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Original Article

Mosaic or melting pot: The use of monogeneans as a biological tag and magnifying glass to discriminate introduced populations of Nile tilapia in sub-Saharan Africa☆

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ABSTRACT

The origin of introduced Nile tilapia stocks in sub-Saharan Africa is largely unknown. In this study, the potential of monogeneans as a biological tag and magnifying glass is tested to reveal their hosts' stocking history. The monogenean gill community of different Nile tilapia populations in sub-Saharan Africa was explored, and a phylogeographic analysis was performed based on the mitogenomes of four dactylogyrid species (*Cichlidogyrus halli*, *C. sclerosus*, *C. thurstonae*, and *Scutogyrus longicornis*). Our results encourage the use of dactylogyrids as biological tags. The magnifying glass hypothesis is only confirmed for *C. thurstonae*, highlighting the importance of the absence of other potential hosts as prerequisites for a parasite to act as a magnifying glass. With the data generated here, we are the first to extract mitogenomes from individual monogeneans and to perform an upscaled survey of the comparative phylogeography of several monogenean species with unprecedented diagnostic resolution.

1. Introduction

Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) belongs to the tribe Oreochromini within Cichlidae Heckel, 1840 [1] and is native to the Nile Basin, several river basins in West Africa, various water bodies of the East African Rift Valley, Lake Tana in Ethiopia, and the Yarkon Basin in Israel [2]. However, for aquaculture purposes, it has been extensively introduced outside its native range [3–5]. In sub-Saharan Africa, Nile tilapia has been imported into most countries and is now the main aquaculture species, alongside species of catfish [6,7]. Aquaculture production of Nile tilapia in this region increased from 7500 to 205,000 t in the past 20 years (numbers retrieved from FishStatJ v4.01.2).

The characteristics that make Nile tilapia a popular fish for fish farmers (i.e. fast growth and reproductive rate, being tolerant to a range of environmental conditions, and being undemanding in terms of food

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source [2,4]), also make it highly invasive. Nile tilapias can escape from aquaculture facilities to local water bodies where they can compete with native fish for food and habitat, predate on plants and small fish, and genetically contaminate local tilapias by their ability to hybridise [3,4,8–13]. Indeed, in several water bodies in sub-Saharan Africa, the introduction of Nile tilapia is already threatening native tilapia species, e.g. in Lake Victoria [14,15], the Kafue Basin [16], the Limpopo Basin [17–19], Lakes Rutamba, Nambawala, and Mitupa [20,21], and the Pangani Basin [21]. In addition, introduced Nile tilapia can co-introduce their parasite fauna and pathogens, which can spill over to native hosts [22]. For example, recent spillovers of monogenean parasites from introduced Nile tilapia to native fish have been reported by Šimková et al. (2019) [23] and Jorissen et al. (2020) [22].

The genetic origin of farmed Nile tilapia populations in Africa is often unknown [24]. One factor complicating the assignment of a given population to a particular strain is the debated taxonomy of Nile tilapia subspecies. In nature, eight different subspecies have been recognised to date [2,25]. However, their status has been disputed by later research [26–30]. Furthermore, in an aquaculture setting, where interbreeding and artificial selection between subspecies occur, this classification into subspecies is not applicable [31]. In addition, many introductions and transfers of aquaculture fish between countries and river basins are not documented, making the identification of these strains and their origin even more difficult. Distinguishing Nile tilapia strains used in aquaculture is not only needed for the management of fish stocks (i.e. a self-reproducing group of fish with similar life history characteristics [32]) but also for conservation purposes given the well-documented negative effects that the introductions of these strains can have on native tilapias [8].

Genetics is routinely used to discriminate between fish populations and strains [33,34]. However, in the case of Nile tilapia, microsatellite analyses show only limited resolving power to differentiate between populations on a local scale [29]. Therefore, new genetic techniques and additional information sources are now used to increase resolution. The rise of next-generation sequencing (NGS) techniques provides one way to assess population structure on a smaller geographic and temporal scale [35–38].

Another way to increase resolution is by using parasites as an additional source of information. Parasites have already proven their value in discriminating between different marine fish stocks [39–41], and can be used as a biological tag by screening a large number of fish for a small number of parasites, or by exploring the whole parasite assemblage of different fish populations [42,43]. In the literature, a number of criteria have been proposed for parasites to serve as biological tags e.g. (1) they should be easily detectable and identifiable, (2) they should have no strong pathological effects on their host, (3) parasite prevalence and infection intensities should vary across different host populations, and (4) they should persist on the host over the timescale of the investigation and for more than a year for stock identification. However, in practice, all of these criteria are rarely met and compromises need to be made [42–45].

Beyond their use as a biological tag, the genotypes of parasites can also be used to discriminate between fish stocks. Due to the usually short generation time and higher mutation rate compared to the host, parasites accumulate genetic mutations much faster, providing a 'magnifying glass' to study their hosts evolutionary history [46–49]. For parasites to act as a magnifying glass, they should have a high degree of host specificity, a direct life cycle (i.e. only needing one host to complete their life cycle), and a limited dispersal potential [46,49]. A number of studies already demonstrated the power of parasites as a magnifying glass. For example, in the steelhead trout *Oncorhynchus mykiss* (Walbaum, 1792), molecular genotyping of the digenean *Plagioporus shawi* (McIntosh, 1939) allowed discrimination of host populations from rivers separated by as little as ca. 50 km, where patterns of host microsatellite diversity failed to resolve such fine-scale population assignment [50].

The use of members of Monogenea Van Beneden, 1858, a taxon of parasitic flatworms (Platyhelminthes) within Neodermata, has already been proposed as an additional source of information in phylogeographic studies on cichlids [51,52]. Most species of Monogenea are ectoparasites infecting mainly freshwater, marine, and brackish water fishes [53]. They have a direct life cycle and a high degree of host specificity [53]. Hence, monogeneans comply with most of the criteria for biological tags. The majority of species are found on gills, fins, and skin, which can efficiently be dissected and preserved [54]. Species are easily recognised, as identification is often based on their sclerotised structures, which are clearly visible under a microscope, also in preserved specimens. Furthermore, monogeneans appear to be rarely pathogenic in natural habitats [55]. Because of these same characteristics, monogeneans also meet the above-mentioned requirements to act as a magnifying glass [53]. Jorissen et al. (2021) already found differences in the monogenean community composition of several introduced and native Nile tilapia populations [56]. These differences illustrate the potential value of these parasites for studying the stocking history of Nile tilapia [56]. Moreover, the cytochrome *c* oxidase I (*COI*) gene of several monogenean species was used to elucidate the introduction history of their host in the Congo Basin [57]. However, a relatively low number of single nucleotide polymorphisms (SNPs) was found to differ between haplotypes [57], highlighting the need for more high-resolution genetic markers to study the stocking history of Nile tilapia.

To date, twenty described species of Monogenea have been recorded on Nile tilapia. These include eighteen species belonging to three genera of ectoparasites, found on the gills and skin of their host (*Cichlidogyrus* Paperna, 1960, *Scutogyrus* Pariselle et Euzet, 1995, and *Gyrodactylus* von Nordmann, 1832), and two species belonging to a genus of endoparasites, living in the stomach and foregut of their host (*Enterogyrus* Paperna, 1963) [53] (Table S1).

In the present study, we evaluate the potential of monogeneans to discriminate between different introduced stocks of Nile tilapia in sub-Saharan Africa. To reach this goal, we explore the monogenean community composition (biological tag) on the gills of Nile tilapia from three countries in Sub-Saharan Africa where Nile tilapia has been introduced and compare their mitochondrial genetic diversity and structure (magnifying glass). We focus on gill parasites within Dactylogyridae Bychowsky 1933, a family of Monogenea in Africa with a direct oviparous life cycle. Currently, Dactylogyridae represents the most species rich family of African monogeneans with more than 400 described species [53] of which 13 have been reported infecting Nile tilapia [53]. Different basins were selected where Nile tilapia has been introduced for aquaculture purposes and where it has established feral populations: the Lualaba, Luapula, and Kasai basins in the DRC, the Zambezi Basin in Zimbabwe, and the Sanaga, Nyong, Kienke, and Ntem basins in Cameroon. We hypothesise that different groups of Nile tilapia can be distinguished from each other based on the community composition of their gill parasites, as reported by Jorissen et al. [56], and that phylogeographic analyses of the parasite mitogenomes will reveal population structuring corresponding to different host populations, rendering them applicable as a 'magnifying glass' of the hosts' stocking history.

2. Methods

2.1. Sampling

Specimens of *O. niloticus* were sampled during three separate field expeditions to the Democratic Republic of the Congo (DRC) in July 2019, Zimbabwe in October–November 2019, and Cameroon in February 2020. Feral Nile tilapias were caught in rivers and lakes using gill nets, seine nets, and dip nets. Additionally, farmed Nile tilapias were purchased from local fish farms who caught the fish by seine netting (Table S2; Fig. 1). Fish were killed by severing the spinal cord. Photos of the left-hand side of each fish were taken and are available in the collection of the Royal Museum for Central Africa (RMCA) in Tervuren (Belgium) under the collection number RMCA 2019.013.P (for fish from

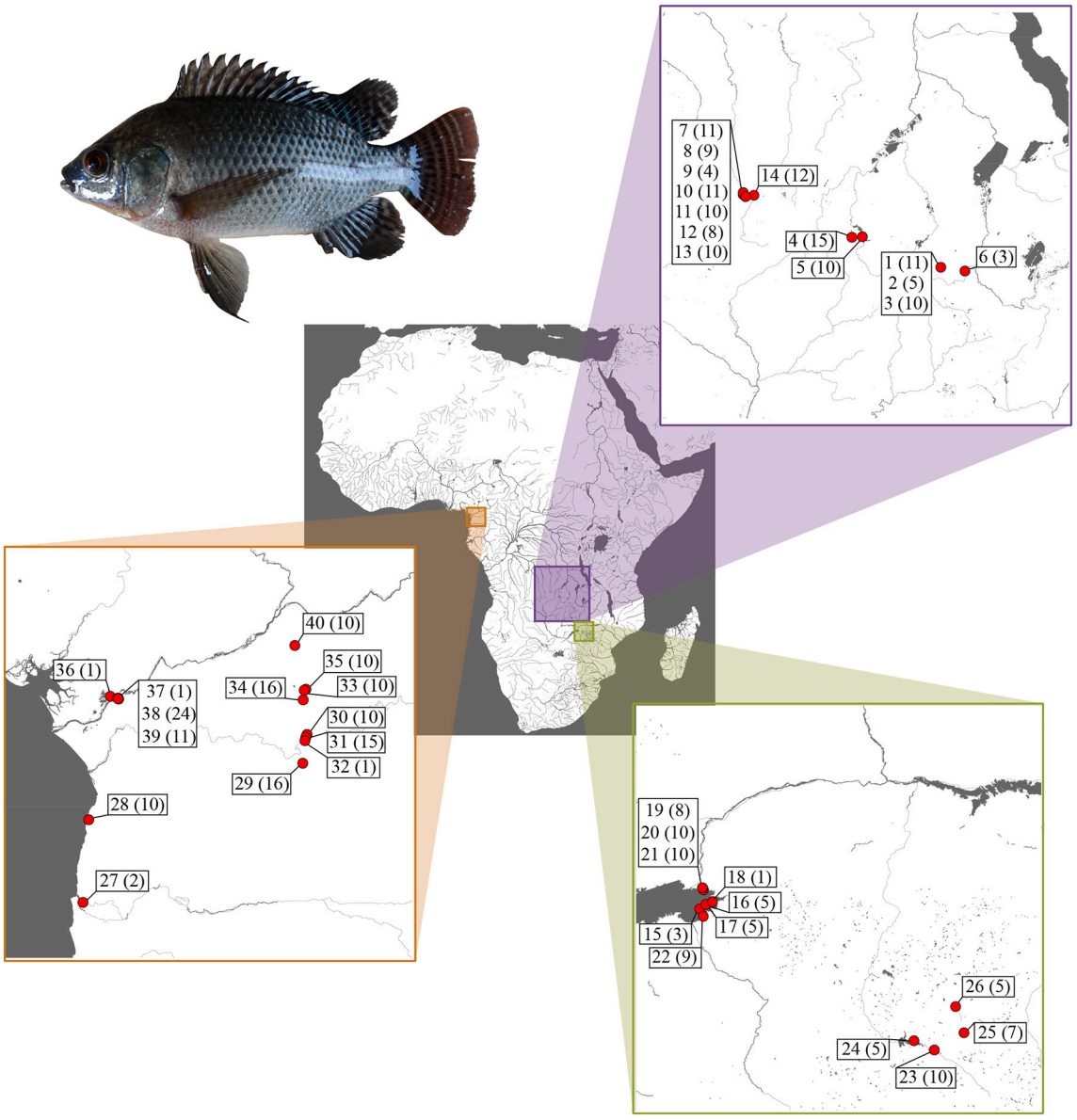

Fig. 1. Different sampling locations in Cameroon, the DRC, and Zimbabwe. A map of Africa (central) with framed regions expanded: sampling region in the DRC in purple, sampling region in Cameroon in orange, and sampling region in Zimbabwe in green. Sampling locations are depicted as red dots. Location labels correspond to the ones in Table S2. Number of hosts sampled at each location are shown between parentheses. A photo of Nile tilapia displayed on the upper left. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the DRC), RMCA 2022.007.P (for fish from Zimbabwe), and RMCA 2022.008.P (for fish from Cameroon). The gills from both gill chambers were dissected and stored in 99% ethanol (*v*/v). From fish from the DRC, only gills from the right gill chamber were dissected, preserving the gills from the left gill chamber to enable morphological vouchering. These fish were fixed in the field with formaldehyde (10%) and later deposited in the ichthyology collection at the RMCA, stored in 70% ethanol under the collection number RMCA 2019.013.P. Gills were inspected exhaustively for monogenean parasites in the laboratory with a Nikon C-DS stereomicroscope using an entomological needle. Parasites were temporarily water-mounted between a microscopic slide and coverslip, and morphologically identified based on their sclerotised parts, i.e. male copulatory organ (MCO), haptor, and vagina (when sclerotised), following Pariselle & Euzet (2009) [58], with a Leica DM2500 microscope with interference contrast, and stored afterwards in 99% ethanol (*v*/v) at 5 °C per species per host.

2.2. Exploring differences in parasite communities

We tested for differences in the parasite infection intensity (total number of parasites per host individual) and species richness (number of parasite species per host individual) [59] between basins and between farmed and feral hosts using single-term deletion of the generalised linear models with a Poisson distribution (count data) and a logarithmic link function in R version 4.1.0 [60]. To test for differences in the parasite infection intensity, the number of parasites present on hosts from the DRC, for which only the gills from the right-hand side of the fish were examined, were doubled assuming that the infection intensity in both gill chambers is similar [61–63]. To address overdispersion, we also applied a negative binomial distribution through the *glm.nb* function in the R package *MASS* version 7.3.54 [64] and a quasipoisson distribution. Model performance was assessed through residuals vs. fitted plots, quantile-quantile (Q-Q) plots, and the Akaike Information Criterion (AIC). A post-hoc analysis using Šidák correction with pairwise

comparisons was performed using the R package *emmeans* version 1.6.3 [65] and visualised using the R package *multcompView* version 0.1.8 [66].

Parasite communities of Nile tilapia were visualised by histograms in Excel version 16.52 grouped by country, basin and the farming history (farmed or feral) of the host. In addition, communities were visualised by a Non-metric Multi-dimensional Scaling (NMDS) plot with the package *vegan* version 2.5.7 [67] in R using the Bray-Curtis dissimilarity index as a distance measure. An ANOSIM test was performed with the same R package to test for a statistical difference between the parasite communities between countries, basins, farming, and farming per country. To explore which parasite species predominate the community composition in a particular country or basin, or in farmed or feral hosts, an Indicatory Species Analysis was performed with the package *indicspecies* version 1.7.9 [68] in R, using 9999 permutations.

2.3. DNA extraction and mitogenome sequencing

Specimens of four species of Dactylogyridae, namely *Cichlidogyrus halli* (Price & Kirk, 1967), *C. sclerosus* Paperna & Thurston, 1969, *C. thurstonae* Ergens, 1981, and *Scutogyrus longicornis* (Paperna & Thurston, 1969), were selected from fish caught in different basins from the three selected countries (Table S3). These species were selected because of their presence in most basins in the three countries (except for *C. sclerosus*, for which only a few specimens were found in Cameroon). All specimens of a certain parasite species were selected from different specimens of Nile tilapia. Before DNA extraction, micrographs of each specimen were taken with the Leica DM2500 microscope and the Leica Application Suite X (LASX) software. Micrographs are available on Morphbank http://morphobank.org/permalink/?P420 under the numbers M840772–M841343. The DNA of these specimens was extracted using a modified salting-out method. The specimen was transferred to a tube with a solution of TNES buffer and 20 mg/mL proteinase *K* for digestion, followed by incubation at 55 °C until complete digestion. Yeast tRNA was added as a carrier after which 5 M NaCl and 96% (*v*/v) ethanol were added. The sample was stored at −20 °C for at least one hour for precipitation of the DNA. The pellet was purified by two rounds of centrifugation, removing the supernatant and adding 70% (v/v) chilled ethanol. The purified DNA was stored in 0.1× TE with 0.02% Tween™ Surfact-Amps™ Detergent Solution at −20 °C. The concentration of DNA from each extraction was quantified with a Qubit® 2.0 Fluometer (Life Technologies, Paisley (UK)). Whole genome amplification was carried out for samples with low DNA concentrations using the Illustra™ Ready-To-Go™ GenomiPhi™ V3 Amplification kit. Libraries were prepared with a Nextera XT DNA Library Preparation Kit and 151 bp paired-end sequenced on an Illumina platform with a HiSeq X system at Macrogen Korea (Seoul, South Korea).

2.4. Mitogenome assembly

The overall quality of the reads was checked with the software FastQC version 0.11.7 [69]. Reads were paired and orphan reads were deleted with Trimmomatic version 0.39 [70]. Before assembly, paired reads were first normalised with the 'Error correct and normalize reads' option in Geneious Prime 2021.2.2 (Biomatters, New Zealand). The 'map to reference' option and the GenBank reference mitogenomes JQ038226.1 (*C. sclerosus*) [71], MG970255.1 (*C. halli*) [72], and MT447060.1 (*S. longicornis*) [73] were used for the assembly of mitogenomes of specimens of *C. sclerosus*, *C. halli*, and *S. longicornis* respectively. The reference MT447060.1 (*S. longicornis*) was used for the assembly of mitogenomes of specimens of *C. thurstonae* because more reads were mapped when using the mitogenome of *S. longicornis* as a reference genome than when using the reference mitogenomes of *C. halli* or *C. sclerosus*.

Assembled mitogenomes were mapped back to the respective reference genome and independently annotated with Geneious Prime using the respective annotated reference genome and with the MITOS webserver [74], applying the echinoderm and flatworm mitochondrial genetic code. Furthermore, tRNAs were predicted with the tRNAscan-SE version 2.0 webserver using the eukaryotic sequence code [75]. Open reading frames (ORFs) were identified with Geneious Prime using the echinoderm mitochondrial genetic code. Boundaries of protein coding genes (PCGs) were determined by matching the annotations with ORFs. In case of discordance between the ORFs and annotations resulting from different software, the proposition with the least number of non-coding regions and the least intergenic overlap was chosen (except for the overlapping *nad*4 and *nad*4L gene). Population genomic analyses were performed using a concatenated dataset of the twelve PCGs. Stop codons were omitted because abbreviated versions of the stop codon cause frameshifts. In addition, non-coding regions were excluded, as well as *12S* rDNA and *16S* rDNA. Non-coding regions were excluded because of their high level of variation (high mutation rate) and the presence of repeats, causing alignment and annotation problems in these regions. The *12S* rDNA and *16S* rDNA genes were omitted because of their relatively slow evolutionary rate, making them less suited for distinguishing between closely related populations [76,77]. The assembled mitogenomes of all included individuals are available under the GenBank accession numbers **ON036422–ON036469** (Table S3).

2.5. Genetic diversity and phylogeographic structure

To assess the genetic diversity of the four selected species, the number (S) and percentage (S%) of polymorphic sites, the number of haplotypes (h), haplotype diversity (Hd), and nucleotide diversity (π), were calculated in DnaSP version 6 using default settings [78]. The genetic structure of parasite populations from different countries and basins was quantified by calculating the pairwise fixation index F_{st} in Arlequin version 3.5.2.2 [79]. To further evaluate the genetic structure of a particular parasite population between different basins, an analysis of molecular variance (AMOVA) with 1000 permutations was conducted using the same software.

2.6. Phylogenetic analyses

The phylogenetic relationships between individuals from different countries and basins were examined by creating a median joining haplotype network for each species with PopART version 1.7 [80]. Additionally, maximum-likelihood (ML) and Bayesian phylogenetic trees were constructed. The best fitting substitution models were selected based on the Akaike Information Criterion with jModelTest2 on the Cipres Science Gateway version 3.3 [81]. For *C. sclerosus* and *S. longicornis*, the generalised time reversible model GTR + G + I was selected, while GTR + G was selected for *C. halli* and *C. thurstonae*.

The ML phylogenetic tree was built in MEGAX 10.2.6 [82] with 1000 bootstraps using an extensive Subtree-Pruning-Regrafting (SPR level 5) method. The Bayesian phylogenetic tree was constructed in BEAST version 1.10.4 [83] with a Markov Chain Monte Carlo (MCMC) approach with the best fitting substitution model, a constant size coalescent tree prior (default), and a strict molecular clock model (default). All other operators and prior distributions were left at default settings. Five independent runs were performed from a random starting tree with one cold and one heated chain (deltaTemperature = 0.1) for 10,000,000 generations with a sampling frequency of 1000. The resulting log files were combined in Tracer version 1.7.2 [84] with a 50% burn-in to check trace plots for convergence. All model parameters had effective sample sizes of well above 200. Tree files were combined in LogCombiner version 1.10.4 (implemented in BEAST) with a 50% burn-in and TreeAnnotator version 1.10.4 (implemented in BEAST) was used to infer a Maximum Clade Credibility tree with default settings. Phylogenetic trees were visualised in FigTree version 1.4.4 [85]. The GenBank reference mitogenome MG970255.1 (*C. halli*) was chosen as an outgroup in the phylogenetic analyses of *C. halli* and JQ038226.1 (*C. sclerosus*) in

the analyses of *C. sclerosus*, while MT447060.1 (*S. longicornis*) was used as an outgroup in the analyses of *S. longicornis* and *C. thurstonae*.

2.7. Checking for a barcoding gap

To genetically verify species identification and to check for the presence of cryptic species, we calculated the intra- and interspecific distances based on the *COI* gene. The intraspecific genetic distance in this gene is usually smaller than the interspecific distance, warranting its role as a general barcoding marker [86,87]. While deemed suboptimal for barcoding in flatworms [88], it has been proven effective for species delineation in Nile tilapia monogeneans by Jorissen et al. (2021) [57]. The Kimura-2-parameter (K2P) distance model [89] was used as in Vanhove et al. (2013) [88], and results were visualised with histograms in R using the script made available by G. Sonet. For the calculation of interspecific distances, *COI* sequences of species of *Cichlidogyrus* and *Scutogyrus* were selected from GenBank (Table S4). Manual trimming and gap removal resulted in alignments of 269 bp.

3. Results

3.1. Monogenean gill parasite community on Nile tilapia

In the DRC, a total of 116 out of 129 screened specimens of *O. niloticus* were infected by ectoparasitic dactylogyrids: 63 out of 75 screened fish from the Kasai Basin, 50 out of 51 screened fish from the Lualaba Basin, and three out of three screened fish from the Luapula Basin. In Zimbabwe, a total of 60 out of 76 screened specimens were infected by ectoparasitic dactylogyrids: 43 out of 49 screened fish from Lake Kariba (Middle Zambezi Basin), 16 out of 22 screened fish from the surroundings of Harare (Middle Zambezi Basin), and one out of five screened fish from Lake Mazowe (Lower Zambezi Basin). In Cameroon, a total of 76 out of 102 screened fish were infected by ectoparasitic dactylogyrids: 49 out of 58 screened fish from the Nyong Basin, 20 out of 31 screened fish from in the Sanaga Basin, six out of 11 screened fish from the Kienke Basin, and one out of two screened fish from the Ntem Basin (Fig. 1; Table S2). From these fish, 1665 gill monogeneans were collected from the DRC, 1758 from Cameroon, and 1408 from Zimbabwe (Fig. 2; Fig. S1). Only a subset of the dactylogyrid species reported from Nile tilapia (Table S1) are found: *C. halli*, *C. sclerosus*, *C. thurstonae*, *C. tilapiae* Paperna 1960, and *S. longicornis* are present in all three countries, though, *C. sclerosus* with a low prevalence in Cameroon. *Cichlidogyrus cirratus* Paperna 1964 was only found in Cameroon. Parasites classified as 'other' comprise species that are naturally not present on Nile tilapia, and may present host switches (these are still under scrutiny and fall outside of the scope of the present study).

Before testing for differences in the parasite infection intensity, we checked the model performance of the negative binomial distribution and quasipoisson distribution because of overdispersion in the Poisson distribution. The negative binomial distribution was selected because of a great reduction in AIC ($\Delta AIC = 4552.6$) and a better fit in the Q-Q plot and residual vs. fitted plot compared to the quasipoisson distribution. Only the farming history of the host, $\chi^2\,(1, N = 2) = 50.819, p < 0.001$, is significantly correlated with the parasite infection intensity (Table S5. a). Notably, the response values for farmed hosts are consistently higher than those for feral hosts within a basin and none of the grouping labels were shared between them (Table S5.b), which means that a farming environment increases the parasite infection intensity on the host.

To test for differences in the parasite species richness, a generalised linear model with a Poisson distribution was applied as no overdispersion was observed. Both the basin ($\chi^2\,(5, N = 6) = 18.784, p < 0.01$) and the farming history of the host ($\chi^2\,(1, N = 2) = 8.128, p < 0.01$) have a significant effect on species richness (Table S6.a). The grouping labels indicate that feral hosts from the Kasai Basin have a significantly lower species richness than farmed hosts from the Lualaba, Sanaga, and Nyong basins, and feral hosts from the Nyong Basin. In addition, the grouping labels indicate that feral hosts from the Middle Zambezi Basin, Lake Kariba, and Lualaba Basin, and farmed hosts from the Kasai Basin have a significantly lower species richness than farmed hosts from the Nyong Basin (Table S6.b).

From the ANOSIM test, there seems to be some indication of a difference in the parasite communities from different countries, basins, and farming per country (Table S7). However, as the test statistic is rather small (Table S7) and the NMDS plots show overlap between different groups (Fig. S2), these results should be interpreted with caution. The results of the Indicator Species Analysis using the country as a grouping factor indicate *C. cirratus* as significantly associated with Nile tilapia from Cameroon, *C. sclerosus* with Nile tilapia from Zimbabwe, and *C. thurstonae* with Nile tilapia from the DRC (Table S8). When using the farming history of the host for each country as grouping factor, *C. cirratus* appears significantly associated with farmed and feral Nile tilapia from Cameroon, *C. sclerosus* with farmed and feral Nile tilapia from Zimbabwe, and *C. thurstonae* with feral hosts from Cameroon and the DRC, and with farmed hosts from the DRC (Table S8).

3.2. Molecular data

In total, the mitogenome of nine specimens of *C. halli,* 17 specimens of *C. sclerosus*, 12 specimens of *C. thurstonae*, and ten specimens of *S. longicornis* were sequenced and assembled (Table S3).

After exclusion of non-coding regions, and *12S* rDNA and *16S* rDNA, a dataset of all twelve concatenated protein coding genes (PCGs) remained. This resulted in a total fragment of 8565 bp for *C. halli*, 9702 bp for *C. sclerosus*, 9218 bp for *C. thurstonae*, and 9757 bp for *S. longicornis*. For the dataset of *C. halli* and *C. thurstonae*, part of the *nad*5 gene was excluded due to low coverage.

3.3. Genetic diversity and phylogeographic structure

A summary of the genetic diversity is represented in Table 1. For all four parasite species, the number of haplotypes matches the number of studied individuals, except for *C. sclerosus*, where where two pairs of individuals share the same haplotype. The genetic diversity is highest for *C. halli* as measured by the number (S) and percentage (S%) of polymorphic sites, and the nucleotide diversity (π). The lowest genetic diversity is found for *S. longicornis*, except in Zimbabwe, where *C. thurstonae* shows the lowest genetic diversity.

The pairwise F_{st} analysis reveals significant differentiation between populations of *C. sclerosus* and *S. longicornis* from the DRC and Zimbabwe. For populations of *C. thurstonae* significant genetic differentiation is present between all three countries with the highest level of genetic differentiation between Zimbabwe and the DRC (Table S9). For none of the parasite species, genetic differentiation is found between populations from different basins (Table S10).

3.4. Phylogenetic analyses

The haplotype networks colouring individuals by country (Fig. 3), show a clear phylogeographic pattern for individuals of *C. thurstonae*, where individuals from each country cluster together. The cluster of individuals from the DRC is situated in the centre of the network from which the clusters of Cameroon and Zimbabwe radiate. The number of mutational steps between the clusters of the DRC and Zimbabwe is higher than those between the clusters of the DRC and Cameroon. Within the DRC, a relatively high number of mutational steps is found between individuals from different basins (Fig. S3). No specific clustering patterns of parasites are visible when considering the farming status of their host (Fig. S4). For *C. halli*, the number of mutational steps between the different individuals is much higher compared to that for the other species, and the haplotype network does not show any clear patterns. The number of mutational steps between specimens from the

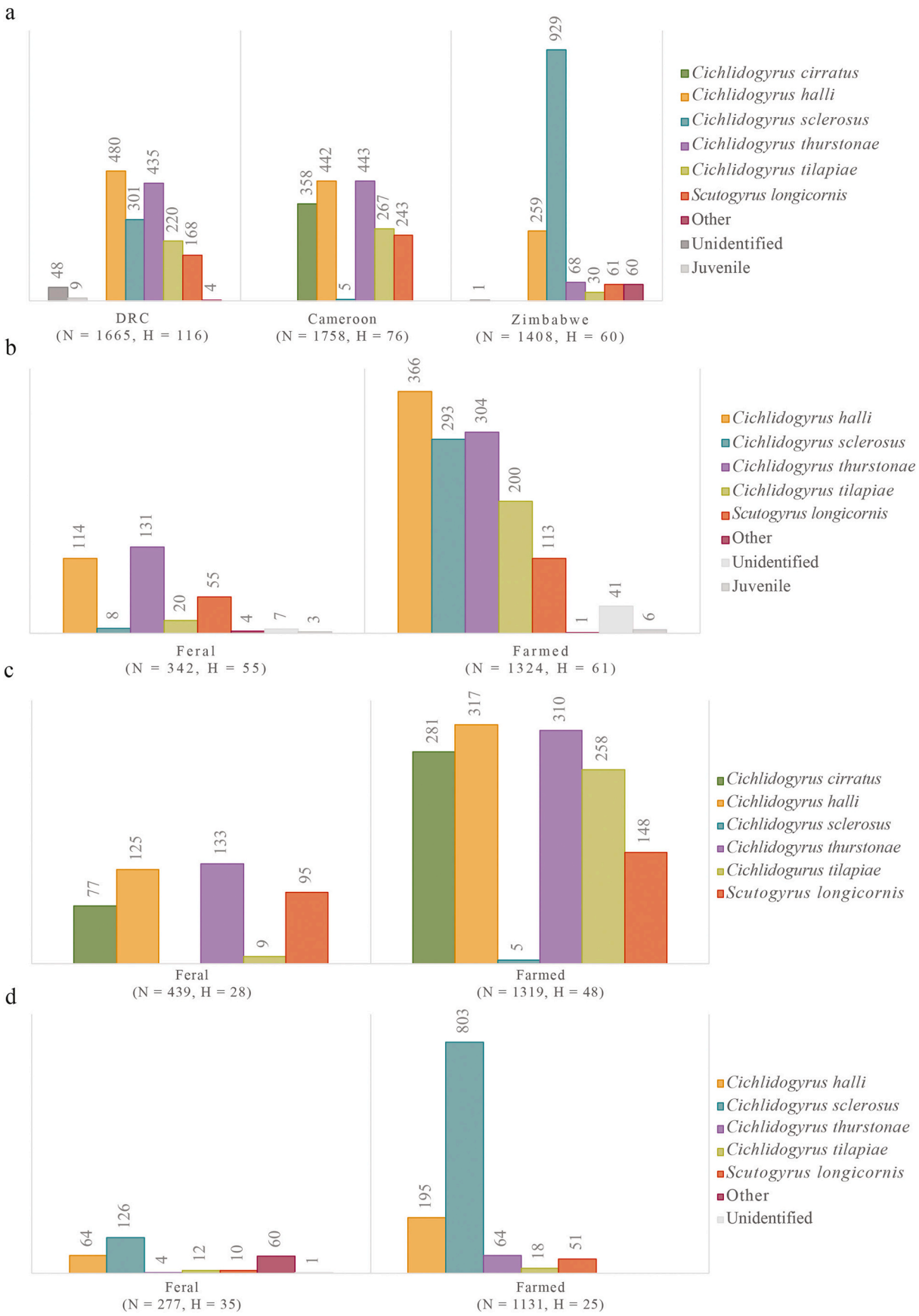

Fig. 2. Histograms of the parasite community on sampled Nile tilapia in the different countries (a), on farmed and feral Nile tilapia in the DRC (b), Cameroon (c), and Zimbabwe (d). Numbers of parasites are shown above each bar. The total number of parasites (N) and sampled hosts (H) are indicated below each graph.

Table 1
Summary of the genetic diversity of *C. halli* (Price et Krik, 1967), *C. sclerosus* Paperna et Thurston, 1969, *C. thurstonae* Ergens, 1981, and *S. longicornis* (Paperna et Thurston, 1969) grouping all individuals per species and country.

Species		*C. halli*	*C. sclerosus*	*C. thurstonae*	*S. longicornis*
	Total number of sites	8565	9702	9085	7016
All individuals	Number of individuals	9	17	12	10
	S	2464	822	596	198
	S%	28.8	8.5	6.6	2.8
	h	9	15	12	10
	Hd	1	0.985	1	1
	π	0.122	0.023	0.023	0.009
Cameroon	Number of individuals	3	/	4	3
	S	1279	/	120	57
	S%	14.9	/	1.3	0.8
	h	3	/	4	3
	Hd	1	/	1	1
	π	0.100	/	0.007	0.005
DRC	Number of individuals	3	8	5	2
	S	1643	559	232	36
	S%	19.2	5.8	2.6	0.5
	h	3	6	5	2
	Hd	1	0.929	1	1
	π	0.133	0.025	0.011	0.005
Zimbabwe	Number of individuals	3	9	3	5
	S	1369	589	15	112
	S%	16.0	6.1	0.2	1.6
	h	3	9	3	5
	Hd	1	1	1	1
	π	0.109	0.019	0.001	0.007

The number of studied sites is given, as well as the number of individuals, the number (S) and percentage (S%) of polymorphic sites, the number of haplotypes (h), the haplotype diversity (Hd), and the nucleotide diversity (π).

same country is sometimes much higher than between specimens of different countries (Fig. 3; Figs. S3 and S4). Also, for *C. sclerosus* no clear pattern is found in the haplotype network when grouping individuals by country, basin, or farming history of their host (Fig. 3; Figs. S3 and S4). Two pairs of individuals share a haplotype, each pair sampled at the same location but from two different hosts. Finally, for *S. longicornis*, individuals from Cameroon cluster together in the haplotype network as well as most individuals from Zimbabwe (Fig. 3) with a small number of mutational steps between two individuals from feral hosts in the Middle Zambezi and Lake Kariba, and between two individuals from a feral and a farmed host from the Middle Zambezi (Figs. S3 and S4).

The clustering of specimens in the phylogenetic trees (Fig. S5) are highly consistent with that in the haplotype networks (Fig. 3). The topology of the Bayesian tree of *C. halli* encompasses three highly supported monophyletic clades including respectively (1) one specimen from Cameroon, two from Zimbabwe, and one from the DRC, (2) two specimens from the DRC and one from Zimbabwe, and (3) two specimens from Cameroon (Fig. S5). In the maximum likelihood (ML) tree, similar groupings are inferred albeit with weaker support. Also, several highly supported groups are inferred in the Bayesian and ML tree of *C. sclerosus*: one encompassing three specimens from the DRC and two from Zimbabwe, one consisting of four specimens from Zimbabwe, one comprising two specimens from Zimbabwe, and one consisting of two specimens from the DRC (Fig. S5). Specimens of *C. thurstonae* form maximally supported clades per country in the Bayesian phylogenetic tree with the Congolese clade being the sister group of the Cameroonian clade (Fig. S5). In the ML phylogenetic tree, the Congolese and Zimbabwean clade form sister groups, though with weak support values (Fig. S5). The topology of the phylogenetic trees of *S. longicornis* suggests a clustering of three groups of individuals encompassing (1) the specimens from Cameroon and one from Zimbabwe, (2) two from Zimbabwe and one from the DRC, and (3) two from Zimbabwe, though the latter with weak support in the Bayesian phylogenetic tree (bootstrap value <0.85) (Fig. S5).

A clear phylogeographical pattern is only illustrated by *C. thurstonae* when colouring the specimens by country. Such a clear pattern is not visible for the other species. Also, no clear patterns are visible in the phylogenetic trees of these species when colouring the specimens by basin or the farming history of the host.

3.5. Checking for a barcoding gap

The intra- and interspecific divergence of *COI* sequences for specimens of *C. halli*, *C. sclerosus*, *C. thurstonae*, and *S. longicornis* are presented in Table S11. A clear barcoding gap is apparent when the intraspecific divergence is calculated for specimens of *C. sclerosus*, *C. thurstonae*, and *S. longicornis*: 3.5–19.9%, 4.6–18.9%, and 1.9–19.4%, respectively. The gap between the intra- and interspecific divergence is considerably lower when calculating the intraspecific divergence for *C. halli*: 15.56–20.89% (Fig. 4). Some overlap between the range of intra- and interspecific divergences is caused by the pairwise interspecific divergence between the GenBank sequences of *C. sclerosus* and *C. mbirizei* Muterezi Bukinga, Vanhove, Van Steenberge & Pariselle, 2012, and between *C. gistelincki* Gillardin, Vanhove, Pariselle, Huyse & Volckaert, 2012 and *C. milangelnari* Rahmouni, Vanhove & Šimková, 2017, which are much lower than other interspecific divergences. This low genetic distance between *C. gistelincki* and *C. milangelnari* is in line with the findings of Rahmouni et al. (2021), reporting a genetic divergence of only 3–3.2% based on the *28S* rDNA region [90]. The low interspecific divergence between the sequences of *C. sclerosus* (derived from pooled samples) and *C. mbirizei* may be ascribed to the incorrect labelling or contamination of individuals in pools. However, additional research is required to explain this low level of divergence.

4. Discussion

We evaluate the potential of monogeneans as biological tags for the assessment of their hosts' stocking history by examining the monogenean parasite community on the gills of Nile tilapia from different countries, basins, and from farmed as well as feral hosts. In addition, we test the magnifying glass hypothesis for four dactylogyrid species, namely *C. halli*, *C. sclerosus*, *C. thurstonae*, and *S. longicornis*, using a concatenated dataset of all twelve mitochondrial PCGs. We hypothesised that the parasite community of different groups of Nile tilapia

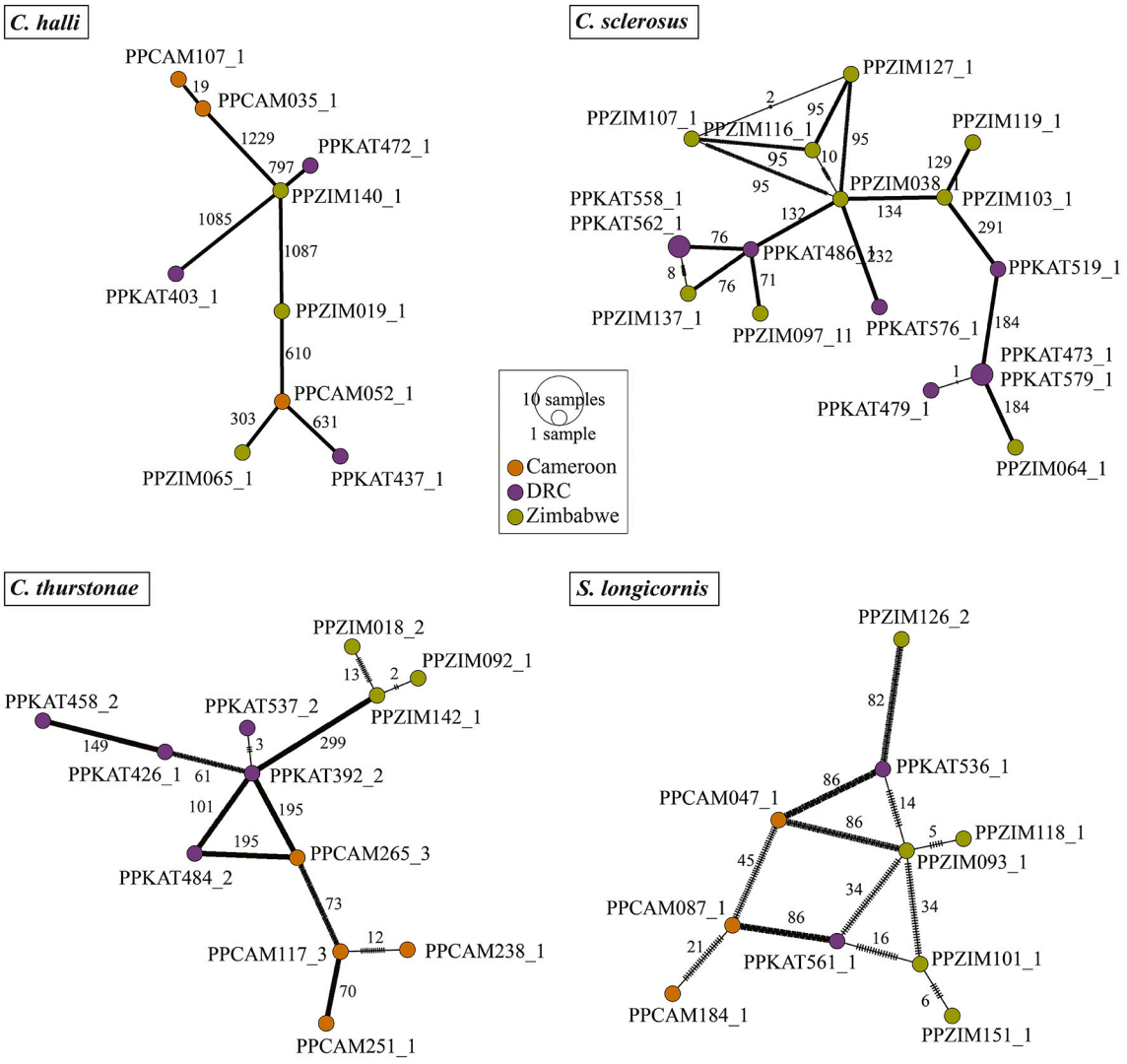

Fig. 3. Median joining haplotype networks based on the mitochondrial PCGs of *C. halli*, *C. sclerosus*, *C. thurstonae*, and *S. longicornis*. The size of the circles in the haplotype networks is proportional to the number of individuals of that haplotype. Circles are coloured according to country. Mutational steps are represented by hatch marks along each branch. For clarity, the number of mutational steps is also mentioned next to each branch.

differs significantly between different Nile tilapia populations (biological tag), and that phylogeographic analyses of these parasites' mitogenomes reveal high resolution population structuring, rendering them applicable as a magnifying glass to learn more about the migration or translocation history of their host.

4.1. Monogeneans as biological tag

The ultimate purpose of a biological tag is to be able to discriminate between different host populations, and, thereby, in case of introduced populations, revealing their origin. In the present study, only a subset of the dactylogyrid species reported from Nile tilapia is detected. The presence of *C. halli*, *C. sclerosus*, *C. thurstonae*, *C. tilapiae*, and *S. longicornis* in all three countries is not surprising as these species have also been found co-introduced with Nile tilapia in different continents, such as North America and Asia [23,63,91–93]. These five species have even been reported by Jorissen et al. (2021) as the most prevalent species with the widest geographic distribution [56]. Other dactylogyrids infecting Nile tilapia (Table S1) have a rather limited distribution area within the hosts' native range [58], e.g. *C. rognoni* Pariselle, Bilong Bilong & Euzet, 2003 has only been reported from Nile tilapia in the Senegal River and on the same host species at the IDESSA Research Station in Bouaké (Ivory Coast) [94]. Therefore, the likelihood of these parasite species being co-introduced with Nile tilapia is lower.

The parasite communities seem to differ significantly between different countries, basins, and between farmed and feral hosts (Table S7). Moreover, several species are identified as indicators for different groups of hosts. This is exemplified by *C. cirratus* as it is only detected on Nile tilapia from Cameroon while the occurrence of *C. sclerosus* in this country is sporadic. The latter species was also absent from farmed Nile tilapia in Yaoundé (Cameroon) according to Tombi et al. (2014) [62]. Also, our findings concur with those of Pariselle et al. (2003), who recorded *C. cirratus* on native Nile tilapia in Western Africa, but not *C. sclerosus* [95]. Therefore, we propose that the Nile tilapia we sampled in the Ntem, Nyong, Kienke and Sanaga basins in Cameroon have a Western African origin. Indeed, an introduction of Nile tilapia from the Chad Basin to Yaoundé (Nyong Basin) has been reported by Thys van den Audenaerde (1966) [96]. Yet, more baseline data on the monogenean community on different native Nile tilapia populations and different aquaculture strains should be collected before we can safely designate *C. cirratus* as an indicator species.

The finding of a higher parasite infection intensity in farmed compared to feral Nile tilapia is in line with the results of Lim et al. (2016) [63] and Ibrahim (2012) [97]. The higher infection level of

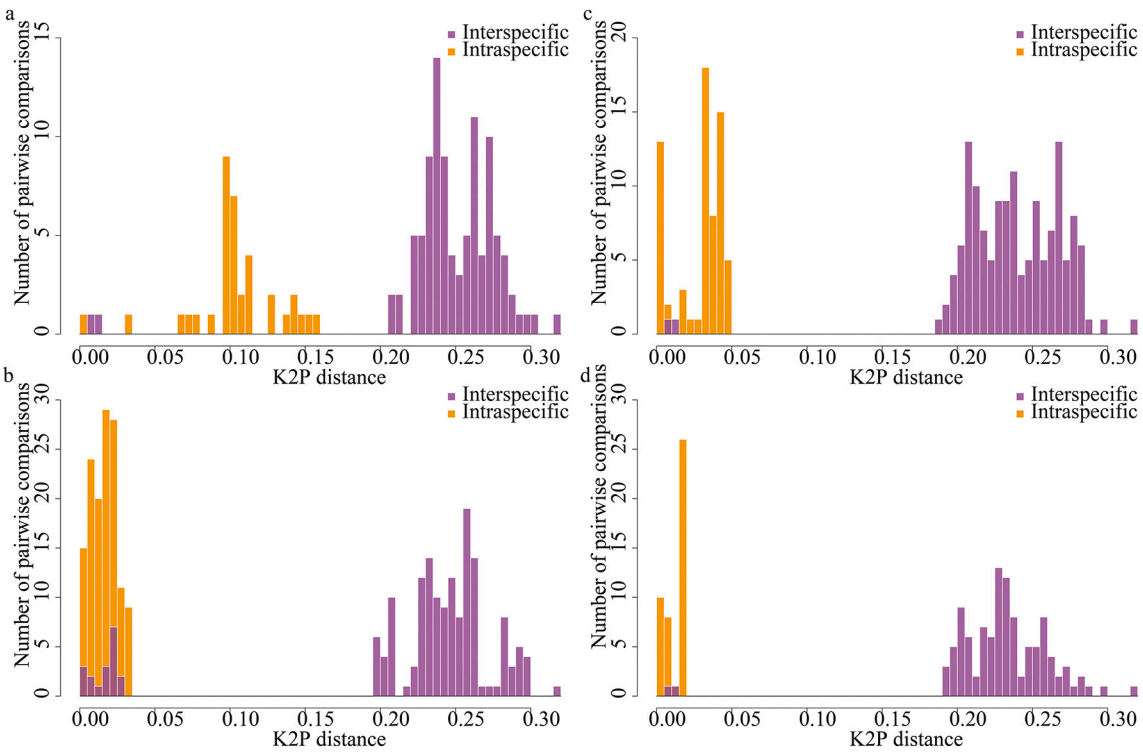

Fig. 4. Frequency distributions of intra- and interspecific genetic divergence for the *COI* gene in specimens of *C. halli* (a), specimens of *C. sclerosus* (b), specimens of *C. thurstonae* (c), and specimens of *S. longicornis* (d).

farmed fish can probably be ascribed to the high density of fish in aquaculture facilities, facilitating the transmission of parasites, and a lower immune response of the hosts due to increased stress [63,97]. Higher transmission is typical for parasites with a direct life cycle as they do not depend on an intermediate host. Also, oviparous species are difficult to treat in aquaculture with anthelmintics as chemical treatment is only effective on attached parasite specimens, allowing eggs to persist, hatch, and reinfect the fish [98].

The results from the present study encourage the use of dactylogyrid monogeneans as biological tags for the discrimination of Nile tilapia stocks. However, more spatiotemporal information on parasite infection of well-delineated fish populations is needed because differences in the parasite communities at different locations can also be attributed to seasonal fluctuations, temperature, the presence of other fish species, the degree of water pollution, etc. [43,45,55,98,99]. Additionally, in case of introduced species, parasite communities of different host populations can also differ due to the absence of intermediate host species in the new location, which negatively impacts the survival of certain parasite species during the invasion process, also known as the enemy release hypothesis [100–102]. Also, co-introduced parasite communities, originating from the same source population, may differ from each other because introduced host populations are small subsets of the native parasite population (the so-called founder effect) [101,103]. Finally, some parasite life history traits can also be at play. Dactylogyrids do not meet all aforementioned criteria for parasites to act as biological tags. They have a relatively short life span estimated between only five and forty days [104], while a longevity of more than one year has been specified by MacKenzie and Abaunza (1998) and Lester and MacKenzie (2009) for a parasite to be of value in stock determination [43,105].

4.2. Phylogeography of Monogenea with unprecedented resolution

To date, phylogeographic and population genetic work on monogeneans is still relatively uncommon and mostly limited to a small set of single markers [106–108] due to the lack of more mitochondrial markers [88]. Increasing the mitogenomic coverage would facilitate the design of new mitochondrial markers but the number of mitogenomes that is available for dactylogyrid species infecting cichlids is currently still limited. Vanhove et al. (2018) assembled the first mitogenomes for representatives of *Cichlidogyrus*, namely *C. halli* and *C. mbirizei* (near-complete mitogenome) [72]. Caña-Bozada et al. (2021) reported the first mitogenome of *S. longicornis* [73], Zhang et al. (Unpublished results) assembled the mitogenome of *C. sclerosus* [71], and Zhang et al. (2019) described the first mitogenome of *Enterogyrus malmbergi* Bilong Bilong, 1988 [109]. Given that species of *Cichlidogyrus* and *Scutogyrus* are small (in most cases less than 1 mm long), it can be challenging to extract enough genetic material from a single individual for NGS. Therefore, all those mitogenomes were assembled using pooled samples. Disadvantages of pooling are, however, the uneven representation and amplification of each individual in the pool and the erroneous degree of population differentiation inferred from these data [110,111]. Additionally, for the purpose of our study, pooling samples from different localities would preclude their use as a magnifying glass and complicate the assessment of intraspecific diversity parameters such as haplotype diversity [110]. In the present study, we managed for the first time to assemble all twelve mitogenomic PCGs of four dactylogyrid species, obtained from a total of 48 individual worms, allowing us to perform a comparative phylogeography of African monogenean species with unprecedented resolution.

4.3. Parasites as magnifying glass

We find a clear geographic signal for *C. thurstonae* with specimens grouping according to country (Table S9; Fig. 3). The highest genetic differentiation is found between specimens from Zimbabwe and the DRC (Table S9) and the highest genetic diversity is found in the DRC, followed by Cameroon and Zimbabwe (Table 1). This result is also evident in the haplotype network, where a high number of mutational steps is visible between individuals from the DRC (Fig. 3). The significant

differentiation of specimens of *C. thurstonae* between countries suggests the use of different sources of Nile tilapia in aquaculture in each of the countries. The high genetic diversity in the DRC implies the use of multiple strains of Nile tilapia in this country originating from different source populations, a hypothesis also suggested by Jorissen et al. [57] who found a high variety of haplotypes, based on the *COI* gene, of *C. thurstonae* within the Congo Basin. Furthermore, the occurrence of multiple stocking events in the DRC has been suggested by Geraerts et al. (Unpublished results), based on RAD-sequencing data of Nile tilapia [38]. Modern aquaculture originated in Lubumbashi (Upper Congo Basin) during colonial times. Besides culturing native tilapias, Nile tilapia was introduced to meet the growing demand for fish [31,112]. Today, most local fish farms are culturing Nile tilapia, often using multiple aquaculture strains such as 'Chitralada' and 'Chirundu' (pers. comm. *A. Chocha* Manda), confirming the use of different stocks of Nile tilapia. Naturally, the denser sampling in the DRC compared to other countries might provide a distorted view of the real genetic diversity in the different countries. Our results should therefore be validated by a larger sampling size including more specimens from multiple basins.

For the other species included in this study (i.e. *C. halli*, *C. sclerosus*, and *S. longicornis*), no clear patterns are uncovered when grouping the individuals according to country, basin or farming history of their host, though, some findings are in accordance to those of *C. thurstonae*. Specimens of *C. sclerosus* and *S. longicornis* also display significant differentiation between the DRC and Zimbabwe. However, differentiation values are lower than those of *C. thurstonae* (Table S9). Also, for these species, a larger sample size is needed to justify these results. The two pairs of specimens of *C. sclerosus* from the DRC sharing a haplotype are sampled in the same farm (location 13 and 15, respectively), explaining their mitogenomic sequence similarity. The high similarity between a specimen from Zimbabwe (PPZIM137_1) and two from the DRC (PPKAT558_1 and PPKAT562_1) (Fig. 3) strongly suggests a common stocking origin, which is in contrast with the genetic differentiation found between these two countries. Nevertheless, it is possible that one of the aquaculture strains used in the DRC is also used in Zimbabwe. In the haplotype network and phylogenetic trees of *S. longicornis* (Fig. 3; Fig. S5), specimens from Cameroon cluster together. This finding suggests a single source of Nile tilapia used in aquaculture, which is also suggested by the clustering pattern of specimens of *C. thurstonae* and by the parasite community suggesting a Western African origin. In Zimbabwe, two pairs of individuals of *S. longicornis* showed a high haplotype similarity. The first pair includes a parasite from a feral host from Lake Kariba and one from a feral host in the Middle Zambezi Basin (Lake Chivero) (about 250 km apart), and a second pair includes a parasite from a host from a fish farm in Harare and one from a feral host in the Middle Zambezi Basin (Lake Chivero) (about 30 km apart). Our results therefore suggest that introduced fish from local farms escaped into the lake and dispersed throughout the rest of the basin, which is quite possible given the many aquaculture settings surrounding the Zambezi Basin [113,114].

The pairwise genetic differentiation of only *C. thurstonae* is consistently higher between countries, and even between most basins, compared to the genetic differentiation of introduced Nile tilapia in the Congo Basin (F_{st} = 0.0367–0.1306) [38], and Tanzania (F_{st} = 0.0367–0.54758 [35]; F_{st} = 0.01–0.44 [36]), confirming the magnifying glass hypothesis for this parasite-host association. However, the question remains how much more resolution the entire mitogenome provides compared to a single mitochondrial gene. Therefore, as a first step towards understanding the added value of utilising the entire mitogenome over rapidly evolving single markers, the same genetic analyses were repeated using the *COI* and *nad*2 genes, two genes with a high intraspecific genetic variability (Tables S9, S13 and S14; Fig. S6) [57,72,115]. From the results of these analyses, similar patterns appear as when using all PCGs in the analyses e.g. *C. thurstonae* is the only parasite species that shows a clear geographical signal when grouping specimens according to country with the highest genetic differentiation between specimens from Zimbabwe and the DRC (Table S9; Fig. S6), and in the haplotype network of *S. longicornis*, specimens from Cameroon cluster together (Fig. S6). However, neither of these two genes give consistently the same level of resolution as the PCGs as a whole.

As mentioned before, for parasites to act as a magnifying glass, they should have a high degree of host specificity [46,49]. A possible reason for *C. halli*, *C. sclerosus*, and *S. longicornis* to fail as a magnifying glass is the presence of other potential host species. Indeed, in Cameroon, *Sarotherodon melanotheron* Rüppell, 1852 was found to be infected with *C. halli*, a reported host for this parasite species [53]. In the DRC, we sampled *Oreochromis aureus* (Steindachner, 1864), *O. macrochir* (Boulenger, 1912), and *Oreochromis mweruensis* Trewavas, 1983, the first being a reported host for *S. longicornis*, the second for *C. halli*, and the third for *C. halli* and *C. sclerosus*. Finally, in Zimbabwe, *Oreochromis mossambicus* (Peters, 1852) and (a hybrid of) *Oreochromis mortimeri* (Trewavas, 1966) were caught, both potential hosts for *C. sclerosus* and *S. longicornis*, and *O. mortimeri* for *C. halli* (Table S12) [53]. Possibly, species of Monogenea from different host species are genetically differentiated depending on the host they infect, an observation already made by Rahmouni et al. (2021) for a species of *Cichlidogyrus* in Lake Tanganyika [116]. The presence of these other potential hosts may obscure the phylogeographic signal due to the transmission of conspecific parasites of different strains between Nile tilapia and other suitable sympatric hosts, a theory already proposed by Kmentová et al. (2021) [115]. For *C. thurstonae*, no reported suitable hosts were sampled in any of the countries, which might explain why this is the only species suitable as a magnifying glass, and which highlights the importance of host specificity or the absence of other potential hosts as a requisite for a parasite to reveal their hosts' phylogeography.

Our sampling also reveals the presence of Nile tilapia parasites on sympatric tilapia species that were previously unrecorded on these hosts (Table S12), which may imply a host switch from introduced Nile tilapia. Additional research, genetically comparing these parasites with those present on Nile tilapia is needed to validate these potential host switches (outside the scope of the present study).

4.4. Checking for species complexes

A much higher genetic diversity is found for *C. halli* compared to that for the other species (Table 1), even though the number of included specimens was lowest for this species. In the haplotype network, the genetic variation of specimens within a country is sometimes even larger than between countries, suggesting the presence of a species complex. These results are in accordance with the findings of Jorissen et al. (2021), who also found a high degree of intraspecific variation in the *COI* gene of representatives of *C. halli* [57].

In the past, the *COI* gene region has repeatedly been used in the species delimitation of flatworms [88,117–119]. The gap between the intra- and interspecific sequence divergence of this gene, also known as the 'barcoding gap', enables the discrimination of even closely related species [87]. In the present study, a clear barcoding gap is found for *C. sclerosus* (3.5–19.9%), *C. thurstonae* (4.6–18.9%), and *S. longicornis* (1.9–19.4%), but not for *C. halli* (Fig. 4). This lack of a barcoding gap for *C. halli* may be caused by lumping together unrecognised cryptic (morphologically indistinguishable) or pseudocryptic (a posteriori distinguishable) species, and, hence, implies the existence of a species complex [120,121]. This renders *C. halli* as a species complex, awaiting taxonomic revision, unfit to serve as a magnifying glass in phylogeographical studies.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2022.110328.

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