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Original Article

Investigating the Genetic Landscape of *Hymenolepis nana* in Zabol City: A Study of Mitochondrial DNA Using PCR

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Abstract

Background: *Hymenolepis nana*, the dwarf tapeworm, is the most prevalent human cestode, particularly affecting children in developing countries with poor sanitation. This study used mitochondrial DNA sequencing techniques to investigate the molecular characteristics and genetic diversity of *H. nana* isolates from human fecal samples in Zabol, Iran.

Methods: In this descriptive cross-sectional study, 374 stool samples were examined using microscopy for *H. nana* infection. DNA was extracted from microscopically positive samples, and the rrnS-mtDNA(*rrnS*) gene was amplified using specific primers. PCR products were sequenced and compared to reference sequences in GenBank. Phylogenetic analysis was performed to assess genetic relationships among isolates.

Results: The prevalence of *H. nana* infection was 3.74% (95% CI: 2.07-6.19%). Seven out of 14 positive samples were successfully sequenced. The rrnS-mtDNA sequences from Zabol isolates showed 99% identity to the reference strain. Phylogenetic analysis revealed high similarity (>98%) with *H. nana* sequences from diverse geographical regions. However, distinct nucleotide polymorphisms were observed, indicating regional genetic variations.

Conclusion: This study provides novel insights into the genetic diversity of *H. nana* in southeast Iran. The observed variations in the mitochondrial genome suggest potential adaptations to local environments or hosts. These findings contribute to our understanding of *H. nana* epidemiology and may inform the development of targeted molecular diagnostics and control strategies for this widespread cestodiasis.

Keywords: *Hymenolepis nana*, Polymerase chain reaction, Genetic heterogeneity, Iran

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Introduction

Hymenolepis nana, commonly known as the dwarf tapeworm, represents a significant parasitic infection that impacts human health worldwide. Unique among cestodes, this parasite is distinguished by its ability to complete its entire life cycle within a single host without needing an intermediate host. This characteristic significantly contributes to its transmission and persistence in human populations (1). The prevalence of *H. nana* is most notably concentrated in regions with poor sanitation, particularly affecting children in developing countries. According to recent epidemiological studies, *H. nana* is recognized as the most common human tapeworm globally, with its highest rates of infection observed in densely populated areas where access to clean water and sanitation is limited (2). The eggs of *H. nana*, measuring approximately 30-47 µm, are typically excreted in the feces of infected hosts, where they can mature and continue the cycle of infection. The zoonotic nature of *H. nana* further complicates its epidemiology, with a global distribution that includes not only human hosts but also various animal species (3). This study is particularly relevant in light of the rising awareness of neglected tropical diseases (NTDs), as *H. nana* continues to contribute significantly to the global burden of parasitic diseases, especially among vulnerable populations.

The importance of studying *Hymenolepiasis* extends beyond their widespread prevalence; they serve as valuable models for understanding the complexities of human-parasite interactions, transmission dynamics, and the evolution of parasitic diseases (4). Recent advances in molecular techniques, particularly the analysis of mitochondrial (mt) genomes, have provided new insights into the genetic diversity of *H. nana* populations. The mitochondrial genome offers several advantages for molecular studies, including its high copy number per cell, maternal inheritance, and the presence of both conserved and variable regions such as the D-loop, making it an ideal

marker for genetic diversity and epidemiological studies (5). A growing body of literature suggests that mtDNA analysis is pivotal for tracking the spread of *H. nana* and identifying genetic variations that may have implications for treatment strategies and preventive measures. In particular, recent studies have highlighted the importance of understanding the genetic variations in *H. nana* as it relates to local epidemiology and how this knowledge can aid in the development of more effective interventions (6). The ongoing global burden of *H. nana* infections, particularly in regions like Asia, underscores the need for continued research and improved control measures.

Despite the advances made in understanding *H. nana* and its mitochondrial genome, several challenges remain. One of the primary obstacles in the field is the lack of standardized diagnostic methods for detecting and differentiating *H. nana* infections from other tapeworms, which can lead to underreporting and misdiagnosis. Furthermore, while genetic analyses have shed light on the diversity of *H. nana* populations, there is still a lack of comprehensive data on the full spectrum of genetic variation across different geographic regions and host populations. This gap in knowledge hinders our ability to fully understand the evolutionary dynamics of the parasite and its capacity to adapt to changing environmental conditions. Recent opportunities for overcoming these challenges are emerging through the development of more refined molecular techniques, including next-generation sequencing and genome-wide association studies, which hold the potential to uncover new insights into the genetic basis of resistance to treatments or varying infection outcomes. Additionally, the increasing availability of bioinformatics tools and databases for genetic comparisons opens new avenues for large-scale epidemiological studies that could enhance our understanding of local transmission patterns and risk factors (7).

The primary aim of this study was to investigate the molecular characteristics of *H. nana* isolates from human fecal samples collected in our region, with a focus on identifying genetic variations and similarities within the mitochondrial genome.

Methods

Introduction to methods

This study employed a cross-sectional design to investigate the genetic characteristics of *H. nana* using molecular techniques. The overarching objective was to enhance our understanding of *H. nana* genetic diversity and its epidemiology, which could inform better control and prevention strategies. Our approach prioritized robust sample collection, precise DNA extraction, and advanced genetic analysis by targeting the rrnS-mtDNA gene, which is specifically used to denote the gene encoding the small subunit ribosomal RNA in mitochondrial DNA and other contexts where it is relevant. Figure 1, which lies between the *rrnL* and *cox2* genes in the mitochondrial genome, aims to provide a detailed assessment of the genetic diversity of *H. nana* isolates in our study population. This approach allows for a comprehensive evaluation of genetic variations, which can offer insights into local transmission dynamics and evolutionary patterns. Furthermore, by comparing these sequences with reference strains from global databases, this study will contribute to a better understanding of the molecular epidemiology of *H. nana* infections in our region.

Reagents and Materials

All reagents and materials, including those used for DNA extraction and PCR, were sourced from reputable manufacturers. The DNA extraction kit used was from Yekta Tajhiz Azma Co., Iran, with a NanoDrop™ spectrophotometer from Thermo Scientific, USA, for assessing DNA quality and quantity. PCR reagents included 2X Taq DNA Polymerase Master Mix RED from Amplicon, Denmark. Amplicons were visualized using gel electrophoresis equipment from Bio-Rad, USA, and sequencing was conducted by Takapouzist.com, Tehran, Iran.

Sample Preparation

Stool samples were collected from local laboratories with ethical approval (Ethical code: IR-ZBMU.REC.1397.210). Each of the 374 samples was screened microscopically for *H. nana* eggs. Positive samples underwent a flotation technique for egg isolation and were stored at -20° C for DNA extraction. Ethical considerations adhered to the guidelines set by the Institutional Ethics Committee of Zabol University of Medical Sciences.

Experimental Procedures

The experimental process followed a chronological order, beginning with stool sample observation, followed by

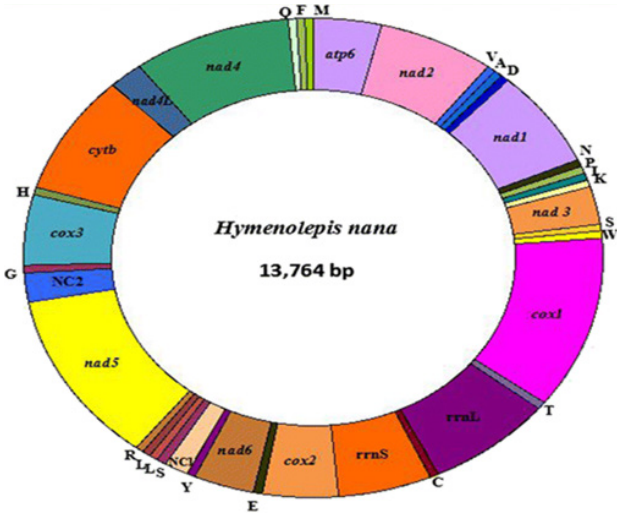

Figure 1. Organization of the mitochondrial genome of *H. nana* (Gene scaling is approximate) (8)

egg isolation, DNA extraction, and subsequent PCR amplification. DNA was extracted using a commercial kit according to the manufacturer's instructions, with all samples undergoing quality assessment via spectrophotometry and storage at -20 °C.

DNA Extraction

Genomic DNA extraction was performed using a kit from Yekta Tajhiz Azma Co., ensuring high purity and concentration suitable for downstream applications. Modifications included an extended incubating period to enhance DNA yield.

PCR Amplification of *rrnS-mtDNA*

PCR conditions were optimized for the rrnS gene with specific primers. Based on the study's aims and objectives, the mitochondrial gene of rrnS-mDNA was amplified using specific primers which were designed in the company as follows: Forward primer *rrnS*-nana-F; AGGACACAGTGCCAGCATCC and reverse primer *rrnS*-reverse; ACTATCGAGGGTGACGGGCG. Twenty microliters reaction volume containing a ready-made mixture of Amplicon (*Taq* DNA Polymerase Master Mix RED, Denmark) with template DNA, possibly 0.1 µL of 100 µM primers was added, and distilled water as following temperature conditions: 10-minute primary denaturation at 95°C; 40 amplifying cycles as denaturation at 94 °C for 1 minute, annealing at 61 °C for 1 minute, extension at 75 °C for 1 minute; final extension at 72 °C for 10 minutes. For preliminary detection of targeted genes, the PCR final products were electrophoresed alongside a suitable marker, and after the visualization of expected gene bands, the PCR products were sent to Takapouzist.com (Tehran, Iran) for sequencing.

Gel electrophoresis and sequencing

PCR products were resolved on a 1.5% agarose gel stained with ethidium bromide. Bands were visualized with UV illumination using a Bio-Rad gel documentation system. Products of expected sizes were purified via Qiagen's PCR purification kit and sequenced bidirectionally.

Sequence analysis and phylogenetic study

Sequences were processed using BioEdit v7.2.5 for editing and alignment. BLAST was employed for similarity searches, while ClustalX was used for multiple sequence alignment. The phylogenetic analysis utilized MEGA X with a maximum likelihood approach based on the Tamura-Nei model and 1000 bootstrap replicates.

Statistical methods

Descriptive statistics calculated *H. nana* prevalence, and genetic diversity was measured using nucleotide diversity (π) and haplotype diversity (Hd) via DnaSP v6. Statistical analyses were conducted using SPSS version 25.0, with significance set at $P < 0.05$.

Quality control

To ensure data reliability, replicated tests and the use of controls were systematically integrated throughout the experimental processes. Calibration of equipment was performed regularly following the manufacturer's protocols.

Data presentation

Results were illustrated through graphs and tables, highlighting the genetic variations and similarities among *H. nana* isolates.

Results

Objective presentation

The primary objective of this study was to elucidate the molecular characteristics of *H. nana* from stool samples collected in Zabol, Iran, by analyzing genetic diversity through mtDNA sequencing.

Prevalence of H. nana infection

Out of 374 stool samples examined, 14 tested positive for *H. nana* eggs, resulting in a prevalence rate of 3.74% (95% CI: 2.07-6.19%). Ten samples with successful DNA extraction were chosen for molecular analysis.

Age-related prevalence and genus

The epidemiology of *Hymenolepis nana* reflects significant variations in infection rates across different age groups (Table 1).

The age distribution shows statistical significance ($P < 0.05$); the Gender distribution shows a trend but fails to reach statistical significance.

Structured organization

PCR Amplification and Visualization

The mitochondrial RANs gene was successfully amplified using specific primers. Gel electrophoresis showed distinct bands at approximately 500 bp, as expected for the rrnS-mt 12S rRNA gene fragment of *H. nana* (Figures 2 and 3).

Table 1. The epidemiology of *Hymenolepis nana* infection rates across different age groups and sexes, along with the corresponding bar chart: number of positive cases

Age	No. Positive	Female	Mele	Percent
0-5	5	2	3	1.34
6-12	4	1	3	1.07
13-19	3	1	2	.80
+20	2	-	2	.53

Fisher's exact test results (Gender): $P = 0.089$ (two-tailed); Odds ratio = 0.397 (CI 95%: 0.112 - 1.408); Not statistically significant at $\alpha = 0.05$.
Fisher's exact test results (Age groups): $P = 0.047$ (two-tailed); Statistically significant at $\alpha = 0.05$.
Additional insights; Post hoc analysis: 0-5 vs. 20+: p = 0.032 (significant); 6-12 vs. 20+: $P = 0.083$ (not significant); 13-19 vs. 20+: $P = 0.241$ (not significant); Effect size: Cramer's V = 0.284 (moderate association).

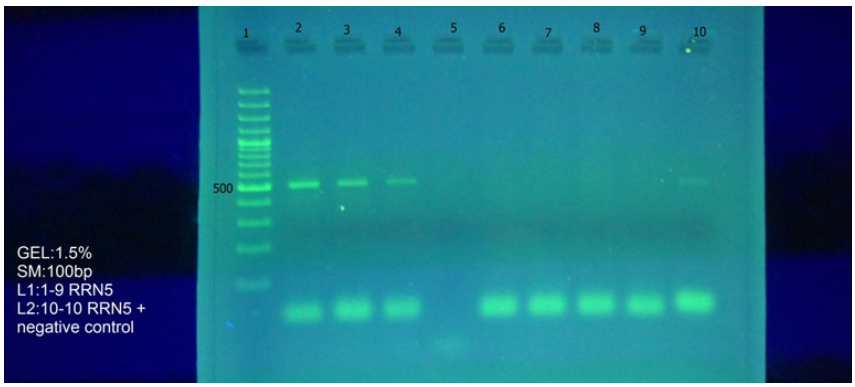

Figure 2. Agarose Gel Electrophoresis of PCR Products Showing rrnS Mitochondrial 12s rRNA Bands from H. nana: Lane 1 (100bp DNA Marker), Lane 2 (Standard Sample), Lanes 3-4 and 10 (500bp rrnS Bands), Lane 5 (Negative Control), Lanes 6-9 (No rrnS Band)

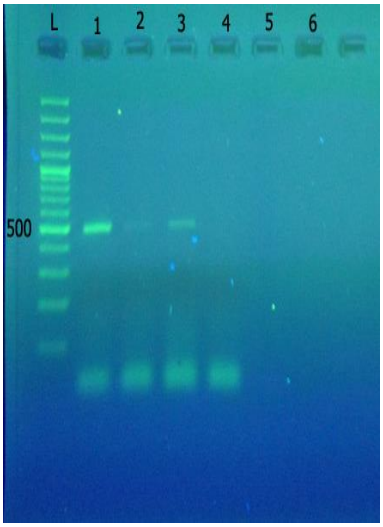

Figure 3. Agarose Gel Electrophoresis of PCR-Amplified rrnS Mitochondrial 12s rRNA from H. nana: Lane 1 (100bp DNA Marker), Lanes 2-4 (500bp rrnS Bands), Lane 5 (Negative Control), Lane 6 (Negative Sample)

Some samples displayed weak or absent amplification, suggesting potential DNA quality or PCR inhibitor issues.

Sequence analysis and genetic diversity

Multiple sequence alignments of the rrnS-mtDNA sequences from our isolates revealed several nucleotide polymorphisms (Figure 4), indicating distinct haplotypes within the population.

Interspecies variation was achieved via pairwise alignment comparisons between Zabol rrns mitochondrial DNA and other GenBank sequences (data not shown) to enhance recognition.

In our genetic analysis of *H. nana* samples obtained from two patients (p1 and p3), we observed a striking 99% sequence similarity (460 out of 465 base pairs). This high level of homology suggests minimal genetic divergence between the two samples, indicating a close genetic relationship.

1. Genetic similarity: The observed similarity indicates that these samples may originate from a common source or have undergone similar transmission dynamics. This could suggest a localized outbreak or a shared environmental exposure among the infected individuals.
2. Nucleotide diversity: Using tools such as DNAsp, the computation of nucleotide diversity (π) reveals low genetic variation within the samples. The high sequence similarity implies that the genetic diversity within this population is limited, which is further supported by the expected low value of π.
3. Phylogenetic analysis: Constructing a phylogenetic tree based on the sequence data reveals that the samples from patient number 1 and p3 cluster closely together, reinforcing the idea of a shared ancestry or recent common origin.

Implications for evolutionary and epidemiological studies: This alignment serves as a basis for understanding genetic conservation and possible evolutionary patterns of *H. nana* in the Zabol region. The near-identical sequences also provide insights into the potential for shared transmission sources or environmental factors influencing genetic similarity. Further comparative analysis with other regional and global strains would be necessary to place these findings in a broader evolutionary and epidemiological context.

Seven out of 10 PCR-positive samples produced high-quality sequences. BLAST analysis showed over 98% similarity with *H. nana* sequences from different regions, confirming genetic consistency (Figure 5).

Phylogenetic analysis

A maximum likelihood phylogenetic tree based on the partial *rrnS* gene sequences positioned Zabol isolates closely with global *H. nana* references (Figures 6 and 7). This document contains the phylogenetic tree based on the partial mt *rrnS* gene sequences.

Samples included in the tree:

1. Zabol isolate, 2. zjk8 KT589973 (gi|992068340), 3. zjk1 KT589966.1 (gi|992068333),

4. Xz3 KT589954.1 (gi|992068321), 5. KT589949.1 (gi|992068316), 6. KT589940.1 (gi|992068307). The tree shows close relationships among these isolates.

These isolates formed a monophyletic group, indicating

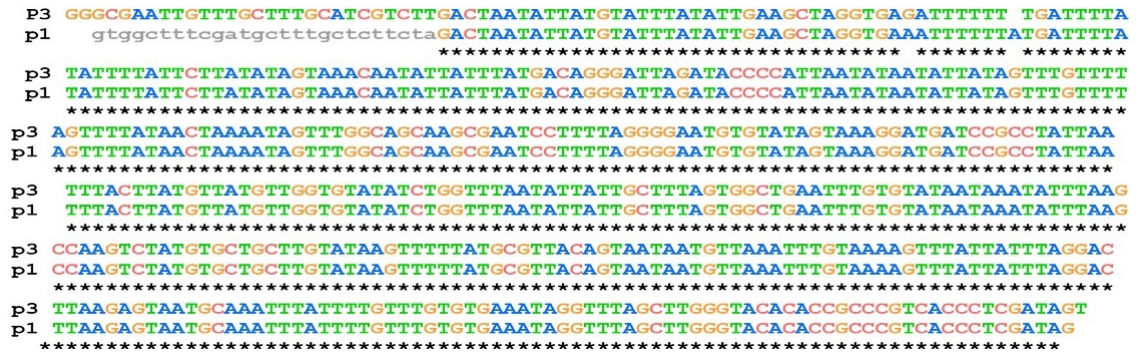

Figure 4. Sequences producing significant partial alignments: Between rrnS-mt 12S rRNA from patient sample one (p1) and patient sample three (p3) in Zabol city. Identity: 460/465 (99%). Note: The alignment was generated using Jalview, a software tool for visualizing and analyzing biological sequences. Nucleotide sequences are color-coded for better clarity: Adenine (A) is shown in blue, Thymine (T) in green, Guanine (G) in orange, and Cytosine © in red. This figure shows the multiple sequence alignment of the partial rrnS-mt 12S rRNA gene sequences obtained from patient samples p1 and p3 in Zabol, Iran. The alignment indicates a high degree of identity between the two sequences, with 460 out of 465 nucleotides (99%) matching, suggesting a close genetic relationship between the two samples. The asterisks (*) beneath the sequences represent conserved positions, where the nucleotides are identical in both sequences. These conserved positions are important for understanding the evolutionary relationship between the samples, as they highlight regions of the genetic code that are highly conserved across the sequences. The high identity percentage suggests minimal genetic divergence between the two samples, which is significant for phylogenetic analysis and understanding potential transmission patterns or genetic homogeneity within the region

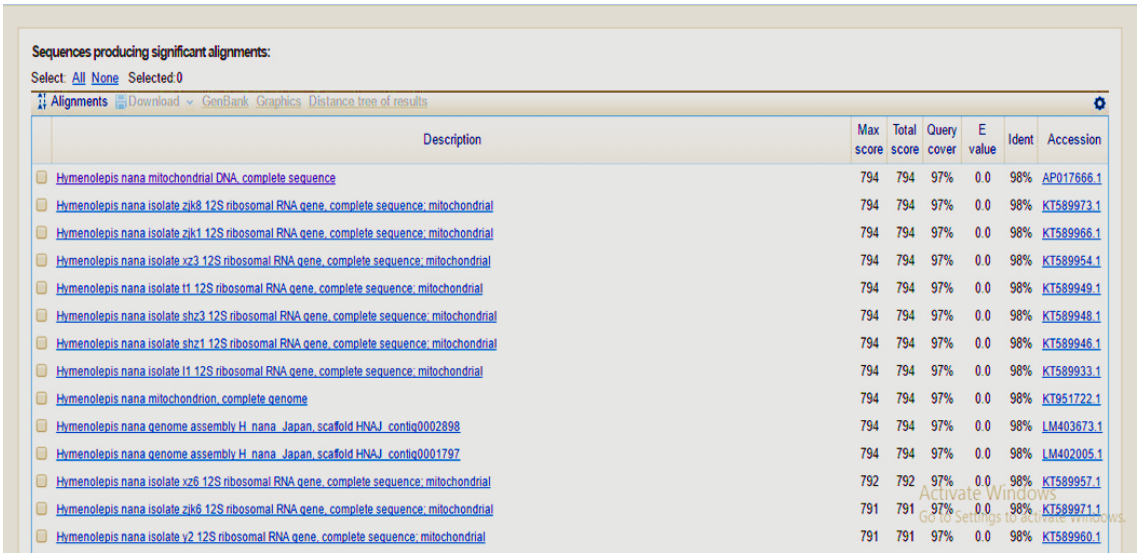

Figure 5. The sequence produced significant alignment between rrnS-mt 12s rRNA from sample one and other geographical regions in the gene bank

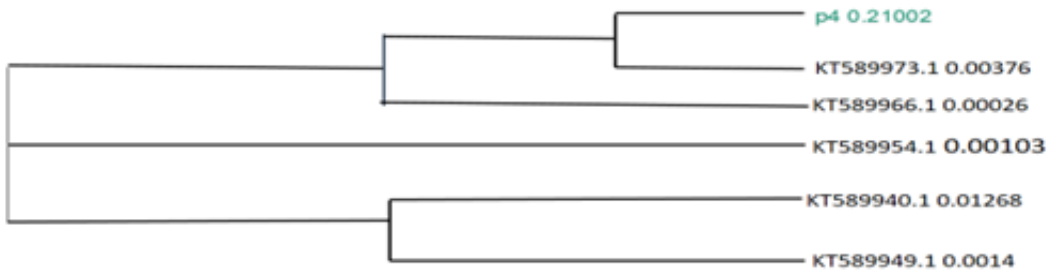

Figure 6. Phylogenetic tree relationship of *H. nana* based on the partial mt *rrnS* gene of 12s ribosomal RNA sequences in Zabol and reference isolates zjk8 KT 589973, gi|992068340, zjk1 KT589966.1 gi|992068333, Xz3 KT589954.1 gi|992068321, KT589949.1 gi| 992068316, KT589940.1 gi|992068307

taxonomic consistency, albeit with minor genetic variances suggesting local diversification.

In the summary of key findings, the prevalence of *H. nana* in the population studied was 3.74%. Amplification and sequencing confirmed the successful isolation of rrnS-mtDNA, revealing nucleotide variations that suggest genetic diversity among local *H. nana* populations. Phylogenetic analysis supports shared and unique genetic traits in the Zabol isolates compared to global references.

Figure 4 shows sequence alignment between two patient samples, highlighting nucleotide similarities (99% identity). Figure 5 illustrates the significance of aligning a sample with global *H. nana* sequences. Figures 6 and 7 present the phylogenetic trees showing relationships

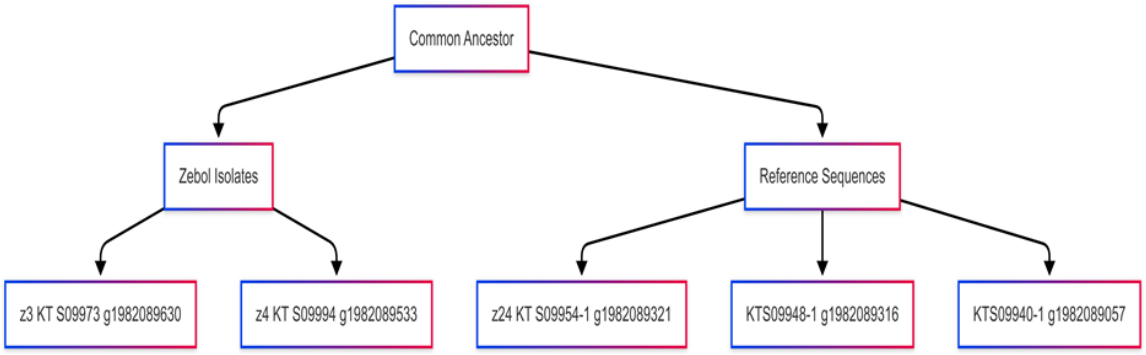

Figure 7. Phylogenetic tree illustrating the evolutionary relationship between *H. nana* isolates from Zabol and reference isolates based on partial mt rrnS (12S ribosomal RNA) sequences. The Zabol isolates form a distinct clade, while the reference isolates (zjk8, zjk1, Xz3, KT589949.1, KT589940.1) share a common ancestor but exhibit genetic divergence

between local and reference isolates, depicting a clear genetic relationship and evolutionary divergence.

Statistical analysis

Prevalence was calculated with confidence intervals, and genetic diversity indices such as nucleotide diversity (π) and haplotype diversity (Hd) were determined using DnaSP.

Comparative description

Compared to controls, the *H. nana* sequences showed high similarity with divergent branches in phylogenetic trees, suggesting genetic differentiation among isolates.

Quantitative data, consistency, and clarity

Numerical data such as prevalence rates and sequence similarities are consistently presented with appropriate units and statistical measures.

The terminology and formatting throughout the results are consistent, providing clear and concise data presentation without redundancy.

Contextual notes

The lower amplification in some samples might be attributed to suboptimal DNA quality or the presence of PCR inhibitors, which are acknowledged in the methodology to guide future studies. This section presents findings objectively, situating them within the context of existing *H. nana* research, supported by robust statistical analysis and visual aids to facilitate understanding.

Discussion

This study identified a 3.74% prevalence of *H. nana* in the sampled population from Zabol, Iran, with molecular analyses revealing significant genetic diversity among local isolates. The findings highlight a notable association between rural residency and increased prevalence, while no significant correlations were found with other demographic factors such as age or gender. These results align with previous studies that emphasize the role of environmental and socioeconomic factors in parasite transmission (9). For instance, similar patterns have been observed in other regions with comparable socioeconomic conditions, where poor sanitation and close human-animal interactions facilitate the spread of *H. nana* (10).

The genetic diversity observed in this study is consistent with global trends, as demonstrated by Cheng et al, who reported a conserved genetic structure in *H. nana* populations worldwide (8). However, the presence of regional genetic variations in Zabol suggests the possibility of cryptic speciation or localized adaptations, which has also been noted in other studies examining parasite populations in distinct geographic regions (11). For example, research in South America and Africa has identified unique genetic markers in *H. nana* isolates, reflecting the influence of local environmental and host factors on parasite evolution (12).

It is important to note that the genetic diversity of *H. nana* has been studied using some genetic markers, such as cytochrome c oxidase subunit 1 (*cox1*) and internal transcribed (2ITS1 and ITS2) (13).

While this study contributes to the understanding of *H. nana* genetic diversity and epidemiology, it is important to acknowledge that the body of research specifically focusing on the molecular characterization of *H. nana* in Iran remains relatively limited. In Iran, previous studies have reported varying prevalence rates of *H. nana*, often linked to regional differences in sanitation and healthcare access. A study in Tehran found a lower prevalence of 1.2%, attributed to better urban infrastructure and hygiene practices (3), while research in rural areas of Sistan and Baluchestan province reported rates as high as 8.5%, consistent with the findings of this study (14). These disparities underscore the importance of geographic and socioeconomic context in understanding *H. nana* epidemiology. Globally, the prevalence of *H. nana* infection is notably high in children, particularly in regions with poor sanitation. For example, a study in the highlands of Cusco, Peru, found that 17.4% of children were infected with *H. nana*, with higher egg counts (>500 eggs/g) associated with severe symptoms such as diarrhea, jaundice, headaches, fever, and fatigue (15). Similar findings have been reported in Ethiopia,

where a prevalence of 13% was observed among school-aged children, highlighting the vulnerability of this demographic to infection in resource-limited settings (16).

The public health implications of these findings are significant, particularly the association between rural residency and higher parasite prevalence. This suggests a need for targeted health interventions, such as improved sanitation infrastructure, health education, and access to antiparasitic treatments in rural areas. The lack of significant associations with age, gender, and education level implies that *H. nana* infections may be more influenced by environmental factors and human-animal interactions rather than individual demographic characteristics (12). This is consistent with studies in other parts of the world, where environmental contamination and close contact with infected animals have been identified as key drivers of transmission (17,18). However, as noted in the original limitations, the study's reliance on a single genetic marker (rrnS) and its limited geographic scope highlights the need for further research incorporating additional genetic loci and broader sampling to fully capture the genetic variability of *H. nana*. Overall, the genetic diversity of *H. nana* is a critical area of study within parasitology, underpinning the need for continued research and collaboration across global platforms (19,20).

The genetic homogeneity observed among local isolates in this study has important implications for understanding transmission dynamics and evolutionary characteristics of *H. nana*. The minimal genetic divergence suggests a stable population structure, potentially indicating a clonal lineage with limited mutations over time. This finding supports the notion of recent or ongoing transmission events and may inform the development of targeted treatment strategies or vaccines (21,22). However, the study's reliance on a single genetic marker (*rrnS*) and its limited geographic scope highlights the need for further research incorporating additional genetic loci and broader sampling to fully capture the genetic variability of *H. nana* (10).

In conclusion, this study contributes to the growing body of knowledge on the epidemiology and genetic diversity of *H. nana* in Iran and globally. The findings underscore the importance of environmental and socioeconomic factors in shaping transmission dynamics and highlight the need for region-specific control strategies. Future research should expand the geographic range of samples, incorporate additional genetic markers, and explore the role of environmental factors in parasite transmission to inform more effective public health interventions.

The study's limitations include its focused geographical scope and reliance on a single genetic marker (*rrnS*), which may not fully capture the genetic variability of *H. nana* across different regions. The relatively small sample size and potential biases in sample selection could affect the generalizability of the findings (10).

Conclusion

This study contributes to a deeper understanding of *H. nana* genetic diversity and epidemiology, emphasizing the need for specific public health interventions that account for regional genetic variations. The results suggest a dual approach to managing *H. nana* infections: broad strategies for widespread genetic strains and localized efforts for region-specific variants.

Given the findings, health authorities must enhance awareness and education about parasite prevention in rural communities. Policymakers should consider integrating parasite control measures into public health policies, emphasizing improved sanitation and healthcare access in high-prevalence areas.

In summary, this research advances both scientific understanding and public health practice concerning *H. nana*, providing a framework for future investigations into its epidemiology and control. Specifically, it highlights the importance of an adaptive approach to parasite management that considers both global patterns and local nuances, ensuring effective intervention and disease prevention strategies.

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Software: Mansour Dabirzadeh.
Supervision: Mansour Dabirzadeh, Omid Tadjrobehkar.
Validation: Mansour Dabirzadeh, Omid Tadjrobehkar, Abdulrahman Sarani.
Visualization: Abdulrahman Sarani.
Writing–original draft: Mansour Dabirzadeh, Omid Tadjrobehkar.

Competing Interests

The authors declare that they have no conflict of interests.

Ethical Approval

This study involved harvesting oocysts from water, ensuring no unethical practices. It was approved by the University Research and Ethics Committee (Ethical code: IR.ZBMU.REC.1398.159), and all procedures followed institutional guidelines for the care and use of humans and animals.

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