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Mitochondrial genome analysis supports zoonotic transmission of triclabendazole-resistant human fascioliasis in Peru

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ABSTRACT

Fascioliasis is a parasitic infection caused by *Fasciola* spp., primarily affecting ruminant animals. These digenean flatworms cause severe liver damage in their hosts, resulting in substantial economic losses within the livestock industry. Human fascioliasis is an emerging public health concern, with an estimated global prevalence of 2.6 million cases. Infection in humans typically occurs through the ingestion of aquatic vegetation or water contaminated with metacercariae. Triclabendazole (TCBZ) remains the only drug recommended by WHO for the treatment of human fascioliasis and is widely used in livestock. However, the increasing prevalence of TCBZ resistance in livestock, along with reports of TCBZ-resistant human infections, poses a growing challenge to disease control. Although it has been suggested that resistant livestock infections may contribute to the emergence of resistance in human populations, this relationship has not been systematically investigated. In this study, we characterized the mitochondrial genomes of TCBZ-resistant and TCBZ-sensitive *F. hepatica* isolates from human infections and conducted a comparative haplotype analysis with *F. hepatica* samples obtained from cattle in the same region of Peru. Maximum-likelihood phylogenetic and haplotype network analyses of 304 animal and 11 human *F. hepatica* samples identified five distinct haplogroups. Mitochondrial haplotypes from human infections clustered into monophyletic groups alongside those from animal hosts, supporting the hypothesis of local zoonotic transmission from animal reservoirs. Additionally, a phylogeographic analysis of global ND1 sequence diversity provided insights into the demographic history of the parasite across pre- and post-domestication periods and revealed genetic signatures of global dissemination that have shaped its present-day distribution.

1. Introduction

Fascioliasis is a zoonotic foodborne trematode infection of the liver. Production parameters in the livestock industry are severely affected by *Fasciola* infection worldwide, while human infection primarily affects tropical and sub-tropical area, particularly in South America [1,2]. In Peru, animal and human infections intertwine, creating a complex problem [3]. Subsistence in rural communities where *Fasciola* is endemic depends on small-scale livestock farming for draft power and the production of milk, wool, and meat [4]. Heavy *Fasciola hepatica* infections may affect up to 90 % of livestock and are associated with decreased carcass weight, threatening income and food security in rural

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communities [5–7]. Human fascioliasis is considered a disease of poverty, affecting an estimated 2.6 million people globally [8]. In the Cusco region of Peru, prevalence is higher in areas with lower socio-economic status [9]. The largest burden of fascioliasis in the region is bearded by school aged children [10], as in the hyperendemic Bolivian Altiplano [11]. Anemia and malnutrition have been associated with subclinical infections among this group [12,13]. Although less studied, fascioliasis is likely to contribute to the burden of chronic liver and biliary tree disease [14].

Triclabendazole (TCBZ), a benzimidazole, is the only medication recommended for the treatment and control of fascioliasis in humans [15]. It is also widely used in livestock because it is efficacious in early acute and chronic stages of infection. The lack of stewardship in TCBZ use has led to the emergence of resistance on farms in both developed and developing countries [16]. Reports of resistance in sheep and cattle have emerged from farms in Australia, the United Kingdom, Spain, Germany, Peru, and Argentina [17–22]. A study by Borgsteede et al. in the Netherlands suggested that once TCBZ resistance emerged on a farm, it persisted for years even after switching to other flukicides [23]. Resistant human infections have been reported from the Netherlands, Portugal, Turkey, Chile, and Peru [15]. Decreased treatment efficacy has been reported in human case series of fascioliasis [24,25]. Triclabendazole selection pressure differs between livestock and human populations [26]. In the former, TCBZ is administered several times a year for treatment and prevention, while in the latter, the drug is mostly prescribed in symptomatic cases that reach the healthcare system [27]. Poor quality of veterinary products and improper dosing are likely drivers of selection in livestock. In humans, few examples of TCBZ mass drug administration have been reported. The Bolivian government implemented a fixed dose mass drug administration program in 2008 that administered TCBZ to all target populations regardless of age or infection status. A study by Mollinedo et al. published in 2019 suggested significant reductions in prevalence among children; however, official data on ongoing coverage and effectiveness remain unavailable [28]. Similar mass TCBZ administration programs in other countries of South America, such as Peru, have been more limited in duration and scope, and none have been active for many years [29]. Although it is probable that resistant infections in livestock are driving the emergence of resistance in humans, this has not been systematically described [15].

Studies evaluating the genetic diversity of *F. hepatica* using mitochondrial genes tracked the introduction of the parasite in different areas and its population dynamics. In Colombia, the analysis of nuclear and mitochondrial genes revealed little genetic diversity in *Fasciola* parasites collected from seven regions [30]. In Uruguay and Peru, the evaluation of mitochondrial genes showed low diversity, suggesting the introduction of *Fasciola* from Europe [31,32]. In our study, we performed a comprehensive analysis of the mitochondrial genome of *F. hepatica* adults collected from naturally infected cattle and eggs collected from humans with TCBZ-resistant (TCBZ-R) or -susceptible (TCBZ-S) infections. Our results indicate that human-infecting parasites are not genetically distinct from those infecting the local cattle population and suggest that resistant infections in humans likely originate from resistant parasites in livestock in Peru. These findings provide insights into the transmission dynamics and population connectivity of *F. hepatica* across host species.

2. Materials and methods

2.1. Collection of Fasciola hepatica from human infections

A TCBZ-R *F. hepatica* specimen was obtained from a female patient diagnosed with chronic fascioliasis by stool microscopy and ELISA serology in Cusco, Peru. She was treated at least twice with TCBZ (Egaten 250 mg tablets, Novartis Pharma AG, Basel, Switzerland) and self-medicated with veterinary TCBZ twice with no response by stool microscopy [33]. She underwent endoscopic retrograde cholangiopancreatography for acute biliary tree obstruction with removal of three adult parasites that were not collected during the procedure. She also developed a subcutaneous nodule near her left breast after TCBZ treatment. Upon excision of the nodule, a 1 cm long *Fasciola* parasite was recovered and processed for DNA sequencing as described previously (SRA BioSample ID: SAMN05173112) [34]. Stool samples from humans with fascioliasis were obtained from the biobank at the Sede Cusco del Instituto de Medicina Tropical of Universidad Peruana Cayetano Heredia. Samples were collected from children aged 3 to 16 years in the Anta province of Cusco. These children participated in epidemiologic studies on fascioliasis, and their parents provided consent to store their deidentified samples for future use [12,25]. All children with fascioliasis were treated with TCBZ following Peruvian Ministry of Health recommendations and tested for cure 30-90 days after treatment. Up to four courses of directly observed TCBZ treatment were provided to children who failed to cure the infection. Diagnosis of fascioliasis, treatment cure, or treatment failure was made using Lumbreras rapid sedimentation and Kato Katz microscopy tests on three stool samples [9]. Children with no *Fasciola* sp. eggs in any of the stool microscopy tests after treatment were considered cured. Children with *Fasciola* sp. eggs in at least one stool microscopy test were considered to have fascioliasis; if this occurred after treatment, they were classified as treatment failures. Follow-up for treatment response was conducted one to three months after treatment to minimize the likelihood of reinfection becoming detectable by stool microscopy. We selected baseline stool samples from children with fascioliasis who cured after treatment and classified them as containing TCBZ-S parasite eggs. Post-treatment samples from children who failed three or more treatment courses were selected and classified as containing TCBZ-R parasite eggs. Five-gram stool samples from children with known treatment outcomes and stored at −80 °C without preservatives were thawed at room temperature, transferred to a new conical cup, and suspended in 200 mL of distilled water. Eggs were enriched in the suspended samples following the same rapid sedimentation technique previously described [35]. Using the final sediment, eggs were captured by aspiration under a stereoscope and transferred to a DNase free tube for DNA extraction. If more than one stool sample was available in the biorepository for an individual, the eggs captured from all the samples were pooled in the same DNase free tube.

2.2. DNA sequencing of Fasciola hepatica eggs from infected humans

We extracted genomic DNA from egg pools using the E.Z.N.A stool DNA kit (Omega-BioTek, Norcross, GA, USA) following the manufacturer's instructions with the following modifications. We added 540 μL of the SLX-Mlus buffer provided in the kit to the egg samples and performed three cycles of heating at 90 °C for 20 min and freezing at −70 °C for 10 min. Then, we added 200 mg of glass beads to the specimens and vortexed them for 20 min. Finally, we added DS buffer and proteinase K and incubated the specimens at 70 °C for 15 min. The resulting lysate was used for DNA extraction according to the manufacturer's protocol. DNA quality and quantity were evaluated using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The DNA was preserved using DNA Stable (Sigma-Aldrich, St. Louis, MO, USA) and shipped to Washington University in St. Louis (Missouri, USA) for DNA sequencing. DNA was recovered in 40 μL of nuclease-free water and used to construct sequencing libraries with the xGen™ cfDNA & FFPE DNA Library Preparation Kit and sequenced on Illumina's NovaSeq platform (2 × 150 bp paired-end reads) by the McDonnell Genome Institute at Washington University in St. Louis.

2.3. Mitochondrial genome consensus calling

The sequencing data quality was assessed using FastQC v0.11.8 [36], and adapter and low-quality sequences were removed using Trimmomatic v0.39 [37]. Human and bacterial reads were removed using

Kraken2 with the *kraken2_standard_20231009* database [38]. Read alignment was performed using BWA v0.7.17 [39] against the *F. hepatica* reference mitochondrial genome (GenBank accession number: NC_002546.1) [40]. Previously published sequencing data from adult *F. hepatica* samples (**Table S1**) were processed identically, but the Kraken2 contaminant removal step was omitted. PCR and optical duplicates were removed using Picard v2.26.2 (http://broadinstitute.github.io/picard/), and mitochondrial genomes were reconstructed for each sample by consensus calling using SAMtools v1.16.1 [41] (consensus -a -m simple -c 0.5 --min-MQ 30). The reference mitochondrial genome of *F. hepatica* (NC_002546) contains a repeat region at the 3′ end that results in low read mapping quality. This region (NC_002546:13,138-14,462) was excluded from all downstream analyses. *F. hepatica* eggs samples from infected humans (**Table S2**) may contain multiple mitochondrial haplotypes because the sequencing libraries were generated from pooled eggs. However, if the frequency of the most abundant mitochondrial haplotype in a sample exceeds 50 %, a consensus calling approach can reconstruct the major haplotype without phasing ambiguities. The frequency of the most abundant haplotype in egg samples was estimated using highly variable "microhaplotype" loci, which contain multiple SNPs located close enough to be genotyped within the same sequencing read. These loci were identified in each egg sample, and allele frequencies were estimated using mhFromLowDepSeq [42] (-af -w 125 -pool). Egg samples were subjected to consensus calling only if the major allele frequency exceeded 50 % across all variable loci in the mitochondrial genome (**Table S3**).

2.4. Phylogenetic and haplotype network analyses

Multiple sequence alignments of the mitochondrial DNA sequences were generated using MAFFT v7.505 [43] (--globalpair --maxiterate 1000). All positions containing gaps in the alignment were removed using trimal v1.4.1 [44]. The resulting multi-FASTA alignment was converted to VCF format using SNP-sites [45], and singleton SNPs (i.e., SNPs present in only one individual sample) were identified using VCFTOOLS v0.1.16 [46] (--singletons). These singleton SNPs were masked by replacing them with 'N' in the multiple sequence alignment. For the analysis of NADH dehydrogenase subunit 1 (ND1) sequences, Jalview v2.11.4 [47] was used to extract the corresponding region (NC_002546.1:5183-5717) from the multiple sequence alignment after incorporating ND1 sequences from previously published studies (**Table S4**) and aligning them using MAFFT v7.505 [43] (--localpair --maxiterate 1000). Maximum-likelihood (ML) phylogenetic trees were constructed using IQ-TREE v2.4.0 [48] with 10,000 bootstrap replications (-B 10000), and the best-fit model was automatically selected using ModelFinder [49]. iTOL v7 [50] was used to visualize the phylogenetic trees. PopArt v1.7 [51] was used for the construction and visualization of median-joining haplotype networks. The command-line arguments used in the analysis are provided in **Text S1**.

3. Results

3.1. Mitochondrial DNA haplogroup identification in Fasciola hepatica

Using all published *F. hepatica* whole-genome sequencing (WGS) data from individual adult fluke samples, we performed reference-guided consensus calling to reconstruct the complete mitochondrial genome for 304 samples (Table 1 and **Table S1**). The dataset included samples from Peru [34,52], Uruguay [34], the United States [53], and the United Kingdom [54,55], as well as an assembled genome from Australia (GenBank accession: NC_002546.1) [40], which was used as the reference for read alignment. A maximum likelihood (ML) phylogenetic analysis of the mitochondrial genomes from adult flukes isolated from animal hosts revealed five distinct haplogroups (Fig. 1A). The branches defining each of the five clades were strongly supported, with bootstrap values ≥90 (Fig. 1A). To systematically classify these haplogroups based on their evolutionary relationships, we calculated ML distances between individual *F. hepatica* samples and *F. gigantica* samples (Fig. 1B and **Table S1**), which represent the closest sister taxon and served as the outgroup (Fig. 1A). Clades with shorter genetic distances to *F. gigantica* were designated as ancestral, while those with greater distances were considered derived. Considering both the phylogenetic topology among the haplogroups (Fig. 1A) and their genetic distances to *F. gigantica* (Fig. 1B), we assigned alphanumeric labels to each haplogroup (Fig. 1C). The 2 earlier diverging basal lineages were denoted A and B, and the haplogroups within each lineage were labeled numerically in ascending order from the most ancestral to the most derived. A median-joining haplotype network was constructed using the mitochondrial genomes, recapitulating the five haplogroups (Fig. 1D). There were a total of 320 segregating sites, with an average pairwise nucleotide diversity of 0.0035. Although the haplotype diversity represented in this analysis was largely based on samples from Peru, samples from other countries also showed close phylogenetic affinity to the identified haplogroups. *F. hepatica* samples from Australia, Uruguay, and the United States belonged to haplogroups B1, A2, and A3, respectively. Samples from the United Kingdom belonged to haplogroups A3 and B1 (Fig. 1D). The average number of genome-wide nucleotide substitutions between haplogroups A and B was approximately 92, corresponding to 0.7 % sequence divergence.

Table 1
Fasciola hepatica samples used in the whole mitochondrial genome analysis.

Host	Country of origin	Sample type		Reference
		Individual adult	Pooled eggs	
Animal	Australia	1	–	[40]
	Peru	286	–	[34,52]
	United Kingdom	11	–	[54,55]
	USA	1	–	[53]
	Uruguay	5	–	[34]
Human	Peru	1	10	[34], This Study
Total		305	10	

3.2. Mitochondrial haplogroup sharing between human and animal Fasciola hepatica infections

Complete *F. hepatica* mitochondrial genome sequences were generated through consensus calling from pooled eggs isolated from ten human fecal samples, with each sequence representing the predominant haplotype within the sample, and from one adult fluke, using previously published WGS data [34]. All eleven human-derived samples were collected from the Cusco region of Peru (**Table S1** and **S2**). This enabled direct comparison with haplotypes obtained from the local cattle population [52], which were used to define the haplogroups (Fig. 1). A maximum-likelihood phylogenetic analysis incorporating both human- and animal-derived sequences revealed that mitochondrial haplotypes from human infections formed monophyletic groups with haplogroups A3 and B1, identified in animal host samples, supported by bootstrap values ≥90 (Fig. 2A). Among the eleven human-derived samples, four distinct mitochondrial haplotypes were identified. Two of these were identical to haplotypes found in animal infections. Of the remaining two, one differed by a single nucleotide and the other by two nucleotides from the closest matching haplotypes in animal-derived samples (Fig. 2B).

3.3. No association between triclabendazole resistance and mitochondrial haplogroup in Fasciola hepatica

Among 285 adult fluke samples collected from cattle in Cusco, Peru, 91 were classified as TCBZ-S and 194 as TCBZ-R based on in vitro assays [52] (Fig. 2B). No significant association was found between the TCBZ-R phenotype and mitochondrial haplogroup distribution (chi-square

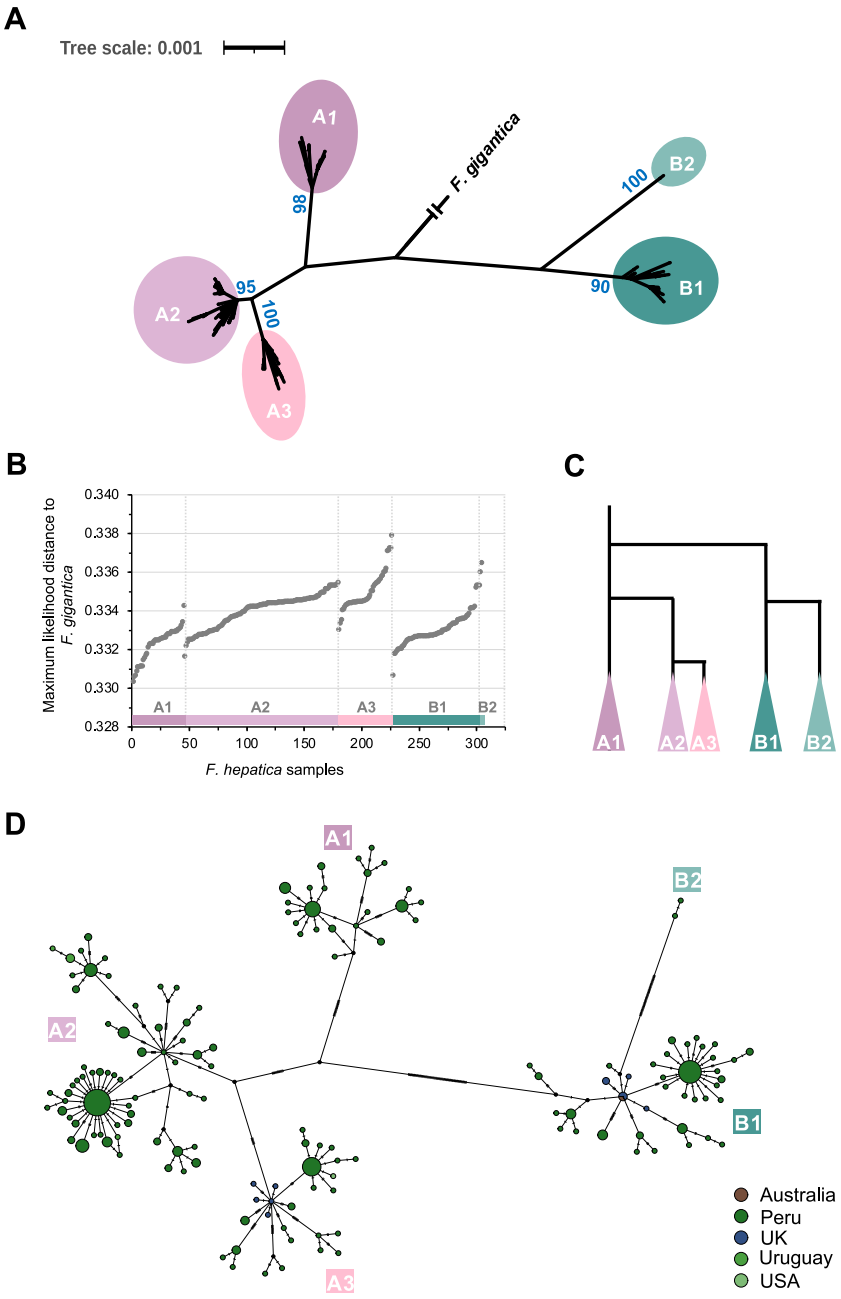

Fig. 1. Mitochondrial genome phylogeny and haplogroups of *Fasciola hepatica* from animal infections. (A) Maximum likelihood phylogenetic tree based on the complete mitochondrial genomes of 304 animal-derived *F. hepatica* samples, including 19 *F. gigantica* outgroup samples. (B) Distribution of the maximum likelihood distances of *F. hepatica* samples to *F. gigantica.* (C) Naming of the mitochondrial haplogroups based on their inferred evolutionary relationship. (D) Median-joining haplotype network based on the complete mitochondrial genomes of 304 animal-derived *F. hepatica* samples, colored by their geographical origin.

statistic = 2.19; *p*-value = 0.70) (Table 2). *Fasciola hepatica* egg and adult samples obtained from human infections were classified as TCBZ-S or TCBZ-R based on treatment history (**Table S1** and **S2).** Of the seven TCBZ-R samples from human infections, four and three had mitochondrial haplotypes belonging to haplogroups A3 and B1, respectively. All four TCBZ-S samples from humans belonged to haplogroup A3. Six of the seven TCBZ-R human samples shared haplotypes with TCBZ-S samples from either human or animal hosts, suggesting no association between mitochondrial haplotype and TCBZ resistance in human infections.

3.4. Global Fasciola hepatica mitochondrial NADH dehydrogenase subunit 1 genetic diversity

The mitochondrial NADH dehydrogenase subunit 1 (ND1) sequence has been widely used for population genetic analysis in *F. hepatica* [9]. Using the 535 bp ND1 region (NC_002546.1:5183–5717), extracted from the complete mitochondrial genomes of all 315 *F. hepatica* samples from animal and human infections (Figs. 1 and 2), we constructed a haplotype network to examine ND1 haplotype topology among the five haplogroups defined using the full mitochondrial genome sequence (**Fig. S1**). A total of 12 unique ND1 haplotypes were identified. Haplogroups A3 and B1 formed distinct clusters, whereas A1, A2, and B2 could not be fully resolved based on ND1 sequence variation. Nonetheless, haplogroups A and B were clearly distinguishable in 313 of the 315 total samples, with the exception of two B2 haplotypes that clustered with A2.

We expanded the median-joining network by incorporating 1093 previously reported ND1 sequences from samples collected in

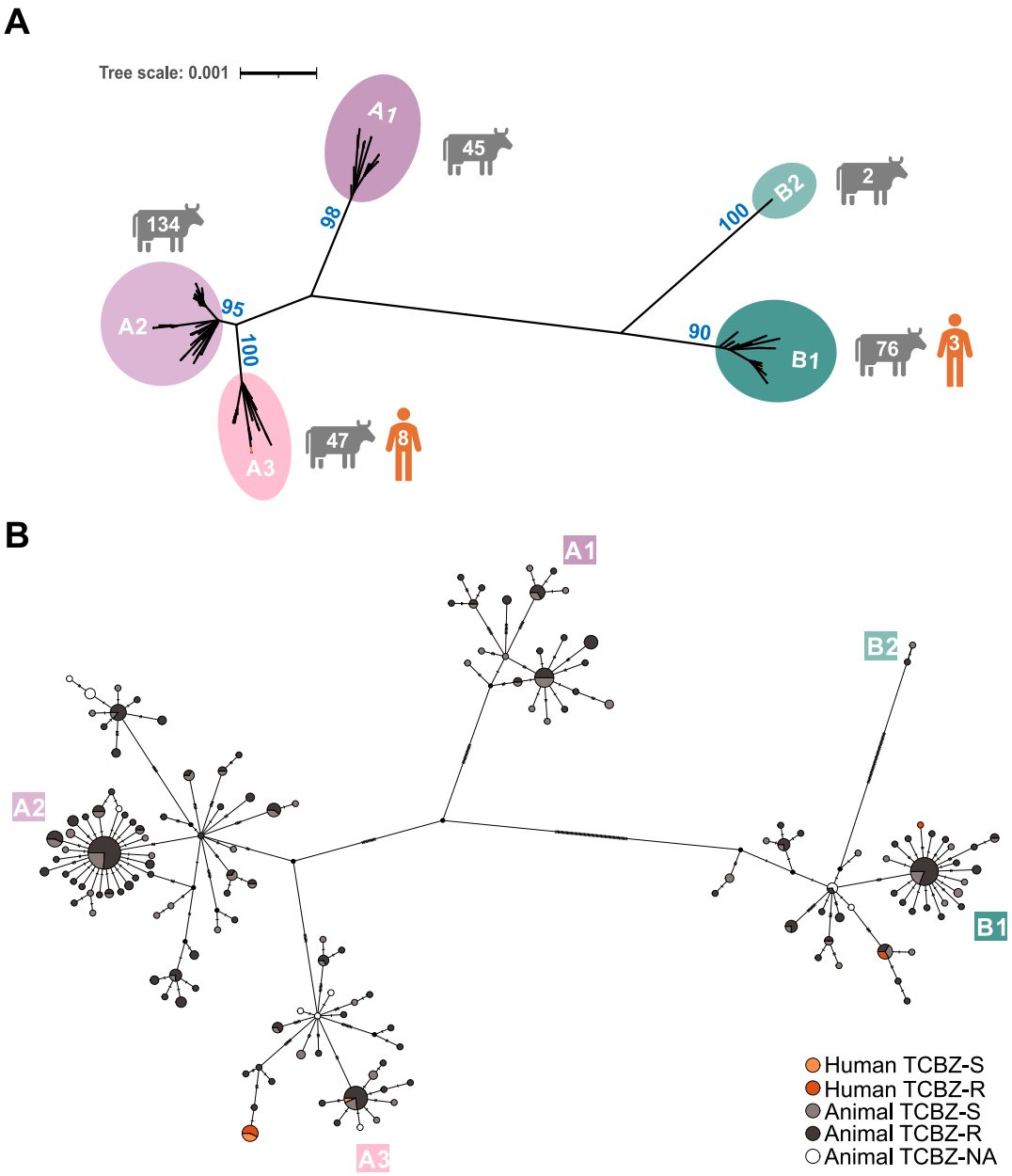

Fig. 2. Phylogenetic placement of *Fasciola hepatica* samples from human infections on a mitochondrial tree and a haplotype network of animal-derived *F. hepatica* samples. *F. hepatica* samples from 11 human infections were placed in a maximum-likelihood phylogeny (A) and a median-joining haplotype network (B) constructed using 304 complete *F. hepatica* mitochondrial sequences from animals.

Table 2
The frequency distribution of triclabendazole sensitivity across mitochondrial haplogroups. No significant association between sensitivity and haplogroup was observed (chi-square statistic = 2.19; p-value = 0.70).

Haplogroup	TCBZ sensitivity			
	Sensitive		Resistant	
	Count	Proportion	Count	Proportion
A1	18	19.8 %	27	13.9 %
A2	37	40.7 %	91	46.9 %
A3	13	14.3 %	28	14.4 %
B1	22	24.2 %	47	24.2 %
B2	1	1.1 %	1	0.5 %

Afghanistan [56], Algeria [57], Armenia [58], Australia [59], Brazil [60], China [61], Ecuador [62,63], Egypt [64], Iran [65–67], Ireland [59], Italy [68], Japan [59], Korea [69], Peru [70], Spain [31,71], and Uruguay [32,59] (**Table S4**). The resulting ND1 haplotype network, comprising 1408 global *F. hepatica* samples, included 127 unique haplotypes, with a nucleotide diversity of 0.0035 and 125 segregating sites (Fig. 3A). Six haplogroups were apparent in this ND1 network. Five corresponded to the haplogroups identified in our full mitochondrial genome-based analysis (**Fig. S1**), while one represented a distinct clade, designated as haplogroup C. Using the haplogroup assignments of the ND1 sequences, we examined the geographic distribution of haplogroups across global regions (Fig. 3B). Across the Eurasian continent, haplogroup A was predominantly distributed in Europe and North Africa, with markedly lower prevalence in Asia. In contrast, haplogroup B was widely distributed across Europe, North Africa, and Asia, with particularly high prevalence in the Far East. Haplogroup C was primarily

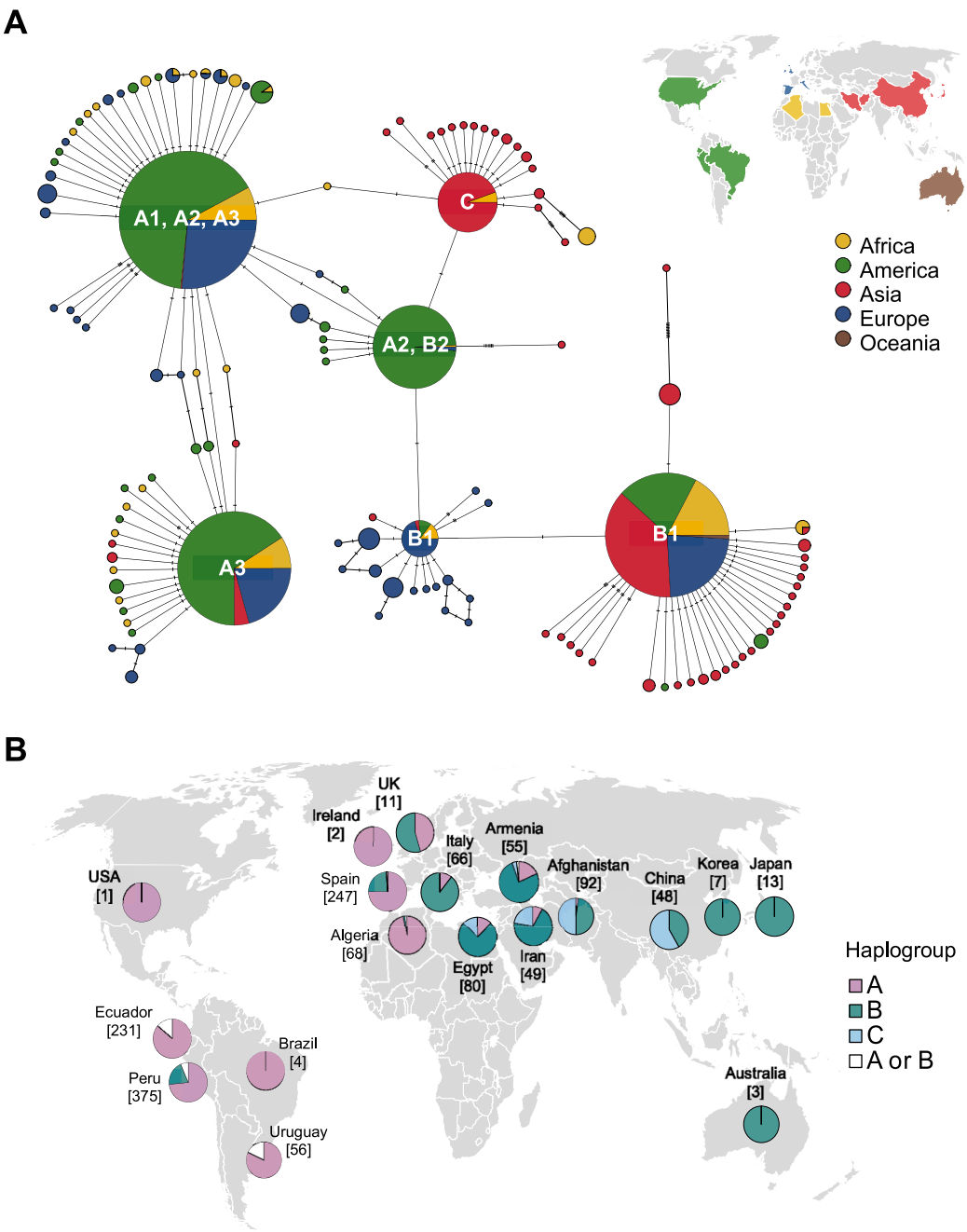

Fig. 3. Median-joining haplotype network of 1408 global *Fasciola hepatica* samples based on the mitochondrial NADH dehydrogenase subunit 1 (ND1) gene. The network incorporates previously published ND1 sequences from North Africa (Algeria, Egypt), the Americas (Brazil, Ecuador, Peru, Uruguay, USA), Asia (Afghanistan, Armenia, China, Iran, Japan, Korea), Europe (Ireland, Italy, Spain, UK), and Oceania (Australia). (A) ND1 sequences are color-coded by geographic origin and labeled by haplogroup. (B) Geographic distribution of haplogroups across global regions. Haplogroup frequencies in each country are represented by pie charts, with sample sizes shown in brackets.

found in Asia, observed at low frequency in Africa, and absent in Europe. In the Americas, both haplogroups A and B were present, whereas in Oceania, only haplogroup B was detected.

4. Discussion

This study provides important insights into the population genetics, transmission patterns, and drug resistance of *F. hepatica* in Peru through a comprehensive mitochondrial genome analysis of samples collected from both human and animal hosts. Our findings provide evidence that human fascioliasis in endemic regions of Peru is zoonotic in origin and lend support to the hypothesis that TCBZ resistance in humans may stem from resistant parasite populations circulating in local livestock.

Mitochondrial whole-genome haplotype analysis identified two basal lineages (A and B) and five well-supported haplogroups (A1–A3 and B1–B2). Notably, mitochondrial haplotypes from human-derived samples in Cusco, Peru, clustered within two haplogroups (A3 and B1) that also contained haplotypes from cattle in the same geographic region (Fig. 2A). The human-derived haplotypes were either identical to or differed by no more than two nucleotides from those found in animals, indicating a shared parasite population between animal and human infections. In the Peruvian highlands, where small-scale livestock farming underpins rural livelihoods, cross-species transmission is likely driven by close human-animal interactions. Our group previously

demonstrated that the proximity of infected livestock to human dwellings was associated with a higher risk of infection among children in the Cusco region [3]. These spatial associations varied by livestock species, with environmental contamination by eggs from cattle stools showing the strongest association with infections in children within households. Sharing a common environment, including water sources potentially inhabited by competent snails, facilitates completion of the *Fasciola* life cycle and transmission of genetically similar parasites across hosts.

Triclabendazole is the only WHO-recommended treatment for fascioliasis in humans and is widely used in veterinary practice. The emergence of TCBZ resistance has become a growing global concern, including in Peru. Our data show that TCBZ-R and TCBZ-S phenotypes are distributed across all identified haplogroups, with no statistically significant association between mitochondrial haplogroup and drug resistance status (Table 2). In human infections, TCBZ-R and TCBZ-S samples clustered within A3 and B1, with multiple resistant haplotypes being genetically indistinguishable from susceptible ones. This suggests that resistance is not linked to mitochondrial inheritance and is likely governed by nuclear genetic variation. Our genome-wide analysis of cattle-derived *F. hepatica* samples in Cusco identified distinct selection signatures in the nuclear genome compared to those observed in the UK, suggesting independent and non-parallel evolution of resistance mutations in geographically diverse *F. hepatica* populations [52].

Our data indicate that *F. hepatica* infecting humans is not genetically distinct from those infecting local cattle, suggesting minimal barriers to gene flow between host species. However, drug selection pressure likely differs between host species, with resistance more readily emerging in livestock populations due to repeated but inconsistent TCBZ exposure. We therefore hypothesize that resistant infections in humans originate from resistant parasite populations circulating in animals. In livestock, TCBZ is frequently administered on a mass scale, often with suboptimal dosing or low-quality formulations, creating conditions that favor the selection and persistence of resistant flukes. By contrast, TCBZ use in humans is most often limited to symptomatic cases, making it unlikely that resistance arises de novo in the human population. These patterns support the interpretation that human TCBZ-R infections are primarily the result of zoonotic spillover from a drug-selected reservoir in livestock.

By placing our mitochondrial genome data in a global context using ND1 sequences from over 1000 additional samples from multiple continents, we identified three broad haplogroups with clear geographic structure (Fig. 3). American samples were confined to haplogroups A and B, with haplogroup A being the most frequent. Haplogroup A was also prevalent among European samples, in contrast to Asian samples, where haplogroup B was highly represented. In addition, a divergent haplogroup C, which was absent in both the Americas and Europe, was found predominantly in Asia, ranging from West Asian countries to China. *Fasciola hepatica* is believed to have originated in the Near East [72] or Asia [73], where it emerged as a distinct species following its divergence from *F. gigantica* or *Fasciola nyanzae* approximately 5 million years ago [34,74]. Haplogroup diversity was highest in Egypt, Armenia, and Iran, where all three haplogroups were detected. This pattern suggests that *F. hepatica* likely originated in a region of the Near East or West Asia near these countries. Its subsequent global spread appears to have involved a reduction in haplogroup diversity, likely driven by founder effects during population expansion. These findings are also consistent with earlier hypotheses suggesting that *F. hepatica* was introduced to the Americas from Europe during the colonial period, followed by localized expansion and limited genetic diversification [31,34,70,72]. The haplogroup frequency distribution in Peru closely mirrors that of Spain, suggesting a strong genetic link between the two populations and reflecting the historical introduction of cattle from Spain [31].

The level of genetic divergence between haplogroups A and B suggests that these lineages emerged prior to the domestication and global spread of ruminant hosts during the Neolithic period (approximately 10,000 years ago), an event that likely facilitated the geographic expansion of *F. hepatica* [72]. A sequence divergence of 0.7 % between haplogroups A and B, based on genome-wide nucleotide substitutions, suggests that these lineages originated between 180,000 and 350,000 years ago, assuming a mitochondrial mutation rate of 2 to 4 % per million years [75]. These estimates are broadly consistent with previous findings based on partial mitochondrial sequences from European and West Asian samples [76–78]. The deep mitochondrial divergence predating the domestication period may have resulted from historical ecological or geographic isolation, although the specific evolutionary processes responsible have yet to be fully understood. This divergence has important implications for the molecular diagnosis of *F. hepatica*, which often relies on mitochondrial DNA markers [79,80]. The presence of highly divergent mitochondrial lineages may reduce the sensitivity of these assays if primers or probes fail to bind all haplotypes effectively, underscoring the need for assay designs that account for the full extent of mitochondrial genetic diversity within the species.

In conclusion, our study indicates that *F. hepatica* infections in humans in Peru are genetically similar to those found in local cattle suggesting zoonotic transmission of the infection, and that TCBZ resistance likely originates from the livestock reservoir. These findings emphasize the importance of integrated One Health approaches and highlight the need for coordinated control strategies and enhanced surveillance encompassing both human and livestock populations.

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Ethics approval statement

The human samples used for this study were stored under future use consent approved by the Research Ethics Institutional Committee of Universidad Peruana Cayetano Heredia (#60574) and by the IRB of University of Texas Medical Branch (#13–080).

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CRediT authorship contribution statement

Pawan Kumar: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Young-Jun Choi:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Martha V. Fernandez-Baca:** Resources, Investigation. **Rodrigo A. Ore:** Resources, Investigation. **Maria L. Morales:** Resources, Investigation. **Pedro Ortiz:** Resources. **Cristian Hoban:** Resources. **Miguel M. Cabada:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization. **Makedonka Mitreva:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

DNA sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive under BioProject PRJNA916254, with sample accessions listed in **Tables S2**.

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