FIRST ISOLATION OF TRICHURIS ARVICOLAE FROM MERIONES MERIDIANUS AND SPERMOPHILUS ALASCHANICUS IN THE HELAN MOUNTAINS, CHINA

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Abstract. Whipworms, parasitic worms found in soil, can infect multiple host species. Our research involved collecting five adult female *Trichuris* from a gerbil (*Meriones meridianus*) and eight from a ground squirrel (*Spermophilus alaschanicus*), captured in September 2022 on the eastern slopes of the Helan Mountains in Ningxia Province. We identified 13 whipworms from the two rodents using morphology and molecular markers. No significant egg size variation was found ($P>0.05$). Phylogenetic analyses, utilizing Cytochrome c Oxidase subunit I (COI) and Internal Transcribed Spacer (ITS) gene sequences, showed both *Trichuris* populations belonged to *Trichuris arvicolae*. Further analysis indicated minimal genetic divergence (Fst=0.23 for COI, 0.00 for ITS) and reproductive isolation (Nm=0.83 for COI, infinite for ITS). Despite gerbils being nocturnal and ground squirrels being diurnal, leading to non-overlapping activity times, both rodents share overlapping habitats. Consequently, the coexistence of these two rodents infected with the same species of *Trichuris arvicolae* is not unexpected.
Keywords: *Trichuris, gerbils, ground squirrels, COI, ITS*

Introduction

Whipworms, belonging to the order Trichinellida, family Trichuridae, genus *Trichuris*, are ubiquitous endoparasites found worldwide. These parasites have a broad host range, infecting various mammalian species including rodents, canines, felines, bovines, humans, nonhuman primates, and other vertebrates (Stephenson et al., 2000; Callejón et al., 2010; Elsemore et al., 2014; Ketzis et al., 2015; Rivero et al., 2020; Zhou et al., 2021). Currently, approximately 80 species of whipworms capable of infecting mammals have been identified (Callejón et al., 2016). Infection with *Trichuris* occurs through direct transmission, typically by ingesting whipworm eggs present in contaminated food, water, or soil. Infected hosts often exhibit common clinical symptoms such as diarrhea, lethargy, dehydration, and in severe cases, death, leading to significant socioeconomic consequences (Pla et al., 2004; Roepstorf et al., 2011).

Trichuris arvicolae can infect Muridae, Arvicolidae, Circetidae rodents, such as *Psammomys obesus*, *Microtus arvalis*, *Arvicola terrestris sherman*, *Cricetulus barabensis*, et al (Feliu et al., 2000; Deter et al., 2007a,b; Callejón et al., 2012; Alshammari et al., 2023). In China, *Trichuris arvicolae* was found in a gerbil (*Meriones meridianus*) and a ground squirrel (*Spermophilus alaschanicus*) belonging to the family of Sciuridae for the first time in current study.

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In contemporary research, the primary methods utilized for identifying whipworm species include morphological and molecular approaches. However, due to their shared status as endoparasites occupying similar ecological niches, different species of whipworms have undergone convergent evolution, resulting in morphological similarities that can be challenging to discern. Many morphological characteristics used for whipworm species identification exhibit overlap (Špakulová, 1994; Robles, 2011). Consequently, alternative methods such as molecular markers have become imperative for accurate *Trichuris* species identification (Liu et al., 2012).

The combination of multiple markers derived from both nuclear and mitochondrial genes has proven to be effective and reliable for *Trichuris* species identification. In our study, we selected both the Cytochrome c Oxidase subunit I (COI) and Internal Transcribed Spacer (ITS) sequences for phylogenetic analysis to identify *Trichuris* species isolated from a gerbil and a ground squirrel. Notably, gerbils, being nocturnal animals, and ground squirrels, being diurnal, both serve as potential vectors for parasites and bacteria in wildlife and humans (Wang et al., 2020; Zhou et al., 2023).

This study aimed to investigate the occurrence and genetic characterization of *Trichuris arvicolae* in two ecologically distinct rodent species (*M. meridianus* and *S. alaschanicus*) within the Helan Mountains, China - a critical ecotone between steppe and desert biomes. Specifically, we sought to: (1) determine whether whipworms from these sympatric but temporally segregated hosts (nocturnal vs. diurnal) represent the same *T. arvicolae* lineage using integrated morphological and molecular approaches (COI/ITS markers); (2) assess potential cross-species transmission mechanisms given their overlapping habitats. As the first report of *T. arvicolae* in these rodent families (Muridae and Sciuridae) from the Helan Mountains, this work expands knowledge of host-parasite associations in arid-land ecosystems and provides baseline data for monitoring wildlife-borne parasitoses.

Materials and methods

A total of eight adult female worms of *Trichuris* were collected from one gerbil, while five were obtained from one ground squirrel (designated as follows: gerbil worms - S1A, S1B, S1C, S1D, S1E, S1F, S1G, S1H; ground squirrel worms - N5A, N5B, N5CT, N5D, N5EH). The sympatric two rodents were captured in September 2022 on the eastern slopes of the Helan Mountains in Ningxia Province. This study's interpretation is constrained by sampling only one individual per host species. Future multi-host, multi-season sampling is needed to validate these preliminary findings.

These worms and their eggs were measured and microphotographed with a differential interference contrast (DIC)-equipped Motic BA410 microscope using ISCpture software (Shanghai Deep Pupil Photoelectric Technology Co., Ltd., Shanghai, China). EXCEL 2016 was used to complete the statistical analysis of worm egg sizes. Then, worms were extensively washed in 0.9% saline solution to remove host contamination and preserved in 70% alcohol at -20 ℃ until required for DNA extraction, PCR and sequencing.

The genomic DNA of thirteen worms was isolated utilizing the TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China), adhering strictly to the protocols provided by the manufacturer. Following the successful extraction, the study focused on the mitochondrial COI gene, a widely recognized molecular marker in phylogenetic studies. The partial sequence of the COI gene was amplified through Polymerase Chain Reaction (PCR), employing the primers HC02198F and CORA (Callejón et al., 2013). The PCR

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amplifications were conducted in a total volume of 25 μl, comprising various components: 8.5 μl ddH_2O, 12.5 μl Premix TaqTM (Ex TaqTM Version 2.0 plus dye, TaKaRa, Japan), 2.0 μl primer mix, 2.0 μl template DNA. The PCR cycling parameters were as follows: initial denaturation at 94 ℃ for 5 minutes, followed by 40 cycles consisting of denaturation at 94 ℃ for 1 minute, annealing at 48 ℃ for 1 minute, and extension at 72 ℃ for 40 seconds, and a final extension phase at 72 ℃ for 7 minutes. Specifically, the ribosomal ITS fragment was amplified following the protocol established by Nissen et al. (2012), utilizing primers and conditions tailored for *Trichuris*.

Each amplicon of the COI and ITS fragments was identified on 1.0% agarose gel to verify that it produced a single band. The PCR products were sent to GENESOUL Technology (Harbin, China) for sequencing. The ITS bands were eluted from the agarose using the TIANgel Purification Kit (Tiangen Biotech, Beijing, China). Then, the isolated DNA was cloned into *Escherichia coli* DH5α using the pMD18-T Easy Vector System (TaKaRa, Japan). Transformed cells were selected by overnight incubation at 37 ℃ on Amp plates. Three positive clones were selected by restriction enzyme digestion analysis and sent to GENESOUL Technology (Harbin, China) for sequencing.

The COI and ITS sequences of thirteen *Trichuris* samples were compared with 12 other *Trichuris* COI and 14 other *Trichuris* ITS sequences (*Table 1*) available in GenBank to conduct phylogenetic analysis.

Table 1. *Information on the Trichuris spp. sequences used to construct the phylogenetic trees*

Species	GenBank accession number	Marker	Host
Trichuris arvicolae	FR851281	COI	*Arvicola terrestris sherman*
Trichuris arvicolae	FR851287		*Arvicola terrestris sherman*
Trichuris discolor	HE653139		*Bos taurus*
Trichuris discolor	HE653141		*Bos taurus*
Trichuris skrjabini	HQ183744		*Capra hircus*
Trichuris skrjabini	AM932679		*Capra hircus*
Trichuris muris	HE653130		*Mus musculus*
Trichuris muris	HE653133		*Mus musculus*
Trichuris ovis	KY656517		*Capra hircus*
Trichuris ovis	MG837081		*Capra hircus*
Trichuris trichiura	OP108822		*Capreolus capreolus*
Trichuris trichiura	OP108824		*Capreolus capreolus*
Trichuris arvicolae	FR849685	ITS	*Microtus arvalis*
Trichuris arvicolae	FR849687		*Microtus arvalis*
Trichuris discolor	HE608854		*Bos taurus*
Trichuris discolor	HE608855		*Bos taurus*
Trichuris skrjabini	AJ489248		*Capra hircus*
Trichuris skrjabini	KT630825		*Ovis orientalis aries*
Trichuris muris	FN543140		*Apodemus flavicollis*
Trichuris muris	FN543194		*Apodemus flavicollis*
Trichuris ovis	JX218218		*Capreolus capreolus*
Trichuris ovis	KT630824		*Capreolus capreolus*
Trichuris trichiura	AM992997		*Homo sapiens*
Trichuris trichiura	AM992998		*Homo sapiens*
Trichuris mastomysi	JX683517		*Mastomys natalensis*
Trichuris mastomysi	JX683523		*Mastomys natalensis*

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A sequence of *Priapulus caudatus* (DQ087502.1) was chosen as the outgroup for the COI gene tree (Callejón et al., 2013), and a sequence of *Ascaris suum* (AB571302.1) was selected as the outgroup for the ITS tree according to Yi (2017). All of the sequences were aligned with ClustalW and phylogenetic trees were created in MEGA v7.0.26 (Kumar et al., 2016). The best fit substitution models of the COI and ITS sequences were T92+I and T92+G+I based on the Bayesian information criterion, respectively. Consensus trees were generated using 1000 bootstrapping replicates based on these models using the maximum likelihood (ML) method.

In addition, 14 COI sequences and 14 ITS sequences, including the *Trichuris* sequences from the present study were selected to analyse the genetic distance of *Trichuris* using MEGA (*Table 1*). Consensus trees were generated using 1000 bootstrapping replicates based on these models using the maximum likelihood (ML) method. Heatmaps of genetic distances were generated using TBtools v2.067 (Zhou et al., 2022). We used DnaSP 5.10 to cauculate average number of nucleotide differences and haplotype diversity. Population genetic and nucleotide diversity was also analysed with the pairwise Fst and Nm values calculated with DnaSP 5.10 (Librado and Rozas, 2009). All final sequences were submitted to GenBank uhder the accession numbers PP493886-PP493898 (COI) and PP495534-PP495546 (ITS).

Results

Out of the thirteen worms examined, eight exhibited complete morphology. Among them, five were obtained from a gerbil, while the remaining three were retrieved from a ground squirrel. Upon morphological assessment, all eight worms were identified as belonging to the genus *Trichuris*. To further characterize these specimens, two whipworms were randomly selected from each rodent. Subsequently, thirty eggs were measured for size from each whipworm, resulting in a total of 120 eggs analyzed.

The eggs from *T. arvicolae* specimens isolated from the single gerbil measured 50.28-59.92×24.46-28.32 μm (55.43×26.39 μm, n=60) (*Figure 1a and 1b*), while those from the single ground squirrel measured 46.87-56.92×23.46-27.21 μm 53.17×25.36 μm, n=60) (*Figure 1c and 1d*). The observed size ranges overlapped substantially between the two host-derived samples. Accurate distinctions cannot be made with the naked eye alone. In addition, it is difficult to identify specific species by morphological methods due to the small differences in interspecies characteristics within the *Trichuris* spp. Therefore, the authors thought that molecular methods were necessary for further species identification.

The COI and ITS sequences of thirteen *Trichuris* samples were successfully amplified and sequenced. Specifically, COI sequences were designated as CS1A, CS1B, CS1C, CS1D, CS1E, CS1F, CS1G, CS1H, CN5A, CN5B, CN5CT, CN5D, and CN5EH, while ITS sequences were labeled as ZS1A, ZS1B, ZS1C, ZS1D, ZS1E, ZS1F, ZS1G, ZS1H, ZN5A, ZN5B, ZN5CT, ZN5D, and ZN5EH. Following sequence editing and alignment, the COI sequences ranged from 387 to 388 base pairs (bp) in length, with a G+C content varying between 38.80% and 39.70%. The average G+C content across all COI sequences was calculated to be 39.18%. Conversely, the ITS sequences exhibited lengths ranging from 1282 to 1287 bp, with a G+C content spanning from 59.80% to 60.00%. On average, the G+C content of the ITS sequences was determined to be 59.92%.

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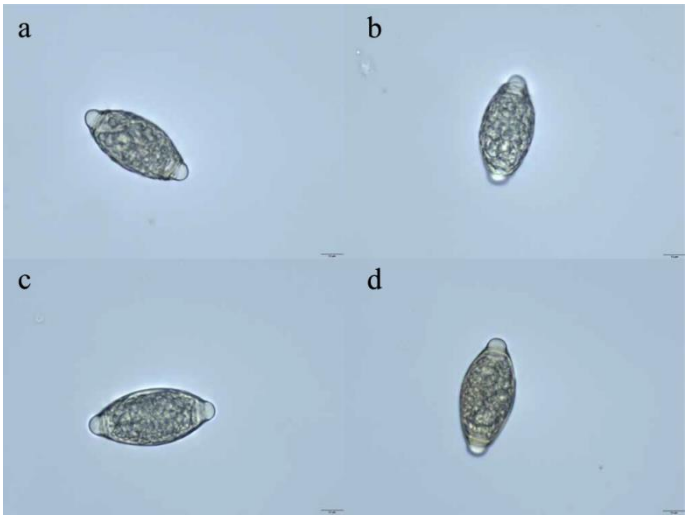

Figure 1. *Photographs of the eggs. a and b whipworm eggs from the gerbil; c and d whipworm eggs from the ground squirrel. Scale bar: 10 μm*

The COI sequences from all thirteen whipworms (8 from the single gerbil and 5 from the single ground squirrel) fell within the *T. arvicolae* clade in phylogenetic analysis (*Figure 2a*). If all the samples are considered to be *T. arvicolae*, then the intraspecifc genetic distances of *T. arvicolae* were 0.000-0.193, and the interspecifc genetic distances of *Trichuris* spp. were 0.225-0.325 (*Figure 3a*). In the ITS sequence tree (*Figure 2b*), the samples were also clustered with known *T. arvicolae*, then the intraspecifc genetic distances of *T. arvicolae* were 0.000-0.371 and the interspecifc genetic distances of *Trichuris* spp. were 0.336-0.772 based on ITS sequences (*Figure 3b*). Therefore, the samples of *Trichuris* from the two rodents were identifed as *T. arvicolae* by combining the analysis of the COI and ITS sequences.

Moreover, the Fst (0.23 for COI; 0.00 for ITS) and Nm (0.83 for COI; infinite for ITS) values between the whipworms from the single gerbil and ground squirrel individuals suggest limited genetic differentiation in these specific specimens. However, the single-host sampling precludes conclusions about population-level gene flow or reproductive isolation in wild *T. arvicolae*. The limited genetic differentiation between worms from these two host individuals (Fst=0.23 for COI) invites further study with adequate sampling to test whether this reflects contemporary cross-host transmission.

The analysis of the thirteen COI sequences revealed three distinct haplotypes. A variable nucleotide position (position 349) were found in three COI sequences (CS1B, CS1D, CS1E; length: 388 bp). The substitution represented a transition (A <-> G). In addition, two variable nucleotide positions (positions 349 snd 388) were found in a COI sequence (CS1C; length: 388 bp). The substitution represented two transitions (A <-> G, T<->C). The nucleotide diversities and haplotype diversities of the COI sequences from the single gerbil and the single ground squirrel were 0.002 and 0.002, and 0.769 and 0.600, respectively.

The ITS sequence analysis revealed considerable genetic variation among the 13 examined *T. arvicolae* (8 from the single gerbil and 5 from the single ground squirrel), with all specimens exhibiting unique haplotypes (25 segregating sites total). The nucleotide variation ranged from 0.003 to 0.005 across these specimens, with maximal haplotype diversity (1.000) observed in both host-derived sample groups. While these

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findings could potentially indicate: (1) authentic population-level diversity in wild hosts, or (2) multiple recent infection events in the sampled individuals, the limited sampling from single host individuals prevents definitive discrimination between these possibilities. The complete haplotype differentiation among specimens suggests either rapid molecular evolution or diverse infection sources, highlighting the need for expanded sampling across multiple host individuals and seasons to properly characterize *T. arvicola*e genetic diversity and transmission patterns in this ecosystem.

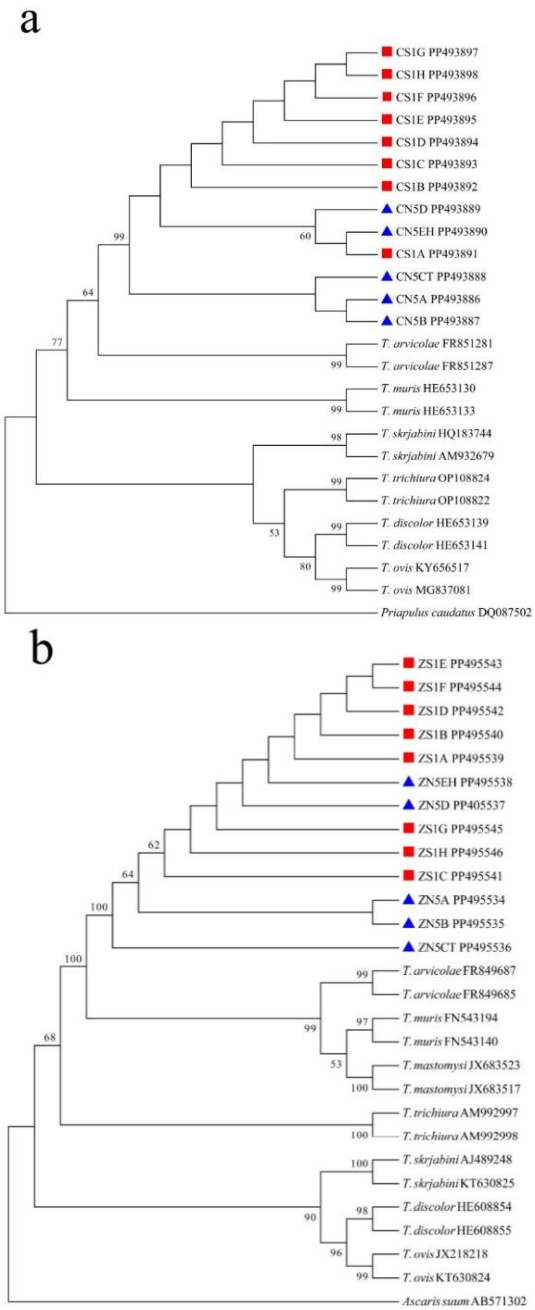

Figure 2. *Phylogenetic tree constructed based on COI sequences (a) and ITS sequences (b) of the thirteen Trichuris specimens (8 from the single gerbil and 5 from the single ground squirrel). Bootstrap support values >50% (shown at nodes) indicate branch reliability. Scale bar represents substitutions per site (■ : Samples from the gerbil; ▲ : Samples from the ground squirrel)*

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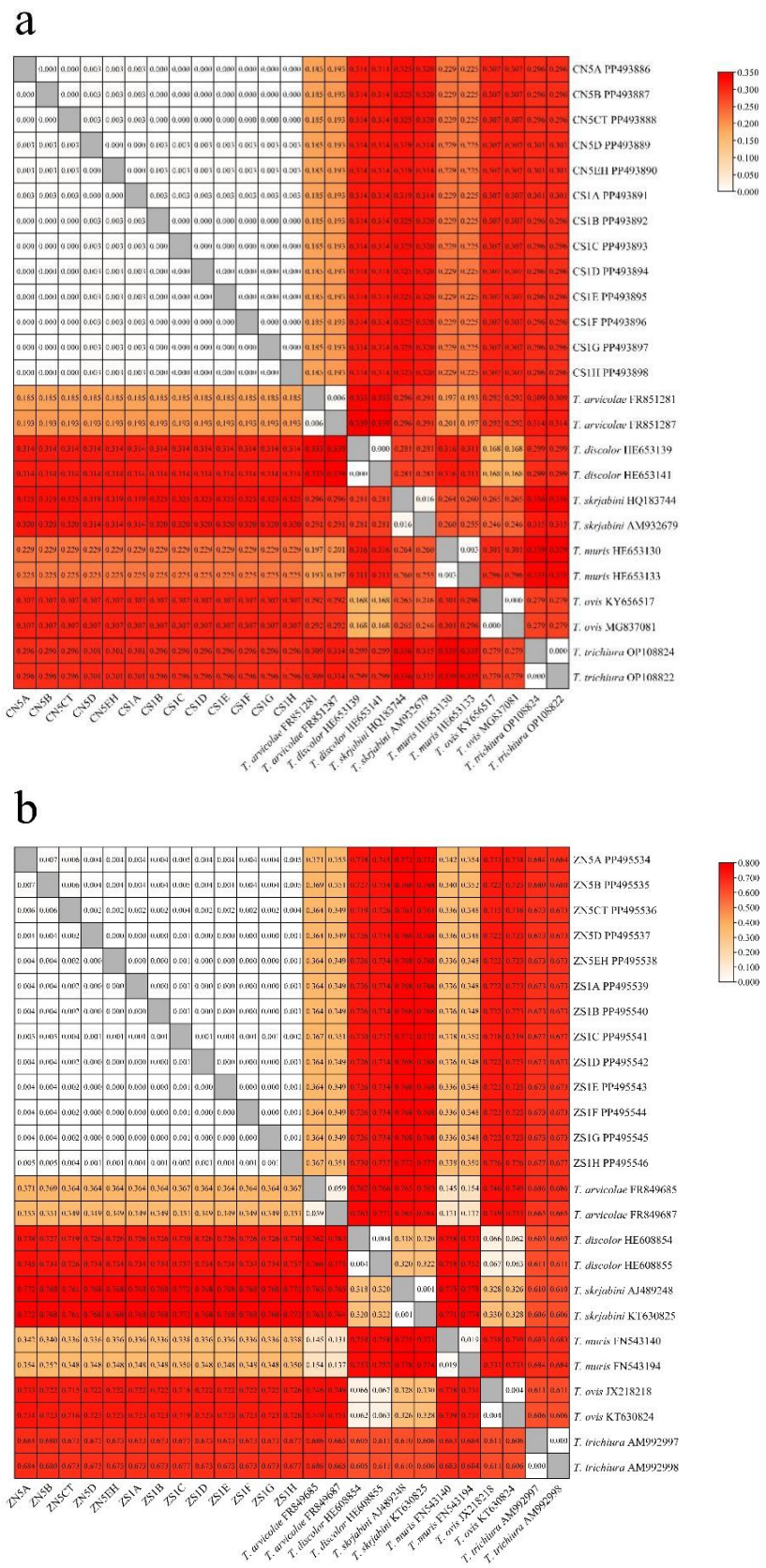

Figure 3. *Heatmap of genetic distances based on the COI sequences (a) and ITS sequences (b) of some trichurids (13 whipworms, 8 from the single gerbil and 5 from the single ground squirrel)*

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Discussion

As shown in *Figure 3*, we can clearly see that the discrepancy observed between the maximum intraspecific genetic distance based on ITS and the minimum interspecific genetic distance contrasts with the results obtained for COI. This inconsistency may be attributed to the differences in the genetic characteristics of COI and ITS. COI, being a gene located on the mitochondria, follows a maternal inheritance pattern and typically evolves at a faster rate compared to the ITS gene, which is located on the chromosome (Cao et al., 2024). As a consequence, the divergence observed among the samples and reference whipworms was more pronounced in the COI gene, while the divergence was less significant in the ITS gene. This disparity underscores the importance of considering the genomic location and inheritance pattern of genes when interpreting genetic distance data.

Combining the results of *Fig. 3a and 3b*, the whipworms identified as *T. arvicolae* in this study are also closely related to *T. muris*, with genetic distances of 0.225-0.229 and 0.336-0.354 for the COI and ITS gene sequences, respectively. This result may be due to the fact that the both *T. arvicolae* and *T. muris* have the ability to infect rodents and share the same ecological niche (Shears et al., 2022). Gerbils (noctural) and ground squirrels (diurnal) activity times do not overlap, the sympatric two rodents were overlap their habitats. Therefore, it is not unexpected that the two rodents were infected with the same species of *T. arvicolae*.

The genetic diversity patterns observed in this study—including COI haplotype variation (3 haplotypes among 8 worms from a single gerbil; 2 among 5 from a single ground squirrel) and complete ITS sequence differentiation (13 unique haplotypes with 25 segregating sites)—should be interpreted as individual-level phenomena rather than population-wide characteristics.

Additionally, it is noteworthy that only female worms (n = 13) were recovered from these two host individuals, with no males detected. This observation suggests an unequal sex ratio in the *T. arvicolae* parasitizing these two hosts, with a strong female bias. The authors hypothesize that this disparity may indicate parthenogenesis in female *T. arvicolae*, particularly in cases where males are scarce or absent. However, due to the small sample size, this finding cannot be generalized to the population level. Future studies with larger sample sizes should validate this possibility through controlled breeding experiments or genomic evidence. Parthenogenesis, a form of asexual reproduction, has been documented in various nematode species such as *Strongyloides* spp., *Diploscapter spp*., and *Panagrolaimus* spp. (Streit, 2008; Lewis et al., 2009; Hiraki et al., 2017), lending credence to the speculation proposed in this study. Investigating the sex-determination mechanisms in whipworms is essential for elucidating this phenomenon and understanding the reproductive strategies employed by these parasites.

While this study provides the first documented isolation of *T. arvicolae* from *M. meridianus* (gerbils) and *S. alaschanicus* (ground squirrels) in the Helan Mountains, several important limitations must be acknowledged. First, the sample was restricted to worms obtained from just one individual of each host species, which precludes meaningful conclusions about: (1) prevalence rates in wild populations, (2) host-specific adaptation patterns, or (3) population genetic structure of *T. arvicolae* in this ecosystem. The genetic diversity metrics (e.g., haplotype diversity = 1.000) and morphological measurements reported here should therefore be interpreted strictly as case-specific observations from these particular host individuals rather than species or population level

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characteristics. Future studies incorporating longitudinal sampling across multiple host individuals, seasons, and geographic locations will be essential to validate whether the patterns observed in this preliminary investigation reflect broader biological trends.

To the best of our knowledge, in the present study, *T. arvicolae* were isolated for the first time in the intestinal tracts of *M. meridianus* and *S. alaschanicus* of the family Sciuridaein the Helan Mountains, China.

All conclusions from this study carry an inherent tentativity due to the case-report nature of sampling single individuals per host species. Rather than establishing definitive patterns, our primary contribution lies in identifying critical questions for future parasitological research in understudied mountain ecosystems.

To validate these preliminary findings, future studies should expand sampling across multiple seasons (minimum n=10 hosts/species) and incorporate advanced methods like soil metabarcoding and host radio-tracking. This is particularly crucial in the climate-vulnerable Helan Mountains ecotone, where understanding *T. arvicolae* transmission dynamics between sympatric rodents requires population-level data to confirm the observed individual-level patterns and assess potential zoonotic risks.

Conclusion

This study provides the first confirmed record of *T. arvicolae* parasitizing both *M. meridianus* (gerbils) and *S. alaschanicus* (ground squirrels) in the Helan Mountains. Through morphological and molecular analyses (COI/ITS markers), we demonstrated that whipworms from these sympatric but ecologically distinct rodents (1) shared overlapping egg dimensions (55.43×26.39 μm vs. 53.17×25.36 μm), (2) exhibited minimal mitochondrial divergence (Fst=0.23), (3) showed complete nuclear haplotype differentiation (13 unique ITS sequences). These findings partially support our initial hypothesis about cross-species transmission in shared habitats while revealing unexpected genetic complexity at the individual host level.

The detection of identical *T. arvicolae* lineages in nocturnal gerbils and diurnal ground squirrels advances understanding of parasite dispersal in temporally partitioned hosts, particularly in arid mountain ecosystems where climate change may alter host-parasite interactions. However, the restricted sample size (single individuals per host species) necessitates caution in extrapolating these patterns to population-level dynamics.

Future research should prioritize longitudinal sampling to validate whether the observed genetic patterns reflect stable transmission routes or ephemeral ecological overlaps. This work establishes critical baseline data for monitoring parasitic nematodes in China's understudied desert-steppe transition zones, addressing the knowledge gap identified in our introduction regarding *Trichuris* host range in mountain ecosystems.

Data availability statement. Representative nucleotide sequences obtained in this study were submitted to the GenBank under the accession numbers PP493886-PP493898 and PP495534-PP495546.

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Conflicts of interest/competing interests. The authors declare that they have no competing interests.

Ethical approval. We declare that the gerbil and ground squirrel samples captured in this study were handled in a manner that ensured the welfare of animals. There is no conflict of interest with species conservation guidelines. The collection of the worms from the gerbil and ground squirrel was approved by

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the local Ningxia government agents. All experimental designs and animal handling were approved by the Institutional Animal Care and Use Committee of Northeast Forestry University (2022WVH03).

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