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Molecular Studies of Gastrointestinal Strongyle Nematodes in Migratory, Resident, and Sedentary Plains Zebras (*Equus quagga*) in Kenya

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Abstract

The molecular identity of gastrointestinal (GI) strongyle larvae recovered from faecal cultures from plains zebras in Kenya was determined using molecular tools. Internal transcribed spacer (ITS) ribosomal DNA (rDNA) extracted from the larvae were amplified using Polymerase Chain Reaction (PCR), sequenced for identification, and compared to sequences in the GenBank to determine their phylogeny. Sixteen sequences were obtained and identified as *Cyathostomum montgomeryi, Cylicostephanus minutus, Poteriostomum imparidentatum, Triodontophorus nipponicus* and *Strongylus vulgaris*. The genetic identity of *P. imparidentatum, T. nipponicus* and *S. vulgaris* from plains zebras in Kenya are reported for the first time in this study. The 16 sequences clustered into 5 clades according to the 5 genera of nematodes identified. The clade having *T. nipponicus* was placed as a sister to the Cyathostominae but was very distinct from the clade having *S. vulgaris*. The close clustering of *T. nipponicus* to the Cyathostominae supports previous suggestions that it belongs to this subfamily rather than Strongylinae. Five sequences of *C. montgomeryi* clustered closely with four sequences of *C. montgomeryi* in the GenBank isolated from zebras in Kenya. The other five sequences were evolutionary distinct. Similarly, two of the *Cy. minutus* sequences clustered with *Cy. minutus* sequences from zebras in Kenya, while the other one, was distinct. These results suggest intra-species genetic polymorphism among the *C. montgomeryi* and *Cy. minutus* isolates. The *Poteriostomum imparidentatum, T. nipponicus,* and *S. vulgaris* sequences were distinct from sequences found in the GenBank. This study contributes to the scanty but growing literature on equine strongyle genetics in zebras.

Keywords

Molecular identity; phylogeny; strongyles; plains zebras

1. Introduction

Infections by parasites are of significant concern to wildlife, as they present the most pervasive challenges to grazing herbivores [1]. Gastrointestinal (GI) helminths are among the most abundant parasites in the world [2]. These parasites are significant in regulating wildlife because of the harm they cause and can act as agents of extinction [3,4]. Studies on helminth infections have increased since the parasites are found in both wild and domestic populations. Interestingly, wildlife has been found to be a reservoir for many livestock

diseases [5], and hence identifying the diverse parasites in wild populations is a key step in reducing the rate of infections across wild and domestic animals.

The Strongylidae family is the most common of the helminth parasites which consist of a diverse group of faecal-orally transmitted nematodes that chronically infect untreated equid hosts [6]. There is a rich and diverse body of research on gastrointestinal parasites of equids spanning over a century [7]. Given their existence as relatively free-ranging, zebras represent equids untouched by parasite control measures such as anthelmintics. Therefore, studies on parasites of zebras may provide foundational information for wildlife and equine parasitology [8].

Equine strongyles (Family Strongylidae) are a significant category of intestinal nematodes. They are among important helminth parasites in the *Equus* gut, and have been found in horses, donkeys, mules and zebras, and inter-species hybrids [9]. Transmission of the parasites is through ingestion of infective larvae (L_3). Most of the information available on these nematodes is from studies in horses, mainly on aspects such as prevalence, morphology, and disease control and prevention [10]. A few studies, based on morphology of adult worms, have reported on prevalence and worm burdens in zebras in Africa [8,11,12]. The equine strongyles are an important cause of illness and mortality in horses and donkeys, while the cyathostominae have shown increased resistance to anthelmintics [13,14]. The pathogenic effects of equine strongyles in zebras have not been documented, but they are potentially harmful and may have consequences on host populations dynamics. The phylogenetic relationship of species within *Equus* is well documented, confirming the close association between zebras, donkeys, and horses [15].

Equine strongyles are classified into two sub-families, the Strongylinae (large strongyles), and the Cyathostominae (small strongyles). The large strongyles are further classified into 5 genera (*Strongylus, Oesophagodontus, Triodontophorus, Bidentostomum*, and *Craterostomum*) and 14 species. The small strongyles are classified into 14 genera (*Caballonema, Coronocyclus, Cyathostomum, Cylicocyclus, Cylicodontophorus, Cylicostephanus, Cylindropharynx, Gyalocephalus, Hsiungia, Parapoteriostomum, Petrovinema, Poteriostomum, Skrjabinodentus,* and *Tridentoinfundibulum*) and 50 species [6].

Most of the equid strongyles can infect multiple equid species [8,16,17]. Identifying equine strongyles using morphological features at some life cycle stages (eggs and larvae (L_3)) is difficult. DNA technology is an alternative approach for identifying these nematodes [18,19]. DNA technology has not been fully exploited in veterinary parasitology. Techniques, like the PCR [20] has opened opportunity for identification of nematode eggs and larvae during diagnosis of parasitic infections. While there is diverse literature on genetic identification of horse strongyles, most of this work is limited to strongyles infecting the domestic horse [9,21,22]. Despite the importance of strongyle nematodes as disease-causing agents in horses and possibly other equids, there is a scarcity of reports on species identification.

There are two species of zebras in Kenya, the plains zebra (*Equus quagga*) and the Grevy's zebra (*Equus grevyi*). The plains zebras are the most common and are found in many parts of the country while the Grevy's zebras are endangered and commonly found in the Northern part of the country. Only one report by [7] on the genetic identity of gastrointestinal nematodes in the plains and the Grevy's zebras in Kenya is available. It has been observed that each year, some of the plains zebras (migratory) in the Masai Mara National Reserve (MMNR) migrate together with wildebeest and other animals between the Serengeti National Park in Tanzania and Kenya's Masai Mara and vice versa in search of pasture and water. The resident zebras in Masai Mara remain in the reserve throughout the year, with an unlimited range (open-sedentary system) of grazing land. Other zebras are in protected fenced parks such as Lake Nakuru National Park (LNNP) and are in a closed sedentary system. In a previous study by [23] gastrointestinal nematodes eggs/larvae in these zebras were identified morphologically to genera level. The study reported that these zebras were infected by different types of helminths including nematodes, trematodes, and cestodes; the strongyle nematodes were the most abundant. The aim of the study was to determine the molecular identity and phylogenetic relationships of GI strongyle (L_3), previously isolated from the three groups of plains zebras in MMNR and LNNP.

2. Materials and Methods

2.1. Study Area and Design

The study area and design were described previously by [23]. In brief, fresh faecal samples were collected in 2014 and 2015 from pastures and resting places for the migratory and resident plains zebras in MMNR, and sedentary plains zebras in LNNP. In total, 867, 732, and 616 samples were collected from the migratory, resident, and sedentary plains zebras, respectively. During the faecal collection, sampling areas measuring 20m x 100m were marked out in the 2 conservation areas. The areas were then walked in a Z pattern, collecting faecal samples using the procedure for collection of herbage samples described in [24]. The samples were examined for helminth eggs using microscopy and those positive for nematode eggs cultured for larvae identification.

2.2. Molecular Techniques

2.2.1. Faecal Culture and Larvae Isolation

GI nematodes were identified using molecular markers, larvae from fresh faecal samples were cultured and harvested using a modified Baermann technique [25]. Composite faecal samples for each of the three groups of zebras (migratory, resident, and sedentary) were placed in different containers, moistened, and gently mixed. They were incubated at room temperature for 12 days with daily monitoring which involved moistening with water and stirring to prevent them from being invaded by fungi.

Larvae (L_3) extraction from the cultures was carried out on the 13th day. The culture jars were filled with lukewarm water, stirred, and inverted on a petri dish. The exposed area of the inverted jar was filled with clean water and left to stand for 12 hours; nematode larvae swam from the cultured faecal mixture into the clean water. The larvae suspension was examined under a microscope at 10X to 40X magnification and identified according to procedure in the MAFF manual [24]. The L_3 culture results were used as a general identification of the worm genera using key characteristics like intestinal cell number, head characteristics, and sheath tail characteristics.

The prevalence and distribution of L_3 isolated from the faecal cultures for the three groups were reported by [23]. L_3 of cyathostomes, *Strongylus* species (spp), and *Trichostrongylus* spp. were recovered, L_3 of the cyatostomes were the most abundant followed by *Strongylus* spp. For each culture per zebra group, that is, the migratory, the resident (R), and the sedentary (S), 200 (L_3) were preserved in 70% ethanol. A total of 600 individuals (L_3) were collected across all the three zebra groups.

2.2.2. DNA Extraction

Ethanol used in preserving the larvae was removed from the (L_3) using a lyophilizer and then washed in Phosphate saline buffer to completely remove ethanol residues. DNA extraction from the 600 L_3 samples was done using Invitrogen PureLink Genomic DNA (gDNA) Mini Kit Cat no. K1820-02 as per manufacturer's protocol. The samples were quantified using the NanoDrop 2000. Eluted gDNA samples were run on 1.5% Agarose Gel using 1X TBE Buffer to check for the integrity of the DNA. The samples were then standardized to 23ng/µl for PCR and stored at -20°C until use. Internal Transcriber Spacer I and II Polymerase Chain Reaction (PCR) Amplification and Sequencing

Each gDNA extract was amplified at the region spanning internal transcriber spacer 1 (ITS1) 5.8S and ITS2 of ribosomal DNA (rDNA) using two sets of primers, first by NC5 (forward, 5' GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (reverse, 5'-TTAGTTTCTTTTCCTCCGCT-3'). Amplification was carried out in 15µ reactions containing 1.5 µl of genomic DNA, 6 µl of 5 PRIME hotmaster mix (Hamburg, Germany), 0.75 µl of each primer (10 µM) and 6 µl of PCR-quality water. Amplification was preceded by a 2-minute polymerase activation step at 90°C, followed by 35 cycles of 45 sec each at 57°C annealing, 72°C extension and 95°C denaturation. Amplification was terminated by a final extension step at 72 °C for 5 minutes. The nested reaction was carried out using primers NC1 (forward, 5'-ACGTCTGGTTCAGGGTTGTT-3') and NC2 (reverse, 5'- TTAGTTTCTTTTCCTCCGCT-3'). The PCR condition for this primer was identical to the (NC5-NC2 primer) conditions except that its annealing temperature was 55°C [26]. PCR amplicons in gels were excised and purified using QIAquick PCR purification Kit (Cat no. 28106) as per the manufacturer's protocol. The purified DNA was sequenced using same forward and reverse PCR primers at Macrogen Europe Laboratories (Amsterdam, the Netherlands).

2.3. Data Analysis

The obtained nematode ITS rDNA nucleotide sequences were viewed and manually verified using chromatogram peaks, edited, and assembled using CLC Main Workbench 6.3.8 software (CLCbio, Qiagen GmbH, Germany) and BioEdit program. Bioinformatics analysis of the sequences was done using the nucleotide Local Alignment Search Tool (BLASTn), multiple sequence alignment, and phylogenetic analysis. Multiple sequence alignment was done using Log-Expectation (MUSCLE) v3.8.31 [27]. Sixteen ITS gene consensus sequences obtained in this study and seventeen reference ITS gene sequences of closely related helminths species retrieved from the GenBank **(Table 1)** were used to reconstruct a phylogenetic tree employing the Maximum Likelihood (ML) algorithm implemented in MEGA X [28]. The best nucleotide substitution model with the lowest BIC (Bayesian Information Criterion) scores of 1724.87 was found to be Tamura 3- parameter model [29] as determined using MEGA X with 1000 bootstrap replicates [30]. The analysis involved a total of 33 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 84 positions in the final data set. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches.

3. Results

3.1. Molecular Detection and Prevalence of Plains Zebra Gastrointestinal Strongyles

DNA was extracted from all the larvae from faecal cultures of the three groups of plains zebra, upon amplification only 104 produced positive amplicons for sequencing. Out of the 104 amplicons sequenced only 32 were of good quality for further analysis. After cleaning and editing, only 16 sequences matched with nematodes in the GenBank. The sequences obtained were distributed among the three groups of zebras: 5 each from the migratory (MMMZ) and resident (MMRZ) plains zebras from MMNR and 6 from the sedentary (LNSZ) plains zebras from LNNP **(Table 2)**.

All the 16 identified (L_3) belonged to the family Strongylidae of which fourteen (87.5%) belonged to the subfamily Cyathostominae while the remaining two (12.5%) belonged to the subfamily Strongylinae as shown in **Table 3**.

Table 1: References for sequences of species of Strongyles and Bunostomum selected from GenBank and included in the phylogenetic tree.

Nematode Name	Host	Country	Gene	Accession No
Cyathostomum montgomeryi	Plains Zebra	Kenya	ITS	MZ435504
Cyathostomum montgomeryi	Grevy's Zebra	Kenya	ITS	MZ435572
Cyathostomum montgomeryi	Plains Zebra	Kenya	ITS	MZ435563
Cyathostomum montgomeryi	Plains Zebra	Kenya	ITS	MZ435583
Cylicostephanus minutus	Horse	China	ITS	MT382658
Cylicostephanus minutus	Horse	Germany	ITS	MH487658
Cylicostephanus minutus	Plains Zebra	Kenya	ITS	MZ435498
Cylicostephanus minutus	Plains Zebra	Kenya	ITS	MZ435536
Poteriostomum imparidentatum	Horse	China	ITS	KY495604
Poteriostomum imparidentatum	Donkey	China	ITS	KP693433
Poteriostomum imparidentatum	Horse	Australia	ITS	Y08590
Triodontophorus nipponicus	Horse	China	ITS	KR296739
Triodontophorus nipponicus	Donkey	China	ITS	KP693437
Triodontophorus nipponicus	Donkey	China	ITS	KU205013
Triodontophorus nipponicus	Donkey	China	ITS	KU205011
Strongylus vulgaris	Donkey	China	ITS	KP693439
Bunostomum phlebotomum	Cattle	Australia	mtDNA	NC_012308

Three species of the subfamily Cyathostominae were identified and distributed as *Cyathostomum montgomeryi* (10; 71.43), *Cylicostephanus minutus* (3; 21.43%), and *Poteriostomum imparidentatum* (1; 7.14%). Species of Strongylinae identified from PCR products were *Triodontophorus nipponicus* (1; 50%) and *Strongylus vulgaris* (1; 50%) **(Table 3)**.

Amplicons belonging to both subfamilies were identified from both the migratory and resident zebras from MMNR, while only sequences belonging to the Cythostominae subfamily were recovered from zebras of Lake Nakuru National Park as shown in **Table 3**.

3.2. Molecular Identities of Detected Cyathostominae and Strongylinae Subfamilies

Results from the local alignment search tool (BLASTn) for all the 16 gastrointestinal Strongylidae nematode larvae ITS rDNA are shown in **Table 4**. The partial ITS rDNA sequences appear in GenBank under the accession numbers; OK235465 to OK235480. BLASTnn analysis revealed that majority, 10 (62.5 %), of the Cyathostominae were similar to *Cyathostomum montgomeryi* with matching identity between 92.26 and 100% to annotated sequences in the GenBank. The five annotated sequences for *C. montgomeryi* in the GenBank having the highest match to sequences obtained in the current study were for nematodes previously reported from zebras in Kenya. The sequences identified as *C. montgomeryi* in the current study were almost equally distributed across the migratory (n=3, isolates ME, MF26, and MO), resident (n=3, isolates R4, R5, and RX), and sedentary (n=4, S3, S4, S7, and S8).

Three (18.75%) of the sequences were similar to *Cy. minutus* with sequence identity of between 91.63 and 99.18%. The three sequences matched two annotated sequences for *Cy. minutus*, previously reported from zebras in Kenya. One sequence was similar to *Poteriostomum imparidentatum* retrieved from GenBank isolated from an adult feral horse (*Equus caballus*) from Australia with sequence identity of 96.77% **(Table 4)**. This is the first report on genetic identity of *P. imparidentatum* species from zebras in Kenya.

Two of the sequences were those of Strongylinae subfamily, one similar to *S. vulgaris* with sequence identity of 90.12% and the other similar to *T. nipponicus* with sequence identity of 96.45% **(Table 4)**. This is also the first report of *S. vulgaris* and *T. nipponicus* in zebras in Kenya.

Table 2: Distribution of 16 rDNA amplicons obtained from the polymerase chain reaction of rDNA extracts from gastrointestinal strongyle nematode larvae isolated from cultures of faecal samples from the three Zebra groups.

Zebra Group	MMMZ (Migratory)	MMRZ (Resident)	LNSZ (Sedentary)
PCR Amplicon	ME/KEN/MMNR/2015	R1/KEN/MMNR/2015	S1/KEN/LLNP/2015
	MF25/KEN/MMNR/2015	R3/KEN/MMNR/2015	S2//KEN/LLNP/2015
	MF26/KEN/MMNR/2015	R4/KEN/MMNR/2015	S3/KEN/LLNP/2015
	MO/KEN/MMNR/2015	R5/KEN/MMNR/2015	S4/KEN/LLNP/2015
	MO2/KEN/MMNR/2015	RX/KEN/MMNR/2015	S7/KEN/LLNP/2015
			S8/KEN/LLNP/2015

Table 3: Species of Cyathostominae and Strongylinae subfamilies identified from PCR amplicons of larvae ITS rDNA from cultures of the three Zebra groups.

Strongylidae nematode larvae	Distribution of nematode species among the zebra groups			Total
	MMMZ (n=5)	MMRZ (n=5)	LNSZ (n=6)	
Subfamily Cyathostominae				
Cyathostomum montgomeryi	3	3	4	10
Cylicostephanus minutus	0	1	2	3
Poteriostomum imparidentatum	1	0	0	1
Subfamily Strongylinae				
Triodontophorus nipponicus	1	0	0	1
Strongylus vulgaris	0	1	0	1

3.3. Phylogenetic Analysis of Zebra Strongyles

Results of the phylogenetic analysis of the strongyle (L_3) rDNA sequences from the plains zebras and those in the GenBank are shown in **Figure 1**. Overall, the 16 sequences clustered into 5 clades, according to the 5 genera of nematode larvae identified. The five clades were (A) having *C. montgomeryi,* (B) having *Cy. minutus,* (C) having *P. imparidentatum,* (D) having *T. nipponicus* and (E) having S. *vulgaris* **(Figure 1)**. The clade having *T. nipponicus* (Clade D), which is classified as a Strongylinae was placed as a sister clade to the one having the true Cyathostominae (*C. montgomeryi, Cy. minutus* and *P. imparidentatum*). The clade having *T. nipponicus* appeared evolutionary distinct from the only other rDNA sequences from a Strongylinae, *S. vulgaris.*

Five of the ten sequences of *C. montgomeryi* from this study clustered closely with four sequences of *C. montgomeryi* in the GenBank. The four sequences were from adult worms previously sampled from zebras in Central Kenya. The other five sequences appeared distinct, but distantly related to those in the GenBank. Two of the three *Cy. minutus* sequences from the current study clustered with two *Cy. minutus* sequences in the GenBank, from the same study in central Kenya. One of the sequences of *Cy. minutus* from the current study appeared distinct (bootstrap value of 96) from the other two sequences and was a sister to a clade having sequences from horses in China (MT382658.1) and Germany (MH487658.1). *Poteriostomum imparidentatum* singleton obtained in this study was distinct from *P. imparidentatum* isolates obtained from the GenBank. The sequences in the GenBank were KY495604.1 and Y08590.1 from horses in China and Australia, respectively, and KP693433.1 from wild donkeys in China.

The *T. nipponicus* sequence identified in this study was distantly related to sequences in the GenBank from horses and wild donkeys from China. Our single isolate of *S. vulgaris* was distinct but related to *S. vulgaris* sequence (KP693439.1), found in the GenBank and isolated from a wild horse in China. No closely related sequences for any previous isolates of *P. imparidentatum, T. nipponicus* or *S. vulgaris* from zebras, donkeys, or horses in Kenya were found in the GenBank.

4. Discussion

This study reports for the first time the molecular diversity in migratory, resident, and sedentary plains zebras in Kenya. Overall, the study revealed that the majority of the sequenced reference samples belonged to the subfamily Cyathostominae and were identified as *Cyathostomum montgomeryi*, *Cylicostephanus minutus,* and *Poteriostomum imparidentatum.* The rest belonged to the subfamily Strongylinae and were *Triodontophorus nipponicus* and *Strongylus vulgaris*. Only one previous report which looked at the genetic identity of GI nematodes from zebras in Central Kenya is currently available in the literature [7]. The study sequenced ninety-one amplicons from adult worms expelled in faeces from plains and Grevy's zebras and identified four genera of equine strongyles from the subfamily Cyathostominae (*Parapoteriostomum* spp, *Cylindropharynx brevicauda, C. intermedia, C. longicauda, Cyathostomum montgomeryi* and *Cylicostephanus minutus*) and one genus from the subfamily Strongylinae (*Craterostomum acuticaudatum*). Our study also reported the occurrence of *C. montgomeryi* and *Cy. minutus* in zebras in Kenya, as was reported by [7].

Table 4: Sixteen Strongylidae infective larvae ITS rDNA sequences from plains zebras, identified using Local Alignment Search Tool (BLASTn), with their corresponding accession numbers, matching sequences, accession numbers of the highest match and percentage identity.

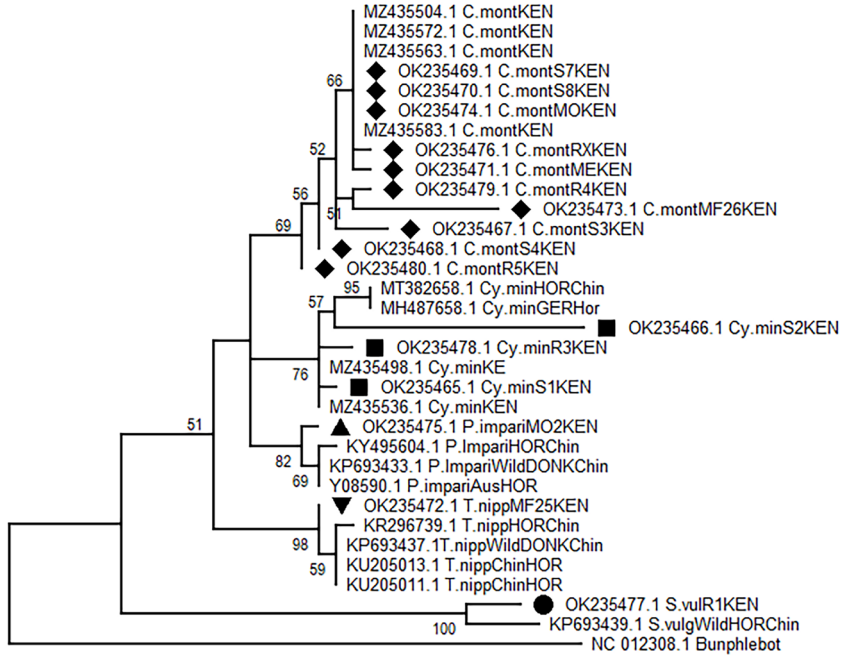
Isolate	Our accession number	Matching sequence	Accession no. of highest match	E-value	Identity (%)
MF26	OK235473	*Cyathostomum montgomeryi*	MZ435563.1	0.0	94.60
ME	OK235471	*Cyathostomum montgomeryi*	MZ435563.1	0.0	99.56
MO	OK235474	*Cyathostomum montgomeryi*	MZ435572.1	0.0	99.69
RX	OK235476	*Cyathostomum montgomeryi*	MZ435583.1	0.0	96.02
R5	OK235480	*Cyathostomum montgomeryi*	MZ435563.1	0.0	97.13
R4	OK235479	*Cyathostomum montgomeryi*	MZ435497.1	0.0	92.29
S4	OK235468	*Cyathostomum montgomeryi*	MZ435583.1	0.0	98.96
S7	OK235469	*Cyathostomum montgomeryi*	MZ435553.1	0.0	99.71
S8	OK235470	*Cyathostomum montgomeryi*	MZ435563.1	0.0	100
S3	OK235467	*Cyathostomum montgomeryi*	MZ435563.1	0.0	95.69
R3	OK235478	*Cylicostephanus minutus*	MZ435498.1	0.0	97.61
S1	OK235465	*Cylicostephanus minutus*	MZ435536.1	0.0	99.18
S2	OK235466	*Cylicostephanus minutus*	MZ435536.1	0.0	91.63
MO2	OK235475	*Poteriostomum imparidentatum*	Y08590.1	0.0	96.77
R1	OK235477	*Strongylus vulgaris*	KP693439.1	0.0	90.12
MF25	OK235472	*Triodontophorus nipponicus*	KU205013.1	0.0	96.45

Results from our study indicated that *C. montgomeryi* was the most abundant equine strongyle, [7] reported the predominance of *Parapoteriostomum* spp. Previous reports **[31,32]** suggest that *C. montgomeryi* is restricted to *Equus* species in Africa. Results from our study support this observation as no matching sequences for this parasite could be found in the GenBank from studies in equines from other parts of the world. The number of amplicons examined in the study by [7] was much larger than the sixteen amplicons examined in our study. They also analyzed adult worms collected from plains zebras, Grevy's zebras, and a Grevy's x plains hybrid zebra at Mpala in Laikipia County. The differences in species of zebras examined, the type of samples used, and the numbers of amplicons analyzed could account for differences in nematode genera identified in this study and that by [7].

The ten ITS gene sequences identified as *C. montgomeryi* in our study had varying species identity (92.26 to 100%) with the five annotated sequences for the same parasite, reported from zebras in Kenya by [7]. Similarly, the three ITS gene sequences identified as *Cy. minutus* in our study had varying species identity (91.63 to 99.18%) with the two annotated sequences for *Cy. minutus* reported from zebras in Kenya by [7]. This data suggests that there is intra-species genetic polymorphism of *C. montgomeryi* and *Cy. minutus* in zebras in Kenya.

The study is the first to report, the genetic identity of *Poteriostomum imparidentatum, Triodontophorus nipponicus,* and *Strongylus vulgaris* from plains zebras in Kenya. Equine strongyles in the genera *Poteriostomum* and *Triodontophorus* have been reported previously in Burchell's zebras in South Africa **[8,33]**, while *P. imparidentatum* and *Strongylus vulgaris* have been reported in plains and Grevy's zebras in Ukraine **[34]**. The three studies were based on morphological identification of adult worms from zebras **[8,33,34]**. Our study confirms the presence of these parasites in plains zebras in Kenya, as has been reported in other parts of the world, and provides information on their genetic identity.

The phylogenetic analysis placed the Cyathostominae (*Cyathostomum, Poteriostomum,* and *Cylicostephanus*) in sister clades, indicating that they were all closely related. This differs from the results of **[10]**, who examined the phylogenetic relationships of twelve protein-coding genes from twenty Strongyloidea nematodes from various hosts and reported that *Poteriostomum* spp was a sister to a clade having species of *Cyathostomum, Cylicostephanus, Triodontophorus,* and others. They concluded that *P. imparidentatum* was evolutionarily distant from other Cyathostominae and *Triodontophorus* spp. Results of our phylogenetic analysis indicate that *Triodontophorus* spp. was a sister to the subfamily Cyathostominae, having clades that had *Cyathostomum, Poteriostomum,* and *Cylicostephanus* species. Although our data and that from **[10]** indicates very close phylogenetic relationships between all four genera of nematodes (*Triodontophorus, Cyathostomum, Poteriostomum,* and *Cylicostephanus)*, the differences in the placing of the parasites in the phylogenetic tree could be explained by differences in the genes used and the number of sequences analyzed in the two studies.



KEY
C.mont refers to different Isolates of *Cyathostomum montgomeryi*; Cy.min refers to different isolates of *Cylicostephanus minutus*; P. impari refers to different isolates of *Poteriostomum imparidentatum*; T. nipp refers to different isolates of *Triodontophorous nipponicus* while S. vulg refers to different isolates of *Strongylus vulgaris*. Bunphlebot refers to *Bunostomum phlebotomum*.

Figure 1: Phylogenetic analysis of ITS segment of the nucleotide sequences obtained in this study marked with different dark shapes black shapes and 17 reference ITS sequences obtained from the GenBank of the family Strongylidae including two sub-families (Cyathostominae and Strongylinae). *Bunostomum phlebotomum* (sub-family Bunostominae) was used as an outgroup.

The close relationship between *Triodontophorus* spp., which is classified in the subfamily Strongylinae and nematodes in the subfamily Cyathostominae, is an interesting finding. Similar findings have been reported by **[10]**, suggesting that *Triodontophorus* is a Cyathostominae rather than a Strongylinae. The study by **[10]**, sequenced complete mitochondrial (mt)genomes of three Cyathostominae species (*Cyathostomum catinatum*, *Cylicostephanus minutus*, and *Poteriostomum imparidentatum*) of horses. The complete mt nucleotide sequence comparison of all Strongylidae nematodes revealed that sequence identity ranged from 77.8 to 91.6%. The mt genome sequences of *Triodontophorous* species had a relatively high identity with Cyathostominae nematodes, rather than *Strongylus* species of the same subfamily (Strongylinae). Phylogenetic analyses using mtDNA data indicated that the *Triodontophorus* species clustered with cyathostominae species instead of *Strongylus* spp **[10]**, which is consistent with the current phylogenetic results. Results of a comparative study of the morphology of the larval stage four (L_4) by **[35]** also revealed more similarity between *Triodontophorus* larvae to those of Cyathostominae than to those of *Strongylus* spp. This observation further supports the suggestion that *Triodontophorus* spp. is a Cyathostominae.

The close clustering of five out of the ten sequences of *C. montgomeryi* from our study with those from the study by **[7]** indicates evolution diversity within the species. One out of the 3 sequences from *Cy. minutus* was a sister to a clade having sequences from horses in China and Germany. Unlike *C. montgomeryi*, *Cy. minutus* appears to be more widespread in equines across the world, with the parasites being phylogenetically diverse within the species. The *Cy. minutus* from horses in China and Germany however appear to be evolutionary distant from our isolates. Sequences from *P. imparidentatum, T. nipponicus,* and S. *vulgaris* from this study were evolutionary distinct from those from either horses or donkeys from other parts of the world that were obtained from the GenBank.

The taxonomic identity and phylogeny of equine strongyles detected in the three zebra groups included species of veterinary significance with little known epidemiology in Kenya. This study through the use of genetic tools was able to differentiate closely related nematode species. The eggs of these nematodes are morphologically indistinguishable and are often included in the generic category of 'strongyle eggs' which reduces the identity and richness of the strongyle nematodes. Identification of infection-causing helminth species is anticipated to progressively become important in veterinary management practices. Parasitic helminths, regardless of species, are potentially a burden to the host. The emphasis was on large and small strongyles since they are more significant in terms of effects on the host.

The relatively small sample of genotyped larvae was a consequence of two main factors that is, loss of larvae during the process of removing ethanol, and low quality of DNA that

led to sequencing errors. In our opinion, the results of this study may not represent the whole species spectrum in the three zebra groups. The study used only one marker (ITS); The ITS region is known to reliably differentiate closely related nematode species [22,26,36–40]. This marker is also popular due to its lower level of intra-species polymorphism compared to mtDNA [22,36–41]. More comprehensive studies are recommended using both the ITS region and a portion of the mtDNA cytochrome oxidase 1 (CO1) gene of the mitochondrial DNA (mtDNA) since this locus undergoes rapid evolution and is good for differentiating cryptic parasite species [40] as well as phylogeographic groups within a single species [40]. However, these genetic markers are rarely used together in a single nematode species yet when used in combination they enhance identification output and provide more genetic information [25,41].

5. Conclusions

This study confirms the occurrence of C. *montgomeryi* and *Cy. minutus* in plains zebras in Kenya with evolution diversity within the species. This is the first report on genetic identity of *P. imparidentatum, T. nipponicus,* and S. vulgaris in the plains zebras in Kenya. These three species appeared evolutionary distinct from previously reported isolates in horses or donkeys from other parts of the world. The placing of *T. nipponicus* as a sister to the Cyathostominae further supports previous suggestions that it is a Cyathostominae rather than a Strongylinae.

Authors' Contributions

Linda G.M. Maina: Writing original draft, methodology, data collection, data analysis, funding acquisition, conceptualization. Ndichu Maingi: Drafting the manuscript, supervision, review, editing. Chege J. Ng'ang'a: Supervision, investigation, review, editing. Robert M. Waruiru and Francis Gakuya: Investigation, review, editing. Esther G. Kanduma: Formal analysis, methodology, review.

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Data Availability

The data supporting the findings of this study is available within the article.

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Conflicts of Interest

The authors declare that there are no conflicts of interest related to this paper.

Ethical Approval

The research approval for the study was obtained from the Kenya Wildlife Service (KWS) REF No: (KWS/BRM/ 5001). Faecal collection was done in adherence to the guidelines and regulations by KWS on conducting research on wildlife. All institutional and national guidelines for the care and handling of animals were observed.

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