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Genetic structure of *Trichinella britovi* populations in wildlife of north and northeast Iran

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ABSTRACT

Trichinella britovi is a parasite prevalent in the temperate regions of the vast Palearctic realm, including Iran. In this study, we investigated *Trichinella* infection in road-killed animals and carcasses in northern and northeastern Iran by artificial digestion. We assessed species identification and intraspecific genetic diversity using the markers 5S ribosomal DNA intergenic spacer (5S rDNA), internal transcribed spacer I (ITS1), and cytochrome *c* oxidase subunit I (*COXI*). Of the 80 encountered carcasses, 10 had *Trichinella* infection, including seven golden jackals, one wolf, one wild cat, and one wild boar. BLAST analysis exhibited the highest similarities with *T. britovi* sequences in the GenBank database, at 99.79%, 99.84%, and 100% for *COXI*, 5S rDNA, and ITS1, respectively. All 5S rDNA sequences were identical, while analysis using DnaSP software identified eight haplotypes in the ITS1 region and six haplotypes in the *COXI* sequences. The phylogenetic analysis based on the *COXI* marker clustered all *T. britovi* sequences, including those from Iran, into a distinct clade. Furthermore, this marker revealed shallow branching, dividing *T. britovi* sequences into two subclades. The first subclade, the "European" group, consisted exclusively of haplotypes from Poland. In contrast, the second subclade, "Euro-Asiatic," included haplotypes of Asian and European origins. The Euro-Asiatic and European populations exhibited a 0.52% genetic distance while showing 0.59% and 0.15% intrapopulation divergence, respectively. Further studies involving specimens from other regions of Iran, particularly the southeast adjoining the Oriental zoogeographical zone, could provide additional insights into the molecular identity and population structures of *T. britovi* and potentially other species in Iran.

1. Introduction

Trichinellosis is a zoonotic foodborne parasitic disease caused by nematodes of the genus *Trichinella*. It is acquired by consuming raw or undercooked meat harboring the infective larvae of the genus *Trichinella* (Gabriël et al., 2022). Until the early 1970s, *Trichinella spiralis* was the only known *Trichinella* species worldwide (Malone et al., 2024; Pozio et al., 1992; Zarlenga et al., 2020). In the early 1990s, allozyme analysis of *Trichinella* isolates from five continents revealed eight distinct gene pools, defined as T1-T8, of which four represented the previously proposed species, *Trichinella spiralis* sensu stricto (T1), *T. nativa* (T2), *T. pseudospiralis* (T4), and *T. nelsoni* (T7). The remaining four entities, T3, T5, T6, and T8, were distinct from previously described species (La Rosa et al., 1992). Soon after, a taxonomic revision based on biochemical markers (allozyme patterns and ribosomal DNA fragments) and biological features added *Trichinella britovi* (T3) to the list as a new species (Pozio et al., 1992). Today, mitochondrial genome analysis has identified 13 taxa within the genus *Trichinella*, divided into two clades. One clade comprises seven encapsulated species and three genotypes that infect only mammals. The other clade includes three

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non-encapsulated species, which infect mammals and birds (one species) or mammals and reptiles (two species) (Korhonen et al., 2016; Mohandas et al., 2014; Sharma et al., 2020).

Studies in Iran conducted before the 1980s reported *T. spiralis* in wildlife, the only known species at the time. In 1983, Shaikenov and Boev (1983) identified both *T. spiralis* and *T. nelsoni* (today recognized as *T. britovi*) in wildlife of Iran by differential infectivity and cross-breeding experiments on laboratory animals. The advent of DNA-based assays marked a significant advancement in identifying *Trichinella* species. Multiplex PCR and similar assays have identified *T. britovi* in various carnivores and omnivores across different regions of Iran (Borji et al., 2012; Mirjalali et al., 2014; Mowlavi et al., 2009; Rostami et al., 2017; Shamsian et al., 2018).

T. britovi is prevalent throughout the temperate regions of the vast Palearctic realm, including Iran. Recently, in Poland, cytochrome *c* oxidase I (*COXI*) sequence analysis revealed significant intraspecific genetic variations in *T. spiralis* and *T. britovi* populations from different geographical regions, which might be linked to the geographical distribution and infectivity of the strains (Bilska-Zając et al., 2019). Similar studies in other distant geographical areas, particularly in Asia, could contribute valuable data and provide a comprehensive view of the diversity and dispersal of *T. britovi* populations.

In this study, we investigated *Trichinella* infection in road-killed animals and carcasses in northern and northeastern Iran by trichinelloscopy and artificial digestion. We also assessed *Trichinella* intra-species genetic diversity using the genetic markers 5S ribosomal DNA intergenic spacer (5S rDNA), internal transcribed spacer I (ITS1), and *COXI*.

2. Materials and methods

2.1. Study area and sample collection

The study area encompassed Golestan, North Khorasan (Khorāsān-e Shomālī), and Razavi Khorasan (Khorāsān-e Razavi) provinces in the north and northeast of Iran. Between 2017 and 2020, we looked for road-killed animals and other carcasses along highways and local roads. Additionally, carcasses were also reported to us by Department of Environment patrols monitoring the area. Upon arrival at the site, photographs of the carcasses were taken, and their coordinates were recorded. Samples were collected from various anatomical parts, including the tongue, shoulder, waist, neck, femur, rib area (intercostal), and diaphragm. The remaining body parts were appropriately buried. The photographs were sent to two zoologists for identification of animal species. All specimens were transported on ice to the Department of Medical Laboratory, Golestan University of Medical Sciences for further analyses.

2.2. Artificial digestion

About 50–100 g of pooled muscle tissues (Gamble et al., 2000; Mirjalali et al., 2014) were ground and digested in 200–400 ml of a digesting solution containing 1% w/v pepsin and 1% v/v hydrochloric acid, along with glass beads, as described elsewhere (Gajadhar et al., 2019; Li et al., 2010) with minor modifications. The mixtures were stirred for 30 min at room temperature and then incubated overnight at 37 °C. After digestion, the suspensions were filtered through a gauze pad into an Imhoff sedimentation cone. Then, equal volumes of normal saline were added to the sediments, followed by 30 min of incubation at room temperature. The aqueous phase was gently discarded, and the sediments were examined for larvae under a stereomicroscope at 40X magnification. Larvae were collected using Pasteur pipettes and preserved in 70% ethanol at 4 °C until DNA extraction was conducted.

2.3. DNA extraction

According to the manufacturer's instructions, DNA was extracted from individuals or pools of 10 larvae using a commercial tissue genomic DNA extraction Mini Kit (Favorgen, Taiwan).

2.4. PCR and sequencing

Three loci - 5S rDNA, ITS1, and *COXI* - were amplified using primers previously designed and employed by others (Table 1). Each reaction included 15 µl of 2X master mix Red (Ampliqon, Denmark), 10 pmol each of forward and reverse primers, 3 µl of extracted DNA, and deionized-distilled water (DDW) to reach a final volume of 30 µl. The amplification of the 5S rDNA sequence began with an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 95 °C for 40 s, 58 °C for 35 s, and 72 °C for 40 s, with a final 5 min extension at 72 °C. The amplification programs for *COXI* and ITS1 were similar, with annealing temperatures adjusted to 57 °C and 55 °C, respectively. The PCR products were resolved on 2% agarose gels stained with Green Viewer (Eco DNA Safe Stain, Kala Zist, Iran) alongside a molecular marker and visualized under UV.

The amplicons were sequenced bidirectionally using the same primers used for amplification in a commercial company (Codon, Iran). The resulting sequences were manually inspected and corrected using the BioEdit Sequencing Alignment Editor software (version 7.0.5) (Hall, 1999). The forward and reverse sequences were assembled and checked for ambiguities using CodonCode Aligner software (CodonCode Corp.), and consensus sequences were generated.

2.5. BLAST analysis

The consensus sequences were compared with similar *Trichinella* species sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) analysis (http://blast.ncbi.nlm.nih.gov) to determine the similarities.

2.6. Single nucleotide variants (SNVs) and haplotypes

The single nucleotide variants (SNVs) in the sequences were identified visually by examining the chromatograms. Haplotypes were then generated using the software DNA Sequence Polymorphism (DnaSP) version 6, utilizing the "Unphase/genotype file data" option (Rozas et al., 2017).

2.7. Phylogenetic analysis

The 5S rDNA and *COXI* sequences generated in this study and sequences obtained from the GenBank database (Tables S1 and S2) were aligned using the Clustal X algorithm (Thompson et al., 1997). Phylogenetic trees were constructed using MEGA X software (Kumar et al., 2018) employing the Maximum Likelihood method under the General Time Reversible (GTR) model with all bases option. The reliability of the inferred tree was evaluated using 1000 bootstrap (BP) replicates. We could not draw a phylogenetic tree for ITS1, as the sequences from all genospecies were unavailable.

Table 1
The primers used for amplification of *COX*I, 5S rDNA, and ITSI sequences.

Target	Sequence (5′ to 3′)	Expected band	Reference
COX1	TACCTATACTACCTAAGAGGATTCGGA CTAGTACTCATAGTATGGCTGGTG	816 bp	(Franssen et al., 2015; Krivokapich et al., 2008)
5SrRNA	GGAACACGCAGTGTCGTAGAC CATCGGTGCAGTTTTGGTGC	615 bp	Mirjalali et al. (2014)
ITS1	GGCTTCGCGCCGGGAAATTTC TTTAAACCTGATGCACAACAC	582 bp	Erster et al. (2016)

2.8. Genetic distance

Genetic distances among *Trichinella* spp. isolates were computed using MEGA 11.0.10 software, employing the Tajima-Nei model with the pairwise deletion option. The analysis included *COXI* sequences generated in the present study alongside 24 other *T. britovi* sequences and representatives of other genospecies from the GenBank database.

2.9. GenBank accession numbers

The generated sequences were deposited in the GenBank under accession numbers OK483202-OK483216 for ITSI, OK514695-OK514704 for 5S rDNA, and OK445686-OK445700 for *COXI*.

3. Results

3.1. Animal carcasses

We encountered 80 animal carcasses from ten mammal and three bird species in the study area. The majority were from golden jackals, *Canis aureus* (n = 40), followed by stray dogs, *Canis lupus familiaris* (n = 11), wild cats, *Felis* sp. (n = 8), and wild boars, *Sus scrofa* (n = 7) (Table 2).

3.2. Artificial digestion

Artificial digestion revealed the presence of *Trichinella* larvae in ten carcasses from four different carnivore species: golden jackal (n = 3), grey wolf (n = 1), wildcat (n = 1), and wild boar (n = 1). Among these, golden jackals exhibited the highest infection rate at 17.5%, corresponding to their greater abundance in the study area (Table 2). In one wild boar, the recovery of larvae was notably low, with only a single larva obtained from 100 g of tissue, requiring approximately 3 kg of meat to yield ten larvae from this specimen. Most *Trichinella*-infected animals were recorded in Golestan Province in 2019 (Fig. 1 and Table 3).

Table 2
Identification of *Trichinella* infection in carcasses from Golestan, North Khorasan, and Razavi Khorasan provinces using trichinelloscopy and digestion methods.

Animal	Golestan		North Khorasan		Razavi Khorasan		Total	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Golden jackal (*Canis aureus*)	4	26	2	5	1	2	7	33
Stray dog (*Canis lupus familiaris*)	0	4	0	5	0	2	0	11
Grey wolf (*Canis lupus*)	1	0	0	0	0	0	1	0
Fox (*Vulpes vulpes*)	0	0	0	1	0	0	0	1
Wild cat (*Felis* sp.)	1	6	0	0	0	1	1	7
Wild boar (*Sus scrofa*)	1	5	0	1	0	0	1	6
Badger (*Meles meles*)	0	1	0	0	0	0	0	1
Otter (*Lutra lutra*)	0	2	0	0	0	0	0	2
Weasel (*Mustela nivalis*)	0	0	0	1	0	0	0	1
Great Gerbil (*Rhombomys opimus*)	0	0	0	1	0	0	0	1
Eagle (*Aquila chrysaetos*)	0	1	0	0	0	0	0	1
Vulture (*Gyps fulvus*)	0	2	0	0	0	0	0	2
Egyptian Vulture (*Neophron percnopterus*)	0	1	0	0	0	0	0	1
Hawk (*Buteo buteo*)	0	3	0	0	0	0	0	3
Total	**7**	51	**2**	14	**1**	5	**10**	70

Pos.: Positive; Neg.: Negative.

3.3. PCR and electrophoresis

PCR assays followed by electrophoresis resolved amplicons of approximately 615 bp for 5S rDNA, 582 bp for ITS1, and 816 bp for *COXI*.

3.4. BLAST analysis

The 10 5S rDNA sequences generated in this study were identical. Over 100% coverage, they exhibited the highest similarity (identity score: 99.51%–99.84%) with *T. britovi* sequences (acc. nos. AY009943 and GU325733) and 94.33%–99.35% with other encapsulated *Trichinella* species. The ITS1 sequences matched 99.84% among themselves. Over 100% coverage, they exhibited the highest identity (99.28%–100%) with *T. britovi* sequences (acc. nos. KU374883 and KU374881) and 88.62%–98.22% with other encapsulated *Trichinella* species. The 816-bp *COXI* sequences exhibited 99.79% similarity among themselves. Over 100% coverage, they exhibited the highest identity (99.70%) with *T. britovi* ISS120 (acc. no. KM537413), 90.07%–94.38% with other encapsulated species, and 85.27%–85.78% with non-encapsulated species (Table S3).

3.5. Single nucleotide variants (SNVs)

All 5S rDNA sequences were identical, whereas the ITS1 and *COXI* sequences exhibited variations and heterozygosity at certain positions, as evidenced by overlapping bases (Tables 4 and 5).

3.6. Haplotypes

The DnaSP software identified eight haplotypes among ITS1 sequences and six haplotypes among *COXI* sequences. Due to heterozygous single-nucleotide polymorphisms (SNPs), some sequences yielded two haplotypes (Table S4).

3.7. Phylogeny

The 5S rDNA tree resolved two primary clades. The first clade comprised nine encapsulated genotypes, including *T. britovi*, *T.* sp. T9, *T. murrelli*, *T.* sp. T8, *T.* sp. T6, *T. nativa*, *T. patagoniensis*, and *T. nelsoni* share a distant common ancestor with *T. spiralis*. Within this clade, *T. britovi* sequences emerged in two subclades, with *T. murrelli* positioned between them. The second clade contained the three non-encapsulated species: *T. pseudospiralis*, *T. zimbabwensis*, and *T. papuae* (Fig. 2). The *COXI* tree grouped the sequences into two main clades. One clade comprised *T. spiralis*, the most basal species, alongside nine other encapsulated genotypes: *T. britovi*, *T. nativa*, *T.* sp. T6, *T. murrelli, T.* sp. T9, *T.* sp. T8, *T. nelsoni*, *T. patagoniensis,* and recently discovered *T. chanchalensis* (T13). The other clade contained three non-encapsulated species: *T. pseudospiralis*, *T. papuae*, and *T. zimbabwensis*. *T.* sp. T8 emerged as the sister clade to *T. britovi*. *T.* sp. T6 and *T.* sp. T9 appeared as sister taxa to *T. nativa* and *T. murrelli*, respectively. *T. britovi* sequences emerged in two closely related subclades: One subclade, "the European," comprised only haplotypes from Poland, specifically Poland, while the other, termed "Euro-Asiatic," comprised both Asian and European haplotypes.

3.8. Genetic distance

The genetic distance among the 13 *Trichinella* genospecies ranged from 0.62% between *T. nativa* and *T.* sp. T6, to 17.76% between *T. patagonensis* and *T. pseudospiralis*. The Euro-Asiatic and European

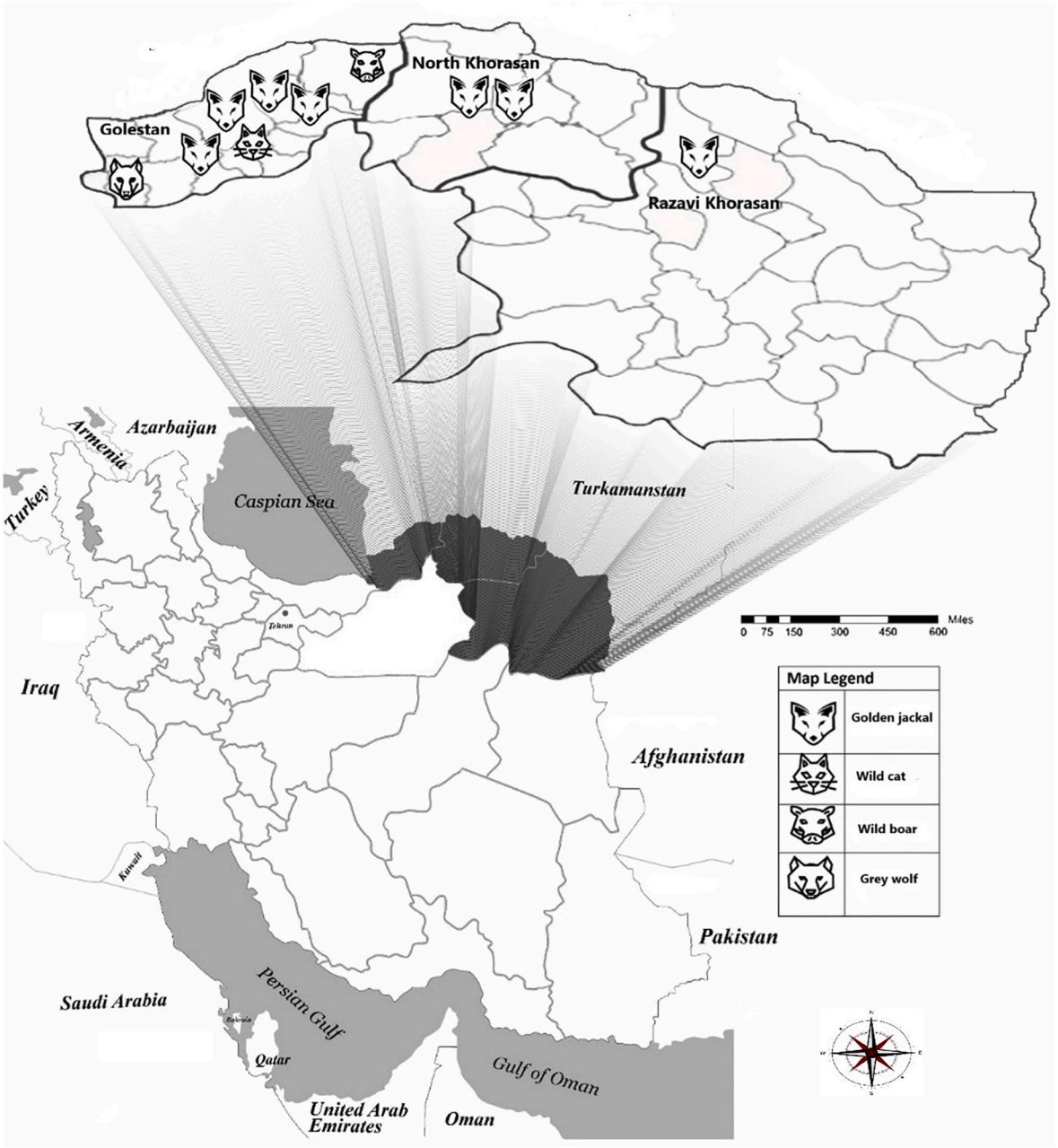

Fig. 1. Map of the study area and *Trichinella*-infected animals.

populations exhibited a 0.52% genetic distance while displaying 0.59% and 0.15% intrapopulation divergence, respectively. They exhibited the least distance (5.89% and 5.65%) from *T.* sp. T8, the genospecies with which they share the most recent common ancestor (Table S5).

4. Discussion

In Iran, trichinellosis surveys of wildlife date back to the early 1960s when *T. spiralis* was the only known species worldwide. In 1960, *Trichinella* larvae were identified in wild boars in northern Iran (Afshar and Jahfarzadeh, 1967). Later, an extensive study from 1967 to 1971 in the same region revealed the larvae in omnivores and carnivores, including wild boars, brown bears, golden jackals, and jungle cats (*Felis chaus*) (Mobedi et al., 1973). In central Iran (Isfahan province), the infection was detected among stray dogs (*Canis lupus familiaris*), golden jackals (*Canis aureus*), red foxes (*Vulpes vulpes*), hyenas (*Hyaena hyaena*), and the great gerbil (*Rhombomys opimus*) (Sadighian et al., 1973). After identifying trichinellosis in wildlife, the disease garnered significant attention, prompting extensive efforts to detect human infections. For instance, the Forensic Medicine Organization of Tehran examined diaphragm muscles from 4838 human cadavers between 1967 and 1970, but no larval infections were found (Eliazian et al., 1976). Recently, anti-*Trichinella* IgG antibodies were detected using Enzyme-linked immunosorbent assays (ELISAs) in communities in northern Iran where hunting and consumption of wild boar meat are practiced (Borji et al., 2012; Koohsar et al., 2021).

With the advent of DNA-based assays, *Trichinella* studies entered a new era. In 2009, multiplex PCR identified *T. britovi* larvae in a leopard (*Panthera pardus saxicolor*) in northwest Iran (Mowlavi et al., 2009) and later in stray dogs and golden jackals in the northeast of the country (Borji et al., 2012; Shamsian et al., 2018). Additionally, other genetic markers such as the internal transcribed spacer 2 (ITS2), mitochondrial small-subunit ribosomal RNA (ssu rRNA), and 5S rDNA confirmed the presence of the same species in wild boars of Mazandaran Province in

Table 3
Details of *T. britovi* isolates in Golestan, North Khorasan, and Razavi Khorasan provinces.

Isolate	Animal	Location	Coordinates	Date of collection
Tb-wG-01	Grey wolf (*Canis lupus*)	Golestan	36.775 N, 54.463 E	Mar. 02, 2019
Tb-jG-02	Golden Jackal (*Canis aureus*)	Golestan	36.816 N, 54.291 E	Jan. 12, 2019
Tb-jG-03	Golden Jackal (*Canis aureus*)	Golestan	37.080 N, 55.151 E	Feb. 10, 2019
Tb-wcG-04	Wild cat (*Felis* sp.)	Golestan	36.853 N, 54.554 E	Mar. 09, 2019
Tb-jG-05	Golden Jackal (*Canis aureus*)	Golestan	36.816 N, 54.291 E	Jan. 12, 2019
Tb-jG-06	Golden Jackal (*Canis aureus*)	Golestan	36.812 N, 54.358 E	Jan. 13, 2019
Tb-wbG-07	Wild boar (*Sus scrofa*)	Golestan	37.369 N, 55.919 E	Jun. 21, 2020
Tb-jNK-08	Golden Jackal (*Canis aureus*)	North Khorasan	37.516 N, 57.507 E	Jun. 17, 2019
Tb-jNK-09	Golden Jackal (*Canis aureus*)	North Khorasan	37.480 N, 57.414 E	Jun. 18, 2019
Tb-jRK-10	Golden Jackal (*Canis aureus*)	Razavi Khorasan	37.000 N, 58.607 E	Nov. 01, 2019

Table 4
SNVs in the Iranian *T. britovi* ITS1 sequences correspond to the base pair numbering in †*T. britovi* iKVI ITS 46 Tb S (Acc. No. KU374883).

Nucleotide positions					
Strain	234	340	375	433	502
†KVI ITS 46 Tb S	T	G	C	G	G
Tb-jNK-08**M**	T	G	C	G	G
Tb-wcG-04**M**	T	G	C	G	G
Tb-wb-07**M**	T	G	C	G	G
Tb-wcG-04**S**	T	G	C	G	G
Tb-jRK-10**S**	T	G	C	G	G
Tb-jNK-09**M**	T	G	C	G	–
Tb-jRK-10**M**	T	A	C	G	G
Tb-JG-03**M**	T	G	C	A	G
Tb-wG-01**M**	T	G	C	G	A
Tb-JNK-08**S**	G	G	C	G	G
Tb-JG-02**S**	**K**	G	C	G	G
Tb-JG-05**M**	G	G	**Y**	G	G
Tb-wG-01**S**	**K**	G	**Y**	G	G
Tb-JG-02**M**	T	G	**Y**	G	A
Tb-JG-06**M**	T	G	**Y**	G	G

S and **M** represent sequences obtained from single and pooled larvae, respectively. **K** = T or/and G; **Y** = T or/and C (according to the IUPAC genetic codes).

the north (Rostami et al., 2017), and golden jackals of Khuzestan Province in southwestern Iran (Mirjalali et al., 2014).

In the present study, we employed three genetic markers to identify the species and characterize the population genetic structure of *Trichinella* nematodes in the north and northeast of Iran. Our generated sequences from these markers indicated the highest identities with *T. britovi*, confirming the presence of this species in the study area. This species has been detected in humans and wildlife in neighboring countries such as Turkey and Armenia (Akkoc et al., 2009; Grigoryan et al., 2020). Consuming raw beef meatballs deceptively mixed with *T. britovi*-infected pork resulted in a trichinellosis outbreak in Izmir, Turkey, in 2004 (Akkoc et al., 2009). We initially extracted DNA from pooled larvae for PCR but found overlapping base pairs in ITS1 and *COXI* sequencing chromatograms. Hence, we decided to check DNA from single larvae to determine whether these variations stem from multi-haplotype infections or were genuine heterozygosity. Iranian isolates showed no divergence at the 5S rDNA locus; all were identical among themselves and with other sequences previously reported from Iran. However, *T. britovi* isolates from Poland exhibited heterogeneity, as defined by SNVs, resulting in seven haplotypes (Bilska-Zając et al., 2019). Nevertheless, variations in ITS1 and *COXI* sequences at specific positions (Tables 4 and 5) resulted in 8 and 6 haplotypes, respectively (Table S4). We observed overlapping base pairs at a few positions in ITS1 and *COXI* sequencing chromatograms from multiple and single larvae DNA (Tables 4 and 5), yielding two haplotypes for some sequences (Table S4). In multiple larvae sequences, overlapping bases might reflect multi-haplotype infections. However, in single larvae, this might suggest heterozygosity in ITS1 and heteroplasmy in *COXI*. The intra-isolate genetic diversity might stem from variation among the mitochondria within an individual larva (Webb and Rosenthal, 2010). Our results revealed some new haplotypes among Iranian sequences, while others were similar to European haplotypes or those previously reported from other regions of Iran. In ITS1, haplotypes Nos. 1 and 3 were identical to haplotypes KVI-ITS-52-Tb-Vv (acc. no. KU374885) and KVI-ITS-46-Tb-Ss (acc. no. KU374883) from Poland. In *COXI*, haplotype No. 2 was similar to haplotype ISS120 (acc. no. KM357413) from Italy and TbS (MH536005) from Poland.

Phylogenetic analysis based on *COXI* differentiated *T. britovi*, including the Iranian isolates, from the closely related species (Fig. 3). However, the 5S rDNA tree showed incongruence with the *COXI* tree, and *T. britovi* sequences branched into two subclades, with *T. murrelli* positioned between them (Fig. 2). This observation aligns with previous reports where *T. britovi* genotypes from Poland appeared in two clades with *T. murrelli* in between, and some genotypes closer to *T.* sp. T9 (acc. no. KP900345) (Bilska-Zając et al., 2019). This marker does not seem helpful for intra-species assessment, particularly when genetically

Table 5
SNVs in Iranian *T. britovi COXI* sequences correspond to the base pair numbering in †*T. britovi* strain ISS120 (acc. No. KM357413).

Strain/Isolates	Nucleotide positions														
	15180	15184	15187	15231	15268	15291	15292	15297	15324	15399	15478	15744	15837	15885	15888
†ISS120	T	T	T	C	C	C	A	T	A	C	T	T	T	T	G
Tb-JNKh-08**M**	T	T	T	C	C	C	A	**Y**	A	**Y**	T	T	T	C	A
Tb-JNKh-08**S**	T	T	T	C	C	C	A	T	A	C	T	T	T	C	A
Tb-JG 02**M**	T	T	T	C	C	C	A	**Y**	A	**Y**	T	T	T	C	A
Tb-JG-02**S**	T	T	T	C	**Y**	C	A	T	A	C	**Y**	T	T	C	A
Tb-JG-03**M**	T	T	T	C	C	C	A	**Y**	A	**Y**	T	T	T	C	A
Tb-JG-05**M**	T	T	T	C	C	C	A	**Y**	A	**Y**	T	T	T	C	A
Tb-JG-06**M**	T	T	T	C	C	C	A	**Y**	A	**Y**	T	T	T	C	A
Tb-wG-01**M**	C	T	T	C	C	C	A	T	A	C	T	T	T	C	A
Tb-wG-01**S**	T	T	T	C	T	C	A	C	A	T	C	T	T	C	A
Tb-wcG-04**M**	T	T	**Y**	C	C	C	A	C	A	T	**Y**	T	T	C	A
Tb-wcG-04**S**	C	T	T	C	C	C	A	T	A	C	T	T	T	C	A
Tb-wbG-07**M**	T	T	T	C	T	C	A	C	A	T	C	T	T	C	A
Tb-jKS-09**M**	–	–	–	C	C	C	A	C	A	T	T	T	T	C	A
Tb-jKR-10**M**	T	T	T	C	**Y**	C	A	C	A	T	**Y**	T	T	C	A

M and **S** represent sequences from multiple and single larvae, respectively. **Y** = T or/and C (according to the IUPAC genetic codes).

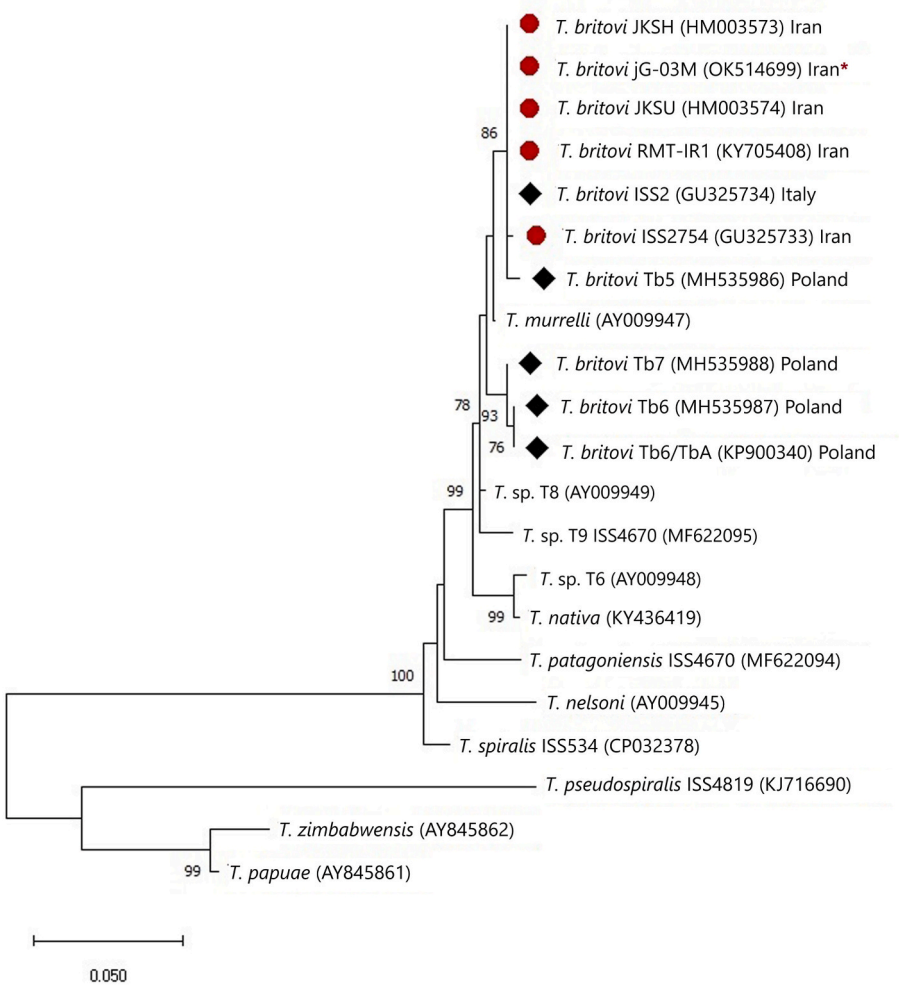

Fig. 2. Maximum Likelihood Tree based on *Trichinella* species 5S rRNA Sequences. The red circles and diamond black squares show Iranian and European haplotypes, respectively. The sequences generated in this study are shown with red asterisks (*). Bootstrap probability (BP) values are indicated on the nodes; BP values (1000 replicates) ≥ 70% are shown at the nodes. The scale bar represents 0.05 estimated nucleotide substitutions per site, indicating evolutionary distances between sequences.

closely related species are assayed alongside. The *COXI* tree *T. britovi* sequences displayed further shallow sub-branching, diverging into two subclades. One subclade, "the European," comprised only haplotypes from Poland, while the other, a mixed group termed "Euro-Asiatic," comprised both Asian (Iran and India) and European (Poland, Italy, and Iran) haplotypes. Based on *COXI*, *T. britovi* isolates from Europe and Asia showed the least genetic distance (5.65–5.89%) with *T.* sp. T8, the genospecies with which they share the most recent common ancestor (Table S5). Previously, cross-breeding assays demonstrated high genetic compatibility between these two genospecies. For instance, cross-breeding between single males and females of *T. britovi* and *T.* sp. T8 resulted in progenies in both directions, whereas hybridization between *T. nelsoni* and *T.* sp. T8 yielded no offspring (Marucci et al., 2009).

The high infection rate of golden jackals (17%), the most encountered road-killed animal in the present study, corroborates previous reports, highlighting this animal as a sentinel species for trichinellosis surveillance in Iran (Mirjalali et al., 2014; Mowlavi et al., 2009). Similar studies in southwest Iran (Khuzestan Province) and northeastern Iran (Razavi Khorasan Province) reported *Trichinella* sp. infection rates of 6.25% and 8.3% among golden jackals (Mirjalali et al., 2014; Shamsian et al., 2018). All molecular surveys conducted in Iran consistently indicated only the presence of *T. britovi*. In some regions of the Middle East, mixed infections of *T. britovi* and *T. spiralis* have been reported (Erster et al., 2016), possibly resulting from *T. spiralis* spillover from pig farms to wildlife.

Further studies involving specimens from other regions of Iran, particularly the southeast adjoining the Oriental zoogeographical zone, could add insights into the molecular identity and population structures of *T. britovi* and potentially other species in Iran.

5. Conclusion

In the present survey, artificial digestion detected *Trichinella* larvae in 10 carcasses from four different carnivore species. The high infection rate (17%) of golden jackals in Iran underscores their role as sentinel animals for trichinellosis surveillance. Among the three genetic markers used for phylogenetic analysis, *COXI* revealed the highest resolving power and differentiated *T. britovi* from its closely related species. Additionally, this marker displayed shallow branching and split *T. britovi* sequences into European and Euro-Asiatic subclades. The 5S rDNA tree, on the other hand, was incongruent with the *COXI* tree, suggesting that this marker may be too conserved for intra-species assessment. Variations in ITS1 and *COXI* sequences at specific positions revealed 8 and 6 haplotypes, respectively, among Iranian isolates.

CRediT authorship contribution statement

Faramarz Koohsar: Visualization, Investigation, Formal analysis.

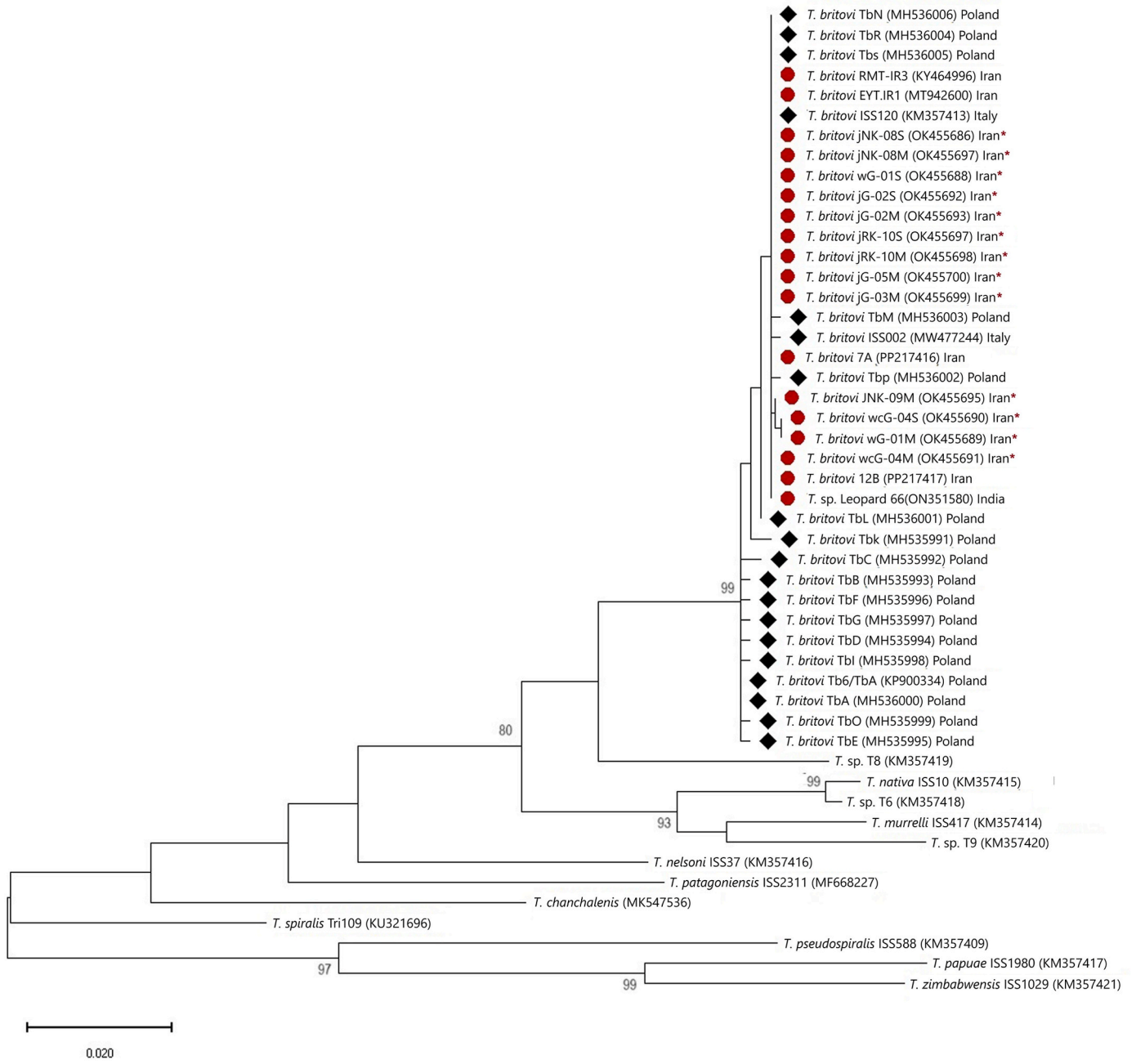

Fig. 3. Maximum Likelihood Tree based on *Trichinella* species *COXI* sequences. The red circles and diamond black squares show Iranian/Asian and European haplotypes, respectively. The sequences generated in this study are shown with red asterisks (*). Bootstrap probability (BP) values are indicated on the nodes; BP values (1000 replicates) $\geq$ 70% are shown at the nodes. The scale bar represents 0.02 estimated nucleotide substitutions per site, indicating evolutionary distances between sequences.

Saied Reza Naddaf: Writing – review & editing, Writing – original draft, Software, Methodology, Data curation, Conceptualization. **Hamed Mirjalali:** Writing – original draft, Methodology, Investigation. **Mehdi Mohebali:** Methodology, Data curation. **Mohammad Bagher Rockni:** Resources, Methodology. **Ahmad Mahmoudi:** Software, Methodology. **Gholamreza Mowlavi:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Conceptualization.

Consent to participate

All authors above consented to the submission and subsequent revisions submitted by the corresponding authors.

Ethics approval

The Center for Research of Endemic Parasites of Iran (CREPI), Tehran University of Medical Sciences approved this project (Code No. IR.TUMS.SPH.REC.1396.3996).

Code availability

The ethical committee of Tehran University of Medical Sciences approved this study (Code No. Ir.tums.sph.rec.1396.3996).

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2024.101032.

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