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Research Article

Molecular Characterizations of Sheeppox Virus Strains

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ABSTRACT

Sheeppox and goatpox viruses cause infections in sheep and goats characterized by pyrexia, generalized or local pox lesions, and lymphadenopathy. The sheeppox virus, goatpox virus and lumpy skin disease virus are ordered in the *Capripoxvirus* genus of *Poxviridae* family. The aim of this study was to assess the genomic relationships of vaccine strains Bakırköy strain and Kenya0240 strain and of other reference strains. To show the genomic relationships, LD116 gene region coding the viral P32 protein and CD47 gene region from membrane protein genes were chosen. The DNA sequences and as references DNA sequences of the genes of the sheeppox, goatpox and lumpy skin disease viruses obtained from the NCBI Gene Bank were analyzed in DNASTAR gene analysis program using the Clustal W analysis method to determine their differences as to nucleotides and aminoacids. For phylogenetic relationships between the viruses and similarity indices the Neighbor-Joining method was used. The sequence analysis of both gene regions showed that Bakırköy strain belonged to the group where there was only sheeppox virus (Lineage I) and that Kenya0240 strain belonged to the group of the other viruses (Lineage II). Potential vaccine strains protecting not only against sheeppox and goatpox, but also against the lumpy skin disease is significantly important in eradicating the disease.

Key Words: Sheeppox virus, sequencing, molecular characterization, phylogenetic analyses

ÖZET

KOYUN ÇİÇEĞİ VİRUS SUŞLARININ MOLEKÜLER KARAKTERİZASYONU

Sheeppox ve goatpox virusları, koyun ve keçilerde pyrexia, generalize veya lokal çiçek lezyonları ve lymphodenopathy ile karakterize bir enfeksiyondur. Sheeppox virusu, goatpox virusu ve lumpy skin disease virusu *Poxviridae* ailesindeki *Capripoxvirus* cinsi içerisinde bulunmaktadır. Bu araştırmada aşı suşu olarak kullanılan Bakırköy suşu ile Kenya0240 suşu arasında ve diğer referans suşlar arasındaki genomik ilişkilerin incelenmesi hedeflendi. Bu genomik ilişkilerin ortaya konulmasında virusun P32 proteinini kodlayan LD116 gen bölgesi ve membran protein genlerinden CD47 gen bölgesi seçildi. DNA dizilimleri ile NCBI Gen bankasından referans gen

olarak seçilen sheppox, goatpox ve lumpy skin diseases viruslarının DNA dizilimleri ClustalW analiz yöntemi kullanılarak DNASTAR gen analiz programında nüklotid ve aminoasit farklılıkları analiz edildi. Viruslar arasındaki filogenetik ilişkiler ve benzerlik indeksleri için Neighbor-Joining yöntemi kullanıldı. Her iki gen bölgesinin sekans analizleri sonucunda Bakırköy suşu sadece koyun çiçek viruslarının bulunduğu grupta (Lineage I) bulunurken, Kenya0240 suşu diğer virusların da içerisinde bulunduğu grupta (Lineage II) olduğu saptandı. Potansiyel aşı suşlarının sadece koyun veya keçi çiçeğine değil aynı zamanda sığırların lumpy skin disease virusuna karşı da koruma sağlayacak nitelikte olması, bu hastalığın eradikasyonu için çok önemlidir.

Anahtar Kelimeler: Koyun çiçeği virusu, sekans, moleküler karakterizasyon, filogenetik inceleme

Introduction

Sheeppox disease virus (SPDV) and goatpox disease virus (GPDV) cause infections in sheep and goats characterized by pyrexia, generalized or local pox lesions, and lymphadenopathy (Rao and Bandyopadhyay, 2000). In areas where the infection is prevalent the infected sheep and goats show significantly low production of milk, regression in gaining body weight, increased rate of abortion, low quality wool, susceptibility to respiratory infections and sometimes even mortality (Babiuk et al., 2008). In Europe the disease was first reported by Norway in 1879. Although the disease has been eradicated first in England and then in other European countries, it still continues particularly in India and Middle-East and also in some Far-East and African countries (Bhanuprakash et al., 2012; Bhanuprakash et al., 2006; Rao and Bandyopadhyay, 2000). Although eradication programs and vaccination are applied in Turkey, the infection is still occasionally reported in sheep and goats (Gulbahar et al., 2006; Oğuzoğlu et al., 2006).

According to the most recent virus classification, SPPV, GPPV and lumpy skin disease virus (LSDV) are ordered in the *Capripoxvirus* genus of *Poxviridae* family (King et al., 2012). All *Capripoxvirus* are enveloped DNA viruses; their DNAs are double-stranded with a length of around 150 kbp. Strains from goatpox and sheeppox share at least 147 genes. Lumpy skin disease virus has an extra 9 genes that are non-functional in GPPV and SPPV; these extra genes are thought to be responsible for the infection in cattle (Tulman et al., 2002).

The varying severity of pox epidemics with years, presence of the disease in different animals in vaccinated regions, and genomic differences between field strains and vaccine strains (Bhanuprakash et al., 2010) have led us to study the genomic differences between the vaccine strains in Turkey and reference strains. The aim of this study was to assess the genomic relationships between vaccine strains Bakırköy strain and Kenya0240 strain and other reference strains. To show the genomic relationships, LD116 gene region coding the viral P32 protein and CD47 gene region from membrane protein genes were chosen. P32 protein is a structural protein found in all species of *Capripoxvirus*, that carries the most important antigenic determinants (Heine et al., 1999; Tian et al., 2010).

Materials and Methods

Viruses: Sheeppox vaccine (Penpox) produced in the Pendik Institute of Veterinary Control, Istanbul, was used as control in the study. Bakırköy strain, isolated from sheep at 1974, used for the production of Penpox vaccine, and Kenya0240 strain obtained from the same institute comprised the sequencing material.

Polymerase chain reaction (PCR): The pair of F primer INS1.1 5-AGA AAC GAG GTC TCG AAG CA-3 and R primer INS1.2 5-GGA GGT TGC TGG AAA TGT GT-3 chosen from the CD47 membrane protein genes of sheeppox virus and copying a region of 289 pb were used in PCR (Rao and Bandyopadhyay, 2000). For viral DNA isolation from the samples the Roche DNA Extraction Kit (catalog number: 11 796 823 001) was used according to the protocol of the manufacturer. The PCR mixture

was prepared with 5 µl DNA, 5 µl flexi color buffer, 5 µl 25mM $MgCl_2$, 1 µl dNTP, 2 µl primers (for each, 10pmol), 0.5 µl Taq DNA polymerase enzyme completed to a volume of 50µl with ultrapure distilled water. The amplification of the targeted gene region was realized by holding the mixture in thermocycler first for 9 min at 95°C, and then for 30 cycles for 1 min at 94°C, 1 min at 55°C, 1 min at 72°C, and finally 7 min at 72°C. Electrophoresis with the PCR product was then carried out in 1.5% agarose gel under 100 volts, and the results were studied under ultraviolet light.

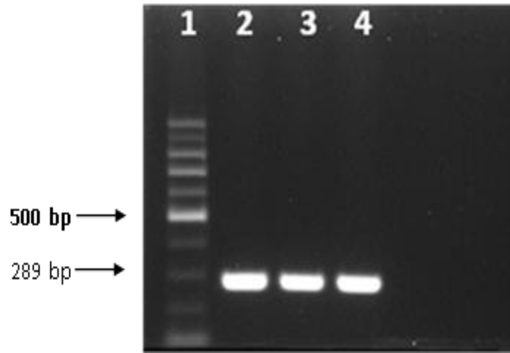
Sequencing: The pair of F primer pMF1 5-GAA TTT AAA GAT GGG TAT GTT G-3 R primer pMR2 5-GAT GTT TCT AAT GCT TTG CTA AT-3 were chosen by us from the P32 protein-coding LD116 RNA polymerase subunit gene region, and copying a region of 196 bp were sequenced. The PCR mixture was prepared with 5 µl DNA, 5 µl flexi color buffer, 5 µl 25mM $MgCl_2$, 1 µl dNTP, 2 µl primers (for each, 10pmol), 0.5 µl Taq DNA polymerase enzyme completed to a volume of 50 µl with ultrapure distilled water. The targeted gene region was amplificated by holding the mixture in the thermocycler first for 3 min at 94°C, and then for 30 cycles for 1 min at 94°C, 1 min at 55°C, 45 seconds at 72°C, and finally 7 min at 72°C. For the purification of PCR product, the Roche PCR Purification Kit (catalog number: 11 732 668 001) was used according to the protocol of the manufacturer. The purified PCR product was then sent to REFGEN Biotechnology Firm (www.refgen.com) for DNA sequencing. The chromotograms of DNA sequences of Bakırköy and Kenya0240 strains sent by the firm were then studied using the Chromast Lite program.

These DNA sequences and the DNA sequences of the reference SPDV, GPDV and LSDV (Table 1) were analyzed in the DNASTAR gene analysis program using ClustalW analysis method, and their differences as to nucleotides and aminoacids were determined. Followingly, in the same gene analysis program their phylogenetic relationships were assessed using the Neighbor-Joining method, and their similarity indices were calculated.

Results

According to the results of PCR studies, when compared with the DNA control marker, the positive-control Penpox vaccine strain and sheeppox virus Kenya0240 and Bakırköy strains showed same length bands (289bp and 196bp) (Figure. 1 and Figure 2). When the DNA sequences of the PCR products of two gene regions were compared with the genes and nucleotides of the reference viruses, the Kenya0240 strain showed an order of nucleotides similar to those in SPDV, GPDV, and LSDV, but the Bakırköy strain only a nucleotide order similar to that in SPDV (Figure 3 and Figure 4). Similar results were obtained also in phylogenetic analyses (Figure 5, A and B). Accordingly, Kenya0240 and Bakırköy strains take place in two different lineage groups, Lineage I and Lineage II, respectively. This was true not only for the LD116 gene region coding the P32 protein, but also true for the CD47 gene region tested.

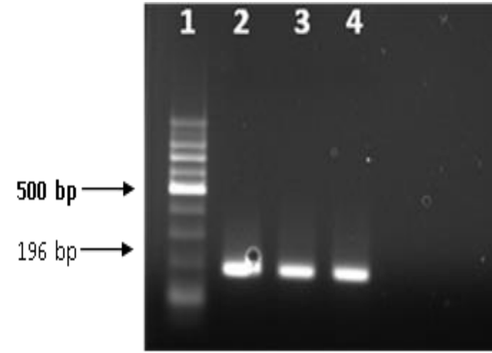
In similarity index studies, as similarity percentages in the LD116 gene region (Table 2), the Bakırköy strain showed identity similar to those strains in Lineage Groups II and I by 100% and 96.9-97.9%, respectively, whereas the Kenya0240 strain to strains in Lineage Groups I and II by 99.3-100% and 97.9%, respectively. As similarity percentages in the CD47 gene region (Table 3), the Bakırköy strain showed identity similar to those strains in Lineage Groups II and I by 100% and 95.9-97.4%, respectively, whereas the Kenya0240 strain to strains in Lineage Groups I and II by 99.5-100% and 96.9%, respectively. When the aminoacids of the Bakırköy and Kenya0240 strains and reference strains were compared, it was observed that each order of nucleotides did not cause a change in the order of aminoacids (Figure 6, A and B), but the order in aminoacids changed with changes in the order of nucleotides which is shown by phylogenetic analysis.



1: DNA marker (Heliosis 100bp), 2: Poxvirus positive control (Penpox vaccine), 3: Sheepopoxvirus Kenya0240 strain, 4: Sheeppoxvirus Bakırköy strain

Figure 1. PCR product of amplified CD47 gene of sheeppox viruses.

Şekil 1. Koyun çiçek virülerinden amplifiye edilmiş CD47 geninin PCR ürünü.



1: DNA marker (Heliosis 100bp), 2: Poxvirus positive control (Penpox vaccine), 3: Sheepopoxvirus Kenya0240 strain, 4: Sheeppoxvirus Bakırköy strain

Figure 2. PCR product of amplified LD116 gene of sheeppox viruses.

Şekil 2. Koyun çiçek virülerinden amplifiye edilmiş LD116 geninin PCR ürünü.

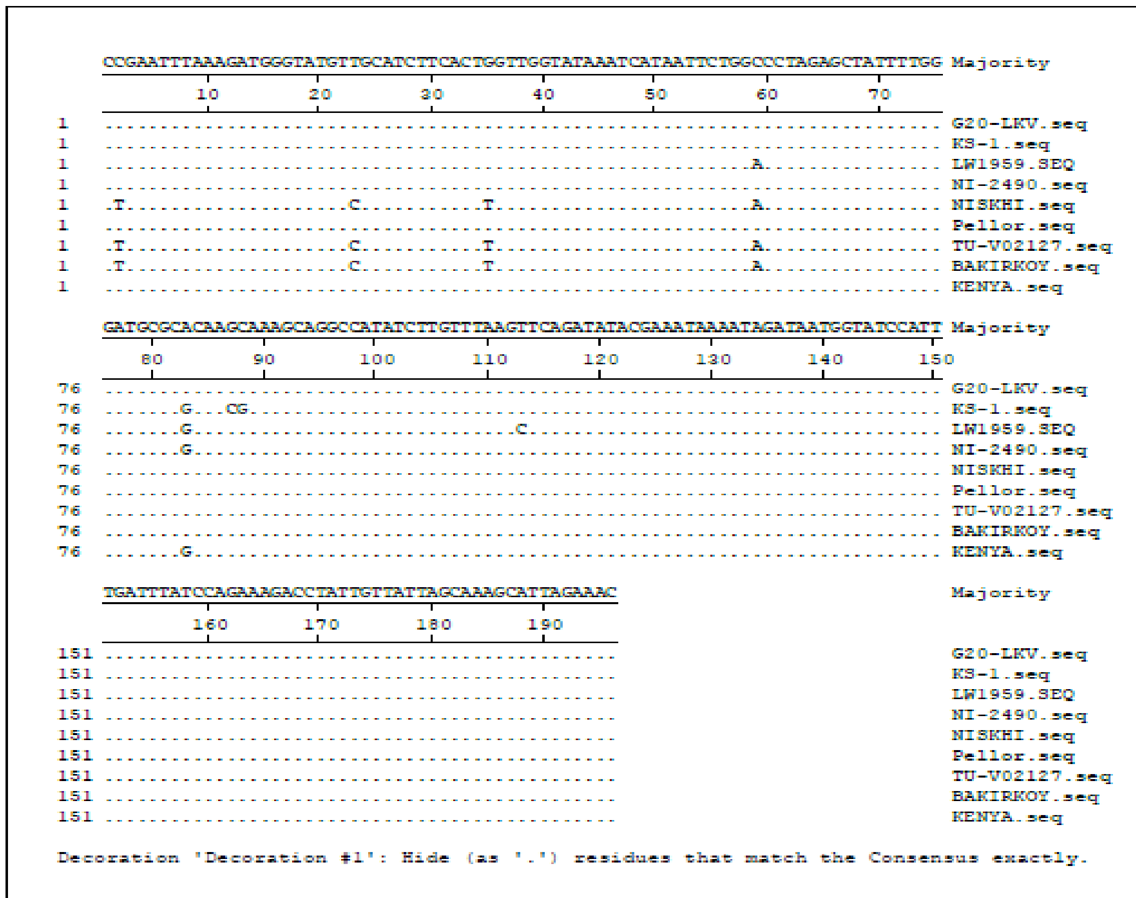


Figure 3. The comparison of LD116 gene nucleotides of sheeppox virus Kenya0240 and Bakirkoy strains, and reference viruses.

Şekil 3. Koyun çiçek virüsü Kenya0240, Bakırköy suşlarının ve referans viruslerin LD116 gen nükleotidlerinin karşılaştırılması.

Table 1. The reference viruses obtained from the NCBI Gene Bank.

Tablo 1. NCBI Gen Bankasından elde edilen referans virüsler.

Strains	Virus	Isolated Year	Country	Accession Number
KS-1	Sheeppox virus	1989	England	M28824
TU-VO2127	Sheeppox virus	1970	Turkey	AY077832
NISKHI	Sheeppox virus	2000	Kazakhstan	AY077834
G20-LKV	Goatpox virus	2000	Kazakhstan	AY077836
Pellor	Goatpox virus	2006	Kazakhstan	AY077835
LW1959	Lumpy skin disease virus	2003	South Africa	AF409138
NI-2490	Lumpy skin disease virus	1958	Kenya	AF325528

Table 2. The percent similarity of LD116 gene nucleotides of sheeppox virus Kenya0240 and Bakirkoy strains, and reference viruses.

Tablo 2. Koyun çiçek virüsü Kenya0240 ve Bakırköy şuşlarına ve referans virüslerine ait LD116 gen nükleotidlerinin benzerlik yüzdesi.

Percent Similarity

Percent Divergence

	1	2	3	4	5	6	7	8	9		
1	***	99.3	98.3	99.3	100	98.3	98.3	98.3	99.3	**1**	G20-LKV
2	0.7	***	99	100	99.3	97.9	97.9	97.9	100	**2**	KS-1
3	1.8	1	***	99	98.3	96.9	96.9	96.9	99	**3**	LW1959
4	0.7	0	1	***	99.3	97.9	97.9	97.9	100	**4**	NI-2490
5	0	0.7	1.8	0.7	***	98.3	98.3	98.3	99.3	**5**	Pellor
6	1.8	2.1	3.2	2.1	1.8	***	100	100	97.9	**6**	TU-V02127
7	1.8	2.1	3.2	2.1	1.8	0	***	100	97.9	**7**	NISKHI
8	1.8	2.1	3.2	2.1	1.8	0	0	***	97.9	**8**	BAKIRKOY
9	0.7	0	1	0	0.7	2.1	2.1	2.1	***	**9**	KENYA0240
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**		

Table 3. The percent similarity of CD47 gene nucleotides of sheeppox virus Kenya0240 and Bakirkoy strains, and reference viruses.

Tablo 3. Koyun çiçek virüsü Kenya0240 ve Bakırköy şuşlarına ve referans virüslerine ait CD47 gen nükleotidlerinin benzerlik yüzdesi.

Percent Similarity

Percent Divergence

	1	2	3	4	5	6	7	8	9		
1	***	98.5	98.5	99.5	97.4	100	97.4	97.4	99.5	**1**	G20-LKV
2	1.5	***	98	99	95.9	98.5	95.9	95.9	99	**2**	KS-1
3	1.5	2.1	***	99	96.9	98.5	96.9	96.9	99	**3**	LW1959
4	0.5	1	1	***	96.9	99.5	96.9	96.9	100	**4**	NI-2490
5	2.1	3.7	2.6	2.6	***	97.4	100	100	96.9	**5**	NISKHI
6	0	1.5	1.5	0.5	2.1	***	97.4	97.4	99.5	**6**	Pellor.seq
7	2.1	3.7	2.6	2.6	0	2.1	***	100	96.9	**7**	TU-V02127
8	2.1	3.7	2.6	2.6	0	2.1	0	***	96.9	**8**	BAKIRKOY
9	0.5	1	1	0	2.6	0.5	2.6	2.6	***	**9**	KENYA0240
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**		

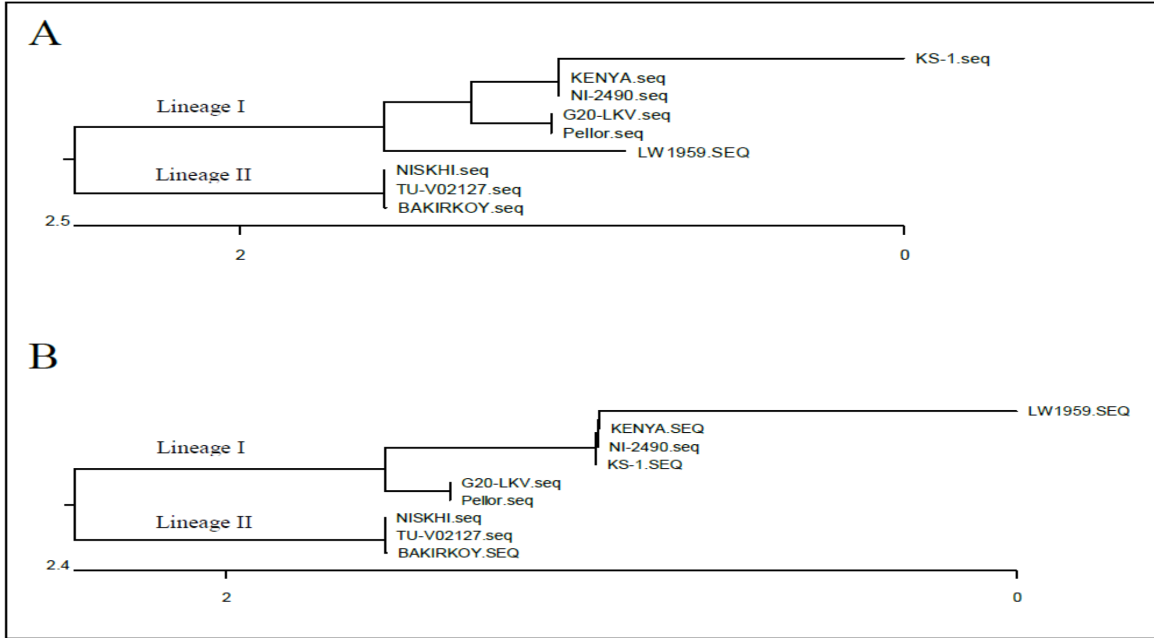


Figure 5. Phylogenetic comparison of LD116 (A) and CD47 (B) gene nucleotides of sheeppox virus Kenya0240 and Bakirkoy strains, and reference viruses.

Şekil 5. Koyun çiçek virüsü Kenya0240 ve Bakırköy şuşlarına ve referans virüslerine ait LD116 (A) ve CD47 (B) gen nükleotidlerinin filogenetik karşılaştırması.

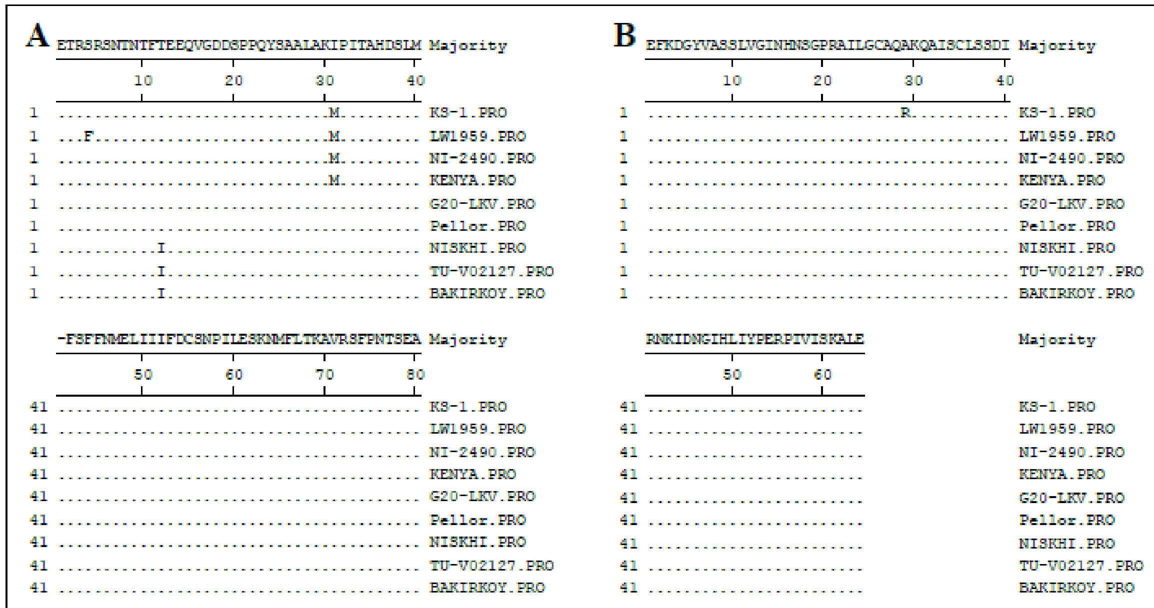


Figure 6. Aminoacid comparison of LD116 (A) and CD47 (B) gene of sheeppox virus Kenya0240 and Bakirkoy strains, and reference viruses.

Şekil 6. Koyun çiçek virüsü Kenya0240 ve Bakırköy şuşlarına ve referans virüslerine ait LD116 (A) ve CD47 (B) genlerindeki aminoasit karşılaştırması.

Discussion

When the 150 kbp whole genomes of the virus isolates in the *Capripoxvirus* genus (SPPV, GPPV, and LSDV) are compared, the isolates show 96% relationship with each other (Tulman et al., 2002). On the other hand, phylogenetic studies, particularly studies comparing the nucleotides of membrane protein P32 show that these viruses form host-specific groups (Hosamani et al., 2004). P32 protein is a structural protein carrying the most important antigenic determinants and present in all species within the *Capripoxvirus* genus (Heine et al., 1999; Tian et al., 2010).

In studies assessing the differences or similarities between the viruses one should not directly use the material or isolates. Although the strains are isolated from sheep or goats, since sheeppox virus may infect goats or goatpox virus may infect sheep, determination of the specific host is not possible. This situation leads to inconsistent results in investigations. For this reason, phylogenetic studies should not use material or isolates directly, but should compare the strains. The differences in morbidity and mortality of sheeppox epidemics in India in 2007 and 2008 were caused by strains with different genomic and so different antigenic structure (Bhanuprakash et al., 2010).

The similarities or differences between the strains determined by DNA sequence analysis contribute significantly to the selection of vaccine strains. With the introduction and use of DNA sequencing, it was determined that vaccines used in many areas did not provide sufficient protection and that vaccine strains capable of protecting against all of SPPV, GPPV and LSDV should be selected like the attenuated KS-1 strain which is recommended (Babiuk et al., 2008). In case of the absence of such a vaccine, use of combined (multivalent) vaccines is recommended (Rao and Bandyopadhyay, 2000). As a result of our phylogenetic assessments and similarity index analyses, when the Bakırköy and Kenya0240 strains were compared, we found that only the Kenya0240 strain was in the same group with all representatives of the *Capripoxvirus* genus (Lineage I). This result was true not only for the LD116 gene region coding P32 protein of the virus, but true also for the CD47 gene region.

As shown by sequence analyses and phylogenetic studies, there exists a stronger relationship between some strains of SPDV and GPDV than between these viruses and LSDV (El-Kenawy and El-Tholoth, 2010). For this reason, potential vaccine strains should protect not only against sheeppox and goatpox infections, but also against lumpy skin disease of the cattle (Kitching, 2003). Vaccination programs of a country is not sufficient to eradicate these diseases because the causative agents could be easily carried by animals movements from neighboring countries or by other ways; control programs on regional basis are required (Babiuk et al., 2008).

Against infections caused by viruses in the *Capripoxvirus* genus (SPDV, GPDV, and LSDV), vaccines prepared from the Kenya0240 strain which is genomically closer to other viruses in the genus may provide a more effective protection.

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