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Genetic characterization of zoonotic hookworms infecting wild felids in northern India

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Abstract

Background Hookworms are the most common soil-transmitted helminths that inhabit the small intestine of various domesticated and wild animals. Despite their conservation status, there is a paucity of research on hookworm infections in wild felids. This study aimed to investigate the prevalence of hookworm infections in wild felids in northern India and to genetically characterize the hookworms. Faecal samples ($n=96$) from wild felids (lion, tiger, leopard, panther, jungle cat, and civet cat) were examined for helminthic infections. Samples positive for hookworms were subsequently subjected to molecular analysis targeting the internal transcribed spacer (ITS) region, followed by sequencing and phylogenetic analysis.

Results Among helminthic infections, *Ancylostoma* spp. ranked second (7.3%) after *Toxocara cati* (13.5%). Molecular analysis identified two species, *A. caninum* and *A. ceylanicum*. Phylogenetic analysis revealed distinct monophyletic clades for each species. *Ancylostoma caninum* formed a large clade with two subclades, one comprising Asian isolates and the other encompassing isolates from the Americas and Australia, whereas *A. ceylanicum* formed a single clade. Nucleotide identities ranged from 97.9 to 100% for *A. caninum* and from 99.1 to 100% for *A. ceylanicum*. Haplotype network analysis revealed eight haplotypes for *A. caninum* and six for *A. ceylanicum*. Genetic diversity correlated with geographic distance for *A. caninum* isolates, with Asian populations exhibiting high haplotype diversity but low nucleotide diversity. Neutrality indices suggested population stability for *A. caninum* and expansion for *A. ceylanicum*. Continent-wise analysis of molecular variance (AMOVA) indicated that 52.66% of the variation occurred within *A. caninum* populations, while 47.34% occurred between populations.

Conclusions This study highlighted the genetic diversity and molecular epidemiology of hookworms in wild felids.

Keywords *Ancylostoma caninum*, *Ancylostoma ceylanicum*, Wild felids, Genetic diversity, Haplotype networking, ITS region

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Background

Hookworms (Nematoda: Ancylostomatidae) are among the most common soil-transmitted helminths, equipped with specialized structures at their anterior end, including teeth, cutting plates, lancets, and a dorsal cone. These worms typically inhabit the small intestine of various domesticated and wild animals, where they feed on blood. The major genera *Ancylostoma*, *Uncinaria*, and *Necator* are of significant medical and veterinary importance due to their high prevalence, widespread global distribution, and zoonotic potential. Within the genus *Ancylostoma*, 14 species have been identified, with *A. caninum* being the most widespread [1]. The species *A. caninum*, *A. duodenale*, *A. braziliense*, and *A. ceylanicum* within the genus *Ancylostoma* are of particular public health concern [2, 3]. These hookworms can cause a range of pathologies in humans, including cutaneous larva migrans (creeping eruptions), eosinophilic enteritis, protein deficiency, and iron deficiency anemia [4]. Heavy infections can lead to intellectual disability in children [5] and increased neonatal mortality in companion animals [6]. The increasing overlap between human, domestic animal, and wildlife populations, exacerbated by factors such as climate change, urbanization, and conservation efforts, has resulted in increased pathogen transmission and disruptions to host dynamics [7, 8]. Consequently, various *Ancylostoma* species have successfully established themselves in diverse wild animal populations worldwide, facilitating the emergence of new host-parasite relationships [9, 10].

The pathologic effects of *Ancylostoma* spp. infection in wild animals include anemia [11], retarded growth [12], tissue damage, inflammation [13, 14], and mortality, particularly in young animals [14, 15]. Hookworms of the genera *Ancylostoma*, *Arthrostoma*, *Galoncus*, and *Uncinaria* have been documented in wild felids worldwide [16]. Due to the conservation status of wild felids, limited studies have been conducted on hookworm infections in these animals. In India, various studies have reported a prevalence of *Ancylostoma* spp. ranging from 3.57 to 100% in wild felids, including Bengal tigers, captive lions, captive leopards, and jungle cats, in different zoos, such as Rajkot Zoo, Gujarat [17], Maharaj Bagh Zoo, Nagpur, Maharashtra [18], Mahendra Chaudhary Zoological Park/Chhatbir Zoo, Zirakpur, Mohali, Punjab [19], Nandankanan Zoological Park, Bhubaneswar, Odisha [20], and V.O.C Park and Mini Zoo, Coimbatore, Kerala [21]. Factors influencing hookworm infections include regional temperature, soil humidity, parasite resistance to environmental conditions, host density, immune status, mode of transmission, and seasonal prevalence [16]. Within the genus *Ancylostoma*, *A. ceylanicum* is capable of inducing patent infections in humans and is highly prevalent in Asian countries [22]. Recently, *A. ceylanicum* has been identified as a novel agent responsible for diarrhea in travelers returning from countries such as Malaysia, Papua New Guinea, Lao People's Democratic Republic, and India [23]. Wild felids, including civet cats, leopards, and Asian golden cats, have been documented to harbor *A. ceylanicum* [24, 25]. However, previous identifications of *A. ceylanicum* relied solely on morphological characteristics of adults and larvae, without the use of molecular diagnostic tools.

Numerous species of hookworms infect wild animals, with *Ancylostoma* spp. being particularly prevalent among them. However, species identification of different *Ancylostoma* spp. cannot be achieved solely on the basis of egg morphology. Therefore, an effort was made to investigate the prevalence of hookworm infections in wild felids housed in biological parks in the northern Indian region. Furthermore, species-level identification was conducted using PCR-sequencing of the internal transcribed spacer regions (ITS1-5.8S-ITS2). Additionally, the phylogenetic position, haplotype distribution, and genetic diversity of the *Ancylostoma* species were determined and compared with sequences available in the GenBank database.

Materials and methods

Sample collection, examination and isolation of genomic deoxyribonucleic acid (DNA)

A total of 96 faecal samples from free-ranging wild felids and feliforms (Table 1) were collected non-invasively from various biological parks in Uttar Pradesh, India. These samples were received at the Division of Parasitology, Indian Veterinary Research Institute, Izatnagar, for

Table 1 Parasitic ova detected in faecal samples of captive wild felids

Host species	Samples screened	Positive samples (%)	Parasitic ova detected
Leopard	19	2 (10.53%)	*Toxocara cati* (01), *T. cati* + *Ancylostoma* spp. (01)
Tiger	28	8 (28.57%)	*T. cati* (06), *T. cati* + *Ancylostoma* spp. (01), *Diphyllobothrium latum* (01)
Lion	32	10 (31.25%)	*T. cati* (05), *Ancylostoma* spp. (05)
Panther	10	2 (20.00%)	*T. cati* (01), *T. cati* + *D. latum* (01)
Jungle cat	4	1 (25.00%)	*Ancylostoma* spp. (01)
Civet cat	3	1 (33.33%)	*Ancylostoma* spp. (01)
Total	**96**	**24 (25.00%)**	***Toxocara cati*(13.5%), *Ancylostoma*spp. (7.3%), *D. latum*(1.04%), *T. cati* + *Ancylostoma*spp. (2.08%), *T. cati* + *D. latum*(1.04%)**

routine parasitological examination between April 2020 and March 2021. Faecal samples found positive for hookworm ova through direct smear examination [26] were stored at 4 °C and subsequently subjected to a flotation technique using saturated sodium chloride solution for hookworm egg isolation, as previously described [26, 27]. The isolated eggs from positive samples, including those from a tiger (Lucknow), a leopard (Kanpur), a jungle cat (Gorakhpur), and a civet cat (Bareilly; Fig. 1), were then subjected to genomic DNA isolation using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany), following the manufacturer's protocol.

Polymerase chain reaction (PCR) assay for the amplification of ITS1-5.8S-ITS2 region of hookworms

A set of three published primers (one common forward primer: RTGHF1 and two sets of reverse primers: RTABCR1 and RTAYR1) that specifically amplify *A. caninum*, *A. ceylanicum*, *A. braziliense*, and *U. stenocephala* were used [28, 29]. The details of the primers, PCR reaction mixture, conditions, and amplicon size are listed in Table 2. In the current study, two PCR reactions were performed for each positive specimen: one using the RTGHF1-RTABCR1 primer pair and another using the RTGHF1-RTAYR1 primer pair. The amplified PCR products were checked using 1.5% agarose gel electrophoresis [30, 31] and documented using a gel documentation system (Syngene, Japan).

Sequencing and phylogeny

Genomic DNA isolated from the hookworm eggs of four different hosts was amplified (545 bp) in bulk, column-purified using a PCR purification kit (Qiagen, Germany), and submitted to Eurofins Genomics India Pvt Ltd, Bangalore, for custom bidirectional DNA sequencing. To ensure accuracy, each isolate was sequenced in triplicate to eliminate any sequencing errors. The obtained sequences were viewed and edited using SnapGene Viewer and then identified based on sequence similarity to known published sequences in the database using tools like BLAST (Basic Local Alignment Search Tool). Subsequently, the sequences were submitted to the GenBank.

For phylogenetic analysis, closely related sequences with 97–100% query coverage (with newly generated sequences) were downloaded from the GenBank. A dataset of 63 sequences, comprising *A. caninum* ($n = 44$), *A.*

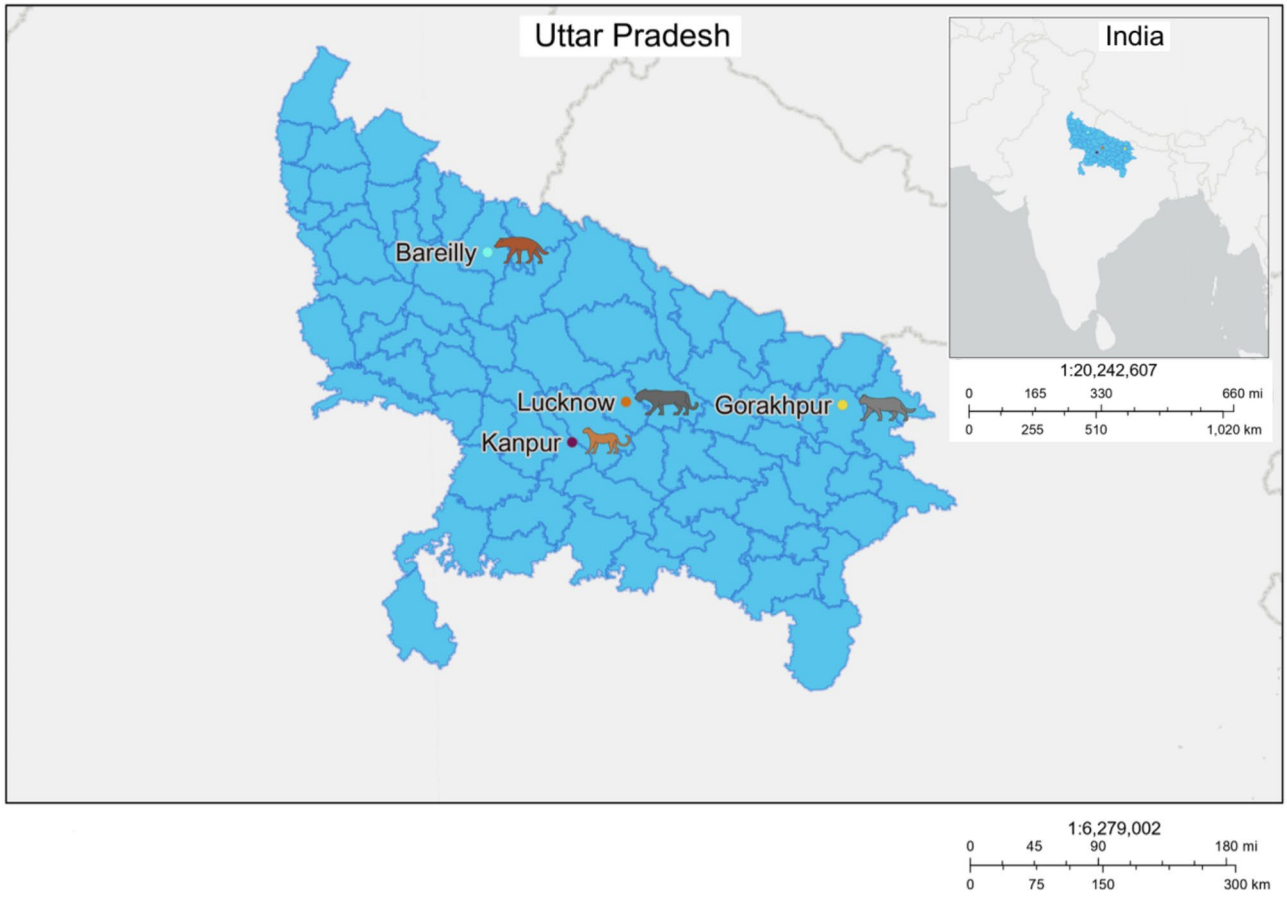

Fig. 1 Map illustrating the sampling sites and hosts for hookworms in Northern India

Table 2 Primers used for amplification of DNA isolated from *Ancylostoma* spp. eggs

Targeted nuclear marker	Primers	Sequence (5' to 3')	PCR reaction mix	PCR reaction conditions	Amplicon size	Parasite amplified	Reference
ITS1-5.8S-ITS2 region	RTGHF1 RTABCR1	CGTGCTAGTCTTCAGGACTTTG CGGGAATTGCTATAAGCAAGTGC	2X Phusion High-Fidelity PCR Master Mix – 12.5 µL Forward primer (10 pmol) – 0.5 µL	98 °C for 1 min, (98 °C for 10 s, 68 °C for 30 s, and 72 °C for 30 s) x 40 cycles, and 72 °C for 10 min	545 bp	*A. caninum, A. ceylanicum, and Uncinaria stenocephala*	28, 29
	RTGHF1 RTAYR1	CGTGCTAGTCTTCAGGACTTTG CTGCTGAAAAGTCCTCAAGTCC	Reverse primer (10 pmol) – 0.5 µL DNA template – 3.0 µL Nuclease free water– up to 25 µL		673 bp	*A. braziliense*	

ceylanicum ($n = 13$), *A. braziliense* ($n = 2$), *A. duodenale* ($n = 2$), and *U. stenocephala* ($n = 2$), was created, with details listed in Table 3. Multiple sequence alignment was performed using ClustalW, and the sequences were trimmed from both ends to ensure uniform start and end positions, resulting in a final length of 542 bp, including gaps [32]. The evolutionary history was inferred using MEGA-X version 10.1.7, employing the Maximum Likelihood tree-building method [33] and the Kimura 2-parameter substitution model [34] with 1000 bootstrap replications. A discrete Gamma distribution was used to model evolutionary rate differences among sites. *Uncinaria stenocephala* (MT345056 and HQ262055) was used as an outgroup (Fig. 2).

Haplotype networking and genetic diversity

Separate haplotype data files were generated from a dataset comprising 44 sequences of *A. caninum* and 13 sequences of *A. ceylanicum*, each spanning a length of 534 bp, using DnaSP 6.0 software [35]. Country- and continent-wise sequence sets were generated for both *A. caninum* and *A. ceylanicum* populations, which were used to generate median-joining haplotype networks (Fig. 3). For each defined population, genetic diversity parameters, including the number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π), and the number of polymorphic or segregating sites (S), were estimated. Additionally, genetic differentiation indices, such as the average number of nucleotide differences in pairs (Kxy), statistics based on haplotypes (Hs), nucleotide sequences (Ks) [36], genetic differentiation index based on the frequency of haplotypes (Gst), and nucleotide-based statistics, were calculated. Neutrality tests, including Fu's Fs, Fu and Li's D, Fu and Li's F, Tajima's D, Raggedness statistic (r), Mean Absolute Error (MAE), and Ramos-Onsins and Rozas' R2, were also performed. The haplotype alignment and trait files were then imported into PopART [37], and haplotype networks (Fig. 3) were constructed for *A. caninum* and *A. ceylanicum* populations using the median-joining network method [38, 39]. For continent-wise (Asia, Australia, and South America) *A. caninum* populations, analysis of molecular variance (AMOVA) and pairwise genetic differentiation values (Fst) were calculated using Arlequin version 3.5.2 to assess the degree of genetic variation among and within different populations [40]. A Mantel test was performed using R Studio 4.3.2 (http://www.rstudio.com) to detect any significant correlation between the genetic and geographic distance of the *A. caninum* isolates.

Results

Prevalence and molecular identification

Examination of faecal samples using different microscopic techniques revealed eggs of multiple parasites, including single and mixed infections. Out of 96 faecal samples examined by the direct smear method, 24 were found positive for various parasitic ova (Table 1). The highest prevalence was recorded for *Toxocara cati* (13.5%), followed by *Ancylostoma* spp. (7.3%) and *Diphyllobothrium latum* (1.04%). Additionally, mixed infections of *T. cati* plus *Ancylostoma* spp. (2.08%), and *T. cati* plus *D. latum* (1.04%) were also recorded in this study. The genomic DNA extracted from *hookworm* eggs of four different hosts produced a 545-bp amplification with the RTGHF1-RTABCR1 primer pair only. The hookworm eggs isolated from a civet cat were confirmed to be *A. ceylanicum* (Accession no. OP715867), whereas the hookworm eggs recovered from a tiger, leopard, and jungle cat were confirmed to be *A. caninum* (Accession no. OL314658, OL314659, and OP715868).

Phylogenetic analysis and sequence similarity based on the ITS region

The maximum likelihood tree (Fig. 2) provided a robust resolution of all the *Ancylostoma* spp., with each forming a distinct clade. The *A. caninum* sequences formed a large monophyletic clade, including the newly generated Indian sequences (OL314658, OL314659, and OP715868), which was further divided into two subclades. One subclade comprised sequences from Australia, North America (USA), and South America (Brazil), whereas the other subclade consisted of sequences from

Table 3 List of *Ancylostoma* spp. sequences of the ITS region used in the phylogenetic analysis and haplotype networking

S. No	Accession No	Host	Parasite	Place	Sequence length (bp)	Nucleotide Position	Reference
1	OL314659	Tiger	*Ancylostoma caninum*	Lucknow, India	544	12–544	Current study
2	OL314658	Leopard	*Ancylostoma caninum*	Kanpur, India	544	12–544	Current study
3	OP715868	Jungle cat	*Ancylostoma caninum*	Gorakhpur, India	544	12–544	Current study
4	LC054295	Dog	*Ancylostoma caninum*	Punjab, India	544	12–544	Unpublished
5	KP844730	Dog	*Ancylostoma caninum*	Australia	752	12–544	[41]
6	KP844731	Dog	*Ancylostoma caninum*	Australia	752	37–569	[41]
7	KP844732	Dog	*Ancylostoma caninum*	Australia	752	37–569	[41]
8	KP844733	Dog	*Ancylostoma caninum*	Australia	752	37–569	[41]
9	KP844734	Dog	*Ancylostoma caninum*	Australia	752	37–569	[41]
10	KP844735	Dog	*Ancylostoma caninum*	Australia	752	37–569	[41]
11	KP844736	Dog	*Ancylostoma caninum*	Australia	752	37–569	[41]
12	KC755026	Cat	*Ancylostoma caninum*	China	738	37–569	[42]
13	KC755029	Dog	*Ancylostoma caninum*	China	738	40–582	[42]
14	AM850105	Dog	*Ancylostoma caninum*	China	738	40–582	[42]
15	AM850106	Dog	*Ancylostoma caninum*	China	738	40–582	[42]
16	DQ438070	Dog	*Ancylostoma caninum*	Brazil	681	40–582	[3]
17	DQ438071	Dog	*Ancylostoma caninum*	Brazil	752	22–554	[3]
18	DQ438072	Dog	*Ancylostoma caninum*	Brazil	724	26–558	[3]
19	DQ438074	Dog	*Ancylostoma caninum*	Brazil	754	40–572	[3]
20	DQ438075	Dog	*Ancylostoma caninum*	Brazil	736	47–579	[3]
21	DQ438077	Dog	*Ancylostoma caninum*	Brazil	753	26–558	[3]
22	DQ438078	Dog	*Ancylostoma caninum*	Brazil	683	40–561	[3]
23	DQ438079	Dog	*Ancylostoma caninum*	Brazil	731	36–568	[3]
24	MT130904	Opossum	*Ancylostoma caninum*	Brazil	710	10–542	[43]
25	MT130905	Opossum	*Ancylostoma caninum*	Brazil	706	13–543	[43]
26	MT130906	Opossum	*Ancylostoma caninum*	Brazil	693	13–543	[43]
27	MT130907	Opossum	*Ancylostoma caninum*	Brazil	698	13–543	[43]
28	MT130908	Opossum	*Ancylostoma caninum*	Brazil	707	13–543	[43]
29	MT130909	Opossum	*Ancylostoma caninum*	Brazil	691	13–544	[43]
30	MT130911	Opossum	*Ancylostoma caninum*	Brazil	709	13–543	[43]
31	MT130912	Opossum	*Ancylostoma caninum*	Brazil	702	13–543	[43]
32	MT130913	Opossum	*Ancylostoma caninum*	Brazil	705	11–540	[43]
33	MT130914	Opossum	*Ancylostoma caninum*	Brazil	712	13–543	[43]
34	MT130916	Opossum	*Ancylostoma caninum*	Brazil	728	13–543	[43]
35	MT130917	Opossum	*Ancylostoma caninum*	Brazil	605	13–555	[43]
36	MT130921	Opossum	*Ancylostoma caninum*	Brazil	716	13–544	[43]
37	MT130922	Opossum	*Ancylostoma caninum*	Brazil	610	13–543	[43]
38	MT130924	Opossum	*Ancylostoma caninum*	Brazil	659	13–544	[43]
39	MT130926	Opossum	*Ancylostoma caninum*	Brazil	716	13–543	[43]
40	MT130930	Opossum	*Ancylostoma caninum*	Brazil	716	13–542	[43]
41	MT130933	Opossum	*Ancylostoma caninum*	Brazil	708	13–544	[43]
42	JQ812694	Dog	*Ancylostoma caninum*	USA	825	13–543	[44]
43	OR827008	Dog	*Ancylostoma caninum*	Indonesia	668	76–609	Unpublished
44	OR827009	Dog	*Ancylostoma caninum*	Indonesia	668	1–533	Unpublished
45	OP715867	Civet cat	*Ancylostoma ceylanicum*	Bareilly, India	544	1–533	Current study
46	LC036567	Human	*Ancylostoma ceylanicum*	Japan	1738	1–544	[23]
47	KF279132	Dog	*Ancylostoma ceylanicum*	China	738	902–1434	[45]
48	KF279133	Dog	*Ancylostoma ceylanicum*	China	738	50–582	[45]
49	KF279134	Dog	*Ancylostoma ceylanicum*	China	738	50–582	[45]
50	KF279135	Cat	*Ancylostoma ceylanicum*	China	738	50–582	[45]
51	KF279138	Cat	*Ancylostoma ceylanicum*	China	738	50–582	[45]
52	KC755027	Cat	*Ancylostoma ceylanicum*	China	738	50–582	[42]
53	DQ381541	Dog	*Ancylostoma ceylanicum*	Australia	681	50–582	[46]

Table 3 (continued)

S. No	Accession No	Host	Parasite	Place	Sequence length (bp)	Nucleotide Position	Reference
54	OR826944	Dog	*Ancylostoma ceylanicum*	Indonesia	669	12–544	Unpublished
55	OR826945	Dog	*Ancylostoma ceylanicum*	Indonesia	668	1–533	Unpublished
56	OR826949	Dog	*Ancylostoma ceylanicum*	Indonesia	670	1–533	Unpublished
57	OR826950	Dog	*Ancylostoma ceylanicum*	Indonesia	668	1–533	Unpublished
58	DQ438060	Dog	*Ancylostoma braziliense*	Brazil	698	1–533	[3]
59	DQ359149	Cat	*Ancylostoma braziliense*	Australia	693	13–534	[46]
60	EU344797	-	*Ancylostoma duodenale*	China	810	13–533	Unpublished
61	MK271367	Dog	*Ancylostoma duodenale*	Kenya	704	52–584	[47]
62	MT345056	Wild Boar	*Uncinaria stenocephala*	France	1325	1–529	Unpublished
63	HQ262055	Island Fox	*Uncinaria stenocephala*	USA	844	64–598	[48]

Asian countries, including China, India, and Indonesia. However, the presence of these two subclades was not supported by high bootstrap values. Furthermore, all *A. caninum* sequences exhibited 97.9–100% nucleotide identity with each other.

Similarly, all *A. ceylanicum* sequences formed a single monophyletic clade, including the newly generated Indian sequence (OP715867), and displayed 99.1–100% sequence identity with each other.

Haplotype networks

A total of eight and six haplotypes were identified from *A. caninum* (Table 4) and *A. ceylanicum* (Table 5) sequences, respectively. Among the *A. caninum* haplotypes, Hap_4 (h = 34) was the most common, followed by Hap_5 (h = 03) and Hap_6 (h = 02). Similarly, Hap_2 (h = 07) followed by Hap_1 (h = 02) were the most common haplotypes of *A. ceylanicum*. All remaining haplotypes of *A. caninum* and *A. ceylanicum* were singleton and unique to one country (Fig. 3). The results of location-wise haplotype network and phylogenetic tree were congruent with each other.

Population genetic analyses

Genetic diversity parameters revealed that both Asian *A. caninum* and *A. ceylanicum* populations exhibited high haplotype and nucleotide diversities. Among Asian countries, the Indian *A. caninum* population displayed the highest haplotype and nucleotide diversities, followed by the Chinese and Indonesian populations (Table 6). Similarly, the Chinese *A. ceylanicum* population exhibited high haplotype and nucleotide diversity (Table 6). Neutrality tests, including Fu's Fs, Fu and Li's D, Fu and Li's F, and Tajima's D, were performed on country- and continent-wise populations, as well as the overall dataset. The results produced non-significant positive and negative values, indicating a constant population size of *A. caninum*. Furthermore, no sequence variations were observed in the Australian and South American populations, precluding the estimation of pairwise differences, DNA polymorphism parameters, and neutrality indices. In contrast, neutrality tests yielded significant negative values for *A. ceylanicum* populations (Table 6), suggesting population expansion due to an excess of low-frequency polymorphisms.

The Asian, Australian, and South American *A. caninum* populations exhibited huge genetic differentiation from each other (Fig. 4), with a low level of gene flow. The Asian and South American populations (Fst = 0.52111) showed the highest genetic differentiation, while the Australian and South American populations (Fst = 0.30192) displayed the lowest genetic differentiation (Table 7). Furthermore, the Mantel test revealed a statistically significant correlation between the genetic distance of *A. caninum* isolates and their geographical location, indicating the presence of geographical structuring.

Mismatch analysis of the Asian and global *A. caninum* populations produced multimodal and bimodal distributions (Fig. 5), respectively, implying a constant population size of *A. caninum*, with each peak representing a cluster of individuals with similar genetic characteristics. In contrast, the Asian and global *A. ceylanicum* populations exhibited unimodal distributions (Fig. 6), suggesting a recent population expansion.

Continent-wise AMOVA revealed that 52.66% of the variation occurred within *A. caninum* populations, whereas 47.34% of the variation occurred between them (Table 8). The *P*-value of the fixation index was less than 0.05, indicating significant genetic differentiation among populations.

Discussion

Hookworms are the most important soil-transmitted nematodes, with *A. caninum*, *A. braziliense*, and *A. ceylanicum* being common species infecting dogs, cats, and other mammals in tropical countries, where environmental conditions favour hookworm survival [28]. In the Americas and Africa, *Necator americanus* and *A. duodenale* are human-infective hookworm species transmitted through anthroponosis [49], whereas in Southeast Asia and the Pacific, *A. ceylanicum* is the most predominant zoonotic hookworm [50]. Rapid urbanization and natural

Fig. 2 Phylogenetic tree based on the ITS region of the hookworms presenting distinct monophyletic clades indicative of each *Ancylostoma* spp. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. The taxon name of each sequence is shown by the accession number followed by the scientific name of the parasite, the host species, the sampling location, if any, and the country of origin. The clades representing *A. caninum* and *A. ceylanicum* are shaded with cyan blue and lavender blue color, respectively. The branch and taxon names representing the outgroup are colored dark blue. The bootstrap values for each node are mentioned in decimals

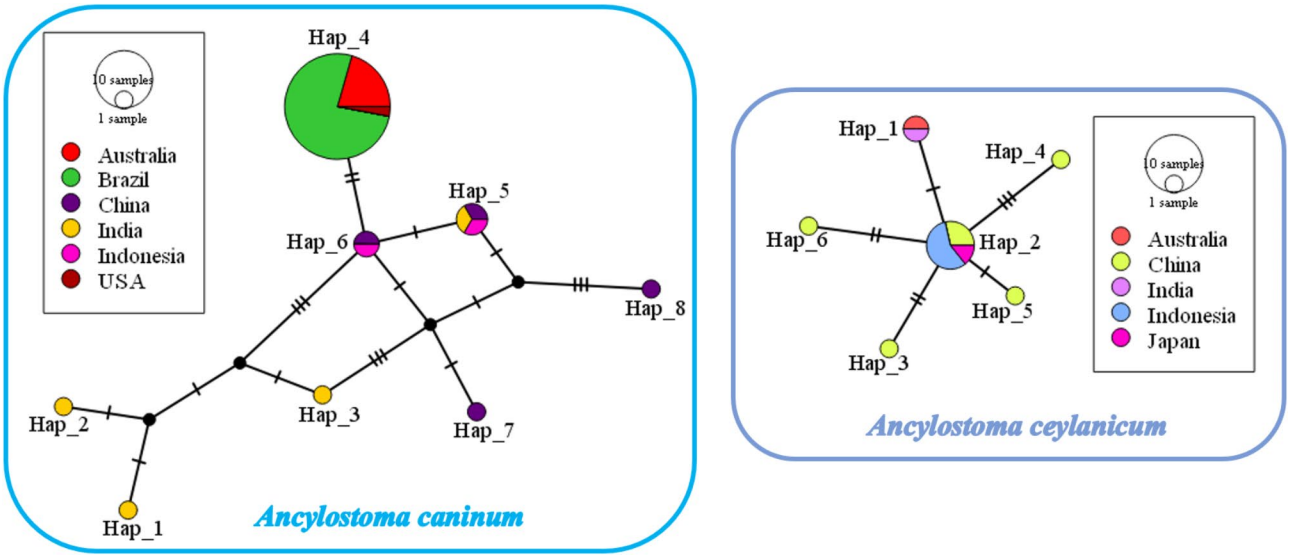

Fig. 3 Location-wise median-joining haplotype networks based on the ITS region of *A. caninum* (cyan blue box) and *A. ceylanicum* (lavender blue box). Each circle represents a unique haplotype, with its size proportional to the haplotype's frequency. Default black nodes serve as connecting nodes within the network. Nucleotide differences are indicated by hatch marks on the connecting lines, with each mark representing a single nucleotide difference

Table 4 List of haplotypes of *Ancylostoma caninum* identified in the current study

Haplotype	Frequency	Sequence accession number (Host and place of isolation)
Hap_1	1	OL314659 (Tiger; Lucknow, India)
Hap_2	1	OL314658 (Leopard; Kanpur, India)
Hap_3	1	OP715868 (Jungle cat; Gorakhpur, India)
Hap_4	34	KP844730, KP844731, KP844732, KP844733, KP844734, KP844735, KP844736 (Dog; Australia); DQ438070, DQ438071, DQ438072, DQ438074, DQ438075, DQ438077, DQ438078, DQ438079 (Dog; Brazil); JQ812694 (Dog; USA); MT130904, MT130905, MT130906, MT130907, MT130908, MT130909, MT130911, MT130912, MT130913, MT130914, MT130916, MT130917, MT130921, MT130922, MT130924, MT130926, MT130930, MT130933 (Opossum; Brazil)
Hap_5	3	LC054295 (Dog; Punjab, India); AM850106 (Dog; China); OR827008 (Dog; Indonesia)
Hap_6	2	KC755026 (Cat; China); OR827009 (Dog; Indonesia)
Hap_7		KC755029 (Dog; China)
Hap_8	1	AM850105 (Dog; China)

Table 5 List of haplotypes of *Ancylostoma ceylanicum* identified in the current study

Haplotype	Frequency	Sequence accession number, host and place of isolation
Hap_1	2	OP715867 (Civet cat; Bareilly, India); DQ381541 (Dog; Australia)
Hap_2	7	LC036567 (Dog; Japan); KF279132, KF279134 (Dog; China); OR826944, OR826945, OR826949, OR826950 (Dog; Indonesia)
Hap_3	1	KF279135 (Cat; China)
Hap_4	1	KF279133 (Dog; China)
Hap_5	1	KF279138 (Cat; China)
Hap_6	1	KC755027 (Cat; China)

habitat destruction have led to the swapping and spillover of parasites like *Ancylostoma* spp. between human, domesticated, and wild animal populations. Hookworms cause neonatal mortality in wild animals, negatively impacting the conservation status of vulnerable and endangered species. Additionally, contaminated soil with hookworm larvae can initiate clinical complications (cutaneous larva migrans) in humans. Given the zoonotic significance of hookworm infections and their potential threats to neonatal wild animals, especially those on the brink of extinction or categorized as vulnerable or endangered, it is imperative to diagnose infections at the species level. Different *Ancylostoma* spp. vary in their primary route of infection, pathogenicity, post-deworming colonization, and zoonotic ability, necessitating species-level identification in domesticated and wild populations to devise effective regional control strategies. Furthermore, understanding the genetic diversity and population

Table 6 Genetic diversity and neutrality indices of *A. caninum* and *A. ceylanicum* haplotypes within described populations

	Ancylostoma caninum								***Ancylostoma ceylanicum***						
Continent	**Australia**	**South America**	**Asia**				**North America**	**Global**	**Australia**	**Asia**					**Global**
Country/Region/State	**Australia**	**Brazil**	**China**	**India**	**Indonesia**	**Total**	**USA**		**Australia**	**China**	**India**	**Indonesia**	**Japan**	**Total**	
No. of sequences (*n*)	7	26	4	4	2	10	1	44	1	6	1	4	1	12	13
No. of mutations	0	0	9	11	3	14		13		8		0		9	9
No of segregating sites (S)	0	0	9	11	3	14		12		8		0		9	9
No. of haplotypes (h)	1	1	4	4	2	8		8		5		1		6	6
Haplotype diversity (Hd) ± SD	0	0	1 ± 0.177	1 ± 0.177	1 ± 0.5	0.956 ± 0.059		0.403 ± 0.093		0.933 ± 0.122		0		0.682 ± 0.148	0.718 ± 0.128
Average no. of nucleotide differences (K)	0	0	5.333	5.833	3	5.422		1.729		2.667		0		1.5	1.513
Nucleotide diversity (π) ± SD	0	0	0.01001 ± 0.00248	0.01094 ± 0.00306	0.00563 ± 0.00281	0.01017 ± 0.00144		0.00333 ± 0.00090		0.005 ± 0.00128		0		0.00281 ± 0.00093	0.00284 ± 0.00086
Fu's Fs	0	0	-0.439	-0.324		-1.77		-1.127		0.973				0.971	-1.898
Fu and Li's D test	0	0	0.86015	-0.27914		0.137		-0.95816		-1.44279		0		-2.49383**	-2.16871
Fu and Li's F test	0	0	0.85393	-0.2808		0.23838		-1.26011		-1.54414		0		-2.69158**	-2.38669
Tajima's D	0	0	0.86015	-0.27914		0.43814		-1.29184		-1.40833		0		-2.01608*	-1.88452*
Raggedness statistic (r)			0.5556	0.3333	2	0.081		0.3713		0.0578				0.0204	0.0204
Mean Absolute Error (MAE)			0.9989	1.2466	1.1563	0.8056		0.7306		0.6824				0.3407	0.3407
Ramos-Onsins and Rozas (R2)			0.2298	0.2059	0.5	0.165		0.0702		0.1382				0.1125	0.1125

*Statistical significance (P < 0.05); **statistical significance (P < 0.01)

Populations with at least four sequences were included for neutrality rests

Populations with at least two sequences were included for mismatch analysis

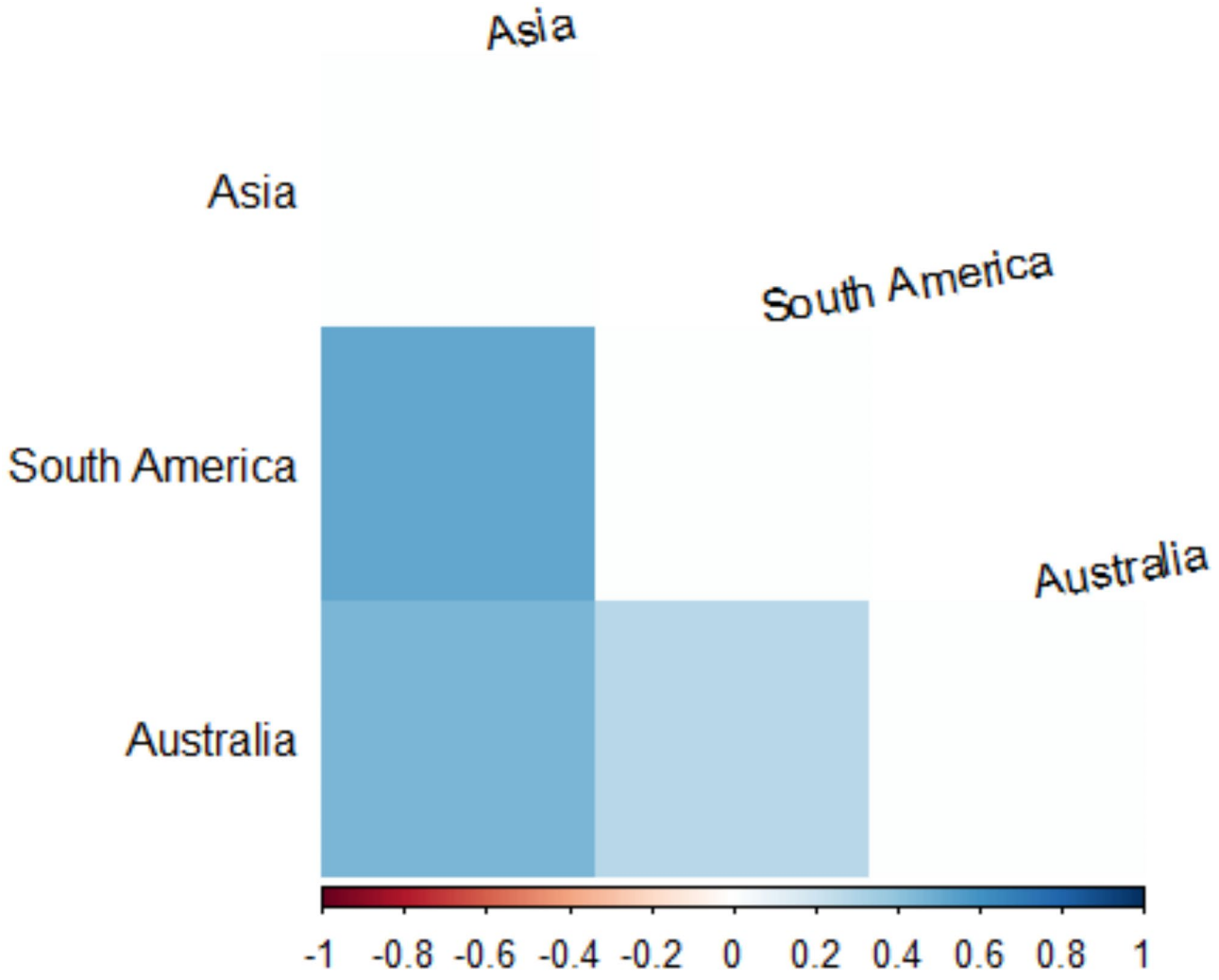

Fig. 4 Matrix showing the degree of genetic association between the different continent-wise *A. caninum* populations, where the intensity of color represented the Fst value. Darker the color, higher is the Fst value and stronger is the genetic differentiation between the populations

Table 7 Pairwise genetic differentiation of *A. caninum* haplotypes between different continents

Population 1	Population 2	Hs	Ks	Kxy	Gst	Fst	Nm
Asia	Australia	0.588	3.1895	4.5	0.3113	0.45029*	0.61039
Asia	South America	0.7904	2.9217	5.7115	0.0781	0.52111*	0.45949
Australia	South America	0.6086	1.5442	1.7692	0.2119	0.30192*	1.15608

Hs: Hudson's haplotype-based statistics; Ks: Hudson's nucleotide sequence-based statistics; Kxy: Average proportion of nucleotide differences between populations; Gst: Genetic differentiation index based on the frequency of haplotypes; Fst: Pairwise genetic distance between populations; Nm: gene flow between populations

*Statistical significance ($P < 0.05$)

structure of zoonotic hookworms is crucial from a One Health perspective.

In this study, the prevalence of *Ancylostoma* spp. infection in wild felids (lion, tiger, leopard, jungle cat, and civet cat) in various zoos of Uttar Pradesh was found to be 7.3%, which is consistent with earlier reports from different zoological parks in India [17–20]. A wide range of wild animals, including wild cat, leopard, Bengal tiger, lion, palm civet, striped hyena, Indian wild dog (dhole), jackal, captive fox, wolf, and black bear residing in various zoological parks across India, have been reported to harbor *Ancylostoma* infections [51].

Molecular identification of *Ancylostoma* spp. can be achieved through PCR amplification of nuclear (ITS region) and mitochondrial (cytochrome oxidase I, *COXI* gene) markers [28, 52–54], followed by sequencing and/or PCR-RFLP [28]. In this study, hookworm species identification was performed using PCR-sequencing of the ITS region. The results revealed that wild animals,

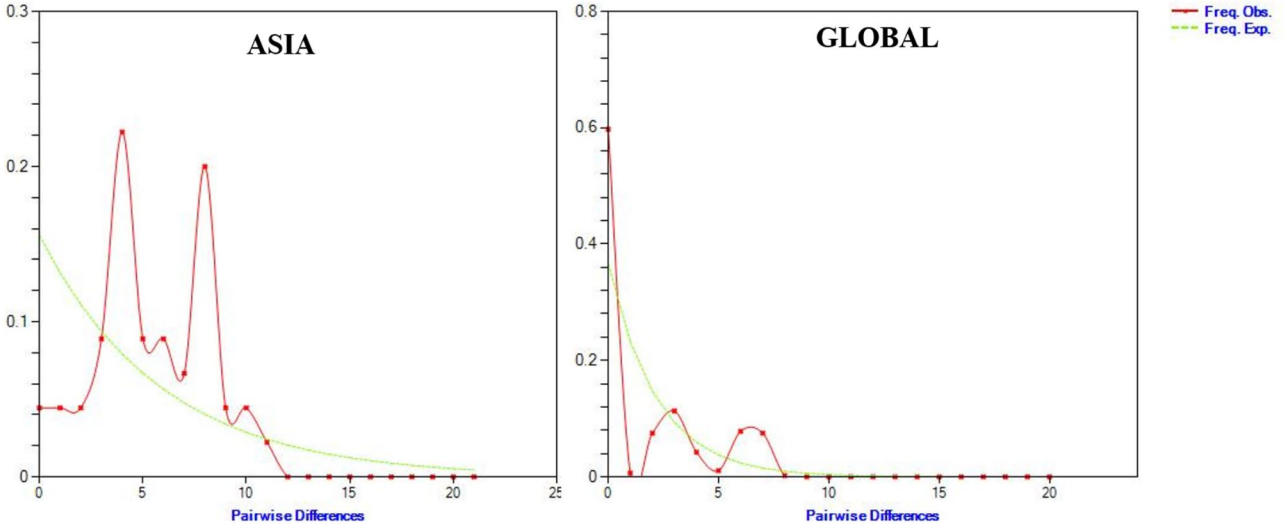

Fig. 5 Representation of the multimodal and bimodal distribution of the Asian and global *A. caninum* populations based on the mismatch analysis

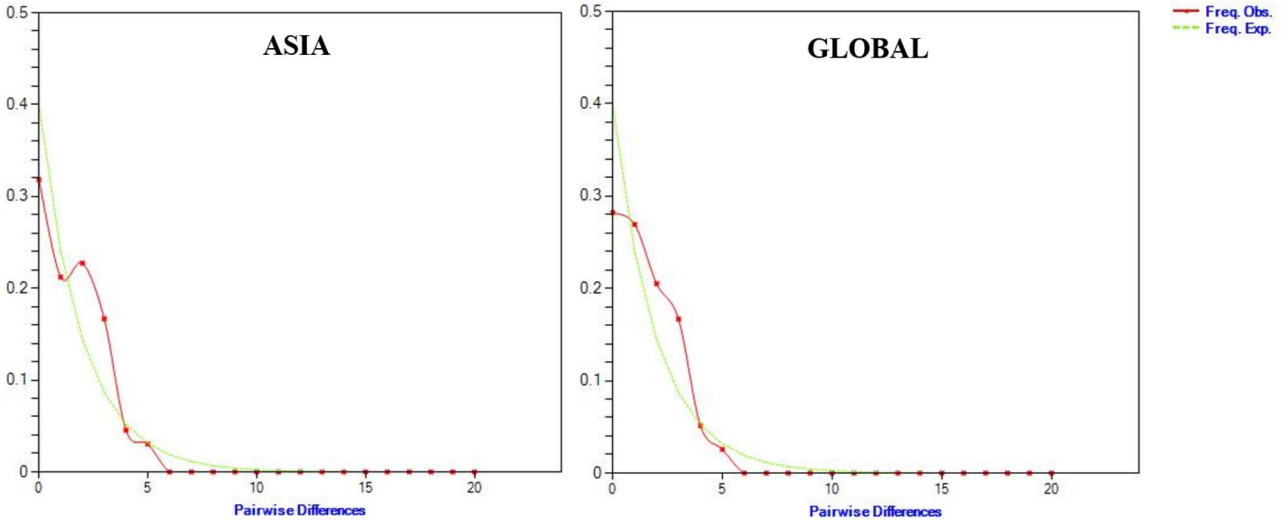

Fig. 6 Representation of the unimodal distribution of the Asian and global *A. ceylanicum* populations based on the mismatch analysis

Table 8 Analysis of molecular variance (AMOVA) of continent-wise *A. caninum* populations

Source of variation	Degree of freedom	Sum of squares	Variance components	Percentage variation	Fixation index (*P*-value)
Among populations	2	28.612	1.09879 Va	47.34	0.47335 ($P=0.00000$)
Within populations	40	48.9	1.2225 Vb	52.66	
Total	42	77.512	2.32129		

including tiger, leopard, and jungle cat, harbored *A. caninum*, whereas a civet cat was infected with *A. ceylanicum*.

Phylogenetic analysis of *Ancylostoma* spp. revealed that the newly generated *A. caninum* sequences from wild felids in India were more divergent from other sequences. The presence of two *A. caninum* subclades indicated considerable genetic variability, a separate evolutionary lineage from their common ancestor, and distinct biogeographic distribution. Moreover, Asian *A. caninum* isolates appeared to be cryptic in nature and displayed a distinct strain. Similar phylogenetic resolutions of *A. caninum* sequences have been previously reported [41, 42, 44]. However, further validation is necessary, which would involve generating more *A. caninum* sequences from domesticated and wild animals, an area currently lacking in research.

The median-joining haplotype network of *A. caninum* revealed continent-wise subgrouping, supporting the results of the phylogenetic analysis. The *A. caninum* sequences from Australia, Brazil, and the USA were

represented as a single haplotype (Hap_4). In contrast, sequences from India (OL314659, Hap_1; OL314658, Hap_2; OP715868, Hap_3) and China (KC755029, Hap_7; AM850105, Hap_8) appeared to be unique and novel, as they were positioned at the periphery of the network.

Similarly, the median-joining haplotype network of *A. ceylanicum* revealed Hap_2 as an ancestral haplogroup comprising haplotypes from China, Indonesia, and Japan. Over time, new and unique haplotypes, viz., Hap_3 (KF279135, cat, China), Hap_4 (KF279133, dog, China), Hap_5 (KF279138, cat, China), and Hap_6 (KC755027, cat, China), appear to have descended from the Chinese dog haplotype (Hap_2, KF279132 and KF279134). Notably, China has been reported as the origin of the first dog isolate of *A. ceylanicum* [41]. The *A. ceylanicum* sequence from India (civet cat) and Australia (dog) shared the same haplotype (Hap_1), suggesting a possible transmission of the parasite through companion animals. Previous studies on genetic diversity and haplotype networking of *Ancylostoma* spp. have focused on the *COXI* gene [44, 53]. This study is the first to elucidate genetic diversity and haplotype networking of *Ancylostoma* spp. based on the ITS region.

In the current study, a remarkably high level of haplotype diversity (Hd = 1.0) was observed among *A. caninum* populations in Asian nations, namely China, India, and Indonesia. In contrast, no appreciable DNA polymorphism based on the ITS region was detected in Australian and Brazilian populations, and hence all sequences belonged to a single haplotype (Hap_4). Conversely, high haplotype diversity was reported in Australian (0.80, $n = 38$), Brazilian (0.88, $n = 164$), and United States (0.904, $n = 60$) *A. caninum* populations based on partial *COXI* gene sequences in previous studies [44, 52, 54]. As reported in the present study, the global haplotype diversity was moderate (0.403) for *A. caninum* and high (0.718) for *A. ceylanicum*. Among *A. ceylanicum* populations, the Chinese population exhibited high haplotype diversity (0.933 ± 0.122), consistent with previous findings of similar haplotype diversity levels (0.9394 ± 0.0577) based on the *COXI* gene [22].

A continent-wise (Asia, Australia, and South America) AMOVA of *A. caninum* populations, along with the Fst value, revealed varying degrees of genetic differentiation: high between Asia and South America, moderate between Asia and Australia, and low between Australia and South America. Similarly, a previous study reported a huge genetic differentiation between North American and Australian *A. caninum* populations, with a consistently low level of gene flow between them, based on the *COXI* gene [53]. Another study found moderate genetic differentiation among Brazilian hookworm populations from five different localities [54]. In the present study, the Mantel test revealed a significant correlation between genetic distance and geographical location of the *A. caninum* isolates. A similar study based on the *COXI* gene sequences of *A. caninum* from the USA, China, Japan, and Australia reported moderate population structuring [44]. This correlation indicates that geographical structure plays a role in shaping the observed patterns of genetic variation in *A. caninum* populations. In our study, the analysis of neutrality indices of country-wise *A. caninum* populations suggested a constant population size with limited gene flow. In contrast, significant values of neutrality tests indicated population expansion in *A. ceylanicum* populations. A previous study found widespread geographical distribution of *A. ceylanicum* haplotypes (based on the *COXI* gene) with gene flow between Asian countries (Thailand, Cambodia, Malaysia, and China) [22].

A notable limitation of this study is the small dataset, which may compromise the robustness and generalizability of the genetic analysis findings. The limited sample size may lead to biased estimates of genetic diversity and population structure, and may not capture the full range of genetic variation within the species. Future studies should prioritize expanding the dataset to include a larger, more geographically diverse collection of sequences.

Conclusion

Sequence data of *Ancylostoma* spp. from both domesticated and wild animals in India were notably lacking. The present study addressed this knowledge gap by incorporating the newly generated sequences of *A. caninum* ($n = 3$) and *A. ceylanicum* ($n = 1$) obtained from the Indian wild felids. Additionally, it provided a global perspective on the genetic structure of *A. caninum* and *A. ceylanicum*, facilitating an understanding of genetic diversity, gene flow patterns between populations, and the correlation between genetic diversity and geographic locations of *A. caninum* isolates. This information will aid in formulating precise intervention strategies crucial for controlling the spread of infection. Furthermore, Indian wild felids harboured the most divergent and unique haplotypes of *A. caninum*.

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Author contributions

R.P. and A.K.N.: Writing- Original draft preparation, Conceptualization, Investigation, Software, Formal analysis, and Methodology.H.R.: Conceptualization, Formal analysis, Writing - review and editing, and Supervision.T.V., M.K. and D.P.P.: Investigation and Methodology.R.G. and A.M.P.: Writing - review and editing.

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Availability of data and materials. The datasets generated and/or analyzed during the current study are available in the GenBank repository (https://www.ncbi.nlm.nih.gov/) and the accession numbers are listed in Table 3.

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Declarations

Ethics approval and consent to participate

No permissions were required for conducting this study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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