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DNA Barcoding Reveals Occurrence of *Cardiocephaloides* sp. (Digenea: Strigeidae) Infecting the Great Cormorant *Phalacrocorax carbo* (L. 1758) in Mwanza Gulf, Lake Victoria

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Abstract

Cormorants (Pelecaniformes) are widely distributed worldwide, occurring as coastal birds in inland waters and marine environments, and have been implicated in the transmission of some aquatic parasites. As such, the objective of the present work was to investigate the occurrence and morphological variations in *Cardiocephaloides* (Digenea: Strigeidae), parasites of the great cormorant *Phalacrocorax carbo*. The *Cardiocephaloides* specimens used for molecular analysis (DNA barcoding (*cox*1) region) were obtained from the intestines of the great cormorants collected from Mwanza Gulf in Lake Victoria. Morphological examination of *Cardiocephaloides* specimens showed the possible co-existence of four morphospecies belonging to the genus *Cardiocephaloides*. However, detailed analyses of the *cox*1 sequences, phylogeny and haplotypes revealed that all four morphospecies belonged to a single unknown species of *Cardiocephaloides*. This paper provides the first report on the great cormorant *Phalacrocorax carbo*, serving as the definitive host for *Cardiocephaloides* in freshwater systems. The findings also reveal that the diversity of *Cardiocephaloides* in Africa is higher than earlier reported. Moreover, it highlights the need for more research in Tanzania to divulge snail and fish species involved in the life cycles of digenean species occurring in birds and other vertebrates in aquatic environments.

Keywords: Digenean trematodes; Strigeidae; *Phalacrocorax carbo*; *Cardiocephaloides*; *cox*1.

Introduction

Cormorants (Pelecaniformes) are widely distributed throughout Asia, Europe, Australia, Africa and America, occurring as coastal birds in inland waters and marine environments. The great cormorant, *Phalacrocorax carbo* (Phalacrocoracidae), which was the focus of the present study, belongs to a group of common migratory birds that reside in all continents apart from South America and Antarctica (Moravec and Scholz 2016). The birds (*P. carbo*) feed on several fish species, especially cyprinids and percids (Martyniak et al. 1997). Furthermore, due to their close association with aquatic habitats, the great cormorants can potentially play significant roles in the life cycles and dispersal patterns of some parasitic organisms like the digenean trematodes.

The digenean trematodes, particularly members of the genus *Cardiocephaloides* (Family: Strigeidae) are common intestinal parasitic worms of fish-eating birds, and their metacercariae have been found in different organs of fish (Niewiadomska 2002). Although *Cardiocephaloides* species are ubiquitous, abundant and diverse, a complete understanding of their biodiversity is challenging, often due to similarities between species, morphological variability among populations, and cryptic species (Niewiadomska 1996, Botros and Eiler 2016, Achatz et al. 2020). The variability of the parasites' morphological traits is subjective to several factors, including the density and age of individuals and the host species infected (Graczyk 1991, Cribb et al. 2022). The interpretation of the morphological features of *Cardiocephaloides* parasites can also be influenced by the approaches used in the preparation and examination of their samples (Bakke 1988, Niewiadomska 1996). Therefore, an experimental establishment of the life cycles is an indispensable prerequisite for reliably identifying digenean species (Niewiadomska 1996). However, establishing a complete experimental life cycle of trematodes is always challenging and time-consuming, and it is often difficult to obtain the parasite's natural definitive host for experimental investigations. As a result, the taxonomic aspect of *Cardiocephaloides* species remains a complex subject (Achatz et al. 2020).

However, due to their advantages over morphology, DNA techniques (Blasco-Costa and Locke 2017) are increasingly used in the taxonomy of strigeids, including *Cardiocephaloides* species (Uzonnah 2017, Achatz et al. 2020, Vermaak et al. 2021). In some cases, such methods have led to the opportunistic discovery of some genetically distinct but morphologically similar species (Chen et al. 2007, Bray et al. 2022). Despite the increasing use of molecular methods to elucidate the identity and classification of strigeid species elsewhere, such methods have not been deployed in Tanzania.

The present study aimed to investigate the occurrence of *Cardiocephaloides* species from the intestinal tracts of the great cormorant *Phalacrocorax carbo* from the Mwanza Gulf of Lake Victoria, using the cytochrome c oxidase 1 gene (*cox*1). In this study, this gene (cox1) also helped to disentangle the morphological variability of four *Cardiocephaloides* morphotypes recovered from the intestinal tracts of the great cormorant from the Mwanza Gulf.

Materials and Methods

Source of genomic DNA

The *Cardiocephaloides* specimens used for molecular analysis in the present study were obtained from the intestines of 12 great cormorants, *Phalacrocorax carbo*, from the Mwanza Gulf of Lake Victoria (Figure 1), between January and December 2012. Prior to DNA extraction in 2021, the worms were frozen in absolute ethanol. The University of Dar es Salaam issued permission to conduct the study on behalf of the Tanzania Commission for Science and Technology.

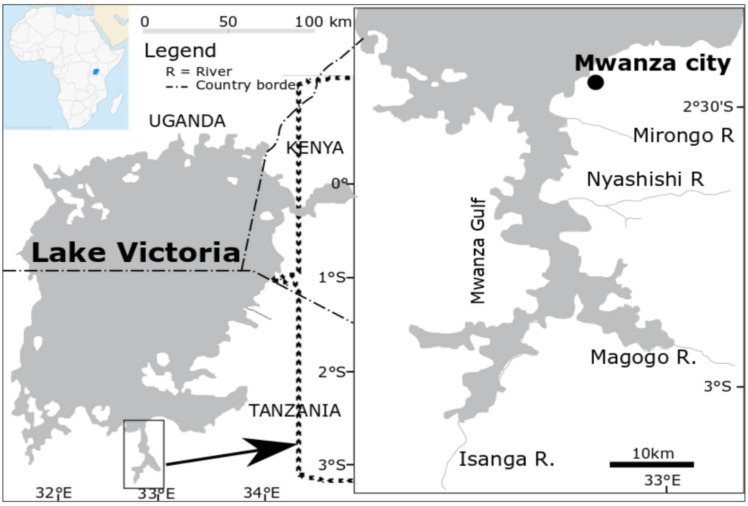

Figure 1: Localities in the Mwanza Gulf in Lake Victoria where the samples of the great cormorant, *P. carbo*, were collected.

Extraction of genomic DNA (gDNA), amplification and sequencing

DNA extraction from *Cardiocephaloides* species specimens (Figure 2) was carried out using the Qiagen DNeasy Tissue and Blood Kits following the manufacturer's protocol (Qiagen 2020, Hilden, Germany). DNA amplification was conducted in the Zoology laboratory at the University of Dar es Salaam using primers MplatCOX1dF/R (Moszczynska et al. 2009). The conditions for PCR amplification were as follows: initial denaturation at 94 °C for 1 min, 5 cycles at 94 °C for 40 sec, 45 °C for 40 sec, and 72 °C for 1 min. This was followed by 35 cycles at 94 °C for 40 sec, 51 °C for 40 sec, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. Finally, sequencing for the barcoding region of cytochrome c oxidase 1 (*cox*1) was carried out at the Incaba Biotec™, South Africa.

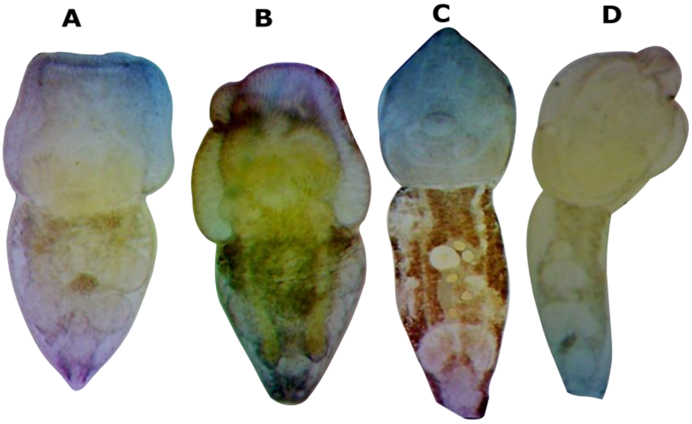

Figure 2: Four morphospecies of *Cardiocephaloides* species that were retrieved from the intestines of the great cormorant, *P. carbo*, in the Mwanza Gulf of Lake Victoria.

Analysis of sequences

BLAST and alignment

Chromatograms of *cox*1 were assembled and edited with Bioedit version 7.0.5.3 (Hall 1999) and adjusted manually as needed. Multiple alignments were carried out with ClustalW tool (Thompson et al. 1994) implemented in BioEdit version 7.0.5.3. The present study produced 17 novel mitochondrial cytochrome c oxidase 1 (*cox*1) sequences of *Cardiocephaloides* sp. specimens recovered from the intestinal tracts of the great cormorant *P.carbo* inhabiting the Mwanza Gulf of Lake Victoria. The novel sequences were compared with each other and with sequences of other *Cardiocephaloides* species accessible in GenBank. More than 600 homologous sequences were retrieved from the NCBI GenBank under the search "Strigeidae and cytochrome c oxidase" and aligned with seventeen (17) novel sequences obtained in this study. However, to improve phylogenies, sequences with less than 300 bp were removed from the alignment (Ranwez and Chantret 2020). The dataset was further reduced to 49 sequences by removing similar sequences of the same haplotype. Table 1 contains information on *Cardiocephaloides* species' sequences retrieved from the GenBank. Furthermore, the novel *cox*1 sequences were deposited in the GenBank database with the accession numbers OP837037-OP837052, and their detail are found in Table 1.

Table 1: Summary data for the isolates of *Cardiocephaloides* sp *cox*1 sequences retrieved from GenBank incorporated in the analysis to identify the present material (A, adult; M, metacercariae).

Species	**Life Cycle stage**	**Host species**	**Locality**	***cox*1 Accession No**	**References**
Cardiocephaloides sp	A	*Phalacrocorax carbo*	Lake Victoria	OP837037	Current study
Cardiocephaloides sp	A	*Phalacrocorax carbo*	Lake Victoria	OP837038	Current study
Cardiocephaloides sp	A	*Phalacrocorax carbo*	Lake Victoria	OP837039	Current study
Cardiocephaloides sp	A	*Phalacrocorax carbo*	Lake Victoria	OP837040	Current study
Cardiocephaloides sp	A	*Phalacrocorax carbo*	Lake Victoria	OP837041	Current study
Cardiocephaloides sp	A	*Phalacrocorax carbo*	Lake Victoria	OP837042	Current study
Cardiocephaloides sp	A	*Phalacrocorax carbo*	Lake Victoria	OP837043	Current study
Cardiocephaloides sp	A	*Phalacrocorax carbo*	Lake Victoria	OP837044	Current study
Cardiocephaloides sp	A	*Phalacrocorax carbo*	Lake Victoria	OP837045	Current study
Cardiocephaloides sp	A	*Phalacrocorax carbo*	Lake Victoria	OP837046	Current study
Cardiocephaloides sp	A	*Phalacrocorax carbo*	Lake Victoria	OP837047	Current study
Cardiocephaloides sp	A	*Phalacrocorax carbo*	Lake Victoria	OP837048	Current study
Cardiocephaloides sp	A	*Phalacrocorax carbo*	Lake Victoria	OP837049	Current study
Cardiocephaloides sp	A	*Phalacrocorax carbo*	Lake Victoria	OP837050	Current study
Cardiocephaloides sp	A	*Phalacrocorax carbo*	Lake Victoria	OP837051	Current study
Cardiocephaloides sp	A	*Phalacrocorax carbo*	Lake Victoria	OP837052	Current study
Cardiocephaloides medioconiger	A	*Thalasseus maximus*	USA	MH581272	Locke et al. 2018
Cardiocephaloides medioconiger	A	*Thalasseus maximus*	USA	MH581273	Locke et al. 2018
Cardiocephaloides medioconiger	A	*Thalasseus maximus*	USA	MH581274	Locke et al. 2018
Cardiocephaloides medioconiger	A	*Thalasseus maximus*	USA	MH581946	Achatz et al. 2020

Species	Life Cycle stage	Host species	Locality	*cox*1 Accession No	References
Cardiocephaloides physalis	A	*Larus argentatus*	Ukraine	MN817944	Achatz et al. 2020
Cardiocephaloides physalis	A	*Larus argentatus*	Ukraine	MN817945	Achatz et al. 2020
Cardiocephaloides physalis	A	*Spheniscus magellanicus*	Chile	MN817947	Achatz et al. 2020
Cardiocephaloides physalis	M	*Spheniscus demersus*	South Africa	MW365507	Vermaak et al. 2021
Cardiocephaloides physalis	M	*Clinus superciliosus*	South Africa	MW365508	Vermaak et al. 2021
Cardiocephaloides physalis	M	*Clinus superciliosus*	South Africa	MW365509	Vermaak et al. 2021
Cardiocephaloides physalis	M	*Clinus superciliosus*	South Africa	MW365510	Vermaak et al. 2021
Cardiocephaloides sp.	M	*Clinus superciliosus*	South Africa	MW365513	Vermaak et al. 2021
Cardiocephaloides sp.	M	*Clinus superciliosus*	South Africa	MW365522	Vermaak et al. 2021
Cardiocephaloides sp.	M	*Clinus cottoides*	South Africa	MW365525	Vermaak et al. 2021

Reconstruction of phylogenetic relationships

The phylogenetic tree of 448 bp *cox*1 was estimated using Maximum Likelihood (ML) and Bayesian Inference (BI) analyses from 33 nucleotide sequences. *C. marcogliesei* (MH581279) was used as an outgroup. The best substitution models were estimated using jModelTest version 2.1.4 (Posada 2008). The Akaike's information criterion (AIC), Corrected Akaike's information criterion (AICc) and Bayesian Information Criterion (BIC) selection models selected Hasegawa-Kishino-Yano modelled using a discrete Gamma distribution (HKY+I+G) as the best substitution model for *cox*1. ML analysis was conducted using MEGA X (Kumar et al. 2018), bootstrapping at 1000 replicates. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The initial trees for the heuristic search were automatically obtained by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with a superior quality log-likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.1572)). All positions containing gaps and missing data were eliminated. Bayesian inference analysis was estimated using MrBayes (Ronquist et al. 2012). Runs consisted of 800000 MCMC generations, sampling every 100th tree. The run was stopped when the average standard deviation of split frequencies for runs 1 and 2 were 0.008462 and 0.008475, respectively. Tracer 1.5 (Rambaut and Drummond 2009) was used to validate the convergence and mix to ensure that all effective sample size (ESS) values were greater than 200. The trees were summarised using samp burnin = 4001 and sumt burnin = 4000, i.e., the burn-in of 50% was used to discard the initial trees.

Results

Occurrence of *Cardiocephaloides* sp in the Mwanza

Out of 12 cormorants examined, 5 were infected with *Cardiocephaloides* sp, making a prevalence of 41.67 %. The minimum and maximum intensities were 1 and 104, respectively. The approximated mean intensity was 50 individuals per infected bird.

*Cox*1 *sequence variation*

The average sequence length was 545 bp (390–635 bp). Out of the 545 positions in the final dataset analysed, only 13 were variable. Among the *cox*1 sequences, the mean intraspecific divergence between species was, on average, 0.00% (range, 0.00% to 0.01%). The pairwise p-distances among the CO1 sequences within the studied trematodes are presented in Table 2. On the other hand, the interspecific variations between sequences of cox1 for *Cardiocephaloides* sp. examined in this study and their congeners were as follows: *Cardiocephaloides* spOK. (11.35–11.75%), C. *medioconiger* (13.75–14.75%), *C. longicollis* (13.35–14.24%) and *C. physalis* (10.91–12.35%).

Furthermore, DNA polymorphism of the novel 17 sequences showed ten (10) haplotypes with haplotype (gene) diversity, Hd: 0.868 ± 0.070, and variance of haplotype diversity of 0.00495. Similarly, network analysis showed low genetic variation in the *cox*1 of *Cardiocephaloides* sp among bird hosts, *P. carbo* (Figure 3). The haplotype frequency distributions of *Cardiocephaloides* sp among bird hosts revealed random infection in *P. carbo*. For instance, bird no. 3 and 9 were infected with more than one haplotype, and parasites identified from bird no. 18 had different haplotypes. Fewer mutation steps indicate negligible differences between one haplotype and the most closely related variant. In other words, the considerable overlap in the haplotypes found in various bird hosts showed little or no variation among the parasites infecting *P. carbo*. These results suggest that the trematodes investigated in this study belong to the same population despite the variability in morphology.

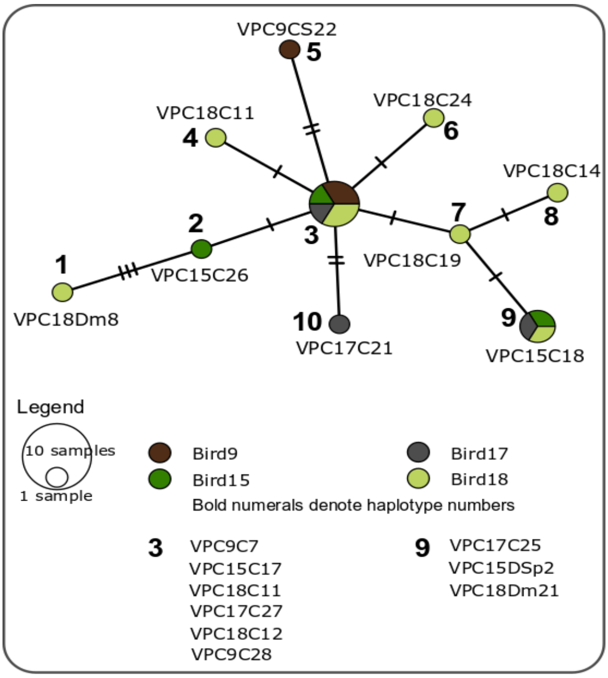

Figure 3: *cox*1 sequences haplotype network for *Cardiocephaloides* sp. isolated from different *P. Carbo* individuals inhabiting the Mwanza Gulf of Lake Victoria. Each mutational step is represented by a single mutational variation and connective lines; circle sizes are proportional to the number of isolates sharing a haplotype. Numbers represent the haplotype code number.

Table 2: Estimates of intraspecific evolutionary divergence between sequences of *Cardiocephaloides* species. Analyses were conducted using pairwise p-distances, which involved 17 nucleotide sequences. All positions containing gaps and missing data were eliminated (pairwise deletion option). The final dataset consisted of 545 positions.

	28	**7**	**22**	**17**	**26**	**18**	**27**	**25**	**21**	**8**	**11**	**12**	**23**	**14**	**19**	**20**	**24**
VPC9C28	0.000																
VPC9C7	0.000																
VPC9C22	0.004	0.004															
VPC15C17	0.000	0.000	0.004														
VPC15C26	0.002	0.002	0.006	0.002													
VPC15C18	0.004	0.004	0.007	0.004	0.006												
VPC17C27	0.000	0.000	0.004	0.000	0.002	0.004											
VPC17C25	0.004	0.004	0.007	0.004	0.006	0.000	0.004										
VPC17C21	0.004	0.004	0.007	0.004	0.006	0.007	0.004	0.007									
VPC18C8	0.007	0.007	0.011	0.007	0.006	0.011	0.007	0.011	0.011								
VPC18C11	0.002	0.002	0.006	0.002	0.004	0.006	0.002	0.006	0.006	0.009							
VPC18C12	0.000	0.000	0.004	0.000	0.002	0.004	0.000	0.004	0.004	0.007	0.002						
VPC18C23	0.000	0.000	0.004	0.000	0.002	0.004	0.000	0.004	0.004	0.007	0.002	0.000					
VPC18C14	0.004	0.004	0.007	0.004	0.006	0.004	0.004	0.004	0.007	0.011	0.006	0.004	0.004				
VPC18C19	0.002	0.002	0.006	0.002	0.004	0.002	0.002	0.002	0.006	0.009	0.004	0.002	0.002	0.002			
VPC18C20	0.004	0.004	0.007	0.004	0.006	0.000	0.004	0.000	0.007	0.011	0.006	0.004	0.004	0.004	0.002		
VPC18C24	0.002	0.002	0.006	0.002	0.004	0.006	0.002	0.006	0.006	0.009	0.004	0.002	0.002	0.006	0.004	0.006	0.000

Phylogenetic relationships

Both maximum likelihood (ML) and Bayesian inference (BI) phylogenetic trees reconstructed from the *cox*1 region strongly confirmed the occurrence of a single species of *Cardiocephaloides* occurring in the intestinal tracts of piscivorous birds, *P. carbo*, from Mwanza Gulf in Lake Victoria area. The combined phylogenetic tree (Figure 4) shows two main clades (ML 100%, BI 1); Clade I (CLI): represents *Cardiocephaloides* species recovered from *P. carbo* in Lake Victoria. This clade consists of a monophyletic population of a single species. Clade II (CLII): embodies the lineage of *Cardiocephaloides* species from elsewhere recovered as adults from birds or as metacercariae from fishes. Clade II exhibited two further branches, explicit enough to separate the subclades into individual species, i.e., *Cardiocephaloides medioconiger*, *C. longicolis*, *C. physalis* and an unknown *Cardiocephaloides* species from fish *Clinus superciliosus* (Figure 4).

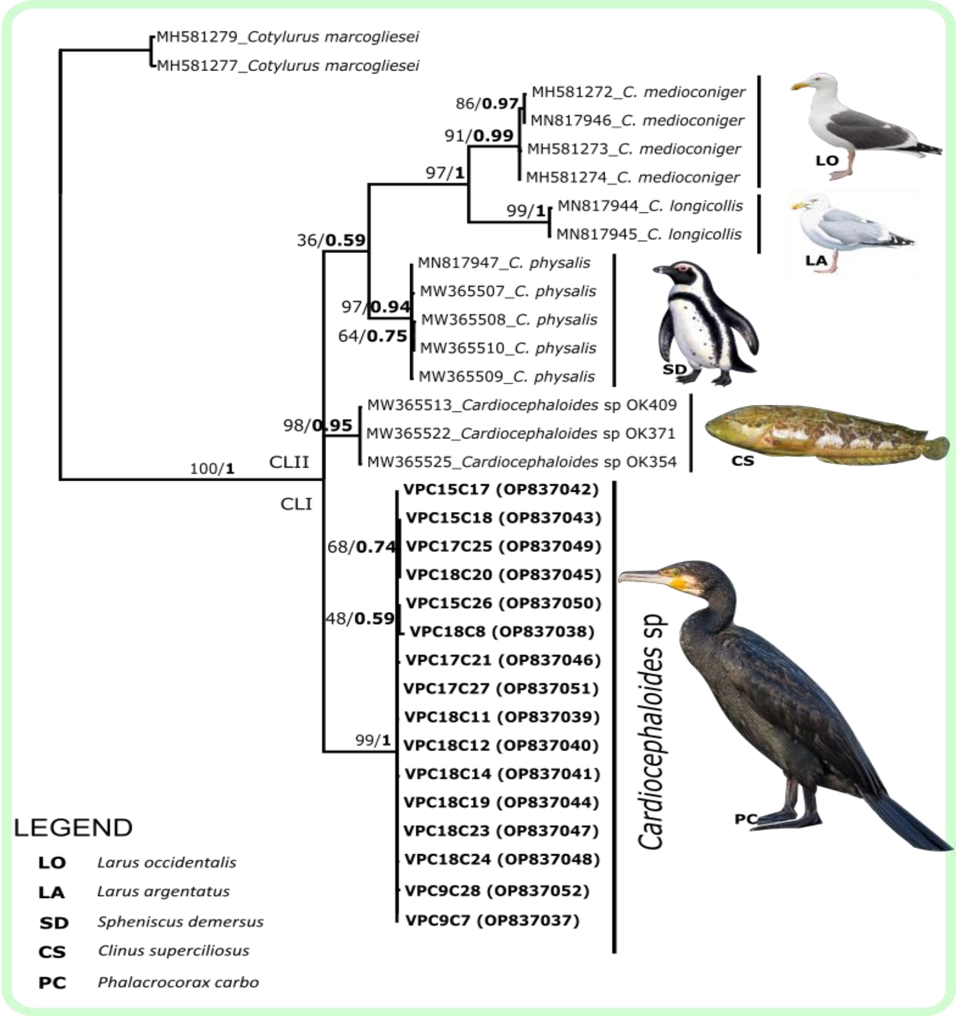

Figure 4: A combined Maximum Likelihood (ML) Bayesian Inference (BI) tree showing an evolutionary relationship for *Cardiocephaloides* sp. infecting the great cormorant, *P. carbo* in Lake Victoria, and their congeners from other hosts elsewhere. Branch supports represent ML (unbolded (%)) and BI (bolded in proportions). Within the phylogeny, the names of the specimens collected in the present study and their GenBank accession numbers are presented in bold.

Discussion

The present study has revealed the occurrence of *Cardiocephaloides* species in the great cormorant *Phalacrocorax carbo* in Lake Victoria. Although species of the genus *Cardiocephaloides* are ubiquitous in marine systems, for instance, their adults have been reported from penguins and seagulls (Horne et al. 2011, Botros and Eiler 2016, Achatz et al. 2020), *Cardiocephaloides* species have never been recorded as adults in freshwater systems of Africa. The occurrence of *Cardiocephaloides* species from *P. carbo* in Lake Victoria is, therefore, regarded as the first record of the genus in great cormorants in the freshwater systems of Africa. However, trematodes surveys elsewhere have reported *Cardiocephaloides* species existing as encysted metacercariae in various organs of fish (Niewiadomska 2002, Chaudhary et al. 2021, Vermaak et al. 2021). As such, a better understanding of the transmission dynamics and pathology of *Cardiocephaloides* sp recovered in this study requires extensive studies of the occurrence, diversity and distribution of *Cardiocephaloides* sp in all potential hosts in the entire Lake Victoria ecosystem.

The present study has also demonstrated that molecular markers, such as cytochrome c oxidase 1 (*cox*1), can be extremely useful in differentiating digenetic flukes. Previously, the barcode region has been used successfully in studying species diversity (Chibwana et al. 2013, Locke et al. 2015, Chibwana and Katandukila, 2021), life cycles (Chibwana et al. 2015, Blasco-Costa and Locke 2017), cryptic species (Georgieva et al. 2014, Cribb et al. 2022) and parasites community composition (Désilets et al. 2013, Locke et al. 2013). In the present study, the *cox*1 region precisely identified the trematodes infecting the cormorant *P. carbo,* in Lake Victoria as belonging to the genus *Cardiocephaloides* and refuted the conclusions previously made by Chibwana (2014) that there were four *Cardiocephaloides* species infecting *P. carbo*. Instead, all the morphotypes identified as distinct species have been confirmed as a single unidentified *Cardiocephaloides* sp.

Cardiocephaloides Sudarikov 1959 is a small digenean genus within the family Strigeidae Railliet 1919, comprising seven species such as *C. longicollis*, *C. hilli*, *C. physalis*, *Cardiocephaloides brandesii*, *C. medioconiger*, *C. megaloconus*, and *C. ovicorpus* (Vermaak et al. 2021). In Tanzania, an adult stage of *Cardiocephaloides* species has been recovered from the intestines of common cormorant *P. carbo* in Lake Victoria. Morphological and molecular analyses (*cox*1 mtDNA) have revealed that the specimens from *P. carbo* in Tanzania belong to an unidentified species of *Cardiocephaloides*. Therefore, the present study provides the first DNA sequences for *Cardiocephaloides* species from Tanzania. Based on DNA sequence data for the barcoding gene (*cox*1), the identity of this species is still unknown due to either belonging to a known species whose cox1 sequences are yet to be submitted to GenBank and bold systems or being a new species hitherto undescribed.

The interspecific variation between sequences of *cox*1 for *Cardiocephaloides* sp. reported in this study, and their congeners were as follows: *Cardiocephaloides* sp. (11.35–11.75%), *C. medioconiger* (13.75–14.75 %), *C. longicollis* (13.35–14.24 %) and *C. physalis* (10.91–12.35 %). These interspecific pairwise nucleotide variations of partial cox1 sequences provided in this study are much higher than previously reported comparisons for *C. medioconiger*, *Cardiocephaloides* sp. (JX977784), *C. medioconiger*, *C. longicollis* and *C. physalis*, which ranged 8.7–11.8% (Achatz et al. 2020). In addition, the observed lower intraspecific variability among the CO1 sequences of different isolates of *Cardiocephaloides* sp. suggests the isolates recovered in the present study belong to one species. Thus, these findings demonstrating a low intraspecific variation in *cox*1 sequences within *Cardiocephaloides* species and high interspecific variations between species is an indication of a novel species. This study also corroborates previous reports that have demonstrated the inability of *Cardiocephaloides* species to exhibit high genetic intraspecific variability (beyond 0.4 – 0.9%) regardless of sampling distance (Achatz et al. 2020, Vermaak et al. 2021).

The observed evolutionary distance between Tanzanian species and their congeners from elsewhere is low, suggesting non-missing data within the evolutionary radiation. The presence of *cox*1 data of *Cardiocephaloides* species from different hosts in the United States of America (North America), Ukraine (Europe) and Chile (South America) (Table 1) coupled with a highly supported tree topology (Figure 4) further suggests a recent monophyletic relationship of *Cardiocephaloides* species irrespective of their geographical distance. The specialisation of parasites on different hosts could lead to divergence and speciation (Summers et al. 2003). Therefore, the small evolutionary distance observed between material from Tanzania and their congeners might be attributed to the lack of specialisation of trematodes in second intermediate and final hosts.

Conclusion

The occurrence of *Cardiocephaloides* sp. from the great cormorant *P. carbo*, in the Mwanza Gulf of Lake Victoria, Tanzania, shows the diversity of digenean trematodes in the freshwater bodies of Tanzania is potentially high. The DNA barcoding gene, cytochrome c oxidase 1 region (cox1), has proved useful in this study. If supplemented with morphological information, the sequencing of the *cox*1 gene can serve as a tool for specialists and non-specialists to identify unknown specimens and discriminate cryptic species or individuals of the same species with different morphological appearances (Hebert et al. 2003). It is, therefore, implied here that morphological attributes alone without molecular data in studies of occurrence, taxonomy, distribution, life cycles of trematodes and ecosystem biodiversity can mislead respective interpretations and conclusions.

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