PHYLOGENETIC RELATIONSHIPS OF THE METAZOAN PARASITES OF THE CLARIID FISHES OF LAKE VICTORIA INFERRED FROM PARTIAL 18S rDNA SEQUENCES

CJ Mwita[†] and G Nkwengulila[2]
[†] Departments of Aquatic Sciences and Fisheries and [2] Zoology and Wildlife Conservation, College of Natural and Applied Sciences, P.O. Box 35064, University of Dar Es Salaam
E-mail: mwitachacha@udsm.ac.tz,
Telephone: 255 22 2410462/255 22 2650011, Fax: 255 22 2410480/2650244

ABSTRACT

*Phylogenetic relationships among twenty two metazoan parasites recovered from seven species of clariid fish from Lake Victoria were analysed using partial 18S rDNA sequences. The 18S rDNA gene was amplified by polymerase chain reaction, directly sequenced, aligned and phylogenies inferred using maximum parsimony. Heuristic bootstrap MP searches yielded one most parsimonious tree (CI = 61%, HI = 39%), which showed that clariid parasites are monophyletic at higher taxonomic levels (Cestodea, Nematodea, Digenea and Crustacea). However the position of two trematodes (*Allocredium mazoensis *and* Clinostomum sp.*) and a nematode (*Contracaecum sp.*) were not stable. Despite the present findings, the study utilised single species from each taxa, hence further analysis with additional sequences from a multitude of species is recommended to further resolve the phylogeny of the parasites of the clariids in Lake Victoria and elsewhere.*

Key words: Clariid's parasites phylogeny, Lake Victoria, Tanzania.

INTRODUCTION

The Clariidae belong to a group of catfishes that exploit a wide range of habitats from streams, rivers and freshwater lakes (Eccles, 1992; Agnése and Teugels 2005). Due to their catholic exploitation of habitats, the clariid catfishes are often infected by a variety of metazoan parasites (Mwita and Nkwengulila, 2004; Barson *et al.* 2008; Mwita and Nkwengulila, 2008), inhabiting a wide range of tissues and organs in their hosts (Williams and Jones 1994, Mwita and Nkwengulila, 2004). Among the clariids, the parasites of *Clarias gariepinus* are the best studied (Mwita and Nkwengulila 2004) and those of other clariid species particularly in Lake Victoria are poorly known, let alone their phylogenetic relationships.

With the advent of molecular systematic methods, phylogenetic studies have been undertaken in a number of fish parasites particularly from the Northern hemisphere (Jovelin and Justine 2001). However, little agreement exists on the relationships among the different groups of parasites, partially because of the poor representation of some groups (Campos *et al.* 1998) and difficulties in identifying parasites to species level at all stages of their life cycle (Chappell *et al*. 1994; Niewiadomska, 1996; Galazzo et al. 2002). The host-induced variations are made worse when parasites for phylogenetic studies are collected from a range of species (Campos *et al*. 1998), which occupy a wide range of geographical habitats (Hansen et al., 2003).

The present study is a link to the previous one (Mwita and Nkwengulila 2008) on the parasites of the clariid catfishes of Lake Victoria from which a total of 32 parasite species were documented from 658 specimens of clariid fishes representing seven different species. Of the parasites recovered only 16 were identified to species level, partially due to the reasons stipulated above as well as artefacts produced during

fixation (Chappell *et al*. 1994). Studies have shown that DNA-approaches provide ways of distinguishing between species when morphological criteria are ineffective (Mariaux, 1998, Galazzo *et al*. 2002). Molecular characters also complement morphological traits to reconstruct phylogenetic relationships among organisms (Carreno and Nadler, 2003). The present study thus aimed at utilizing the 18S rDNA gene to elucidate the phylogenetic relationships of the common parasites of the clariid catfishes in Lake Victoria for two major reasons: (1) the 18S rDNA gene is widely used in phylogenetic related works particularly those involving parasites, hence suited for comparative studies, and (2) like cytochrome b, part of the 18S rDNA is known to evolve fast enough to permit its use in resolving recent evolutionary history.

MATERIALS AND METHODS

Study Area

Lake Victoria, the largest tropical lake in the world, is shared between Tanzania, Uganda and Kenya. The lake lies in a shallow continental sag between the two arms of the Great Rift Valley, 1170 m above sea level. The lake has a maximum depth of 84 m, a volume of 2750 km^3, and a surface area of 68,800 km^2. Primary inflows to the basin include rivers such as the Kagera in the west and the Mara in the east. All outflows are to the north along the Nile through Lake Kyoga. The mean surface temperature is about 25 ^{o}C while the temperature of deeper layers is about 1 to 2 degrees lower (Witte &Van Densen 1995). The present study covered the surroundings of Bukoba town, the Mwanza Gulf, Speke Gulf, parts of Ukerewe Island and the delta of Mara River (Fig. 1).

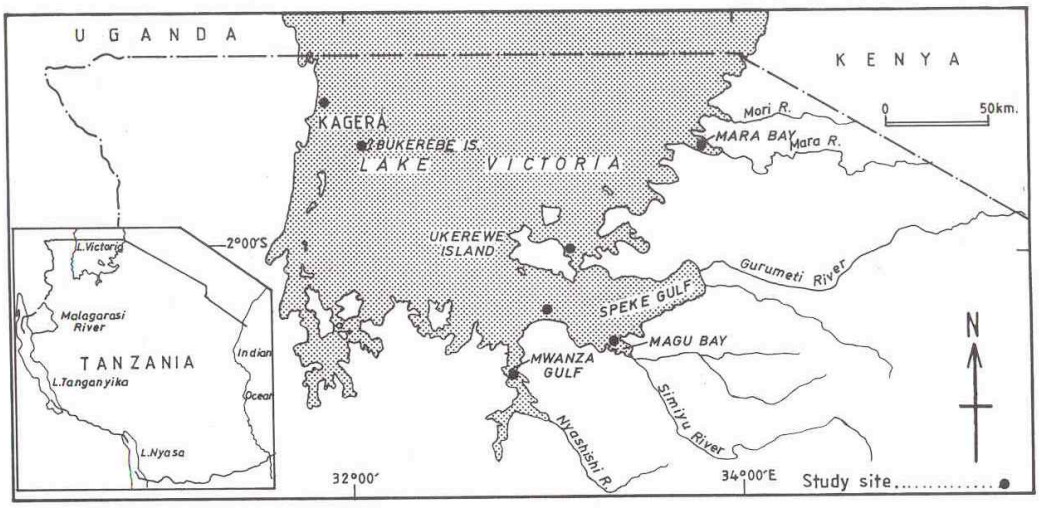

Figure: A map of Lake Victoria showing the study sites

METHODS

Fish collection, examination for parasites and identification followed standard procedures (Anderson 1992, Khalil *et al*. 1994 and Gibson *et al*. 2002). Parasite samples for DNA analysis were initially soaked in a 2 ml vial containing 95% ethanol and left to stand for 1 hr, then transferred into another similar vial with fresh 95% ethanol and stored at 4 ^{o}C until required for DNA extraction. A total of 22 parasites species (identified to genus or species level) with sufficient biomass were considered for DNA analysis (Table 1).

Table 1: Parasite species analysed in the present study.

Family/Species	Acc.no	Host
Nematoda		
Paracamallanus cyathopharynx	DQ813445	C. werneri
Neogoezia sp.	DQ813444	*C. liocephalus*
Procamallanus laevionchus	DQ813446	C. alluaudi
Spinitectus petterae	DQ813447	*C. werneri*
Quimperia sp.	DQ813448	*C. liocephalus*
Rhabdochona congolensis	DQ813457	*C. gariepinus*
Contracaecum sp.	DQ813456	C. gariepinus
Trematoda		
Diplostomum mashonense	DQ813458	*C. gariepinus*
Tylodelphys sp.1	DQ813454	*C. gariepinus*
Tylodelphys sp.2	DQ813455	*C. gariepinus*
Eumasenia bangweulensis	DQ813461	C. liocephalus
Astiotrema reniferum	DQ813459	*C. gariepinus*
Astiotrema sp.	DQ813460	*C. gariepinus*
Phylodistomum folium	DQ813462	*B. docmac*
Allocredium mazoensis	DQ813450	C. gariepinus
Clinostomum sp.	DQ813463	*C. alluaudi*
Monobothrioides woodlandi	DQ813449	*C. werneri*
Cestoda		
Proteocephalus sp.1	DQ813465	*C. gariepinus*
Proteocephalus sp.2	DQ813451	*H. longifilis*
Polyonchobothrium clarias	DQ813464	*C. liocephalus*
Crustacean		
Argulus monody	DQ813452	*H. longifilis*
Dolops ranarum	DQ813453	*C. gariepinus*

DNA Extraction

DNA extraction was performed with one/individual specimen, for the large worms such as nematodes, cestodes, crustaceans and leeches, and with several specimens for smaller worms like digeneans. Total genomic DNA was obtained with 20 µl of proteinase K in 180 µl of extraction buffer (Qiagen Inc. Mississauga, Ontario) incubated for 4 to 5 hours in a heating block at 56 ^{o}C with intermittent shaking. Samples were then heated to 70 ^{o}C for 10 minutes and total gDNA extracted by the Qiagen column (Qiagen Inc. Mississauga, Ontario) according to manufacturer's protocol and kept frozen until further analysis.

PCR Amplification

Amplification of 18S ribosomal DNA fragments from all parasites was carried out in an Eppendorf Mastercycler Thermal cycler using primers JLR24, 5' –CGG AAT TCG CTA GAG GTG AAA TTC TTG G-3' and JLR25, 5'-CCG AAT TCC GCA GGT TCA CCT ACG G-3' as described by Campos *et al.* (1998). The DNA amplification reactions contained 2.5 µl 10x PCR buffer (4.0 mM $Mgcl_2$), 0.5 µl of 10 mM dNTPs, 1µl of each primer (10 mM),

1.0 U HotStar *Taq* polymerase and 5 µl gDNA in a total of 25 µl reaction volume.

PCR conditions varied slightly depending on the type of worms; annealing temperature was 48 °C for the nematodes, crustaceans and hirudineans, and 52 °C for the flatworms. Generally however, the PCR reactions were characterised by an initial denaturation for 15 min at 95 °C, followed by 40 cycles at 93 °C for 30 sec, 48 °C/52 °C for 90 sec, 72 °C for 2 min, with a final extension at 72 °C for 5 min. Samples were then held at 4 °C. The PCR fragments were then run through an electrophoresis using 2% agarose gel with ethidium bromide and visualised under UV light. PCR products were then purified using the QIAquick PCR purification kit (Qiagen) following the manufacturer's protocols (Qiagen Inc. Mississauga, Ontario).

Sequencing and Sequence Analysis

DNA was sequenced with the dideoxy-termination method with fluorescent-labelled primers using an ABI 377 Prism Automated DNA Sequencer. Both strands were sequenced using each of the two initial PCR primers, JLR24 and JLR25. The sequencing products were then purified via isopropanol precipitation and snap cooled before being subjected to sequencing electrophoresis reaction. The automated sequence data were analyzed using the Sequencher v. 3.0 software (Gene Codes Corporation, Inc.). Chromatograms were visually inspected and consensus sequences were aligned manually prior to further analysis (Plate 1).

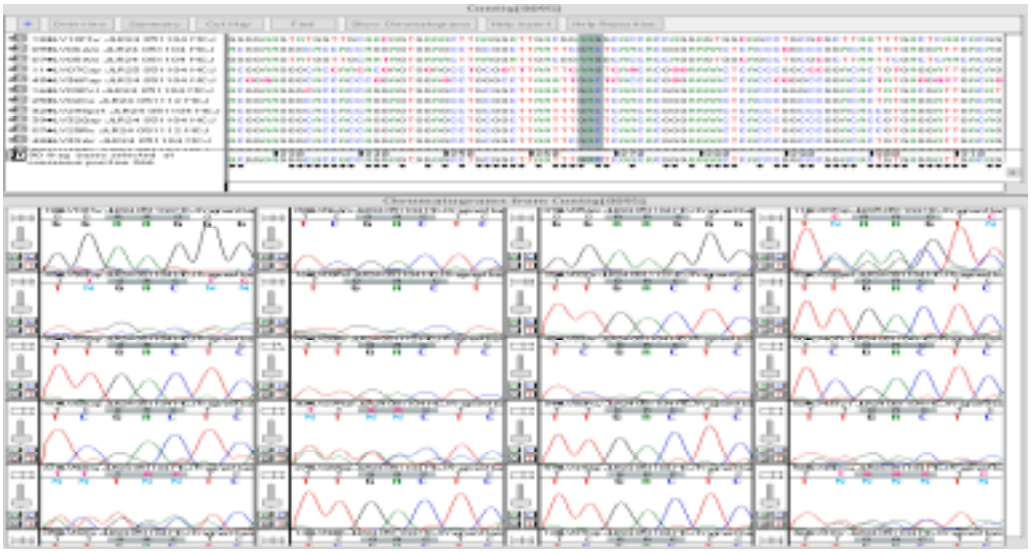

Plate 1: Chromatogram for the clariid parasites DNA sequences from automated DNA Sequencher

Phylogenetic relationships were inferred using the neighbour-joining method (NJ) based on the Hasegawa, Kishino and Yano (1985) [HKY85] distances that estimate a transition/transversion ratio and base frequencies. The minimum evolutionary (ME) rate assumed gamma distribution with a shape parameter of 0.5. Starting tree(s) were obtained via neighbour joining with tree-bisection-reconnection (TBR) branch swapping algorithm. Maximum parsimony (MP) searches were performed using

heuristic method with TBR. Gaps were treated as missing, multistate taxa interpreted as uncertainty and starting tree obtained via stepwise addition with addition sequences obtained randomly in a 10x replicates. All the analysis were performed as implemented in PAUP* v.4.b10 for Macintosh (Swofford 2000). Reliability of each clade was assessed statistically using 1000 replicates of bootstrap resampling (Felsenstein 1985).

RESULTS

The 18S rDNA fragments analyzed consisted of 530 nucleotide characters long of which 133 characters were constant. 180 variable characters were parsimonious uninformative and 217 characters were parsimonious informative. Nucleotide frequencies were relatively equal in distribution such that A (25.2%), C (22.3%), G (28.4%) and T (24.1%), the lowest sequence divergences observed among the nematodes was 0.95% and *Monobothrioides woodlandi* was arbitrarily used as an outgroup due to difficulties in obtaining a proper outgroup for a study involving all groups of parasites.

Heuristic MP searches yielded one most parsimonious tree (CI = 61%, HI = 39%) (Fig. 2), the topology of which did not conflict much with that of the NJ tree (Fig. 3). In both the MP and NJ trees all the parasites analyzed formed monophyletic clades at the higher taxonomic level such that cestodes, nematodes, digeneans and crustacean were each recovered as a separate clade supported by high bootstrap values above 50% (Figs. 2 and 3).

The MP tree (Fig. 2) excluded *A. mazoensis*, *Clinostomum* sp., *D. mashonense* and *Tylodelphys* sp. 1 and 2 from the clade containing the trematodes, and *Contracaecum* sp. from the clade with the nematodes. On the other hand, NJ searches yielded a tree that separated each parasite into its respective higher taxon i.e. nematodes, cestodes, trematodes and crustaceans. However, this tree slightly differed from the MP tree on the position of *Clinostomum* sp. (Trematoda), which is excluded from a clade containing the trematodes (Fig. 3).

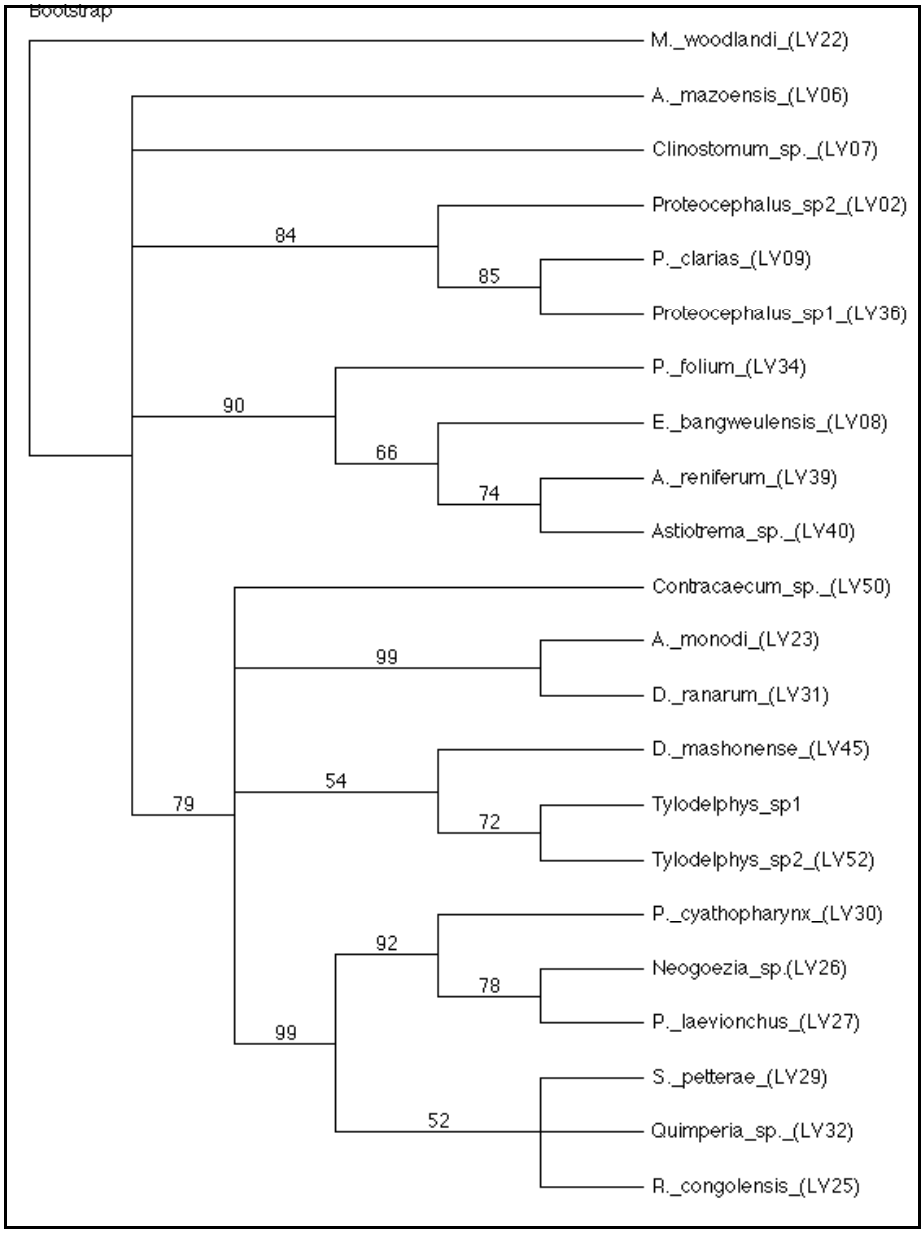

Figure 2: Consensus tree based on maximum parsimony (MP) with 1000 replicates bootstrap values (numbers in branches).

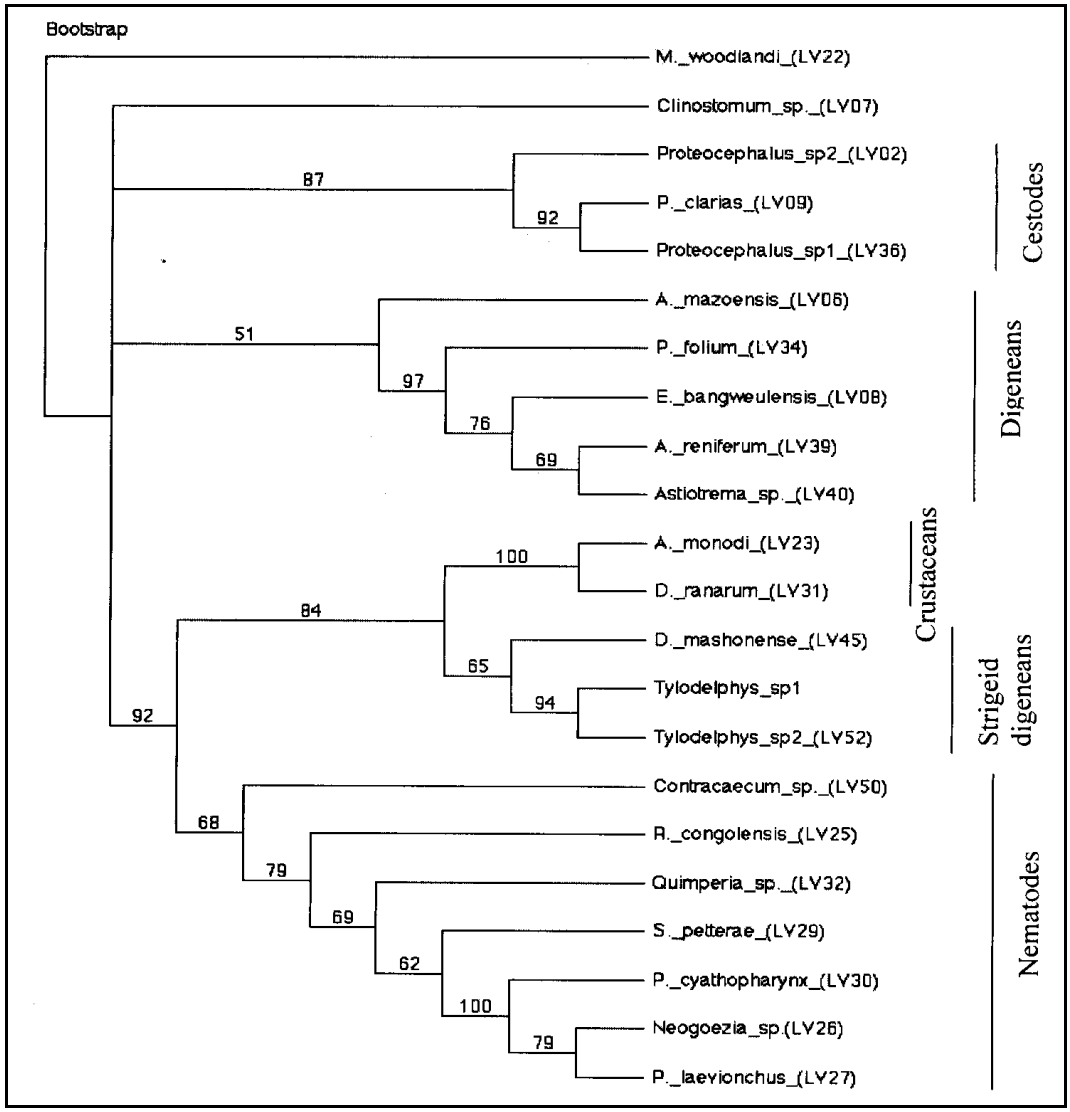

Figure 3: A consensus tree based on the Neighbour Joining (NJ) method with bootstrap values indicated in branches (1000 replicates).

DISCUSSION

The relationships among the parasites infecting fish of the family Clariidae of the Lake Victoria basin were deciphered by partial 18S rDNA gene sequences. The use of the 18S rDNA gene was based on its conserved nature and potential to provide many informative characters for comparative analysis (Carreno and Nadler 2003). Based on the said gene the present analysis was able to separate the four major parasite taxa infecting the clariid fish species from the Lake Victoria basin. The cestodes, trematodes, crustaceans and nematodes each emerged as an independent group.

Despite the rise in the number of phylogenetic studies on the invertebrates

groups (Brooks 1979, Brooks and McLennan 1993, Olson *et al*. 2001), very few have investigated groups comprising of flatworms (Platyhelminthes), roundworms (nematodes) and crustaceans in one study (Campos *et al*. 1998). Few still analyzed such groups from closely related host species such as fish from the family Clariidae as investigated in the present study (Barson *et al*. 2010). The majority of studies analyzed the so called composite communities of parasitic and free-living invertebrates from different forms of life such as birds, mammals and fish (Olson *et al*. 2001). Nevertheless, despite such a wide variation the results of the present study closely agreed with previous investigators whom utilized the 18S rDNA to resolve the phylogenetic relationships of various forms of parasites (Ehlers, 1986; Campos et al., 1998) at least at the higher organizational level.

The results of the present study showed that trematodes infecting the clariids in the Lake Victoria basin are not monophyletic but comprise of three independent groups namely; clinostomidae, digeneans and diplostomids. In the NJ and MP trees Clinostomidae and Allocreadiidae (*A. mazoensis*) are placed close to the cestodes. This is not surprising as both cestodes and trematodes belong to the same phylum – the Platyhelminthes (Campos *et al*. 1998, Olson *et al*. 2001, Littlewood 2006).

Most of the digeneans analyzed in the present study share a common body form with regard to arrangement of internal structures such as position of the oral sucker, ventral sucker, posterior location of the egg filled uterus and the presence of the vitelline follicles (Gibson *et al*. 2002, Mwita 2006). These structures and their respective arrangements form an important feature in the classification of digeneans, hence therefore, may also indicate the possibility of common or closely related ancestors among the trematodes as revealed by the results in this study.

The above body form however, slightly differs from that of diplostomids, in that the body of a diplostomid is divided into the dorsoventrally flattened anterior region and the conical hind body. In addition to the ventral sucker, the diplostomids have a tribocytic or Brandes organ located in the forebody, posterior to the ventral sucker, near the border with the hind body. Gonads are located in the hind body as opposed to those of other trematodes arranged in tandem just behind the ventral sucker. Differences in body morphology therefore, are key factors in separating trematode groups.

The diplostomid body form is quite distinct from that of a dorsoventrally flattened crustacean (Argulus and Dolops species), the dorsum of which is covered by a carapace and the ventral side with paired appendages with hair-like projections for swimming (Mwita 2006). The close genetic relationship observed for these two groups is highly unexpected. The two groups however, separated independently in the MP tree.

The families Diplostomidae and Clinostomidae analyzed in the present study comprise a group of allogenic parasites that utilise the clariid fishes as secondary intermediate hosts before maturing in fish eating birds (Paperna, 1980; Williams and Jones 1994). The other group of monophyletic trematodes analysed are autogenic parasites of the Clariidae and other fish families (Williams and Jones 1994). Whether these life forms (allogenic or autogenic) are partially or wholly controlled by the 18S rDNA gene, such that a trematode is adapted to life in a secondary fish host and later identifies a fish eating bird as a final host, requires further investigations. Similar results were observed by Campos et al. (1998) where the Schistosomatidae, Diplostomidae and Fellodistomidae were separated as a monophyletic clade.

Trees estimated in the present study strongly supported monophyletic relations among the nematode families infecting the clariid fish species of Lake Victoria. In the NJ tree, *Contracaecum* sp. was in the most basal position among the nematodes analysed. However, this was not the case for the MP tree in which *Contracaecum* sp. was located basal to the rest of the groups including Diplostomidae and crustaceans. Poor resolution with respect to the phylogenetic relationship of *Contracaecum* sp. among the nematodes has been reported by (Nadler and Hudspeth, 1998, Szostakowska and Fagerholm 2007).

The MP tree divided the nematodes into two groups; the Camallanidae / Heterocheillidae (Anisakidae) and the Rhabdochonidae / Quimperidae. Like the trematodes, the nematodes differ in certain aspects of their morphology, the Camallanidae, for instance, have an elongate oesophagus divided into anterior muscular and posterior glandular. The buccal capsule is lined with chitinous plates. The second group of Rhabdochonidae / Quimperidae however have few structures in common and this is supported by poor bootstrap values based on genetic relationships.

In a similar manner to the trematode clade, *Contracaecum* sp. is an allogenic parasite and all the other nematodes analysed are autogenic parasites of the clariids in Lake Victoria and elsewhere. Whether the same reason(s) as for the trematodes above apply in the division of nematodes into groups observed is not immediately apparent, therefore needs further investigation. However, the fact that the allogenic nematodes and trematodes share the same secondary intermediate host (clariid fish) and the final bird hosts such as cormorants herons, pelicans, egrets and darters (Barson and Marshall, 2004), renders support that these parasites have a common gene that enables the two groups to utilise the same ecological environment as observed in the present study. So the evolutionary relationship is rather tied to the feeding ecology and life history traits, which evolve over a long period, rather than merely genetics.

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