologies, VSA). Drie nucleaire DNA-sequenties werden gebruikt om de afbakening van soorten vast te stellen, de kleine en grote ribosomaal subunit genen (18S en 28S rDNA), de internal transcribed spacer 1 (ITS-1) en cytochroom oxidase subunit 1 gen (COI). Verschillende PCR's werden uitgevoerd met verschillende primer sets. Omdat formaldehyde kan leiden tot DNA-fragmentatie van de stalen (Heindler et al., 2008), hebben we ook een DNA library preperation protocol uitgevoerd voor next generation sequencing gebruik makende van de Nextera DNA Flex library prep kit. Na het vaststellen van de DNA-kwaliteit en kwantiteit, werden de stalen opgestuurd naar MACROGEN (Zuid-Korea) voor sequencing.

Twee nieuwe soorten van *Cichlidogyrus* werden gevonden op *C. kingsleyae* en *C. elongata*. *Cichlidogyrus* sp. 'diglossiae' komt op beide gastheren voor, terwijl *C.* sp. 'gnomon' alleen op *C. elongata* voorkomt. De twee soorten verschillen in morfologie van de ventrale ankers en de dorsale

staaf. *Cichlidogyrus sclerosus* Paperna & Thurston en *C. amphoratus* Pariselle & Euzet, 1995 gelijken *C.* sp. 'diglossiae' in meerdere delen van de haptor en *C. fontanai* Pariselle & Euzet, 1997 gelijkt op *C.* sp. 'gnomon'. Deze soorten infecteren respectief *Oreochromis mossambicus* (Peters, 1852), *Tilapia louka* (Thys van den Audenaerde, 1969) and *Sarotherodon occidentalis* (Daget, 1962). Het resultaat van de principal component analyse, gebaseerd op haptor variabelen, laat een onderscheid zien tussen de twee soorten.

De morfologie van de haptor van de twee soorten verschillen en beide morfologieën komen terug in soorten die basale groepen infecteren, zoals Oreochromini, Hemichromini en Tilapinii. Soorten die Tropheini infecteren, wat een meer afgeleide groep is, vertonen ook gelijkenissen in de haptor met de nieuwe soorten. De MCO van beide soorten is uniek in het hebben van een vleugelvormig structuur. Deze structuur verschilt in de twee soorten. In *C.* sp. 'diglossiae' de vleugelvormige structuur vertoont groeven, dit is niet het geval in *C.* sp. 'gnomon'. Geen enkele beschreven soort van *Cichlidogyrus* vertoont sterke gelijkenissen met deze morfologie van het MCO. Ook werd er aanzienlijke variatie geobserveerd in het MCO van *C.* sp. 'diglossiae' in de vorm van de touwachtige structuur. Rekening houdende met het lage aantal gastheren dat onderzocht werd, de gastheer specificiteit van *C.* sp. 'diglossiae' is genus specifiek en *C.* sp. 'gnomon' is soortspecifiek. De gelijkenissen in de haptor morfologie met soorten die soorten van Tilapiini, Oreochromini, Haplochromini en Tropheini infecteren, kunnen verklaard worden door een gelijkend bauplan van de basale groepen (Vignon et al., 2011) of convergente evolutie met de soorten die meer afgeleide groepen infecteren.

Cichlidogyrus sp. 'diglossiae' en *C.* sp. 'gnomon' zijn de eerste parasieten die beschreven worden van *C. kingsleyae* en *C. elongata*. In deze studie wordt er nieuwe informatie over de diversiteit van parasieten van cichliden soorten die voorkomen in de Nyanga en Congo bassin. De morfologie van het aanhechtingsorgaan is belangrijk in het reflecteren van de fylogenie van deze parasieten. Extra stalen en genetisch materiaal is nodig in toekomstig onderzoek zodat de hypotheses die gemaakt worden in deze studie kunnen verworpen of geaccepteerd worden.

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