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Genetic identification of *Stephanofilaria sp.* isolated from ulcerative dermal lesions in black rhinoceros

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Research Paper

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Abstract

Stephanofilaria is a genus of nematodes that cause ulcerative dermal lesions in large mammals. However, there is a dearth of knowledge on the molecular genetics of *Stephanofilaria* species infecting critically endangered rhinoceros. This study employed genetic barcoding genes to identify *Stephanofilaria* species and to determine its genetic diversity and evolution. Phylogenetic analyses on partial genes of the second internal transcribed spacer Ribosomal DNA (ITS-2) and cytochrome c oxidase subunit 1 (Cox-1), revealed a 77% and 93% bootstrap support at the Cox-1 and ITS-2 loci respectively to a clade containing previously identified *Stephanofilaria* species. Morphological examination also confirmed features diagnostic of *Stephanofilaria dinniki* previously known to infect rhinoceros. Gene diversity of Cox-1 was 0.931 ± 0.030 and 0.579 ± 0.104 for the ITS-2, whereas nucleotide diversity was 0.008 ± 0.002 and 0.00197 ± 0.0016 for the Cox-1 and ITS-2 genes respectively. Neutrality tests (Fu and Li's D* and Fu and Li's F*) were significantly negative ($p<0.05$) at all loci, whereas Tajima D and Fu's FS were each statistically significant ($p<0.05$) at the Cox-1 and ITS-2 loci respectively. The high gene diversity, low nucleotide diversity and negative neutrality tests are consistent with positive selection at the Cox-1 gene. *Stephanofilaria* infection among rhinoceros is currently restricted to highland sanctuaries compared to a widespread distribution in both lowlands and highlands in the 1960s suggesting an adaptation to vectors thriving in cooler highland temperatures. This is the first genetic identification of *S. dinniki*, in rhinoceros and will aid in diagnosis, treatment, studies, and rhinoceros conservation.

Introduction

Stephanofilaria is an economically important genus of nematodes (order: Spirurida, family: Filariidae) that causes ulcerative dermal lesions in several species of large mammals globally. About a dozen species have been morphologically described in this genus (Hodda 2022). These include *Stephanofilaria dinniki*, which infects the critically endangered black rhinoceros (Round, 1964); *Stephanofilaria thelazioides*, which infects the hippopotamuses (Boomker *et al.* 1995); *Stephanofilaria boomkeri*, which infects domestic and wild African suids (Bain *et al.* 1996); *Stephanofilaria zaheeri*, which infects water buffalo *Bubalus bubalis*; and *Stephanofilaria srivastavai*, which infects the Asian elephant, *Elephas maximus* (Bhattacharjee 1967). In addition, several species of *Stephanofilaria* that parasitize domestic bovids including cattle in different parts of the world have been described. *Stephanofilaria stilesi* (Chitwood 1934), has been reported in the USA and Canada, *Stephanofilaria* sp. in Australia, *Stephanofilaria assamensis* (Singh 1958) in India and Russia, *Stephanofilaria kaeli* (Buckley 1937) in Malaysia, *Stephanofilaria dedoesi* (Buckley 1937) in Indonesia, and *Stephanofilaria okinawaensis* (Ueno *et al.* 1977; Ueno and Chibana 1978) in Japan.

Stephanofilarial infections are well described in cattle. The key features of stephanofilarial infections in cattle include acanthosis, spongiosis, ulcers, eosinophilia, and fibrosis. Ulcerated lesions vary in size from 5 × 4 cm to 36 × 10 cm and are well defined, with crusted edges and a purulent exudate (Islam *et al.* 2018; Loke and Ramachandran 1967; Matos *et al.* 2022). In Australian cattle, the size of lesions was dependent on whether they were positive or not for stephanofilarian worms. Specifically, *Stephanofilaria* sp positive lesions were larger (ranging from 7.02 to 54.44 cm^2) and had significantly higher scores of alopecia (>80% of the lesion area affected) and hyperkeratosis than lesions without Stephanofilarial worms (Naseem *et al.* 2023). Moreover, like previous studies, Stephanofilaria-infected lesions had significantly higher inflammation and higher scores of eosinophil, macrophage, and lymphocyte infiltration than lesions without Stephanofilaria worms (Naseem *et al.* 2023). In rhinoceros, lesions caused by *Stephanofilaria dinniki* are similar to Stephanofilarial lesions in cattle; they are erosive, crater-like dermal ulcerations, 2–3 cm deeper than the surrounding skin with crusting, and have edges raised

above the normal skin (Tremlett 1964). The average diameter of these lesions varies between 5–7 cm and 15–20 cm in black rhinoceros populations (Hitchins and Keep 1970; Mutinda *et al.* 2012; Plotz 2014; Tremlett 1964). Although the prevalence of stephanofilarial ulcerative lesions is less in white compared to black rhinoceros, white rhinoceros appear to experience severe ulceration (King'ori *et al.* 2024). In Meru National Park, the stephanofilarial lesions were 23 cm in diameter in white rhinoceros, and 15 cm in black rhinoceros (Mutinda *et al.* 2012). Lesions in black rhinoceros are typically on the shoulder and ventral thorax, while in white rhinoceros, lesions are commonly seen at the rump (Mutinda *et al.* 2012). Although these stephanofilarial infections are considered benign in black rhinoceros, a similar disease in cattle causes delayed puberty, reduced milk yields, and longer inter-calving intervals in affected cattle (Rai *et al.* 2010). Recent studies have shown that these hemorrhaging lesions are associated with a loss in body condition, anemia, elevated stress, and mortality in black rhinoceros (Mutinda *et al.* 2012; Plotz 2014) and may be chronic or recrudescent (Kock and Kock 1990). These hemorrhagic skin lesions can also cause black rhinoceros' mortality through systemic secondary bacterial infection (Clausen and Ashford 1980). Flaring up of wounds can also be induced by translocation-associated stress, particularly in artificially managed rhinoceros metapopulations where translocation is a common practice (Hitchins and Keep 1970). Hemorrhaging lesions can also affect the aesthetic values of the rhinos.

Despite the economic importance of the genus *Stephanofilaria* to the livestock industry, molecular genetic studies and genetic identification have gained attention only recently (Lui *et al.* 2023; Naseem *et al.* 2021). However, no studies have focused on the genetic identification or molecular characterization of *Stephanofilaria* species infecting the critically endangered rhinoceros populations. Previous studies on this genus have focused on morphological features for species identification. However, diagnostic morphological features for species identification can be limited to specific life stages or sexes, and if these life stages or sexes are rare or absent or the key species diagnostic features of specimens are badly distorted, specimen identification can be impossible to achieve. Moreover, paucity of suitable keys remains a huge gap between the number of taxa treated in keys and the number of species for which gene sequence data are available (Will and Rubinoff 2004). Molecular methods such as DNA-barcoding offer potentially efficient alternative approaches to species identification and studies of their ecology. This is because molecular techniques are sensitive in the detection of small quantities of nematode DNA, regardless of the developmental stage or sex. Molecular barcoding may help to identify cryptic and polymorphic species and give means to associate life history stages of unknown identity (Schander and Willassen 2005). Molecular genetic identification is also useful for the investigation of aspects of nematode biology, including understanding the life cycle and the identification of potential vectors. Genes used in molecular barcoding offer quick and reliable means of species identification and for detecting the transmission, spread, and the evolution of nematode species (Powers 2004). Developing genetic data of pathogens can also be useful in inferring their transmission, reconstructing their epidemiological history, and identifying physical and environmental drivers of disease spread. Molecular methods such as DNA-barcoding beyond species detection can offer potentially efficient alternative approaches to studying the nematode disease ecology and evolution.

In this study, we isolated worms from ulcerative dermal lesion scrapings of black and white rhinoceros and conducted preliminary morphological characterization to corroborate previous descriptions of *Stephanofilaria dinniki* in rhinoceros. We also extracted DNA, carried out PCR amplification, and sequenced two gene fragments – the second internal transcribed spacer Ribosomal DNA (ITS-2) and the cytochrome c oxidase subunit 1 (Cox-1) genes – and used this for genetic identification and molecular characterization of *Stephanofilaria dinniki* for the first time. The second internal transcribed spacer (ITS-2) has a high degree of hyper-variability useful in discriminating populations of the same species, while the cytochrome c oxidase subunit 1 (Cox-1) gene is also useful in demonstrating finer intra-species level variation in many nematodes. Lastly, the patterns of sequence variation in the Cox-1 and ITS-2 were used to infer evolutionary and demographic signatures of the *Stephanofilaria* sp. infecting rhinoceros.

Materials and methods

Ethics statement

This study was approved by the Ethics Committee of the Kenya Wildlife Service (KWS/BRM/5001), the Institution mandated to protect and conserve Wildlife in Kenya. Stephanofilarial worms were collected from rhinoceros ulcerated skin lesions during immobilization of rhinoceros for ear notching, translocation, clinical management of injuries (due to snares and intraspecific fights), and clinical infections. Immobilization and translocation of rhinoceros are undertaken by experienced veterinarians guided by the Kenya Wildlife Service protocol for rhinoceros' immobilization and translocation (https://www.kws.go.ke/file/3239/download?token=5F1MI56-) and guidelines on Wildlife Veterinary Practice 2018 and the Veterinary Surgeons and Veterinary Para-professionals Act Cap 366 of the Laws of Kenya that regulate veterinary practice in Kenya.

Study area

We collected worms from rhinoceros in two sanctuaries: Meru Rhino Sanctuary (MRS) and Ol Jogi Conservancy (OLJ) (Figure 1). MRS is a fenced portion in the western part of Meru National Park covering 38.8 km^2 and is located between 36° 40′ E–37°00′ E and 0°02′ S–0°07′ N. Meru National Park covers an area of 870 km^2 and receives rainfall ranging from 635–762 mm in the west of the park to 305–356 mm in the east. The habitat of MNP varies from woodland to open grasslands intersected by permanent rivers and associated riverine vegetation. The southern portion of the rhino sanctuary is dominated by forests, while the rest of the sanctuary is dominated by thickets and grassland interspersed with several rivers such as Makutano, Kanjoo, and Rojaweru. Meru National Park has a rich diversity of wild animals including elephants, hippopotamuses, leopard, cheetah, black rhinoceros, and some rare antelopes, with incursions from cattle, camel, goats, and sheep. The Meru National Park is a home for 40 black rhinos and 79 white rhinos.

Ol Jogi is on private land located in central Kenya between 37° 00′E–37°05′E and 0°15′N–0^{0}20′N. Founded in 1960, it forms part of the Laikipia-Samburu-Meru-Marsabit ecosystem. It ranges between 1800 m and 1920 m in altitude and lies on the Laikipia plateau near Mount Kenya with an area of 235 km^2 and provides a safe habitat for indigenous species. Rainfall averages 460 mm per year. The vegetation is a mosaic of grassland, Acacia woodland, and shrubs. In 2005, Ol Jogi expanded its 50-km^2 rhino sanctuary to the entire conservancy and developed a fence, enabling rhinoceros to be

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protected within its boundary while allowing the free migration of all other species.

Isolation and morphological examination of worms

Skin scrapings from filarial wounds were taken from immobilized rhinos during routine notching exercises in MRS and OLJ. Filarial wounds were washed of mud, and a clean sterile scalpel blade was used for scraping (Figure 2). The scraped tissue was collected into a sterile fecal pot (Figure 2). Scrapings were collected from seven black rhinoceros in OLJ, four black rhinoceros from MRS, and three white rhinoceros from MRS during ear notching exercises. Ear notching was carried out in 2021 at MRS and in 2022 at OLJ. The scraping was done deep into the filarial wound crust until blood oozed. Later, the wound was dressed with a topical antibiotic spray. The scraping was then transferred to a 5-ml cryovial, labeled, and preserved in liquid nitrogen. Data on specific rhino identity, time of capture, date, and GPS coordinates were recorded for every scraped individual rhino. The samples were then transported to the laboratory for analysis. In the lab, frozen scraping samples were thawed and poured into a clean glass petri dish and examined using a Leica EZ4D stereo microscope to detect filarial worms. Detected worms were picked using a pair of pointed forceps, transferred on a clean glass slide, and observed for finer details on Leica DM 500 microscope. Following observation under a light microscope, each worm was then macerated using a clean scalpel blade and transferred to a 1-ml Eppendorf tube awaiting DNA extraction. Worms were successfully isolated in 7 of 11 black rhinoceros, but nothing was isolated from 3 white rhinoceros.

DNA extraction from the isolated worms

DNA was extracted from 25 worms from five rhinoceros sampled from OLJ, and from five worms extracted from two rhinoceros sampled from MRS. Each isolated worm was macerated and homogenized in 360 µl phosphate-buffered saline (PBS) (pH =7.4) and vortexed for 90 seconds. The extraction of total nucleic acids was done from 200 µl of the homogenate using DNeasy blood and tissue kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. A total number of 35 worms were analyzed at the ITS-2 and Cox-1 loci. All the 35 worm samples were amplified at both loci and used for molecular identification of filaria worm. A 700 bp fragment of mitochondrial Cox-1 gene and a 900 bp of the ITS-2 gene-fragment were obtained using the primers sets: St_CO1F (5'- ATACTGTKAATCATAAGACTATTGG -3') and St_CO1R: (5'- GACCAAAAAATCAAAACAAATGCTG -3') (Naseem *et al.* 2021) and S_5.8S_29F: (5'- TAGCGGTGGATCACTTGGCTCG -3') and 28S_400R: (5'- CAACTTTCCCTCACGGTACTTGT -3') (Naseem *et al.* 2021). The amplification of the mitochondrial Cox-1 and the ITS-2 gene-fragments was carried out in a total volume of 25 µl that consisted of 1 µl DNA template, 12.5 µl of OneTaq® Quick-Load® 2X Master Mix with Standard Buffer (New England Biolabs-NEB, Massachusetts, USA), and 0.5 µl of 10 mM each forward and reverse primers. As a negative control, molecular grade nuclease-free water was used. The following cycling conditions were used for PCR amplification in a SimpliAmp thermal cycler (Life Technologies): an initial denaturation at 94°C for 1 minute followed by 35 cycles of denaturation at 94°C for 20 seconds, annealing at 46°C for 20 seconds, and 1 minute extension at 68°C. The final extension was at 68°C for 5 minutes.

The PCR amplicons were resolved in a 1% (W/V) agarose gel by electrophoresis with a 1x TAE running buffer at 90 V for 35 min. Gelpilot 1000 bp plus ladder (Qiagen, Germany) was used as a molecular size DNA marker. Ethidium Bromide-stained gels were visualized under UV trans-illumination. All the amplicons with the expected band size were submitted for sequencing in both forward and reverse directions at Macrogen Europe B.V. DNA chromatograms and sequences were visualized and edited using Geneious v11 (Kearse *et al.* 2012) software. ITS-2 sequences were manually edited for polymorphic or double chromatogram peaks. Consensus worm-sequences for ITS-2 and Cox-1 genes were generated from forward and reverse sequence data and exported as fasta files to DNAsp 6 (Rozas *et al.* 2017) for diphasing and haplotype extraction, in the case of nuclear ITS-2. Clean edited sequences for the ITS-2 locus were obtained for 11 worms from three rhinoceros in OLJ and three worms from two rhinoceros sampled in MRS. For the Cox-1 locus, clean edited sequences were obtained for 19 worms from four rhinoceros in OLJ and for three worms from two rhinoceros in MRS. The remaining sequences with low quality chromatograms were discarded.

Genetic identification and phylogenetic analyses

The trimmed and edited DNA sequences were used to detect similarities with other available sequences in GenBank using BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 26 October 2023).

Phylogenetic analyses using Cox-1 and ITS-2 sequences of *Stephanofilaria* worms were carried out to identify the evolutionary clades they fall into using orthologous gene sequences of identified worms available from GenBank. The method of maximum likelihood and the best nucleotide substitution model estimated with ModelFinder (Kalyaanamoorthy *et al.* 2017) were used to infer evolutionary relationships between species using the IQTree software (Nguyen *et al.* 2014). Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the best nucleotide substitution model for the data and then selecting the topology with a superior log-likelihood value. To evaluate statistical support for clades to which stephanofilarial worms were assigned to, the ultrafast bootstrap support approximation from 1,000 replicates using the UFBoot algorithm was used (Hoang *et al.* 2018).

The Cox-1 maximum likelihood tree was fitted using the GTR +F+I+G4 nucleotide substitution model selected based on the Bayesian Information Criteria (BIC). The model had a rate heterogeneity of Gamma with four categories. The proportion of invariable sites was 0.423, and the Gamma shape parameter alpha was 0.808 for the Cox-1 locus. Twelve haplotypes obtained in this study (GenBank Accession numbers PP749201 — PP749212) and 32 orthologous sequences from known nematode species from GenBank, including two recent sequences of *Stephanofilaria* species, were used. The sequences consisted of 649 nucleotide sites with 325 (50.08% of all sites) constant sites and the 275 parsimony informative sites. The tree was rooted using *Protospirura numidica* as an out group. The robustness of the topology was tested using 1,000 bootstraps implemented with the UFBoot algorithm in the IQTree software program. The ITS-2 maximum likelihood tree was also fitted using a GTR+F+I+G4 substitution model selected based on the BIC. The model had a rate heterogeneity with four categories. The proportion of invariable sites was 0.334, and the gamma shape parameter alpha was 0.757. Eight haplotypes obtained in this study (GenBank Accession numbers PP892684 — PP892691) and 27 orthologous sequences from known nematode species including sequences of *Stephanofilaria* genus were used. The sequences consisted of 424 nucleotide sites with

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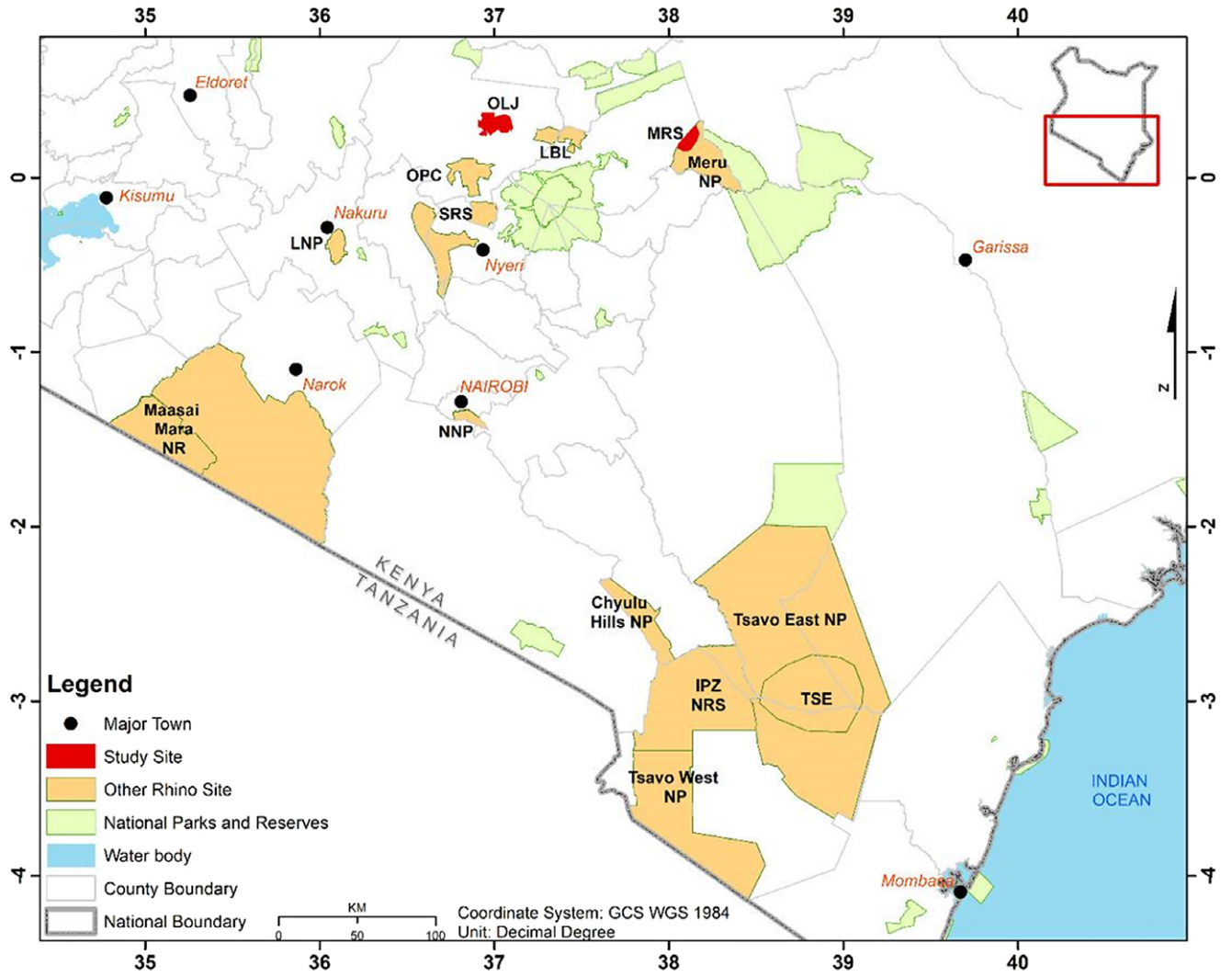

Figure 1. Map of Kenya showing rhinoceros sanctuaries and study sites (rhinoceros sanctuaries) sampled in this study.

212 (50%) constant sites and 171 (40.30%) parsimony informative sites. The tree was rooted using *Stegophorus macronectes* as an out group. The robustness of the topology was tested using 1,000 bootstraps implemented with the UFBoot algorithm in the IQTree software program.

Figure 2. A photo showing a collection of scrapings from a filarial wound on a black rhino in Ol Jogi wildlife conservancy.

Genetic diversity, natural selection, and demography

Genetic diversity indices including haplotype diversity, nucleotide diversity, number of haplotypes, and segregating sites were determined for the mitochondrial Cox-1 and the nuclear ITS-2 genes using DNASP 6.0 software (Rozas *et al.* 2017). Haplotype diversity is the probability that two randomly chosen haplotypes are different (Nei and Roychoudhury 1974; Nei and Tajima 1981), whereas nucleotide diversity is the average number of nucleotide differences per site, π, between any two randomly chosen DNA sequences from a population (Nei 1987). Numerous tests were conducted to determine signatures of departures from neutral expectations and large and stable population sizes (Fu's Fs and Tajima's D, Fu and Li's D*, Fu and Li's F*, Ramos-Onsins and Rozas's R2, Raggedness r) (Ramos-Onsins and Rozas 2002a) on the patterns of polymorphism of the Cox-1 and ITS-2 sequence data using DNASP 6.0 (Rozas *et al.* 2017). Tajima D (Tajima 1989), Fu and Li's D* and F* (Fu and Li 1993) tests are sensitive to selection, whereas Fu's FS (Fu 1997), Ramos-Onsins and Rozas R2 (Ramos-Onsins and Rozas 2002a), and a raggedness index r (Harpending 1994) are sensitive to changes

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in demography (Ramos-Onsins and Rozas 2002a). Moreover, demographic changes are expected to affect the genome more evenly than selective pressures (Galtier *et al.* 2000; Hahn *et al.* 2002), so analyses of the empirical distribution of Tajima's D for the mitochondrial Cox-1 and nuclear ITS-2 regions could help decipher between selection and demography as explanations for the observed deviation from neutral expectation.

Results

Morphology of adult worms, larvae, and eggs

Several female worms and a single male were recovered from suspected filarial wound lesion scrapings. The single male recovered measured 2.6 mm in length and 0.02 mm in maximum thickness (Table 1). In addition, we observed a spicule on this male measuring 0.054 mm in length (Figure 3). Recovered adult female worms measured between 2.71 mm and 3.49 mm in length and 0.044 mm and 0.079 mm in maximum thickness (Table 1). Some females were observed with their uterine tubes containing either embryonated eggs or developed microfilaria (Figure 3). The eggs measured from 52 to 54 micrometers by 30 to 34 micrometers (Table 1). Microfilaria (L1) measured between 0.199 mm and 0.21 mm in length and 0.01 mm in diameter (Table 1). The body of the larvae (L1) is cylindrical in shape, the head blunt and the tail tapering to a point (Figure 3). Larvae, recovered from lesions likely at the L5 stage, measured 1.6 mm in length (Table 1, Figure 3). The cuticle throughout the length of the body of adult female worms was finely striated transversely (Figure 3). There was a lack of spines on the transverse striations and the oral opening was surrounded by numerous cuticularized spines (Figure 3). The head had a terminal peri-buccal ring of cuticular spines that we could not determine the number. Behind this ring is a circle of eight cephalic spines, arranged in pairs. The tails of adult females were prominently curved ventrally (Figure 3).

Genetic identification and phylogenetic analyses

Twenty-two worms from six rhinoceros were successfully amplified and sequenced at the Cox-1 locus comprising 649 bp after primer trimming, while 14 worms from six rhinoceros were amplified and sequenced at the ITS 2-locus comprising 424 sites resulting in 28 diphased sequences with 36 segregating sites.

Blast results were inconclusive due to lack of a barcoding gap between the taxa constituting the top four hits on GenBank based on BLASTn matching our orthologous sequences. Specifically, 10 of 12 Cox-1 haplotypes best matched *Thelazia callipaeda* with a similarity between 81% and 82.6% and sequence coverage of 71% to 97% followed by 9 of 12 haplotypes matching *Serratospiculum tendo* at a similarity of 81% and a sequence coverage of 97%.

Table 1. Measurements of larva, adult, and egg dimension of *Stephanofilaria* sp isolated from black rhinoceros

Host ID/worm number	Body length	Body width	Tail width	Head width	Spicule length
Adult (Measurements are in mm)					
Female worms					
Rhino No. 20_AW1	2.72	0.044	0.012	0.031	NA
Rhino No. 85_AW1	2.71	0.056	0.020	0.020	NA
Rhino No. 84_AW1	3.49	0.079	0.067	0.036	NA
Mean	*2.97*	*0.059*	*0.033*	*0.029*	*NA*
Male worms					
Rhino No. 553_AW1	2.60	0.019	0.011	0.0062	0.054
Larvae L (Measurements are in mm)					
Rhino No. 20_WL2	1.603	0.0394	0.0236	0.0228	NA
Rhino No. 20_WL4	1.656	0.0415	0.0203	0.0166	NA
Mean	*1.6295*	*0.04045*	*0.02195*	*0.0197*	NA
Micro larvae ML (measurements are in micrometers, μm)					
Rhino No. 88 WmL1	208.16	11.13	2.47	5.88	NA
Rhino No. 88 WmL 2	198.74	10.66	3.49	5.66	NA
Rhino No. 88 WmL 3	208.86	10.87	4.82	5.96	NA
Mean	*205.25*	*10.87*	*3.59*	*5.83*	*NA*
Egg (Measurement in micrometers, μm)					
Rhino No. 88 Egg 1	54.11	34.38	NA	NA	NA
Rhino No. 88 Egg 2	53.08	32.08	NA	NA	NA
Rhino No. 88 Egg 3	52.71	36.00	NA	NA	NA
Rhino No. 88 Egg 4	53.08	33.67	NA	NA	NA
Mean	*52.96*	*33.92*	*NA*	*NA*	*NA*

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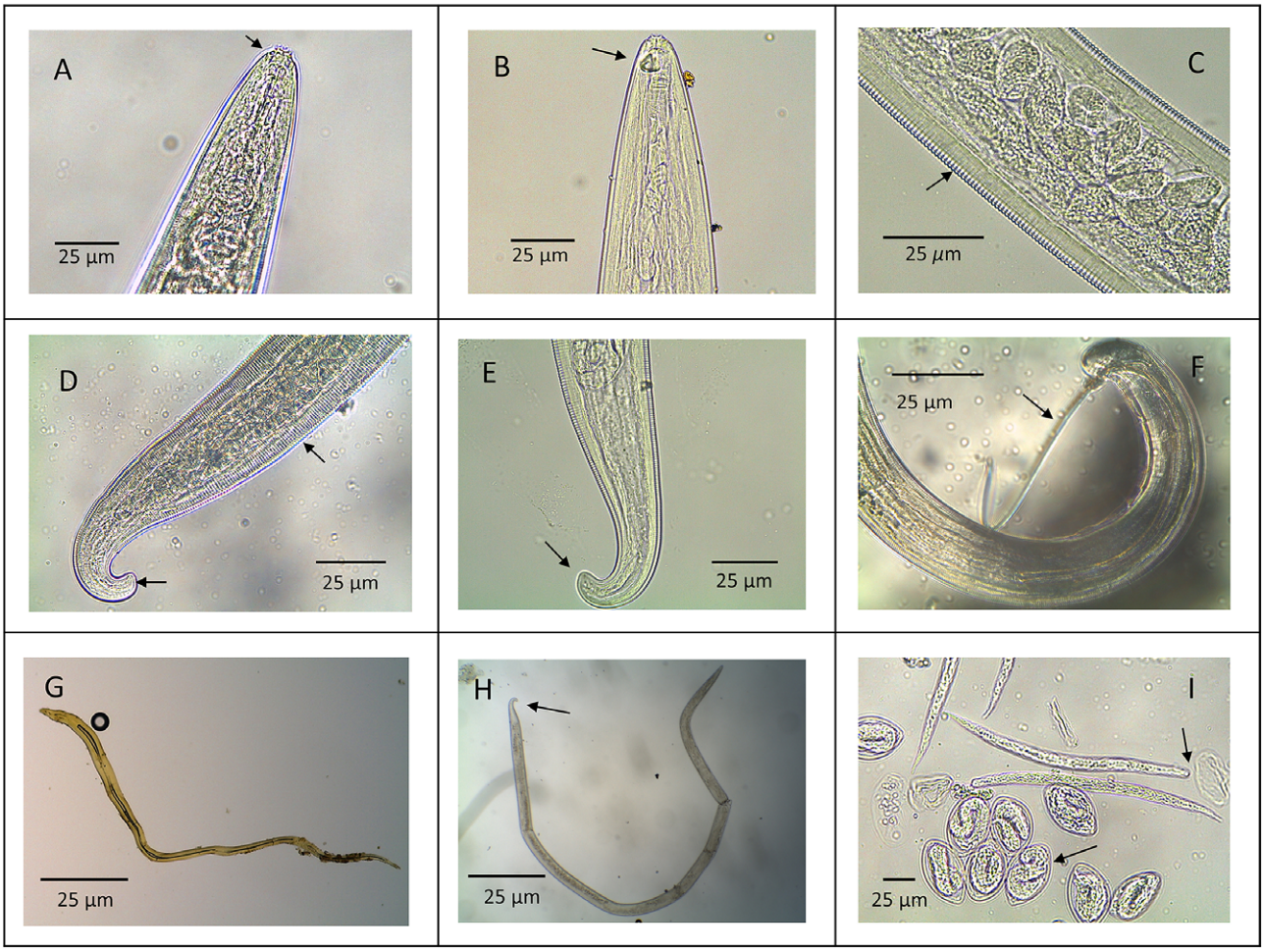

Figure 3. *Stephanofilaria dinniki* showing key features, anterior portion (A), cleaned with glycerol (B), showing an oral opening surrounded by numerous cuticularized spines terminal peri-buccal ring of cuticular spines. The mid portion (C) shows fine transverse striations. Posterior portion (D), cleaned with glycerol (E), showing a curved tail, A male spicule is shown in (F), L5 Larvae in (G), a whole worm with a curved tail and L1 and embryonated eggs (I).

However, when orthologous sequences are matched against *Stephanofilaria* genus sequences on GenBank using BLASTn, all the 12 haplotypes sequences matched to *Stephanofilaria stilesi* isolated from American cattle with a percent similarity of 81.18% to 82.93% and a coverage of 51% to 62% to *Stephanofilaria sp* with a percent sequence identity of 79.04% to 80.69% and coverage of 93%. For the ITS-2 haplotypes, the best match identified by BLASTn using the GenBank database was *Thelazia callipaeda* isolate from a red fox with a 91.21% to 91.86% similarity and a sequence coverage of 75% and to *Stephanofilaria sp* isolated from Australian cattle by 88.66% to 89.00% similarity and sequence coverage of 71%. Other top similarity hits were *Ochocerca gibsoni* and *Loloa loloa* with a percentage identity of 85.29% to 85.67% and sequence coverage at about 83%.

Phylogenetic analyses at the Cox-1 locus (Figure 4) and the ITS-2 locus (Figure 5) provided support that sequences from this study formed a single cluster. The cluster had 100% bootstrap support at the Cox-1 locus and 97% bootstrap support at the ITS-2 locus for belonging to the same cluster. Similarly, *Stephanofilaria dinniki* samples formed a cluster with other *Stephanofilaria* species. Specifically, there was 77% bootstrap support at the partial Cox-1 gene and a 93% support at the partial ITS-2 gene for a *Stephanofilaria* genus cluster.

Genetic diversity, demography, and evolution

Twenty-two worms from six rhinoceros were successfully amplified and sequenced at the Cox-1 locus comprising 649 bp after primer trimming. About 54.5% of the 22 sequences were unique (i.e., 22 sequences comprised of twelve distinct haplotypes obtained) with 32 segregating sites. All the variations were synonymous substitutions. Haplotype diversity (Hd ± Sd) was high (0.930 ± 0.030), nucleotide diversity, PiT, was low (0.008 ± 0.002), and the average number of nucleotide differences, Kt, was 5.277 ± 2.650 (Table 2).

Fourteen worms from six rhinoceros were amplified and sequenced at the ITS-2 locus comprising 406 sites after trimming resulting in 28 diphased sequences with six segregating sites. There were eight distinct haplotypes (or 28.6% unique sequences) with Haplotype diversity, Hd ± Sd being 0.579 ± 0.104, nucleotide diversity, PiT, was 0.00197 ± 0.0016, and the average number of nucleotide differences, Kt, was 0.799 ± 0.595 (Table 2).

Tajima's D and Fu and Li's D* and F* for the parasite Cox-1 locus were negative and significantly different from neutral expectation and a large and stable population, but Fu's Fs, Ramos-Onsins and Rozas's R, and raggedness index r were not significantly different from neutral expectation. Similarly, neutrality tests such as Tajima's D, TD, Fu and Li's D*, FLD*, Fu and Li's F*, FLF* and

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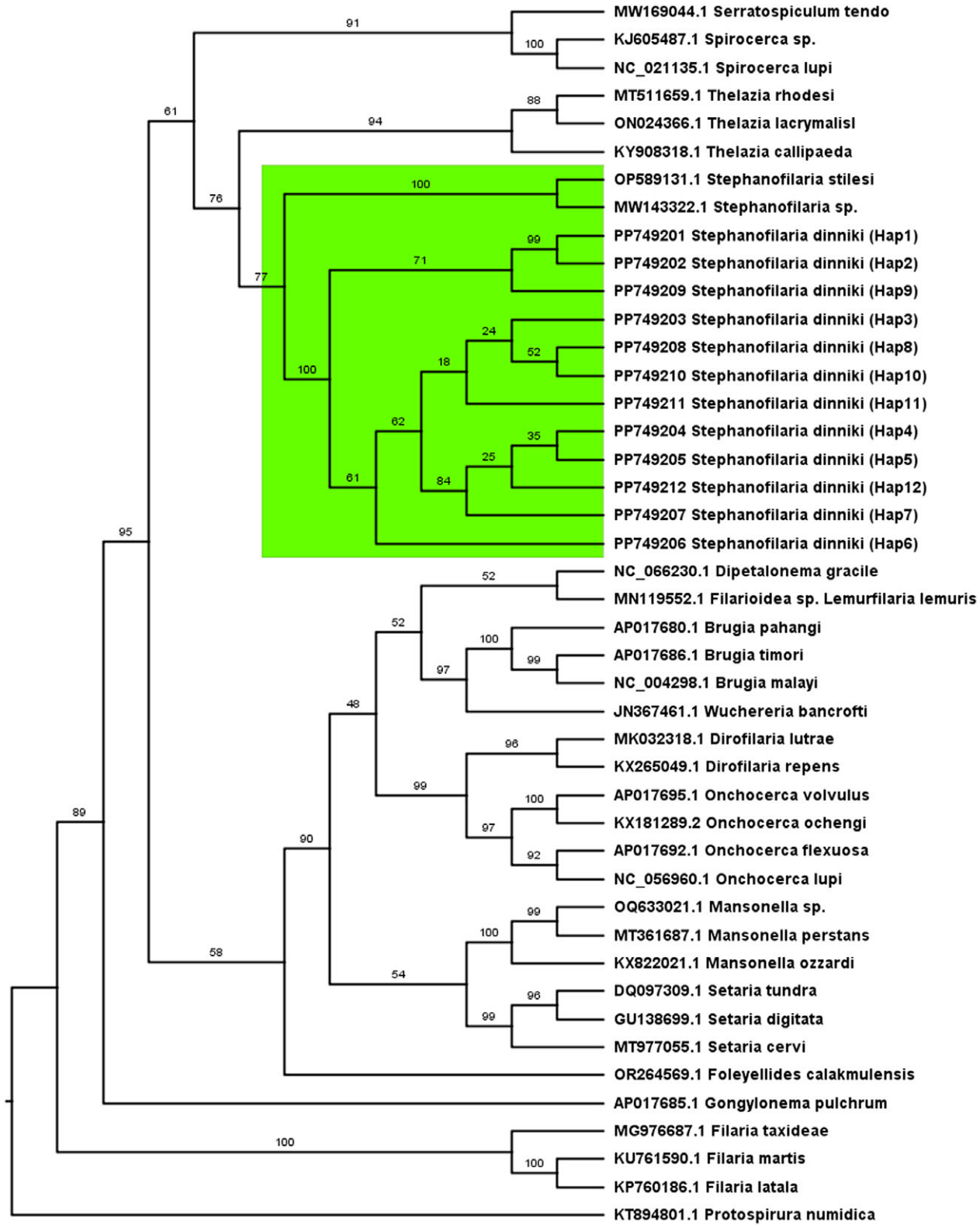

Figure 4. Maximum likelihood tree based on partial Cytochrome c oxidase 1 gene showing a *Stephanofilaria* clade coloured green. Best-fit substitution model according to BIC: GTR +F+I+G4, Model of rate heterogeneity: Invariant + Gamma with 4 categories, Proportion of invariable sites = 0.423, Gamma shape alpha: 0.808. Bayesian information criterion (BIC) score: 15133.14. Input data: 44 sequences with 649 nucleotide sites; Number of constant sites: 325 (= 50.08% of all sites); Number of invariant (constant or ambiguous constant) sites: 325 (= 50.08% of all sites); Number of parsimony informative sites: 275, Number of distinct site patterns: 346.

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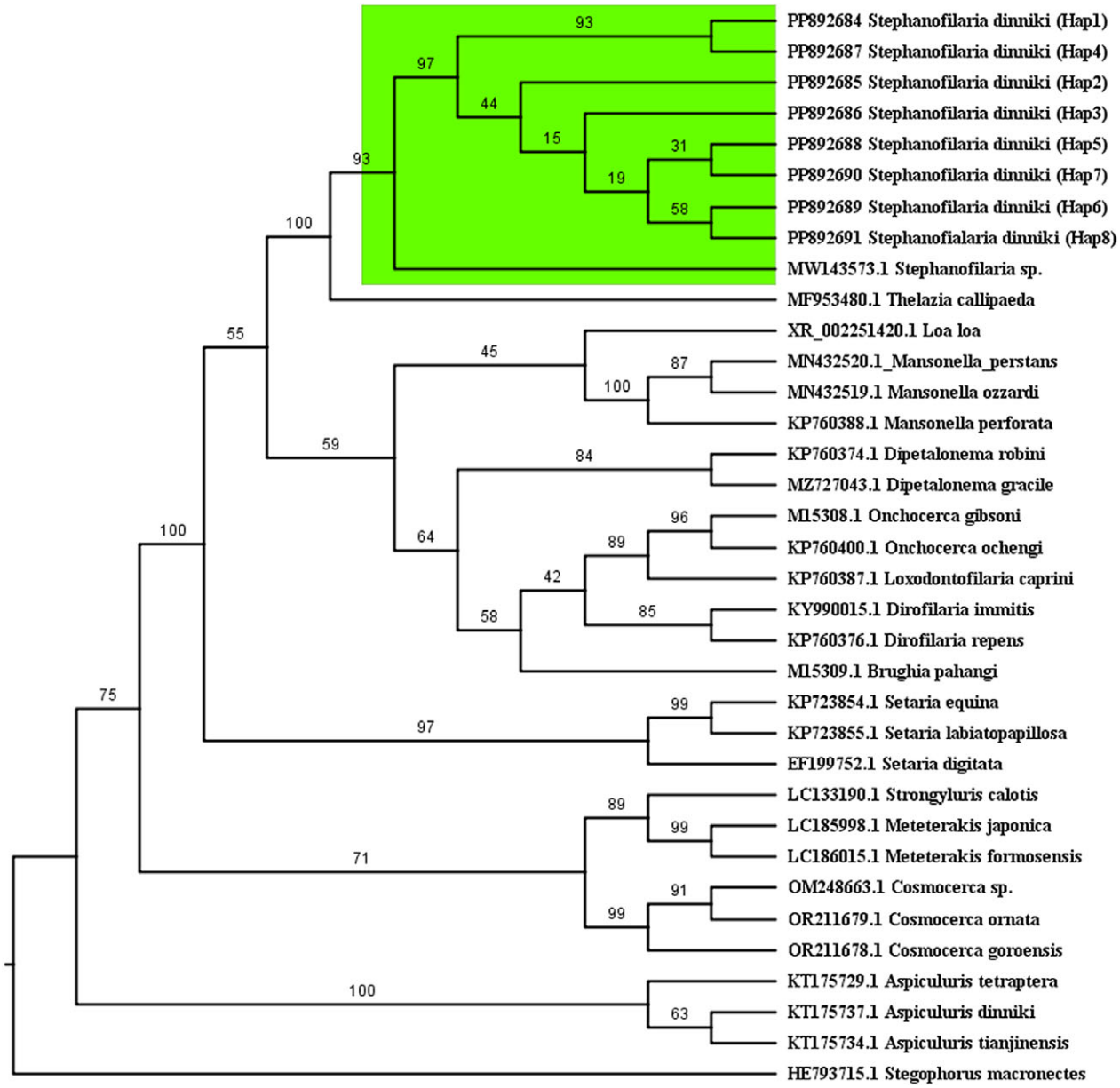

Figure 5. Maximum likelihood tree based on partial ITS-2 gene showing a *Stephanofilaria* clade coloured green. Best-fit model according to BIC: GTR+F+I+G4. Model of rate heterogeneity: Invar + Gamma with 4 categories, Proportion of invariable sites: 0.3344, Gamma shape alpha: 0.757, Input data: 35 sequences with 424 nucleotide sites, Number of constant sites: 212 (50% of all sites), Number of parsimony informative sites: 171 (40.3% of all sites), Number of distinct site patterns: 244, Log-likelihood of the tree: -2961.660 (s.e. 125.580). Bayesian information criterion (BIC) score: 6389.149.

Fu's FS were negative, but only Tajima's D was not statistically significant from neutral expectation at the ITS-2 locus (Table 2).

Discussion

Morphological examination of worms recovered from black rhinoceros skin scrapings revealed the presence of diagnostic traits for the filaroid worm, *Stephanofilaria dinniki*. These traits include, among others, the possession of a cuticle along the entire length of the body with fine transverse striations, a cuticularized ring of numerous cuticular spines around the oral opening, and tails that are curved ventrally (Round 1964) in both males and females. In addition, female worms had uterine tubes containing either embryonated eggs or developed microfilaria (Hitchins and Keep 1970). The generic diagnostic features for *Stephanofilaria* include presence of the oral aperture surrounded by a protruding cuticular rim with a denticulate edge (Bain *et al.* 1996; Boomker *et al.* 1995; Watrelot-Virieux and Pin 2006). Notable morphological deviation from previous studies was on size of the adult worms and larvae; the females from this study were smaller than in previous studies. For example, adults and micro larvae were 2.71–3.49 mm and 0.199–0.209 mm in this study compared to 4.6–5.7 mm and 0.12–0.15 mm from a previous study (Round 1964), respectively. Intraspecific size variation during various life stages has not been explicitly examined in this species, as only a single study has examined the body length variation in this species (Round 1964).

Phylogenetic analyses of the COX-1 and ITS-2 genes revealed a close relationship between Stephanofilaria worm sequences from

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Table 2. Evolutionary and demographic signals on Cox-1 and ITS-2 for *Stephanofilaria dinniki* worms from Black rhinoceros

Statistic	Observed Value	Mean expected value (95% LCI–UCI)	Probability
Cox–1			
Tajima's D, TD	**–1.460**	**–0.064 (–1.419–1.374)**	**0.042**
Fu and Li's D*, FLD*	**–2.277**	**–0.063 (–1.871–1.232)**	**0.022**
Fu and Li's F*, FLF*	**–2.177**	**–0.067 (–1.799–1.239)**	**0.020**
Fu's FS	–1.965	–0.009 (–3.877–3.788)	0.189
Ramos-Onsins and Rozas's R2	0.109	0.129 (0.083–0.183)	0.287
Raggedness, r	0.027	0.082 (0.024–0.203)	0.081
ITS–2			
Tajima's D, TD	–1.399	–0.038 (–1.527–1.623)	0.058
Fu and Li's D*, FLD*	**–1.934**	**–0.053 (–2.244–1.151)**	**0.050**
Fu and Li's F*, FLF*	**–1.881**	**–0.051 (–2.182–1.251)**	**0.044**
Fu's FS	**–5.165**	**–0.609 (–3.280–1.630)**	**0.002**
Ramos-Onsins and Rozas's R2	0.088	0.141 (0.069–0.243)	0.089
Raggedness, r	0.073	0.250 (0.000–0.862)	0.152

this study and previously identified *Stephanofilaria* species. Using the Cox-1 sequences, stephanofilarial worms formed two sister clades: the *Stephanofilaria dinniki* clade and the *Stephanofilaria stilesi* clade (*Stephanofilaria sp* isolated from Australian cattle and *Stephanofilaria stilesi* isolated from American cattle) each with 100% bootstrap support. The two *Stephanofilaria* clusters formed a single clade with 77% bootstrap support. Similarly, phylogenetic analyses of the ITS-2 sequences also revealed that sequences of Stephanofilaria worms from rhinoceros clustered with known *Stephanofilaria* species with a 93% bootstrap support. Phylogenetic analyses at both the Cox-1 and ITS-2 loci suggest a closer relationship of the genus *Stephanofilaria* with Thelazioidea, rather than the family Filariidae (Filarioidea), in which it has been historically assigned to (Saparov *et al.* 2014). The close affinity to Thelazioidea is supported by recent genetic studies of this genus (Lui *et al.* 2023; Naseem *et al.* 2021).

For the Cox-1 gene, Tajima's D, and Fu and Li's D* and F* were negative and statistically significant, but the Fu's FS, Fu and Li's D*, and Fu and Li's F*, but not Tajima's D, were negative for the ITS-2 gene. Negative and significant values indicate an excess of rare polymorphisms, which suggests positive or purifying selection, genetic hitchhiking, and a recent spatial expansion or increase in population size. Statistical tests like Tajima's D, Fu and Li's D* and F*, and Fu's FS can be significantly negative under purifying selection, population expansion, or selective sweeps, although each statistic may be best at detecting one of these forces (Braverman *et al.* 1995; Fu 1998; Fu 1995; Simonsen *et al.* 1995). Tajima's D statistic and Fu and Li D* and F* tests are the most powerful in detecting a selective sweep and genetic hitchhiking (Simonsen *et al.* 1995). However, Fu's FS, Ramos-Onsins and Rozas's R2, and raggedness r tests are sensitive in detecting population growth (Ramos-Onsins and Rozas 2002b). The significance of Tajima's D but not Fu's FS at the Cox-1 locus suggests that this gene is under purifying selection. Demographic changes, however, would be expected to affect the genome more evenly than selection, particularly the ITS-2 genes, which are known to evolve neutrally subject to secondary structure constraints (Prahl *et al.* 2021). Indeed, the ITS-2 gene had a negative and statistically significant Fu's FS statistic, indicative of a population expansion after a reduced ancestral effective population size (Avise 2000).

Purifying or positive selection occurs when an allele is favored by natural selection. The frequency of the favored allele increases creating an excess of rare polymorphism from prior standing genetic variation in the population. Cytochrome c oxidase subunit I (Cox-1) is one of the major proteins responsible for oxidative phosphorylation (OXPHOS), a process that releases ATP in eukaryotes. It is usually subject to strong purifying selection in response to higher energy requirements or limited oxygen availability (Boratynski *et al.* 2014; Shen *et al.* 2010; Tomasco and Lessa 2011) driven usually by altitudinal changes in temperature and associated hypoxia. Such environmental pressures have been suggested to cause changes in the structure and function of proteins associated with oxidative phosphorylation in mitochondria including the Cox-1, CytB, and the NADH dehydrogenase complex (Bartáková *et al.* 2021). A similar evolutionary pattern for mitochondrial OXPHOS genes has been observed in deep sea fishes and subterranean mammals (Shen *et al.* 2019; Tomasco and Lessa 2011), vertebrates in environments that have total darkness, cold, scarce food, and low oxygen. Selected sweeps can occur as adaptation to new environments and habitats (Wei *et al.* 2023). A recent study revealed that *Stephanofilaria* infections in the Kenyan rhinoceros population is higher in sanctuaries with low minimum temperatures compared to the 1960s when it was more widespread including lowland sanctuaries with higher minimum temperature (King'ori *et al.* 2024). The more recent restricted distribution of *Stephanofilaria* is possibly due to the extinction of *Rhinomusca brucei* Malloch (Parsons and Sheldrick 1964), a biting fly previously associated with skin lesions caused by *Stephanofilaria dinniki* (Round 1964) in lowland areas (Mihok *et al.* 1996). This extinction occurred due to reduction of rhinoceros numbers and the amalgamation of all remnant rhinoceros populations into a few populations in cooler highland locations. Moreover, *Rhinomusca dutoiti*, a species of fly that appears adopted to cooler temperatures in arid and semi-arid locations of other rhinoceros reserves, may have taken advantage of increased rhino densities to proliferate and become the dominant vector for *Stephanofilaria dinniki*.

Genetic diversity in this study was high (0.930 ± 0.030) for the Cox-1 gene and moderate (0.579 ± 0.104) for the ITS-2 gene. Nucleotide diversity was generally low, at 0.008 ± 0.002 for the Cox-1 gene and 0.00197 ± 0.0016 for the ITS-2 gene. The Cox-1 gene has been shown to display wider genetic variation among nematode species and similar patterns of diversity recorded in this study have been observed elsewhere. In the mulberry root-knot nematode, *Meloidogyne enterolobii* in China, haplotype diversity of 0.90 (Shao *et al.* 2020) was observed for the Cox-1 gene. Similarly, the Cox-1 gene of the pinniped hookworms *Uncinaria lucasi* was reported to have high haplotype diversity ranging between 0.96 and 0.98 and a high nucleotide diversity at 0.014 (Davies *et al.* 2020). A study of *Haemonchus placei* and *Haemonchus contortus* also revealed high haplotypic diversity (0.98–0.99 and 0.98–1) and nucleotide diversity (0.016–036, 0.009–0.01) at the Cox-1 locus, respectively, from several locations in Brazil (Brasil *et al.* 2012). In contrast, several studies have also revealed moderate to low gene diversity at the Cox-1 gene. For example, in a study of *Ascaris spp* infecting humans, moderate diversity (0.616) was recorded in selected municipalities in Brazil (Monteiro *et al.* 2019). In another study on *Heterotakis gallinarum* infecting chicken in Tunisia, low

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genetic diversity at the Cox-1 gene ranging from 0.12 to 0.42 was observed at different locations (Amor *et al.* 2018). Similar levels of nucleotide diversity at the Cox-1 gene (0.006 + 0.0006) have been observed in *Thelazia callipaeda* from China (Zhang *et al.* 2018). Lower levels of nucleotide diversity were observed at the Cox-1 gene (0.00147 ± 0.00051) for *Setaria digitata*, a nematode causing ocular lesions in horses (Junsiri *et al.* 2023). The ITS-2 gene also displays greater variability in gene and nucleotide diversity among nematodes. Gene diversity of the ITS-2 gene of *Onchocerca volvulus* in Africa and Brazil was 0.983, and nucleotide diversity was 0.0073 (Morales-Hojas *et al.* 2007). The mean gene diversity in 12 *Haemonchus contortus* populations from Thailand was 0.724 ± 0.025, while average nucleotide diversity was 0.007 ± 0.004 (Mangkit *et al.* 2014). A study on *Heterotakis gallinarum* infecting chickens in Tunisia revealed low to moderate gene diversity ranging from 0.35 to 0.59 at the ITS genes (Amor *et al.* 2018). The high gene diversity and low nucleotide diversity (Hd: 0.930 ± 0.030, PiT: 0.008 ± 0.002) at the Cox-1 observed in this study indicate a high number of closely related haplotypes, and suggest that the parasite population may have undergone a recent expansion following a bottleneck (Mendez-Harclerode *et al.* 2007; Zhang *et al.* 2021). The parasite population likely expanded following the amalgamation of rhinoceros to cooler highland sanctuaries, an increase in rhinoceros population size and proliferation of rhinoceros sanctuaries in highland locations. These results are also consistent with a soft selective sweep as discussed earlier.

This study provides the first genetic characterization of *Stephanofilaria dinniki* and sheds light into the molecular diagnosis of *Stephanofilaria dinniki* in rhinoceros. Accurate identification of rhinoceros Stephanofilaria is crucial for its diagnosis, treatment, epidemiological studies, and control. This study also corroborates recent genetic studies, which suggested that this genus is phylogenetically closer to Thelazioidea rather than the family Filariidae (Filarioidea), where it is currently placed.

Data availability statement. All the relevant data has been deposited in Genbank or included in the main manuscript.

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