Molecular footprint of parasite co-introduction with Nile tilapia in the Congo Basin

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Abstract
Nile tilapia, one of the most popular aquaculture species worldwide, has been introduced into the Congo Basin several times for aquaculture purposes. Previous studies based on morphological features showed that some of the monogenean gill parasites were co-introduced with Nile tilapia and some spilled over to native Congolese cichlids. In this study, we genetically investigated the co-introduced monogeneans of Nile tilapia from three major parts of the Congo Basin: Upper, Middle and Lower Congo. We sequenced 214 specimens belonging to 16 species of Monogenea, collected from native and introduced tilapia species from Congo, Madagascar and Burundi. We evaluate their position in a phylogeny including 38 monogenean species in total. Our results confirm the co-introductions in the Congo Basin and suggest one unreported parasite transmission from introduced Nile tilapia to native Mweru tilapia in Upper Congo, which was undetectable with a morphological study alone. Shared parasite COI haplotypes between Madagascar and the Congo Basin illustrate how anthropogenic introduction events homogenize parasite communities across large geographical distances and thereby disrupt isolation by distance patterns. Contrary to our expectation, the parasite populations co-introduced in the Congo Basin reveal a high COI diversity, probably resulting from multiple Nile tilapia introductions from different geographic origins. Additionally, we tested the barcoding gap and the performance of mitochondrial COI and nuclear ribosomal ITS-1, 28S and 18S markers. We found a significant barcoding gap of 15% for COI, but none for the other markers. Our molecular results reveal that Cichlidogyrus halli, C. papernastrema, C. tiberianus, C. cirratus and C. zambezensis are in need of taxonomic revision.

Keywords Invasive species · Monogenea · Co-introduction · Oreochromis niloticus · Barcoding · Marker performance
Introduction

Human-mediated species translocations are ubiquitous and form a major challenge to global biodiversity today (Pimentel et al., 2001; Tollefson, 2019). As a result of invasive species, the abundance of native plants, insects and other animals has fallen by an estimated 20% since 1900 (Tollefson, 2019). Of these translocated species, tilapias are among the most widely introduced aquaculture species and are now found in over 140 countries (Deines et al., 2016). Cultured tilapia comprises species of African cichlids mainly from Oreochromis Günther 1889, Coptodon Gervais 1848, Tilapia Smith 1840 and Sarotherodon Rüppel 1852 and is estimated to produce over 5 million tonnes yearly globally (Deines et al., 2016; FAO, 2017). Of these, Nile tilapia, Oreochromis niloticus (Linnaeus, 1758), is the most popular and makes up over 75% of the cultured tilapias in Sub-Saharan Africa (FAO, 2017). This species has been deliberately introduced in many African countries, despite the presence of ample native tilapias. In the DRC, culture of non-native Nile tilapia started after the Second World War (Micha, 2013). Production spread rapidly throughout the country until 1960 after which production almost halted (Micha, 2013). In 1964, the first feral Nile tilapia population was found in the North-East of the basin (Thys van den Audenaerde, 1964). From 1996 onwards, Nile tilapia production increased steadily to 3000 tons/year in 2010 (El-Sayed, 2013; Toguyeni, 2004).

Negative effects of Nile tilapia introductions are reported worldwide, including competition with and subsequent decline of native fish species (Canonico et al., 2005; Shipton et al., 2008; Šimková et al., 2019), hybridization with other tilapias (Deines et al., 2014; Firmat et al., 2013; Nyingi & Agnèse, 2007; Trewavas, 1983), stimulation of phytoplankton growth (Starling et al., 2002), predation on eggs of aquatic organisms (Alcaraz et al., 2015) and co-introduction of parasites (Jiménez-García et al., 2001; Jorissen et al., 2020; Šimková et al., 2019; Soler-Jimenez et al., 2017; Zhang et al., 2019). One of the dangers of co-introducing parasites is that these parasites might switch hosts and successfully infect native species (‘spillover’, Goedknegt et al., 2016), possibly increasing the extinction risk of native species.

A previous study identified parasite co-introductions with Nile tilapia and spillover to native cichlids in the Congo Basin (Jorissen et al., 2020). The respective parasites belong to Cichlidogyrus Paperna 1960 and Scutogyrus Pariselle & Euzet, 1995. They are monogenean flatworms (Dactylogyridae Bychowsky, 1937) that mainly infect the gills of African mainland cichlids (Pariselle & Euzet, 2009), where they feed on mucus, skin and possibly blood of the hosts (Gonçalves et al., 2009). Currently, 130 valid species of Cichlidogyrus and seven of Scutogyrus have been described (WoRMS, 2021) and DNA sequences of 31 and three, respectively, are available on GenBank (NCBI, 2021). These sequences are generally limited to partial fragments of 28S or 18S+ITS1 (internal transcribed spacer 1 region) rDNA. The genes coding for 18S and 28S are the most conservative markers currently available (VanhoVE et al., 2013) and are used for phylogenetic reconstructions (Mendlová & Šimková, 2014; Mendlová et al., 2012; Pouyaud et al., 2006; Wu et al., 2007). However, there is a lack of more variable molecular markers for barcoding flatworms (Littlewood, 2008; Moszczynska et al., 2009; VanhoVE et al., 2013) and a lack of sequenced species. For example, only five Cichlidogyrus and Scutogyrus species have been sequenced for the mitochondrial cytochrome c oxidase subunit I (COI) gene.

The first goal of this study is to aid in filling this knowledge gap by adding sequences of Congolese native and introduced species and to evaluate the position of these previously unsequenced species in a phylogenetic tree. Additionally, we will discuss marker performance for mitochondrial COI, ITS-1 rDNA, 18S rDNA and 28S rDNA.

Secondly, because of this molecular knowledge gap, these monogeneans are predominantly diagnosed based on the morphology of their sclerotized hard parts from the reproductive organs and the attachment organ (haptor). However, out of the five morphospecies of Cichlidogyrus and one of Scutogyrus that were co-introduced with Nile tilapia into the Congo Basin (Jorissen et al., 2020), we suspect that three are in need of taxonomic revision: Cichlidogyrus halli (Price and Kirk 1967), C. cirratus Paperna 1964 and C. tilapiae Paperna 1960. A subspecies of C. halli has been described in the past (Paperna, 1979), but was later revoked (Pariselle & Euzet, 2009). Additionally, we discovered a morphotype of C. halli native to Bangweulu-Mweru, in the Upper Congo Basin, infecting the native Oreochromis mweruensis Trewavas, 1983 (Jorissen et al., 2018a). However, genetic information on this morphotype is missing and would be helpful to decide on its taxonomic status. Pouyaud et al. (2006) found three molecular variants of C. halli based on 18S and 28S rDNA, but the low molecular variation did not indicate the need of taxonomical revision at the time. The morphological and molecular variation within C. cirratus could also be underestimated as this species was recently reported in new areas and on new hosts (Jorissen et al., 2020; Zhang et al., 2019 and unpublished data). Finally, Pouyaud et al. (2006) suggested that C. tilapiae might constitute a species complex of morphologically closely resembling taxa. They found larger intraspecific distances based on 18S and 28S rDNA within C. tilapiae than between some specimens of C. tilapiae and C. cubitus Dossou 1982.

Our second goal is therefore to evaluate the species status of these three species through molecular data.
Thirdly, genotyping the co-introduced monogeneans of Nile tilapia could offer a more precise picture of the invasive history of the fish. Indeed, parasites can, due to their faster generation time compared to their host, shed more light on the evolutionary and biogeographical history of their hosts, as predicted by the magnifying glass concept (Nieberding & Olivieri, 2007). Moreover, genotyping can reveal biological phenomena like hybridization, as was the case for catfish parasites, which in turn implied historical contact between the respective host species that currently live in allopatry (Barson et al., 2010). Also the lack of genetic variation can be informative on host biogeography (Hayward et al., 2003). *Gyrodactylus anguilae* Ergens, 1960 collected from eel populations from three different continents share identical ribosomal DNA sequences, as the result from recent intercontinental live eel trade (Hayward et al., 2001).

In this study, we focus on the Congo Basin because it is the largest African basin where Nile tilapia was introduced and because the country has a historical tradition of tilapia culture (Welcomme, 1988; Micha, 2013). Additionally, molecular data from monogenean parasites within the Congo Basin, apart from Lake Tanganyika, is largely lacking. Our expeditions took place in Upper Congo (Bangweulu-Mweru) in the southeast of the basin, Middle Congo around Kisangani (DRC) and Lower Congo downstream of Boma and the tributaries of the Congo around Mbanza-Ngungu (DRC). The boundaries between these three parts are Pool Malebo around Kinshasha and Boyoma Falls upstream from Kisangani (Fig. 1; Alter et al., 2015). We included parasite populations from introduced Nile tilapia from Madagascar because Nile tilapia is well-established there, and mostly the same monogenean species have been co-introduced there as in the Congo Basin (Šimková et al., 2019). Lastly, we sampled a native population of Nile tilapia from a pool next to Lake Tanganyika (Burundi) because it is geographically the closest native population of Nile tilapia to the DRC. According to the concept of isolation by distance (IBD), the genetic similarity between populations should decrease with increasing geographic distance (Avise, 1994; Poulin & Krasnov, 2010). However, introduction events can blur this signal and can also lead to lower genetic diversity in introduced populations compared to their source populations. This can lead to potential founder effects (Avise, 1994; Mayr, 1942). Therefore, we expect a low diversity in the introduced parasite populations from Congo and Madagascar, compared to the native parasite population from Burundi. Also, if Nile tilapia in Congo and Madagascar originate from a common source of introduction, they will share parasite haplotypes and no signal of IBD will be found.

**Material and methods**

**Data collection**

A total of 214 specimens of parasites belonging to 16 described and one undescribed species, of which 15 of *Cichlidogyrus* and two of *Scutogyrus*, were collected from seven host species (Table 1) during three field expeditions to the Democratic Republic of the Congo: Kisangani in May–June 2014, Bangweulu-Mweru in August–September 2014 (Jorissen et al., 2018a), Lower Congo in June 2015 (Jorissen et al., 2018b); one to Burundi in September 2013 (Rahmouni et al., 2017); and one to Madagascar in April 2016 (Šimková et al., 2019). Fish from Lake Kariba in Zimbabwe were caught in September 2016. Fish were collected in the wild, from aquaculture stations or bought at local fish markets and killed with an overdose of MS222 (tricaine mesylate). Specimens and sample localities are listed in Addendum 1 and shown in Fig. 1. The gill arches of the right side were dissected in the field and stored in pure ethanol. The left side of the fish was left intact for ichthyological research. Parasites were isolated in the lab using an entomologi-
cal needle and a Wild M5 stereomicroscope (Heerbrugg, Switzerland). Parasites were cut in half with a scalpel; the anterior body part was fixed in Hoyer's medium (Humason, 1979) and sealed on a slide with glyceel (Bates, 1997) for morphological identification. The pictures of *C. cf. halli* 'Burundi' were taken with a Zeiss Axio Imager Z1 microscope at a magnification of 100 × (oil immersion, 10 × ocular) under differential interference contrast, with an AxioCam MR3 camera and AxioVision v.4.2.8 software. The posterior body part of the parasite was placed in an Eppendorf tube filled with 180 µl of T1 buffer, Nucleospin Kit, Macherey Nagel, Düren, Germany, for DNA extraction and stored at −21 °C if extraction was not carried out immediately (see Addendum 1 for the collection numbers of fish hosts, parasite vouchers and GenBank accession numbers of the parasite DNA sequences generated in this study). Parasite slides and fish hosts were both stored in the collections of the Royal Museum for Central Africa, Tervuren, Belgium (RMCA). Fish from the Madagascar expedition and Upper Congo (Bangweulu-Mweru) were stored under collection 2016–15-P. Fish from Middle and Lower Congo were stored under collections 2014–XX-P and 2015–30-P, respectively. Parasite slides are found under RMCA_VERMES_MT (see Addendum 1).

### Molecular analyses

DNA was extracted using the Nucleospin kit (Macherey–Nagel, Düren, Germany). In the final step, 60 µl of elution buffer was added instead of 100 µl. DNA extracts were stored at −21 °C. We used the primers listed in Table 2 for the amplification of fragments of the mitochondrial cytochrome *c* oxidase subunit 1 (COI, 314 bp), complete ITS-1 + partial 18S (702 bp) and partial

### Table 1

Overview of the sampling design with parasite species listed per host species and per region. I/N represents the introduced (I) or native (N) status of the host in the region. Last column depicts “the total number of sequenced specimens: 18S + ITS-1/28S/COI sequences”

| Host species       | Region         | I/N | Parasite species                                                                 | #Sequences |
|-------------------|----------------|-----|----------------------------------------------------------------------------------|------------|
| *Coptodon rendalli* (Boulenger, 1897) | Lower Congo | I   | *C. berradae*                                                                    | 4:1/4/0    |
|                   | Upper Congo   | N   | *C. dossouei*, *C. quaestio*, *C. tiberianus*                                    | 1:1/1/1    |
|                   |               |     |                                                                                 | 2:0/2/0    |
|                   |               |     |                                                                                 | 2:1/2/0    |
| *Hemicromis stellifer* (Loiselle, 1979) | Madagascar   | I   | *C. sp.2*                                                                        | 2:0/2/0    |
| *Oreochromis mweruensis* (Trewavas, 1983) | Lower Congo | N   | *C. cirratus*, *C. halli* morphotype 2, *C. papernastrema*, *C. tilapia*, *S. gravivaginus* | 2:1/2/0    |
|                   |               |     |                                                                                 | 2:0/2/0    |
|                   |               |     |                                                                                 | 1:0/1/0    |
|                   |               |     |                                                                                 | 1:1/1/0    |
| *Oreochromis niloticus* (Linnaeus, 1758) | Lake Tanganyika, Burundi | N   | *C. cf. halli* 'Burundi', *C. tilapia*                                           | 1:1/1/1    |
|                   |               |     |                                                                                 | 6:0/3/3    |
|                   | Lower Congo   | I   | *C. halli*, *C. sclerosus*, *thurstonae*, *C. tilapia*                          | 14:6/9/6   |
|                   |               |     |                                                                                 | 3:0/1/3    |
|                   |               |     |                                                                                 | 34:5/17/16 |
|                   |               |     |                                                                                 | 22:5/13/10 |
|                   | Middle Congo  | I   | *C. halli*, *C. sclerosus*, *thurstonae*, *C. tilapia*                          | 1:0/0/1    |
|                   |               |     |                                                                                 | 1:0/1/1    |
|                   |               |     |                                                                                 | 1:0/1/1    |
|                   |               |     |                                                                                 | 3:0/0/3    |
|                   | Upper Congo   | I   | *C. halli*, *C. sclerosus*, *thurstonae*                                        | 8:0/0/8    |
|                   |               |     |                                                                                 | 6:0/0/6    |
|                   |               |     |                                                                                 | 1:0/0/1    |
|                   | Madagascar    | I   | *C. halli*, *C. sclerosus*, *thurstonae*, *C. tilapia*, *S. longicornis*        | 5:2/3/1    |
|                   |               |     |                                                                                 | 1:0/0/1    |
|                   |               |     |                                                                                 | 4:0/2/2    |
|                   |               |     |                                                                                 | 2:1/1/2    |
|                   |               |     |                                                                                 | 1:0/1/0    |
| *Sargochromis mellandi* (Boulenger, 1905) | Upper Congo | N   | *C. consobrini*, *C. zambezensis*                                               | 2:1/2/0    |
|                   |               |     |                                                                                 | 1:1/1/0    |
| *Serranochromis macrocephalus* (Boulenger, 1899) | Middle Zambezi, Zimbabwe | N   | *C. zambezensis*                                                                | 3:1/3/0    |
| *Tilapia sparrmanii* (Smith, 1840) | Upper Congo   | N   | *C. dossouei*, *C. papernastrema*                                               | 1:0/0/1    |
|                   |               |     |                                                                                 | 4:0/3/1    |
28S (653 bp). For COI, if initial amplification was unsuccessful we tried again with nested primers, resulting in the 314 bp mentioned above. Protocols of DNA amplification are included in Table 3. PCR products were run on 1.5% agarose gels stained with GelRed (Biotum Inc., Fremont, California). Successful amplifications were purified with EXOSAP-IT (Thermo Fisher, Waltham, Massachusetts) in a 5/2 (product/EXOSAP) ratio and incubated at 37 °C for 4 min followed by 80 °C for 1 min. A total of 3.2 µM forward primer was added to the purified PCR products in a 7/5 (purified PCR product/3.2 µM primer) ratio and sent to Macrogen Europe under the EZ-seq service for single direction Sanger sequencing.

Phylogenetic analyses

A total of 38 species were included to build the phylogeny from ribosomal markers; for 12 of these, we present the first sequences. All sequences are submitted to GenBank and accession numbers are available in Addendum 1. Our tree was rooted on Cichlidogyrus pouyaudi Pariselle and Euzet (1994) as previous phylogenetic research found that it is the most basal taxon of the group (Mendlová & Šimková, 2014; Mendlová et al., 2010; Pouyaud et al., 2006; Wu et al., 2007). All sequence chromatograms were visually inspected for sequencing errors and blasted individually on the NCBI website (http://www.ncbi.nlm.nih.gov). The resulting sequences were aligned with MUSCLE (Edgar, 2004a, 2004b) under default settings, edited in MEGA 7.0.18 (Kumar et al., 2016) and cleaned-up with Gblocks 0.91b under default parameters (Castresana, 2000; Talavera & Castresana, 2007). The 28S and 18S + ITS-1 sequences were concatenated using SequenceMatrix (Vaidya et al., 2011). To test whether a concatenation was possible we performed a partition-homogeneity test in PAUPUP 1.0.3.1 (Swofford, 2003) using the GTR + G + I model. A median-joining haplotype network (Bandelt et al., 1999) was constructed with PopART 1.7 (Leigh & Bryant, 2015) with ε = 0 separately for C. sclerosus (Paperna and Thurston, 1969), C.thurstonae and C. tilapiae based on 9, 20 and 17 COI sequences, respectively. COI alignments were translated to amino acid sequences with MEGA 7.0.18 for information on (non-)synonymous mutations and to check for nuclear

### Table 2

| Gene                  | Primer                  | Direction | Sequence (5′-3′)                      | Reference                      |
|-----------------------|-------------------------|-----------|--------------------------------------|--------------------------------|
| COI                   | ASmit1                  | F         | TTTTTTGCGATCTGAGGTAT                 | Littlewood et al., 1997        |
| COI                   | Cox1_Schisto_3          | R         | TAATGCATMGGAAAAAACA                  | Lockyer et al., 2003           |
| COI                   | ASmit2 (nested)         | R         | TAAAGAAGAAACATAATGAAAATG             | Littlewood et al., 1997        |
| 18S + partialITS-1    | S1                      | F         | ATTCCGATAACGACGGACT                  | Sinappah et al., 2001          |
| 18S + partialITS-1    | IR8                     | R         | GCTAGCTTCGTTCTTCATCG                 | Šimková et al., 2003           |
| 28S                   | C1                      | F         | ACCCGCTGAAATTAAGCAT                  | Hassouna et al., 1984          |
| 28S                   | D2                      | R         | TGTTCCGTGTTTCAAGAC                   | Hassouna et al., 1984          |

Haplotype networks and distance matrices

Distance matrices were calculated in PAUPUP 1.0.3.1 (Swofford, 2003) using the GTR + G + I model. A median-joining haplotype network (Bandelt et al., 1999) was constructed with PopART 1.7 (Leigh & Bryant, 2015) with ε = 0 separately for C. sclerosus (Paperna and Thurston, 1969), C. thurstonae and C. tilapiae based on 9, 20 and 17 COI sequences, respectively. COI alignments were translated to amino acid sequences with MEGA 7.0.18 for information on (non-)synonymous mutations and to check for nuclear
mitochondrial DNA copies (numts). To estimate a barcoding gap and species partitioning we used ASAP: Assemble Species by Automatic Partitioning (Puillandre et al., 2020) calculated with the K2P-distance model (Kimura, 1980). Nucleotide diversity and polymorphic sites were calculated with DnaSP V.6.12.03 (Rozas et al., 2017). Distance matrices are available in addenda.

### Results

The sequencing of the partial COI gene resulted in 67 sequences of 314 bp of 13 species after trimming and clean-up in Gblocks; none showed stop codons. We opted for the nested fragment to include the most specimens in the analyses. The partition homogeneity test allowed the concatenation of 18S, ITS-1 and

| Primer set                     | PCR condition | Mastermix               |
|-------------------------------|---------------|-------------------------|
| **ASmit1 – Schisto3 (COI)**    | 94 °C: 3 min  | 1X PCR Buffer           |
|                               | 94 °C: 30 s   | 1.5 mM MgCl₂            |
|                               | 44 °C: 30 s   | 0.2 mM dNTP mix         |
|                               | 72 °C: 60 s   | 0.4 µM F-primer         |
|                               | 72 °C: 7 min  | 0.6 µM R-primer         |
|                               | 14.8 µl ddH₂O | 1.2 µl Template         |
|                               | 1008          | 3 Units/reaction Taq    |

| Primer set                     | PCR condition | Mastermix               |
|-------------------------------|---------------|-------------------------|
| **ASmit1 – ASmit2 (COI, nested PCR)** | 94 °C: 3 min  | 1X PCR Buffer           |
|                               | 94 °C: 30 s   | 1.5 mM MgCl₂            |
|                               | 50 °C: 30 s   | 0.2 mM dNTP mix         |
|                               | 72 °C: 60 s   | 0.4 µM F-primer         |
|                               | 72 °C: 7 min  | 0.4 µM R-primer         |
|                               | 15 µl ddH₂O   | 1.2 µl Template         |
|                               | 1008          | 3 Units/reaction Taq    |

| Primer set                     | PCR condition | Mastermix               |
|-------------------------------|---------------|-------------------------|
| **S1 – IR8 (18S + ITS-1)**    | 94 °C: 2 min  | 1X PCR Buffer           |
|                               | 94 °C: 60 s   | 1.5 mM MgCl₂            |
|                               | 50 °C: 60 s   | 0.2 mM dNTP mix         |
|                               | 72 °C: 90 s   | 0.4 µM F-primer         |
|                               | 72 °C: 10 min | 0.6 µM R-primer         |
|                               | 14.8 µl ddH₂O | 1.2 µl Template         |
|                               | 1008          | 3 Units/reaction Taq    |

| Primer set                     | PCR condition | Mastermix               |
|-------------------------------|---------------|-------------------------|
| **C1 – D2 (28S)**             | 94 °C: 2 min  | 1X PCR Buffer           |
|                               | 94 °C: 20 s   | 1.5 mM MgCl₂            |
|                               | 50 °C: 30 s   | 0.2 mM dNTP mix         |
|                               | 72 °C: 90 s   | 0.4 µM F-primer         |
|                               | 72 °C: 10 min | 0.4 µM R-primer         |
|                               | 15 µl ddH₂O   | 1.2 µl Template         |
|                               | 1008          | 3 Units/reaction Taq    |
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28S fragments with a p-value of 0.95 (anything above 0.05 was sufficient for concatenation). Our concatenated sequences consisted of 1362 bp divided in fragments of 429, 275 and 658 bp of 18S, ITS-1 and 28S rDNA, respectively. We generated 31 sequences of 10 morphospecies for 18S + ITS-1 rDNA and 85 sequences of 17 morphospecies for 28S rDNA. The ITS-1 fragment was the most variable (60.2% maximum variance, Addendum 2) followed by COI (44.3%, Addendum 3), 28S (11.9%, Addendum 4) and 18S (4.8%, Addendum 5–7). For the COI alignment, the topology of both the ML and Bayesian trees was not well supported, especially the deeper branches lacked support. We therefore used the COI dataset to compare haplotypes through median-joining haplotype networks (Fig. 2a–c). For C. halli the genetic distance between specimens was too large for a median-joining haplotype network. The COI dataset included 15 morphospecies and was divided in 18 species by ASAP and between 22 and 28 by bPTP. The concatenated dataset included 37 morphospecies and was divided in 34–38 species by ASAP and between 22 and 47 by bPTP. The species divisions in the COI dataset between ASAP and bPTP corresponded well and were largely well-supported with bPTP. However, for the concatenated ribosomal dataset with bPTP, the species divisions were largely unsupported.

Co-introduced parasites of Nile tilapia

Four of the co-introduced species of *Cichlidogyrus*, *C. halli*, *C. thurstonae*, *C. tilapiae*, and *C. sclerosus* showed intraspecific variation in COI (Addendum 3). The variation within the halli group was so high (0.6–30.1%, 104 polymorphic sites, Pi = 0.1463) that it did not allow a median-joining haplotype network. Within *C. halli*, we observed nine groups, each separated by more than ten to 30 mutations. The locality Bumaki in the Upper Congo has the most haplotypes of *C. halli* and these cluster in three groups. Furthermore, the native *C. cf. halli ‘Burundi’* and *C. halli* morphotype 2 *sensu* (Jorissen et al., 2018a) showed the highest distance to the introduced specimens (0.2–3.2% 18S, 3.2–10.1% ITS-1, 1.1–1.9% 28S, 24.7–36% COI), with large distances within these native representatives for COI also, but not for the rDNA fragments (0.2% 18S, 2.4% ITS-1, 1% 28S, 23.1–29.5% COI). The COI sequence of *C. cf. halli ‘Burundi’* has three non-synonymous mutations compared to introduced *C. halli* and the two specimens of *C. halli* morphotype 2 have two and four non-synonymous mutations respectively. One non-synonymous mutation was shared between these three native specimens and two non-synonymous mutations between the two specimens of *C. halli* morphotype 2. Within the introduced specimens of *C. halli* in Congo, three of Upper Congo and one from Middle Congo shared the same non-synonymous mutation. Other co-introduced species, *C. sclerosus* (1–2.7% COI, 16 polymorphic sites, Pi = 0.01776), *C. tilapiae* (0.3–3.4% COI, 10 polymorphic sites, Pi = 0.01409) and *C. thurstonae* (0.3–3.4% COI, 13 polymorphic sites, Pi = 0.01706) show more modest variation (Addenda 3, 6).

For *C. sclerosus*, each locality was characterized by a unique parasite haplotype, with the highest variation within Upper Congo. Only in one locality in Upper Congo a non-synonymous mutation was found. The haplotype from the Middle Congo was more closely related to the one from Madagascar than to the haplotypes from Upper Congo (Fig. 2a). For *C. thurstonae* on the other hand, the highest diversity was found in Lower Congo and haplotypes are shared by parasites from Madagascar and Upper Congo (Fig. 2b). Specimens

![Fig. 2 Median-joining haplotype networks constructed using COI sequences (314 bp) for a Cichlidogyrus sclerosus, b Cichlidogyrus thurstonae and c Cichlidogyrus tilapia collected in DRC, Madagascar and Burundi](image-url)
from the Upper and Middle Congo cluster furthest apart, each separated by at least five mutations respectively from the nearest *C.thurstonae* specimen from Lower Congo, of which three were non-synonymous. Thus, the three regions of the Congo Basin do not share any *C.thurstonae* haplotype. This in contrast to *C. tilapiae*, where apart from a few unique sequences, haplotypes are shared between the Lower and Middle Congo and between Lower Congo and Madagascar (Fig. 2c). The native parasite specimens from Burundi were clearly distinct from the introduced specimens in Congo and Madagascar. However, the amino acid sequence of these Burundese specimens is identical to all other specimens of *C. tilapiae*, except for one specimen from Middle Congo, which has two non-synonymous mutations.

**Phylogenetic relationships among native and introduced tilapia parasites and evaluation of species status**

A Bayesian phylogram constructed with 28S and 18S + ITS-1 sequences of *Cichlidogyrus/Scutogyrus* representatives of native and introduced tilapia parasites and sequences from GenBank is shown in Fig. 3. The topologies of the BI and ML trees were very similar (ML tree not shown). Minor differences were on the level of poorly supported clades (bootstrap support < 70), or unresolved intraspecific relationships. The basal topology is unclear from our analyses as we observe six monophyletic groups, between which the relations are unresolved. To facilitate the interpretation of the phylogram, we named each of these monophyletic groups after the oldest described species within it, following Pouyaud et al. (2006). These are artificial ‘species groups’ and the name of these groups does not infer anything to the evolutionary relationships or taxonomic status of the taxa within.

The “halli” group has a reference sequence of *C. halli* from Senegal (Addendum 1) at the base followed by a polytomy of 18 haplotypes of co-introduced *C. halli* from Nile tilapia in the Congo basin and Madagascar (0–2.9% 18S, 0.4–7.2%ITS-1, 0–1.4% 28S 1.6–21.9% COI, see Addenda 2–7). More derived of this group are three sequences of *C. halli* from Upper Congo and a hybrid *Oreochromis* host (Vanhove et al., 2018). *Cichlidogyrus halli* forms its own clade and the closest relative was not revealed by phylogenetic analysis.

The “papernastrema” group houses apart from *Cichlidogyrus papernastrema* Price, Peebles and Bamford 1969 also *Cichlidogyrus zambezensis* (Douëllou, 1993). Our two specimens of *C. papernastrema* (intraspecific distance of 1.8% 28S, 29.9% COI) do not cluster together. *Cichlidogyrus zambezensis*, on the contrary, is monophyletic and the distance (0.6% 28S) between specimens on different host species is larger than between two specimens of the same host species (identical 28S), although they all are from the same geographic region. Furthermore, both ASAP and bPTP split *C. zambezensis* in two species.

The “tiberianus” group has *C. cubitus* at the basal position followed directly by *C. tiberianus* Paperna 1960 (reference sequence from Senegal). The group then splits up in three lineages, one of which includes a polytomy of 23 haplotypes of co-introduced *C. thurstonae* from Nile tilapia, the reference of *C. thurstonae* from Madagascar and *C. ergensi* Dossou 1982. Distances within this lineage are 0–1.7% 18S, 0–4.4% ITS-1, 0–0.6% 28S, 0.3–4.8% COI (Addenda 2–7). However, the support for this group is very low (53 posterior probability and < 50 bootstrap value). Directly related to *C. thurstonae* and *C. ergensi* are *Cichlidogyrus dossoi* (Douëllou, 1993) and *C. tiberianus* from Upper Congo. Our specimens of *C. tiberianus* from Upper Congo do not cluster with the reference of *C. tiberianus* from Senegal. The bPTP analysis confirms this result. The distance of *C. tiberianus* between Upper Congo and Senegal is 0.2–0.5% 18S, 8.5–9% ITS-1 and 1.4–28S.

The second lineage in the “tiberianus” group has *Cichlidogyrus aegypticus* Ergens 1981 at the base followed by *C. arthracanthus* Paperna 1960 (Senegal) and two specimens of *C* sp. 2 from Congolese Redbreast tilapia, which are morphologically similar to *C. arthracanthus* (Jorissen et al., 2020). Pairwise distances amount to 1–1.4% for 28S between *C* sp. 2 and *C. arthracanthus* and 0.5% within *C* sp. 2. Within this group we have a well-supported monophyletic group which includes *Cichlidogyrus agnesi* (Pariselle & Euzet, 1995) and *C. gallus* (Pariselle & Euzet, 1995) as sister species and the *C. bilongi* Pariselle and Euzet 1994 and *C. flexicolpos* (Pariselle & Euzet, 1995) as sister species (only supported by PP), respectively. The last lineage includes *Cichlidogyrus douellouae* Pariselle, Bilong Bilong, Euzet 2003.

In the “cirratus” group, we find *Cichlidogyrus njinei* Pariselle, Bilong Bilong and Euzet 2003 at the base followed by *C. acerbus* Dossou 1982 and *C. cirratus* (Senegal) and lastly two specimens of *C. cirratus* from native Mweru tilapia from Bangweulu-Mweru. Distances within *C. cirratus* are 0.5% 18S, 9.8% ITS-1, 0–2.8% 28S with the 28S sequences from Mweru tilapia being identical. Furthermore, bPTP suggests that our specimens of *C. cirratus* belong to a different species than the reference.

In the “tilapiae” group we find all sequences of *C. tilapiae* from both native and introduced hosts (n=24, with p-distances ranging from 0 to 1.7% 18S, 0.7 to 2.7% ITS-1 and 0 to 1%
28S, 0.3–3.4% COI), with no apparent structure other than that the reference sequence from Senegal is the earliest diverging (Addendum 1).

The “longicirrus” group is split up in three clades and contains species that infect Hemichromis spp. (Dossou & Birgi, 1982; Pariselle & Euzet, 2004; Jorissen et al., 2018a) One clade is well-supported and contains a representative of Cichlidogyrus falcifer (Dossou & Birgi, 1984) from Lower Congo, which clusters with the reference from Senegal and the recently discovered C. polyenso Jorissen, Pariselle and Vanhove 2018 from Lower Congo which clusters with C. longicirrus Paperna 1965.

Another poorly supported clade contains three smaller well-supported groups. The first contains our specimens of C. sclerosus (identical 28S, 3% COI), with their reference sequence (distance of 1.4–1.6% 28S) and a reference of C. amphotaratus (Pariselle & Euzet, 1995). The recently discovered C. consobrini Jorissen, Pariselle and Euzet 2018 clusters with the reference from Senegal and C. irenae Gillardin, Vanhove, Pariselle, Snoeks, Huyse and Volckaert 2011 and C. casuarinus Pariselle, MutereziBUkinga and Vanhove 2015. These three species have not been considered closely related to each other before (Jorissen et al., 2018a; Pariselle et al., 2015) Furthermore, both C. irenae and C. casuarinus were found in Lake Tanganyika but on different host species, Gnathochromis pfefferi (Boulenger, 1898) and representatives of Bathybates Boulenger 1898, respectively (Gillardin et al., 2012; Kmentová et al., 2016a, b; Pariselle et al., 2015), whilst C. consobrini is native to the Upper Congo and found on Sargochromis mellandi and Orthochromis katumbii Schedel, Vreven, Manda, Abwe, Manda, Schliwien, 2018 (Jorissen et al., 2018a). Furthermore, we have a clade that consists of representatives of Scutogyrus.

Finally, we have a well-supported clade with two specimens of C. berradae (Pariselle & Euzet, 2003) (0.2% 28S), C. quaestio (Douéllou, 1993), C. digitatus Dossou 1982 and C. yanni (Pariselle & Euzet, 1995). The 28S sequence of C. yanni was identical to C. berradae and was left out of the tree. Jorissen et al. (2018a) predicted all these species were closely related to each other based on the morphology of the sclerites.

Discussion

Through molecular identification, we were able to confirm the parasite introductions into the Congo Basin and Madagascar observed in Jorissen et al. (2020). Additionally, we discovered a parasite transmission (spillover) of C. tilapia from introduced Nile tilapia to native Mweru tilapia in Upper Congo. In Jorissen et al. (2020) we considered all C. tilapia on Mweru tilapia native to the Upper Congo, but looking at the haplotype network (Fig. 2c) we find that all sequenced specimens of C. tilapia belong to an introduced strain, even the ones on native hosts. This is the first record of a ‘cryptic invasion’ that we propose. Whether the native strain of C. tilapia persists or is completely replaced on native hosts in the Congo Basin is unknown as we did not find the native strain.

Co-introduced parasites of Nile tilapia

The COI haplotype networks constructed for the monogenean parasites visualize the high diversity for each of the three species. There are many unique haplotypes, but with some notable exceptions. For example, The Lower and Middle Congo share a haplotype for C. tilapia, but this could possibly be explained by natural gene flow, given the connectivity of both sites despite the large distance. More striking is the sharing of parasite haplotypes between Madagascar and Congo, which are geographically far apart. Specimens of C.thurstonae from Madagascar appeared identical to a specimen from Upper Congo (Fig. 2b) whilst Madagascar and the Lower Congo also share identical C. tilapia haplotypes (Fig. 2c). Also, the C.thurstonae haplotype from Upper Congo that is identical to the one from Madagascar is highly divergent from specimens collected in the Lower and Middle Congo (Fig. 2b). This apparent lack of isolation by distance (IBD) typically reflects recent introduction events, which blurs geographic signals, as found in the study of Hayward et al. (2001). In that study, the monogenean eel parasite G. anguillae had identical rDNA sequences (ITS-1, 5.8S, ITS-2) in North America, Europe and Australia as a result of live eel trade that started forty to fifty years ago (Hayward et al., 2001). Similarly, Gyrodactylus cichlidarum Paperna, 1968 was co-introduced into Mexico with Nile tilapia after fish introductions started in the 1940s (García-Vásquez et al., 2017). The ITS-1 sequences of G. cichlidarum specimens that spilled over to Mexican poeciliids were almost completely identical to G. cichlidarum from Nile tilapia in Ghana (García-Vásquez et al., 2017). Our results, therefore, strongly point to an identical or geographically close source of introduction of Nile tilapia in Madagascar and Nile tilapia in the Upper and Lower Congo.

The high haplotype diversity of C. sclerosus within the Upper Congo (Fig. 2a) can partly be explained by the sampling bias (relatively more specimens were sequenced from this locality), but the divergent haplotypes strongly suggest that multiple introductions have taken place in this area, from different geographic source populations. Similarly, also C. tilapia and C.thurstonae show high haplotype diversity. For C. tilapia, it appears that the introduced populations in Congo show a higher diversity compared to the population of C. tilapia on native Nile tilapia in Burundi. Even though more samples are needed to validate these trends, our results appear to refute our initial hypothesis that introduced parasite populations suffer bottlenecks.
Similar scenarios have been described for other biological invasions, where introduced populations could maintain a high diversity because of multiple introductions from different source populations (Genton et al., 2005; Kolbe et al., 2004), sometimes followed by intraspecific hybridization (Rosenthal et al., 2008).

**Marker performance and barcoding gap**

Based on our histograms (Fig. 4), we find a significant ($p < 0.05$) barcoding gap for COI at 15% (Fig. 4a) but none for 28S, 18S or ITS-1 (Fig. 4b–d). Visually, there is a second gap between 7 and 11% for COI (Fig. 4a) but this was not found significant by ASAP. It should be noted that our fragment of the COI gene covers just less than a quarter of the total COI gene and constitutes the most variable part (unpublished data). Additionally, the COI dataset itself was the smallest of the four markers because hardly any references were available on GenBank and the amplification success of COI was lower than that of the rDNA markers. Whether 15% is representative for *Cichlidogyrus/Scutogyrus* should be investigated in the future by including more species.

We can find some clues in the literature to a possible barcoding gap for the rDNA markers, since we did not find a significant one within our dataset. Within *Cichlidogyrus* from Lake Tanganyika, Rahmouni et al. (2022) found

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**Fig. 4** Histogram of K2P model-corrected distances for COI, 18S, ITS-1 and 28S made with ASAP. Red line indicates a significant ($P < 0.05$) barcoding gap. $X$-axis represents the genetic distance; $Y$-axis the number of distances.
have interspecific variation < 6% for COI and around 1% for Dactylogyridae: Monogenea), which infect siluriforms, representatives of the three rDNA fragments are higher in our study. Repre-

sentatives of Trinigyrus Hanek, Molnar and Fernando, 1974 (Dactylogyridae: Monogenea), which infect siluriforms, have interspecific variation < 6% for COI and around 1% for 28S (Franceschini et al., 2020). In Dactylogyridae Diesing, 1850 (Dactylogyridae: Monogenea), which infect European cyprinids, the cut-off value was set at 1.4% for a fragment consisting of partial 18S, complete ITS-1 and partial 5.8S (Šimková et al., 2004). Rahmouni, Řehulková, et al. (2017) found 1% for 28S, 0.4% for 18S and 4.3% for ITS-1 of Dac-
tylogyrus parasitizing North-African congeneric cyprinids. From all these, we learn that the barcoding gap for 28S may be around 1% and for 18S below 1%. For ITS-1, this is likely higher than 1% but most probably lower than the 15%, which we found for COI of the included species of Cichlidogyrus.

For 28S sequences specifically, we observe that they can be identical over large geographic distances (C. thurstonae, C. sclerosus and C. falcifer) between introduced and native populations (C. tilapiae) and even between species (C. berradae and C. yanni; Scutogyrus gravivaginus (Paperna & Thurston 1969), S. baillonii (Pariselle & Euzet, 1995) and S. longicornis (Paperna & Thurston, 1969) (Addendum 4). However, whether 28S can be identical between species of Cichlidogyrus/Scutogyrus is uncertain as the obtained references from GenBank can be morphologically misidentified. New sequences of C. berradae, C. yanni, S. gravivaginus, S. longicornis and S. baillonii are needed to verify this. In other groups of closely related monogeneans, the rDNA fragments can be conserved also, between different hosts (Kmentová et al., 2016a, b) or between closely related species (Marchiori et al., 2015). Therefore, there is potential for the rDNA fragments to be used for species delineation in Cichlidogyrus/Scutogyrus, as shown for Gyrodactylus spp. (Matějusková et al., 2001; Mendoza-Palmero et al., 2019) and Dactylogyridae spp. (Benovics et al., 2018). However, at this time, we need to include other methods such as bPTP to interpret results from the rDNA fragments about species status. What we can conclude so far is that the conservative rDNA fragments (18S and 28S) are good for constructing higher-level phylogenies and the COI fragment is suited for population level studies and species delineation, which has also been stated in previous studies.

Phylogenetic relationships among native and introduced tilapia parasites and evaluation of species status

Previous phylogenetic studies showed that Scutogyrus forms a monophyletic group within Cichlidogyrus, rendering Cichlidogyrus paraphyletic (Mendlová & Šimková, 2014; Mendlová et al., 2012; Pouyaud et al., 2006; Wu et al., 2007). Representatives of Cichlidogyrus formed several well-supported monophyletic groups, but the relation between some of these groups were unresolved (Mendlová & Šimková, 2014; Mendlová et al., 2012; Pouyaud et al., 2006; Wu et al., 2007). We focus on and add species from the Congo Basin, which were until now sparsely represented in phylogenetic studies of Cichlidogyrus. Our results largely correspond with the previously published analyses on Cichlidogyrus/Scutogyrus (Mendlová & Šimková, 2014; Mendlová et al., 2012; Pouyaud et al., 2006; Wu et al., 2007).

Cichlidogyrus halli forms a species complex (see Jorissen et al., 2018a) supported by molecular data in the present study. ASAP suggests the “halli” group to consist of at least three species: firstly, the native specimen from Burundi, secondly the native specimens from Mweru tilapia from Upper Congo together with the specimen from the O. niloticus × mweruensis hybrid from Upper Congo, and thirdly all introduced specimens of C. halli. The genetic distances within the “halli” group are indeed large (2.1% for 28S; 3.2% for 18S; 10.1% for ITS-1 and 36% for COI). This variation is higher than all other intra/interspecific boundaries stated above. The bPTP method is inconclusive for the rDNA markers, where C. halli is divided in six species, including morphotype 2 and C. cf. halli ‘Burundi’, but the support for these divisions is very low. The divisions in COI are better supported and correspond with ASAP.

Morphotype 2 of C. halli (sensu Jorissen et al., 2018a) from Upper Congo, as drawn and discussed by Jorissen et al. (2018a), corresponds in locality and host species with C. halli ex O. mweruensis on the tree (Fig. 3). Therefore, we suggest that morphotype 2 should be elevated to species level. Similarly, the specimens of C. cf. halli ‘Burundi’ from Lake Tanganyika, Burundi, belong to the third species within C. halli, as suggested by ASAP. From the same Burundese population, we found a specimen of C. cf. halli ‘Burundi’ with elongated and thickened hooklets pair I compared to C. halli (Addendum 10). However, we strongly believe that the species delineation within the “halli” group should be based on morphology and genetics together. Therefore, new morphological material is needed to resolve this. For species within Cichlidogyrus/Scutogyrus, the genital sclerites are important for species identification (see diagnosis in Pariselle & Euzet, 2009). Therefore, for our study, we decided to only keep the body part with the genital sclerites and use the body part with the haptor for genetic analysis (Jorissen et al., 2018a). However, recent work on Kapentagyrus and Cichlidogyrus shows that closely related species might first diverge in haptor morphology before genital sclerites (Kmentová et al., 2016a; Messu Mandeng et al., 2015). This implicates the evolution of these parasites is related strongly.
to microhabitat (attachment site) and host species (Messu Mandeng et al., 2015, Gobbin et al., 2020). We conclude that morphological features of the haptor are important in this complex for species delimitation.

*Cichlidogyrus zambezensis* and *C. papernastrema* together form a clade. However, both species belong to different groups within the genus based on the morphology of the haptor hooklets. *Cichlidogyrus zambezensis* has seven pairs of small hooklets (group A sensu Vignon et al., 2011), whilst in *C. papernastrema* the first pair is thick and elongated (group B sensu Vignon et al., 2011). Pariselle and Euzet (2003) suggested a division of species of *Cichlidogyrus* in three groups based on the morphology of haptor hooklets (uncini in the source). Additionally, Vignon et al. (2011) found a high congruence between these morphological groups and the molecular phylogeny, meaning that hooklet morphology is phylogenetically constrained. However, our result suggests that the division between groups A and B might not be supported by phylogenies. This is possible because Pariselle and Euzet (2003) and Vignon et al. (2011) included a subset of specimens in their analyses. Vignon et al. (2011) even omitted *C. arthracanthus* from their analysis because it did not fit any of the three groupings. In conclusion, this could mean that firstly, haptor morphology is not as phylogenetically constrained as previously thought (see the ‘halli’ group above) and secondly, that the division in three groupings – whilst useful for morphological identification (see Pariselle & Euzet, 2009) – does not cover the morphological evolution of the haptor within *Cichlidogyrus* fully.

Both *C. papernastrema* and *C. zambezensis* have a copulatory tube with a bulbous thickening in the middle and this could be a synapomorphy for the “papernastrema” group instead of characters of haptor morphology. Other species with a bulbous thickening of the copulatory tube and thus possibly belonging to this group are *Cichlidogyrus halinus* Paperna 1969, *Cichlidogyrus sanjeani* Pariselle and Euzet 1997, *Cichlidogyrus philander* (Douéllou, 1993), *Cichlidogyrus bulbophallus* Geraerts and Muterezi Bukinga 2020, *Cichlidogyrus pseudozambezensis* Geraerts and Muterezi Bukianga 2020 and *Cichlidogyrus ranula* Geraerts and Muterezi Bukianga 2020. Within these candidate species are several representatives from haplochromine cichlids and others from southern Africa. *Cichlidogyrus zambezensis* is monophyletic, but we observe variation between specimens of different host species. Douéllou (1993) reports infraspecific morphological variation between specimens from different hosts in *C. zambezensis*. *Cichlidogyrus zambezensis* is known from four cichlid hosts, belonging to three cichlid lineages (Douéllou, 1993; Jorissen et al., 2018a; Vanhove et al., 2013). Additionally, the bPTP analysis of COI splits our samples of *C. zambezensis* and the reference as different species. Therefore, *C. zambezensis* is in need of further study and might consist of multiple species. The monophyly of *C. papernastrema* is not supported. Even more, the genetic distance between the two specimens of *C. papernastrema* is larger than between this species and *C. zambezensis* and above 1% for 28S and 15% for COI. Therefore, it is likely that both specimens belong to different species. Jorissen et al., (2018a, 2018b) redescribed *C. papernastrema* and noted large variation in thickness of the copulatory tube between specimens. It would be worthwhile to check whether this variation is a good diagnostic character to delineate species in tandem with genetic distances.

In the “tiberianus” group, *C. tiberianus* from Bangweulu-Mweru does not cluster with the reference sequence from Senegal (Mendlová et al., 2012) and the bPTP analysis suggests both sequences belong to different species. *Cichlidogyrus tiberianus* infects representatives of *Coptodon* ranging from Senegal to Zimbabwe (Douéllou, 1993; Jorissen et al., 2018a; Mendlová et al., 2012; Pariselle & Euzet, 1995, 1996, 2009). This is a native range of over 7000 km, spanning different ichthyographic provinces and basins. The genetic distances between *C. tiberianus* of Senegal and Upper Congo are above 1% for 28S and well below 1% for 18S. *Cichlidogyrus tiberianus* requires a species status re-evaluation backed by genetic data from across its native range and different host species. Fannes et al. (2017) used SEM to investigate the sclerotized parts of *C. dossoui* and *C. tiberianus* from Upper Congo because both species are morphologically quite similar and share hosts. The COI genetic distances are smaller between *C. tiberianus* and *C. dossoui* from Upper Congo than within *C. tiberianus*. Therefore, it is not surprising that *C. dossoui* from Bangweulu-Mweru appears as the sister species to *C. tiberianus* from Bangweulu-Mweru.

Furthermore, in the “tiberianus” group, *C. ergensi* is situated within *C.thurstonae*, but without support (53 posterior probability and not supported in the ML analysis), thus we do not make inferences to this result. All species in the “tiberianus” group belong to group C based on the morphology of the haptor hooklets (Pariselle & Euzet, 2003; Vignon et al., 2011), except for *C. arthracanthus* and *C. sp. 2*, which fall outside of the classification in three main groups. Here again, the division by Pariselle and Euzet (2003) is not completely supported.

*Cichlidogyrus cirratus* was found to be monophyletic (100 posterior probability and 90 bootstrap support value, Fig. 3). However, the branch lengths within *C. cirratus* are much longer than for example between the different species of *Scutogyrus*. The genetic distance between *C. cirratus* from Bangweulu-Mweru and Senegal is 0.5% 18S, 9.8% ITS-1 and 2.8% for 28S (Addenda 2, 4, 5). This indicates that our samples might represent two separate species. It is also debated whether *C. cirratus* and *C. mbirizei* Muterezi Bukinga, Vanhove, Van Steenberge, Pariselle, 2012, are conspecific (Zhang et al., 2019). Scanning electron microscopy revealed that the distinguishing characters between *C. cirratus* and *C. mbirizei* (Muterezi Bukinga et al.,
2012) on specimens of *C. cirratus* from China (introduced) could be transformed by turning the specimens over (Zhang et al., 2019). In conclusion, we deem it likely that *C. cirratus* and possibly *C. mbirizei* consist of multiple species and that this should be investigated further genetically. Subsequently, an evaluation of the morphological characters within *C. cirratus* and *C. mbirizei* is needed.

In *C. tilapiae*, the reference sequence from Senegal appears basal to all other specimens of native and introduced hosts in the Congo Basin (Fig. 3). We do not find any evidence to contest the species status of *C. tilapiae* as opposed to Pouyaud et al. (2006) who suggested it is a species complex based on ribosomal DNA.

**Conclusion**

Our results strongly point to an identical or geographically similar source of introduction of Nile tilapia in Congo and Madagascar, as both regions share identical COI parasite haplotypes. The high haplotype diversity of *C. sclerosus* within the Upper Congo strongly suggests that multiple introductions have taken place in this area, from different geographic source populations. This refutes our initial hypothesis that introduced parasite populations would suffer genetic bottlenecks. Also, the strong differentiation between parasites from the Upper Congo compared to those from the Middle and Lower Congo suggests different sources of introduction for the latter two regions. Finally, shared parasite haplotypes between the Lower and Middle Congo suggest that natural gene flow is possible at this scale, or it could point to a shared source of introduction or human-assisted dispersal.

Considering the genetic markers, we find a barcoding gap at 15% variation for COI, but not for the other markers. This value is quite high compared to other dactylogyrid monogeneans, but it aligns with the only other available study within *Cichlidogyrus*, which suggests a barcoding gap above 12% (Rahmouni et al., 2022). However, we want to stress that sequences of more species are needed to have a more complete overview of the phylogeny of the group and to estimate a barcoding gap more precisely. Based on our study, we suggest the need of taxonomic re-evaluation for *C. halli*, *C. papernastrema*, *C. zambezensis*, *C. tiberianus* and *C. cirratus* as they all potentially represent at least two species. Additionally, within *C. halli*, we find that closely related species can first diverge in haptor morphology and later differentiate in the genital sclerites. Lastly, the grouping of *C. papernastrema* with *C. zambezensis*, *C. arthracanthus* and *C. sp. 2* within the “tiberianus” group shows that the division of the genus by Pariselle and Euzet (2003) based on haptor configuration does not explain the variation within the group fully and that this division is not always phylogenetically supported. We utter the need for a revision of morphological features corresponding with the larger clades in the phylogenetic tree of *Cichlidogyrus/Scutogyrus*.

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**Data availability** All data generated or analysed during this study is included in this manuscript and its supplementary files. All generated sequences can be found on GenBank (see Addendum 1).

**Declarations**

**Ethics approval** The field expedition and sampling in Burundi were approved by Ethics Committee of Masaryk University, approval number CZ01308. Fieldwork was carried out under permission 06/AR.ED./15 from the General Directorate for Fishery Resources and Fisheries, Ministry of Fisheries Resources and Fisheries of Madagascar, and mission statements 863/2014 (Faculté des Sciences Agronomiques, Université de Lubumbashi), C/075/2015/I.S.P./MBNGU/AUT. AC and AC/076/2015/I.S.P./MBNGU/AUT.AC.

**Conflict of interest** The authors declare no competing interests.
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