TITLE PAGE

Occurrence and genotypic characterization of *Diplostomum* species (Digenea: Diplostomidae) in *Oreochromis niloticus* L. and snail vectors in Kisumu municipality

Authors

Violet M. Ndeda: Department of Zoology, Maseno University. **Email**: vmmbone@yahoo.

Dickson Owiti: Department of Zoology, Maseno University. **Email**: otienowiti@yahoo.com

Ben A. Ochieng': Department of Zoology, Maseno University. **Email**: akechben@yahoo.com

Geraldine Matolla: University of Eldoret, Chepkoilel. **Email**: gmatolla@yahoo.com

David M. Onyango: Department of Zoology, Maseno University.

Email:dvdonyango7@gmail.com.

Correspondence author: Violet M. Ndeda

Correspondence address: Maseno University, Department of Zoology, P. O. Box 333 - 40105, Maseno, Kenya.

Correspondence Email: vmmbone@yahoo.com

ABSTRACT

Background

Diplostomum species metacercariae, are trematodes that pose economic threat to aquaculture practice globally. Despite their diversity, species identification is difficult owing to their striking morphological similarities, especially at the metacercarial stage. The problematic nature of *Diplostomum* species identification represents a major impediment for the assessment of their actual role in fish populations, advancement of the knowledge of parasite biology and evolutionary aspects of the host-parasite relationships.

Methodology

A total of 1728 Nile tilapia fish were sampled from at least three fish farms in three main settlement areas of Kisumu municipality between December, 2011 and February, 2012. *Diplostomum* parasites were detected in fish eyes using microscopy to ascertain parasitic indices in the different farms. Snails were identified based on shell morphology while shedded cercaria from snails were distinguished based on cercarial movement and morphology. Physico-chemical characteristics of the water including pH and temperature were determined using a pH meter and a temperature probe. Genetic discrimination of *Diplostomum* species was assessed using sequences of the internal transcribed spacer region (ITS1–5.8S–ITS2) and 18S ribosomal genes in 23 diplostomoids.

Results

52.3% of the fish sampled were positive of *Diplostomum* parasites. Parasite mean intensities ranged between 8 - 12 parasites per host fish. Analyses of ITS rDNA gene dataset revealed five genetically distinct *Diplostomum* species (*D. mashonense*, *D. baeri*, *D. pseudospathaceum*, *D. paracaudum* and *D. mergi*) in the fish populations studied. Molecular identification using18S

rDNA sequences revealed three genetically distinct *Diplostomum* species (*D. compactum*, *D. phoxini* and *D. spathaceum*) which did not support the delineation detected by ITS1-5.8S-ITS2. Distribution of vector snails indicated presence of *Lymnea*, *Biomphalaria*, *Bulinus* and *Cerratophalus* snail species, however; *Diplostomum* larval communities were reported only in *Biomphalaria* spp. at a prevalence rate of 21.7%.

Conclusion

Abundance of *Biomphalaria* snail vectors was positively correlated to *Diplostomum* parasite abundance, pH and temperature as well as vegetation cover. Species heterogeneity of cercariae in *Biomphalaria* snails was likely similar to species heterogeneity of trematodes in host fish. Such community-level interactions have rarely been demonstrated and have implications for epidemiological surveys and ecosystem management.

Key words

Prevalence, Intensity, *Diplostomum*, ITS, 18S rDNA, *Biomphalaria* snails, Nile tilapia

BACKGROUND

The trematode genus, *Diplostomum* represents a large group of widely distributed parasites with complex lifecycles involving: freshwater snails as first intermediate hosts, fish as second intermediate hosts and fish-eating birds as definitive hosts [1, 2]. Metacercariae in the eyes of freshwater fish are considered major pathogens since heavy infections may be a source of substantial losses of wild and farmed fish. Nile tilapia (*Oreochromis niloticus* L. 1758) ranks the most commercialized fish in western Kenya. However, unrelenting low production of pond reared Nile tilapia in Kisumu municipality has been recorded in the past 3 – 5 years [3]. This has possed severe threats to the aquaculture component of the stimulus package on fish farming by the Kenyan government initiated to revamp economy among riparian communities. Farmers are shying away from the initiative due to lack of economic returns as a result of decline in fish production [4] as well as reduced average size of fish caught in the farms [3]. Information regarding the epidemiology of diplostomiasis infection among cultured fish in the region, is absent. Although, studies by [1], [5] and [6] reported reduced growth, loss of vision, emaciation, reduced fish crypsis as well as escape response and massive deaths as a result of fish invasion with *Diplostomum* digenetic trematodes. These parasites are morphologically indistinct in their hosts because of overlap in morphological characteristics, lack of identification keys devoted to morphological stages and phenotypic plasticity induced by age, host and fixation procedures. The problematic nature of *Diplostomum* species identification represents a major impediment for the assessment of their actual role in fish populations, and the advancement of the knowledge of parasite biology and evolutionary aspects of host-parasite relationships of *Diplostomum* spp. Different *Diplostomum* species impose different pathogenicity to the host, and little is known about the diversity of diplostomid species affecting fish, leading to misidentification of host - parasite interaction in relation to host behaviour.

To date, 41 nominal species of *Diplostomum* have been described within the Palaearctic regions, predominantly from Europe; of these, 25 were considered valid in the latest taxonomic revision of the genus according to [7] and [8]. However, a combination of identification and taxonomic problems, have led to the biological paradox of a large number of *Diplostomum* spp. described and recorded in fish eating birds, intermediate fish and snail hosts. So far, only scanty of molecular data on *Diplostomum* spp. is available in Africa [9], this has hampered large scale screening of natural *Diplostomum* infections in fish and therefore calls for more research to update the taxonomic database in Africa. Altogether, aspects of species richness in the first intermediate host are important ecologically as well as in understanding evolutionary changes in parasites that enable transmission to the next trophic level. Few studies have focussed on the genetic diversity of cercariae in the snail host [10]. Studies conducted in Europe [11, 12, 13, 14] provided an approximate ratio of 9: 3: 2 *Diplostomum* species in birds, fish and snails respectively. The low species richness recorded in snails reflect the scarcity of data on spatial distribution of *Diplostomum* spp in snails compared to the numerous data recorded in fish. This however, is important if distribution and gene flow in the ecological system of a given area is to be understood. This is an indication that a large proportion of the *Diplostomum* species diversity was probably missed due to identification failure.

The principal goal of this study was to genetically identify *Diplostomum* spp. parasitising populations of *Oreochromis niloticus* L. and vector snails in fish ponds within Kisumu municipality in order to achieve a comprehensive interpretation of gene flow in the ecological system. The study went further to determine the epidemiology of diplostomiasis in the region, identified *Diplostomum* vector snails found in ponds and finally assessed the environmental and physicochemical factors that influence snail distribution and *Diplostomum* development.

MATERIALS AND METHODS

The study was conducted in three locations within Kisumu municipality (Fig. 1), which borders Lake Victoria in western Kenya, from December, 2011- February, 2012. Kisumu was preferred for the study because of the Economic Stimulus Program (ESP) initiated by the government in 2009 that targeted fish farmers in the region and led to the construction of over 300 fish ponds in the municipality. In addition, Kisumu experiences four distinct seasons, i.e. two rainy seasons and two dry seasons. The rainy seasons are further sub-divided into the long rainy season and the short rainy season [15]. Likewise, the dry season is also subdivided into a long dry season, and a short dry season. The long rainy season usually begins in March through to May. This is normally followed by a long dry spell, which starts in June and ends in August. The short rainy season starts in October and lasts for two months until November, followed by the long dry spell which starts in December through to February [16]. Period of sampling for this study (December 2011- February 2012) was preferred based on previous literature that reported diplostomid transmission patterns to be highly seasonal [17] and higher during dry seasons compared to wet seasons. Maximum temperatures in Kisumu occur in the long dry spell with an annual maximum temperature range of about 27^0C to about 32^0C [18]. Minimum temperature ranges from 14^0C to 18^0C, with the peak minimum temperature recorded in August through September [18]. At least three farms were selected per location based on their location near a hatchery centre which serves as sources of the much required quality fingerlings for supply to prospective farmers. These locations border each other hence ease of accessibility.

Fish sampling procedure and transportation

Fish sample size for Nile tilapia used in this study was estimated according to the formula by [19]. Sampling was done in three main settlement areas of Kisumu after every three weeks for a

period of three months (December 2011 – February 2012). Sixty four (64) Nile tilapia fish were randomly sampled per pond, for every three ponds per farm using a seine net of 1.5m diameter and 6mm mesh. Sampled fish were then transported in iced cool boxes at 8^0C to Maseno University, Zoology department laboratory for analysis.

Sampling of snail population and transportation

A minimum of 100 snails were randomly collected each farm using a scoop or by hand collection from the ponds. Sample size was estimated as described by [20]. Sampling was performed between 08:30 h and 10:30 h. At each collection time, snails from each site were appropriately labelled and transported in separate perforated plastic containers to Maseno University, Zoology department laboratory for analysis.

Laboratory procedures

Examination of fish specimens for *Diplostomum* parasites

Fish eyes were dissected and then examined for metacercariae with a stereoscopic microscope using procedures as described by [21] and [22]. The metacercariae extracted from each eye were counted as separate lots, and placed in a petri dish containing saline solution before storing in 95% ethanol. Isolated metacercariae were stored in viols at 4^0C and labeled according to their collection sites.

Confirmation of snail infection

By 11:00 h, snails were rinsed and placed individually in 24-well culture plates (Corning Glass Works, Corning, NY, USA) containing 1 ml of filtered Dechlorinated water for a period of 24 hours. The snails were exposed to a 15-h light/9-h dark lighting regimen. After 24 hours, plate

wells were examined with a dissecting microscope for presence of cercariae shed by the snails. Identification of cercariae was made according to their morphology and behaviour, as strigeoid cercariae form a distinctive right-angle resting position and the furcae spread apart at an angle of 180^0 [23]. Isolated cercariae were picked and stored in vials containing 95% ethanol and stored at 4^0C.

Physicochemical characteristics of the pond water

We chose physicochemical parameters of the water suggested to be important for snail distributions and *Diplostomum* development in fish. In this regard, we recorded pH and temperature. Water pH and temperature were measured directly in the field using a pH meter (3071 Jenway) and a mercury thermometer, respectively.

Genetic characterization of *Diplostomum* species

DNA was extracted from individual diplostomoids following the method of [24]. DNA amplification of the 18S rDNA sequence was performed according to [25] protocol in 25 µl volumes via the polymerase chain reaction (PCR) in MJ Gradient thermocycler (Gene amp. PCR system 9700, Applied Biosystems U.S.A). Each PCR reaction consisted of: 10.25 µl sterilized distilled H_2O, 2.5 µl 10X (-$MgCl_2$) PCR reaction buffer, 2.5 µl $MgCl_2$ (25 mM), 0.5 µl dNTP (10 mM; Fermentas #R0191), 1.25 µl of the forward and reverse PCR primers and 0.25 µl *Taq* DNA polymerase. 18.5 µl of the above PCR master mix was aliquoted into each 1.5 µl PCR reaction tube after vortexing. 6.5 µl (5ng) of sample DNA was added into each tube to bring the total reaction volume to 25µl. The mixture was then vortexed slightly and placed into a PCR machine for amplification. Primer sequences of 18S9F (5′TGATCCTGCCAGTAGCATATGCTTG - 3′); 18S300R [5′TCAGGCTCCCTCTCCGG - 3′(400nt)] and 18S637R [5′

TACGCTATTGGAGCTGGAGTTACCG-3′ (600nt)] were used. Amplification of the portion of the rDNA that included the complete ITS1–5.8S–ITS2 region was done via the polymerase chain reaction (PCR) in MJ Gradient thermocycler (Gene amp. PCR system 9700, Applied Biosystems U.S.A) according to [26] protocol. The PCR primers designated D1 (5′-AGG AAT TCC TGG TAA GTG CAA G-3′) and D2 (5′- CGT TAC TGA GGG AAT CCT GGT-3′) were employed. The product was run through electrophoresis using a 1% agarose gel containing ethidium bromide (0.5 µg/mL) alongside 0.5 µg/µl; Fermentas: GeneRuler$_{TM}$ 1kbp DNA Ladder and visualized under Ultra Violet light. PCR products were then purified using the Gene – Jet PCR purification kit (Fermentas, No. K 0701) following the manufacturer's protocol.

DNA sequencing

Sequencing was performed at the Inqaba laboratories in South Africa. ITS1–5.8S–ITS2 region was sequenced using primers BD1 (5′-GTC GTA ACA AGG TTT CCG TA-3′) and BD2 (5′-TAT GCT TAA ATT CAG CGG GT-3′) of [27] which were used as the forward and reverse primers, respectively. Sequencing for 18S rDNA gene was performed using the forward PCR primer only.

Data analysis

Analysis of parasite occurrence among fish farms

Prevalence (%) of *Diplostomum* parasites was estimated as the ratio between the number of infected fish and the number of examined fish expressed in percentages. The mean intensity (M.I) was determined as the ratio between the total number of parasites in a sample and the number of infected fish in a sample. The mean abundance (M.A) was determined as the ratio

between the total number of parasites in a sample and the total number of fish examined (infected + uninfected).

One-way ANOVA was used to test for differences in abundance of parasites among the farms. To determine the possible correlation between the parasite number and snail abundance, Pearson's linear correlation ''r,'' was used. Kruskall-Wallis H test was used to test for differences in parasite number among the host length classes. Comparison for prevalence of infection between the snail populations among the farms was performed using Fisher's exact test. Associations between snail abundance and physicochemical variables were determined using spearman correlations (rs). Results from various statistical tests were considered significant at $p \leq 0.05$ using SPSS v. 17.00 (USA) software packages.

Sequence analysis

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.

***Diplostomum* species identification, delineation and diversity**

Metacercariae were identified to family or genus based on morphology, using the keys of [21] and [22]. Contiguous sequences of the small subunit region and internal transcribed spacer regions of ribosomal DNA from 21 specimens were created from forward and reverse chromatograms and edited using DNABaser version 2.7. Multiple alignments of the contigs was conducted using Muscle 3.8.31 multiple alignment software. Nucleotide sequence data of ITS rDNA and 18S rDNA sequences from all clusters were submitted to Basic Local Alignment Search Tool (BLAST,www.ncbi.nlm.nih.gov/blast) for similarity searches in Gen Bank with the

aim of matching our representative sequences with those published in other studies of diplostomoids. *Diplostomum mashonense* MS14 18S ribosomal RNA gene was used as a reference sample. Potential species were distinguished by clustering in Neighbour-Joining (NJ) phenograms using MEGA version 4.0.2 [28]. The reliability of internal branches in the Neighbour-Joining trees was assessed using bootstrap analysis with 1,000 replicates. The resulting networks were rooted with the out-group taxa.

RESULTS

Metacercariae of *Diplostomum* spp. were found actively moving in the vitreous humor and lens of the sampled fish. Prevalence, mean abundance and mean intensity of parasitic indices were investigated. Prevalence (%) among fish from East Kisumu, Kajulu west and Kajulu East locations was 47.4%, 43.2 % and 66.1% respectively (Table 1). *O. niloticus* from East Kisumu location had a mean intensity of 12 parasites per host fish with an abundance range of between 5 and 8 parasites. Sampled fish from Kajulu West fish ponds had a mean intensity of 11 parasites per fish with an abundance range of between 4 and 8 metacercariae. Sampled fish from Kajulu East fish ponds had a mean intensity of 9 parasites per fish and abundance range of between 5 and 7 parasites (Table 1).

One way ANOVA computed to compare the number of *Diplostomum* parasites infesting fish among the farms indicated that there was a statistically significant difference among the farms $p < 0.0001$. A Post hoc comparison test of the Tukey HSD indicated a statistical significant difference between farms in Kajulu west and Kajulu East ($p = 0.050$; < 0.05). However, farms in East Kisumu ($M = 3.49$, $S.D = 5.00$) did not significantly differ from farms in the two locations hence the difference in parasite number among the farms. With regard to vector - parasite distribution, a total of 1359 snails were collected from all the sampled sites. Out of these, 124

(9.12%) snails were infected with trematodes representing only two species of the freshwater snail community. The general prevalence of natural infection in host snails was, 0.64% (1) in *Bulinus spp* and 12.58% (123) in *Biomphalaria spp.* (Table 2). The larval trematode community was depauperate and composed of 3 species, (based on cercarial morphology), namely: xiphidiocercaria type I, amphistomes and strigeoid cercariae. No individual snail was infected with more than one species of digenea (multiple infections). The most prevalent cercariae were strigeoid cercariae (21.69%) (which contain genus *Diplostomum*) recovered from snails of *Biomphalaria* spp. Fish farms from East Kisumu location had the highest snail infection prevalence (10.42%) compared to Kajulu west fish farms (5.56%) and Kajulu East fish farms (5.71%). There was a marginal difference in snails abundance among the different sites ($F_8, 77 = 2.15$, $p = 0.056$).

Environmental factors

The most common vegetation cover identified in close proximity to the fish ponds in all the study sites was papyrus reeds and sugarcane plantation. Qualitative data indicated that the vegetation cover contributed to the occurrence of snails in the farms. Farms in Kajulu East were situated in close proximity to papyrus reeds which acted as the source of water for the farms. The papyrus reeds were also a home for several birds. The birds namely; cormorants, kingfisher, egrets and eagles were observed visiting the farm as well as the papyrus reeds. Farms in East Kisumu and Kajulu west were in close proximity to each other (< 1km) compared to the location of fish farms in Kajulu East (> 5 km away), and were surrounded by sugarcane plantation which also acted as a hideout to several birds such as cormorants, egrets, eagles and open bill storks.

Physicochemical parameters of water

There was a monthly variation in water temperature in all the sites. The mean values of water temperature ranged between 26.4 - 32.4°C with a recorded mean of 29.4 ± 1.4°C. This significantly influenced the overall snail abundance ($F_{2, 900} = 49.47$, $p < 0.0001$). There was a positive association between water temperature and overall snail abundance ($r = 0.3$, $p = 0.01$). The pH levels of water did not vary greatly at the sites. The mean pH of water from farms in East Kisumu was 6.62 ± 1.1 (range = 5.2 - 8.2), Kajulu west fish farms was 5.34 ± 1.2 (range = 5.1 - 5.38) and Kajulu East fish farms was 5.15 ± 0.31 (range = 5.1 – 5.3). pH was positively associated with snail abundance from all the sites ($r = 0.733$, $p<0.001$).

Variation of parasite number with fish size

Total number of parasites increased with fish length, with the highest number of parasites observed between the sizes of 10.1 – 15.0 cm (Figure 2). Contrary to the parasite increase, a gradual decrease in the numbers was observed among fish ≥15 cm in all the farms. Kruskal-Wallis test did not reveal any significant effect of fish size grouping on value [χ^2 (3) = 6.59, $p = 0.086$]. Because the overall test was not significant, pair wise comparisons among the four groups could not be completed. Figure 2 shows the result where total parasite number was varied by host length class.

Association of parasite abundance with snail abundance

Pearson Correlation used to examine the relationship between mean *Diplostomum* parasite abundance and mean snail abundance revealed highly significant difference ($p < 0.001$) among the nine sites examined with a small correlation ($R^2 = 0.2417$) recorded between the two factors.

Neighbour - Joining analyses of ITS rDNA and 18S rDNA sequences from *Diplostomum* specimens collected from fish in Kisumu municipality

Phylogenetic analyses were conducted based on the alignment of partial and complete sequences of ITS rDNA and 18S rDNA using the NJ method. The resultant tree presented bootstrap consensus values of >50% for almost all branches confirming that the samples were indeed members of the *Diplostomum* genus (Fig. 3).

The NJ analyses of ITS rDNA (Fig. 4) and 18S rDNA (Fig. 5) sequences confirmed the presence of single species of *Ichthyocotylurus, Strigidae* and *Bolbophorus,* two species of *Apharyngostrigea* and *Posthodiplostomum,* three species of *Alaria* and *Tylodephys* and at least 8 species of *Diplostomum* in the database. The resultant tree presented bootstrap consensus values of >50% for almost all branches. The bootstrap [29] consensus tree was inferred from 1000 replicates and taken to represent the relationship of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. In all cases sequences of the same genus were grouped into two strongly supported clusters representing 13 species from the Diplostomidae and 4 species from the Strigeidae.

PCR micrographs of cercariae by use of 18S rDNA gene sequence

Analysis of PCR products of cercariae from snails by electrophoresis using 1% agarose gel showed successful DNA amplification. PCR products of expected size were observed at 400 and 600 bp from the infected samples as shown in figures 6 and 7. Loaded wells that did not show any band were considered negative for *Diplostomum* infection.

DISCUSSION

Prevalence of *Diplostomum* infection in Nile tilapia

This study has been novel in attempting to elucidate the prevalence and diversity of *Diplostomum* species infecting pond reared Nile tilapia in Kisumu. Results from the present study indicated that *Diplostomum* infection is present in Kisumu municipality at a prevalence rate of 52.3%. Prevalence of the infection varied among the locations being higher in fish farms from Kajulu west (66.1%) and moderately low in East Kisumu and Kajulu east (47.4% and 43.2% respectively) fish farms (Table 1). These findings are in agreement with [30] who reported 55% prevalence of *Diplostomum* infection for tilapias *Oreochromis aureus* and *O. mossambicus.* Similarly, a study conducted by [31] in Kenyan earth pond-based farms in Sagana area reported 1 – 4 metacercariae in farmed *O. niloticus* and 40.7% prevalence. Occurrence of *Diplostomum* infection in the farms could be related to the presence of a high abundance of *Biomphalaria* spp snails shedding cercariae of *Diplostomum* parasites in all the sites (Table 2). These findings concur with the suggestion by [32] that snails serve as important intermediate hosts in *Diplostomum* life cycle. In addition, presence of a high bird density observed visiting the farms (personal observation, December, 2011 – February, 2012) might have contributed immensely to the completion of *Diplostomum* lifecycle. As was suggested by [32] and [17], fish eating birds and snails serve as important intermediate hosts in population dynamics of *Diplostomum* spp.

According to [33], temperature and pH play a significant role in snail and parasite development. This study demonstrated that these physicochemical parameters of water appeared to be the key determinant of increased trematode prevalence among the snails. The pH of water in all the farms (range 5.1 – 6.6) was lower than the recommended range of pH for cultured fish (range 6.8 – 8.7), while the association with snail abundance was ($r = 0.733$, $p<10^{-3}$) positive. Although tilapia can survive in pH ranging from 5 to 10, they do best in a pH range of 6 to 9. Reduced pH

of the pond water was likely related to sugarcane plantation found within the vicinity of the farms. Researches done in sugarcane plantations, point reduction of soil pH to the use of nitrogenous fertilizers which reduce soil pH during ammonification and nitrification processes [34, 35]. These heavy metals become soluble in the soil and are readily transported by surface runoffs and leaching to aquatic environments where they accumulate. The presence of association between pH and snail abundance has also been reported previously by [33] and suggested that pH may be a key determinant of snail abundance in aquatic ecosystems.

Equally, water temperature appeared to be a key determinant of snail abundance. The positive association between snail abundance and water temperature observed in our study ($r = 0.3$, $p = 0.01$) is in agreement with observations that demonstrated *Biomphalaria pfeifferi* grew and survived better at 25°C than at 19°C [36]. Likewise, in temperate regions, occurrence of diplostomid trematodes in fish is primarily temperature dependent [37] with higher abundance occurring in autum and summer. The present study was conducted between December and February, these months were dry and hot with recorded temperature range of the pond waters being 26^0C - 32^0C. These results are typical of what was found in a previous study by [17] who reported high abundance of *Diplostomum* in fish during summer and autum and attributed this scenario to reduced volumes of pond water and also slow water movements which offer a stable environment for snails to lodge onto surfaces and not be washed away.

Although there was a high prevalence of *Diplostomum* infection in fish, prevalence of trematodes in the snails seemed low with the highest prevalence (10.42%) observed in snails collected from fish farms in East Kisumu. This is typical of infection by digenean larvae [38]. High prevalence of trematode parasitism in snail population renders a remarkable proportion of snails infertile due to the host-castrating effect of trematodes. As a result, the host density is reduced [39, 40]. This

suggestion concurs with the low number of infected snails evidenced in this study. Snail prevalence of less than 10% of *Diplostomum* infections have similarly been reported in lymnaeid snails in both Finland [41, 42, 14] and other European countries [12, 43]. Nonetheless, possible explanation for the low number of snails shedding cercariae in the present study could also be related to the season when the snails were sampled (December – February). Similar findings have been reported along the Kisumu beach where cercarial shedding for mammalian schistosoma was observed to be lowest during the month of February [44] with snails shedding cercariae ranging between 0 and 5.

Genotypic identification of *Diplostomum* species in fish population

The principal findings of this study indicated that eight species infections of *Diplostomum* were common in the fish community. These findings were revealed using ITS rDNA and 18S rDNA. These species were closely related to findings of [45] and [26].

However, identification of only eight *Diplostomum* species might represent an underestimate of the true diversity of this genus in this study area because interpretation of our data was limited to phenetic method of analysis as opposed to cladistic method. ITS sequence data of specimen D32 was closely related to *D. mashonense* (Beverley-Burton, 1963), and *Tylodelphis* spp. This is in agreement with the observation by [45] who pointed out striking similarity between *D. mashonense* (Beverley-Burton, 1963) and *Tylodelphys* spp. 1 and 2, and later discriminated *D. mashonense* (FJ 470402) from *Tylodephys* spp. using morphometric variability analysis. This suggests a strong similarity between *Diplostomum* spp studied in Tanzania and *Diplostomum* in the current study.

Genotypic identification of *Diplostomum* species in fish population using ITS

Phylogenetic analysis of ITS rDNA sequence data from adult forms of *Diplostomum* by Gallazo *et al*. (2002) lends support to 4 sequences from this study which demonstrates highly similar consensus sequences to *D. baeri* (GenBank: JQ 665460), *D. mergi* (GenBank: JX 494233, JX 494231) *D. pseudospathceum* (GenBank: JQ 665456, JX 494232) and *D. paracaudum* (GenBank: JQ 665457) classified as European or American species (Fig. 4). Specimens closely related to these species include D42, D52, D35, D57, D44, D26, and D46 which were equally assessed by ITS rDNA and strongly supported by a high bootstrap value (99%). Similarly, [46] reported similar relationships using ITS 1 rDNA sequences.

The present study provides a preliminary confirmation of diplostomoid species residing in both continents with a possibility of recent divergence or hybridization. This is because, according to [47] some European material from which sequences deposited in the GenBank were obtained are misidentified and the reliability of otherwise unpublished records is difficult to evaluate. This study further lends support to the idea of similarities in the geographical distribution of species within the *Diplostomum* genus, since *D. mashonense* from Tanzania, exhibited the greatest genetic similarity to the present material.

Similarly, [46] reported *D. paracaudum* from European flounder, *Platichthys flesus* (Linnaeus, 1758), whose geographical distribution was limited to European waters to have similar genetic similarities to another European species reported to be *D. paracaudum* by [26]. Thus, the pattern of parasite distribution in the fish host species might be as a consequence of co-evolutionary interactions or evolutionary history associated with geographical divergence of the species. However, on the contrary, [47, 48] suggested that the presence of closely similar species in the same environment, do not support an evolutionary history associated with geographical divergence of the species, given the mobility of the avian definitive hosts. This study supports

390 the hypothesis by [47, 48] which suggests that there does not appear to be an evolutionary
391 separation of the species; however, different kinds of data should be considered for accurate
392 identification of diplostomid metacercariae at the specific level.

393 **Genotypic identification of *Diplostomum* species in fish population using 18s rDNA**

394 Three species of *Diplostomum* {*D. compactum* (GenBank: AY 245764), *D. phoxini* (GenBank:
395 AJ 287503, AY 222090) and *D. spathaceum* (GenBank: AY 245761)} were closely related to
396 specimens studied herein using 18S rRNA because the resultant tree presented bootstrap
397 consensus values of >50% for almost all branches (Fig 3 and Fig. 5).

398 There are several possible ecological and evolutionary mechanisms that could underlie the
399 observed patterns of *Diplostomum* spp. in the sampled fish. First, the patterns could be
400 determined by the ecology of the different hosts [49]. In our study system, *Biomphalaria* spp.
401 vector snails were observed to harbour furcocercariae and xiphidiocercarie, however, infection
402 patterns to establish reasons for specialization of the cercariae to this intermediate host amidst
403 several other vectors was not established. [50] likewise reported cercariae resembling those of
404 the Diplostomidae from *Biomphalaria pfeifferi* snails at Mindu dam in Tanzania. The present
405 study went further to conduct genetic analysis based on 18S rDNA on the shedded cercariae to
406 establish species heterogeneity between the snails and fish (first and second intermediate hosts
407 respectively). Presence of *Diplostomum* cercariae in *Biomphalaria* snails from the study ponds
408 was demonstrated by microscopy and confirmed by PCR. The parasite DNA fragments of
409 expected sizes (bands with corresponding sizes of about 400 and 600 bp) were obtained (Fig.6.0
410 and Fig. 7.0 respectively). However, further sequence analysis of the cercariae was not
411 successful because of decreased DNA quantities. Nevertheless, results from this study
412 demonstrated a high possibility of similar genotype in the snail and fish parasite and a

preliminary confirmation that the parasites belonged to the genus *Diplostomum* and needed further analysis for species identification.

In addition to ecological factors and evolutionary processes, lens-infecting Diplostomid species have been shown to exhibit a wider range of harboured parasite species than what can be found in other tissues [47,48]. Most of the specimens analysed molecularly in this study were obtained from the lens tissue. This is most likely because metacercariae of the lens-infecting species have less interaction with their hosts, as they are protected from the immune system once they have reached the lens. Hence the increase in community species composition recorded.

Conclusion

This study indicates that although very low proportion of infected *Biomphalaria* snails were reported, this was sufficient enough to translate to high levels of diplostomiasis infection in the fish farms. On the other hand, snail abundance was directly associated with vegetation cover surrounding the ponds environment as well as pH and temperature of the water. Therefore, preventive measures such as vegetation control and removal of snail populations in and around ponds are needed to prevent any possible diplostomiasis outbreaks in culture systems. Regarding diversity of *Diplostomum* parasites; eight species of *Diplostomum* (*D. baeri*, *D. pseudospathaceum*, *D. paracaudum*, *D. mergi*, *D. compactum*, *D. phoxini*, *D. spathaceum* and *D. mashonense*) were found to closely resemble our present material, although the latter species depicted 100% similarity. These results are novel in demonstrating the genetic and taxonomic diversity of *Diplostomum* spp. in the natural parasite populations of pond reared Nile tilapia in western Kenya. However, for a better understanding of the genotypic and phenotypic complexity within the genus *Diplostomum*, more variable genes such as the mitochondrial cytochrome *c* oxidase subunit 1 (cox1) barcode region should be used. The platyhelminth cox1 gene exhibits a

much higher variation compared with the ITS regions and may thus serve as a more suitable marker for species detection.

Abbreviations

ITS: Internal Transcribed Spacer; rDNA: Ribosomal DNA

Author's contributions

VMN: Main person in the design of the study, collected and prepared the samples for the study, compiled the data, performed statistical analysis and wrote the manuscript.

BAO: Participated in the design of the study, assisted in statistical analysis and helped to draft the manuscript

DO: Provided scientific guidance in data collection, analysis of data and compiling of the data

GM: Revised the manuscript for important intellectual content

DMO: Was involved in the design of the study, analysis of the results, helped to draft and revise the manuscript and provided financial support.

All authors read and approved the final manuscript.

Author details

VMN: MSc student Maseno University

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identification of *Diplostomum* parasites in the field. I would like to thank Ibrahim Ndung'u, Mercy Macharia, Winnie Akoth, Elly and Benjamin Opot for their technical support during molecular work.

List of figures

Figure 1 : Map showing Kisumu Municipality and the study area (marked in box)

Figure 2: Total *Diplostomum* parasites as observed between class lengths (cm) of *Oreochromis niloticus*

Figure 3: Neighbour-Joining tree of sequences constructed in this study in comparison with representatives in the *Diplostomum* spp. as inferred from 8 ITS and 18S rDNA sequences. Numbers at the nodes represent bootstrap values. *Tylodelphys spp.* was set as an out group.

Figure 4: Neighbour - Joining tree depicting the relationships between *Diplostomum* spp. as inferred from 8 ITS rDNA sequences. Tree is drawn to scale. Numbers at the nodes represent the bootstrap values. *Tylodelphys spp.* was set as out group

Figure 5: Phylogenetic tree depicting the relationships between *Diplostomum* spp. as inferred from fifteen 18S rDNA sequences using the NJ method. Numbers at the nodes represent bootstrap values. *Bolbophorus spp* was set as out group.

Figure 6: Agarose gel showing the amplicons produced in PCR for cercariae using the specific primers for 18S rDNA (600nt). Lanes M: DNA size markers (100bp); Lanes C1-C4: Cercariae positive for 18S (600nt) gene; C5 and C6: Cercariae negative for 18S gene.

Figure 7: Agarose gel showing the amplicons produced in PCR for cercariae using the specific primers for 18S rDNA (400nt). Lanes M: DNA size markers (100bp); Φ: Negative control lane; Lanes C1- C4: Cercariae positive for 18S (400nt) gene. C5: Cercariae negative for 18S (400nt) gene.

Table 1: Prevalence, mean abundance and mean intensity of *Diplostomum* parasites from fish farms in three locations within Kisumu

Location	Fish Examined	Fish infected	Prevalence (%)	Parasites recovered	Mean Abundance	Mean Intensity
East Kisumu	576		47.4	1101	5.7	12.1
Kajulu west	576		43.23	934	4.9	11.3
Kajulu East	576		66.1	1228	6.4	9.7
Total	**1728**		**52.3**	**3726**	**6.5**	**12.4**

Prevalence is the proportion of infected hosts among all hosts examined at a particular time.
Mean abundance is the mean number of parasites found in all hosts.
Mean intensity is the mean number of parasites found in infected hosts.

Table 2: Summary of the distribution of snails collected in three locations within Kisumu municipality

Site	Type of snail species	Number of snails collected	Number of snails infected with:			
			Mammalian cercariae	Xiphidiocercariae	Amphistomes	Strigeoid cercariae
East Kisumu	*Biomphalaria* spp	288	0	0	0	30 (10.42%)
	Lymnea spp	180	0	0	0	0
	Cerratophalus	0	0	0	0	0
	Bulinus spp	6	0	0	0	0
Kajulu west	*Biomphalaria* spp	270	0	18(6.67%)	24(8.89%)	15 (5.56%)
	Bulinus spp	156	0	0	1 (0.64%)	0
	Lymnea spp	12	0	0	0	0
Kajulu East	*Biomphalaria* spp	420	0	0	12 (2.86%)	24 (5.71%)
	Lymnea spp	3	0	0	0	0
	Bulinus spp	24	0	0	0	0

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Figure 1 : Map showing Kisumu Municipality and the study area (marked in box)

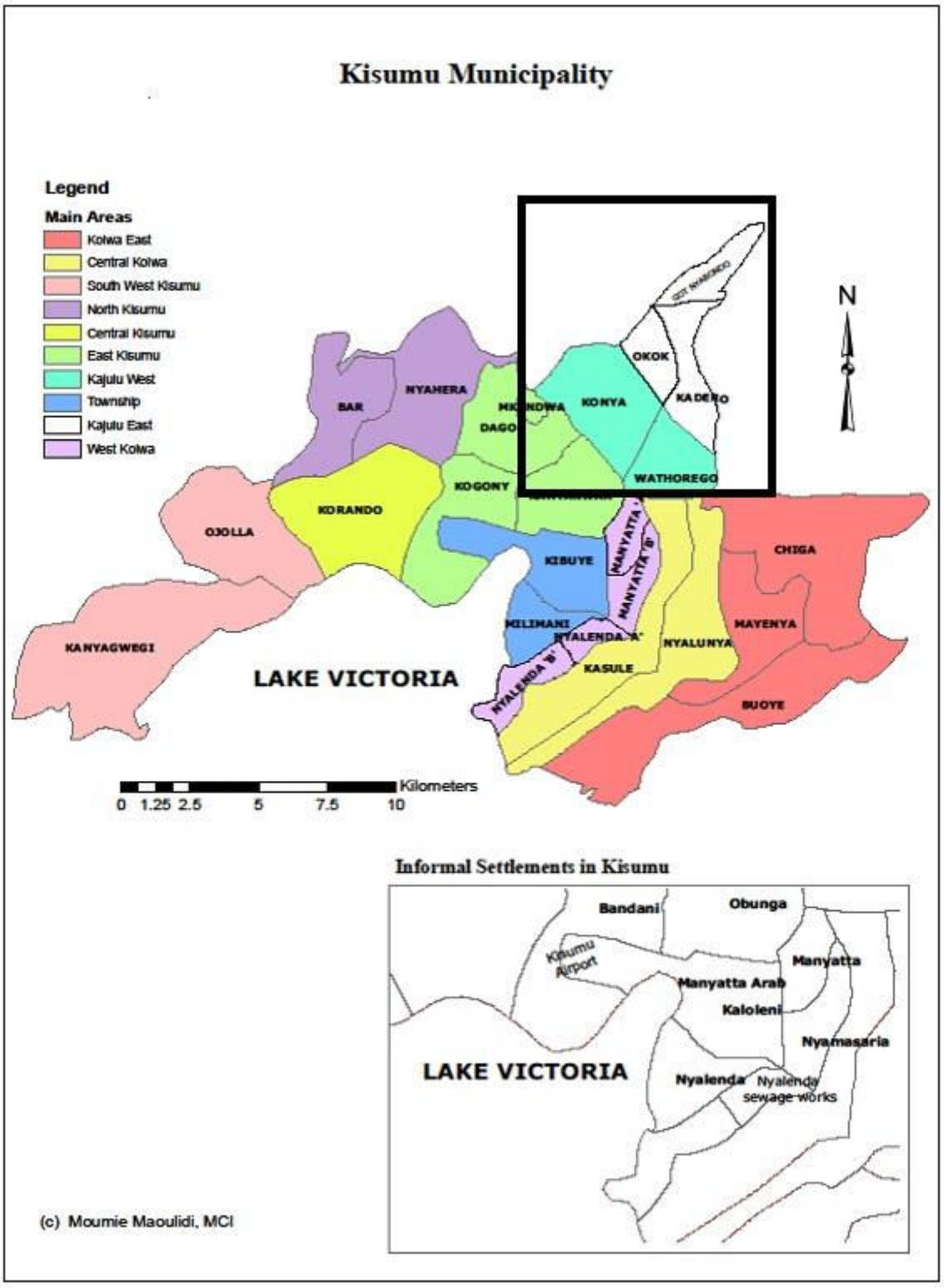

Figure 1

Figure 2: Total Diplostomum parasites as observed between class lengths (cm) of Oreochromis niloticus

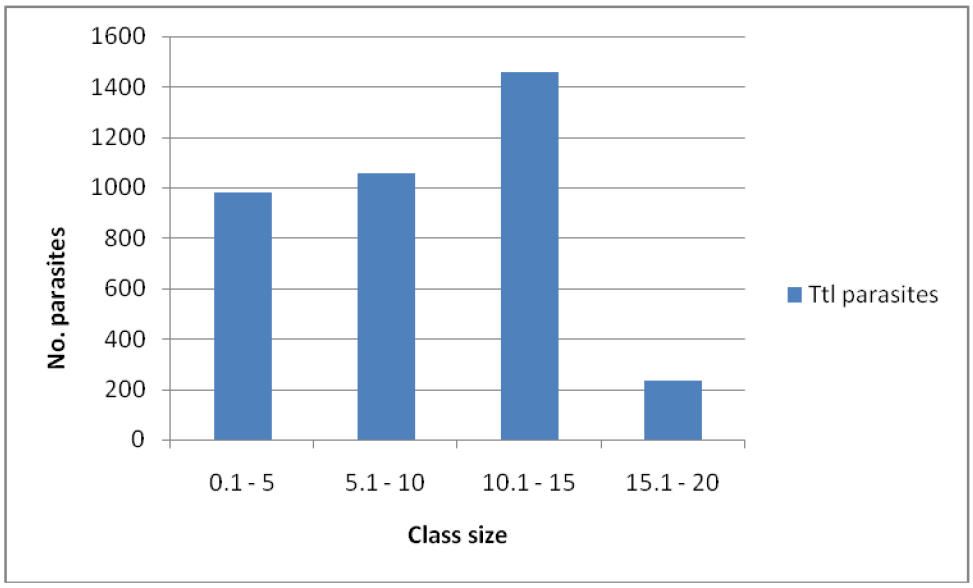

Figure 3: Neighbour-Joining tree of sequences constructed in this study

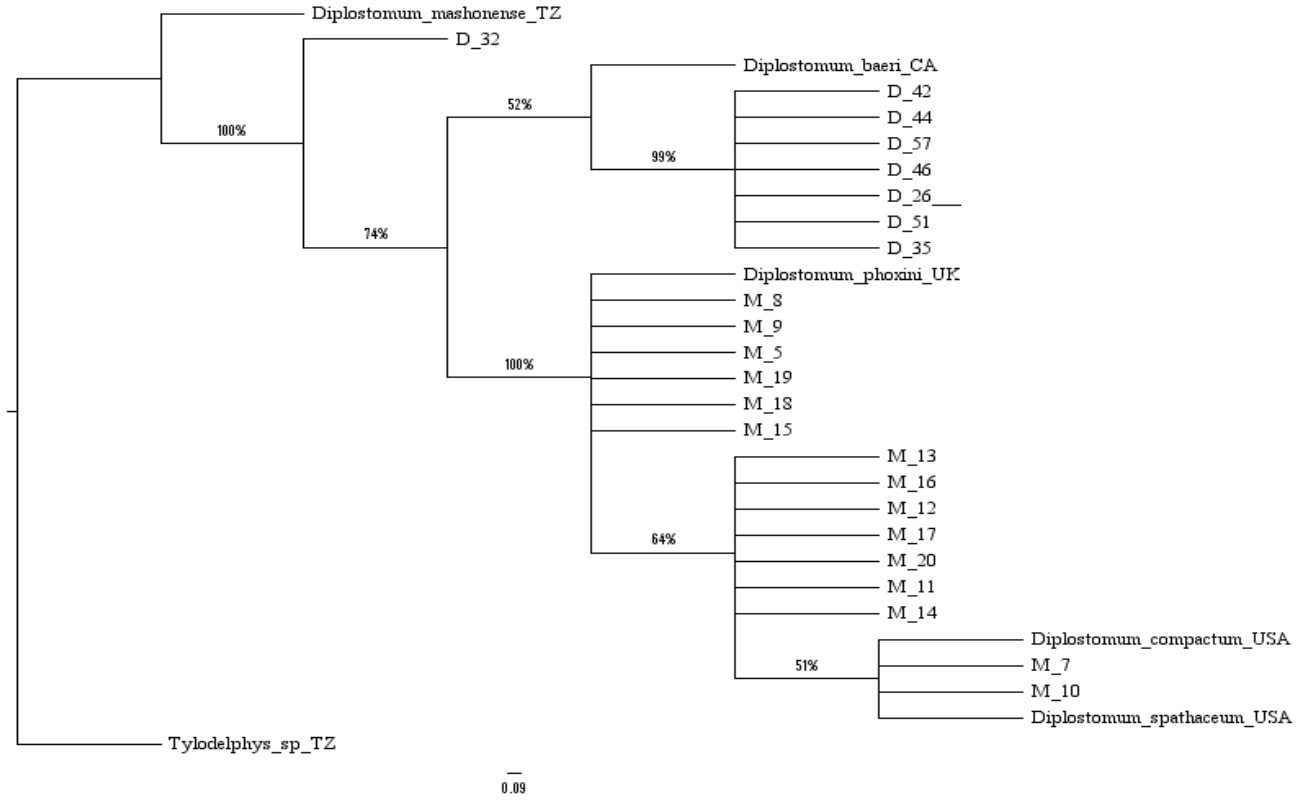

Figure 4: Neighbour - Joining tree depicting Diplostomum spp. as inferred from 8 ITS rDNA sequences.

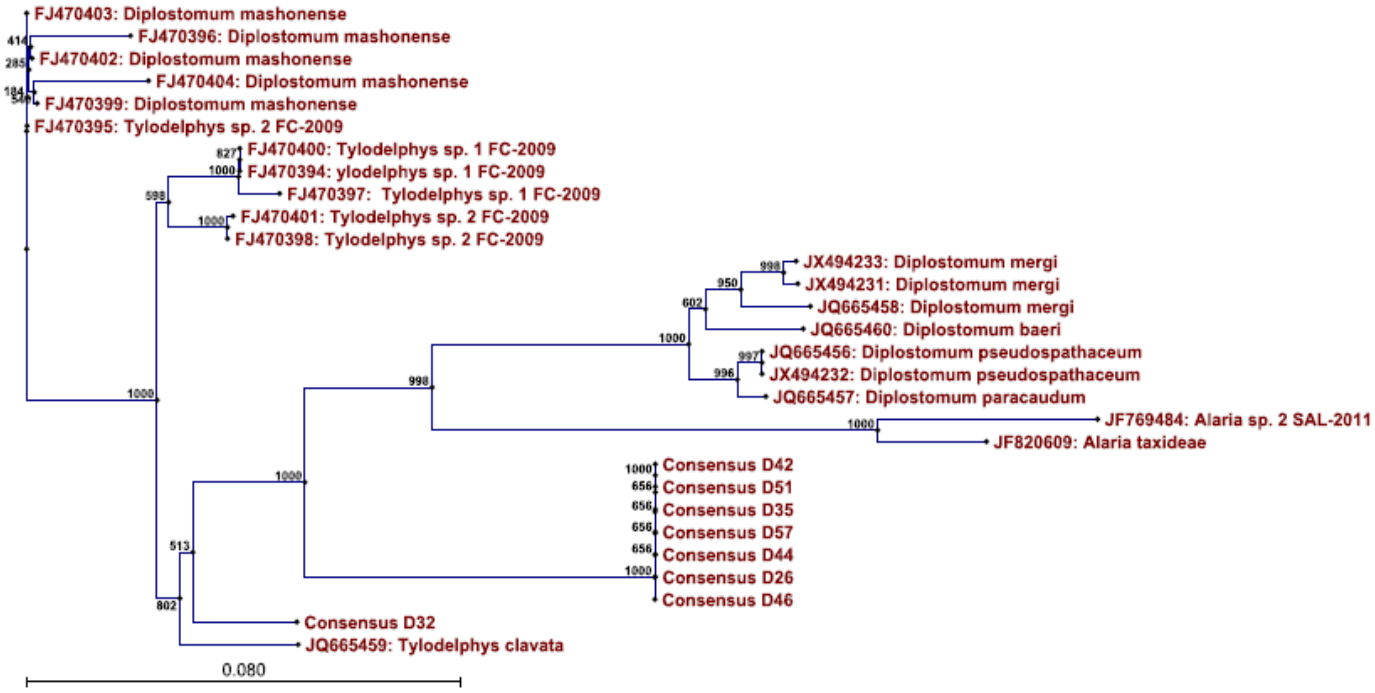

Figure 5: Neighbour - Joining tree depicting Diplostomum spp. as inferred from 18S rDNA sequences.

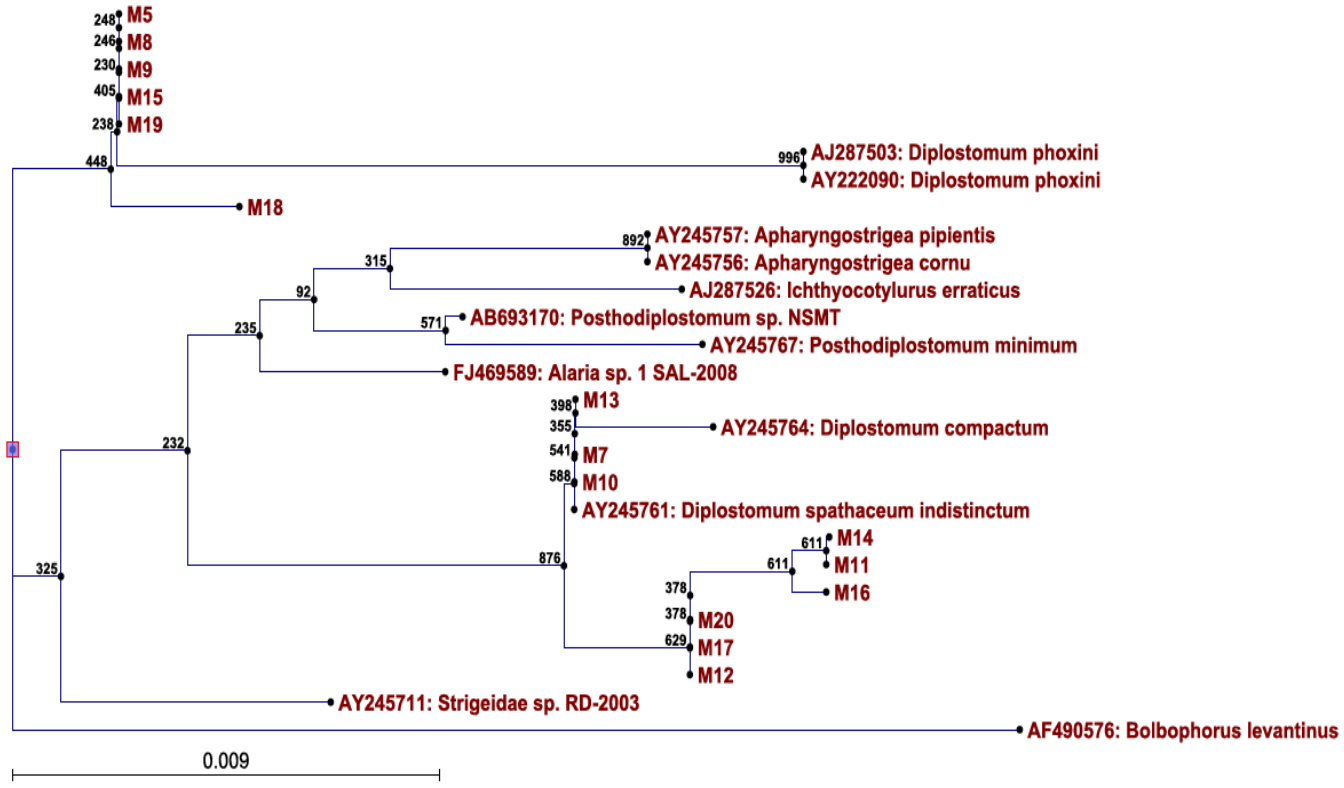

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Figure 6: Amplicons produced in PCR for cercariae using the specific primers for 18S rDNA (600nt).

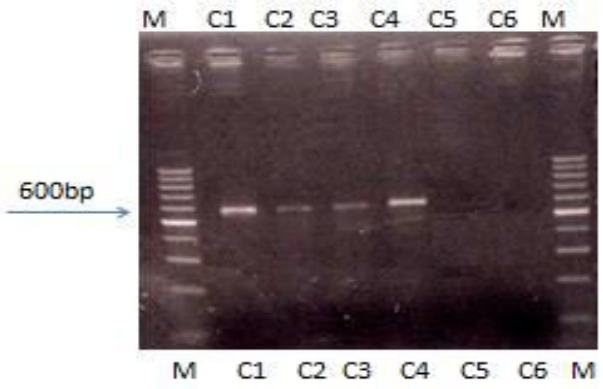

Figure 7: Amplicons produced in PCR for cercariae using the specific primers for 18S rDNA (400nt).