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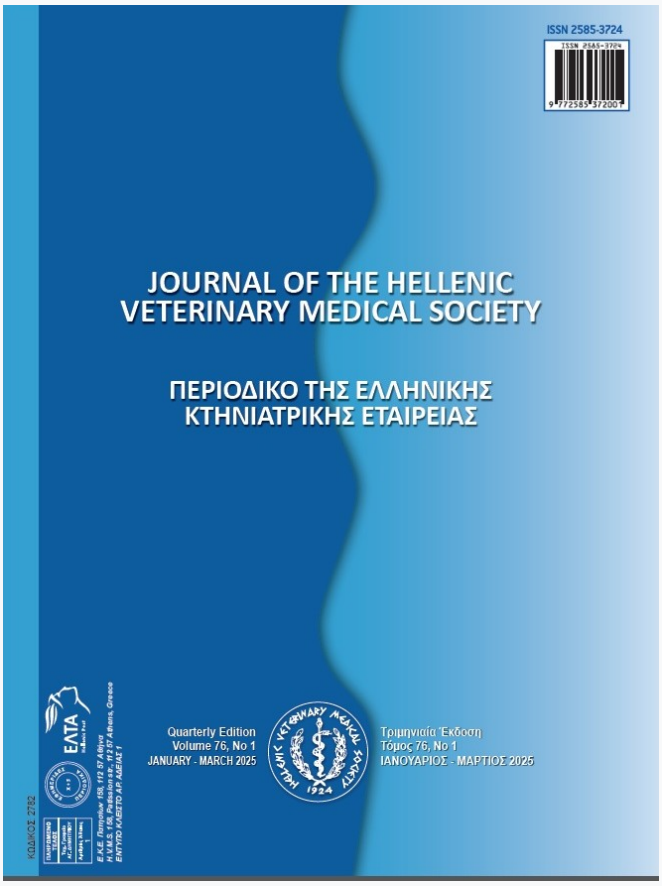

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Research article
Ερευνητικό άρθρο

Genetic Variability and Haplotypes of *Echinococcus equinus* in a Donkey in Türkiye

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ABSTRACT: Cystic echinococcosis (CE) is a parasitic zoonosis caused by *Echinococcus granulosus* sensu lato larva. This study was conducted for the molecular characterization of CE cysts obtained from a donkey naturally infected with CE cysts, and for the determination of haplotype diversity. Ten individual CE cysts localized in a donkey's liver were investigated. Genomic DNA was extracted from each individual cyst isolate. PCR product of 875 bp fragment of the mitochondrial cytochrome c oxidase subunit 1 (mt-CO1) gene was amplified by using specific primers. The PCR products were purified and sequence analysis was performed. The partial sequences of the mt-CO1 gene belonging to the CE cyst isolates were compared with published reference sequences. Partial sequences of mt-CO1 were identified as *E. equinus*. After haplotype analysis, two different haplotypes (Hap01 and Hap02) were identified. Nine sequences obtained from this study resided in the main haplotype (Hap01); however, another donkey sequence from this study was located in Hap02 with a nucleotide difference. Although *Echinococcus granulosus* s.s. (G1 and G3) is reported to be the dominant species, and the current findings showed that *E. equinus* (G4) and its haplotypes could also circulate in donkeys from Türkiye.

Keywords: *Echinococcus equinus;* Donkey; Haplotype; Türkiy,

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INTRODUCTION

Cystic echinococcosis (CE) is a zoonotic disease caused by the larval form of *Echinococcus granulosus* sensu lato (s.l.) (Romig et al. 2017), which affects livestock and causes economic losses in farm animals (Torgerson 2003), wild animals, and humans. Molecular and taxonomic studies conducted in recent years have shown that *Echinococcus* genus comprises five species: *E. granulosus* sensu lato, *E. multilocularis*, *E. oligarthrus*, *E. vogeli* and *E. shiquicus*. *E. granulosus* s.l. complex includes *E. granulosus* s.s. (G1/G3), *E. equinus* (G4), *E. ortleppi* (G5), *E. canadensis* (G6/7 and G8-G10) and *E. felidis* species (Vuitton et al., 2020). The studies have shown that *E. granulosus* s.s. (G1 and G3) are the dominant species, and *E. equinus* (G4), *E. ortleppi* (G5), and *E. canadensis* (G6/G7) have been reported in Türkiye (Utuk et al. 2008, Saarma et al. 2009, Simsek et al. 2010, Simsek et al. 2015, Kinkar et al. 2016, Kinkar et al. 2018, Kinkar et al. 2018, Avcioglu et al. 2021). Till date only four studies have been conducted on molecular epidemiology and genotyping of CE in equine from Türkiye (Kesik et al. 2019). Initially, *E. granulosus* s.s. was reported in a horse isolate (Utuk and Simsek 2013). Then, *E. equinus* was found in mule (Simsek and Cevik 2014) and later in donkeys (Simsek et al. 2015, Kesik et al. 2019). Notably, there have been some reports on the occurrence and molecular characterization of CE cysts in equids. The prevalence of CE in horses is less than 1% in Italy (Varcasia et al. 2008), whereas the prevalence in donkeys is 2% in Iran (Eslami et al. 2014). *Echinococcus equinus* (G4) was detected in 11 horses in Spain (González et al. 2002), and *E. granulosus* s.s. (G1) and *E. equinus* (G4) were determined in 10 donkeys in Tunisia (Boufana et al. 2014), and *E. equinus* (G4) has been identified in horses in the Sardinia, Tuscany, and Sicily island of Italy (Scala et al. 2006, Varcasia et al. 2008). It has been reported that genetic diversity is greater in *E. granulosus* s.s. (Utuk et al. 2008, Šnábel et al. 2009, Simsek et al. 2010, Simsek et al. 2011, Simsek et al. 2015, Kinkar et al. 2016). Importantly, most studies regarding genetic variability in *E. granulosus* s.s. have focused on the examination of a single cyst sample from each intermediate host (Konyaev et al. 2013, Roinioti et al. 2016, Dán et al. 2018, Yan et al. 2018) overlooking the possibility that naturally infected intermediate hosts may have multiple CE cysts. Hidalgo et al. (2020) previously asserted that the occurrence of multiple haplotypes in the same intermediate host could be beneficial for recognizing the transmission dynamics of *E. granulosus* s.s. in highly endemic areas. They showed for the first time that more than five mt-CO1 haplotypes of *E. granulosus* s.s. can be present in the same animal as CE. In population genetics, computation of gene flow index among different endemic foci of *Echinococcus* isolates can be provided a valuable data concerning epidemiological drift of parasite, allele frequencies and speciation (Spotin et al. 2017). Similarly, the possible presence of different haplotypes in the same host may be useful for identifying the transmission dynamics of *E. equinus*. Naturally infected hosts may have multiple CE cysts, and we hypothesized that donkeys may harbor more than one haplotypes in an area endemic to Türkiye. Moreover, we could not find any reports of haplotype differences among CE cysts located in the same organs in donkeys. Therefore, the aim of the current study was to evaluate the molecular characteristics and haplotype diversity of the cysts collected from a donkey naturally infected with CE cysts in Türkiye.

MATERIALS AND METHODS

Sample Collection

Ten individual CE cysts were isolated from the liver of a dead donkey naturally infected with CE at the Pathology Department of the Veterinary Faculty of Firat University. The germinal membranes were collected separately and preserved in 70% ethanol. The cyst material was sent to the Faculty of Veterinary Medicine's Parasitology Department at Firat University for further analysis. Each collected cyst was stored in a different Eppendorf tube to check for any nucleotide differences among the cysts that were stored at -20 °C until use.

Genomic DNA Isolation

Total genomic DNA was isolated from each individual cyst sample using the Hibrigen Tissue Kit (Hibrigen, Türkiye), according to the manufacturer's instructions, with some modifications. The germinal membranes were sliced using a lancet, transferred to 1.5 ml eppendorf tubes, and washed with PBS at least five times to remove traces of ethanol. The cyst tissues were digested with lysis buffer by adding 20 µl (20 mg/ml) proteinase-K, then incubated overnight at 65°C. The gDNA was then isolated using the kit procedures and stored at -20°C until use.

PCR Amplification

The partial mt-CO1 gene was amplified using the

primer sets F/CO1 (5'-TTGA ATTTGCCACGTTT-GAATGC-3') and R/CO1 (5'-GAACCTAACGA-CATAACATAATGA -3') (Nakao et al. 2000). PCR reaction was carried out in a final volume of 50 µl, containing 5 µl of 10× PCR buffer, 5 µl of 25 mM $MgCl_2$, 400 µM of each dNTPs, 20 pmol of each primers, 0.2 µl of TaqDNA Polymerase (1.25 IU), 28.8 µl PCR grade water and 5 µl of gDNA. Amplification was performed according to the following thermal profile: an initial denaturation step of 10 min at 94 °C; 30 cycles of 30 s at 94 °C, 45 s at 52 °C, and 1 min at 72 °C; and a final extension step of 10 min at 72 °C. PCR was performed using a thermal cycler (SensoQuest GmbH, Germany) and the products were separated by electrophoresis on a 1.4% agarose gel. The amplified bands were cut from the gel under UV light and purified using the GeneJET Gel Extraction Kit (Thermo Scientific). Unidirectional sequence analysis was performed using a forward primer (F/CO1) (BM Labosis, Türkiye).

Phylogenetic Analysis

The chromatogram quality was checked using FinchTV 1.4.0 (Geospiza Inc., Seattle Washington, USA) (http://www.geospiza.com). The sequence ends were trimmed to a final length of 779 bp by comparing the published sequences in PubMed. Sequence alignment was performed with the use of "CLC Sequence Viewer 8" (Knudsen et al. 2007). The alignment was performed using published reference sequences retrieved from NCBI PubMed which were shown in Fig. 2. Sequences were analyzed with MEGA X (Kumar et al. 2018). ClustalW was used to generate various output formats, including PHYLIP, NEXUS, and FASTA, which were suitable for sequence alignment and subsequent analysis. The most suitable model for the phylogenetic tree was determined using MEGA X (Kumar et al. 2018). The phylogenetic tree was then inferred using the Maximum Likelihood method and Tamura-Nei and Gamma distribution (TN93+G) model (Tamura and Nei 1993). Statistical support for specific clades was obtained via 1,000 bootstrap replicates.

Haplotype Networks, Nucleotide Polymorphism, Diversity and Neutrality Indices

PopART- 1.7 (Population Analysis with Reticulate Trees) software was used for the detection of haplotype diversity (Leigh et al. 2015). Haplotypes were created using Median Joining or Minimum Spanning Networks and the relationships among haplotypes were displayed. The data were then added to DnaSP 6 (Rozas et al. 2017). Population diversity indices, such as the number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π), and neutrality indices, including Tajima's D (Tajima 1989), Fu's Fs, Fu's LD, and Fu's LF, were calculated using DnaSP 6 (Rozas et al. 2017). The statistical significance of Tajima D, FLD, and FLF was determined using 10.000 coalescent simulations in DnaSP.

RESULTS

Ten individual CE cysts were isolated from the liver of a naturally infected donkey (Fig 1). All isolates were successfully amplified by PCR using mt-CO1 primers as previously described. After BLAST search

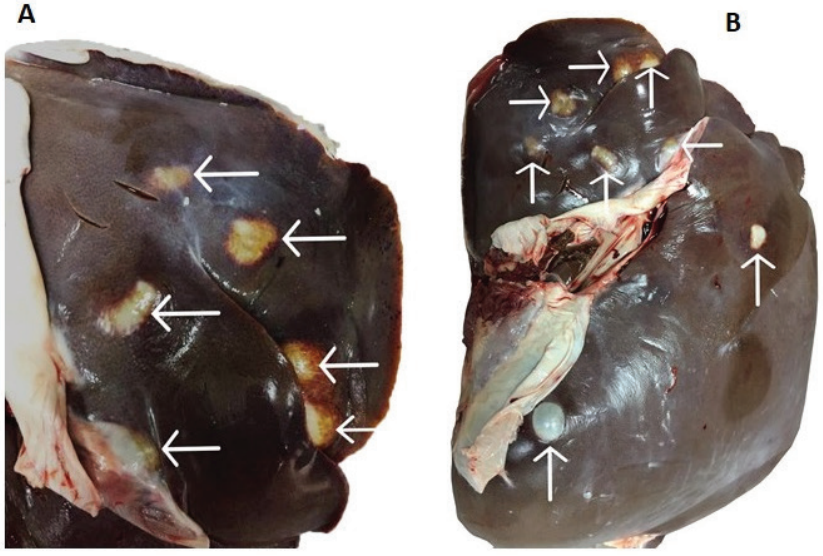

Figure 1. Hydatid cysts in the liver of a donkey. A: Diaphragmatic surface; B: Visceral liver surface.

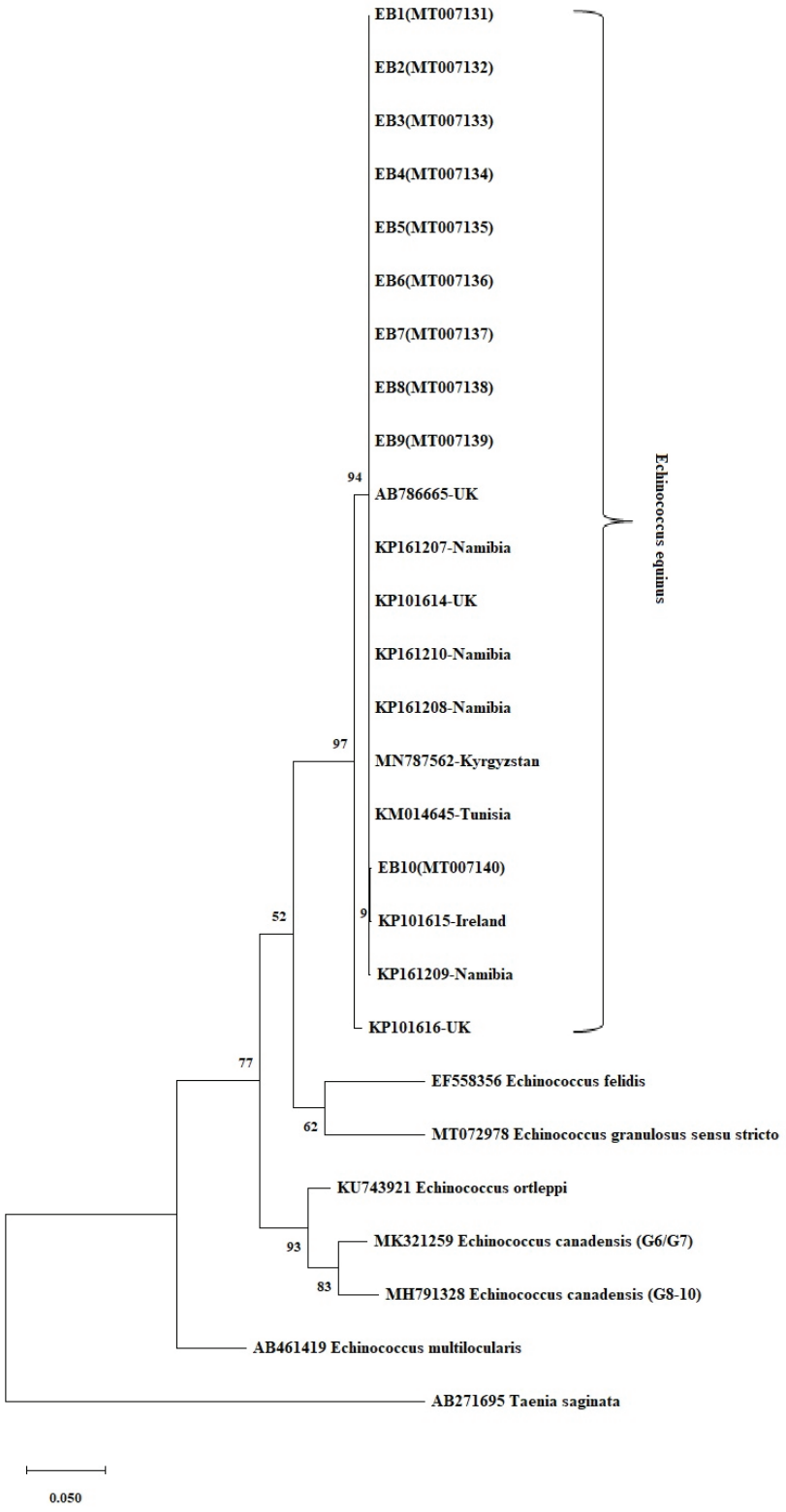

Figure 2. Phylogenetic tree of the isolates using partial mt-CO1 (875 bp) sequences. EB1-EB10: Sequences of donkey isolates in the current study. *E. equinus* (AB786665-UK, KP161207-Namibia, KP101614-UK, KP161210-Namibia, KP161208-Namibia, MN787562-Kyrgyzstan, KM014645-Tunisia, KP101616-UK, KP101615-Ireland, KP161209-Namibia), *E. felidis* (EF558356), *E. granulosus* s.s. (MT072978), *E. canadensis*, G6/G7 (MK321259), *E. canadensis*, G8/G10 (MH791328), *E. ortleppi* (KU743921), *E. multilocularis* (AB461419) and *Taenia saginata* (AB271695) were used as the reference sequences. MEGA X was used to construct a Maximum Likelihood tree based on the TN93+G model. The reliability of the tree was assessed using 1,000 bootstrap replications.

and sequence analysis, 100% identity was detected in nine sequences (EB1-9), while the similarity was 99.87% in the other sequence (EB10). A polymorphic site was detected in the cyst (EB10) in the mt-CO1 sequence of *E. equinus*. All mt-CO1 sequences were deposited in GenBank (accession nos. MT007131-MT007140). The alignment of the sequences, including the reference sequences, is shown in Suppl. Fig. 1. Our sequences (EB1-EB10) and previously published sequences of *E. equinus* (AB786665-UK, KP161207-Namibia, KP101614-UK, KP161210-Namibia, KP161208-Namibia, MN787562-Kyrgyzstan, KM014645-Tunisia, KP101616-UK, KP101615-Ireland, and KP161209-Namibia) were found in the same cluster in the phylogenetic tree. The second-closest group was *E. granulosus* s.s. and *E. felidis*, whereas the farthest was *Taenia saginata* (Fig. 2). Within the partial mt-CO1 sequences of *E. equinus*, 10 sequence data (EB1-EB10) from the current study and another 10 sequences downloaded from GenBank were used to create the haplotype network, and five different haplotypes were determined (Table 1 and Fig. 3). Nine sequences (EB1-EB9) (MT007131-MT007139) obtained in this study resided in the main haplotype (Hap01) together with the other reference sequences (AB786665-UK, KP161207-Namibia, KP101614-UK, KP161210-Namibia, KP161208-Namibia, MN787562-Kyrgyzstan, KM014645-Tunisia) however, another donkey sequence (EB10) (MT007140) from this study was located in Hap02 with one nucleotide exchange. A sequence from the UK (KP101616) was observed in Hap03, a sequence from Ireland (KP101615) in Hap04 and a sequence from Namibia (KP161209) was shown to be present in Hap05. Fur-

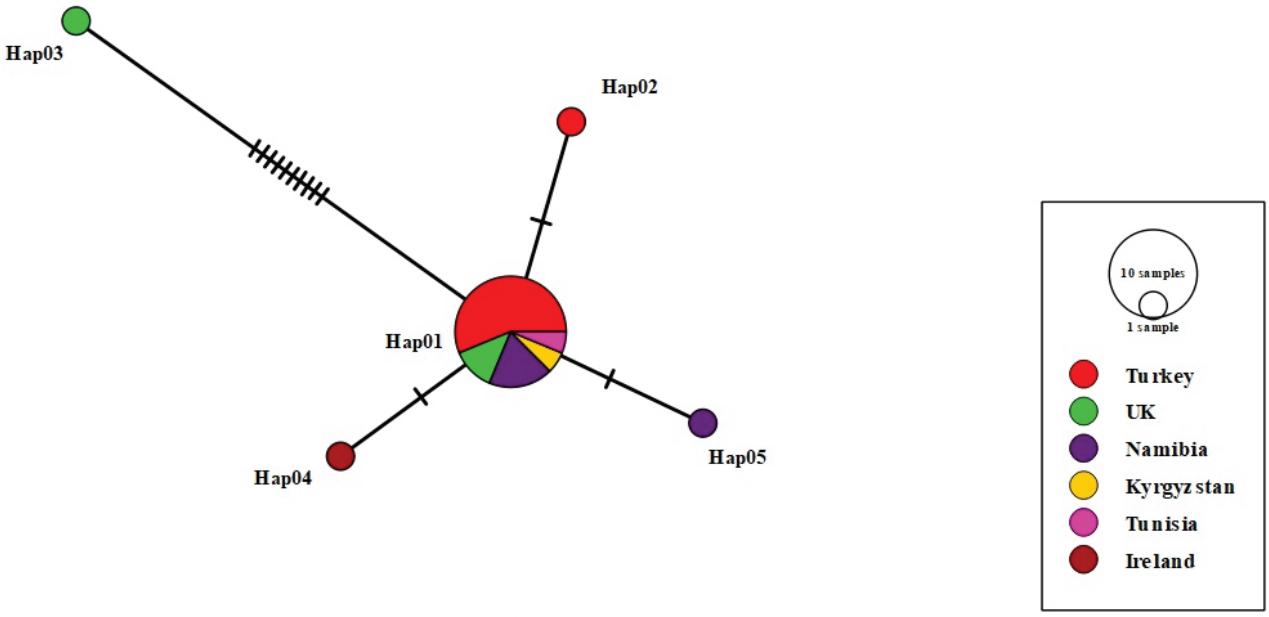

Figure 3. Appearance of mt-CO1 haplotypes of donkey isolates in the current study and published *Echinococcus equinus* sequences. The size of the circles was related to haplotype frequency. The number of mutations distinguishing the haplotypes is shown as hatch marks.

Table 1. Grouping haplotypes of mt-CO1 sequences of *Echinococcus equinus* and accession numbers of isolates (EB: Sample names in the current study).

Haplotype names	Number of isolates	Accession Numbers
Hap01	16	EB1 (MT007131), EB2 (MT007132), EB3 (MT007133), EB4 (MT007134), EB5 (MT007135), EB6 (MT007136), EB7 (MT007137), EB8 (MT007138), EB9 (MT007139), AB786665-UK, KP161207-Namibia, KP101614-UK, KP161210-Namibia, KP161208-Namibia, MN787562-Kyrgyzstan, KM014645-Tunisia
Hap02	1	EB10 (MT007140)
Hap03	1	KP101616-UK
Hap04	1	KP101615-Ireland
Hap05	1	KP161209-Namibia

Table 2. Diversity and neutrality indices obtained by using nucleotide data of partial mt-CO1 gene of *Echinococcus equinus*.

mt-DNA	n	hn	hd ± SD	πd ± SD	Tajima's D	Fu's Fs	FLD	FLF
CO1	20	5	0,368±0,135	0,00167±0,00113	-2,34049 (P<0.01)	-0,446 (P=0,205)	-3,51348 (P<0.02)	-3,68144 (P<0.02)

n: Number of isolates, hn: number of haplotypes; hd: haplotype diversity; πd: nucleotide diversity; SD: standard deviation; FLD: Fu and Li's D test statistic; FLF: Fu and Li's F test statistic

Table 3. Nucleotide variation positions of the mt-CO1 (779 bp) gene among 5 haplotypes analyzed.

Nucleotid position	9	12	21	27	39	334	401	543	585	606	642	704	777
NC_020374 (Reference sequence)	A	T	T	A	G	C	T	T	A	T	T	G	T
Hap01	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap02	.	.	.	.	.	.	.	.	.	.	.	.	A
Hap03	G	A	G	G	.	T	A	A	G	G	.	A	.
Hap04	.	.	.	.	.	.	.	.	.	.	A	.	.
Hap05	.	.	.	.	T	.	.	.	.	.	.	.	.

thermore, a difference of ten nucleotides was detected between Hap03 and the main haplotype (Hap01). In this study, we searched for the occurrence of genetic changes within *E. equinus* isolates using four neutrality tests (Fu's Fs, Tajima's D, Fu's LD, and Fu's LF values). The diversity and neutrality indices are listed in (Table 2). The Tajima D, Fu's LD, and Fu's LF values were negative and not statistically significant. The nucleotide variation positions of the mt-CO1 (779 bp) gene among the 5 haplotypes analyzed based on the reference sequence are shown in Table 3.

DISCUSSION

Four *Echinococcus* species, *E. granulosus* s.s. (G1 and G3), *E. equinus* (G4), *E. ortleppi* (G5), and *E. canadensis* (G6/G7) have been circulating in intermediate and definitive hosts of *E. granulosus* s.l. in Türkiye. However, studies have shown that *E. granulosus* s.s. is the dominant species (Utuk et al. 2008, Šnábel et al. 2009, Simsek et al. 2010, Simsek et al. 2011). *E. granulosus* s.s. (G1 and G3) were detected in horses, whereas the presence of *E. equinus* in donkeys and mules was confirmed by mitochondrial gene sequences (Utuk and Simsek 2013, Simsek and Cevik 2014, Simsek et al. 2015). However, there are no reports on the haplotypes of *E. equinus* in Türkiye. Although there are several published reports on CE from equine hosts, reports on the haplotypic profiles of *E. equinus* are limited. In Tunisia, Boufana et al. (2014) performed genetic diversity and haplotype analyses on 174 CE cyst isolates, 35 of 174 belonged to a donkey. Considerable genetic variation was observed only in the mt-CO1 sequences of *E. granulosus* s.s. (G1 and G3), and only a single haplotype (EqTu01) was identified in the mt-CO1 nucleotide sequence of *E. equinus* (Boufana et al. 2014). In the current study, 10 CE cysts localized to the liver of a donkey were identified after necropsy. After mt-CO1 sequence analysis of our samples, *E. equinus* was identified in all isolates, and two haplotypes (Hap01 and Hap02) were detected.

In the current study, it was concluded that having more than one haplotype in a single organ can be more complex. Theoretically, this can be explained by the exposure to subsequent infections throughout the life of the intermediate host. Some reports support this theory. Shariatzadeh et al. (2015) reported a mixed infection with G1/G3 and G6 genotypes of *E. granulosus* in stray dogs. In addition, two different genotypes (G1 and G6) have been identified in a single dog (Kamenetzky et al. 2002, Bart et al. 2006). Hidalgo et al., (2020) found 16 different haplotypes in 66 echinococcal cysts from 10 animals (cattle and sheep) and stated that both cattle and sheep can accommodate up to five different *E. granulosus* s.s. haplotypes at the same time. In this study, we report that two distinct mt-CO1 haplotypes of *E. equinus* are found in the liver of a naturally infected donkey. The existence of multiple haplotypes in the same host can be explained by consecutive infections of the intermediate host during its life or by a unique infection from the stool of a final host simultaneously harboring adult worms of multiple haplotypes.

Although *E. granulosus* s.s. (G1 and G3) is reported to be the dominant species, and the current find-

ings showed that *E. equinus* (G4) and its haplotypes could also circulate in donkeys from Türkiye. More comprehensive studies on equidae will provide more comprehensive data on haplotype diversity and new data on the genetic variation of *E. equinus*.

ACKNOWLEDGEMENT

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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Supplementary material

Supplementary Figure 1. Alignment of different isolates using partial mt-CO1 (875 bp) sequence. EB1-EB10: Sequences of donkey isolates in the current study. *E.equinus* (AB786665-UK, KP161207-Namibia, KP101614-UK, KP161210-Namibia, KP161208-Namibia, MN787562-Kyrgyzstan, KM014645-Tunisia, KP101616-UK, KP101615-Ireland, KP161209-Namibia), *E. felidis* (EF558356), *E. granulosus* s.s. (MT072978), *E. canadensis*, G6/G7 (MK321259), *E. canadensis*, G8/G10 (MH791328), *E. ortleppi* (KU743921), *E. multilocularis* (AB461419) and *Taenia saginata* (AB271695) were used as the reference sequences.