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Molecular characterization of ovine parapoxviruses in Türkiye: phylogenetic overview

Caracterización molecular de parapoxvirus ovinos en Türkiye: descripción filogenética

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ABSTRACT

The genus *Parapoxvirus* of the family *Poxviridae* is the causative agent of the Ecthyma Contagiosum (Orf virus) disease, which is widespread in sheep and goats around the world. The Orf virus is also recognized as an occupational zoonotic agent, causing auto limited lesions in humans. The Orf virus has an affinity for epithelial tissue and causes proliferative lesions around the lips and nose, udder, and hairless areas of the skin. In this study, the positivity of the virus was investigated by PCR in samples collected from several provinces in different regions of eastern and western Türkiye. Molecular characterization of the samples identified as positive by PCR was performed based on the B2L gene region. A phylogenetic tree was constructed by comparing the obtained partial B2L gene sequences with the reference parapoxvirus strains obtained from GenBank. It was found that the strains obtained in the study were close to Iranian and Sudanese strains. When the deduced amino acid sequences of the strains obtained with the reference strains taken from GenBank were compared, amino acid changes were detected at two different points. The phylogenetic map showed that different variants were likely to have circulated in different parts of the country. This study provided up-to-date information on Orf virus strains circulating in different regions of the country.

Key words: Ectima contagiosum, molecular characterization, Orf, Parapoxviruses, phylogenetic analysis

RESUMEN

El género *Parapoxvirus* de la familia *Poxviridae* es el agente causante de la enfermedad Ecthyma Contagiosum (virus Orf), que está muy extendida entre ovejas y cabras en todo el mundo. El virus Orf tambien es reconocido como un agente zoonótico ocupacional que causa lesiones autolimitadas en humanos. El virus Orf tiene afinidad por el tejido epitelial y causa lesiones proliferativas alrededor de los labios y la nariz, la ubre y las áreas desprovista de pelo en la piel. En este estudio, se investigó la positividad del virus mediante PCR en muestras recolectadas de varias provincias en diferentes regiones del este y oeste de Turquia. La caracterización molecular de las muestras identificadas como positivas mediante PCR se realizó en base a la región del gen B2L. Se construyó un árbol filogenético comparando las secuencias parciales del gen B2L obtenidas con las cepas de parapoxvirus de referencia obtenidas de GenBank. Se encontró que las cepas obtenidas en el estudio eran similares a las cepas iraníes y Sudanesas. Cuando se compararon las secuencias de aminoácidos de las cepas obtenidas con las cepas de referencia tomadas del GenBank, se detectaron cambios de aminoácidos en dos puntos diferentes. El mapa filogenético mostró que probablemente habrían circulado distintas variantes en diferentes partes del país. Este estudio proporcionó información actualizada sobre las cepas del virus Orf que circulan en diferentes regiones del país.

Palabras clave: Ectima contagiosa, caracterización molecular, Orf, Parapoxvirus, análisis filogenético

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INTRODUCTION

Ectima contagiosum infection, also known as "Orf", is an infection of wild and domestic animals {primarily sheep and goats, but also camels *(Camelus bactrianus)*, deer *(Cervus elaphus)*, dogs *(Canis lupus familiaris)*, cats *(Felis catus)*, and squirrels *(Sciurus Vulgaris)*} caused by the genus *Parapoxvirus* in the subfamily *Chordopoxvirinae* of the family *Poxviridae*, which presents with non-systemic skin lesions [1].

The parapoxvirus genus also includes bovine pseudocowpox virus (PCPV), papular stomatitis virus (BPSV), squirrel parapoxvirus (SPPV), and New Zealand deer parapoxvirus [2, 3]. Infections caused by parapoxviruses, which have a broad host spectrum, have also been reported in several pinniped species, including seals [4]. The pathogen, which has an epitheliotropic character, causes proliferative lesions on the skin. Skin lesions of an edematous proliferative nature occur around the lips and nose, on the udder and on hairless areas of the skin and are generally much more severe in goats than in sheep [5].

ORFV is endemic throughout the world and has highly variable virulence for a variety of hosts, including sheep, goats, dogs and humans. Among small ruminants, ecthyma contagiosum is one of the most prevalent skin diseases. This disease has a very high morbidity rate in sheep and goats, which results in severe productivity losses [6]. In addition, this infection causes significant productivity losses in the sheep industry, particularly lamb mortality [7]. Direct or indirect contact with infected materials can spread the disease. Of course, re-infection can occur months later in animals that have recovered from the infection or have been vaccinated. Lesions on the tongue and gums of affected animals may rarely occur in internal organs [6, 8]. Young animals have been reported to be more susceptible to infection than adults. While the morbidity of the disease is 100%, the mortality can reach 15%, especially in young animals, due to malnutrition, immunosuppression and secondary infections. The virus is transmitted directly and indirectly between animals. In addition, mortality increases in lambs and kid goats during the lactation period due to dehydration and starvation as pain and deformities of the lips and mouth reduce suckling [9, 10, 11]. As a consequence, production decreases drive serious economic impact [7].

Particularly in the spring months, which include the lambing and shearing periods, it has been reported that there is an increased spread and risk of infection for those in close contact with animals, such as veterinarians and farmers [12]. The zoonotic virus creates lesions in humans which appear as painful, big nodules on the hands and face [6, 13, 14]. The zoonotic infection is transmitted to humans through contact with infected animals and causes lesions on the hands, fingers, and rarely the face [15, 16, 17, 18].

The Orf virus (ORFV) genome consists of double-stranded linear DNA, approximately 138 kb-140 kb in size, has a high GC content of approximately 63.5%, and encodes 132 genes. It is known that 88 of these genes are protected in PPVs (*Parapoxviruses*) [19]. Centrally placed, relatively conserved genes are necessary for both viral morphology and replication. While genes necessary for virulence, pathogenesis and immune regulation are found in more variable terminal portions of the genome, highly conserved genes involved in viral replication and structure development can be found in the central region [6, 20, 21].

PPVs are among the smallest of the genera in the subfamily *Chordopoxvirinae* (approximately 260 nm in length), and although their genome is similar to other genera, they have significant differences from other genera due to variations in the G+C content, virion shape, and the existence of a sizable number of PPV-specific genes [22]. The B2L gene (~1137 bp), the major envelope gene of the ORFV, encodes an important immunogenic protein that elicits a strong antibody response. The major envelope protein is encoded by the virus's envelope gene (B2L), which is a highly immunogenic protein with a molecular weight of 42 kD [23]. Due this gene is highly conserved it is often used for detection and genetic characterization of the virus by molecular methods [9, 11, 23, 24].

Although clinical findings are critical to the diagnosis of infection, laboratory diagnosis is important because the symptoms can be confused with infections such as the bluetongue virus, sheep pox virus, foot-and-mouth disease, and type 2 bovine herpesvirus [25, 26]. Because molecular techniques are faster and more sensitive, they are preferred to other techniques (virus isolation, histopathology, etc.) in the diagnosis of the virus, and PCR is a specific method that is widely used in the diagnosis and molecular characterization of infection [23, 27, 28, 29].

The aim of this study was to perform molecular characterization of the virus in crust samples collected from several provinces in the eastern and western regions of Türkiye.

MATERIAL AND METHODS

Ethical statement

The Balikesir University Animal Experiments Local Ethics Committee provided ethical approval for this investigation, which was carried out with their consent dated 07/09/2023 and numbered 2023/7-4.

Animal and sample collection

The research material consisted of crust samples collected between October and December 2023 from sheep *(Ovis aries)* in Balıkesir (4 samples), Tunceli (7 samples) and Bitlis (6 samples) provinces with suspected Ectima Contagiosum infection. For laboratory studies, dried skin crust samples were transferred to sterile tubes containing 2 mL transport medium-PBS. The tubes were vortexed, homogenized, and centrifuged (Espresso-11210801, Thermo Scientific, China) at 1610 G for 10 min. The upper supernatant was transferred to 2.5 mL storage tubes and prepared for viral DNA extraction. Samples were stored at -20°C until assayed.

DNA extraction and Polymerase Chain Reaction (PCR)

Using a commercially available viral nucleic acid isolation kit (Jena Bioscience, Viral RNA+DNA Preparation Kit, Germany), viral DNA was extracted from samples in accordance with the kit's instructions. The resulting templates were stored at -20°C (Hotpoint-Ariston ENTM 18211 F (TK), Italy) until PCR studies were performed. Primers specific for the B2L gene region of Orf virus were used for PCR [30]. For this purpose, the rapid PCR reaction was performed with PPP1 (5'-GTCGTCCACGATGAGCAGCT-3') and PPP4 (5'-TACGTGGGAAGCGCCTCGCT-3') primers. The result of this reaction was a 594 bp product. A total of 30 µl PCR master mix was prepared containing 3 µL DNA, 20 mM $NH_4(SO_4)_2$, 75 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 10 pmol primers, 0.2 mM dNTP's and *Taq* DNA polymerase was prepared at 0.5 U (MBI, Fermentas, Lithuania). The following heating programs were used in the thermal cycler (Prima-Trio, Himedia, India) 9 min at 95°C, followed by 1 min at 94°C, 1 min

at 55°C and 1 min at 72°C, a final extension was performed at 72°C during 10 min. DNA products were analysed by a GelRed stained 1% agarose gel electrophoresis. Amplified products were visualized under UV light (MaestroGen UltraBright® UV Transilluminator MB-21, Taiwan) on a gel imaging device.

Sequencing and Phylogenetic Analysis

Strong positive amplified PCR products were chosen for sequencing. DNA was purified using a commercial purification kit, and then sequenced using the Sanger method with the primers used in the PCR procedure. For this purpose, the capillary was subjected to electrophoretic separation (Nanopac-300, Fisher Scientific, England) in a specialized laboratory (Eurofins Laboratory, Germany).

This gene region is highly conserved and has been widely used in molecular detection and genetic characterization of species within the parapoxvirus genus [6, 24]. In this study, this gene region was selected for genetic characterization of the virus.

The sequences obtained were manually verified by visual analysis of the electrophoregram using Bioedit version.7.0.5.3 software [31] and compared with the GenBank database using the NCBI Blast search tool. All sequences were aligned using the MUSCLE algorithm [32], with sequences trimmed at the start and end positions after alignment in MEGA-X. (Molecular Evolutionary Genetic Analysis, version 10.2.6) [33]. Phylogenetic trees were constructed using maximum likelihood methods, with bootstrap analysis based on 1000 replicates. Based on the findings of the investigation for MEGA X's best DNA/protein model features, the phylogenetic analysis models were chosen. Using the Tamura 3-parameter model with gamma distribution, the maximum likelihood (ML) approach was applied to create phylogenetic trees (T92+G) [34]. Phylogeny was tested by 1000 bootstrap replicates. Interactive Tree of Life (iTool) was used to generate a circular phylogenetic tree diagram [35].

RESULTS AND DISCUSSIONS

In this study, PCR was used for virus detection. In the study, 12 of the samples collected from 17 sheep showing clinical signs of infection were found to be positive (FIG. 1).

Electrophoretic bands of expected size (594bp) were visualized. Since sequence analysis was performed in the study and the data obtained were revealed by phylogenetic analysis, no positive control was used. Sequence analysis was performed on 7 of the samples from different regions.

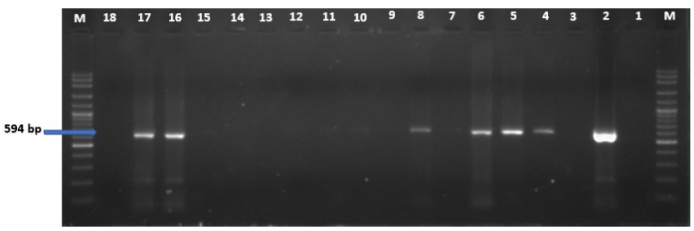

FIGURE 1. PCR amplification products using Orf virus primers. Lane M: 100-bp DNA ladder marker (Fermentas); lanes 2,4,5,6,7,8,10,11,15,16,17: positive amplification PCR products; lane 18: negative control (distilled water); lanes 1,3,9,12,13,14: negative amplification PCR product

The samples evaluated after sequence analysis were registered in GenBank and accession numbers were obtained. Sequences that were listed in the GenBank nucleotide sequence database provided the data for the phylogenetic analysis (TABLE I).

TABLE I
Province, strain ID, GenBank accession numbers of samples infected with ORFV

No	Province	Strain ID	Accession No
1	Tunceli	TR/ORFV02/TUN/2023	PP317136
2	Tunceli	TR/ORFV04/TUN/2023	PP317137
3	Tunceli	TR/ORFV05/TUN/2023	PP317138
4	Bitlis	TR/ORFV06/BIT/2023	PP317139
5	Balıkesir	TR/ORFV08/BAL/2023	PP317140
6	Bitlis	TR/ORFV16/BIT/2023	PP317141
7	Bitlis	TR/ORFV/17/BIT/2023	PP317142

A phylogenetic tree was created by comparing the partial B2L gene sequences obtained from the GeneBank with the sequences obtained in our study (FIG. 2). Three of the ORFV sequences obtained in the study (Acces. Num. PP317136, PP317137, PP317138) showed high similarity to each other (from Tunceli), while the other three (Acces. Num. PP317139, PP317141, PP317142) showed high similarity to each other (from Bitlis). Sequence PP317140 (from Balıkesir) was found to be on a separate branch and close to the second three sequences. The mean genetic distance in pairwise analysis was calculated to be 0.015. The genetic distance between PP317136, PP317137, PP317138 and PP317139, PP317141, PP317142 was 0.015. The genetic distance between PP317140 and PP317139, PP317141, PP317142 was 0.003. The maximum genetic distance was observed between PP317140 and PP317136, PP317137, PP317138 with 0.019.

ORFV is endemic in all regions of Türkiye, especially in sheep and goat herds [23, 24, 27, 36]. When all previously reported ORFV sequences in Turkey are examined, it is found that they show great diversity among themselves and are distributed in different clusters. The partial B2L sequences obtained in our study were found to be far away from the previous Turkish sequences in the phylogenetic tree. The disease has a worldwide distribution and has been reported in China [37], Ethiopia [38], South Korea [39], Egypt [40], India [41], Greece [12], Finland [42], Sudan [43], Nigeria [44] and USA [45]. In the worldwide search, there are many strains recorded in GenBank. It was found that the strains obtained in the study were more similar to Iranian and Sudanese strains. While the genetic distance between strains PP317136, PP317137, PP317138 and AY958203 (Iranian strain) was 0.007, the genetic distance between them and MN701771 (Sudanese strain) was determined to be 0.015. On the other hand, the genetic distance between strain PP317140 and both strains AY958203 (Iran strain) and MN701771 (Sudan strain) was determined to be 0.011. Strains PP317139, PP317141, PP317142 were found to be similar to strain KX013765 (Turkey-Van strain) previously identified in Türkiye, and the genetic distance between them was determined to be 0.003. Considering that these strains were obtained from Bitlis province, the geographical proximity between Bitlis and Van provinces explains this similarity.

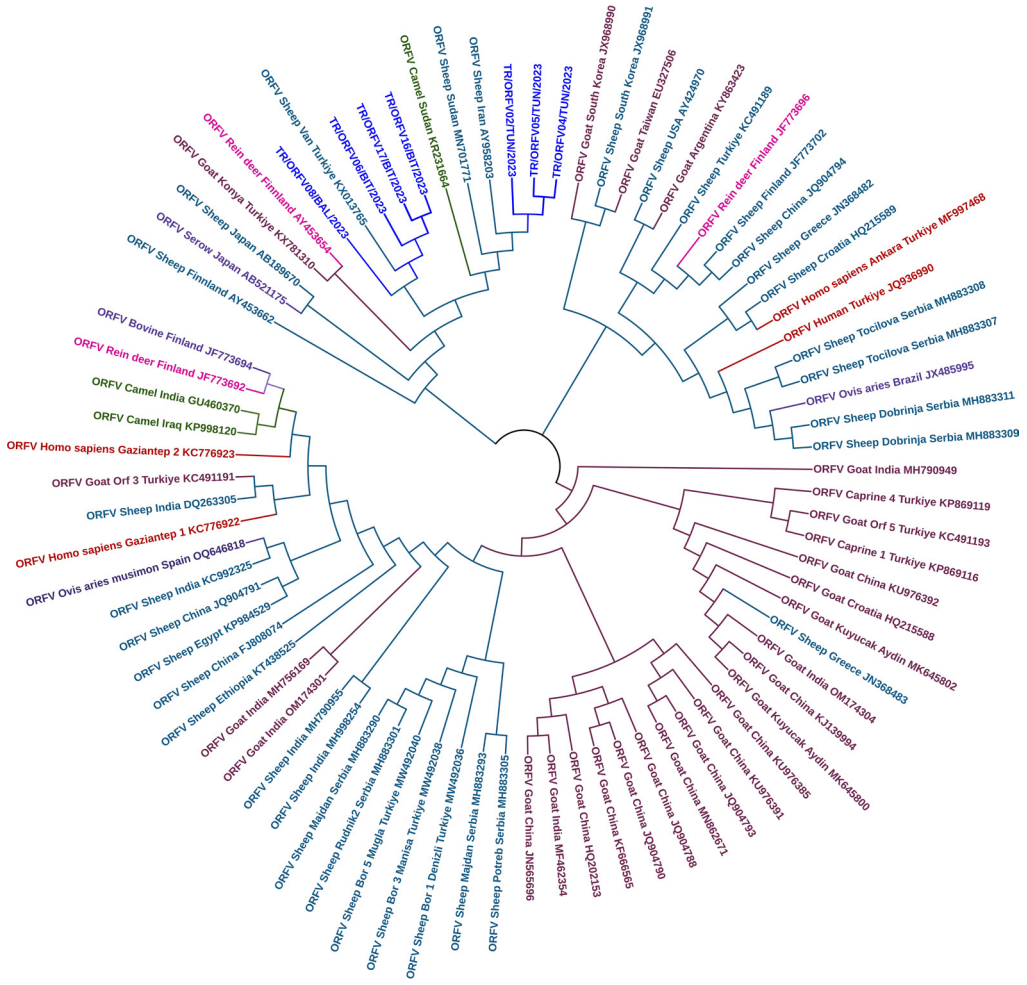

FIGURE 2. Phylogenetic analysis of the ORFV B2L gene

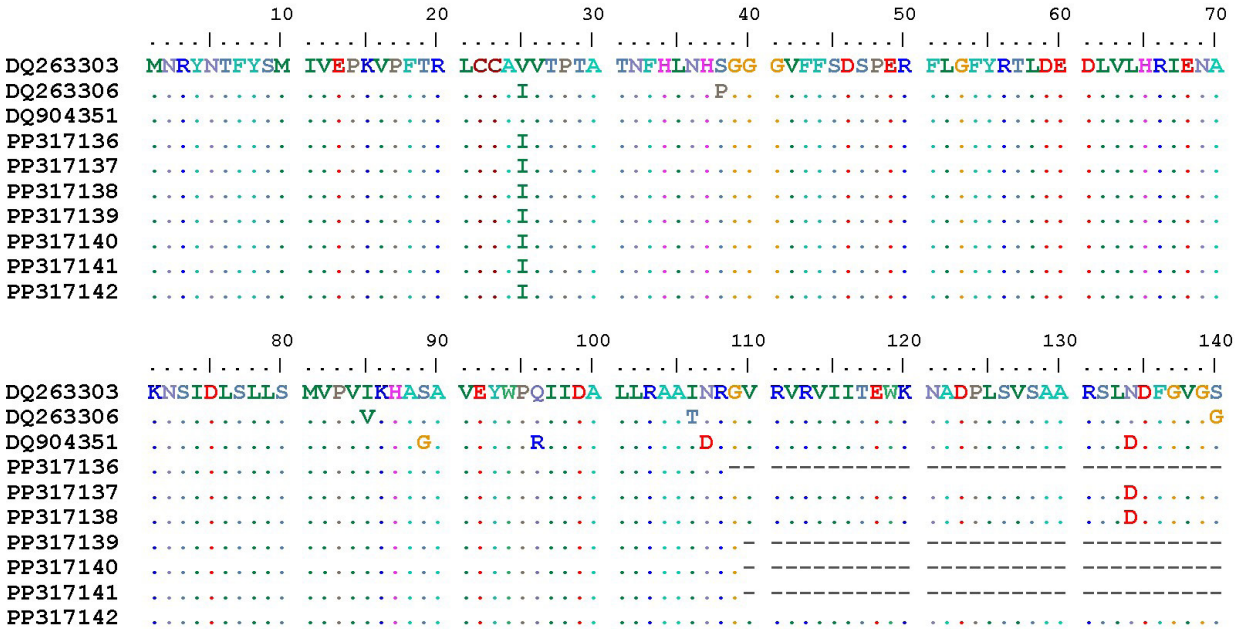

FIGURE 3. Comparison of amino acid sequences of the strains obtained from the study with reference strains

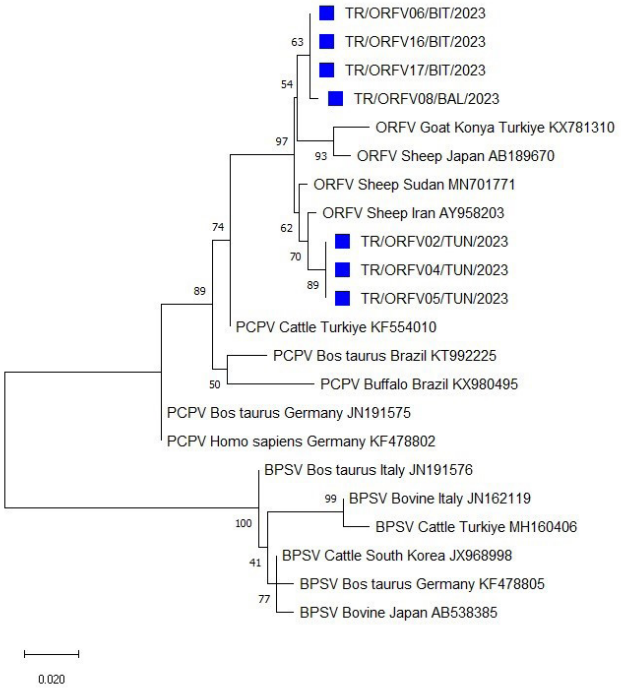

FIGURE 4. The place of our strains in the genus Parapoxvirus. Strains from our study are marked with a square

The amino acid sequences of the strains obtained from the study were compared with the reference strains [6, 46] from GenBank determined in the partial B2L gene of the ORFV genome and amino acid changes were detected at two different points (FIG. 3). *Valine* (val–V) was shown to be transformed to *Isoleucine* (ile–I) at amino acid position 25 in Fig 3. (185th amino acid position in the B2L gene sequence) in every strain. Furthermore, it was found that in the PP317137 and PP317138 strains, *Aspartic acid* (asp–D) replaced *Asparagine* (asn–N) at amino acid position 134 in Fig. 3 (294th in the B2L gene sequence). It was noted that this change was also present in the DQ904351 (Taiwan) reference strain. The B2L gene encodes immunogenic proteins expressed on extracellular enveloped virions and is translated late in the infection process. The B2L gene is a palmitoylated protein that is crucial for the viral envelope and is one of the main antigens on the surface of enveloped virions. Because of its various roles in the host environment, it is also one of the primary envelope proteins that the host animals (goats and sheep) generate an effective immune response against [47]. It has been used in phylogenetic research aimed at the evolutionary and genetic connections between parapoxvirus genus outbreaks in various animals [6, 24]. In the study, limited mutations and amino acid changes were detected on this gene. Nonetheless, no distinct changes of amino acids were detected, which could indicate that the strains of ORFV are closely linked antigenically. Although the B2L gene is a highly conserved gene, it encodes a highly immunogenic protein. It has also been reported that unique host–specific residues identified in the B2L protein contribute to determining the host range [41]. Therefore, it is thought that significant mutations that may occur in the gene may affect the host spectrum of the virus, its pathogenicity and the effectiveness of vaccines.

The place of the strains obtained in our study among the parapoxviruses was shown by constructing a separate phylogenetic tree (FIG. 4).

This study aimed to perform the molecular characterization of the Orf virus. Typing the resulting strains and classifying parapoxviruses into different types, including BPSV, PCPV, ORFV and others, is possible with molecular methods and genetic analysis. In addition, natural host range, clinical symptoms and serological findings are also used in the classification of parapoxviruses [26]. Genetic studies on the B2L gene of ORFV, BPSV and PCPV have shown that this gene is a well–conserved gene region. It has been determined that the central region of the gene is better protected than the terminal regions [6]. By building a distinct phylogenetic tree, the position of the strains found in our investigation within the parapoxvirus family was displayed. It was found that all of these strains are ovine parapoxviruses as it was shown in FIG. 2.

Previous studies have shown that the rate of mutation accumulation in poxviruses varies significantly between different poxvirus genera and strains. Parapoxviruses have a high GC content (64.0–64.5%) in their genomic DNA, in contrast to the low GC content of other poxvirus genera (25.0–43.6%). Low and high GC ratios have been used to differentiate poxviruses, and PCR techniques need to be modified depending on whether this ratio is high or low [45]. Although parapoxvirus is a DNA virus, mutations and changes can occur and this situation results in the occurrence of new gene structure and new virus strains. In addition, it has been reported that the reason for the failure of vaccines may be related to the long–term adaptation of the virus to tissue culture and that the recombinant DNA vaccines developed are more effective [48]. Newly discovered strains will provide progress in producing new vaccines and preventing epidemics.

CONCLUSIONS

In the study, molecular characterization based on the B2L gene region of ORFV strains obtained from different regions was performed, and up–to–date information was obtained by revealing their genetic relationships with other parapoxviruses. As a result of the phylogenetic analysis obtained as a result of the study, it is noteworthy that strains originating from different geographies are in circulation in Türkiye. In the study, strains were obtained from the eastern and western regions of country and were found to be close to eastern strains. The importance of animal mobility and Türkiye's intercontinental geographical location should be taken into account in the spread of many animal diseases, including ORFV infection, which is important for livestock

In several countries, ORFV infection is an economically serious problem for the commercial breeding of sheep and goats. To date, various strategies have been investigated to develop effective antiviral drugs and prophylactic vaccines to control ORFV infection, most of which are derived from immunogenic envelope proteins. It should be taken into consideration that periodic prevalence and current molecular studies involving different gene regions to investigate this disease of zoonotic importance will provide important information for human and animal health and will contribute to the creation of effective prevention and control programs regarding the disease.

Availability of data and materials

The data used and analysed in this study are available from the corresponding author on reasonable request.

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There is no funding support for the study.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author contributions

ZK performed the molecular analyses, MG contributed to the sequence and phylogenetic analyses, ZK and MG drew the figures, wrote and edited the manuscript. Both authors read and approved the final version of the manuscript.

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