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Phylogenetic analyses of *P32*, *RPO30*, *GPCR*, *ORF117*, and *Kelch-like* genes of Tunisian sheeppox virus isolates

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Abstract: Sheeppox is an economically important disease of small ruminants caused by the sheeppox virus (SPPV). Sheeppox virus (SPPV), goatpox virus, and lumpy skin disease virus are capripoxviruses, antigenically related but genetically distinct. Their differentiation requires analysis at a molecular level. Eleven sheeppox virus isolates from five different regions of Tunisia were collected between 2010 and 2016. SPPV was detected by real-time PCR using SYBR Green technology. Primers amplifying the entire *Kelch-like* gene were designed. Then the *GPCR*, *RPO30*, *P32*, *ORF117*, and *Kelch-like* genes were molecularly analyzed to show the different specific lineage patterns of each species. Phylogenetic analyses revealed three monophyletic and distinct specific lineage clusters as per their host origins. Our study proved the efficiency of using these five genes for the identification and differentiation of capripoxvirus species, and the *GPCR* gene is recommended for phylogenetic studies.

Key words: *GPCR*, *Kelch-like*, *ORF117*, *P32*, *RPO30*, sheeppox virus

1. Introduction

Sheeppox is a skin disease of sheep caused by a pox virus. It is characterized by an eruption of vesicles covered with a dry scab. The disease was eradicated in many countries but is still present in Africa north of the equator, West Asia, India, China, and Bangladesh and it is spreading in West Europe, especially in Turkey and Greece [1]. In Tunisia, the disease generally has an enzootic appearance. It poses a threat to the development of the agricultural sector, causing economic losses related to mortality of lambs and a fall in reproduction and production in adults; morbidity and mortality rates are 4.98% and 0.55%, respectively [2].

The sheeppox virus belongs to the *Capripoxvirus* genus, which is one of the eleven genera of the subfamily *Chordopoxviruses* of the family *Poxviridae*. The genus *Capripoxvirus* consists of the sheeppox virus (SPPV), goatpox virus (GTPV), and lumpy skin disease virus (LSDV), which are sources of dermatological diseases in sheep, goats, and cattle, respectively [3].The viral genome is a double-stranded linear DNA of 150 kbp [4]. Each of the three species of the genus *Capripoxvirus* infects their natural hosts and is named according to the host origin; nevertheless, cross-species infections among sheep, goats, and cattle have been detected [5,6]. This makes differential diagnosis more difficult, and serological diagnosis based on detection of antibodies by immunofluorescence, ELISA, or gel immunodiffusion is inefficient [7]. In addition, serological methods cannot distinguish sheeppox infection from lumpy skin or goatpox disease [8]. Only molecular methods allow distinction between all these species through sequencing and molecular analysis of some viral genes [9,10].

Several trials have been carried out to establish the relationship among these virus species by constructing phylogenetic trees after sequencing *RPO30*, *P32*, *ORF117*, and other genes [5,11–13]. None of these genes have given the consistent results required to create a reference candidate to anchor phylogenetic classification within the genus *Capripoxvirus*. Therefore, it is essential to find a common gene that allows more relevant phylogenetic classification and distinction between these virus species without producing discrepancies between studies.

2. Materials and methods

2.1. Sample collection

Skin specimens with crusted scab lesions from the axilla and/or tail were collected from sheep suspected to be infected with pox virus (Table 1). Eleven samples were received from different regions of Tunisia and collected between 2010 and 2016, a period in which more than 344 outbreaks were reported [2].

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2.2. Virus isolation and titration

Primary cell cultures were prepared from the testes of unvaccinated lambs between 3 and 4 months of age. A cell suspension was prepared, filtered, and suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal calf serum (FCS). Ten million cells suspended in DMEM and supplemented with 15% FCS were then distributed to 25-cm^2 flasks, which were incubated at 37 °C with 5% CO_2. Confluent primary lamb testis cell monolayers reaching 90% confluency were inoculated with each isolate. The cytopathic effect (CPE) was observed on the 11th or 12th day after inoculation. The cells and the supernatants were collected and frozen, then thawed and centrifuged at 3000 rpm for 30 min. Virus titration was performed in a 96-well microtiter plate. Serial dilutions ranging from 10^{-1} to 10^{-9} of each isolate were prepared and inoculated into lamb testis cell cultures, which were examined daily for CPE. The virus titer was determined and expressed as the tissue culture infection dose ($TCID_{50}$) according to the Reed–Muench method [14] and expressed as $\log_{10}$ ($TCID_{50}$/mL).

2.3. Genomic DNA extraction

Given the high sensitivity of real-time PCR, sheeppox virus detection was performed from viral DNA extracted directly from collected scabs using QIAamp DNA mini kits (QIAGEN), in accordance with the manufacturer's instructions.

2.4. SYBR Green-based real-time PCR assay

SYBR Green real-time PCR assay was performed to detect the presence of sheeppox virus DNA by amplifying a partial conserved region of the *P32* gene of the sheeppox virus [15] with KAPA SYBR FAST qPCR kit Master Mix (2X) Universal (Kapa Biosystems, USA).

2.5. Design of *Kelch-like* gene primers

The reference sequence used for the design of primers that amplify the entire *Kelch-like* gene (1710 bp) was selected from GenBank (accession number: NC_004002). This reference sequence was introduced into Primer3 software (http://primer3.ut.ee/) and generated two pairs of primers; the first was 767 bp and amplified the first part of the gene, and the second was 1183 bp and amplified the second part of the gene. The two amplified sequences have an overlapping portion, which serves for the assembly and generation of a single sequence.

The specificity of the selected primers was tested by comparing the sequences of each primer with those listed in the GenBank database using the nucleotide BLAST program (http://blast.ncbi.nlm.nih.gov/).

Finally, NetPrimer (http://www.premierbiosoft.com/netprimer/) was used to verify that the selected primers did not form secondary structures such as primer dimers or hairpins. The sequences of the primers and their positions are listed in Table 2.

Table 1. Sample data.

Year of outbreak	Samples	Nature	Sex	Age	Origin	Race
2016	09/16	Scabs	N.S.	2 months	Bizerte	Barbarine
2016	10/16	Scabs	N.S.	1.5 months	Bizerte	Black thibar
2016	36/16	Scabs	♀	3 years	Ben Arous	Western thin tail
2015	13/15	Scabs	♀	5 years	Ben Arous	Western thin tail
2015	14/15	Scabs	♀	2 years	Ben Arous	Croisée
2014	279/14	Scabs	♀	3 years	Mannouba	Barbarine
2014	204/14	Scabs	♂	8 months	Ariana	Western thin tail
2012	1517/12M	Scabs	♂	N.S.	N.S.	N.S.
2012	1517/12F	Scabs	♀	N.S.	N.S.	N.S.
2012	05/12	Scabs	♀	4 years	Mannouba	Barbarine
2010	3337/10	Scabs	♀	10 years	Kairouan	N.S.

Table 2. Primer sequences designed for the *Kelch-like* gene.

Primer sequence 5'-3'	Position	Size (bp)	References
KL1-F AGCAAAAGCAGACAACCGTAT KL1-R GGTGGTGTGGATAAATACTTACG	11651–11672 12395–12417	767	Designed in this study
KL2-F CGTAAGTATTTATCCACACCACCT KL2-R GGGCGTTATTTCTAACAAAGACGA	12395–12418 13554–13577	1183	

2.6. Conventional PCR amplification

The PCR reactions were conducted in a thermal cycler (Bio-Rad T100) as described by Zhou et al. [16] for *P32*, *RPO30*, and *GPCR* gene amplifications. A conventional PCR assay was conducted for the *ORF117* gene as described by Dashprakash et al. [13]. For *Kelch-like* gene amplification, one cycle of 3 min at 95 °C was performed, followed by 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 56 °C, and elongation for 1 min at 72 °C and then a final extension step for 5 min at 72 °C.

2.7. Sequencing of *GPCR*, *RPO30*, *P32*, *ORF117*, and *Kelch-like* genes

The PCR products of *GPCR*, *RPO30*, *P32*, *ORF117*, and *Kelch-like* genes of each tested isolate were sequenced in both directions using the Big Dye Terminator v.3.1 Cycle Sequencing Kit following the manufacturer's instructions and analyzed with an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems).

2.8. Molecular and phylogenetic analyses

Sequences of the *GPCR*, *RPO30*, *P32*, *ORF117*, and *Kelch-like* genes of SPPV, GTPV, and LSDV strains, a Morocco vaccine strain (GU119920), and a vaccine strain in use in Tunisia [RM-65 (KJ818284)] were selected from GenBank and included in the study.

The quality of each obtained sequence was analyzed by the BioEdit program (version 7.2.5.0), and the sequence similarities were checked against sequences deposited in GenBank using the NCBI BLAST search (http://www.ncbi.nlm.nih.gov). ClustalW was used for sequence alignment, and phylogenetic trees were constructed using the maximum likelihood method with 1000 bootstrap replicates in MEGA 6. The sequences obtained from the *P32*, *RPO30*, *GPCR*, *ORF117*, and *Kelch-like* genes of the eleven isolates were submitted to GenBank.

Phylogenetic trees were constructed to study the genetic relationships between the Tunisian strains and other capripoxviruses. Phylogenetic analysis of the nucleic acid sequences of the *GPCR*, *RPO30*, *P32*, *ORF117*, and *Kelch-like* genes was then performed.

3. Results

3.1. Virus isolation and titration

Examination of inoculated cell layers showed some refractive cells with partial lysis that became more extensive on day 7 after inoculation (Figure 1). On day 14 the cell layers were detached, and the cells were reduced to a shriveled and pyknotic core, indicating the cell degeneration of the necrosis phase. The titer of the virus suspension was around $10^{-5.5}$ ($TCID_{50}$/mL).

3.2. Viral genome detection

All samples tested for the SPPV *P32* conserved region were positive. Melting curve analysis confirmed the specific amplification of all isolates, which shared the same melting temperature (75.9 °C).

3.3. Multiple alignment analysis of nucleotide and protein sequences

The sequencing of the eleven tested isolates produced sequences of 1125 bp for the *GPCR* gene, 585 bp for the *RPO30* gene, 972 bp for the *P32* gene, 474 bp for the *ORF117* gene, and 1170 bp for the *Kelch-like* gene. These sequences were introduced to BLAST to retrieve reference sequences. The results demonstrated that the eleven

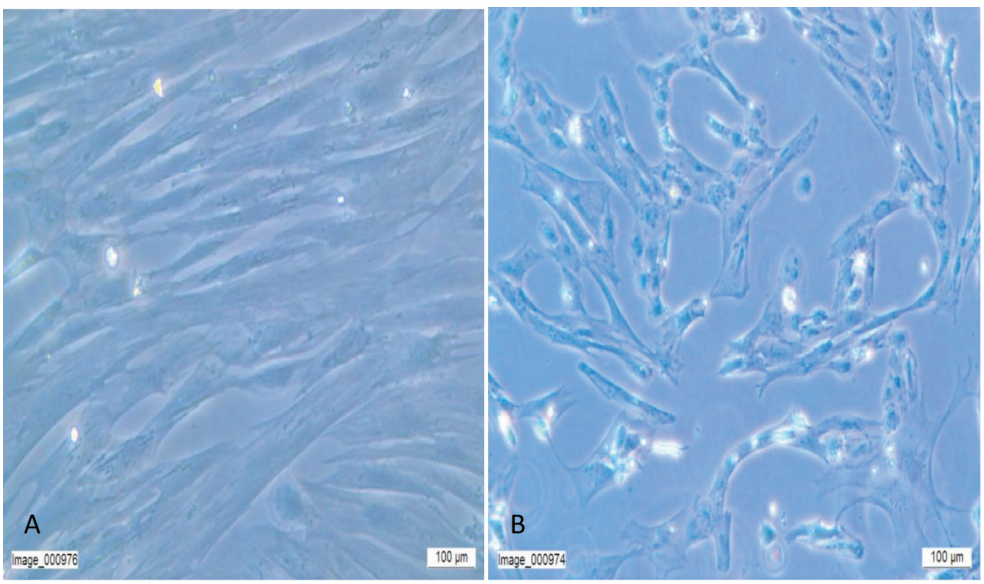

Figure 1. Cytopathic effect induced by isolate 279/14 of sheeppox virus observed in lamb testis cell cultures. A) Uninoculated cell layer. B) Cytopathic effect induced by sheeppox virus.

Tunisian isolates' nucleotide sequences are 100% identical for each tested gene.

3.3.1. *GPCR* sequence analysis

The obtained nucleotide sequences were aligned and the existing variation in *Capripoxvirus* was examined. It appeared that the first 23 nucleotides and 12 others, located at positions 73 to 84, are missing in all LSDV strains. The absence of these nucleotides has led to variation in the size of the *GPCR* gene (1090 bp for LSDV, and 1125 bp for SPPV and GTPV). This implied that the amino acids at positions 1–8 and 25–28 are missing in all strains of LSDV, reducing the size of the GPCR protein for this species. Thus, the GPCR protein in the LSDV strain is made of 363 amino acids, unlike the SPPV and GTPV strains, which have 374 amino acids. Twenty-eight positions in the *GPCR* gene sequence constitute the pattern of the SPPV strains (Table 3). In most of these positions, the LSDV strains have the same nucleotides as those in the GTPV strains. Noncommon nucleotides between GTPV and LSDV constitute their respective patterns (Table 3). Conversion of nucleotide sequences into amino acid sequences confirmed the specificity of each strain of SPPV (Table 4).

3.3.2. *RPO30* sequence analysis

Multiple alignments of nucleotide sequences of the *RPO30* gene showed a size variation among species of capripoxviruses (CaPVs). For the GTPV and LSDV strains, the *RPO30* gene is 606 bp, and for the SPPV strain it is 585 bp. This variability occurred in the SPPV strain with the deletion of the first 21 nucleotides. Specific nucleotide patterns of SPPV were noticed at 15 positions in the *RPO30* gene (Table 3). Conversion of the nucleotide sequences into amino acid sequences resulted in a protein with 195 amino acids in SPPV and 202 amino acids in GTPV and LSDV. The RPO30 protein sequence of SPPV was characterized by the absence of the first 7 amino acids and the presence of amino acids D_{85} and E_{89} (Table 4).

In addition to the absence of 21 nucleotides at the 5' end in specific molecular patterns of SPPV, other patterns were observed and are reported in Table 3. The sequences of the resulting RPO30 amino acids of SPPV, GTPV, and LSDV showed specific patterns for each species. Specific patterns in GTPV were D_{128} and Q_{148}; in LSDV they were D_{95} and H_{134} (Table 4).

3.3.3. *P32* sequence analysis

When comparing the SPPV sequences of the Tunisian isolates with those of the LSDV and GTPV strains, it appeared that they differed by only a few bases, which are identified as the patterns of each virus. The following signatures were characteristic of the SPPV nucleotide sequence: G_{163}, A_{164}, T_{165}, A_{186}, A_{654}, C_{395}, T_{401}, A_{654}, G_{657}, C_{664}, and A_{672} (Table 3). The protein sequence of SPPV is manifested by the presence of the amino acids L_{62}, S_{132}, and I_{134}. These amino acids represented the pattern for SPPV strains as other isolated CaPVs showed different amino acids at these positions, such as D_{55}, which is present only in SPPV. Multiple alignments of P32 amino acid sequences revealed specific GTPV patterns represented by the presence of G_{26}, K_{46}, V_{93}, Y_{136}, M_{290}, and V_{323}. However, LSDV-specific patterns are represented by F_{49} and D_{305}.

Nucleotide alignment showed variability in the size of the *P32* gene from one species to another, and it produced a product of 972 bp for SPPV and 969 bp for GTPV and LSDV. Such size shortening is caused by the absence of three nucleotides in both sequences of GTPV and LSDV strains. The resulting proteins are made of 323 amino acids in SPPV and 322 amino acids in GTPV and LSDV.

3.3.4. *ORF117* sequence analysis

Analysis of the *ORF117* gene sequence revealed the presence of a substitution (A/G) at position 27 in the Tunisian isolates that characterizes and differentiates them from other SPPV isolates. However, this mutation is silent. We also showed that SPPV isolates differ from GTPV and LSDV isolates by 7 patterns dispersed in the *ORF117* gene (Table 3). For most positions, GTPV isolates have the same nucleotides as LSDV isolates. However, they differ at position 291: GTPV has A while LSDV has G. The in silico translation of the *ORF117* gene sequence of CaPV isolates made it possible to obtain a sequence of 148 amino acids. The SPPV fusion protein sequence is distinguished from that of GTPV and LSDV by the presence of N_{28}, N_{36}, N_{36}, and V_{97} (Table 4).

3.3.5. *Kelch-like* sequence analysis

The *Kelch-like* gene has 31 specific signature characteristics of SPPV (Table 3). All tested SPPVs showed complete Kelch-like proteins with 569 amino acids, with the exception of the single Chinese strain (SPPV strain Jilin), which presents a nonfunctional protein with 509 amino acids. The Kelch-like protein sequence of SPPVs has a pattern that differentiates it from other CaPVs (Table 4). Thus, the size of SPPV is 1710 nucleotides, GTPV varies from 1689 to 1690, and LSDV varies from 1709 to 1712.

3.4. Phylogeny

Phylogenetic trees were constructed to study the genetic relationships between the isolated Tunisian strains and various CaPVs. Phylogenetic analysis of the nucleic acid sequences of the *GPCR*, *RPO30*, *P32*, *ORF117*, and *Kelch-like* genes was performed (Figures 2a–2e, respectively). Taking into account the branch length of trees and the node locations, the various isolates were divided into 3 groups regardless of their geographical origin. The phylogenetic tree showed that the viral strains LSDV, GTPV, and SPPV are separated according to their respective hosts, and they formed three distinct groups with strong bootstrap values. The LSDV and GTPV strains formed two monophyletic

Table 3. Representative table of nucleotide motifs of the *GPCR*, *RPO30*, *P32*, *ORF117*, and *Kelch-like* genes.

Nucleotide positions in *GPCR* gene																
	12	18	79	80	94	136	152	156	171	177	206	261	276	348	424	432
SPPV	T	A	A	G	A	G	A	A	C	C	A	T	A	G	A	A
GTPV	G	C/T	G	T	C	A	C	C	T	T	G	G	T	A	G	C
LSDV	-	-	-	-	C	A	C	C	T	T	G	G	G	A	G	C
	550	556	573	574	590	594	632	690	691	870	963	1082				
SPPV	A	T	A	A	A	C	T	A	C	A	T	A				
GTPV	G	C	T	T	G	T	C	G	T	G	G	G				
LSDV	G	C	G	C	G	T	C	G	T	G	G	G				
Nucleotide positions in *RPO30* gene																
	22	23	24	25	28	29	168	222	240	276	288	318	546	564	582	
SPPV	A	T	G	G	G	A	T	G	G	T	G	A	C	A	A	
GTPV	T	C	A	T	A	G	A	A	A	G	T	T	T	G	G	
LSDV	T	C	A	T	A	G	A	A	A	G	T	G	T	G	G	
Nucleotide positions in *P32* gene																
	163	164	165	186	395	401	654	657	664	672						
SPPV	G	A	T	A	C	T	A	G	C	A						
GTPV	-	-	-	C	T	C	G	A	T	C						
LSDV	-	-	-	C	T	C	G	A	T	C						
Nucleotide positions in *Kelch-like* gene																
	48	107	135	186	192	204	319	380	455	460	528	597	648	669	672	688
SPPV	T	T	T	T	G	T	T	T	T	A	A	A	G	A	G	A
GTPV	G	C	A	G	T	G	G	A	G	G	T	G	A	G	C	C
LSDV	G	C	A	G	T	G	G	G	G	G	C	G	A	G	C	C
	712	822	871	913	948	1000	1009	1010	1182	1188	1247	1264	1514	1548	1602	
SPPV	T	T	A	A	A	G	A	A	A	T	A	A	A	A	T	
GTPV	G	G	G	-	T/C	A	G	C	T	C/A	G	C	C	G	C	
LSDV	G	G	G	G	T/C	A	G	C	T	C	G	C	C	G	C	
Nucleotide positions in *ORF117* gene																
	82	83	93	108	165	291	375									
SPPV	A	A	T	T	T	T	G									
GTPV	G	G	G	A	G	A	A									
LSDV	G	G	G	A	G	G	A									

The SPPV signature patterns are colored green, the identities between LSDV and GTPV are orange, the signature patterns of the GTPVs are blue, and the signature patterns of the LSDVs are yellow.

Table 4. Representative table of protein signatures of the GPCR, RPO30, P32, ORF117, and Kelch-like proteins.

Amino acid positions in the GPCR protein																	
	4	6	27	32	46	51	69	92	142	184	186	191	192	197	211	231	321
SPPV	T	R	R	K	A	K	N	L	N	I	Y	I	T	Q	I	H	I
GTPV	T	S	V	Q	T	T	S	F	D	V	H	I	S	R	T	Y	M
LSDV	-	-	-	Q	T	T	S	S	D	V	H	M	P	R	T	Y	M
	361																
SPPV	Q																
GTPV	R																
LSDV	R																
Amino acid positions in the RPO30 protein																	
	85	89	95	128	134	148											
SPPV	D	E	N	N	Y	P											
GTPV	E	D	N	D	Y	Q											
LSDV	E	D	D	N	H	P											
Amino acid positions in the P32 protein																	
	26	46	49	55	62	93	132	134	136	290	305	323					
SPPV	D	N	L	D	L	A	S	I	H	I	N	I					
GTPV	G	K	L	-	F	V	L	T	Y	M	N	V					
LSDV	D	N	F	-	F	A	L	T	H	M	D	I					
Amino acid positions in the Kelch-like protein																	
	16	36	62	68	107	127	152	154	199	205	214	220	230	238	278	291	298
SPPV	N	L	N	N	S	I	I	N	I	A	K	L	T	Y	N	N	N
GTPV	K	S	T	K	A	K	S	D	M	A	Q	L	P	D	N	D	K
LSDV	K	S	K	K	A	R	S	D	M	S	K	R	P	D	D	D	N
	312	334	337	352	353	360	363	368	416	422	475	505	541	549			
SPPV	S	A	N	L	R	A	V	R	K	I	A	H	G	L			
GTPV	T	T	A	S	Q	T	A	R	R	L	T	P	G	L			
LSDV	S	T	A	L	R	A	A	K	R	L	A	P	E	I			
Amino acid positions in the fusion protein																	
	23	28	29	36	42	55	64	69	75	97							
SPPV	R	N	D	N	D	N	N	N	N	V							
GTPV	I	G	D	K	D	K	S	N	N	L							
LSDV	R	G	E	K	E	K	N	D	D	L							

SPPV signature patterns are colored green, signature patterns of the GTPVs are blue, and signature patterns of the LSDVs are yellow. The identities between LSDV and GTPV are orange, the identities between SPPV and GTPV are pink, and the identities between SPPV and LSDV are red.

subgroups for the *GPCR*, *RPO30*, *ORF117*, and *Kelch-like* genes, respectively (Figures 2a, 2b, 2d, and 2e). However, SPPV and LSDV strains segregated together for *P32* (Figure 2c), suggesting that SPPV is genetically closer to LSDV than GTPV.

4. Discussion

Sheeppox is one of the major notifiable infectious diseases of sheep reported by the veterinary services in Tunisia [2,17]. Therefore, early detection of the virus by rapid techniques such as real-time PCR is required. This would facilitate

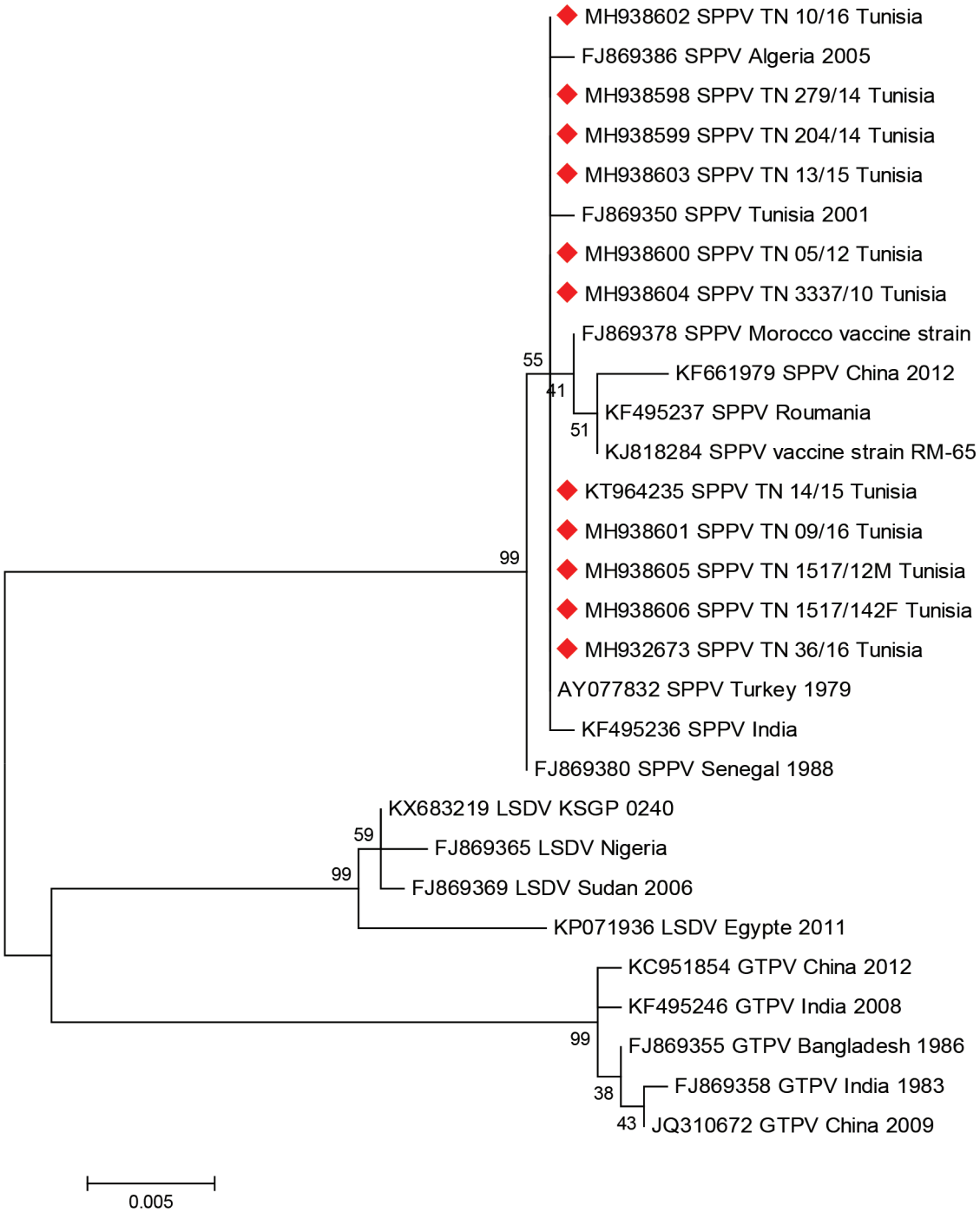

Figure 2a. Phylogenetic analysis of nucleotide sequences of the *GPCR* gene. Sequences were aligned using ClustalW with default settings in BioEdit (version 7.2.5.0). The phylogenetic tree was constructed by the maximum likelihood method with 1000 bootstrap replicates using MEGA 6.0.6. Tunisian strains are marked with a bold red diamond.

epidemiological surveillance of suspected infections in sheep. In the present study, we used SYBR Green-based real-time PCR for molecular diagnosis of SPPV. However, a molecular analysis of different genes is necessary for the dynamic monitoring of viral gene evolution and the classification of SPPV. Many studies have been undertaken for the classification of SPPV based on one or more different genes, which provided different classifications and did not reveal consistent results for the phylogenetic classification of SPPV. In this study we compared different

Figure 2b. Phylogenetic analysis of the nucleotide sequences of the *RPO30* gene. Sequences were aligned using ClustalW with default settings in BioEdit (version 7.2.5.0). The phylogenetic tree was constructed by the maximum likelihood method with 1000 bootstrap replicates using MEGA 6.0.6. Tunisian strains are marked with a bold red diamond.

phylogenetic classifications based on *GPCR*, *RPO30*, *P32*, and *ORF117*. The current study also included the *Kelch-like* gene, as no data had been previously gathered for this gene despite its important role as a determinant of virulence for SPPV [18]. Indeed, repression the of *Kelch-like* gene contributes to a complete attenuation of the virus [18]. The poxvirus Kelch-like protein is thought to mediate viral interaction with specific cellular components. It is known that poxviral *Kelch-like* genes are generally present as multiple, divergent, and occasionally fragmented genes, located in variable and nonessential regions of the genome near the genomic termini, suggesting that they function in aspects of virus–host interaction [19].

Our phylogenetic study confirmed that CaPVs can be divided into three distinct groups using comparison of the complete sequences of the *GPCR*, *RPO30*, *P32*, *ORF117*,

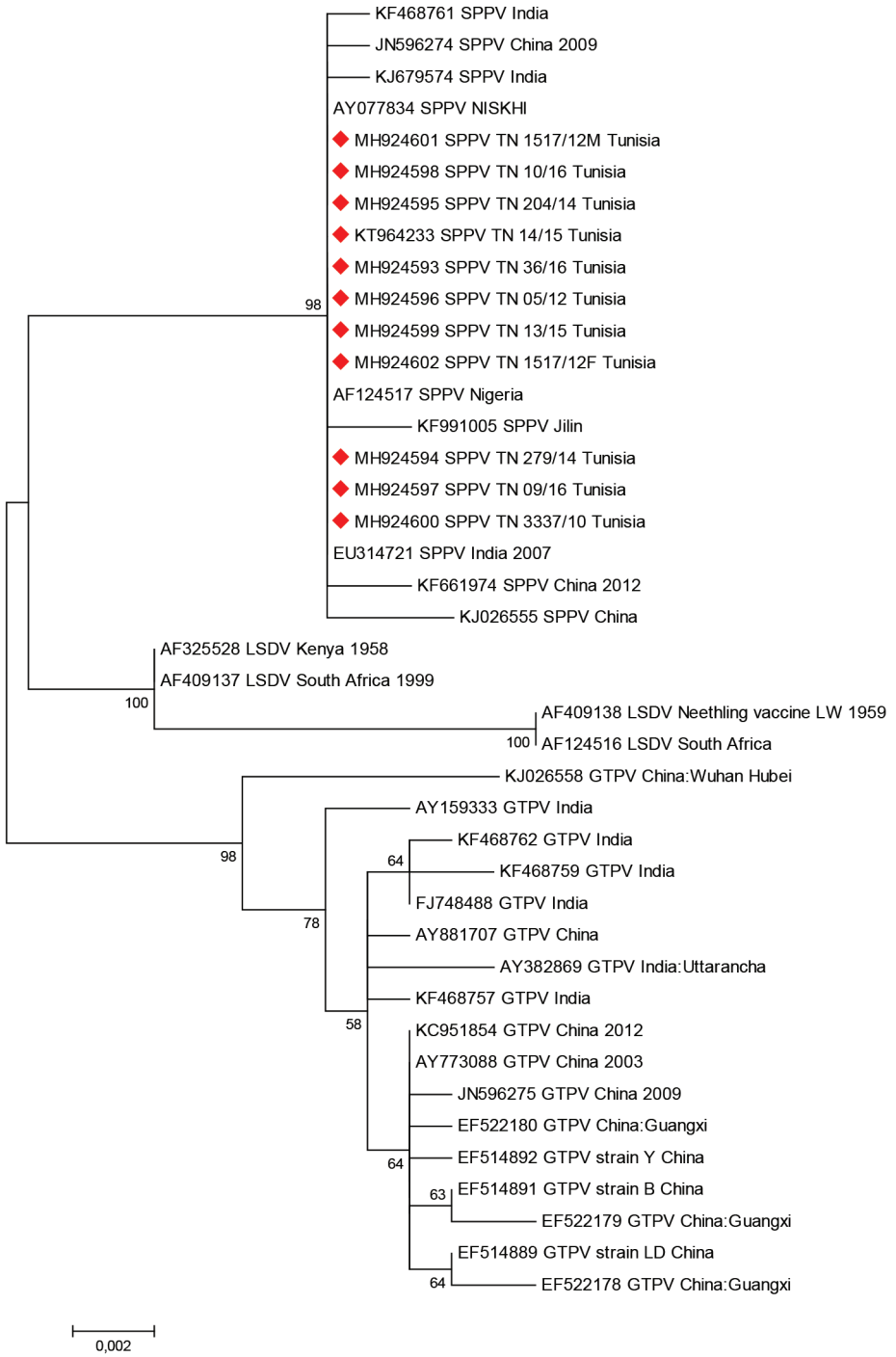

Figure 2c. Phylogenetic analysis of the nucleotide sequences of the *P32* gene. Sequences were aligned using ClustalW with default settings in BioEdit (version 7.2.5.0). The phylogenetic tree was constructed by the maximum likelihood method with 1000 bootstrap replicates using MEGA 6.0.6. Tunisian strains are marked with a bold red diamond.

and *Kelch-like* genes. Based on the *P32* and *RPO30* genes, phylogenetic analysis revealed a discrepancy in phylogenetic relationships between capripoxvirus species. Hosamani et al. [10], Zhu et al. [20], and Maksyutov et al. [12] found that GTPV and LSDV are more closely related to each other than to SPPV when using the *P32* gene. Venkatesan et al. [21] obtained a phylogenetic tree that grouped SPPV and LSDV together, and our study confirmed that of Venkatesan et al. [21] and demonstrated that SPPV and LSDV are clustered in a single group. Our

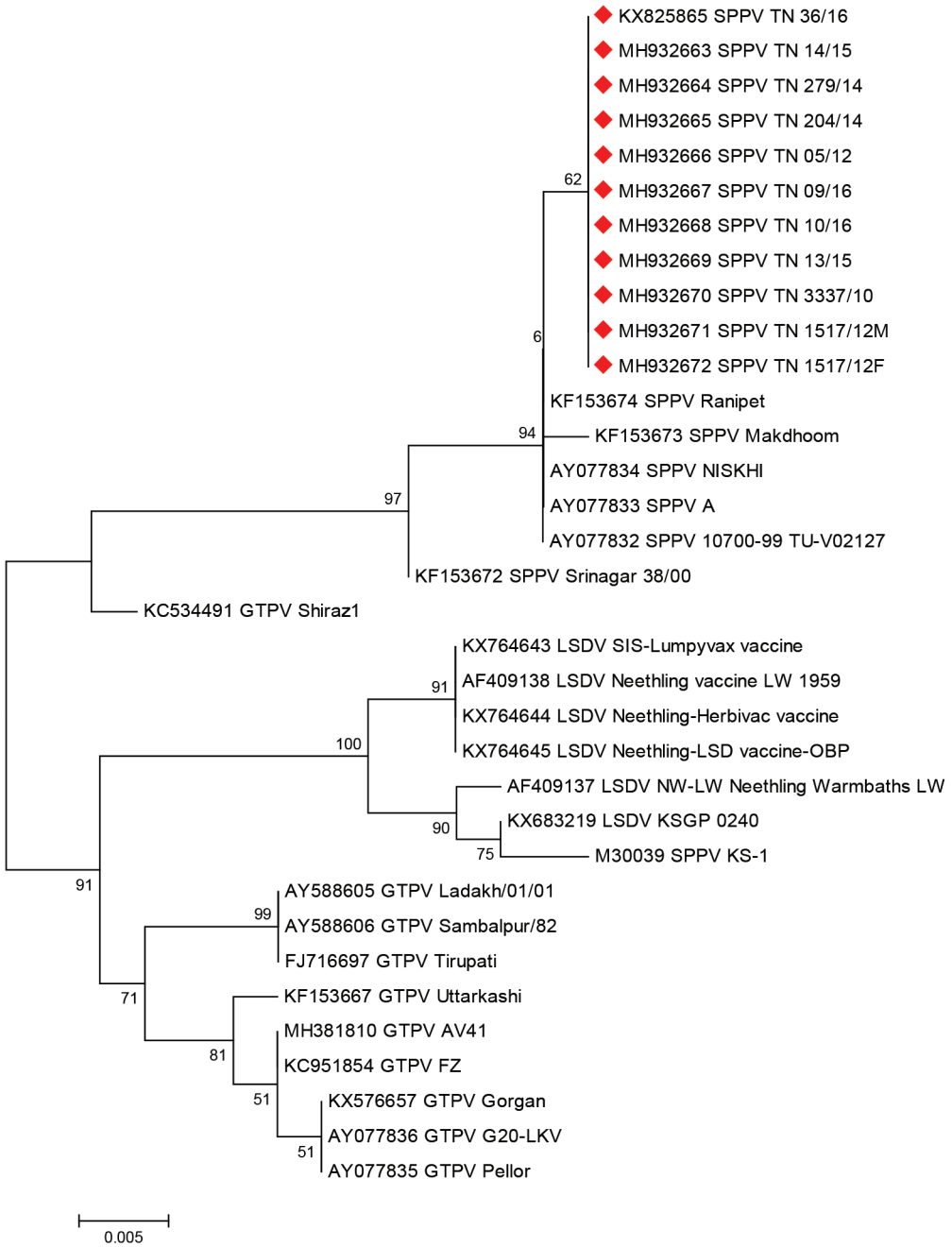

Figure 2d. Phylogenetic analysis of nucleotide sequences of the *ORF117* gene. Sequences were aligned using ClustalW with default settings in BioEdit (version 7.2.5.0). The phylogenetic tree was constructed by the maximum likelihood method with 1000 bootstrap replicates using MEGA 6.0.6. Tunisian strains are marked with a bold red diamond.

study and that of Santhamani et al. [5] found a very close relationship by using used the *RPO30* gene; in addition, we discovered that GTPV and LSDV are more closely related to each other than to SPPV, in contrast to Zhou et al. [16]. These results suggest that the genes *RPO30* and *P32* cannot be considered as robust support for phylogenetic studies of the genus *Capripoxvirus*. We have found that LSDV and GTPV are more closely related to each other than to SPPV in terms of the *ORF117* gene. This result was confirmed by Dashprakash et al. [13], who reported that GTPV and LSDV were grouped in a common cluster, while SPPV emerged alone. Studies using the *GPCR* gene have shown consistent results as reported by Le Goff et al. [22] and Santhamani et al. [5], making it the best candidate for

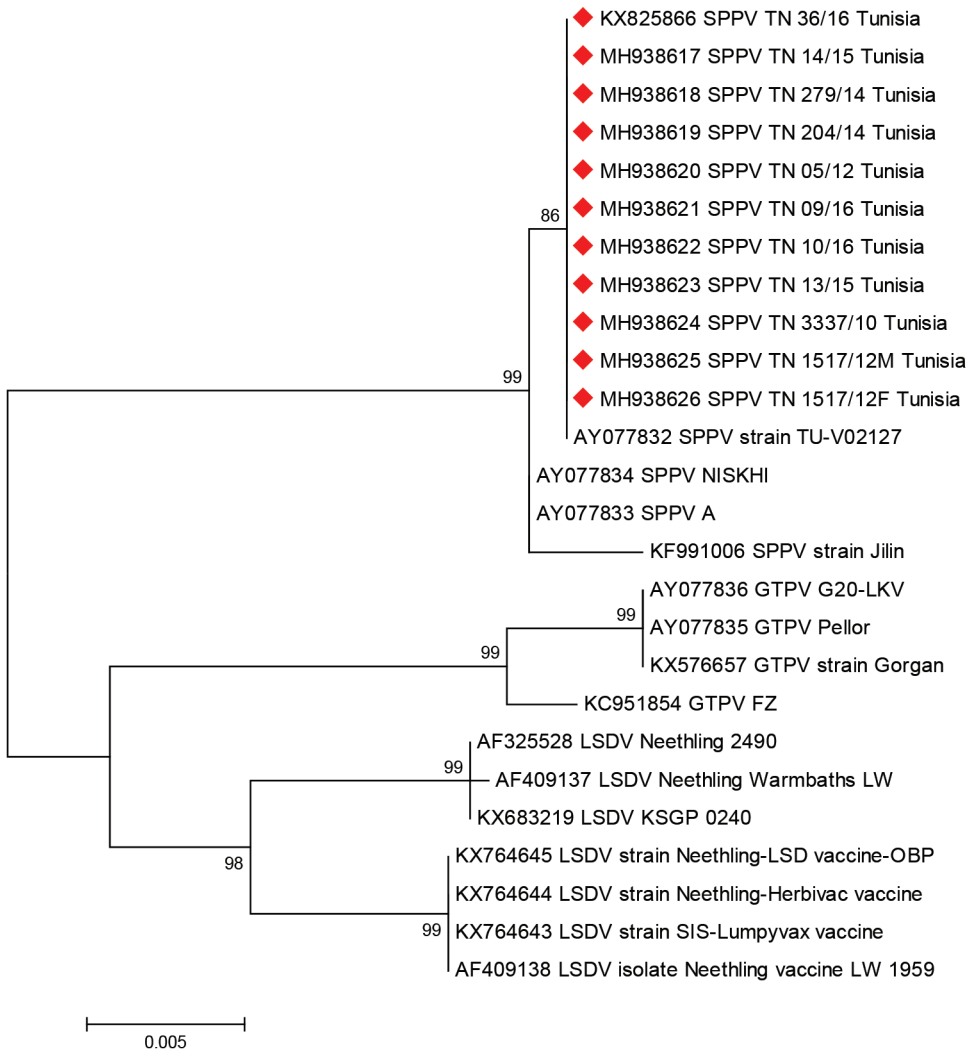

Figure 2e. Phylogenetic analysis of nucleotide sequences of the *Kelch-like* gene. Sequences were aligned using ClustalW with default settings in BioEdit (version 7.2.5.0). The phylogenetic tree was constructed by the maximum likelihood method with 1000 bootstrap replicates using MEGA 6.0.6. Tunisian strains are marked with a red diamond.

phylogenetic analysis. This would support the hypothesis that GTPV and LSDV emerged from a common ancestor [23]. Indeed, previous molecular studies have shown that SPPV can be distinguished genetically from LSDV and GTPV [24,25]. It has also been shown that the *GPCR* gene, encoding for a transmembrane protein, is one of the most variable genes among the species of *Capripoxvirus* [24], making it a suitable target for SPPV isolate typing. Molecular characterization of the *GPCR* gene revealed the presence of the largest specific patterns (n: 28), following the *Kelch-like* gene, which distinguishes SPPV from other species of the genus *Capripoxvirus*. Variations between the three CaPV species in the N-terminal region of the GPCR protein are probably related to the specificity of the host and the optimization of their interactions with the immune system of the corresponding host [22]. *GPCR* results were confirmed by those obtained for the *Kelch-like* gene, demonstrating that GTPV and LSDV may be clustered in a common group, making it a potential candidate for phylogenetic analysis studies. The *Kelch-like* gene is the most representative of nucleotidic "31" and proteic "18" signatures, allowing for differentiation between the three CaPV species and making it a good candidate gene for differentiation. Following alignment of the nucleotide sequences of the *Kelch-like* gene it was shown that it is possible to distinguish between the 3 main species of the genus *Capripoxvirus*. However, further studies are needed to support these results. This study provided the characterization of 11 isolates obtained after outbreaks over a period of 6 years, revealing that it is probably the same

strain that is circulating. Based on sequence alignment and phylogenetic analysis of five genes (*P32*, *RPO30*, *ORF117*, *GPCR*, and *Kelch-like*), the present work has elucidated the genetic relationships between different Tunisian isolates and other *Capripoxvirus* isolates. This study represents a first initiative to look for a determinant gene that would allow for distinction between the three viral species and provide a comprehensive view of CaPV genomics.

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