# 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. In previous morphological studies, we 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 investigated the co-introduced monogeneans of Nile tilapia genetically from three major parts of the Congo Basin; Upper, Middle and Lower Congo. We generated sequences of Congolese native and introduced monogeneans from native and introduced tilapias and evaluate their position in a phylogeny. Additionally, we generated sequences of the same species of monogeneans co-introduced with Nile tilapia in Madagascar and of a native population of Nile tilapia from Burundi. Our results confirm the co-introductions in the Congo. We found that co-introduced parasites are less genetically diverse than native ones, and that there was no geographical pattern between introduced populations. Furthermore, our COI haplotype networks suggest multiple introduction events of Nile tilapia into the Congo Basin. 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 intra/interspecific 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.


# Introduction

Human-mediated species translocations are ubiquitous and form one of the major challenges to global biodiversity today (Pimentel 2001 et al., 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* (Linneaus, 1758), is the most popular and makes up over 75% of the cultured tilapias in Sub-Saharan Africa (FAO 2017). Although Sub-Saharan Africa houses many native tilapias, others were introduced anyway for aquaculture purposes, such as Nile tilapia, which started being cultured after the Second World War in the Democratic Republic of the Congo (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 fish production increased steadily again to modern levels (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 (Trewavas 1983, Nyingi and Agnèse 2007, Firmat et al. 2013, Deines et al. 2014), stimulation of phytoplankton growth (Starling et al. 2002), predation on eggs of aquatic organisms (Alcaraz et al. 2015) and co-introduction of parasites (Soler-Jimenez et al. 2017, Jiménez-

García et al. 2001, Šimková et al. 2019, Zhang et al. 2019, Jorissen et al. 2020). 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), further increasing the extinction risk of native species. For example, co-introduced blood flukes in Spain spilled over from invasive turtles to native European pond turtles. The native pond turtles experienced increased mortality rates and loss of mobility in tail and limbs because of these newly acquired blood flukes (Iglesias et al. 2015).

Our 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 and Euzet 1995. They are monogenean flatworms (Dactylogyridea) that mainly infect the gills of African mainland cichlids (Pariselle and 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 7 of *Scutogyrus* have been described (WoRMS 2021) and DNA sequences of 31 and 3, 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 (Pouyaud et al. 2006; Wu et al. 2007; Mendlová et al. 2012; Mendlová and Šimková 2014). However, there is a lack of molecular markers for flatworms (Littlewood 2008, Moszczynska et al. 2009, Vanhove et al. 2013) and a lack of sequenced species. For example, only for five species within *Cichlidogyrus* and *Scutogyrus* partial or complete sequences of the more variable mitochondrial cytochrome *c* oxidase subunit I (COI) gene are available (Genbank accession numbers: KT037411.1, MG288510.1, MG970255.1, JQ038226.1, MG970257.1). 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 phylogenetic analyses. 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 and 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 *Cichlidogyrus cirratus* could also be underestimated as this species was recently reported in new areas and on new hosts (Zhang et al. 2019, Jorissen et al. 2020 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 geographical history of their hosts, as predicted by the magnifying glass concept (Nieberding and 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 catfish host species that currently live in allopatry (Barson et al. 2010). Also the lack of genetic variation can be informative on transmission routes (Hayward et al., 2003). *Gyrodactylus anguillae* Ergens, 1960 collected from eel populations from three different continents share identical ribosomal DNA sequences, as the result from recent live eel trade (Hayward et al., 2003).

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 include 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 sample 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 and 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 (Mayr 1942, Avise 1994). 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 & Methods

*Data collection*

A total of 146 specimens of parasites belonging to 16 described and one undescribed species, of which 15 of *Cichlidogyrus* and two of *Scutogyrus* were collected from 7 host species (Table 1). Samples were

collected 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. 2017a); and one to Madagascar in April 2016 (Šimková et al. 2019). Samples 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. 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 entomological 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 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 x (oil immersion, 10 x ocular) under differential interference contrast, with an AxioCamMR3 camera and AxioVision v.4.2.8 software. The posterior body part of the parasite was placed in an Eppendorf tube filled with 180µl of T1 buffer, Nucleospin Kit, Macherey Nagel, 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_XXX (see Addendum 1).

*Molecular analyses*

DNA was extracted using the Nucleospin kit (Macherey-Nagel). In the final step 60µl of elution buffer was added instead of 100µl. DNA extracts were stored at -18°C. We used the primers listed in Table 2 for the amplification of fragments of the mitochondrial cytochrome *c* oxidase subunit 1 (COI, 314bp), complete ITS-1+partial18S (702bp) and partial 28S (653bp). For COI, if initial amplification was unsuccessful we tried again with nested primers, resulting in the 314bp 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.). Successful amplifications were purified with EXOSAP-IT (Thermo Fisher) in a 5/2 (product/EXOSAP) ratio and incubated at 37 °C for 4 minutes followed by 80 °C for 1 minute. 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, of which 12 we present the first sequences. All sequences are submitted to Genebank and accession numbers are available upon acceptance. 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 (Pouyaud et al. 2006;

Wu et al. 2007; Mendlová et al. 2010; Mendlová and Šimková 2014). 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). From some specimens either 28S or 18S+ITS-1 were successfully amplified and these sequences were aligned with the concatenated sequences to include the maximum number of species and specimens. A total of 30 reference sequences of congeners were obtained from GenBank (see Table 4). JModelTest2 was used to determine the most appropriate model of nucleotide evolution (Darriba et al. 2012) with default parameters, employing the Akaike Information Criterium (AIC). The GTR+G+I model was found as the optimal model for the concatenated dataset with gamma being 0.65 and the proportion of invariant sites 0.29. For the COI dataset TIM1+G+I was the optimal model, but we used GTR+G+I as it was the most similar model being available in MrBayes and RAxMLHPC2. Gamma was 0.2 and the proportion of invariant sites 0.2. Maximum Likelihood (ML) analyses were carried out with RAxMLHPC2 (Stamatakis 2014) via the CIPRES webserver with the GTR+G+I model and 1000 iterations. Branch support was estimated by bootstrapping with 1000 replicates. The Bayesian inference of phylogeny (BI) was performed in MrBayes 3.2.7 (Ronquist & Huelsenbeck 2003) with 4.000.000 generations sampled every 1000 generations and four chains (three cold and one hot). One fourth of the topologies were discarded as burn-in as the standard deviation of split frequencies was below 0.01 for both the concatenated dataset and for the COI dataset. Figtree 1.4.4. (Rambaut 2018) and TreeGraph 2 (Stöver and Muller 2010) were used to visualize and edit the trees. Additionally, we identified phylospecies and evolutionary species with a coalescent tree-based Poisson tree process model and its Bayesian implementation (bPTP, Zhang et al. 2013) on the web server (http://species.h-its.org/ptp/) under default parameters and 0.2 burnin. Trees will be available on TreeBASE upon acceptance.

*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. For *C. halli* the genetic distance between specimens was too large for a median-joining haplotype network, see results. 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 will be available on TreeBASE upon acceptance.

# 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 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). The COI dataset included 15 morphospecies and was divided in 18 species by ASAP and between 22–28 by bPTP. The concatenated dataset included 37 morphospecies and was divided in 34–38 species by ASAP and between 22–47 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 instraspecific 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* Ergens 1981 (0.3–3.4% COI, 13 polymorphic sites, Pi=0.01706) show more modest variation (Addendum 3, 6).

For *C. sclerosus*, each locality was characterized by a unique parasite haplotype, with the highest variation within Upper Congo. Only one locality in Upper Congo had one non-synonymous mutation. 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 from Upper and Middle Congo cluster furthest apart, each separated by at least five 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* haplotypes. 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 topology of 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. We named each of these groups after the oldest described species within it, following Pouyaud et al. (2006).

The “halli” group has a reference sequence of *C. halli* from Senegal (Table 4) 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 native specimens of Nile tilapia from Burundi, Mweru tilapia 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 *Cichlidogyrus 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 dossoui* 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 distance of *C. tiberianus* between Upper Congo and Senegal is 0.2–0.5% 18S, 8.5–9% ITS-1, 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 and Euzet 1995 and *C. gallus* Pariselle and Euzet 1995 as sister species and the C. *bilongi* Pariselle and Euzet 1994 and *C. flexicolpos* Pariselle and 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 0–1.7% 18S, 0.7–2.7% ITS-1, 0–1% 28S, 0.3–3.4% COI), with no apparent structure other than that the reference sequence from Senegal is the earliest diverging (Table 4).

The "longicirrus" group is split up in three clades and contains species that infect *Hemichromis* spp. (Dossou and Birgi 1982, Pariselle and 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. amphoratus* Pariselle and Euzet 1995. The recently discovered *C. consobrini* Jorissen, Pariselle and Euzet 2018 clusters with *C. irenae* Gillardin, Vanhove, Pariselle, Snoeks, Huyse and Volckaert 2011 and *C. casuarinus* Pariselle, Muterezi Bukinga and Vanhove 2015. These three species have not been considered closely related to each other based before (Pariselle et al. 2015, Jorissen et al. 2018a). 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, Pariselle et al. 2015, Kmentová et al. 2016), whilst *C. consobrini* is native to the Upper Congo and found on *Sargochromis mellandi* and *Orthochromis katumbii* Schedel, Vreven, Manda, Abwe, Manda, Schliewen, 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* Douëllou 1993, *C. digitatus* Dossou 1982 and *C. yanni* Pariselle and 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

In this study, we aim to compare haplotypes of monogenean parasites from introduced populations of Nile tilapia from three regions of the Congo Basin and one from Madagascar. In addition, we generated haplotypes of the parasites from a native population of Nile tilapia in Burundi. We expect the introduced parasite populations from Congo and Madagascar to be low in genetic variation. Additionally, if they share many of the same haplotypes they might have a common introduction origin. Our second goal is to evaluate the species status of co-introduced monogeneans of Nile tilapia through four molecular markers. Lastly, this study aims to add sequences of Congolese native and introduced species (Table 1, addendum 1) and evaluate the position of these previously unsequenced species in phylogenetic analyses.

# Co-introduced parasites of Nile tilapia

From the COI haplotype networks it can be inferred that the variation within countries is sometimes higher than between countries. For example, *C. thurstonae* specimens from Upper Congo and Madagascar are identical but they are different from specimens collected in the Lower and Middle Congo (Fig. 2b). This lack of isolation by distance (IBD) typically reflects recent introduction events, which blurs geographic signals (Hayward et al. 2001). For example, *Gyrodactylus anguillae* Ergens, 1960 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 similar source of introduction of Nile tilapia in the Upper Congo and Madagascar, and a different source (or sources) for the other Congolese regions.

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 it also strongly suggests that multiple introductions have taken place in this area, from different geographic source populations. Indeed, the variation is higher compared to that of the population of *C. sclerosus* on native Nile tilapia in Burundi. Aquaculture of Nile tilapia in the DRC started in the Lubumbashi area, Upper Congo, in the late 1940s (Micha 2013). However, there has also been a period of very low aquaculture activity until 1996 (Toguyeni 2004) and it is not known whether Nile tilapia or its parasites from before 1996 still persist in the basin. In any case, our results 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 (Kolbe et al., 2004; Genton et al., 2005), sometimes followed by intraspecific hybridization (Rosenthal et al., 2008).

Finally, the *C. tilapiae* haplotypes shared by the Lower and Middle Congo suggest that natural gene flow is possible at this scale, or it could point to a shared introduction source (Fig. 2c).

# 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 7–11% 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.

As for the other markers we can find some clues in the literature to a possible barcoding gap. Within *Cichlidogyrus*, Rahmouni et al. (2021) found intraspecific variation of ITS-1 in *C. nshomboi* Muterezi Bukinga, Vanhove, Van Steenberge and Pariselle 2012, up to 1.1% and interspecific variation starting at 3.5%. The 18S sequences of *C. nshomboi* and *Cichlidogyrus casuarinus* Pariselle, Muterezi Bukinga, Van Steenberge, Vanhove 2015, were identical and the 28S sequences differed 0.13% between the species. COI intraspecific variation within *C. nshomboi* amounted to 12.2%. This distance for COI roughly corresponds with what we find in our dataset, but the observed distances for the three rDNA fragments are higher in our study. Representatives of *Trinigyrus* (Dactylogyridae: Monogenea), which infect siluriforms, have interspecific variation <6% for COI and around 1% for 28S (Franceschini et al. 2020). In *Dactylogyrus* (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 et al. (2017b) found 1% for 28S; 0.4% for 18S and 4.3% for ITS-1 of *Dactylogyrus* 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 probably lower than the 15%, which we found for COI of the included species of *Cichlidogyrus*.

Furthermore, 28S sequences 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. bailloni* 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. bailloni* are needed to verify this. Conversely, rDNA fragments can appear conserved

in other closely related monogeneans. Kmentová et al. 2016a found identical 28S and 18S+ITS-1 sequences of *Cichlidogyrus casuarinus* from Lake Tanganyika from different hosts (*Bathybates* and *Hemibates* Regan 1920), although intraspecific morphological variation was observed. The COI fragments, on the other hand, were highly differentiated, with distances between haplotypes reaching 4.7% (Kmentová et al. 2016a). For *Kapentagyrus tanganicanus* Kmentová, Gelnar and Vanhove 2018 infecting Lake Tanganyika sardines, morphological intraspecific variation was found based on host species (phenotypic adaptation for attachment), COI fragment and possibly seasonality, but this was not reflected in the 28S or 18S+ITS-1 sequences (Kmentová et al. 2018). Benovics et al. (2017) observed identical 18S+ITS-1 sequences between *Dactylogyrus vastator* Nybelin 1924, from the Po River, Italy and Šuica River, Bosnia and Herzegovina. Marchiori et al. (2015) found identical 18S sequences for different, but closely related species of *Ligophorus* infecting Mugilidae in Brazil (Dactylogyridae: Monogenea), whilst 28S differed 0.2% and ITS-1 0.4%. In conclusion, rDNA sequences might be conserved in conspecific monogeneans over large geographic distances and between conspecifics from different host species also, but it is unlikely for different species to have identical rDNA sequences. Therefore, there is potential for the rDNA fragments to be used for species delineation in *Cichlidogyrus*/ *Scutogyrus*, as shown for *Gyrodactylus* spp. (Matejusová et al. 2001, Mendoza-Palmero et al. 2019) and *Dactylogyrus* 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 the rDNA fragments are good at right now are constructing higher-level phylogenies and the COI-fragment is suited for population level studies and species delineation.

# 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 (Pouyaud et al. 2006; Wu et al. 2007; Mendlová et al. 2012; Mendlová and Šimková 2014). Representatives of *Cichlidogyrus* formed several well-supported monophyletic groups, but the relation between some of these groups were unresolved (Pouyaud et al. 2006; Wu et al. 2007; Mendlová et al. 2012; Mendlová and Šimková 2014). 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* (Pouyaud et al. 2006; Wu et al. 2007; Mendlová et al. 2012; Mendlová and Šimková 2014).

*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* x *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 a 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* (Fig. 5). However, we strongly feel 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 (Messu Mandeng et al. 2015, Kmentová et al. 2016a). 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 *Cichlidogyrus papernastrema* together form a clade. However, both species belong to different groups within the genus based on the morphology of the haptoral 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 haptoral hooklets (uncinuli 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, the well-supported clade including *C. papernastrema* and *C. zambezensis* has a representative of group A and group B. This suggests this group division might not be supported by phylogenies. Pariselle and Euzet (2003) and Vignon et al. (2011) included a subset of species 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 and 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* Douëllou 1993, *Cichlidogyrus bulbophallus* Geraerts and Muterezi Bukinga 2020,

*Cichlidogyrus pseudozambezensis* Geraerts and Muterezi Bukinga 2020 and *Cichlidogyrus ranula* Geraerts and Muterezi Bukinga 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. Douëllou (1993) reports intraspecific morphological variation between different hosts in *C. zambezensis*. *Cichlidogyrus zambezensis* is known from four cichlid hosts, belonging to three cichlid lineages (Douëllou 1993, Vanhove et al. 2013, Jorissen et al. 2018a). 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. (2018) 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). This species infects representatives of *Coptodon* ranging from Senegal to Zimbabwe (Douëllou 1993, Pariselle and Euzet 1995, 1996, 2009, Mendlová et al. 2012, Jorissen et al. 2018a). This is a native range of over 7000 km, including different ichthyographic provinces and bassins and it is, therefore, plausible that *C. tiberianus* might consist of multiple species within this range. Additionally, the genetic distances between *C. tiberianus* of Senegal and Upper Congo is above 1% for 28S which also point to multiple species; however the distance in 18S is well below 1%. 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. *Cichlidogyrus tiberianus* requires a species status re-evaluation backed by genetic data from across its native range and different host species.

Furthermore, in the “tiberianus” group, *C. ergensi* is situated within *C. thurstonae*, but with low 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 haptoral hooklets (Pariselle and 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 *Cichlidogyrus 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.

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. 2021). 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-evalution 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 et al. 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*.

# Declarations

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## Statements and declarations

The authors have no competing interests to declare that are relevant to the content of this article.

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request. All input data is available through public repositories (see material and methods).

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# Tables

***Table 1.*** *Overview of the sampling design with parasite species listed per host species and per region. Last column depicts "the number of sequenced specimens: 18S+ITS-1/28S/COI sequences".*

| Host species | Generic name | Region | Introduced/native | Parasite species | # |
|---|---|---|---|---|---|
| *Coptodon rendalli* (Boulenger, 1897) | Redbreast tilapia | Lower Congo | introduced | *C. berradae* | 4:1/4/0 |
| | | Upper Congo | native | *C. dossoui*, | 1:1/1/1 |
| | | | | *C. quaestio*, | 2:0/2/0 |
| | | | | *C. tiberianus* | 2:1/2/0 |
| | | Madagascar | introduced | *C.* sp.2 | 2:0/2/0 |
| *Hemichromis stellifer* Loiselle, 1979 | / | Lower Congo | native | *C. falcifer*, | 1:1/1/1 |
| | | | | *C. polyenso* | 1:0/1/0 |
| *Oreochromis mweruensis* Trewavas, 1983 | Mweru tilapia | Upper Congo | native | *C. cirratus*, | 2:1/2/0 |
| | | | | *C. halli* morphotype 2, | 2:0/2/0 |
| | | | | | 1:0/1/0 |
| | | | | | 1:1/1/0 |
| | | | | *C. papernastrema*, | 1:0/1/0 |
| | | | | *C. tilapiae*, | |
| | | | | *S. gravivaginus* | |
| *Oreochromis niloticus* (Linnaeus, 1758) | Nile tilapia | Lake Tanganyika, Burundi | native | *C.* cf. *halli* 'Burundi', | 1:1/1/1 |
| | | | | *C. tilapiae* | 6:0/3/3 |
| | | Lower Congo | introduced | *C. halli*, | 14:6/9/6 |
| | | | | *C. sclerosus*, | 3:0/1/3 |
| | | | | *C. thurstonae*, | 34:5/17/16 |
| | | | | *C. tilapiae* | 22:5/13/10 |
| | | Middle Congo | introduced | *C. halli*, | 1:0/0/1 |
| | | | | *C. sclerosus*, | 1:0/1/1 |
| | | | | *C. thurstonae*, | 1:0/1/1 |
| | | | | *C. tilapiae* | 3:0/0/3 |
| | | Upper Congo | introduced | *C. halli*, | 8:0/0/8 |
| | | | | *C. sclerosus*, | 6:0/0/6 |
| | | | | *C. thurstonae* | 1:0/0/1 |
| | | Madagascar | introduced | *C. halli*, | 5:2/3/1 |
| | | | | *C. sclerosus*, | 1:0/0/1 |
| | | | | *C. thurstonae*, | 4:0/2/2 |
| | | | | *C. tilapiae*, | 2:1/1/2 |
| | | | | *S. longicornis* | 1:0/1/0 |
| *Sargochromis mellandi* (Boulenger, 1905) | Snaileater | Upper Congo | native | *C. consobrini*, | 2:1/2/0 |
| | | | | *C. zambezensis* | 1:1/1/0 |
| *Serranochromis macrocephalus* (Boulenger, 1899) | Purpleface largemouth | Middle Zambezi, Zimbabwe | native | *C. zambezensis* | 3:1/3/0 |
| *Tilapia sparrmanii* Smith, 1840 | Banded tilapia | Upper Congo | native | *C. dossoui*, | 1:0/0/1 |
| | | | | *C. papernastrema* | 4:0/3/1 |

***ble 2.*** *List of primers used to amplify the gene fragments used in this study.*

| Gene | Primer | Direction | Sequence (5’-3’) | Reference |
|---|---|---|---|---|
| COI | ASmit1 | F | TTTTTTGGGCATCCTGAGGTTTAT | Littlewood et al. 1997 |
| COI | Cox1_Schisto_3 | R | TAATGCATMGGAAAAAAACA | Lockyer et al. 2003 |
| COI | ASmit2 (nested) | R | TAAAGAAAGAACATAATGAAAATG | Littlewood et al. 1997 |
| 18S+partialITS-1 | S1 | F | ATTCCGATAACGAACGAGACT | Sinappah et al. 2001 |
| 18S+partialITS-1 | IR8 | R | GCTAGCTGCGTTCTTCATCG | Šimková et al. 2003 |
| 28S | C1 | F | ACCCGCTGAATTTAAGCAT | Hassouna et al. 1984 |
| 28S | D2 | R | TGGTCCGTGTTTCAAGAC | Hassouna et al. 1984 |

***ble 3.*** *PCR conditions and master mix for the different molecular markers used in the phylogenetic analyses. Taq is Platinum Taq DNA polymerase by Invitrogen (PlatTaq).*

| Primer set | PCR condition | Mastermix |
|---|---|---|
| ASmit1 – Schisto3 (COI) | 94 °C: 3 min<br>94 °C: 30 s<br>44 °C: 30 s 39 x<br>72 °C: 60 s<br>72 °C: 7 min | 1X PCR Buffer<br>1.5 mM $MgCl_2$<br>0.2 mM dNTP mix<br>0.4 µM F-primer<br>0.6 µM R-primer<br>14.8 µl $ddH_2O$<br>1.2 µl Template<br>3 Units/reaction Taq |
| ASmit1 – ASmit2 (COI, nested PCR) | 94 °C: 3 min<br>94 °C: 30 s<br>50 °C: 30 s 39 x<br>72 °C: 60 s<br>72 °C: 7 min | 1X PCR Buffer<br>1.5 mM $MgCl_2$<br>0.2 mM dNTP mix<br>0.4 µM F-primer<br>0.4 µM R-primer<br>15 µl $ddH_2O$<br>1.2 µl Template<br>3 Units/reaction Taq |
| S1 – IR8 (18S + ITS-1) | 94 °C: 2 min<br>94 °C: 60 s<br>50 °C: 60 s 39 x<br>72 °C: 90 s<br>72 °C: 10 min | 1X PCR Buffer<br>1.5 mM $MgCl_2$<br>0.2 mM dNTP mix<br>0.4 µM F-primer<br>0.6 µM R-primer<br>14.8 µl $ddH_2O$<br>1.2 µl Template<br>3 Units/reaction Taq |
| C1 – D2 (28S) | 94 °C: 2 min<br>94 °C: 20 s<br>50 °C: 30 s 39 x<br>72 °C: 90 s<br>72 °C: 10 min | 1X PCR Buffer<br>1.5 mM $MgCl_2$<br>0.2 mM dNTP mix<br>0.4 µM F-primer<br>0.4 µM R-primer<br>15 µl $ddH_2O$<br>1.2 µl Template<br>3 Units/reaction Taq |

***ble 4.*** *Reference sequences of* Cichlidogyrus *species used in the phylogenetic analyses.*

| Parasite | Host | Genbank Accession 18S+ITS-1 | Genbank Accession 28S | Locality | Reference |
|---|---|---|---|---|---|
| *Cichlidogyrus acerbus* | *Sarotherodon galilaeus* | HE792780.2 | HQ010036.2 | Senegal | Mendlová et al. 2012, 2010 |
| *Cichlidogyrus aegypticus* | *Coptodon guineensis* | HE792781.1 | HQ010021.1 | Senegal | Mendlová et al. 2012, 2010 |
| *Cichlidogyrus agnesi* | *Coptodon guineensis* | AJ920286.1 | | Ebrie Lagoon, Ivory Coast | Pouyaud et al. 2006 |
| *Cichlidogyrus amphoratus* | *Coptodon guineensis* | HE792782.1 | HE792772.1 | Senegal | Mendlová et al. 2012 |
| *Cichlidogyrus bilongi* | *Coptodon guineensis* | AJ920287.1 | | Ebrie Lagoon, Ivory Coast | Pouyaud et al. 2006 |
| *Cichlidogyrus arthracantus* | *Coptodon guineensis* | HE792783.1 | HQ010022.1 | Senegal | Mendlová et al. 2012, 2010 |
| *Cichlidogyrus casuarinus* | *Bathybates fasciatus* | KX007788.1 | KX007814.1 | Uvira, Lake Tanganyika, DRC | Kmentová et al. 2016a |
| *Cichlidogyrus cirratus* | *Oreochromis niloticus* | HE792784.1 | HE792773.1 | Senegal | Mendlová et al. 2012 |
| *Cichlidogyrus cubitus* | *Coptodon guineensis* | HE792785.1 | HQ010037.1 | Senegal | Mendlová et al. 2012, 2010 |
| *Cichlidogyrus digitatus* | *Coptodon guineensis* | HE792786.1 | HQ010023.1 | Senegal | Mendlová et al. 2012, 2010 |
| *Cichlidogyrus douellouae* | *Sarotherodon galilaeus* | HE792787.1 | HE792774.1 | Senegal | Mendlová et al. 2012 |
| *Cichlidogyrus ergensi* | *Coptodon guineensis* | HE792788.1 | HQ010038.1 | Senegal | Mendlová et al. 2012, 2010 |
| *Cichlidogyrus falcifer* | *Hemichromis fasciatus* | HE792789.1 | HQ010024.1 | Senegal | Mendlová et al. 2012, 2010 |
| *Cichlidogyrus flexicolpos* | *Coptodon guineensis* | AJ920283.1 | | Ebrie Lagoon, Ivory Coast | Pouyaud et al. 2006 |
| *Cichlidogyrus gallus* | *Coptodon guineensis* | AJ920285.1 | | Ebrie Lagoon, Ivory Coast | Pouyaud et al. 2006 |
| *Cichlidogyrus halli* 1 | *Oreochromis niloticus* x *Oreochromis mweruensis* | MG973075.1 | MG973075.1 | Mweru-Luapula | Vanhove et al. 2018 |
| *Cichlidogyrus halli* 2 | *Sarotherodon galilaeus* | HE792790.1 | HQ010025.1 | Senegal | Mendlová et al. 2012, 2010 |
| *Cichlidogyrus halli* 3 | *Oreochromis niloticus* | MH767392 | MH767403.1 | Madagascar | Šimkov et al. 2019 |
| *Cichlidogyrus irenae* | *Gnathochromis pfefferi* | KT692939.1 | MH708145.1 | Mukuruka, Lake Tanganyika, Burundi | Kmentová et al. 2016b |
| *Cichlidogyrus longicirrus* | *Hemichromis fasciatus* | HE792791.1 | HQ010026.1 | Senegal | Mendlová et al. 2012, 2010 |
| *Cichlidogyrus njinei* | *Sarotherodon galilaeus* | HE792792.1 | HE792775.1 | Senegal | Mendlová et al. 2012 |
| *Cichlidogyrus pouyaudi* | *Tylochromis intermedius* | HE792793.1 | HQ010039.1 | Senegal | Mendlová et al. 2012, 2010 |
| *Cichlidogyrus sclerosus* | *Oreochromis niloticus* | MH767390.1 | MH767401.1 | National Park Ankafarantsika Madagascar | Šimkov et al. 2019 |
| *Cichlidogyrus thurstonae* | *Oreochromis niloticus* | MH767394.1 | MH767406.1 | Anjingo River, Antsohihy, Madagascar | Šimkov et al. 2019 |
| *Cichlidogyrus tiberianus* | *Coptodon guineensis* | HE792796.1 | HE792776.1 | Senegal | Mendlová et al. 2012 |
| *Cichlidogyrus tilapiae* | *Hemichromis fasciatus* | HE792797.1 | HQ010029.1 | Senegal | Mendlová et al. 2012, 2010 |
| *Cichlidogyrus yanni* | *Coptodon guineensis* | HE792798.1 | HE792777.1 | Senegal | Mendlová et al. 2012 |

| | | | | | |
|---|---|---|---|---|---|
| *Scutogyrus bailloni* | *Sarotherodon galilaeus* | HE792799.1 | HE792778.1 | Senegal | Mendlová et al. 2012 |
| *Scutogyrus longicornis* | *Oreochromis niloticus* | HE792800.1 | HQ010035.1 | Senegal | Mendlová et al. 2012, 2010 |
| *Scutogyrus minus* | *Sarotherodon melanotheron* | HE792801.1 | HE792779.1 | Senegal | Mendlová et al. 2012 |

# Figures

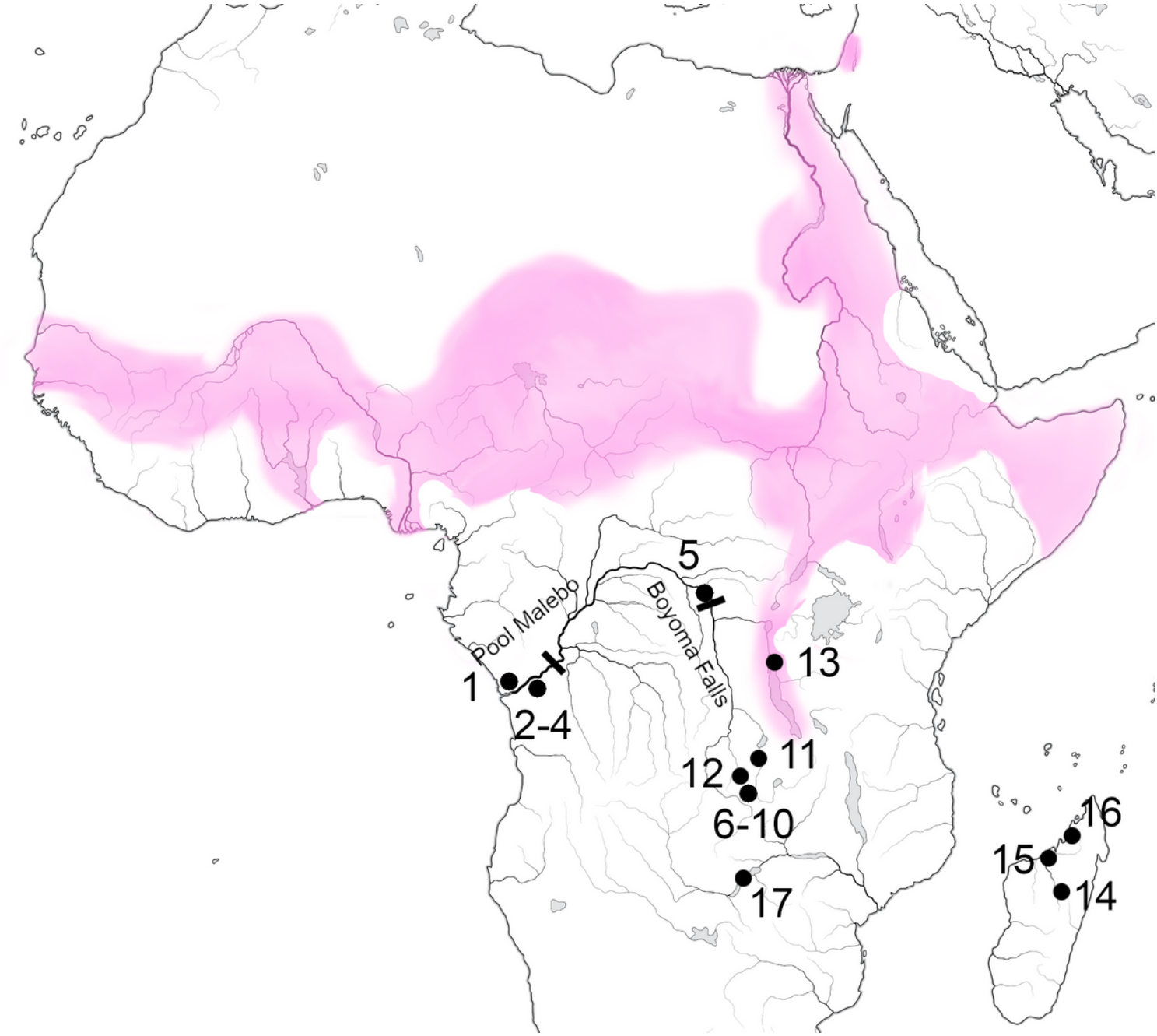
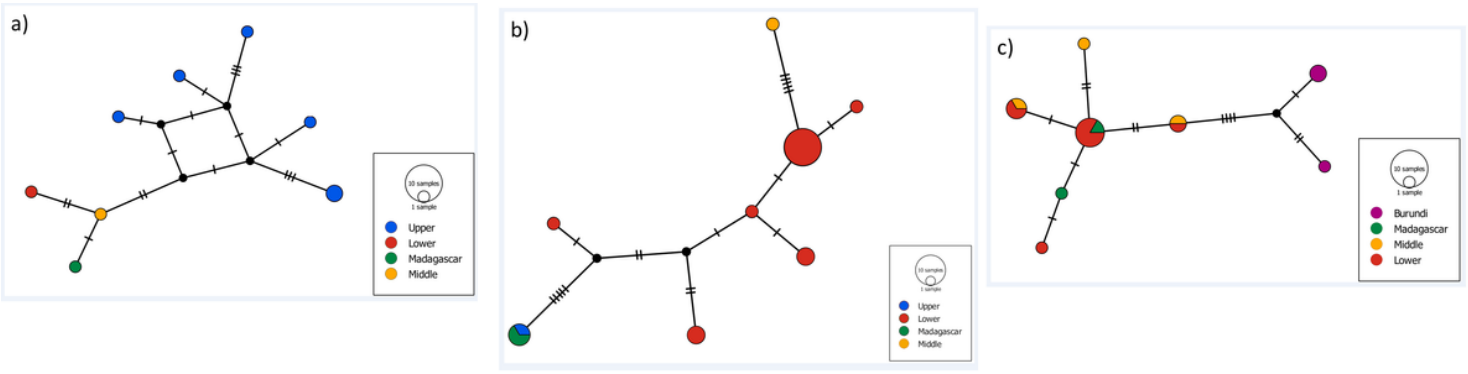


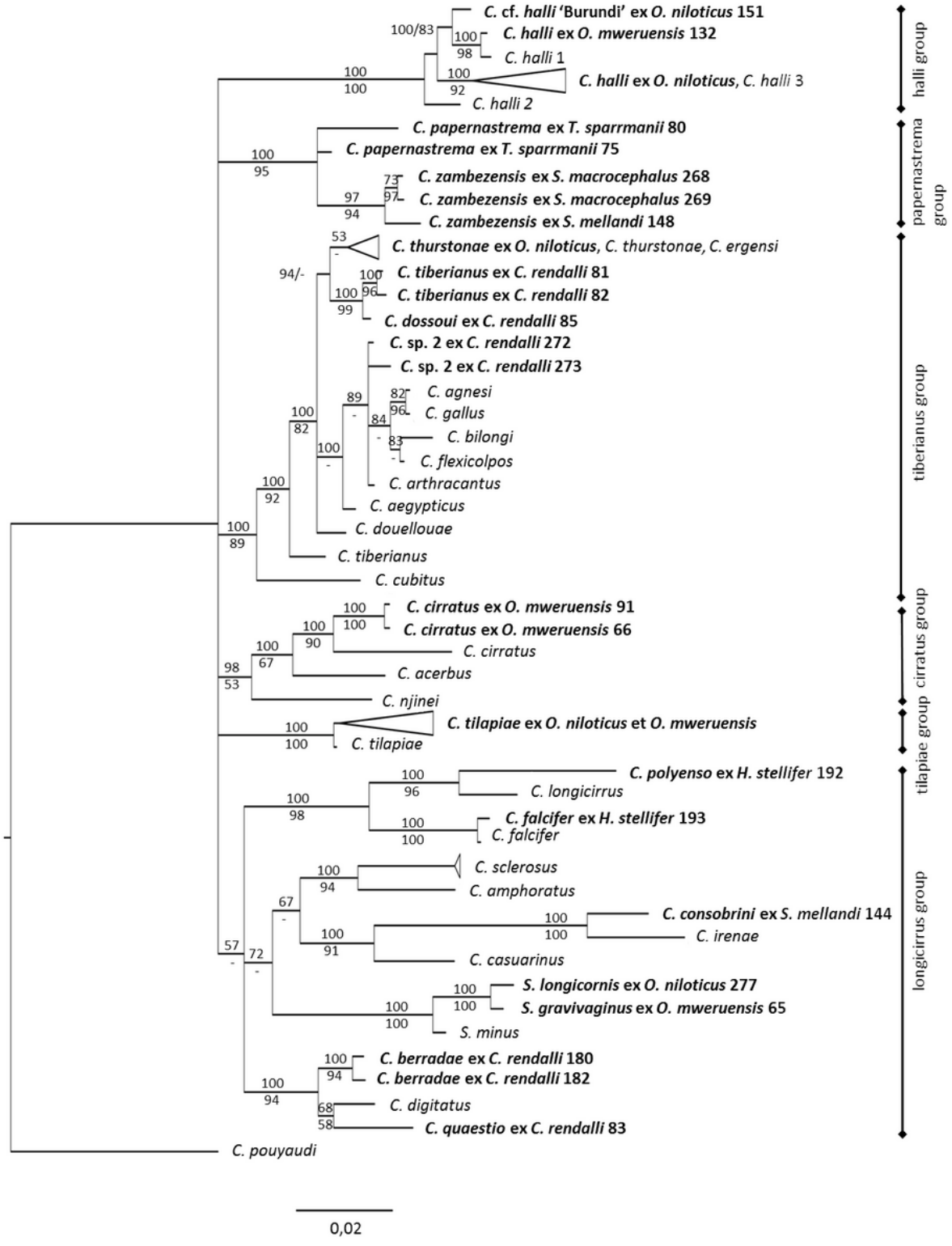

Figure 1

`Map of sampling localities with the native range of Nile tilapia in pink (Teugels and Thys van den Audenaerde 2003, Trewavas and Teugels 1991). Numbers of sampling localities correspond with Table 1. Localities 1–4 are from Lower Congo, 5 from Middle Congo and 6–12 from Upper Congo. Pool Malebo and Boyoma Falls divide the Congo in these three sections.




## Figure 2

Median-joining haplotype networks constructed using COI sequences (314 bp) for a) Cichlidogyrus sclerosus b) Cichlidogyrus thurstonae and c) Cichlidogyrus tilapiae collected in DRC, Madagascar and Burundi.




## Figure 3

Phylogram based on Bayesian inference using a concatenated 18S, ITS-1 and 28S dataset (1362 bp). Posterior probabilities are shown above branches and bootstrap values from the Maximum Likelihood analyses below branches. Nodes with probabilities lower than 50 are collapsed. Newly generated sequences in this study in bold with mention of the host species; other sequences are GenBank reference sequences listed in Table 4.

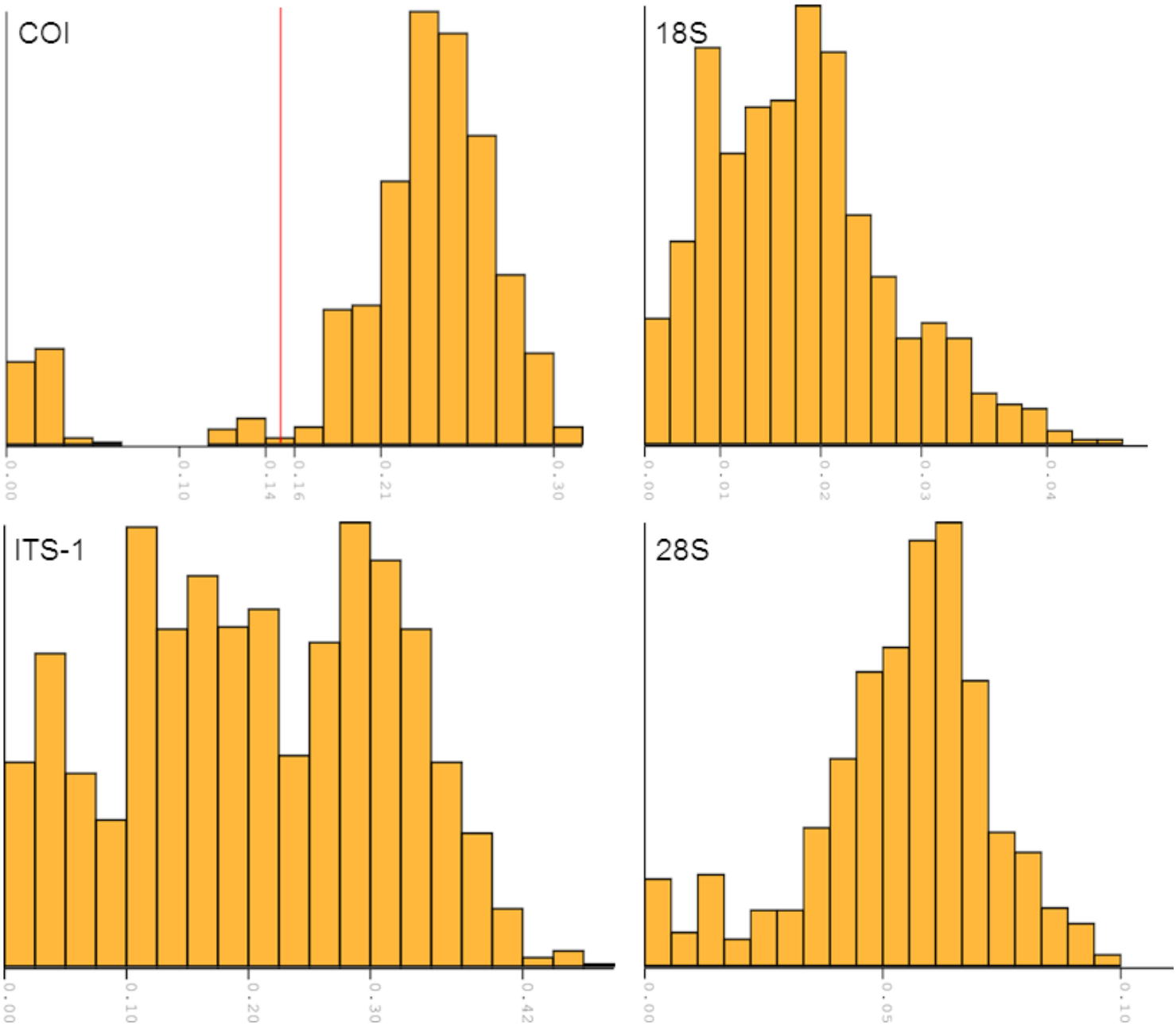



**Figure 4**

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.

**Figure 5**

Differential interference contrast micrographs of the sclerotized structures of C. cf. halli ‘Burundi’ from Nile tilapia. Left: male copulatory organ: AP accessory piece, CT copulatory tube and He heel. Right: sclerotized haptoral structures: I-VII hooklets from pairs I¬-VII. Note that hooklets pair I are elongated and thickened. Hooklets of pair II are very small and associated with the ventral hook (VH), but this is normal

for all species within Cichlidogyrus/Scutogyrus. DH dorsal hook, DB dorsal bar, VB ventral bar. Scale 20µm.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Add1sampleinventory.pdf
- Add2ITS1matrix.xlsx
- Add3COImatrix.xlsx
- Add428smatrix.xlsx
- Add518smatrix.xlsx
- Add6ITS1COI.xlsx
- Add718S28Smatrix.xlsx