

## SHORT COMMUNICATION

# Molecular Epidemiology of Rabies in the Eastern and Southeastern Anatolian Regions of Türkiye, 2016-2021

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## Abstract

Rabies can be transmitted through biting, contact of infected saliva with damaged skin, and high levels of aerosol exposure. Dog and fox-mediated rabies are endemic in Turkey. In this study, the N gene and the G-L intergenic region sequences were obtained from rabies-positive brain samples from Eastern and Southeastern Anatolia (2016-2021). These sequences were analyzed with sequences available in GenBank. They were used to create phylogenetic trees, demonstrating that all sequences from this study belong to the three different subclades (ME1a, ME2 and, CA2) of Cosmopolitan clade. This study provides a better understanding of the molecular epidemiology of rabies virus in the mentioned regions will have benefits for continuing comprehensive rabies surveillance, prevention and control in Türkiye.

**Keywords:** Eastern and Southeastern Anatolia, G-L intergenic region, Rabies virus, N gene

## INTRODUCTION

Rabies is a zoonotic infection that is transmitted from infected animals to humans through biting, contact of infected saliva with damaged skin or mucous membranes, and rarely, through high levels of aerosol exposure to the virus. Human-to-human transmission of rabies is an exceptional occurrence and has been reported only in cases of tissue and organ transplantation<sup>[1]</sup>.

Rabies virus (RABV) belongs to the *Mononegavirales* order, *Rhabdoviridae* family, and *Lyssavirus* genus. Lyssaviruses are divided into seven genotypes and two phylogroups, comprising 17 species. The rabies virions are bullet-shaped, have a non-segmented, single-stranded negative RNA genome of about 12 kb, are enclosed within a helical nucleocapsid and surrounded by an envelope with glycoprotein spikes measuring 5-10 nm in size. Like all lyssaviruses, rabies virus contains five main genes, which are flanked by non-coding intergenic regions in a conserved order, namely 3'-N-P-M-G-L-5'<sup>[2]</sup>. The

N gene, being highly conserved and easily detectable through RNA-based methods, is the most common target for diagnostic tests and phylogenetic typing. Among the non-coding intergenic regions, the G-L intergenic region is located between the G and L genes, which is highly susceptible to mutations. There is no immunological selection pressure on the G-L region, and the most variable region of the rabies virus genome. In general, studies of RABV phylogeny and dispersion have been performed through analysis of the complete G gene, G-L intergenic region, and/or partial or complete analysis of N gene<sup>[3-9]</sup>.

For a long time in Türkiye, stray dogs have been considered the main reservoir of rabies virus<sup>[6,7]</sup>. It is known that interactions between stray animals and wild animals in the same areas create opportunities for transmission to different hosts<sup>[6,7]</sup>. In our country, rabies cases in red foxes (*Vulpes vulpes*) have been reported consistently, with occasional occurrences<sup>[7,8]</sup>. However, the Aegean region drew attention in 1996 when there was a small but continuous increase in rabies cases in foxes. Initially,



it was unclear whether this phenomenon occurring in wildlife was part of an endemic cycle in the wild [6]. Subsequently, it was reported that there was spillover from dogs to foxes, leading to the endemic establishment of fox rabies in Türkiye [7]. According to that situation, the oral vaccination campaign was initiated in 2008 in the western cities of Türkiye such as Manisa and İzmir [7]. The vaccination programmes currently continue for the prevention of rabies in both domestic animals and wildlife. Unfortunately, rabies, despite all efforts, remains endemic in Türkiye and poses a threat to public health.

It is known that molecular and phylogenetic analysis of RABV isolates from in field outbreaks are an important tool to determine the origin of the viruses and helps to predict its future occurrence cases and thus allows the adoption of prevention and control measures. The available information is relatively restricted to a few studies analyzing a limited number of viruses in Türkiye [6-8]. Moreover, very few of them belong to the viruses circulating in Eastern and Southeastern Anatolia regions sharing a land border with some countries where rabies is endemic.

This study presents the distribution of clades/subclades of rabies viruses from different animal species in the Eastern and Southeastern Anatolia regions of Türkiye between 2016 and 2021.

## MATERIAL AND METHODS

### Ethical Approval

This study was conducted with the permission of the Ministry of Agriculture and Forestry, General Directorate of Food and Control (permit number: E-71037622-806.01.03-1035577, dated 01.04.2021) and in accordance with the decisions of the Local Ethics Committee for Animal Experiments at Ankara University (AÜHADYEK; decision no. 2022-3-30, dated 02.02.2022).

### Materials

The study utilized brain samples sent to the Rabies Diagnostic Laboratory of the Etlik Veterinary Control Center Research Institute, a national reference laboratory, from the Veterinary Control Institutes in the Eastern and Southeastern Anatolia regions between 2016 and 2021. Study materials (n=121) were selected from among the ones confirmed as positive for rabies virus according to their location (province), animal species and year.

### RT-PCRs

RNA extracts, prepared using a commercial kit (MagNa Pure Compact Nucleic Acid Isolation Kit I; Roche, Germany), were subjected to RT-PCR using primers sets targeting the N gene and G-L intergenic region, as

described elsewhere [3,9]. For the reverse transcription of viral RNA, cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). RT-PCR was applied to obtain relevant products from the N gene and/or GL gene intergenic region of the rabies virus.

For the N gene region, the PCR protocol consisted of an initial denaturation at 95°C for 3 min, followed by a total of 35 cycles, each comprising denaturation at 95°C for 30 sec, annealing at 51°C for 30 sec, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. For the GL intergenic region, the PCR protocol included an initial denaturation at 94°C for 3 min, followed by a total of 35 cycles, with denaturation at 95°C for 45 sec, annealing at 51°C for 30 sec, extension at 72°C for 1 min and 30 sec, and a final extension at 72°C for 10 min.

After PCR, the obtained amplification products were visualized under UV light following agarose gel electrophoresis with 1% SafeView™ Classic (ABM, Canada). The same primers used for amplification were used for bidirectional sequencing of the obtained PCR products. Relevant sequences were obtained from the GenBank database for rabies viruses detected in Türkiye and other countries using the BLAST engine. The obtained sequences were aligned with the genomic sequences of reference viruses from GenBank and reported local viruses from other countries, using Aliview [10] and MUSCLE (Multiple Sequence Comparison by Log-Expectation) software [11]. For phylogenetic analysis based on nucleotide sequences, the MEGA X program [12] was used. A Maximum-Likelihood method with the K2+I model was used to construct the phylogenetic tree for the N gene, and bootstrap analysis (1000 replicates) was performed. For the G-L intergenic region, a Maximum-Likelihood method with the K2+G model was used to construct the phylogenetic tree, and bootstrap analysis (1000 replicates) was performed.

The nucleotide(nt) identities were calculated by using the SIAS online tool (<http://imed.med.ucm.es/Tools/sias.html>).

## RESULTS

Out of 121 samples tested RT-PCR, 88 from 21 different provinces (cities) produced the expected size amplicons, 1372 bp and 879 bp, of the N gene and/or G-L intergenic regions, respectively.

The phylogenetic analysis data reveal that rabies viruses circulating in domestic and/or wild animals in the Eastern and Southeastern Anatolia regions of Türkiye belong to three different subclades within the Cosmopolitan clade. These subclades are Middle East 1a (ME1a) (n=45), Middle East 2 (ME2) (n=20), and Central Asia 2 (CA2) (n=23). The distribution of rabies virus subclades

<b>Table 1. The distribution of Rabies virus subclades identified according to the provinces</b>							
City	Species (n)	Years					
		2016	2017	2018	2019	2020	2021
Adıyaman	Cattle (1)					ME1a	
	Dog (1)				ME1a		
Ağrı	Dog (3)			ME2	ME2		ME2
	Cattle (1)						ME1a
Ardahan	Dog (3)		ME2*	ME2			
	Wolf (1)		ME2				
Bayburt	Dog (1)			ME1a			
Bingöl	Cattle (2)						ME1a, CA2
	Dog (2)		ME1a		ME2		
	Wolf (1)			ME1a			
Bitlis	Cattle (2)						ME1a,CA2
	Dog (1)		ME1a				
Diyarbakır	Cattle (1)						CA2
	Dog (6)		CA2	ME1a	CA2		CA2**
	Jackal (1)			ME1a			
	Fox (1)		ME1a				
Elazığ	Cattle (1)						ME1a
	Dog (5)		ME1a*	ME1a	CA2		CA2
	Fox (1)		ME1a				
	Cat (1)		ME1a				
Erzincan	Dog (1)						ME1a
Erzurum	Dog (3)			ME1a			ME2, CA2
	Cat (1)			ME1a			
	Fox (1)			ME1a			
Gaziantep	Dog (3)					ME1a	ME1a, ME2
İğdır	Dog (1)						ME2
	Cattle (1)				ME2		
Kahramanmaraş	Cattle (1)					ME1a	
	Goat (1)					ME1a	
	Dog (1)						ME2
	Fox (1)				ME1a		
Kars	Dog (3)			ME2	ME2		ME2
Malatya	Dog (5)	ME1a	ME1a	ME1a	ME1a		ME1a
	Fox (2)		ME1a			ME1a	
	Marten (1)				ME1a		
Mardin	Dog (3)		ME1a	ME1a			CA2
	Wolf (1)		ME1a				
Muş	Dog (2)			ME1a			ME2
Şanlıurfa	Dog (5)				CA2	CA2	CA2**
	Cattle (4)					ME1a	CA2, ME1a*
	Goat (1)					ME1a	
	Donkey (1)						CA2
	Horse (1)						ME1a
Tunceli	Sheep (1)				ME1a		
	Dog (1)			ME1a			
Van	Dog (6)			ME2	ME2	CA2	ME2, CA2*

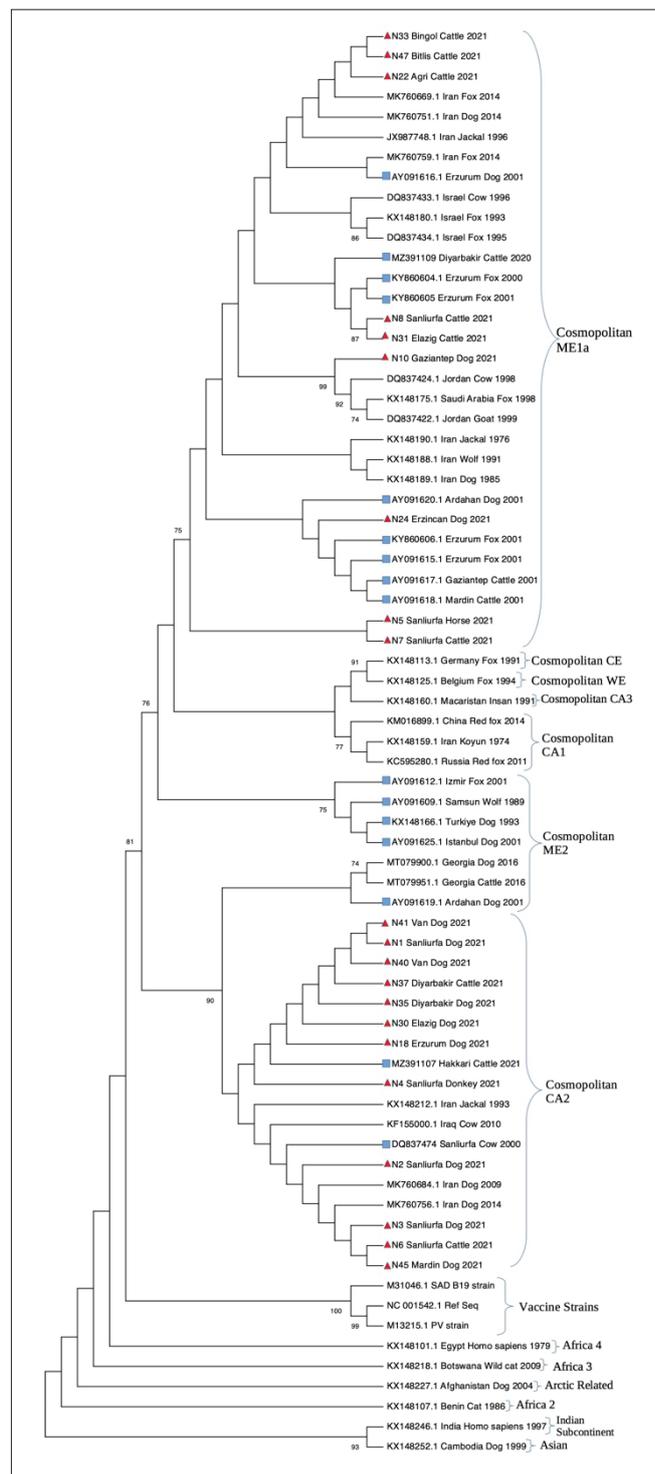
\* n=2 \*\* n=3

**Table 2.** The distribution of Rabies virus subclades identified according to animal species (n)

Species	Subclade (n)	Year						
		2016	2017	2018	2019	2020	2021	
Dog (n=57)	ME2	18		2	4	4		8
	ME1a	21	1	6	8	2	1	3
	CA2	18		1		3	2	12
Cattle (n=13)	ME2	1				1		
	ME1a	8					2	6
	CA2	4					1	3
Fox (n=6)	ME1a	6		3	1	1	1	
Wolf (n=3)	ME2	1		1				
	ME1a	2		1	1			
Cat (n= 2)	ME1a	2		1	1			
Horse (n= 1)	ME1a	1						1
Donkey (n= 1)	CA2	1						1
Marten (n= 1)	ME1a	1				1		
Jackal (n= 1)	ME1a	1			1			
Sheep (n=1)	ME1a	1				1		
Goat (n=2)	ME1a	2					2	
<b>Total</b>		<b>88</b>	<b>1</b>	<b>15</b>	<b>16</b>	<b>13</b>	<b>9</b>	<b>34</b>

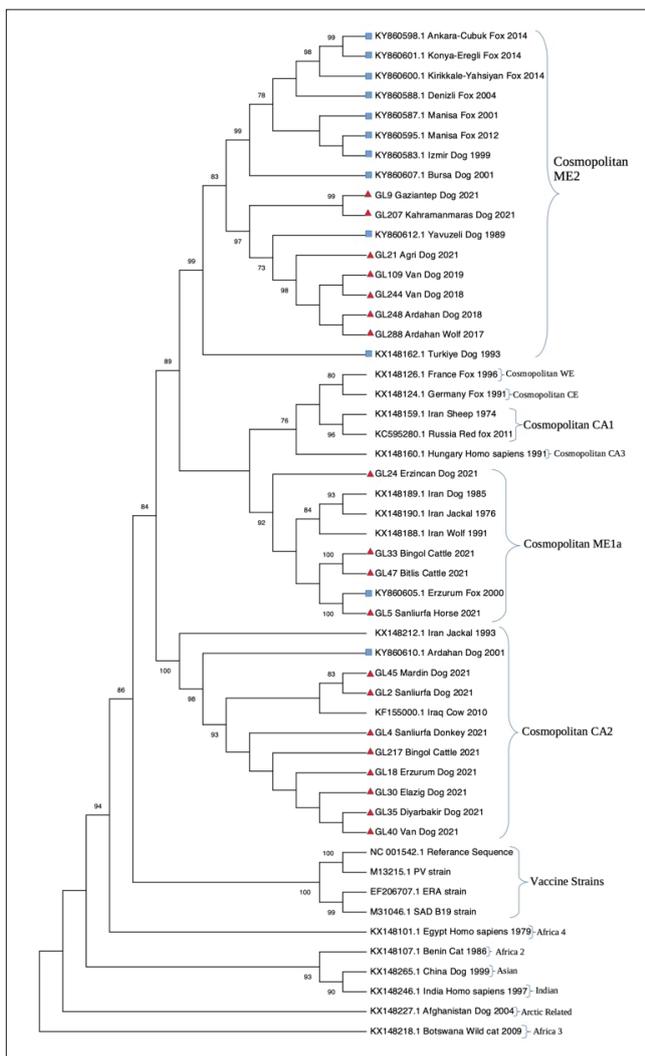
identified were shown in *Table 1* and *Table 2*, according to provinces and animal species, respectively. Additionally, the phylogenetic trees constructed using sequences of rabies viruses from selected animals representing different species, locations and years are shown in *Fig. 1* and *Fig. 2*.

In this part, the pairwise comparison of nucleotide sequences of our strains and strains deposited Genbank were examined in detail for every rabies subclades detected. The identities of the N gene nucleotide sequences of the rabies viruses obtained in this study with the reference strain NC\_001542 were 87.46-92.96%. The local rabies viruses studied in this research shared 95.71-100% sequence identity to each other. Additionally, 93.27-100% and 81.34-100% nucleotide sequence identity to sequences previously reported from Türkiye and other countries sequences used in the tree (*Fig. 1*), respectively. Our Turkish ME1a subclade and CA2 subclade rabies virus nucleotide sequences from shared 95.71-100% and 96.33-100% nucleotide identity among themselves. Also, nucleic acid sequences of the G-L intergenic region were aligned to obtain information on the genetic diversity of the RABVs detected in this study. Our strains shared 89.57-100% nt sequence identity with each other. In addition, they displayed 83.23-84.74% nt sequence identity with the reference strain NC\_001542. They shared 88.99-99.22% nt and 71.42-99.42 nt sequence identity to the other RABVs previously identified in Türkiye and other countries used



**Fig 1.** Phylogenetic tree based on the nucleotide (327 bp) of N gene of RABV. The phylogenetic trees were constructed using the Maximum Likelihood method with bootstrap of 1000 replicates. Numbers to the left of node indicate bootstrap values. Bootstrap values < 70% are not shown. Our strains and Turkish strains previously deposited in GenBank are indicated by red triangles and blue squares, respectively

in the tree (*Fig. 2*), respectively. For the G-L gene region nucleotide identities among the RABVs identified as ME1a, ME2 and CA2 subclades in this study were 90.34-99.8%, 97.1-100% and 89.57-100%, respectively.



**Fig 2.** Phylogenetic tree based on the nucleotide (518 bp) of G-L intergenic region of RABV. The phylogenetic trees were constructed using the Maximum Likelihood method with bootstrap of 1000 replicates. Numbers to the left of node indicate bootstrap values. Bootstrap values < 70% are not shown. Our strains and Turkish strains previously deposited in GenBank are indicated by red triangles and blue squares, respectively

## DISCUSSION

Globally, rabies infection still causes the human deaths, with the majority being children. In developing countries today, dogs, including wildlife species, are considered to be primarily responsible for rabies cases and the spread of the disease. Currently, 99% of human deaths worldwide due to rabies are attributed to dog-mediated transmission [1].

In 2015, the World Health Organization (WHO), Food and Agriculture Organization (FAO), World Organization for Animal Health (WOAH), and the Global Alliance for Rabies Control (GARC) came together to prioritize the fight against rabies with a one health approach. The goal of this platform is to eliminate human deaths from dog-mediated rabies by the year 2030. Consequently, each country will plan its rabies control efforts considering its national and regional characteristics. Due to the limited

availability of data, the inclusion of disease monitoring and surveillance has become a crucial component in the implementation of rabies programs [1].

Studies have revealed that there is a strong correlation between genetic and geographical criteria, with rabies virus isolates forming genetic clusters based on the geographical regions [4-6]. In a previous study [6], Turkish rabies viruses, identified as Genotype 1 based on N gene sequences (327 bp) data, were classified in three different branches according to their geographical origins: the “Western Branch” from materials obtained from the western region of the country, the “Eastern Branch” and the “Northeast-Caucasian Branch” from different isolates from the eastern provinces and the Ardahan province, respectively. Currently, there is a limited number of sequences available for rabies viruses originating from Türkiye in the GenBank database [6-8]. For this reason, although full-length sequences of the N gene region were obtained, to supplement the data with relevant sequence information from previous studies conducted in the Eastern and Southeastern Anatolia regions, Fig. 1 was prepared using partial sequences (327 bp) of the N gene region. The phylogenetic analysis of rabies virus from rabid animals in the Eastern and Southeastern Anatolia regions revealed the presence of three different clades (Table 1, Fig. 1 and Fig. 2), which can be evaluated based on the previously reported articles [6,13]. Specifically, the ME2 clade was associated with the Western branch, the ME1a clade with the Eastern branch, and the CA2 clade with the Northeastern-Caucasian branch. We believe that it will be more accurate to use subclade names in future research and epidemiological evaluations.

This study reports that rabies viruses identified in different species within the domestic and wildlife of the Eastern and Southeastern Anatolia regions exhibit distribution in 3 different subclades, distinct from other geographical regions as deposited GenBank and also a comprehensive project aiming at the molecular epidemiology of rabies virus in Türkiye, which we are currently carrying out (data not shown). This should raise a question about the geographic location of these regions where shares land borders with Georgia, Armenia, Azerbaijan to the northeast, Iran to the east, and Iraq and Syria to the southeast. Thus, rabies viruses identified in this study were clustered with those reported from neighboring countries in the phylogenetic trees (Fig. 1, Fig. 2) as described in previous studies [6,13]. As a matter of fact, the identities of N gene sequences between some of our isolates from dog and cattle sampled from different cities as Ağrı, Bingöl, Bitlis, Şanlıurfa and Mardin in 2021 and Iranian isolates (MK760669 from fox in 2014, MK760684 from dog in 2009 and MK760756 from dog in 2014) were 100%. Similarly, the identities of G-L gene regions sequences between some of our isolates

(from dog sampled from different cities as Mardin, Şanlıurfa, Erzurum, Elazığ, Diyarbakır, Van, and from a cattle from Bingöl in 2021) and Iraq isolates (KF155000; from a cattle sampled 2010) were 99.42%.

Another issue to be noted is the results of CA2 subclade of rabies virus. *Table 1* and *Table 2* shows that there are the increasing rates the CA2 subclade in dogs in the years 2019-2021, and, similarly, its detection in some domestic species (cattle and donkey) particularly in the year 2021. Although data from a limited number of materials could be used, it is our assessment that these results is related to the circulation of rabies virus in these regions, particularly through stray dogs.

In addition to canine-mediated rabies, the epidemiology of rabies in Türkiye is further complicated by the presence and geographical distribution of various reservoir species in wildlife that can transmit rabies. The data about wild animals from this study can be briefly summarized as follows. The viruses detected in wildlife animals (n=11) are all of the ME1a subclade, except for one (ME2) detected in a wolf in the year 2017. It is obviously that the limited number of viruses from animals in wildlife restricts the assessment of virus transmission between domestic and wildlife animals. However, it is possible that the prevalences of ME1a in samples from wildlife animals and dogs (*Table 2*), particularly in 2017-2018, raises the question of the rabies virus cycle between wildlife and domestic carnivores, which warrants further investigation through advanced analyses.

It is generally accepted that the rabies can be controlled and eliminated by mass vaccination of reservoir animal populations. In Türkiye, the Ministry of Agriculture and Forestry initiated an oral vaccination campaign against rabies in wildlife in 2008. As a continuation of the oral vaccination campaigns in the Aegean region, 2008-2010, the “Rabies Disease Control Project in Türkiye,” supported by the European Union, was implemented in 2014. The aerial vaccination was conducted once a year during the years 2014, 2015, and 2016. As a continuation of the mentioned project, aerial vaccination campaigns were conducted in the fall of 2019<sup>[14]</sup>. Finally, in open sources, the statement reports that the rabies cases in the Eastern and Southeastern regions of Türkiye are originating from wildlife (foxes), and the oral vaccination is planned in the mentioned regions<sup>[15]</sup>. There is good synchronization between this statement and this study, which aims to report on subclades of rabies viruses in the geographical regions mentioned and raise some questions, for further studies, as: What species is the main factor of rabies transmission in the region? To what extent do stray dogs pose a threat to humans? What is the importance of stray dogs in the fight against rabies? It is highly likely that the rabies virus, which is transmitted from foxes to

dogs, poses a significant threat to public health in the form of dog-associated rabies cases. Further studies on molecular epidemiology of rabies viruses from humans and, both wild and domestic animals, will contribute to the answering all these questions.

In conclusion, the oral vaccination campaigns conducted by the Ministry of Agriculture and Forestry, as well as the vaccination campaigns for owned/stray dogs and educational activities for the public, are of great importance in the context of controlling rabies. For the follow-up and success of vaccination studies, studies that present up-to-date data on the rates of rabies in different species and the molecular analysis of the circulating viruses etc. should be done continuously. We believe that people with knowledge about rabies, as well as official regulations, are an important component of the success of rabies eradication efforts in Türkiye.

#### Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author (D.A.Y.) upon reasonable request.

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#### Ethical Approval

This study was conducted with the permission of the Ministry of Agriculture and Forestry, General Directorate of Food and Control (permit number: E-71037622-806.01.03-1035577, dated 01.04.2021) and in accordance with the decisions of the Local Ethics Committee for Animal Experiments at Ankara University (AÜHADYEK; decision no. 2022-3-30, dated 02.02.2022).

#### Conflict of Interest

The authors declared that there is no conflict of interest.

#### Author Contributions

DAY and FA conceived and planned the study design. DAY conducted the experiments and performed the molecular biology and bioinformatic analyses (alignments, phylogeny). DAY and FA interpreted the obtained data. DAY drafted and wrote the manuscript; FA reviewed and edited the manuscript. All authors read and approved the final manuscript.

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