MOLECULAR CHARACTERISATION OF LUMPY SKIN DISEASE VIRUS IN CATTLE USING P32 AND RPO30 GENES IN DUHOK PROVINCE, KURDISAN REGION OF IRAQ.

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ABSTRACT

Lumpy skin disease (LSD) is a contagious viral infection of cattle caused by lumpy skin disease virus (LSDV). This infection has a huge economic impact on the cattle industry as a result of skin damage, inflammation of mammary glands and decrease milk production, reduces reproduction and even death as a consequence of secondary bacterial infection. Iraq is considered as an endemic country by OIE that continues breaking throughout different parts of the country. The aims of this research work was to detect and molecular characterization of LSDV in Duhok province for the first time through using a phylogenetic approach of P32 and RPO30 genes. The results showed that LSDV was detected and identified over using PCR and sequencing and the isolate 1 LSDV/Duhok/Kurdistan/2019, isolate 2 LSDV/Duhok/Kurdistan/2019 and isolate 3 LSDV/Duhok/Kurdistan/2019 were clustered in LSDV lineage for both P32 and RPO30 genes. The phylogenetic analysis of these isolates showed a close relationship with the previously published isolates based on P32 gene sequences, while this study is the first study to publish genetic information on RPO30 gene of the Iraqi isolates and compare them to the other publicly available data sets of other countries. To sum up, the findings of this paper could be beneficial to illustrate the spreading nature of LSDV in Iraq and mainly in the Kurdistan region of Iraq. Furthermore, offer the theoretical references for control and prevention of LSDV infections in future.

INTRODUCTION

Lumpy skin disease (LSD) is considered as a viral infection of cattle caused by a lumpy skin disease virus (LSDV) and it is believed that blood-feeding arthropods are the main mechanical carriers of the virus [1,2]. LSDV belongs to the genus *Capripoxvirus* (CaPV) in the family of *Poxviridae* [3]. LSD is characterized by the development of nodules on the skin that appears over all parts of the body along with widespread enlarged lymph nodes [4]. This infection leads to a huge economic impact as a result of skin damage, inflammation of mammary glands and decreases milk production, reduces reproduction and even death as a consequence of secondary bacterial infection [5]. There is a significant variation in morbidity and mortality rates due to the activity of arthropod carriers, vulnerability and the state of the defence system of the host. Morbidity varies significantly from 2%-85% and some cases are even higher. However, the mortality rate is low 1% -5% and could reach up to 40% in some hosts, specilly if secondary microbial infection occur. The disease was reported for the first time in African countries in 1929 and was detected outside the Africa in 1989 in Israel and afterward was diagnosed in Kuwait, Lebanon, Jordan, Turkey, Bahrain, Yemen and Oman [4,6]. In 2013, the LSDV infection was reported for the first time in Iraq [7]. Iraq was listed by OIE in 2015 in LSD affected countries in 2014 and there has been reports of LSD detection since that time [7,8,9].

The LSD is identified and diagnosed based on the appearance of a specific clinical disease of the infection, viral isolation, detection by electron microscope, observation of histopathological changes, immunoassay beside the molecular detection [10]. Besides, the genes P32, RPO30 and GPCR that encode for envelope protein, envelope RNA polymerase subunit 30 kD and G-protein, respectively. These are most commonly used genes for the molecular identification and characterization of *Capripoxviruses* . These genes are greatly conserved in *Capripoxviruses*. The sequence data of them can be considered for differentiating of Sheeppox virus (SPV), Goatpox virus (GPV) and Lumpyskin disease virus (LSDV), and further presenting the genetic relatedness between varied virus strains [9,11]. The aim of this study work was the detection and molecular characterization of LSDV in Duhok province through using a phylogenetic approach of P32 and RPO30 genes.

MATERIALS AND METHODS

Study area and clinical sample collection: A total of 20 scab samples were collected from LSD suspected cattle and calves from different locations in Duhok province during summer 2019. The scabs of raptured nodules were taken in equal amount of glycerol and phosphate-buffered saline (PBS) solution. The samples were immediately taken to the Duhok research center at the University of Duhok where they were stored below -20 C^{o} for later testing.

Sample preparation and DNA extraction: The samples containing scab of skin were homogenized by the tissue homogenizer then centrifuged at 8000 rpm for 2 minutes then 100ml were taken for DNA extraction. The DNA of LSDV was extracted from clinical samples by RIBO-prep nucleic acid extraction kit (REF K2-9-Et-50-CE AmpliSens, Moscow, Rusia) according to the manufacturer's instructions.

Oligonucleotide primers:

In this study, the genes P32 and RPO30 were used for both identification and phylogenetic analysis of LSDV. The primers forward (B68) CTAAAATTAGAGAGCTATACTTCTT and reverse (B69) CGATTTCCATAAACTAAAGTG were used for partial amplification of P32 gene [12]. In addition, another two set of primers CpRPO30-OL1F CAGCTGTTTGTTTACATTTGATTTTT and CpRPO30-OL1R TCGTATAGAAACAAGCCTTTAATAGA (pair1) ,CpRPO30-OL2F TTTGAACACATTTTATTCCAAAAAG and CpRPO30-OL2R AACCTACATGCATAAACAGAAGC (pair 2) were used to amplify the two overlapping fragments of RPO30 gene in order to generate a full length-sequence [13]. All primers of this study were made by Macrogen (Korea).

Polymerase chain reaction For the primers (B68 and B69), the PCR was performed by using 40 µl total reaction volume that composed of 20 µl of Prime Taq premix 2X Master Mix (GeNet Bio), 2 µl of 10 pmol of each primer (B68 and B69), 2µl of the DNA template, then the reaction was made upto 40 µl by adding 14 µl of DNA-RNA free water. The amplification conditions were: an initial denaturation at 94 °C for 5 min, followed by 40 cycles of a denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and primer extension at

72 ° C for 30s. The reaction was then held at 72 °C for 5 min. The expected PCR product for this primer set was 390 bp. while for the other two primers sets (CpRPO30-OL1F, CpRPO30-OL1R (pair 1), CpRPO30-OL2F and CpRPO30-OL2R (pair 2) same volume of amplification reaction were used and the quantities of all PCR components were as mentioned in the first reaction. The thermo cycler conditions for these two pairs of primers were: an initial denaturation at 95 °C for 4 min, 40 cycles of denaturation 95 °C for 30 s, annealing 55 °C for 30 s, extension 72 °C for 45 s, and final extension at 72 °C for 7 min. The expected PCR products for these two primer pairs were: 554 bp (pair 1) and 520 bp (pair 2), respectively. The PCR amplicons of all samples were visualized on a 1.5% agarose gel.

Sequence determination : Three representative samples were considered for sequencing for both partial P32 gene and overlapping CpRPO30 gene. A total of 30 µl of PCR products for positive samples were sent to Macrogen (Korean) for sequencing along with all primer sets used in this study. Moreover, the obtained sequence reads were submitted to the Genbank database. For the RPO30 sequences, the raw reads were assembled through the Staden software (version 2.0.0b8). The accession numbers received from NCBI were MN871848, MN871849, MN871850 for P32 gene, while MN871851, MN871852 and MN871853 for CpRPO30 gene.

Phylogenetic analysis Nucleotide reference sequences of both P32 and CpRPO30 genes of LSD strains were retrieved from NCBI database and multiple sequence alignments were done separately for each gene using bioedit [(14)]. Phylogenetic and molecular evolutionary analyses were performed by MEGA X [(15)]. A neighbour-joining phylogenetic tree was created and evaluated with 1000 bootstrap replicates as employed in the program.

RESULTS

Detection of Capripoxvirus

All 20 clinical samples were shown to be positive by detection of the partial P32 gene amplified fragments. The detected amplicon size was 390bp figure 1; the identity of the detected fragments was confirmed over sequencing. Besides, CpRPO30 gene was also successfully amplified in all samples by detecting amplicons of 554 bp and 520 bp for primers sets pair 1 and pair 2 respectively.

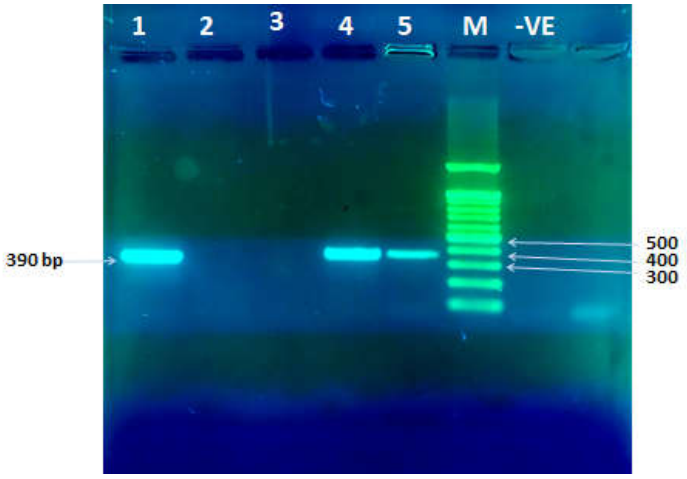

Figure 1: Agarose gel electrophoresis shows a product of 390 bp from amplification of the P32 gene. Lane M: 100 bp DNA ladder, lanes 1–5: LSDV isolates, lane –ve: negative control.

Sequence analysis of P32 and RPO30 genes

The sequences of both P32 and RPO30 genes of the three isolates were subjected to analysis as well as alignment with those reference sequences retrieved from GeneBank. For P32 gene, the isolate 1 LSDV/Duhok/Kurdistan/2019 (MN871848), isolate 2 LSDV/Duhok/Kurdistan/2019 (MN871849) and isolate 3 LSDV/Duhok/Kurdistan/2019 (MN871850) were aligned and showed matching with each other except isolate 1 had a mismatching at the position 295 compared to the other two isolates (Figure 2). The blast search revealed that the isolate 1 LSDV/Duhok/Kurdistan/2019 had a very close relationship with sequences Slemani/Kurdistan/2014 (KM047052 LSDV), LSDV/Slemani/Kurdistan/2013 (KF996498), LSDV Slemani/Kurdistan/2014 (KM047053), LSDV IRQ/2015 (KU720359), LSDV Sulaimani/Kurdistan/2014 (KP313621) and the sequence identity was clearly 100% with these sequences. Nevertheless, the isolate 2 LSDV/Duhok/Kurdistan/2019 (MN871849) and isolate 3 LSDV/Duhok/Kurdistan/2019 (MN871850) shared sequence identities of 100% with these sequences: LSDV Kubash/KAZ/16 (MN642592), LSDV isolate Kenya (MN072619), LSDV isolate 155920/2012 (KX894508), LSDV Russia/2015 (MH893760), isolate Evros/GR/15 (KY829023), isolate SERBIA/Bujanovac/2016 (KY702007). On the other hand, the isolates for the CpRPO30 gene were also analysed based on sequence similarities with the NCBI data sets. The LSDV isolate Duhok 1 (MN871851) and LSDV isolate Duhok 3 (MN871853) were identical by 100% (Figure 5).

Furthermore, when compared to the publicly published references they showed a 100% sequence identity with this sequences: LSDV Kubash/KAZ/16 (MN642592), LSDV Kinelsky (MK452256), LSDV Massalamia/P04/1971 (MK302113), Galesa/B12/2008 (MK302112), Chilimo/B11/2008 (MK302111), LSDV Ginchi/B10/2008 (MK302110), LSDV Holeta/B9/2008 (MK302109), LSDV Ambo/B8/2008 (MK302108), LSDV Gindo/B7/2008 (MK302107). While LSDV isolate Duhok 2 (MN871852) had a relationship with sequences LSDV Kubash/KAZ/16 (MN642592), LSDV Kinelsky (MK452256), LSDV Massalamia/P04/1971 (MK302113), Galesa/B12/2008 (MK302112), Chilimo/B11/2008 (MK302111), LSDV Ginchi/B10/2008 (MK302110), LSDV Holeta/B9/2008 (MK302109), LSDV Ambo/B8/2008 (MK302108), LSDV Gindo/B7/2008 (MK302107) and the sequence identities were 99.83% with all this reference.

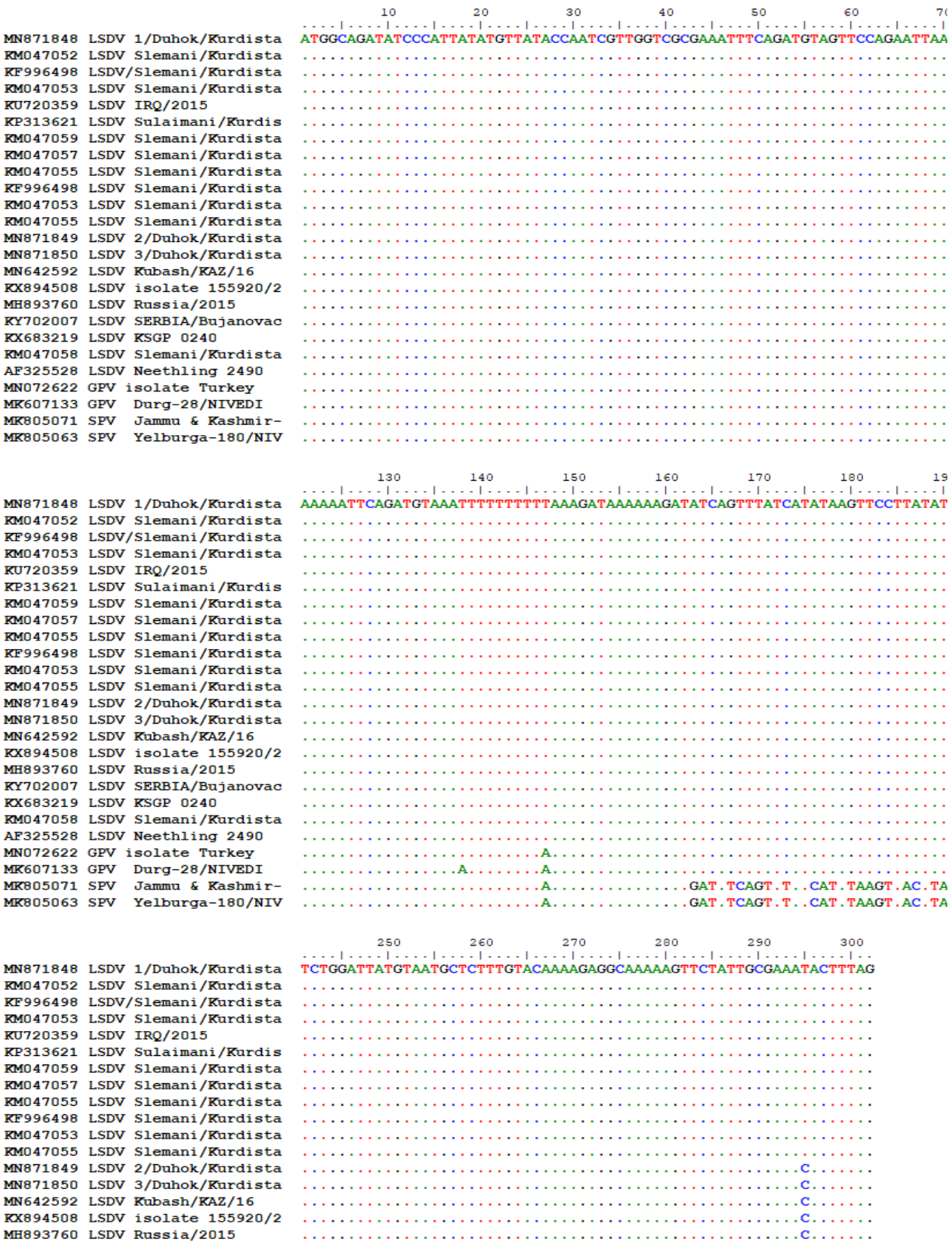

Figure 2: sequence alignment of P32 gene isolates of this study with NCBI references

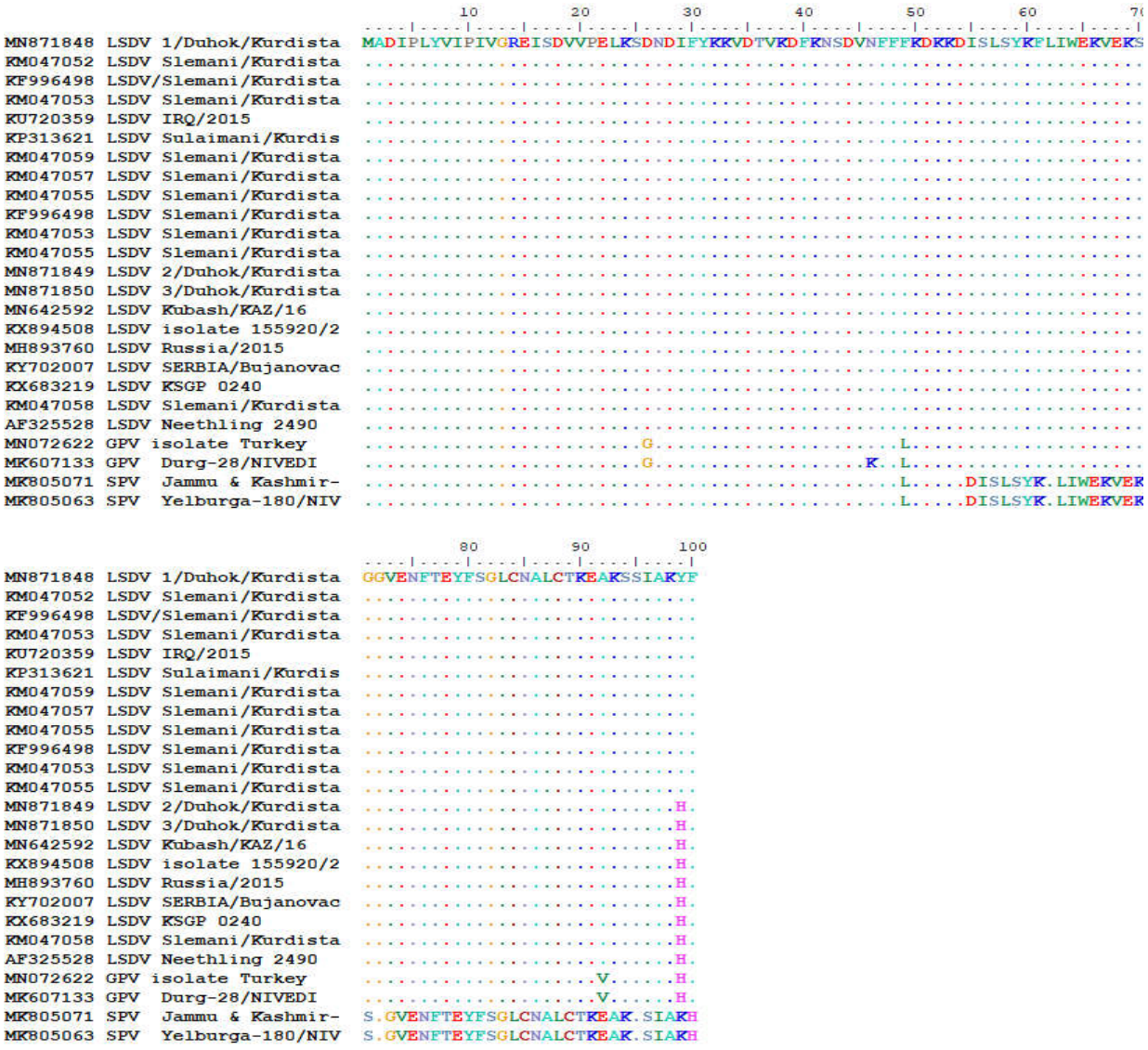

Figure 3: sequence alignment of envelope protein sequence (P32 gene) isolates of this study with NCBI references

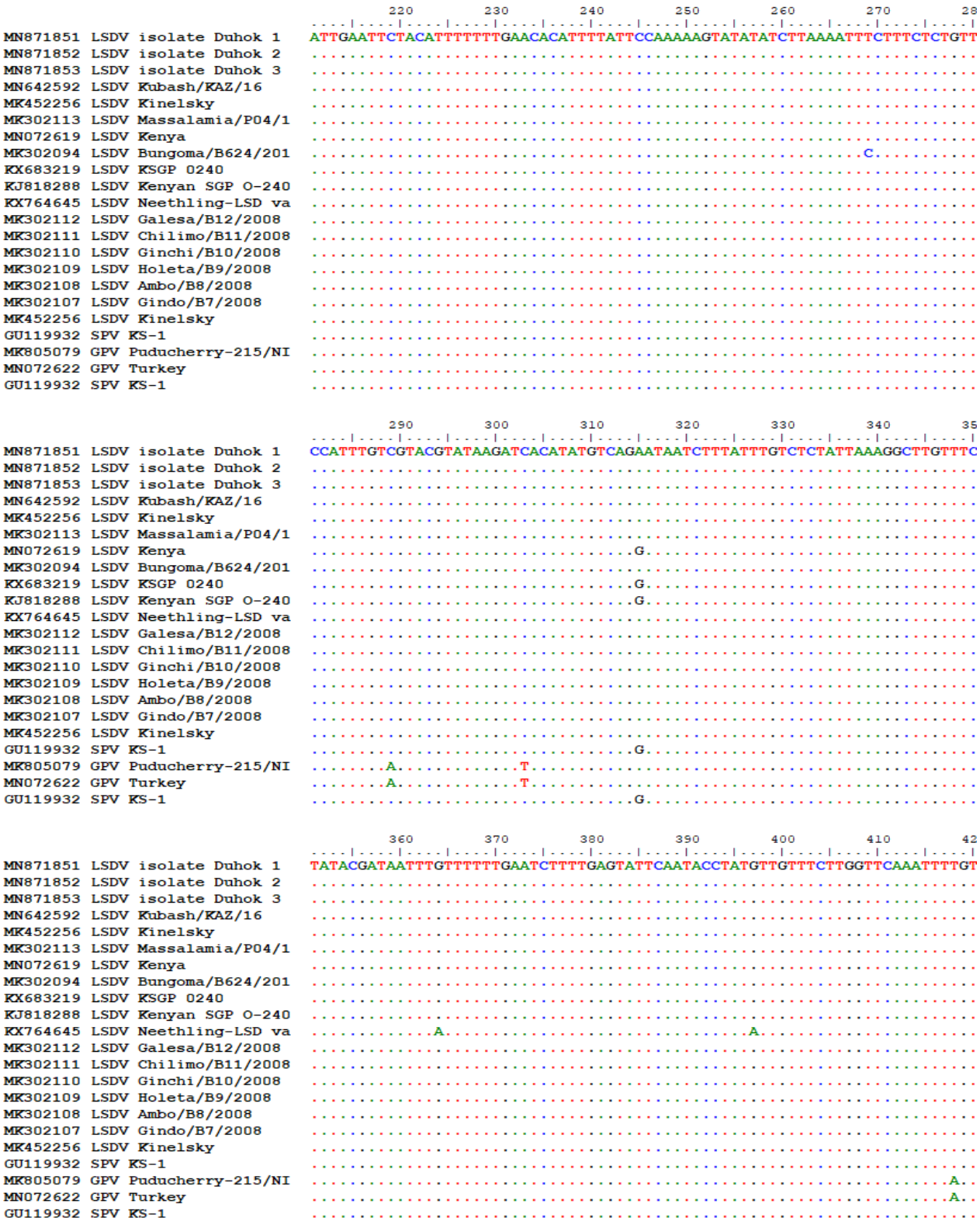

Figure 4: sequence alignment of envelope RPO30 gene isolates of this study with NCBI references

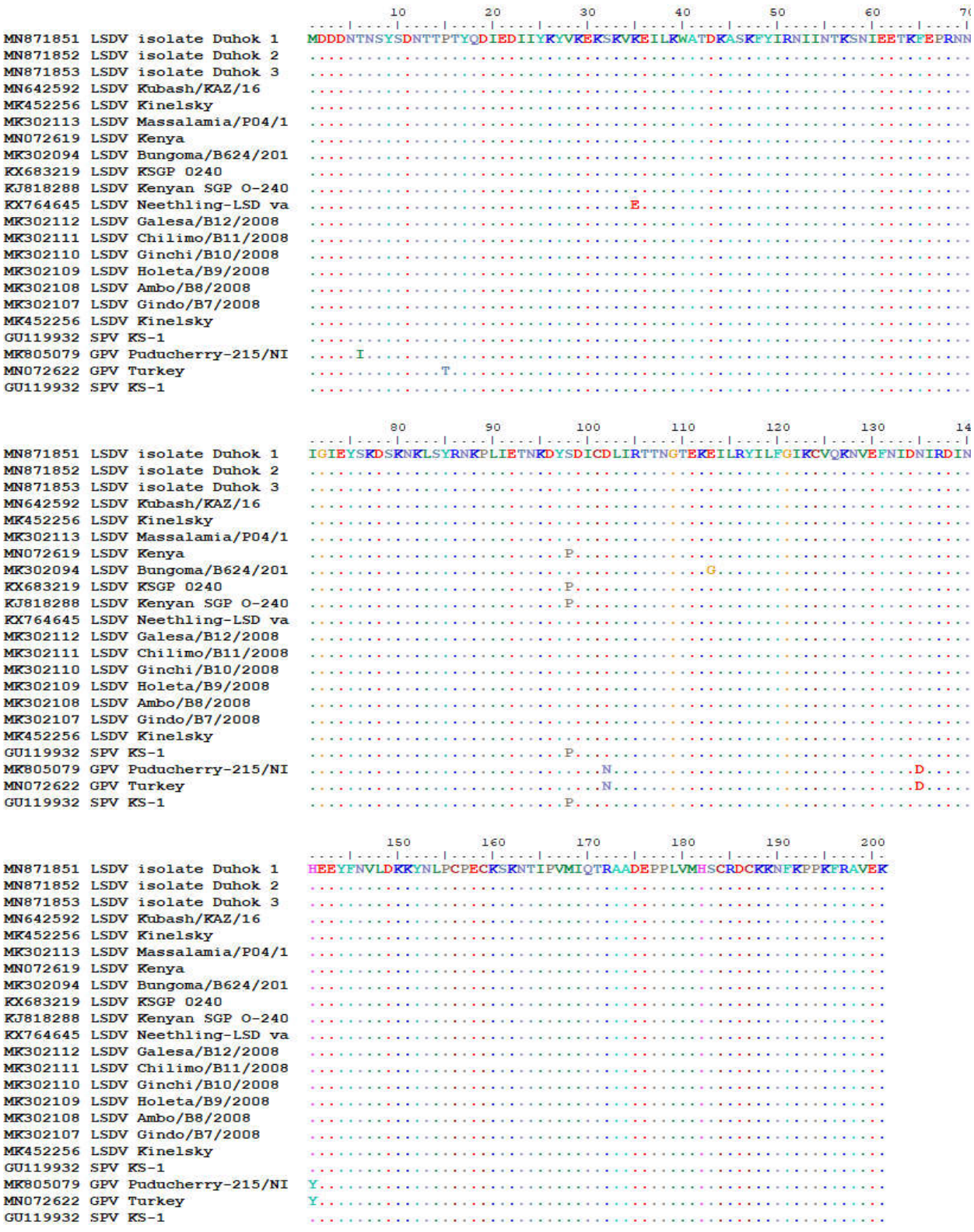

Figure 5: sequence alignment of envelope RNA polymerase subunit 30 kD (RPO30 gene) isolates of this study with NCBI references

Phylogenetic analysis

Phylogenetic tree was constructed to illustrate the genetic relationship of the LSDV isolate of this study with the other *Capripoxvirus* isolates obtained from the NCBI database. The analysis of either partial P32 gene and the full length RPO30 gene sequence of the three isolates of this study were clustered in LSDV lineage (Figures 6, 7). The resulted phylogenetic trees were completely agreed with the genotyping results of the conventional analysis. The Multiple sequence alignment was done for all sequences by using bioedit through the ClustalW method as implemented in the program [14] . Phylogenetic tree was constructed by MEGA X program using neighbour-joining method with the maximum composite likelihood nucleotide substitution model and the pairwise deletion options [15].

The phylogenetic tree was tested for reliability by 1000 bootstrap replicates as employed in the program. The resulted tree for P32 gene, the 1 LSDV/Duhok/Kurdistan/2019 was clustered with other Iraqi isolates like Slemani/Kurdistan/2014 (KM047052 LSDV), LSDV IRQ/2015 (KU720359) as shown in (figure 6). While the isolate 2 LSDV/Duhok/Kurdistan/2019 (MN871849) and isolate 3 LSDV/Duhok/Kurdistan/2019 (MN871850) along with the other Iraqi isolate LSDV Slemani/Kurdistan/2014 (KM047058) were together cluster with a foreign isolates mostly African isolates LSDV Kubash/KAZ/16 (MN642592), LSDV isolate Kenya (MN072619), LSDV isolate 155920/2012 (KX894508), LSDV Russia/2015 (MH893760), isolate Evros/GR/15 (KY829023) (figure 6). On the other hand, the phylogenetic tree of RPO30 gene showed that LSDV isolate Duhok 1 (MN871851) and LSDV isolate Duhok 3 (MN871853) were clustered with LSDV isolates LSDV Kubash/KAZ/16 (MN642592), LSDV Kinelsky (MK452256), LSDV Massalamia/P04/1971 (MK302113). Moreover, the isolate Duhok 2 (MN871852) was grouped separately with LSDV Bungoma/B624/2010 (MK302094) isolate (figure 7). The three isolates sequence of this study of both genes is marked by black triangle (figure 6, 7).

DISCUSSION

Lumpy skin disease virus is a highly contagious viral infection of cattle leading to considerable economic impact on cattle industry [1,2]. The disease is endemic in many

countries like African countries, Israel, Kuwait, Lebanon, Jordan, Turkey, Bahrain, Yemen and Oman [4,6]. In Iraq, LSDV was detected for the first time in 2015 and there have been

Many subsequent reports of LSDV outbreaks throughout the country [7,8,9] To the extent of our knowledge this is a first study to detect and characterize LSDV virus isolates in Duhok province of Iraq.

The objective of this study was to detect and determine the phylogenetic relationship of circulating LSDV isolate in Duhok province with the other strains worldwide. In this study, the genes P32 and RPO30 that encode for envelope protein and an envelope RNA polymerase subunit 30 KD, respectively were used for either detection or phylogenetic analysis of Duhok LSDV isolates. The sequences of these two genes are very distinguished and are used to differentiate the Sheep pox virus, Goat pox virus and Lumpy skin disease virus as well as the relationship between varied strains [9 11].

The phylogenetic analysis of these two genes can arrange the *Capripoxviruses* in a three lineages; LSDV, SPV and GPV. All isolates of the present study for both P32 and RPO30 genes were clustered in LSDV lineage (Figures 6, 7) [10]. However, by looking at the phylogenetic tree of P32 gene, the isolate 2 LSDV/Duhok/Kurdistan/2019 and isolate 3 LSDV/Duhok/Kurdistan/2019 were subclustered with a foreign isolates mostly of African origins and other countries (figure 6).

These two later isolates have an exclusive amino acid substitution with Amino acid Histidine (H) at residue position 99 of the sequences, which is obvious by Tyrosine (Y) amino acid in isolate 1 LSDV/Duhok/Kurdistan/2019 as well as other Iraqi isolates (figure 3). On the other side, the phylogenetic results of RP030 gene sequences of this study showed a homology of 100% with many other LSDV references of the Genbank as all of them are clustered under LSDV lineage (Figure 7). The amino acid sequence of RPO30 gene of all three Duhok/Kurdistan/2019 showed no substitution at the amino acid level that suggests more stability of this gene comparing to the P32 gene (Figure 5).

Furthermore, These findings agreed with an earlier study regardless with the close relationship among the Capv, they have a distinct phylogenetic features [16] and the LSDV signature is clearly noted for both P32 and RP030 gene sequences (Figures 2, 3, 4, 5). Moreover, the topology of the phylogenetic analysis constructed on either nucleotide or amino acid sequences for both P32 and RPO30 genes showed that LSDV and GPV have

a closer genetic relatedness than with SPV (Figures 6, 7). These findings are similar to the results published by Zhu *et al.*, (2013).

In contrast, these results differ from some other previously published studies [11,17] . Comparing the sequences of both RPO30 and P32 genes, P32 gene is more appropriate for epidemiological studies on Iraqi strains due to its abundant existing published data since this is a first study to report information on RPO30 gene of Iraqi LSDV isolates. However, taking only one gene for phylogenetic analysis and genetic relationship among the CaPV is not sufficient due to their huge size genomes [10] .

To conclude, this study reported and identified three isolates of LSDV in Duhok province of Iraq. Depending on the phylogenetic analysis, this study revealed the genetic relatedness among Iraqi LSDV isolates and comparing them with isolates of other countries. To sum up, the findings of this paper could be beneficial to illustrate the spreading nature of LSDV, mainly in the Kurdistan region of Iraq. Furthermore, offer the theoretical references for controling and prevention of LSDV infections in future.

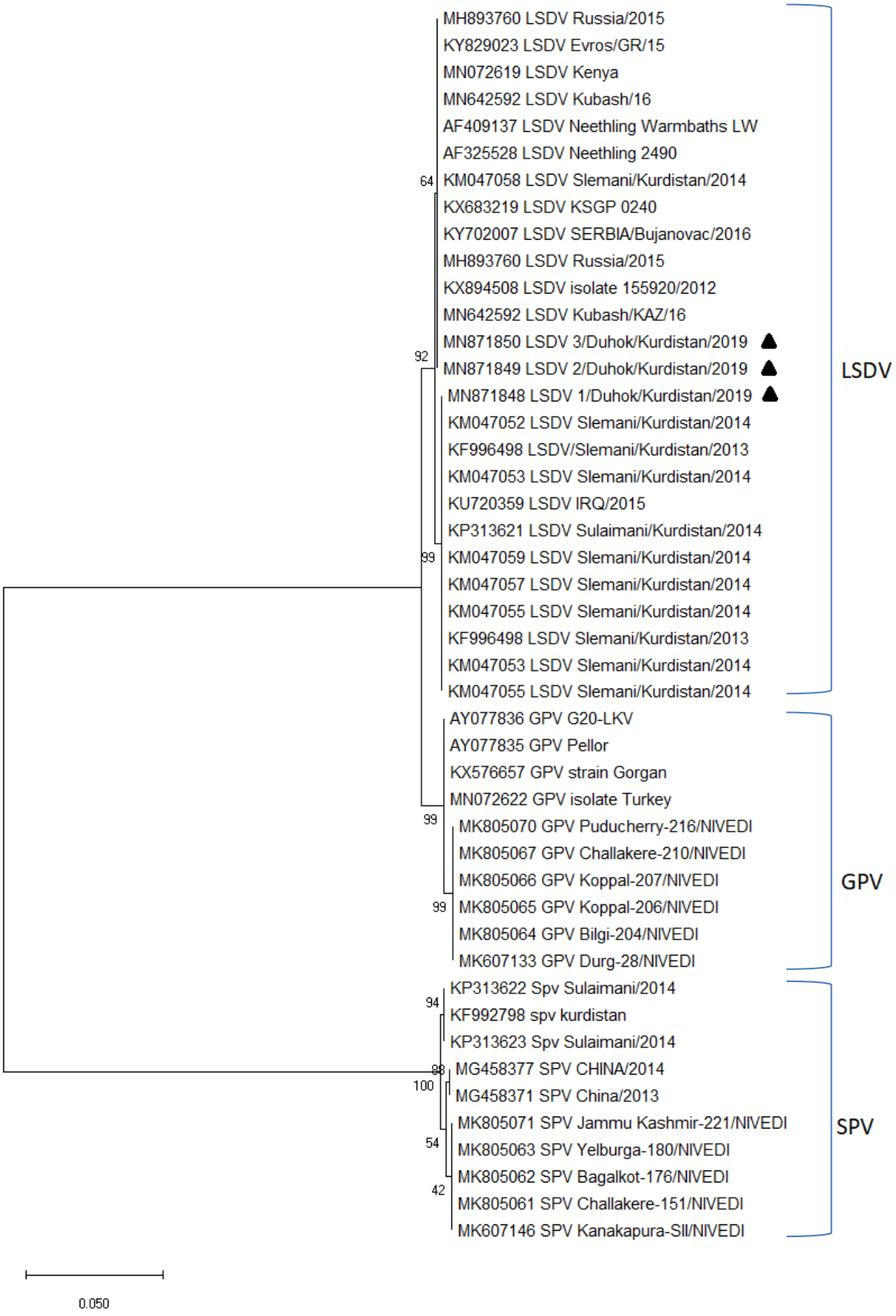

Figure 6: Neighbour-joining tree illustrating phylogenetic relationships of the LSDV isolates of this study and other *Capripoxvirus* references based on P32 gene (Envelop protein) nucleotides sequences.

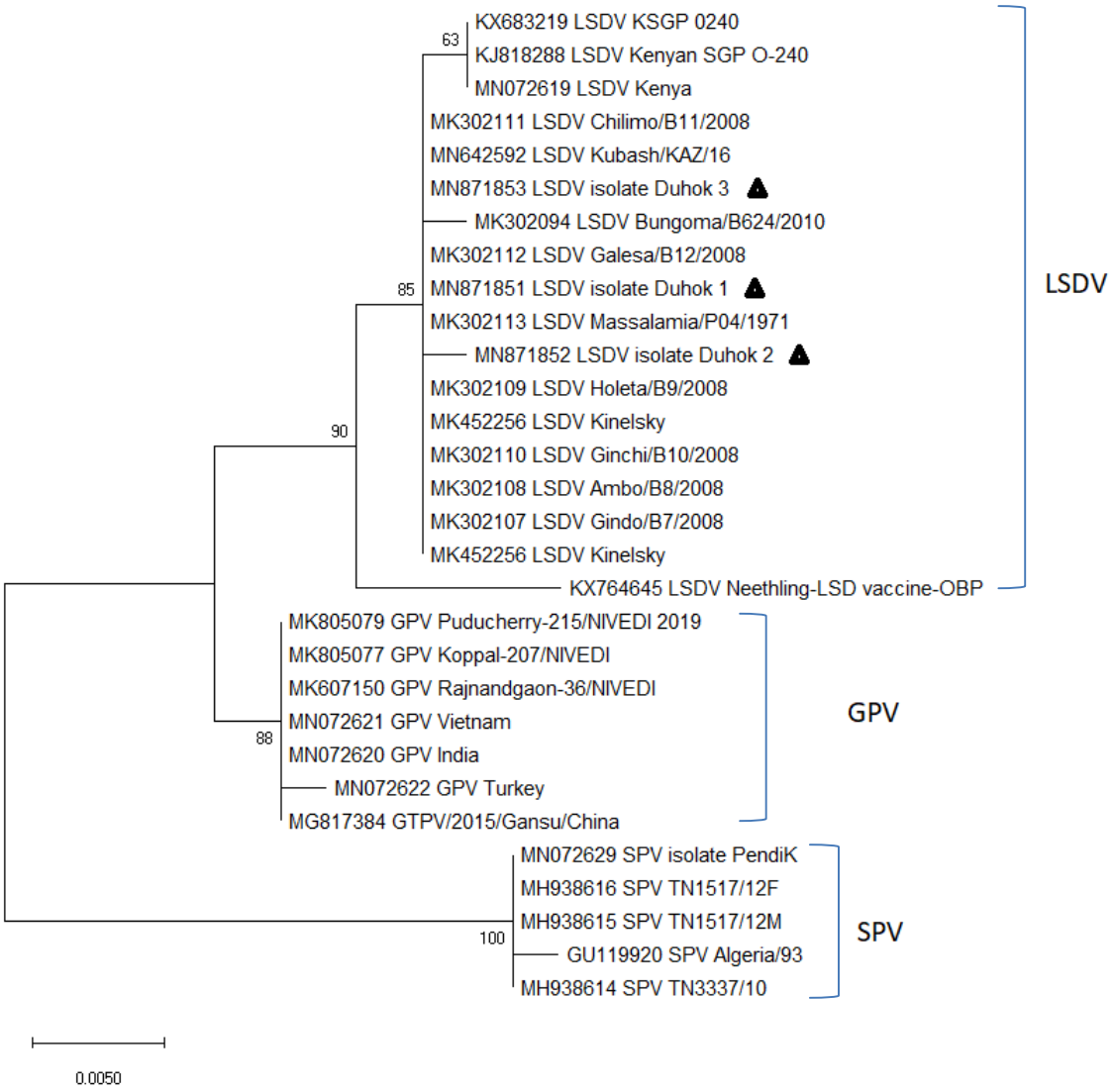

Figure 7: Neighbor-joining tree illustrating phylogenetic relationships of the LSDV isolates of this study and other *Capripoxvirus* references based on RPO30 gene (RNA polymerase subunit 30 kD) nucleotides sequences.

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الوصف الجزيئي لفايروس التهاب الجلد العقيدي في المجترات باستخدام جينات P32 وRPO30 في محافظة دهوك- إقليم كوردستان العراق.

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الخُلاصة

يعد التهاب الجلد العقيدي (LSD) من الأمراض الفيروسية المعدية في المجترات يسببها فايروس التهاب الجلد العقيدي (LSDV). أذ يسبب هذا المرض خسائر اقتصادية كبيرة في مجال تربية المجترات نتيجةً لتلف الجلد، التهاب الضرع، انخفاض انتاج الحليب، وانخفاض نسبة التكاثر، وقد تؤدي الى النفوق نتيجةً للعدوى لعدوى الجراثيم الثانوية. يعتبر العراق من البلدان المستوطنة لمرض التهاب الجلد العقيدي حسب المنظمة العالمية لصحة الحيوان بسبب استمرار تفشي المرض وفي مناطق مختلفة منه. كان الهدف من الدراسة هو الكشف والتوصيف الجزيئي لفيروس التهاب الجلد العقيدي في محافظة دهوك وللمرة الأولى من خلال طريقة التطور الجيني للجينات P32 وRPO30 وباستخدام تقنية تفاعل البلمرة المتسلسل PCR والتسلسل الجيني. أظهرت النتائج عن كشف وتوصيف ثلاث عزلات من فيروس التهاب الجلد العقيدي، العُزلة الأولى: LSDV/Duhok/Kurdistan/2019 ، العُزلة الثانية: LSDV/Duhok/Kurdistan/2019، والعُزلة الثالثة: LSDV/Duhok/Kurdistan/2019. اظهر تحليل التطور الجيني لهذه العُزلات العلاقة الوطيدة مع العُزلات السابقة المنشورة والمُعتمدة على جين P32، بينما هذه الدراسة تُعد الأولى لنشر المعلومات الوراثية لجين RPO30 في العُزلات العراقية وايضاً مقارنة مع بقية العُزلات المنشورة في البلدان الأُخرى. نستنتج ان هذه الدراسة يمكن ان تكون ذات فائدة لتوضيح طبيعة انتشار فايروس التهاب الجلد العقيدي في العراق وبخاصة في إقليم كوردستان، إضافة الى انها تقدم مراجع نظرية للسيطرة والوقاية من الإصابة ي المستقبل.

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