Acta Tropica 258 (2024) 107344

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Detection and characterization of vector-borne parasites and *Wolbachia* endosymbionts in greater one-horned rhinoceros (*Rhinoceros unicornis*) in Nepal

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ARTICLE INFO

Keywords:
Mansonella
Nepal
Onchocerca
Rhinoceros unicornis
Theileria
Wolbachia

ABSTRACT

Vector-borne parasite infections affect both domestic and wild animals. They are often asymptomatic but can result in fatal outcomes under natural and human-induced stressors. Given the limited availability of molecular data on vector-borne parasites in *Rhinoceros unicornis* (greater one-horned rhinoceros), this study employed molecular tools to detect and characterize the vector-borne parasites in rescued rhinoceros in Chitwan National Park, Nepal. Whole blood samples were collected from thirty-six *R. unicornis* during rescue and treatment operations. Piroplasmida infections were first screened using nested polymerase chain reaction (PCR) targeting 18S ribosomal RNA gene. *Wolbachia* was detected by amplifying 16S rRNA gene, while filarial nematodes were detected through amplification of 28S rRNA, COI, *myo*HC and *hsp*70 genes. Our results confirmed the presence of *Theileria bicornis* with a prevalence of 75% (27/36) having two previously unreported haplotypes (H8 and H9). *Wolbachia* endosymbionts were detected in 25% (9/36) of tested samples and belonged to either supergroup C or F. Filarial nematodes of the genera *Mansonella* and *Onchocerca* were also detected. There were no significant association between *T. bicornis* infections and the age, sex, or location from which the animals were rescued. The high prevalence of *Theileria* with novel haplotypes along with filarial parasites has important ecological and conservational implications and highlights the need to implement parasite surveillance programs for wildlife in Nepal. Further studies monitoring vector-borne pathogens and interspecies transmission among wild animals, livestock and human are required.

1. Introduction

The greater one-horned rhinoceros (*Rhinoceros unicornis*) (hereinafter rhinoceros) is the second largest extant megaherbivore in South Asia, specifically inhabiting the floodplains of the Ganges, Brahmaputra, and Sindh rivers and their large tributaries, stretching from the Indo-Burmese border in the east to the Pakistan border in the west (Dinerstein, 2003). Rhinoceros are categorized as vulnerable on the IUCN red list and are currently restricted to small, isolated protected areas of less than 20,000 km^2 due to habitat loss, fragmentation, and poaching (Ellis and Talukdar, 2019; Jhala et al., 2021). The major population of rhinoceros in Nepal resides in Chitwan National Park (CNP) (694 out of 752), according to a recent census carried out in 2021 with an annual growth rate of less than 2.5%, which is half of that in the previous census carried out in 2015 (~ 5%). Currently, the population of rhinoceros in CNP has been rejuvenated from < 100 individuals during the 1960s with

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https://doi.org/10.1016/j.actatropica.2024.107344
Received 12 June 2024; Received in revised form 25 July 2024; Accepted 30 July 2024
Available online 2 August 2024

continuous conservation efforts including the establishment of the national park in 1973 and strict law enforcement (Dinerstein, 2003). Since 2015, the mortality of rhinoceros has spiked with an average of ~ 25 deaths annually (Bhandari et al., 2022). Although incidences of poaching have considerably declined due to rigorous conservation efforts, a rise in mortalities due to unknown or natural causes has reduced the annual growth rate of the population. Conservationists are increasingly concerned about the potential impact of pathogen on the health and mortality of Nepal's rhinoceros populations.

Piroplasmida infections have been associated with morbidity and mortality in rhinoceros population in various countries. *Theileria bicornis* was first described from the black rhinoceros in Africa (Nijhof et al., 2003). It was subsequently found in white rhinocersos in Kruger National Park (Govender et al., 2011) and in black and white rhinos in Kenya (Otiende et al., 2016). Besides free-ranging rhinoceros, *T. bicornis* was also detected in captive rhinoceros in Australia (Yam et al., 2018). Additionally, *T. bicornis* was reported in other wildlife in South Africa (Pfitzer et al., 2011) and in cattle in Uganda (Muhanguzi et al., 2010). Three haplotypes (H1, H3 and H4) of *T. bicornis* reported from black rhinoceros and haplotype H2 reported from white rhinoceros (Otiende et al., 2016; Yam et al., 2018).

Filarioids are parasitic nematodes belonging to the superfamily Filarioidea and can infect a wide range of hosts, including humans. These parasites can be transmitted by an extensive range of arthropod vectors including mosquitoes and ticks (Otranto et al., 2011; Otranto and Deplazes, 2019; Perles et al., 2023; Pupić-Bakrač et al., 2021; Shelley and Coscarón, 2001). Epidemiological studies of filarioids have primarily focused on domestic animals (Grácio et al., 2015; Otranto and Dantas-Torres, 2010). *Dirofilaria immitis* has been reported in wild carnivores in different regions of the world (Otranto et al., 2015), while *Mansonella* spp. have been recorded in ring-tailed coatis (Perles et al., 2023), deer (Uni et al., 2004), and bears (Masatani et al., 2021). However, the epidemiology of filarioids in megaherbivores is still largely unknown. Interestingly, some filarial parasites harbor *Wolbachia* as an endosymbiont, some of which are involved in the reproduction, development, and survival of the host nematode (Martin and Gavotte, 2010; Taylor et al., 2005).

In Nepal, several studies have examined the vector-borne parasitic infections in livestock and ticks. Of note, *Theileria* infection was commonly observed in cattle (Shrestha et al., 2017; Poudel et al., 2023) and ticks (Gupta et al., 2013). Until now, however, no information has been available on *Theileria* infections in wild animals in Nepal. Additionally, there is no data available on filarial infections in livestock and wildlife in Nepal. In this study, molecular techniques were employed to identify and characterize the vector-borne parasites in free-ranging rhinoceros population in Nepal.

2. Materials and methods

2.1. Study area and sample collection

CNP lies in the south-central part of Nepal with an area of 952.63 km^2 and a buffer zone area of 729.37 km^2. The landscape of CNP is a matrix of grassy flood plains, and tropical evergreen Sal Forest while the buffer zone is highly settled and heavily cultivated. The climate in CNP is subtropical monsoon with three distinct seasons: monsoon (June – October), cool-dry (October – February), and hot-dry (February – June). Mean annual temperature ranges between 8 °C in January and 36 °C in April and rainfall of 2036 ± SE 64 mm per year (>80% in monsoon) (Subedi, 2012). A total of 68 mammal species, 544 bird species, 56 reptile and amphibian species, and 126 species of fish are recorded in the park (CNP, 2012).

Samples from 36 rhinoceros (19 from the core area and 17 from the buffer zone) were collected opportunistically during rescue operations between 2015 and 2022. Rhinoceros were rescued when they entered human settlements and were returned to their natural habitat. Some were injured in territorial fights with conspecifics, while the floods forcibly expelled others from their original habitat. Rhinoceros were immobilized jointly by the Department of National Park and Wildlife Conservation and the National Trust for Nature Conservation (NTNC) using a standard protocol with a combination of etorphine hydrochloride and acepromazine delivered by dart gun and reversed by diprenorphine. The blood samples were collected from an auricular vein and stored at −20 °C in the NTNC– Biodiversity Conservation Center (NTNC-BCC), molecular laboratory, Sauraha, Chitwan. The chief of the CNP office granted legal permission for rhinoceros capture and handling. Ethical approval for the study was obtained from the Ethical Clearance Committee of Nepal Veterinary Council (Ref No: 56/2079.80).

2.2. DNA extraction and PCR

Extraction of genomic DNA from whole blood was carried out using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. A nested polymerase chain reaction (PCR) for *Babesia, Theileria,* and *Hepatozoon* species (BTH) was performed to amplify almost a section of the 18S ribosomal RNA gene (rDNA) (1400–1600 bp) (Masatani et al., 2017). The primer sets of BTH 1st F/BTH 1st R and BTH 2nd F/BTH 2nd R were used for the first and second PCRs, respectively.

The presence of Anaplasmataceae was first screened with the primer set (EHR16SD and EHR16SR) amplifying 16S ribosomal RNA gene (rDNA) (Parola et al., 2000). The positive samples were further characterized by running additional PCRs amplifying heat shock protein (*groEL*) and citrate synthase (*gltA*) genes of *Anaplasma* and *Ehrlichia* and 16S rDNA of *Wolbachia* using the primers listed in Table 1.

Samples positive for *Wolbachia* were subjected to PCR to amplify 28S rDNA and cytochrome c oxidase subunit I (COI) gene of *Onchocerca* and heat shock protein 70 (*hsp*70) and myosin heavy chain (*myo*HC) genes of *Mansonella*.

2.3. In-Fusion cloning

Samples showing mixed signals in the sequencing analysis of *Wolbachia* 16S rDNA PCR products were subjected to In-Fusion cloning. First, PCR primers (InFusion_EHR16SD and InFusion_1513R) were designed based on the alignment of *Wolbachia* 16S rDNA sequences available in the database. The purified PCR products amplified by the newly designed primers were cloned into pUC19 vector (TaKaRa Bio Inc, Japan). The infusion reaction was performed in a total volume of 10 μL, containing 2.0 μL of 5X In-Fusion HD Enzyme Premix, 1 μL of PCR-linearized pUC19 vector, 200 ng of each purified PCR fragment, and dH2O from the In-Fusion HD PCR Cloning Kit (TaKaRa Bio Inc, Japan). The infusion reaction mix was incubated at 50 °C for 15 min, and then placed on ice. Following incubation, the heat-shock transformation was conducted by adding 2.5 μl of the infusion reaction mixture to the competent cells, *Escherichia coli* DH5α (Nippon Gene, Tokyo, Japan). Transformed cells were plated on LB agar plates containing ampicillin and incubated at 37 °C for 24 h. The transformants were screened with colony PCR.

2.4. Sanger sequencing

The PCR amplicons were purified using ExoSAP-IT™ Express PCR Product Cleanup Reagent (Thermo Fisher Scientific, Waltham, MA, USA). The sequencing reaction was conducted using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). Sequence data editing and trimming of primer annealing sites was done using ATGC software (GENETYX Corporation, Tokyo, JP) and the consensus sequences were extracted for phylogenetic analysis. The sequences generated in this study were submitted to the DNA Data Bank of Japan (DDBJ) under the accession numbers: LC817751 to

Table 1
Primers used in this study.

Primers	Sequence (5′–3′)	Target organism(s)	Target gene	Purpose	Product size	Reference
BTH 1st F	GTGAAACTGCGAATGGCTCATTAC	*Babesia/ Theileria/ Hepatozoon*	18S rDNA	1st PCR	1400–1600	(Masatani et al., 2017)
BTH 1st R	AAGTGATAAGGTTCACAAAACTTCCC					
BTH 2nd F	GGCTCATTACAACAGTTATAGTTTATTTG	*Babesia/ Theileria/ Hepatozoon*	18S rDNA	2nd PCR	1400–1600	(Masatani et al., 2017)
BTH 2nd R	CGGTCCGAATAATTCACCGGAT					
BTH18SinterF	ATTTTCCGACTCCTTCAGCA	*Babesia/ Theileria/ Hepatozoon*	18S rDNA	Sequencing		(Ogata et al., 2021)
BTH18SinterR	AACTAAGAACGGCCATGCAC					
EHR16SD	GGTACCYACAGAAGAAGTCC/	Anaplasmataceae	16S rDNA	PCR	345	(Parola et al., 2000)
EHR16SR	TAGCACTCATCGTTTACAGC					
HS1-F	CGYCAGTGGGCTGGTAATGAA	Anaplasmataceae	*groEL*	1st PCR	1300	(Rar et al., 2011)
HS6-R	CCWCCWGGTACWACACCTTC					
HS3-F	ATAGTYATGAAGGAGAGTGAT	*Anaplasma*	*groEL*	2nd PCR	1256	(Liz et al., 2000)
HSV-R	TCAACAGCAGCTCTAGTWG					
groEL_fwd3	TGGCAAATGTAGTTGTAACAGG	*Ehrlichia*	*groEL*	2nd PCR	1100	(Gofton et al., 2016)
groEL_rev2	GCCGACTTTTAGTACAGCAA					
F4b	CCAGGCTTTATGTCAACTGC	Anaplasmataceae	*gltA*	1st PCR	800	(Inokuma et al., 2005)
R1b	CGATGACCAAAACCCAT					
EHR-CS136F	TTYATGTCYACTGCTGCKTG	*Anaplasma* and *Ehrlichia*	*gltA*	2nd PCR	650	(Inokuma et al., 2005)
EHR-CS778R	GCNCCMCCATGMGCTGG					
EHR16SD	GGTACCYACAGAAGAAGTCC	*Wolbachia*	16S rDNA	PCR	1300	(Moustafa et al., 2022)
1513R	ACGGYTACCTTGTTACGACTT					
Nematode1	GCGGAGGAAAAGAAACTAA	Nematode	28S rDNA	PCR	765	(Sehgal et al., 2005)
Nematode2	ATCCGTGTTTCAAGACGGG					
COIintF	TGATTGGTGGTTTTGGTAA	*Onchocerca*	COI	PCR	650	(Casiraghi et al., 2001)
COIintR	ATAAGTACGAGTATCAATATC					
MyManF	GAAGCTGAGGCTCAAGCAAT	*Mansonella*	*myo*HC	PCR	554	(Moraes et al., 2022)
MyManR	TCTGTTTTGCTCATCGCATT					
h70ManF	TGAGACAGCTGGAGGTGTTATG	*Mansonella*	*hsp*70	PCR	484	(Moraes et al., 2022)
h70ManR	ATCTTTCTGTGCCTCATCATCTG					
InFusion_EHR16SD	AAACGACGGCCAGTGGTACTCACAGAAGAAGTCCTGGC	*Wolbachia*	16S rDNA	cloning	1300	This study
InFusion_1513R	GACCATGATTACGCCACGGCTACCTTGTTACGACTTCAC					

Note: PCR, Polymerase chain reaction; NA, not applicable.

LC817777 for 18S rDNA of *T. bicornis,* LC817778 to LC817789 for 16S rDNA of *Wolbachia*, LC817841 to LC817843 for 28S rDNA of nematodes, LC817844 to LC817849 for COI of *Onchocerca* sp., LC817850 to LC817856 for *myo*HC gene of *Mansonella* sp., and LC817857 to LC817863 for *hsp*70 gene of *Mansonella* sp.

2.5. Phylogenetic analysis

We aligned the obtained sequences with those available in the database using MAFFT v7.450 software (Katoh and Standley, 2013). After visual inspection, we performed the necessary trimming and editing of the sequences using MEGA7 (Kumar et al., 2016). Completely identical sequences were detected and brought together using the "pgelimdupseq" command in Phylogears2 ver. 2.0 (Tanabe, 2008), followed by the best-fit model estimation based on the Bayesian information criterion by Kakusan4 (Tanabe, 2011), choosing the K80 with gamma distribution for 16S rDNA of *Wolbachia*, the Hasegawa Kishino Yano (HKY) model with gamma distribution for 18S rDNA, and the general time-reversible (GTR) model with gamma distribution for 28S rDNA. Codon-proportional models were selected for *hsp*70, *myo*HC, and COI genes.

The phylogenetic analysis was conducted using MrBayes v3.2.6 (Ronquist et al., 2012) with 1000,000–5000,000 generations at a sample frequency of 500 discarding the first 10% of trees as a burn-in. The consensus tree was visualized using FigTree v1.4.4 (Rambaut, 2012).

To visualize the relationships between the *T. bicornis* haplotypes based on the 18S rDNA sequences, a median-joining haplotype network analysis was performed using PopART v1.7 (Leigh and Bryant, 2015). To compare our data with those previously reported from other regions, we added haplotypes from Australia (H2 and H4), Kenya (H1, H2 and H3), South Africa (H1 and H3).

2.6. Statistical analysis

The association between Piroplasmida infection and age, sex, and habitat was assessed using GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Fisher's exact test was utilized for this analysis. Odds ratios (OR) along with their corresponding 95% confidence intervals (95% CI) were computed. A significance level of $P < 0.05$ was used to determine statistical significance.

3. Results

3.1. Detection and characterization of Theileria bicornis

A total of 27 out of 36 (75%) rhinoceros were positive for *Theileria* infection by BTH PCR. All PCR amplicons were successfully sequenced, and two sequence types of *T. bicornis* were identified.

Out of the 27 sequences we obtained, 25 were completely identical to each other (LC817751-LC817757, LC817759-LC81771 and LC817773-

LC817777) and another two were identical to each other (LC817758 and LC817772). The sequences obtained from this study were distinct from those of previously published haplotypes (H1 to H4) from African rhinoceros species and (H2 to H7) reported from *Amblyomma thollonii.* We have designated the two haplotypes of greater one-horned rhinoceroses as H8 and H9 (Fig. 1). The geographic distribution of each haplotype is shown in Fig. 2. The H8 haplotype was evenly distributed throughout CNP, while the H9 haplotype occurred only in one specific location.

The prevalence of infection in relation to sex, age and location is shown in Table 2. We found that the positive rate increased with age, but there was no statistically significant association observed. There was no association between *Theileria* infection and sex or sampling location.

3.2. Detection and characterization of anaplasmataceae

A total of 9 out of 36 (25%) rhinoceros were positive for Anaplasmataceae by PCR. None of the samples were positive for *groEL* and *gltA* PCRs amplifying the genera *Anaplasma* and *Ehrlichia*. Instead, all nine samples were positive for 16S rDNA PCR for *Wolbachia*. As the direct sequencing analysis of the PCR amplicons showed mixed signals (presumably due to mixed infections), the samples were subjected to In-Fusion cloning to separate the sequences. Two distinct sequence types of *Wolbachia* were identified in two samples. Phylogenetic analysis suggested that these two *Wolbachia* sequences belonged to supergroups C and F (Fig. 3). Specifically, one sequence type (LC817778-LC817783) is closely associated with the *Wolbachia* endosymbiont of the genus *Onchocerca* belonging to supergroup C, whereas the other (LC817784-LC817789) is closely linked to the *Wolbachia* endosymbiont of the genus *Mansonella* belonging to supergroup F. The blast result showed that one sequence type has 99% nucleotide identity with the *Wolbachia* endosymbiont of *Onchocera eberhardi* (KU255248) and the other shows 99% nucleotide identity with the *Wolbachia* endosymbiont of *Mansonella ozzardi* (AJ279034).

3.3. Detection and characterization of nematodes

All samples positive for *Wolbachia* by PCR were also positive by PCR for the 28S rDNA locus of nematodes. Sequence analysis of the amplified products identified two different sequence types. The phylogenetic tree revealed that the obtained sequences were clustered together either with the genera *Onchocerca* and *Mansonella* (Fig. 4). To characterize *Onchocera* sp., the mitochondrial COI gene was amplified, and the sequence was compared with those in the database. The phylogenetic analysis showed that the sequence obtained in this study is not clustered with validated species of the genus *Onchocerca* (Fig. 5). Likewise, the phylogenetic trees constructed for *hsp*70 and *myo*HC genes of *Mansonella* revealed that the sequences obtained from rhinoceros belong to a discrete taxon within the genus *Mansonella* clade (Figs. 6 and 7).

4. Discussion

Considering the limited knowledge regarding vector-borne infections in the vulnerable populations of greater one-horned rhinoceroses in Nepal, we first aimed to screen for a panel of common tick-borne pathogens including Piroplasmida and Anaplasmataceae. In addition to the first detection of *T. bicornis* in Asian rhinoceroses, our efforts led to the discovery of a poorly characterized groups of filarial parasites through their association with *Wolbachia* endosymbionts.

Our study detected *T. bicornis* in greater one-horned rhinoceros with an prevalence of 75%, which is higher than those reported from Kenya with infection rate of 43% and 66% in black and white rhinoceroses, respectively (Otiende et al., 2015) and from South Africa with a prevalence rate of 23.7% and 36.4% in black and white rhinoceros, respectively (Govender et al., 2011; Zimmermann et al., 2021). Additionally, lower prevalence rate of *T. bicornis* were reported from Nyala antelopes (52.6%) in South Africa (Pfitzer et al., 2011) and from cattle (3.8%) originating from western Uganda (Muhanguzi et al., 2010). These variations in infection rate of *T. bicornis* between different studies may be attributed to species differences, geographic locations, and availability of tick vectors.

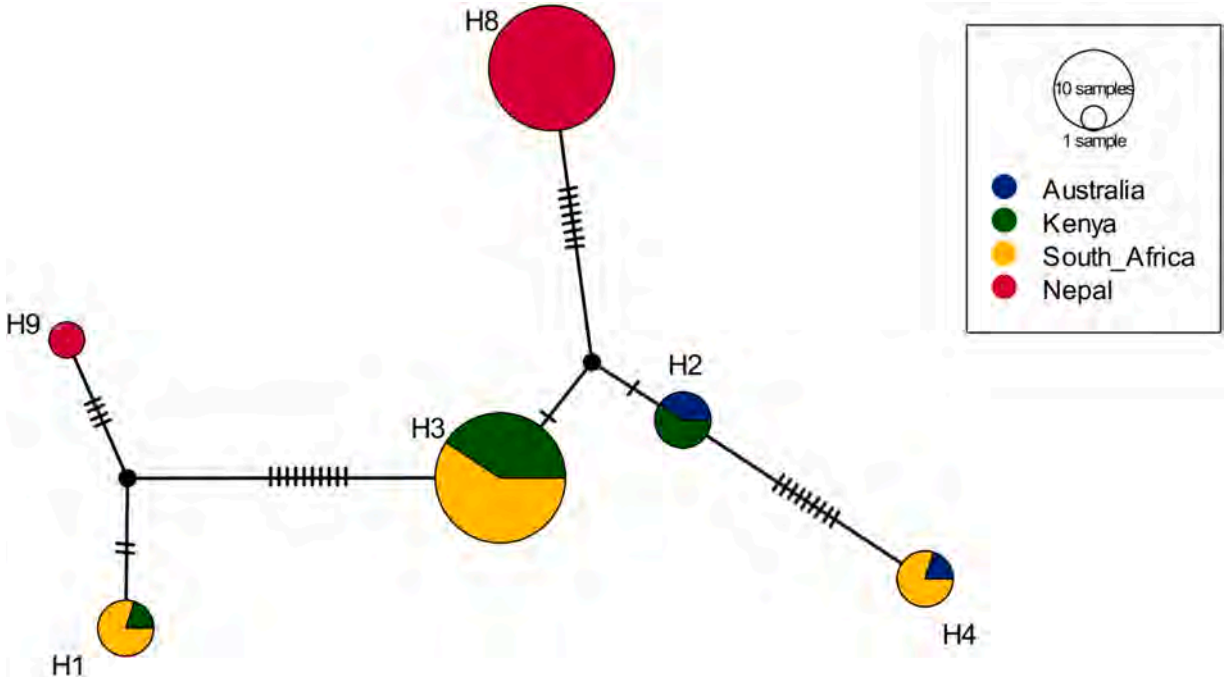

Fig. 1. Visualization of haplotype network of *Thelieria bicornis* using 18S rDNA gene sequences. Median-joining haplotype networks was constructed from 18S rDNA sequences. The circles represent the *T. bicornis* haplotypes. The color and size of each circle correspond to the location where the haplotype was detected and the haplotype number. For the haplotype network of *T. bicornis* sequences of haplotypes from Australia (H2 and H4), Kenya (H1, H2 and H3), South Africa (H1 and H3) and Nepal (H8 and H9) were included.

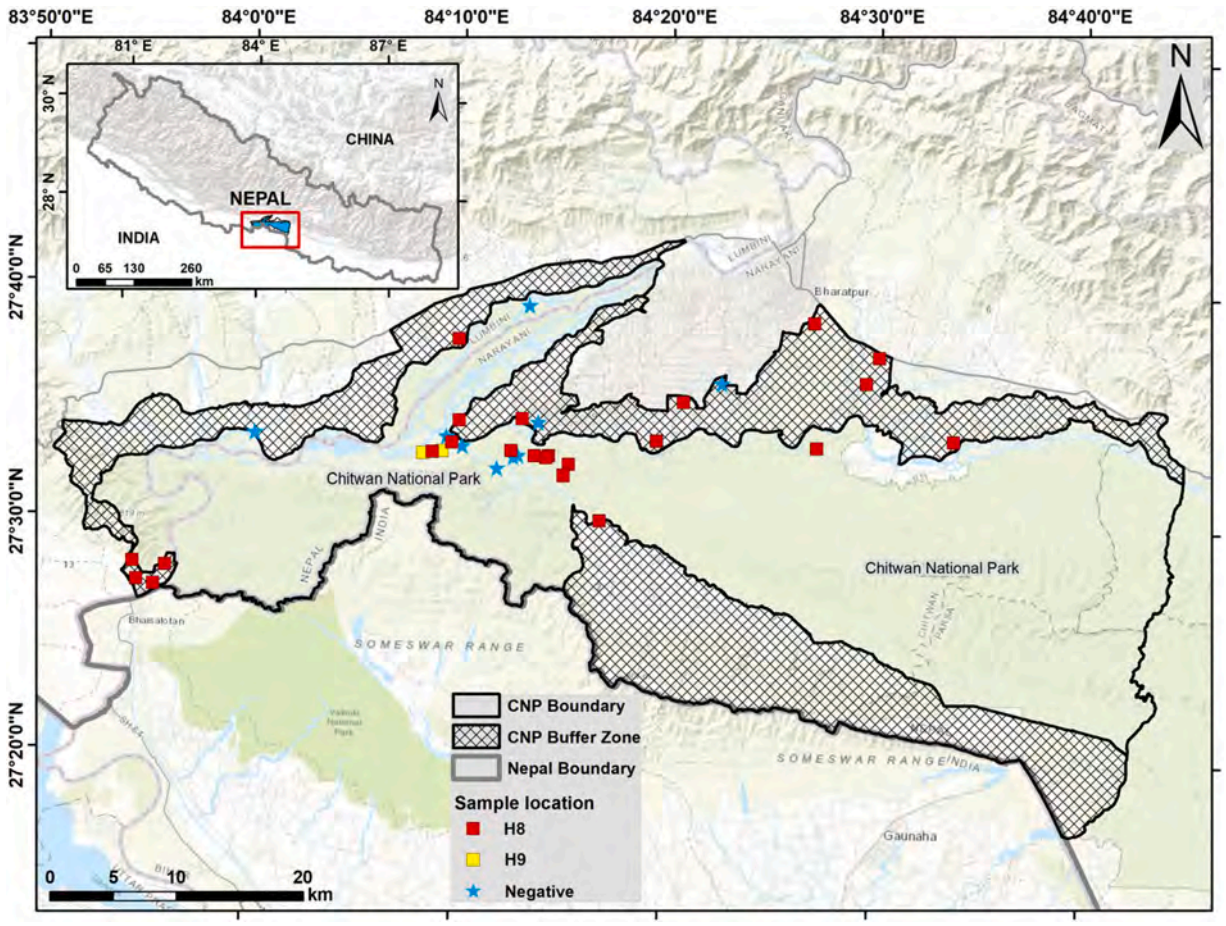

Fig. 2. Geographical distribution of *Theileria bicornis* haplotypes H8 and H9 in Chitwan National Park of Nepal.

Table 2
Theileria bicornis detected in greater one-horned rhinoceroses in Nepal.

Variable	Category	No. tested	Positive no. (%)	Odds ratio (95% CI)	P-value
Age	Young (0 year)	6	4 (67)	Reference	0.62
	Subadult (3–6 years)	4	3 (<1)	1.5 (0.09–25.41)	
	Adult (>6 years)	26	20 (77)	1.67 (0.24–11.45)	
Location	National park	19	13 (68)	0.46 (0.09–2.25)	0.45
	Buffer zone	17	14 (82)	2.15 (0.44–10.44)	
Sex	Female	15	11 (73)	0.86 (0.19–3.94)	1
	Male	21	16 (76)	1.16 (0.25–5.34)	
Total		36	27(75)		

The rhinoceros included in this study were rescued from both the core area and buffer zones. Moving rhinoceroses from the buffer zone back into the core area may increase the risk of introducing new pathogens to the potentially naïve rhinoceros population in the core area. These rescued rhinoceroses often came into contact with livestock in the buffer zone, which can serve as hosts for ticks and haemoparasites. This practice raises the likelihood of spillover of *T. bicornis* from alternative hosts to naïve rhinoceros populations. The precise transmission dynamics and specific vectors involved in the spread of *T. bicornis* remain unknown. *Theileria bicornis* (H2-H7) has been identified in *A. tholloni* ticks collected from African elephants in Kenya (King'ori et al., 2019), suggesting that *Amblyomma* spp., which are widely distributed in Nepal, could act as vectors of *T. bicornis* (Bohara and Shrestha, 2015). Consequently, molecular investigations are needed to identify *T. bicornis* or its tick vectors in livestock populations within Nepal.

Subadult rhinoceros were more likely to experience stress-related changes, including reproductive maturity, courtship, mating, and territorial competition, which could increase their vulnerability to *Theileria* infections (Govender et al., 2011). Nevertheless, our study showed no significant relationship between *T. bicornis* infection and age, which is consistent with the findings from a previous study by Otiende et al. (2015). However, our results may be influenced by sampling bias because a substantial proportion of the rhinoceros enrolled in our study were adults, which may have masked the relationship between infection status and age. Our investigation revealed no significant association between *T. bicornis* infection and sex, suggesting that males and females were at approximately equal risk of acquiring the infection. This finding may be attributed to the animals sharing the same habitat and being exposed to the same vector communities, as well as experiencing similar stress factors in the study area. These results are in line with previous studies conducted by Govender et al. (2011) and Otiende et al. (2015). Other investigators, however, showed that parasitism is sometimes sex-biased with males being infected more often with TBPs than females, at least in dogs (Chatanga et al., 2021).

Globally there are five different rhinoceros species: Africa is home to two species, black and white rhinoceros, while three species, greater one-horned, Sumatran and Javan rhinoceros, are native to Asia. The network analysis revealed that the population of *T. bicornis* in the greater one-horned rhinoceroses in Nepal had two distinct haplotypes from those reported from African rhinoceroses (Yam et al., 2018; Zimmermann et al., 2021). This fact suggests that *T. bicornis* may have

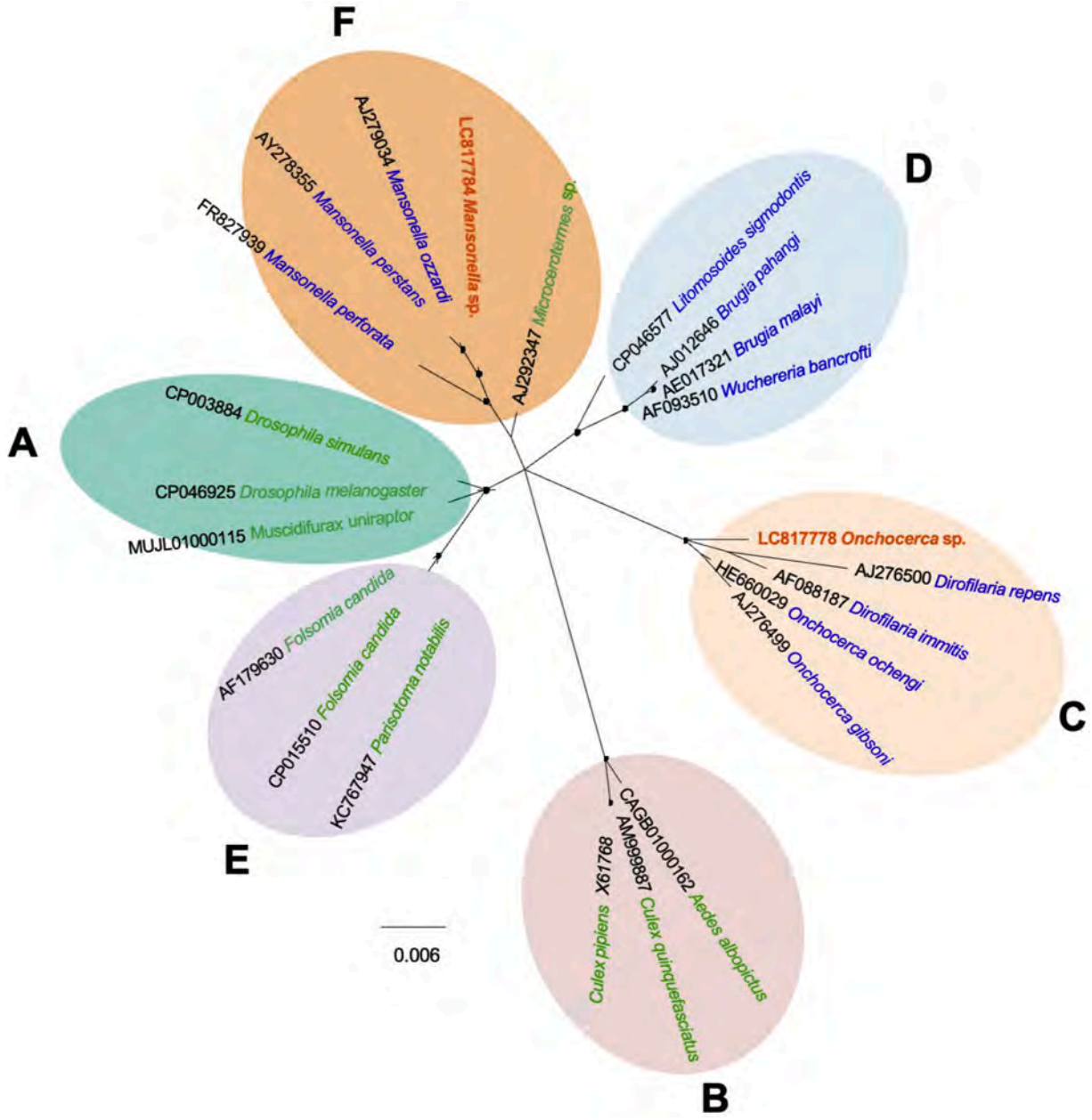

Fig. 3. Bayesian phylogenetic tree based on 16S rDNA gene sequences of *Wolbachia*. Nodes with posterior probabilities greater than 0.75 are indicated by black dots. Tip labels display GenBank accession numbers and their host, with green indicating arthropod hosts and blue indicating filarial nematode hosts and red indicating sequence obtained in this study. The alphabets A-F represent super group of *Wolbachia.*

co-evolved with their animal hosts, leading to an equilibrium state in which infected rhinoceroses do not develop disease from *T. bicornis* infections (Otiende et al., 2015). However, stressors that disrupt host-parasite equilibrium can result in clinical infections. For example, certain species of *Theileria*, such as *Theileria velifera* in cattle and the *Theileria orientalis* genotype buffeli (Kamau et al., 2011; Mans et al., 2015) can establish lifelong infections within their hosts. These infections typically do not result in clinical symptoms unless the animals experience immunosuppression or undergo stressful conditions such as pregnancy, translocation, or suboptimal rearing conditions (Sugimoto and Fujisaki, 2002). While our findings suggest that haplotypes H8 and H9 are native to South Asia and are presumably avirulent in greater one-horned rhinoceroses, we cannot exclude the possibility that these haplotypes are virulent to other rhinoceros species. Asymptomatic infections with tick-borne pathogens are common in wildlife but clinical manifestations sometimes only appear when these hosts are exposed to stressors such as co-infections with other pathogens (Munson et al., 2008), translocations (Höfle et al., 2004; Nijhof et al., 2005) malnutrition, and extreme environmental conditions (Nijhof et al., 2003).

We identified, for the first time, *Mansonella* sp. and *Onchocerca* sp. in rhinoceros in Nepal. Further species identification was impossible due to lack of parasite specimens for morphological examinations. However, this is not the first report on the detection of *Onchocerca* in blood samples. The detection of *Onchocerca* sp. in blood was previously reported in humans and donkeys in Venezuela and Italy, respectively (Botto et al., 1984; Papini et al., 2020). Clinical manifestation of filarial parasites have been previously reported in other species of rhinoceros in Africa, including hemorrhagic ulcerative filarial lesions in black rhinoceros (Schulz and Kluge, 1960). Similarly, Mutinda et al. (2012) reported the first case of putative filariasis outbreak in white and black rhino in Kenya which may have been a result of an immunocompromised condition caused by food shortage. More recently, King'ori et al., 2024

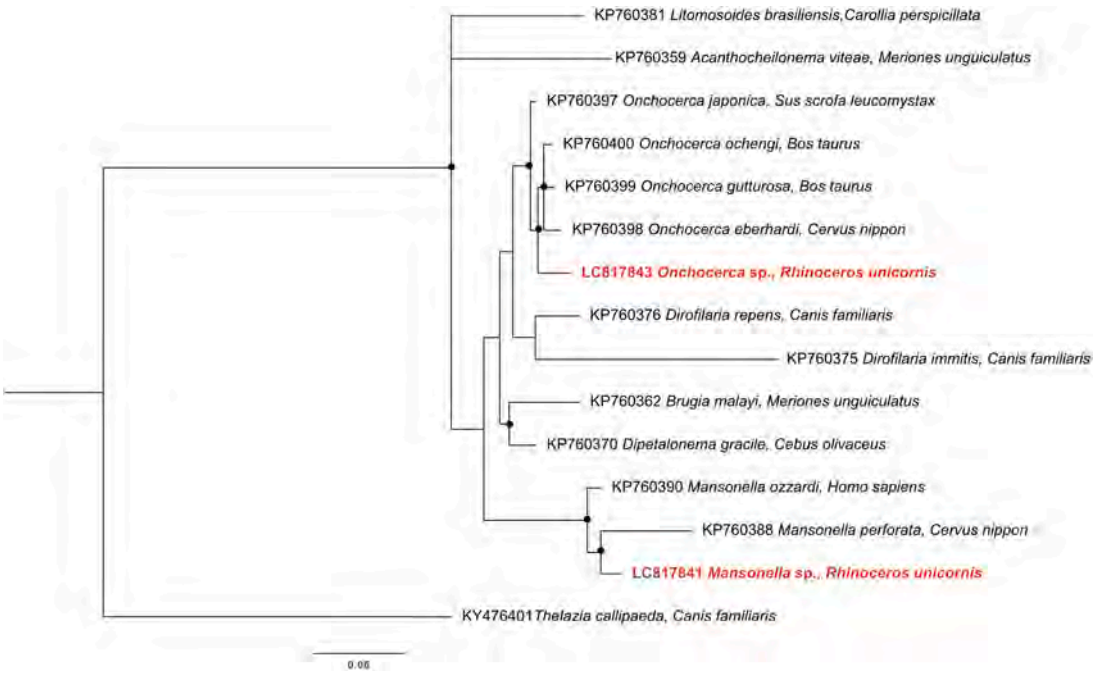

Fig 4. Bayesian phylogenetic tree based on 28S rRNA gene sequences of filarial nematodes. Black dots indicate nodes with posterior probability of > 0.70.

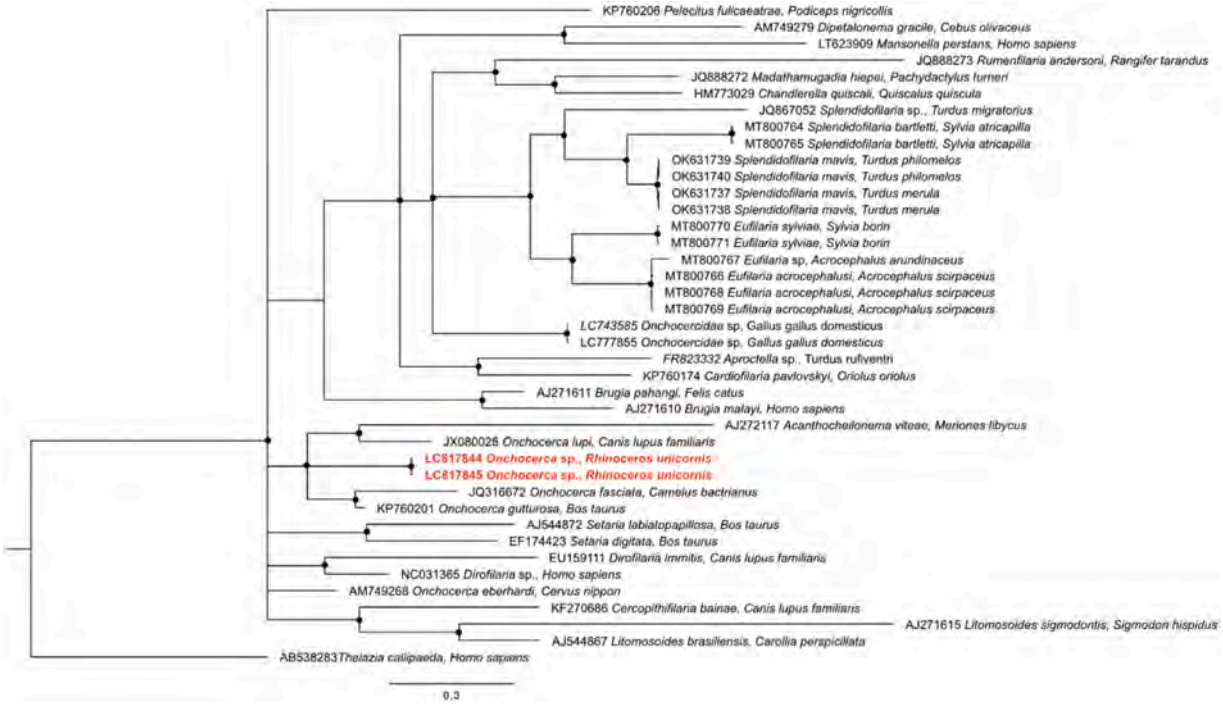

Fig 5. Bayesian phylogenetic tree based on COI gene of filarial nematodes. Black dots indicate nodes with posterior probability of > 0.70.

reported that ulcerative filarial skin lesions caused by *Stephanofilaria dinniki* are a common disease in black and white rhinoceroses. Considering the detrimental impact of filarial infections on the black and white rhinoceroses during immunocompromised conditions, the clinical significance of filarial parasites in greater one-horned rhinoceroses in Nepal cannot be rule out.

Filarial parasites including *Mansonella* and *Onchocerca* may also pose a zoonotic risk (Cambra-Pellejà et al., 2020; Mediannikov and Ranque, 2018). Given that several species of these parasites have been identified in wild animals (Bain et al., 1993; Masatani et al., 2021; Perles et al., 2023; Uni et al., 2015, 2007; Yagi et al., 1994), the close contact between wild animals, livestock and humans may also favor the potential transmission of filarial parasites from animals to humans. Therefore, precise species identification of filarial parasites is crucial for evaluating their zoonotic potential. Characterizing the lifecycle of these parasites, including their definitive hosts, vectors, and developmental stages, provides invaluable insights into their transmission and host-switching potential. Such knowledge is essential for developing effective control measures to mitigate the risk of zoonotic filarial diseases.

Wolbachia species are bacterial endosymbionts vertically transmitted

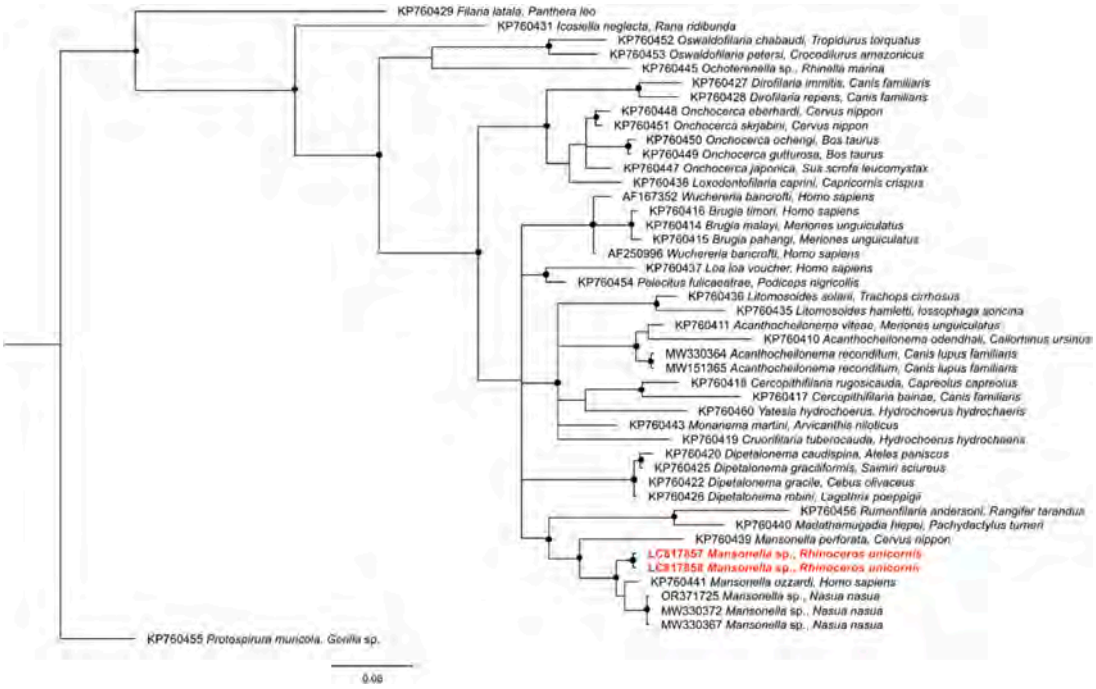

Fig 6. A Bayesian Inference phylogenetic tree of *hsp*70 gene of *Mansonella* spp. Black dots indicate nodes with posterior probability of > 0.75.

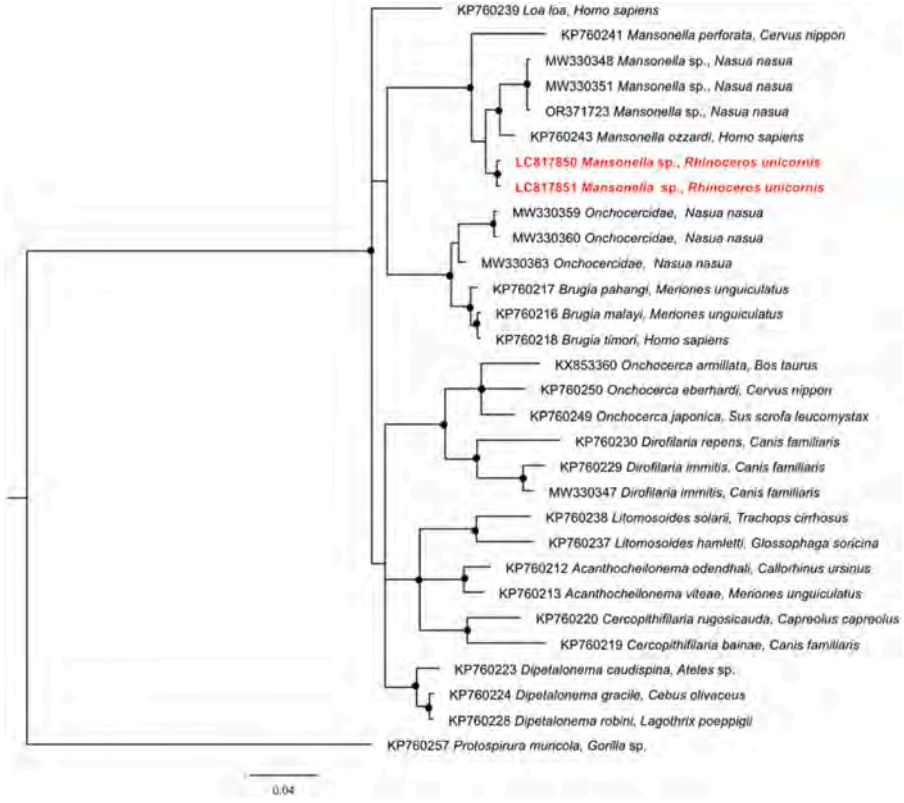

Fig 7. A Bayesian Inference phylogenetic tree of *myo*HC gene sequences of *Mansonella* spp. Black dots indicate nodes with posterior probability of > 0.75.

in arthropods and nematodes (Correa and Ballard, 2016; Manoj et al., 2021). They can be classified into different supergroups in arthropods (A, B, E, H, I, K, and S), and nematodes (C, D, and J), with supergroup F being common to both arthropods and filarial nematodes (Kaur et al., 2021). In our study, *Wolbachia* detected in rhinoceroses fell into two distinct supergroups: C and F. Similar findings were reported by Perles et al. (2023), where *Mansonella*-associated *Wolbachia* in the supergroup F were detected in a blood sample from a ring-tailed coati (*Nasua nasua*) in Brazil. The detection of *Wolbachia* of different supergroups in rhinoceros samples can be attributed to multiple acquisition events by different major lineages of filarial nematodes (Manoj et al., 2021) . *Wolbachia* can serve as a diagnostic marker to detect the presence of

filarial infections as suggested previously (Akter et al., 2019; Qiu et al., 2016). Additionally, a deeper understanding of the symbiotic relationship between *Wolbachia* and their filarial hosts may provide valuable insights into the biology, and evolution, and potential therapeutic targets for treatment of these filarial infections (Turb et al., 2012).

5. Conclusion

This is the first report detecting *T. bicornis* with two new haplotypes in the Asian rhinoceroses. High infection rates suggested that this parasite is well established in the rhinoceros population in Nepal; however, its clinical impact, especially on those individuals under the pressure of ecological stressors, remains unclear. The current investigation revealed that rhinoceroses in Nepal are infected with two different filarial nematodes through their association with *Wolbachia* endosymbionts. Further studies are needed to determine the taxonomical position and life cycle of these filarial parasites together with their zoonotic potential. Given the frequent rescue of rhinoceroses from human settlements and their cohabitation with livestock, disease spillover between these two host species is likely to occur. Therefore, understanding the epidemiology of vector-borne organisms necessitates a thorough investigation of the interactions between multiple hosts that share habitat.

CRediT authorship contribution statement

Gita Sadaula Pandey: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Prajwol Manandhar:** Writing – review & editing, Investigation. **Bijaya Kumar Shrestha:** Resources. **Amir Sadaula:** Writing – review & editing, Resources. **Naoki Hayashi:** Writing – review & editing, Software, Formal analysis. **Abdelbaset Eweda Abdelbaset:** Writing – review & editing, Formal analysis. **Pradeepa Silwal:** Investigation. **Toshio Tsubota:** Writing – review & editing. **Mackenzie L. Kwak:** Writing – review & editing. **Nariaki Nonaka:** Writing – review & editing, Supervision. **Ryo Nakao:** Writing – review & editing, Validation, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no competing interest.

Data availability

Data will be made available on request.

Acknowledgments

The author thanks all the staff of the Chitwan National Park and National Trust for Nature Conservation for their assistance with sampling.

This study was supported in part by the Japan Society for the Promotion of Science (JSPS) KAKENHI, Japan (JP20KK0151 and JP23K23770); the Japan Agency for Medical Research and Development (AMED), Japan (JP23wm0225034).

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