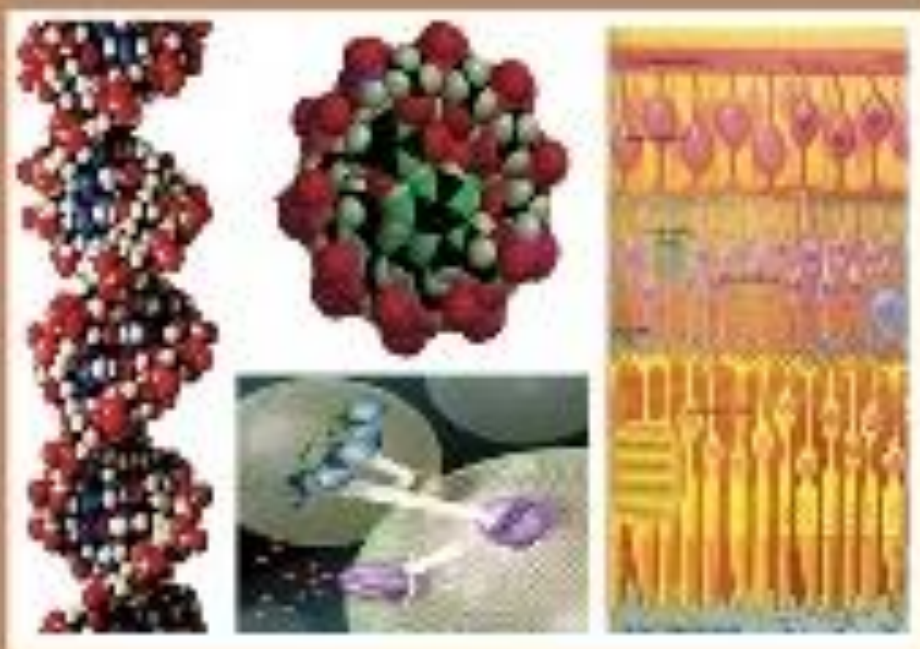




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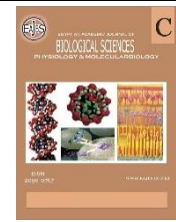
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**Molecular Characterization and Phylogenetic Analysis of Nematodes of *Phataginus tricusps* (Pholidonta: Manis) (Weber, 1904) and *Thryonomys swinderianus*, Temminck 1827 (Rodentia: Thryonomyidae)**

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**ABSTRACT**

This study conducted molecular characterization of nematode parasites in three wild mammals—*Philantomba maxwelli*, *Phataginus tricusps*, and *Thryonomys swinderianus*—collected from bushmeat markets in Lagos and Oyo states, Nigeria. Utilizing primers targeting the 28S rRNA (LSU391/501) and 18S rRNA/ITS regions (VrainF/AB28), nematodes from *P. tricusps* and *T. swinderianus* were successfully amplified, while those from *P. maxwelli* showed poor amplification. Sequencing and phylogenetic analysis revealed *Setaria digitata* in *P. tricusps* (99.37% homology with Japanese isolates) and *Chabertia ovina* in *T. swinderianus* (99.77% homology with Canadian isolates). The overall parasite prevalence was 23.2%, with *P. maxwelli* exhibiting the highest infection rate (41.7%), followed by *P. tricusps* (27.8%) and *T. swinderianus* (14.5%). Nematodes dominated infections (86.8%), with co-infections of cestodes observed in *P. tricusps*. Phylogenetic trees constructed using neighbor-joining methods and p-distance algorithms strongly supported the cladal grouping of *S. digitata* with reference sequences from Japan and Thailand, while *C. ovina* clustered with isolates from Canada and China.

**INTRODUCTION**

Integration of wildlife protection and conservation is an important issue (Pacelle, 1998). Investigating wildlife infectious diseases are essential because some harbor pathogens which can affect domestic animals and may be zoonotic which involves humans too. This is the core of the One Health approach. Parasitic diseases are among the most prevalent and important infectious diseases in wildlife. As far as free-ranging and wild animals are concerned, parasitism is the norm rather than the exception (Choquette, 1956; Thompson, 2013). Although parasites are usually in balance with the host, causing little damage or clinical impact (Choquette, 1956; Thompson, 2013). Evaluation of parasitism in wild mammals can be described in three points: the effect and importance of the parasites on the hosts, parasite transmissibility to domestic animals, and the relationship to public health (Choquette, 1956).

Molecular biology techniques are currently used widely to diagnose parasite structures in order to enhance the identification and characterization of parasites (Tavares *et al.*, 2011). The use of genetic markers in mitochondrial (mt) genomes and the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (rDNA) have been used effectively to identify and differentiate parasites of different groups (Jabbar *et al.*, 2013; Liu *et al.*, 2013; Liu *et al.*, 2012; Blouin, 2002). Studies showed that mitochondrion genomes are useful genetic markers for the identification and differentiation of closely-related species of nematodes (Lin *et al.*, 2013; Liu *et al.*, 2012). 18S rDNA has been widely used for the identification and diversity analyses of eukaryotes; this is because it is well conserved among species and it contains variable regions (Hadziavdic *et al.*, 2014; Hugerth *et al.*, 2014).

Several studies have shown the potential of wildlife harbouring ectoparasites and endoparasites (Futagbi *et al.*, 2010; Okorafor *et al.*, 2012; Okoye *et al.*, 2015). Nematodes have been a major parasite reported infecting wild mammals. Olayemi (2011) reported the presence of 9 species of nematodes in faecal samples of domesticated *T. swinderianus* in southwestern states in Nigeria; Manganga *et al.* (2023) also identified 9 species of nematodes in wild animals from the Zadié department in Gabon. However, most parasitological studies depend on using morphological details by microscopically identifying parasites in the faecal and gastrointestinal tract. Studies have reported nematodes from wild mammals in Nigeria such as *Ascaris* sp., *Bunostomum* sp., *Strongyloides* sp., *Trichostrongylus* sp., *Oesophagostomum* sp., *Trichuris* sp., *Haemonchus* sp., *Nematodirus* sp., and *Strongylus* sp. (Ugiagbe and Awharitoma, 2015; Olayemi, 2011; Opara and Fagbemi, 2008). There's a gap in molecular knowledge about identified parasites. Thus, the need for the study to molecularly characterize selected nematodes of wild mammals in Nigeria.

## MATERIALS AND METHODS

### Sample Collection and DNA Extraction:

Gastrointestinal tracts of *Philatomba maxwelli*, *Phataginus tricuspis*, and *Thryonomys swinderianus* purchased by consumers at Epe and Odo-ona bush meat markets in Lagos and Oyo states, Nigeria were incised to collect parasites. Parasites mainly nematodes recovered from wild mammals were fixed in 70% methanol and transported to the laboratory for further studies. The most abundant nematode species of each mammal were used for molecular assay.

Each nematode was freeze-dried in liquefied Nitrogen and crushed to powdered form for DNA extraction. The powdered form parasite was transferred to a 1.5ml microcentrifuge tube. DNA was extracted using an E.Z.N.A.<sup>®</sup> Tissue DNA kit (Omega Bio-tek, Inc) procedures were followed according to the manufacturer's instructions.

### DNA Quality Assessment:

The purity level of the eluted DNA was assessed using a nanodrop spectrophotometer. The spectrophotometer was blanked with an elution buffer before measurement. 1.5µL of each sample was dropped on the nanodrop spectrophotometer and measured.

### PCR Amplification:

Amplification of genes from eluted DNA was done using two primers: LSU 391 (5'-AGCGGAGGAAAAGAACTAA-3') and LSU 501 (5'-TCGGAAGGAA CCAGCTACTA-3') targeting 28S ribosomal RNA (rRNA); Vrain-F (5'-TTGATTACGTCCCTGCCCTTT-3') and AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3') targeting 18S rRNA, the internal transcribed spacer 1 (ITS1), the 5.8S rRNA, and ITS 2 and part of the 28S rRNA. Amplification was done using a gradient master thermocycler (Clever Scientific Limited). Eluted DNA was amplified at 95°C for 5 minutes, 95°C for 30 seconds, 54°C for 45 minutes, 72°C for 1 minute 30 seconds, 72°C for 10 minutes, and 4°C for ∞.

**Sequence and Phylogenetic Analyses:**

Sequencing was done using the BrilliantDye™ Terminator v3.1 Cycle Sequencing on Applied Biosystems ABI3500XL genetic analyzer. Ten PCR amplicons of the LSU391/LSU501 primer pair and eight PCR amplicons of the VrainF/AB28 primer pair were sequenced using the LSU391 and VrainF primers respectively. The raw sequences were trimmed and edited (base calling) using Sequencer 5.4.6. Build 46289 and Geneious Prime® 2023.0.4 Build 2023-01-24 15:21. Sequence homology was done using the NCBI Nucleotide BLAST tool and sequence alignment using ClustalW in MEGA 7. Phylogenetic analyses were done using the p-

distance algorithms of the Neighbour-Joining method considering 1000 bootstrap replications in MEGA 11.

**Nucleotide Sequence Accession Numbers:**

The 28S rRNA and 18S rRNA gene sequences were submitted to the NCBI-GenBank for comparative identification and accession numbers have been issued.

**RESULTS****Prevalence of Infection:**

*T. swinderianus* is the most abundant mammal in the markets followed by *P. maxwelli*. The overall prevalence was low (23.2%). The prevalence of infection was higher in *P. maxwelli* and the least in *T. swinderianus* as shown in Table 1.

**Table 1:** Prevalence of infection among the mammals.

Mammals	Number Examined	Number Infected	Prevalence (%)
<i>P. maxwelli</i>	96	40	41.7
<i>P. tricuspis</i>	18	5	27.8
<i>T. swinderianus</i>	214	31	14.5
Total	328	76	23.2

Table 2, showed the distribution of infection among the mammal at both markets. Nematodes (86.8%) were the dominant parasites in the three mammals examined in

the study. *P. maxwelli* dominant parasites were nematodes. Coinfection of nematode and cestode was majorly observed in *P. tricuspis* as shown in Table 2.

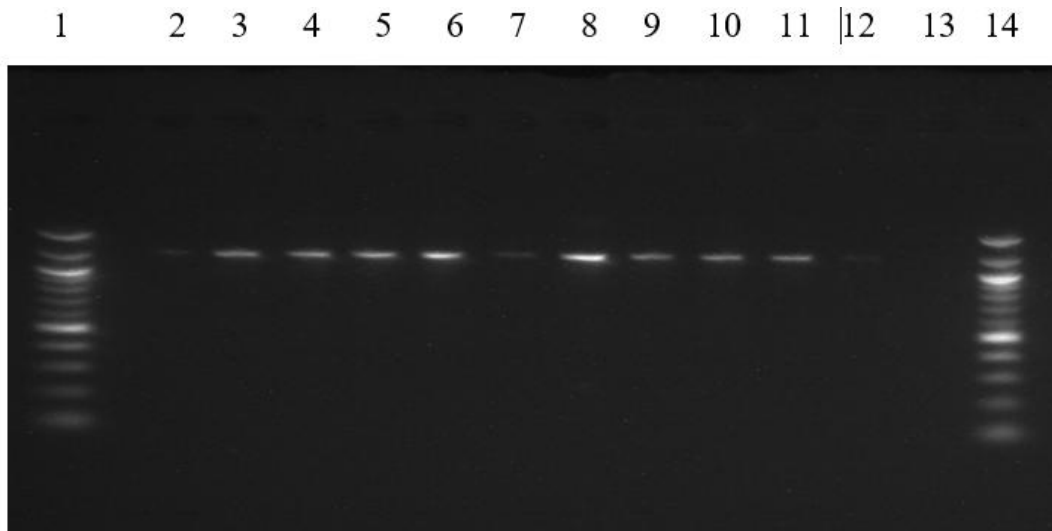
**Table 2:** Distribution of infection among mammals.

Mammals	Number Infected	Nematodes (%)	Cestodes (%)	Coinfection (%)
<i>P. maxwelli</i>	40	100	0	0
<i>P. tricuspis</i>	5	40	40	20
<i>T. swinderianus</i>	31	77.4	19.4	3.2
Overall	76	86.8	9.2	3.9

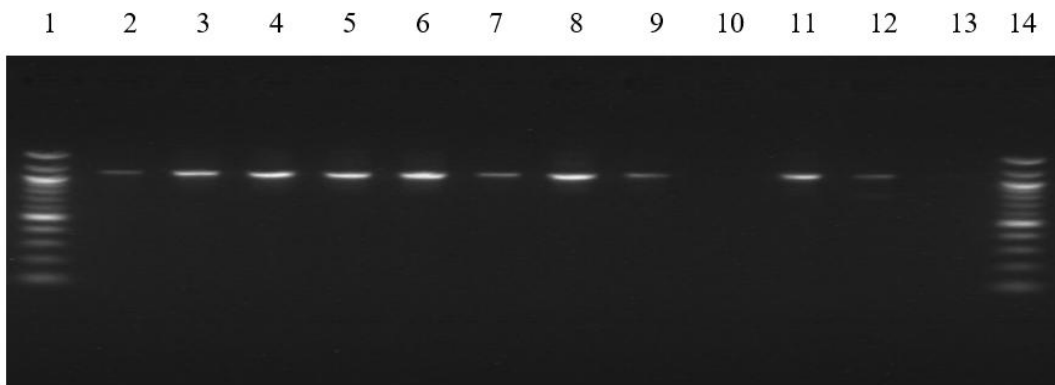
**Molecular Results:**

Amplification of parasites recovered from *Phataginus tricuspis* and *Thryonomys swinderianus* confirmed it to be nematodes using LSU 391/501 and Vrain-F/AB28. Products were successfully amplified at 1100bp and 1050bp respectively. Eight out of ten parasites of *Phataginus tricuspis* were fully amplified using LSU 391/501 while nine

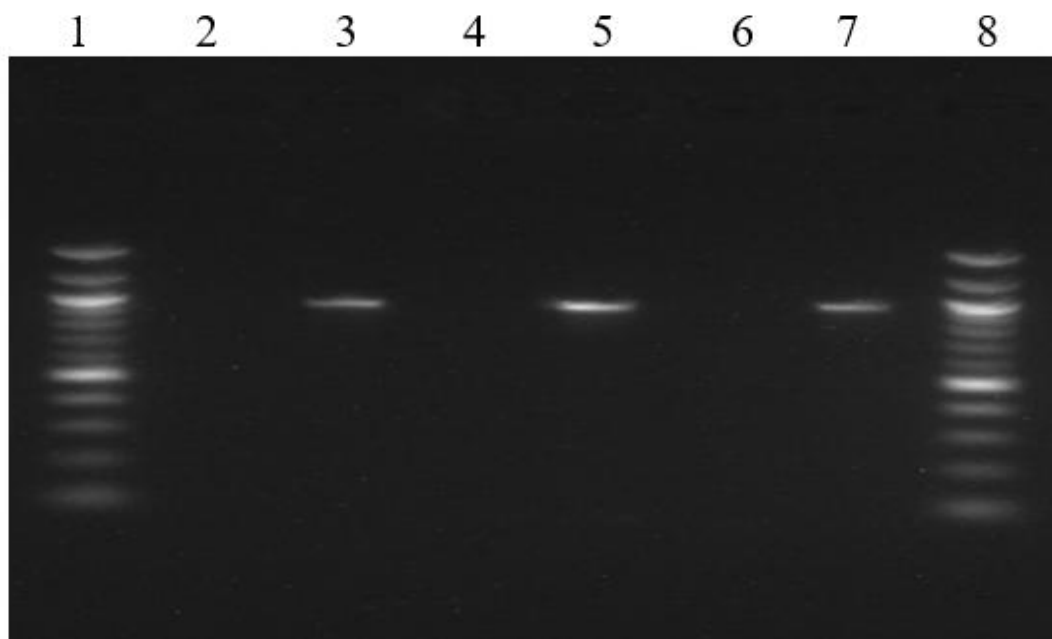
were well amplified using Vrain F/AB28. In *Thryonomys swinderianus*, three parasites out of six were amplified by both primer pairs. However, nematodes 2 and 5 of *Thryonomys swinderianus* were amplified by both primer pairs. Nematodes of *Philatomba maxwelli* were not well amplified by the two primer pairs.



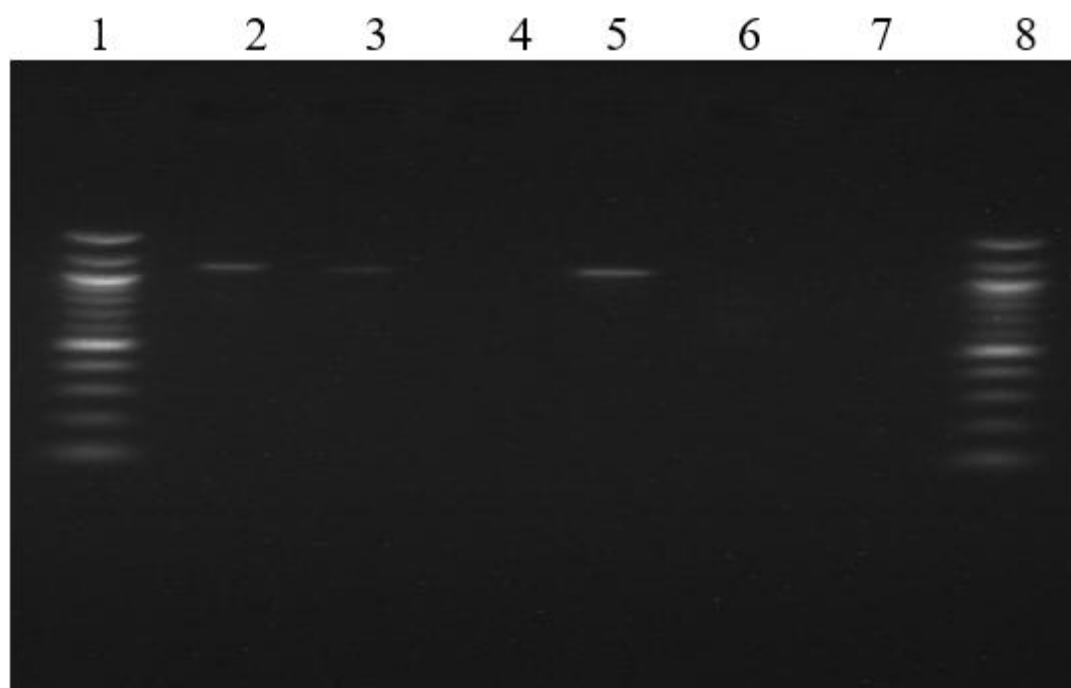
**Fig. 1:** DNA gel for *P. triscupis* and *P. maxwelli* nematodes for LSU 391-501, 28S rRNA gene  
Key: Well 1, 14 – Ladder; 2-11 – nematodes in *P. triscupis*, 12-13 – nematodes in *P. maxwelli*.



**Fig. 2:** Gel pictures of Vrain F-AB28 for *P. triscupis* and *P. maxwelli* nematodes  
Key: Well 1, 14 – Ladder; 2-11 – nematodes in *P. triscupis*, 12-13 – nematodes in *P. maxwelli*



**Fig. 3:** DNA gel for *T. swinderianus* nematodes for LSU 391-501  
Key: Well 1, 8 – Ladder; 2-7 – nematodes in *T. swinderianus*



**Fig. 4:** Gel pictures of Vrain F-AB28 for *T. swinderianus* nematodes  
Key: Well 1, 8 – Ladder; 2-7 – nematodes in *T. swinderianus*.

#### Sequencing Result:

The amplicons of eleven nematode parasites of *Phataginus tricuspis* and three nematode parasites of *Thryonomys swinderianus* were sequenced. The sequenced amplicons of *Phataginus tricuspis* and *Thryonomys swinderianus* were identified as *Setaria digitata* and *Chabertia ovina* respectively using the combination of LSU391/LSU501 primer pair which targets the 28S rRNA region and the VrainF/AB28 primer pair which targets the parts of the 18S rRNA, ITS1, the 5.8S rRNA, and ITS2 and part of the 28S rRNA regions as shown in **Table 3**. Based on partial sequences of the 28S

rRNA region, isolates of *Phataginus tricuspis* shared the closest homology with *Setaria digitata* isolate SL/Sd/10 while isolates of *Thryonomys swinderianus* shared the closest homology with *Chabertia ovina*. Based on sequences of the 18S rRNA, ITS1, and 5.8S rRNA regions, isolates of *Phataginus tricuspis* shared closest homology with *Setaria digitata* isolate ST5. Based on sequences of the 18S rRNA, ITS1, the 5.8S rRNA, and ITS2 regions, the isolate of *Thryonomys swinderianus* shared the closest homology with *Chabertia ovina* isolate CHO5 (Table 3).

**Table 3:** Locations of Strains with Closet Homology from NCBI-BLAST.

S/N	Strains Code	Sequencing Primer	Organism with Closet Homology	Location Isolated	% Identity/ Homology
1	1B ( <i>Pt</i> )	LSU391	<i>Setaria digitata</i> isolate SL/Sd/10	Japan	99.37
2	1C ( <i>Pt</i> )		<i>Setaria digitata</i> isolate SL/Sd/10	Japan	99.37
3	1D ( <i>Pt</i> )		<i>Setaria digitata</i> isolate SL/Sd/10	Japan	99.37
4	1E ( <i>Pt</i> )		<i>Setaria digitata</i> isolate SL/Sd/10	Japan	99.37
5	1G ( <i>Pt</i> )		<i>Setaria digitata</i> isolate SL/Sd/10	Japan	99.37
6	1H ( <i>Pt</i> )		<i>Setaria digitata</i> isolate SL/Sd/10	Japan	99.37
7	1J ( <i>Pt</i> )		<i>Setaria digitata</i> isolate SL/Sd/10	Japan	99.37
8	2B ( <i>Ts</i> )		<i>Chabertia ovina</i>	Canada	99.77
9	2D ( <i>Ts</i> )		<i>Chabertia ovina</i>	Canada	99.77
10	2F ( <i>Ts</i> )		<i>Chabertia ovina</i>	Canada	99.54
11	1B ( <i>Pt</i> )	VrainF	<i>Setaria digitata</i> isolate ST5	Thailand	99.85
12	1C ( <i>Pt</i> )		<i>Setaria digitata</i> isolate ST5	Thailand	99.69
13	1D ( <i>Pt</i> )		<i>Setaria digitata</i> isolate ST5	Thailand	99.54
14	1E ( <i>Pt</i> )		<i>Setaria digitata</i> isolate ST5	Thailand	99.69
15	1G ( <i>Pt</i> )		<i>Setaria digitata</i> isolate ST5	Thailand	99.69
16	1H ( <i>Pt</i> )		<i>Setaria digitata</i> isolate ST5	Thailand	99.69
17	1I ( <i>Pt</i> )		<i>Setaria digitata</i> isolate ST5	Thailand	99.69
18	2D ( <i>Ts</i> )		<i>Chabertia ovina</i> isolate CHO5	China	99.67

Key: *Pt* – *Phataginus tricuspidis*; *Ts* – *Thryonomys swinderianus*

All nucleotide sequences submitted to the NCBI-GenBank have the Accession numbers for the partial 28S rRNA sequences and the VrainF/AB28 primer pair amplicons which targeted the 18S rRNA, 5.8S rRNA, and ITS regions (Table 4). LSU 391 shared a

homology at 319bp while VrainF shared at 646-651bp for *P. tricuspidis* (*Pt*). LSU 391 shared a homology at 872bp, 878bp, and 882bp while VrainF shared at 605bp for *T. swinderianus* (*Ts*) (Table 4).

**Table 4:** NCBI-GenBank Submission Information.

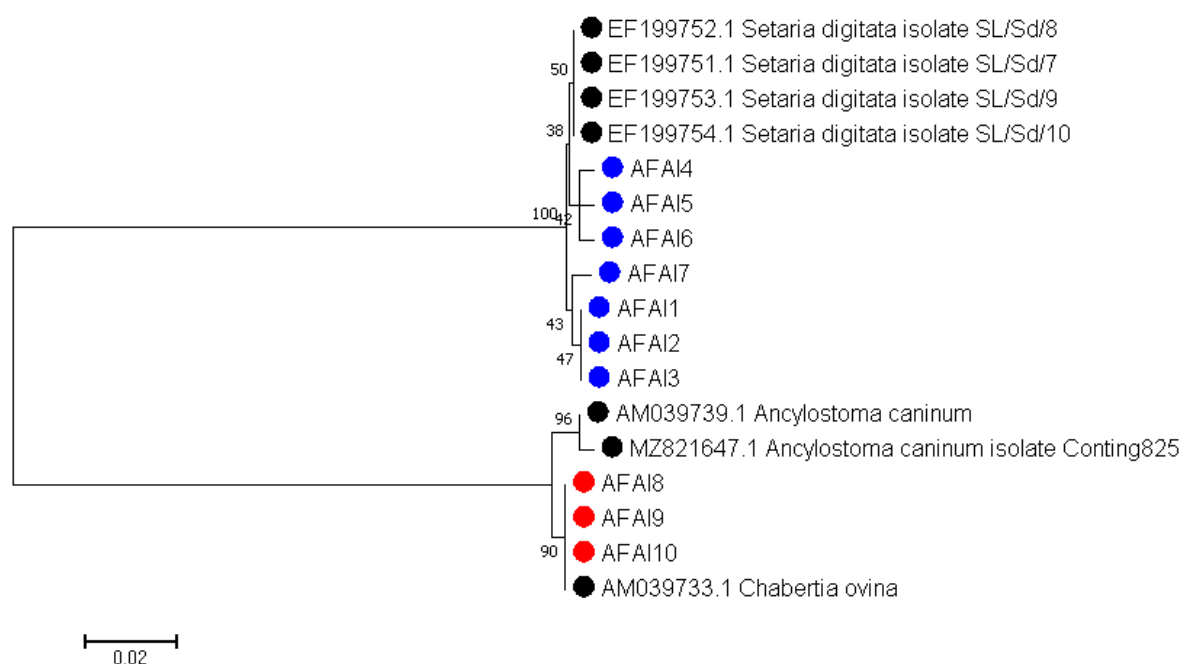
S/N	Isolate Code	Primer	Identity and NCBI-GenBank Code	Nucleotide sequences (bp)	Accession Number
1	1B ( <i>Pt</i> )	LSU391	<i>Setaria digitata</i> strain AFAI1	319	OQ352299
2	1C ( <i>Pt</i> )		<i>Setaria digitata</i> strain AFAI2	319	OQ352300
3	1D ( <i>Pt</i> )		<i>Setaria digitata</i> strain AFAI3	319	OQ352301
4	1E ( <i>Pt</i> )		<i>Setaria digitata</i> strain AFAI4	319	OQ352302
5	1G ( <i>Pt</i> )		<i>Setaria digitata</i> strain AFAI5	319	OQ352303
6	1H ( <i>Pt</i> )		<i>Setaria digitata</i> strain AFAI6	319	OQ352304
7	1J ( <i>Pt</i> )		<i>Setaria digitata</i> strain AFAI7	319	OQ352305
8	2B ( <i>Ts</i> )		<i>Chabertia ovina</i> strain AFAI8	878	OQ352306
9	2D ( <i>Ts</i> )		<i>Chabertia ovina</i> strain AFAI9	882	OQ352307
10	2F ( <i>Ts</i> )		<i>Chabertia ovina</i> strain AFAI10	872	OQ352308
11	1B ( <i>Pt</i> )	VrainF	<i>Setaria digitata</i> strain AFAv1	651	OQ361931
12	1C ( <i>Pt</i> )		<i>Setaria digitata</i> strain AFAv2	651	OQ361932
13	1D ( <i>Pt</i> )		<i>Setaria digitata</i> strain AFAv3	651	OQ361933
14	1E ( <i>Pt</i> )		<i>Setaria digitata</i> strain AFAv4	649	OQ361934
15	1G ( <i>Pt</i> )		<i>Setaria digitata</i> strain AFAv5	650	OQ361935
16	1H ( <i>Pt</i> )		<i>Setaria digitata</i> strain AFAv6	651	OQ361936
17	1I ( <i>Pt</i> )		<i>Setaria digitata</i> strain AFAv7	646	OQ361937
18	2D ( <i>Ts</i> )		<i>Chabertia ovina</i> strain AFAv8	605	OQ361938

Key: *Pt* – *Phataginus tricuspidis*; *Ts* – *Thryonomys swinderianus*

**Phylogenetic Relatedness:**

Phylogenetic relatedness based on LSU 391 primer, our sequence of nematodes of *P. tricuspis* (AFAI1-7) was grouped with sister clade EF199752.1 *S. digitata* isolate SL/Sd/8, EF199751.1 *S. digitata* isolate SL/Sd/7, EF199753.1 *S. digitata* isolate SL/Sd/9, EF199754.1 *S. digitata* isolate

SL/Sd/10 with 100% statistical support. However, the sequence of nematodes of *T. swinderianus* AFAI8-10 was grouped with AM039733.1 *C. ovina* at 90% statistical support. The clade appeared as a sister group to AM039739.1 *Ancylostoma caninum* and MZ821647.1 *A. caninum* isolate Conting825 at 96% support (Fig. 5).

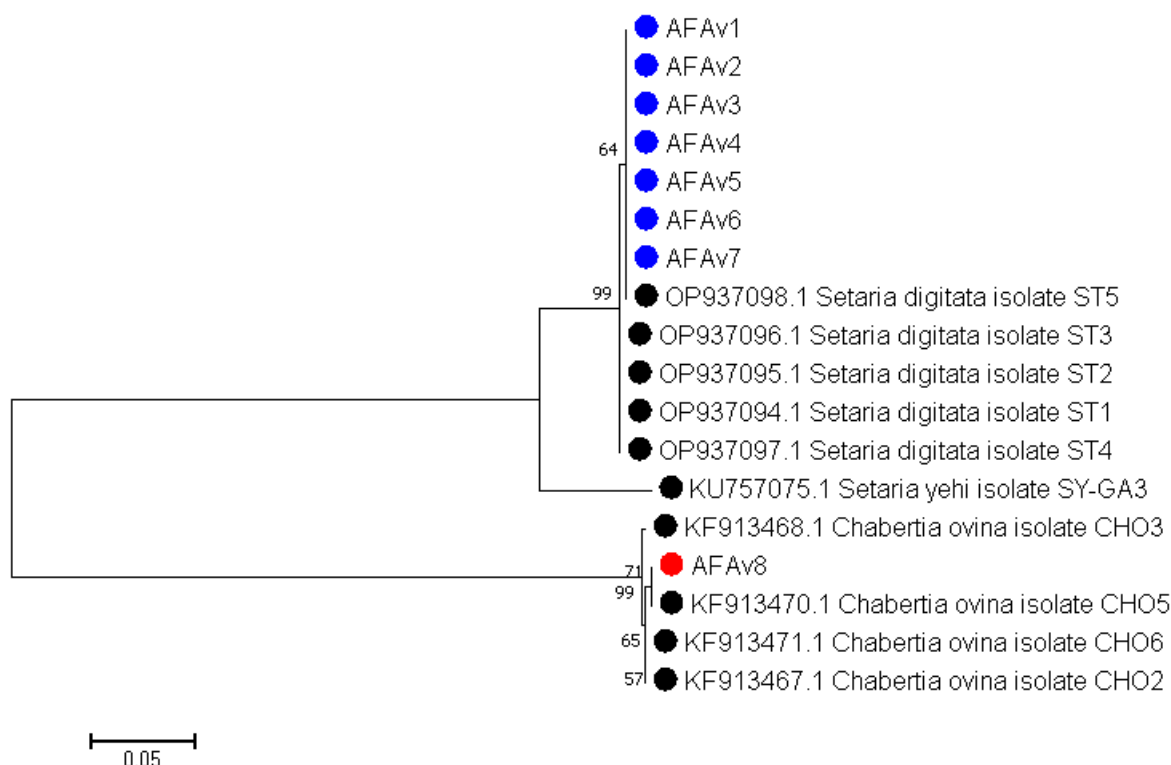


**Fig. 5:** Phylogenetic analysis of LSU 391 rDNA sequence of *P. tricuspis* nematode isolates and *T. swinderianus* isolates obtained in this study and reference sequences retrieved from GenBank. The tree was constructed using the neighbor-joining method and p-distance Tree model in MEGA7 software.

In a comparative approach for the certainty of homology, VrainF primer sequences also affirm the phylogenetic relatedness of the nematodes as shown in Figure 7. AFAv1-7 of *P. tricuspis* showed 64% homology with sister clades OP937098.1 *Setaria digitata* isolate ST5 and 99% with OP937094.1 *Setaria digitata* isolate ST1 - OP937097.1 *Setaria digitata*

isolate ST4 and KU757075.1 *Setaria yehii* isolate SY-GA3. AFAv8 of *T. swinderianus* showed relatedness with sister clade at 71% with KF913468.1 *Charbetia ovina* isolate CHO3, 99% with KF913470.1 *Charbetia ovina* isolate CHO5, 65% with KF913471.1 *Charbetia ovina* isolate CHO6 and 57% with KF913467.1 *Charbetia ovina* isolate CHO2 (Fig. 6).





**Fig. 6:** Phylogenetic analysis of VrainF rDNA sequence of *P. tricuspis* nematode isolates and *T. swinderianus* isolates obtained in this study and reference sequences retrieved from GenBank. The tree was constructed using the neighbor-joining method and p-distance Tree model in MEGA7 software.

## DISCUSSION

The study shows that wild mammals are predominantly infected by nematodes (86.8%). Nematodes are a highly diverse group of organisms. Parasitic nematodes are diverse in their biology and life histories. The prevalence obtained in the study is related to previous surveys which established the susceptibility of wild mammals to parasitic infection. This finding agrees with Dawet *et al.* (2022) which observed 64.84% prevalence of nematodes in their study on the gastrointestinal parasites (GIP) of wild animals found within the surrounding forests of Zuku and Rumfan Gwamna villages in Bassa Local Government Area, Plateau State, Nigeria. Okorafor *et al.* (2012) reported a prevalence of 100% while working with *T. swinderianus* in Oyo State. Opara and Fagbemi (2008) observed a 98% prevalence of nematode infection of *T. swinderianus* in Southeast, Nigeria. Okonkwo and Okaka (2018) observed an overall prevalence of 100% in *Manis*

*temminckii* in Anambra State, Nigeria. The free-ranging capacity of wild mammals exposes them to infective stages of nematode parasites. The mammals may have come in contact with another infected or probably have eaten food contaminated by egg, cysts and larvae of parasites.

Nematodes have a conservative morphology making traditional approaches to their taxonomy tasking thus the need for molecular study. DNA sequence-based methods have been widely applied over recent years for species identification, classification, and evaluation of phylogenetic relationships among nematodes (Camp *et al.*, 2018; Yang, 2012). The use of 28S rRNA and 18S rRNA enhances the amplification of parts of the nematodes. This is because they are universal primers designed for eukaryotes, particularly parasite taxa (Kounosu *et al.*, 2019; Hadziavdic *et al.*, 2014). However, partial amplification was observed in the nematodes of *P. maxwelli*. This may be due to the degenerative activities of the primer.

Sharifdinni *et al.* (2021) successfully used the partial *Cox1*, LSU rDNA, and ITS-rDNA genesto amplify *Baylisascaris devosi* Sprent in Pine marten in Iran.

*Setaria digitata* is a filariae worm of domestic mammals and wild ungulates. In this study, it was identified as a parasite of *Phataginus tricuspis*. All isolates shared a homology highly similar to *S. digitata* isolates from Japan and Thailand. *S. digitata* affects the nervous system of the host and might result in substantial economic losses in animal husbandry. This study was the first to record *Setaria* infection in wild non-ungulate in Nigeria. Nabie *et al.* (2017) reported a rare case of subconjunctival setariasis in a 15-year-old girl by *S. equina* in Northwest Iran.

*Chabertia ovina* is a parasite of ruminant animals (Sheep, Goats, wild ruminants). The phylogenetic relatedness of the nematodes of *T. swinderianus* to *C. ovina* showed that both ruminant and non-ruminant mammals are prone to the infection. *T. swinderianus* may be infected during their feeding activities. Fawole *et al.* (2023) observed *Chabertia* sp eggs in faecal samples of white-bellied Pangolins in Southwestern, Nigeria. The presence of this parasite in *T. swinderianus* may affect the productivity of domesticated wild ones. There's a phylogenetic relatedness between *C. ovina* and *Ancylostoma caninum*, indicating they belong to the same order - Rhabditida.

**Conclusion:** The study has established the need for the conservation of wild mammals and public health intervention. Wildlife studies should be studied at the molecular level to ascertain the risk factors the animals were exposed to for a better life in the wild.

**Declarations:**

**Ethical Approval:** The study did not involve direct interaction with live animals, as the samples were collected from dead hosts.

**Conflict of Interests:** The authors declare no conflicts of interest.

**Authors Contributions:** All authors contributed equally, and have read and agreed to the published version of the manuscript.

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**Availability of Data and Materials:** The data presented in this study are available on request from the corresponding author.

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