Infection, Genetics and Evolution 76 (2019) 104071

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Genetic studies of terminal regions of vaccine and field isolates of capripoxviruses

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ARTICLE INFO

Keywords:
Sheeppox virus
Goatpox virus
Capripoxvirus
Host-range genes
Virulence genes

ABSTRACT

Sheeppox and goatpox are two of the most important diseases associated with significant economic loss and impact on animal trade. In spite of the use of vaccines, outbreaks are being reported on several occasions. Therefore, deciphering the host specificity and virulence of sheeppox virus (SPPV) and goatpox virus (GTPV) is important in developing effective vaccines. It is opined that genes located in the terminal regions play a major role in determining host range and/or virulence. In the present study, nine isolates (6 GTPV and 3 SPPV; included both vaccine and virulent viruses) were genetically characterized by targeting 11 genes (7 host-range and 4 virulence genes) which are located in the terminal regions of capripoxviruses. In the genetic analyses, it was observed that there are several nucleotide and amino acid signatures which are specific for either SPPV or GTPV. However, surprisingly, none of the 11 genes could be able to differentiate the vaccine and field viruses of GTPV and SPPV. Our study indicates that the genes of the terminal regions may have a role in determining the host-specificity but the involvemet in determinatin of virulence/attenuation is not certain at least for the isolates used in the current study. Therefore, it is likely that some other genes located in terminal/central regions may also play a role in determination of virulence and pathogenesis which needs to be confirmed by whole-genome sequencing of several vaccine and virulent viruses.

1. Introduction

Sheeppox and goatpox are highly contagious and economically important diseases of sheep and goats. They are endemic in many parts of the world and transmission occurs through aerosol or close contact with infected animals or through abrasions (Babiuk et al., 2008; Bowden et al., 2008; Kitching and Mellor, 1986; Tuppurainen et al., 2017). Sheeppox and goatpox exhibit similar clinical signs that are typical of generalized pox diseases including pyrexia, cutaneous lesions, and the development of lung lesions. Although once it was believed that sheeppox and goatpox infection is confined to domestic ruminants, recently GTPV caused havoc in a wild Red Serow population in Mizoram State of India (Dutta et al., 2018).

Sheeppox virus (SPPV), goatpox virus (GTPV), and Lumpy skin disease virus (LSDV) are the members of the genus *Capripoxvirus* (CaPV) in the subfamily *Chordopoxvirinae* of the family *Poxviridae*. The capripoxviruses have a brick-shaped structure (Babiuk, 2018). The genome is a double-stranded DNA of ~150 kbp in size with 147-151 open reading frames/putative genes (Tulman et al., 2002; Zhao et al., 2017; Zheng et al., 2007). A most recent study on complete genome sequencing of Chinese isolate showed that the GTPV_FZ strain contains 151 putative genes of 42–2008 amino acids in length. Two putative genes each appear twice because they are completely located within inverted terminal repeat regions (ORFs V001, 002,146,151) (Zeng et al., 2014). The central genomic core region (ORFs 022 to 122) contains 101 genes, most of which are involved in basic replicative mechanisms and morphogenesis of intracellular mature and extracellular enveloped virions (Gershon and Black, 1989). Terminal genomic regions (ORFs 001 to 021 and 123 to 151) contain genes likely affecting pathogenesis and host range functions. These include genes potentially involved in host range (ankyrin repeat proteins: ORFs 010, 138, 140, 145,141.2), of which most are located at the right terminal

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https://doi.org/10.1016/j.meegid.2019.104071
Received 2 August 2019; Received in revised form 7 October 2019; Accepted 11 October 2019
Available online 15 October 2019
1567-1348/

Table 1
Details of virus isolates used and passage history.

Sl. No	Isolate/Strain	Year of isolation	Passage history	Gene sequenced with GenBank accession number										
				ORF 3	ORF 10	ORF 12	ORF 13	ORF 16	ORF 137	ORF 138	ORF 140	ORF 141	ORF 142	ORF 144
1	GTPV Akola	2008	P3 (PLT P1 + Vero P2)	MF629069	MF629123	MF629051	MF629139	MF629100	MF629078	MF629132	–	MF629114	MF629087	MF629060
2	GTPV Mukteswar (Challenge virus)	1946	P19 (scab)	MF629070	MF629124	MF629052	MF629140	MF629101	MF629079	MF629133	–	MF629115	MF629088	MF629061
3	GTPV Mukteswar	1946	P21 (scab P19 + VeroP2)	MF629071	MF629125	MF629053	MF629141	–	MF629080	–	–	MF629116	MF629089	MF629062
4	GTPV Uttarkashi	1978	P6 (Vero)	MF629072	MF629126	MF629054	MF629142	MF629102	MF629081	MF629134	MF629108	MF629117	MF629090	MF629063
5	GTPV Uttarkashi (Vaccine virus)	1978	P62 (Vero)	MF629073	MF629127	MF629055	MF629143	MF629103	MF629082	MF629135	MF629109	MF629118	MF629091	MF629064
6	GTPV Uttarkashi (Vaccine virus)	1978	P100 (Vero)	MF629074	MF629128	MF629056	MF629144	MF629104	MF629083	MF629136	MF629110	MF629119	MF629092	MF629065
7	SPPV Pune	2008	P4 (Vero)	MF629075	MF629129	MF629057	MF629145	MF629105	MF629084	–	MF629111	MF629120	MF629093	MF629066
8	SPPV Srinagar (Challenge virus)	2000	P7 (PLT P4 + Vero P3)	MF629076	MF629130	MF629058	MF629146	MF629106	MF629085	MF629137	MF629112	MF629121	MF629094	MF629067
9	SPPV Srinagar (Vaccine virus)	2000	P42 (PLT P10 + Vero P32)	MF629077	MF629131	MF629059	MF629147	MF629107	MF629086	MF629138	MF629113	MF629122	MF629095	MF629068

PLT Primary Lamb Testicle cells; – not characterized.

regions; genes affecting virulence including interleukin (IL) 18 (ORF012), IL-10 (ORF003), EGF-like protein (ORF013), and serpin (ORF142) (Zeng et al., 2014).

As the SPPV and GTPV show a high degree of genetic and antigenic relatedness, they cannot be distinguished based on clinical signs, lesions, electron microscopy or by conventional serological methods. Therefore, recently many laboratories adapted the molecular-based differentiation of SPPV and GTPV. Several effective vaccines are available for controlling capripoxviruses including LSDV. However, there are occasions that vaccine failure due to partial cross-protection (Tuppurainen et al., 2014) or no cross-protection (Boshra et al., 2015). In the case of sheeppox and goatpox, the cause of vaccine failures may be due to the use of vaccine virus strains with unknown host-specificity (Gelaye et al., 2015). There are reports demonstrated that cross-species infecting capripoxviruses are harboring among sheep and the goat population (Bray, 2007; Ramakrishnan et al., 2017). Therefore, characterizing vaccine and field viruses is important in developing improved vaccines and assessment of vaccination failure. In the current study, we have genetically characterized the GTPV and SPPV (both vaccine and field strains) by targeting terminal variable regions.

2. Materials and methods

2.1. Viruses

A total of nine isolates (6 GTPV and 3 SPPV) which were available at Pox Laboratory of Division of Virology, Indian Veterinary Research Institute, Mukteswar, Uttarakhand were used. The details of virus isolates and passage history are presented in Table 1. Viruses were revived by the co-culture method in Vero cells using Eagle's Minimum Essential Medium (EMEM) with 10% horse serum and antibiotics-antimycotic solution (100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B). Virus-infected cells were harvested when cells showed ~80% cytopathic effect (CPE).

2.2. PCR and sequencing

DNA was extracted from virus-infected cell pellets using DNeasy Blood & Tissue kit (Qiagen, USA). The primers used for PCR and sequencing of host-range and virulence genes of CaPV are provided in Table 2. The cyclic conditions were optimized using either Terra™ PCR Direct Red Dye Premix (TAKARA) or EmeraldAmp® PCR Master Mix (TAKARA) and presented in Table 2. The amplified products were gel-purified (QIAquick® Gel Extraction Kit; Qiagen, USA), cloned into the TA cloning vector (pTZ57R/T; InsTAclone PCR Cloning Kit - Thermo, USA) and commercially sequenced.

2.3. Genetic and phylogenetic analyses

The multiple alignments of nucleotides (nts) and their deduced amino acids (aa), as well as phylogenetic reconstruction, were carried out in MEGA 6. The tree was constructed by the neighbor-joining method with the Kimura-2 parameter model and 1000 bootstrap replicates. The position of nt and aa was presented according to the GTPV-FZ (GenBank Accession No. KC951854).

3. Results

3.1. Genetic analysis

Nucleotide and their deduced amino acid of host-range and virulence genes revealed several unique nucleotides (nts) and amino acids (aa) signatures between SPPV and GTPV. The host-range genes analyzed in the present study are either of Kelch-like (ORFS – 016,137, and144) or Ankyrin repeats (ORFs 010, 138, 140, and 141) families. The virulence genes analyzed are – ORFs 003, 012, 013 and 142. The

Table 2
Details of primers used for PCR and Sequencing, and optimized annealing temperature.

ORF/ Gene	Name of primer	Sequences (5′ – 3′)	Amplicon size (~ bp)	Annealing temp. (°C)	ORF length	PCR enzyme*
ORF3	GTPV-FZ-2046-F GTPV-FZ-2953-R	atgacatattggttggttgtttt (PCR) acaacacatgatgatagtggtatttat (PCR)	908	53	513	E
ORF10	GTPV-FZ-7853-F GTPV-FZ-8857-R	tgctaataattgtagtagctatagtgg (PCR) agaaatgtacgtaggaaactcttat (PCR)	1005	48.6	636	T
ORF12	CaPV- 9890-F CaPV-10677-R	acaagtgcttccaaaccatagt (PCR) agtataaatttttgtttaaatggtggt (PCR)	788	53	486	E
ORF13	CaPV- 10458-F CaPV-10991-R	gtcagacaaatgactactttcttga (PCR) aatgatgatttagcactaaatgagct (PCR)	534	53	GTPV 285** SPPV 303	E
ORF16	GTPV-FZ-11745-F GTPV-FZ-13650-R GTPV-FZ-13129-R GTPV-FZ-12316-F	cagtttagcaaaagcagacaa (PCR) gttatttctaacaaagacgataatgtt (PCR) tgtacgatgttgcagataaata (Seq.) acactttctccaaatgaccaa (Seq.)	1906	51.2	GTPV 1689 SPPV 1710	T
ORF 137	GTPV-FZ-134973-F GTPV-FZ-136784-R GTPV-FZ-135417F GTPV-FZ-136131R	aataactcataactaagaggyrata (PCR) gcgtdaygtaatcgtataacaa (PCR) agattttaataattgtctcgatg (Seq.) accaccaattargtacatttta (Seq.)	1812	48.6	1644	T
ORF138	GTPV-FZ-136651-F GTPV-FZ-138813-R GTPV-FZ-137503-F GTPV-FZ-137634- F GTPV-FZ-137666-R	ttttgtaatttaawtaactcaaagc (PCR) cttcgtracaatctttcataat (PCR) aaatcaaaaatcagaaagtgg (Seq.) atgaaactttgttacatatttacttac (Seq.) acctacgtaagtaaatatgtaacaaag (Seq.)	2163	48.6	1905	T
ORF140	GTPV-FZ-139966-F GTPV-FZ-141648-R GTPV-FZ-141194-R	caccaaaagataataatgaaaa (PCR) ctaatgtattccatggtgtc (PCR) aacctcgtaaaagtttttgtaa (Seq.)	1683	51.2	1497	T
ORF141	GTPV-FZ-141519-F GTPV-FZ-143072-R GTPV-FZ-142190-F GTPV-FZ-142422-R	tatttagatttttcagactttgatac (PCR) gatatagaaacaggagaaattaacaca (PCR) aaggaagatattttcttatccgcatt (Seq.) gtttctccacatgatgttgttatattt (Seq.)	1554	51.2	1344	T
ORF142	CaPV- 142893-F CaPV-144205-R	actggaatacattaccaacagaga (PCR) tcaactttaccaccattaaatctcca (PCR)	1313	55.8	1014	E
ORF144	GTPV-FZ-144477-F GTPV-FZ-146431-R GTPV-FZ-145098-F GTPV-FZ-145868-R	catacaaaaatagatattgtaaattgc (PCR) gaatctcgttgtaagtatttttg (PCR) cgttgcatcggaaaatag (Seq.) ggtgctacgttatcccact (Seq.)	1955	48.6	1659	T

* E = EmeraldAmp® PCR Master Mix; T = Terra™ PCR Direct Red Dye Premix.ORF length of GTPV Mukteswar P19 (scab) and GTPV Mukteswar P21 (scab P19 + VeroP2) is 306 nts.
Primers used for PCR or Sequencing (Seq.) are provided in the parentheses. In addition, M13F and M13R primers were also used for sequencing.

Table 3
Unique nucleotide/amino acid signatures observed in ORF137.

Sl. No.	Nucleotide position and difference				Amino acid position and difference (nonsynonymous variation only)			
	Position	GTPV	SPPV	LSDV	Position	GTPV	SPPV	LSDV
1.	34	C	T	C				
2.	90	A	G	A				
3.	102	C	T	T				
4.	120	T	G	G	40	I	M	M
5.	130	A	G	A	44	K	E	K
6.	197	C	T	C	66	A	V	V
7.	234	A	T	G				
8.	238	A	T	T	80	I	L	L
9.	248	C	G	T	83	T	S	T
10.	346	A	G	A	116	I	V	I
11.	399	T	C	C				
12.	402	T	A	A	134	F	L	L
13.	417	A	G	G				
14.	526	T	G	G	176	L	V	V
15.	604	A	G	G	202	N	D	D
16.	611	T	C	T	204	L	S	L
17.	614	G	A	A	205	R	K	K
18.	639	A	G	A				
19.	753	G	T	G				
20.	754	A	T	T				
21.	756	T	A	T	252	I	L	F
22.	799	A	G	A	267	I	V	I
23.	845	T	C	C	282	I	T	I
24.	861	C	G	G				
25.	900	C	T	T				
26.	926	G	T	G	309	S	I	S

(continued on next page)

Table 3 (*continued*)

Sl. No.	Nucleotide position and difference				Amino acid position and difference (nonsynonymous variation only)			
	Position	GTPV	SPPV	LSDV	Position	GTPV	SPPV	LSDV
27.	984	A	G	A				
28.	999	C	A	A				
29.	1120	T	C	T	374	Y	H	Y
30.	1176	G	T	G				
31.	1213	G	A	A				
32.	1215	G	T	G	405	V	I	M
33.	1332	C	T	T				
34.	1347	C	A	C				
35.	1356	T	G	T				
36.	1360	G	A	G	454	V	T	A
37.	1361	T	C	C				
38.	1368	A	C	C				
39.	1377	C	A	C				
40.	1440	A	G	A				
41.	1524	T	A	A	508	N	K	K
42.	1546	A	G	A	516	N	D	N
43.	1552	T	C	T	518	S	P	S
44.	1572	A	G	G	524	I	M	M
45.	1576	G	T	G				
46.	1577	A	G	G	526	D	C	G
47.	1598	T	A	T	533	I	N	I
48.	1633	G	A	G	545	V	I	V

Table 4
Unique nucleotide/amino acid signatures observed in ORF144.

Sl. No.	Nucleotide position and difference				Amino acid position and difference (nonsynonymous variation only)			
	Position	GTPV	SPPV	LSDV	Position	GTPV	SPPV	LSDV
1.	7	T	G	G	3	S	A	A
2.	24	A	G	G				
3.	41	C	A	C	14	T	K	T
4.	54	T	C	C				
5.	105	G	A	A				
6.	129	A	C	C	43	K	N	N
7.	242	G	A	G	81	S	N	R/G
8.	326	C	T	C	109	A	V	A
9.	346	G	A	G	116	D	N	D
10.	352	A	G	G	118	N	D	D
11.	434	G	C	G	145	C	S	C
12.	469	A	G	A	157	T	A	T
13.	502	C	A	C	168	L	I	L
14.	507	T	C	T				
15.	512	A	G	A/G	171	K	R	K/R
16.	556	C	G	G	186	L	V	V
17.	559	A	C	A	187	S	R	S
18.	612	C	A	C				
19.	654	A	C	A	218	K	N	K
20.	672	C	A	C				
21.	700	G	A	A	234	E	K	K
22.	703	C	A	A				
23.	719	A	G	–	240	K	R	–
24.	731	A	G	A	244	N	S	N
25.	756	A	T	A				
26.	784	T	C	C	262	S	P	P
27.	787	G	A	A	263	D	N	N
28.	801	C	T	C				
29.	820	T	A	G	274	S	T	A
30.	831	T	G	G				
31.	876	G	T	G	292	Q	H	Q
32.	948	A	G	G				
33.	966	C	T	C				
34.	1144	A	G	G	382	N	D	D
35.	1149	C	A	T/C				
36.	1192	G	A	A	398	D	N	N
37.	1258	G	A	G	420	D	N	D
38.	1267–1268	T C	G A	A/G C	423	S	D	T/A
39.	1293	G	T	G				
40.	1320	T	C	C				

(*continued on next page*)

Table 4 (*continued*)

Sl. No.	Nucleotide position and difference				Amino acid position and difference (nonsynonymous variation only)			
	Position	GTPV	SPPV	LSDV	Position	GTPV	SPPV	LSDV
41.	1356	G	A	G				
42.	1385	G	A	G	462	R	H	R
43.	1474	A	G	A	492	I	V	I
44.	1503	G	A	G				
45.	1506	C	T	C				
46.	1539	C	T	T				
47.	1615	C	A	C	539	L	I	L

Table 5
Unique nucleotide/amino acid signatures observed in ORF016.

Sl. No.	Nucleotide position and difference				Amino acid position and difference (nonsynonymous variation only)			
	Position	GTPV	SPPV	LSDV	Position	GTPV	SPPV	LSDV
1.	48	G	T	G	16	K	N	K
2.	107	C	T	C	36	S	L	S
3.	135	A	T	A				
4.	171	T	C	C				
5.	185–186	CG	AT	AG	62	T	N	K
6.	192	T	G	T				
7.	204	G	T	G	68	K	N	K
8.	288	A	T	T				
9.	305	C	A	A	102	T	K	K
10.	319	G	T	G	107	A	S	A
11.	359	T	A	A	120	I	N	N
12.	380	A	T	G	127	K	I	R
13.	455	G	T	G	152	S	I	S
14.	460	G	A	G	154	D	N	D
15.	521	T	C	C				
16.	528	T	A	C				
17.	570	T	C	C				
18.	597	G	A	G	199	M	I	M
19.	624	C	T	T				
20.	640	C	A	A	214	Q	K	K
21.	648	A	G	A				
22.	660	G	A	G				
23.	669	G	A	G				
24.	672	C	G	C				
25.	688	C	A	C	230	P	T	P
26.	712	G	T	G	238	D	Y	D
27.	724–726	ACA	–	–	242	T	–	–
28.	825	G	T	A				
29.	874	G	A	G	292	D	N	D
30.	Between 892 and 893	–	AAA ACA AT T GTA AT G AAA TT G ATA	AAA ACA AT T GTA AT G AAA TT G ATA	Between 298 and 299	–	N N C N E I D K	
31.	897	A	C	C	300	K	N	K
32.	914–915	CC	GT	GT	306	T	S	S
33.	927	C	A	C/T				
34.	979	A	G	A	328	T	A	T
35.	988	G	A	G	331	A	N	A
36.	989	C	A	C				
37.	1020	G	T	T				
38.	1034	C	T	T	346	S	L	L
39.	1057	A	G	G	354	T	A	A
40.	1067	C	T	T	357	A	V	A
41.	1119	T	C	C				
42.	1161	T	A	T				
43.	1164	T	A	A				
44.	1226	G	A	G	410	R	K	R
45.	1243	C	A	C	416	L	I	L
46.	1401	C	T	C				
47.	1402	A	G	G	469	T	A	A
48.	1479	A	C	C				
49.	1493	C	A	C	499	P	H	P
50.	1527	G	A	G				
51.	1581	C	T	C				

Table 6
Unique nucletide signatures observed in ORF010.

Sl. No.	Nucleotide position and difference				Amino acid position and difference (nonsynonymous variation only)			
	Position	GTPV	SPPV	LSDV	Position	GTPV	SPPV	LSDV
1.	94	A	C	C	32	K	Q	Q
2.	162	G	T	G				
3.	223	G	A	G	75	E	K	E
4.	230	T	G	G	77	V	G	G
5.	234	T	C	C				
6.	235	G	A	G	79	D	N	D
7.	300	A	G	G				
8.	306	C	T	C				
9.	387	C	T	C				
10.	510	T	C	T				
11.	559	T	G	G	187	Y	D	D
12.	572	C	A	C	191	T	K	T
13.	591	T	G	G	197	N	K	K
14.	629	T	A	A	210	I	K	K

Table 7
Unique nucleotide/amino acid signatures observed in ORF138.

Sl. No.	Nucleotide position and difference				Amino acid position and difference (nonsynonymous variation only)			
	Position	GTPV	SPPV	LSDV	Position	GTPV	SPPV	LSDV
1.	24	A	C	T				
2.	30	T	C	T				
3.	90	A	G	A				
4.	102	T	C	C				
5.	135	C	T	C				
6.	309	G	A	G				
7.	338	T	C	C	113	L	S	S
8.	360	A	G	A				
9.	361	A	G	A	121	S	G	S
10.	387	C	T	C				
11.	513	C	T	C				
12.	547	C	T	T	183	L	F	F
13.	555	T	C	C				
14.	564	G	A	G	205	S	N	N
15.	567	G	A	A				
16.	614	G	A	A				
17.	629	C	T	C	210	T	I	T
18.	648	T	C	C				
19.	651	A	C	C				
20.	675	C	T	C				
21.	699	A	G	A				
22.	795	A	G	A				
23.	920	T	C	C	307	L	S	S
24.	928	G	A	A				
25.	929	T	C	C	310	V	T	T
26.	980	G	A	A	327	R	K	K
27.	1016	A	G	A	339	N	S	N
28.	1044	T	G	G				
29.	1080–82	AGT	AGT	ATG				
30.	1089	G	A	G	363	M	I	R
31.	1102	G	T	G	368	A	S	A
32.	1137	T	C	C				
33.	1292	G	A	G	431	S	N	S
34.	1308	A	G	T				
35.	1425	T	A	T				
36.	1428	T	C	C				
37.	1454	G	A	A	485	R	K	K
38.	1482	G	A	A				
39.	1535	C	T	C	512	S	L	S
40.	1542	C	T	C				
41.	1545	T	A	A	515	Y	E	K
42.	1551	T	C	C				
43.	1589	A	G	G	530	N	S	S
44.	1602	G	A	G				
45.	1606	G	A	G	536	V	I	V
46.	1609	T	C	T	537	S	P	S
47.	1617	T	C	T				
48.	1618	G	A	G	540	D	N	D

(*continued on next page*)

Table 7 (*continued*)

Sl. No.	Nucleotide position and difference				Amino acid position and difference (nonsynonymous variation only)			
	Position	GTPV	SPPV	LSDV	Position	GTPV	SPPV	LSDV
49.	1662	A	G	A				
50.	1671	G	A	G	557	M	I	M
51.	1678	A	G	A				
52.	1679	T	G	G	560	I	G	R
53.	1692	A	G	A				
54.	1706	C	G	C	569	A	G	A
55.	1764	T	C	C				
56.	1770	C	T	C				
57.	1854	G	A	G				
58.	1875	C	A	C				
59.	1888	G	T	G	630	V	F	V
60.	1895	T	A	T				
61.	1897	T	A	T				
62.	1901	C	A	C				

Table 8
Unique nucleotide/amino acid signatures observed in ORF140.

Sl. No.	Nucleotide position and difference				Amino acid position and difference (nonsynonymous variation only)			
	Position	GTPV	SPPV	LSDV	Position	GTPV	SPPV	LSDV
1.	21	C	T	C				
2.	24	T	C	C				
3.	28	C	A	C	10	H	N	H
4.	49	A	G	G	17	N	D	D
5.	147	G	T	G				
6.	156	T	C	T				
7.	164	G	A	G	55	R	K	R
8.	175	G	A	G	59	E	K	E
9.	186	C	T	T				
10.	241	T	C	T	81	Y	H	Y
11.	280	C	T	C				
12.	300	A	T	G				
13.	301	A	G	G	101	N	D	D
14.	342	T	G	G	114	I	M	M
15.	387	G	A	G				
16.	408	A	T	A				
17.	438	G	A	A/G				
18.	453	C	T	T				
19.	472	C	T	T	158	L	F	F
20.	492	C	T	T				
21.	501	C	T	T				
22.	552	G	T	G				
23.	571	G	A	G	191	D	N	D
24.	669	A	G	G				
25.	679	T	C	C				
26.	690	C	T	C				
27.	748	A	T	T	250	I	L	L
28.	763	G	A	G	255	E	K	E
29.	873	T	C	T				
30.	876	T	A	A				
31.	885	A	C	C				
32.	931	G	T	G	311	A	S	A
33.	937	A	G	G	313	K	E	E
34.	942	A	C	C				
35.	1019	G	A	G	340	S	N	S
36.	1077	A	G	G				
37.	1141	A	A	G	381	K	K	E
38.	1170	G	T	G	390	K	N	K
39.	1218	A	G	A				
40.	1309	G	A	A	437	D	N	N
41.	1322	G	A	G	441	R	K	R
42.	1359	T	G	G				
43.	1366–1368	ATA	TAT	TAT	456	I	Y	Y
44.	1417	C	T	C	473	H	Y	H

Table 9
Unique nucleotide/amino acid signatures observed in ORF141.

Sl. No.	Nucleotide position and difference				Amino acid position and difference (nonsynonymous variation only)			
	Position	GTPV	SPPV	LSDV	Position	GTPV	SPPV	LSDV
1.	16	G	A	G	6	D	N	D
2.	24	A	C	A	8	E	D	E
3.	25	G	A	G				
4.	39	G	A	G				
5.	107	C	A	C	36	T	K	T
6.	161	T	G	G	54	I	S	S
7.	338	C	A	C	113	T	K	T
8.	355	A	G	G				
9.	357	C	T	C	119	N	D	D
10.	376	T	C	T/C				
11.	402	T	G	G	134	N	K	K
12.	442	A	G	G	148	N	D	D
13.	507	T	G	T/G	169	D	E	D/E
14.	654	C	T	T				
15.	684	A	C	C				
16.	708	A	G	A				
17.	714	A	T	A				
18.	717	A	G	A				
19.	756	T	C	T				
20.	758	G	A	G	253	S	N	S
21.	789	T	A	T/C				
22.	827	C	T	C	276	T	I	T
23.	870	C	A	C				
24.	887	G	A	G	296	C	Y	C
25.	896	G	A	A	299	S	N	N/D
26.	899	A	T	A	300	Y	F	Y
27.	907	G	A	G	303	D	N	D
28.	958	G	A	G	320	E	K	E
29.	997	A	G	G	333	K	E	E
30.	1005	C	T	C				
31.	1063–1064	AA	GG	AG	355	N	G	S
32.	1078	G	A	G	360	D	N	D
33.	1121	G	T	G	374	R	M	R
34.	1124	A	G	A	375	Y	C	Y
35.	1211	A	G	G	404	K	R	R
36.	1230	T	G	G	410	N	K	K
37.	1246	G	A	A	416	D	N	N
38.	1252	T	G	G/A				
39.	1296	C	A	C				
40.	1308	A	G	G				
41.	1334	C	T	C	445	A	V	A

detailed nucleotides (nts) and amino acid (aa) change observed between GTPV and SPPV are presented in Tables 3 to 13.

In brief, in the Kelch-like family, ORF137 (GenBank Accession nos. MF629078-MF629086) showed nts changes in 48 positions between SPPV and GTPV which leads to aa changes in 25 positions. ORF144 (MF629060- MF629068) showed nts changes in 47 positions between SPPV and GTPV which leads to aa changes at 28 positions. ORF016 (MF629100-MF629107) showed nts changes at 51 positions between SPPV and GTPV which leads to aa changes at 27 positions. In case of ORF016, all GTPV had an ORF length of 1689 nts whereas all SPPV had a length of 1710 nts which results in insertion of 24 nucleotides (AAA ACA AT T GTA ATG AAA TTG AT A) between position 892 and 893 (insertion of 8 aa between positions 298 and 299; NNCNEIDK). Similarly, all the SPPV had a deletion of three nucleotides (723 to 724 - AAC) which leads to the deletion of the single amino acid at position 242 (T).

In Ankyrin repeats family, ORF010 (MF629124-MF629131) showed nts

Table 10
Unique nucleotide/amino acid signatures observed in ORF3.

Sl. No.	Nucleotide position and difference				Amino acid position and difference (nonsynonymous variation only)			
	Position	GTPV	SPPV	LSDV	Position	GTPV	SPPV	LSDV
1.	11	G	A	A				
2.	46–51	AGTTTG	–	AGTTTG	16–17	SL	–	SL
3.	55	G	C	G	19	V	L	V
4.	67	G	A	G	23	V	T	A/V
5.	68	T	C	C				
6.	78	T	T	G				
7.	96	T	C	C				
8.	140	T	G	G	47	I	S	S
9.	172	C	A	C	58	Q	K	Q
10.	207	G	A	G				
11.	219	T	T	G				
12.	234	C	T	T				
13.	249	C	T	T				
14.	303	A	C	C	101	Q	H	H
15.	306	A	A	G				
16.	314	T	A	A	105	I	K	K
17.	321	C	T	T				
18.	366	G	A	G				
19.	391	A	C	C	131	N	H	H
20.	406	A	G	A	136	T	A	T
21.	415	G	A	A	139	A	T	T
22.	432	G	A	A				
23.	477	C	T	C				
24.	484	T	C	T	162	Y	H	Y
25.	487	G	T	T	163	V	L	L
26.	489	T	A	A				
27.	509	C	A	A	170	T	K	K

Table 11
Unique nucleotides/aminoacids signatures observed in ORF012.

Sl. No.	Nucleotide position and difference				Amino acid position and difference (nonsynonymous variation only)			
	Position	GTPV	SPPV	LSDV	Position	GTPV	SPPV	LSDV
1.	15	G	A	G				
2.	19	G	A	G	7	V	I	V
3.	24	A	C	C				
4.	46	T	A	A	16	S	T	T
5.	147	C	T	T				
6.	174	G	T	G				
7.	181	C	T	C				
8.	222/223	T/G	C/A	C/G	75	D	N	D
9.	227	A	T	T	76	N	I	I
10.	238	C	A	C				
11.	273	C	A	C				
12.	294	A	G	G				
13.	387	G	A	G				
14.	390	T	C	C				
15.	391/393	G/T	A/C	G/T	131	D	N	D
16.	420	C	T	C				
17.	423	G	T	G	141	E	D	E
18.	453	C	T	T				
19.	455	C	T	C	152	S	L	S
20.	465	G	T	G	155	E	D	E

Table 12
Unique nucleotide/amino acid signatures observed in ORF142.

Sl. No.	Nucleotide position and difference				Amino acid position and difference (nonsynonymous variation only)			
	Position	GTPV	SPPV	LSDV	Position	GTPV	SPPV	LSDV
1.	15	G	A	G/A				
2.	18	C	T	C				
3.	23	T	C	C	8	L	S	S
4.	29	T	A	T	10	I	K	I
5.	35	T	C	C	12	F	S	S

(*continued on next page*)

Table 12 (*continued*)

Sl. No.	Nucleotide position and difference				Amino acid position and difference (nonsynonymous variation only)			
	Position	GTPV	SPPV	LSDV	Position	GTPV	SPPV	LSDV
6.	75	G	A	G				
7.	107	G	A	A	36	S	N	N
8.	136	C	G	G	46	L	V	V
9.	144	A	G	A				
10.	150	G	A	G				
11.	155	G	A	G	52	S	N	S
12.	161	A	G	G	54	Y	C	C
13.	210	A	T	A	70	K	N	K
14.	219	C	A	C	73	F	L	L
15.	226	C	A	C	76	Q	K	Q
16.	253	C	A	C	85	I	L	L
17.	320	A	G	G	107	K	R	R
18.	333	C	T	C				
19.	348	A	G	G				
20.	419	T	G	G	140	I	S	S
21.	427	G	A	G	143	D	N	D
22.	466	A	G	A	156	N	D	N
23.	564	C	T	T				
24.	624	T	C	T/A				
25.	627	G	A	G				
26.	638	G	A	A	213	S	N	N
27.	647	C	T	T	216	S	L	L
28.	657	A	C	C	219	E	D	D
29.	783	G	T	G				
30.	811	G	A	G	271	V	I	V
31.	834	C	T	C				
32.	843	A	G	A				
33.	855	A	G	A/G				
34.	859	G	A	G	287	A	T	A
35.	870	T	C	C				
36.	905	G	T	G	302	C	F	C
37.	948	C	A	C	316	F	L	F/L
38.	976	C	A	C	326	L	I	L
39.	984	T	A	C				
40.	638	G	A	A	213	S	N	N
41.	647	C	T	T	216	S	L	L
42.	657	A	C	C	219	E	D	D
43.	783	G	T	G				
44.	811	G	A	G	271	V	I	V
45.	834	C	T	C				
46.	843	A	G	A				
47.	855	A	G	A/G				
48.	859	G	A	G	287	A	T	A
49.	870	T	C	C				
50.	905	G	T	G	302	C	F	C
51.	948	C	A	C	316	F	L	F/L
52.	976	C	A	C	326	L	I	L
53.	984	T	A	C				

Table 13
Unique nucletide signatures observed in ORF013.

Sl. No.	Nucleotide position and difference				Amino acid position and difference (nonsynonymous variation only)			
	Position	GTPV	SPPV	LSDV	Position	GTPV	SPPV	LSDV
1.	49	G	A	A	17	V	V	I
2.	57	G	T	G	19	M	I	M
3.	84	T	C	C				
4.	93	C	T	C				
5.	118	G	A	G	40	D	N	D
6.	161	C	A	A	54	T	K	K
7.	153	G	A	A				
8.	207	C	T	C				
9.	209	G	A	G	70	R	K	R
10.	231	C	A	C				
11.	248	A	G	G	83	K	R	R
12.	270	T	A	A				
13.	280	T	T	T				
14.	281	G	A	A				

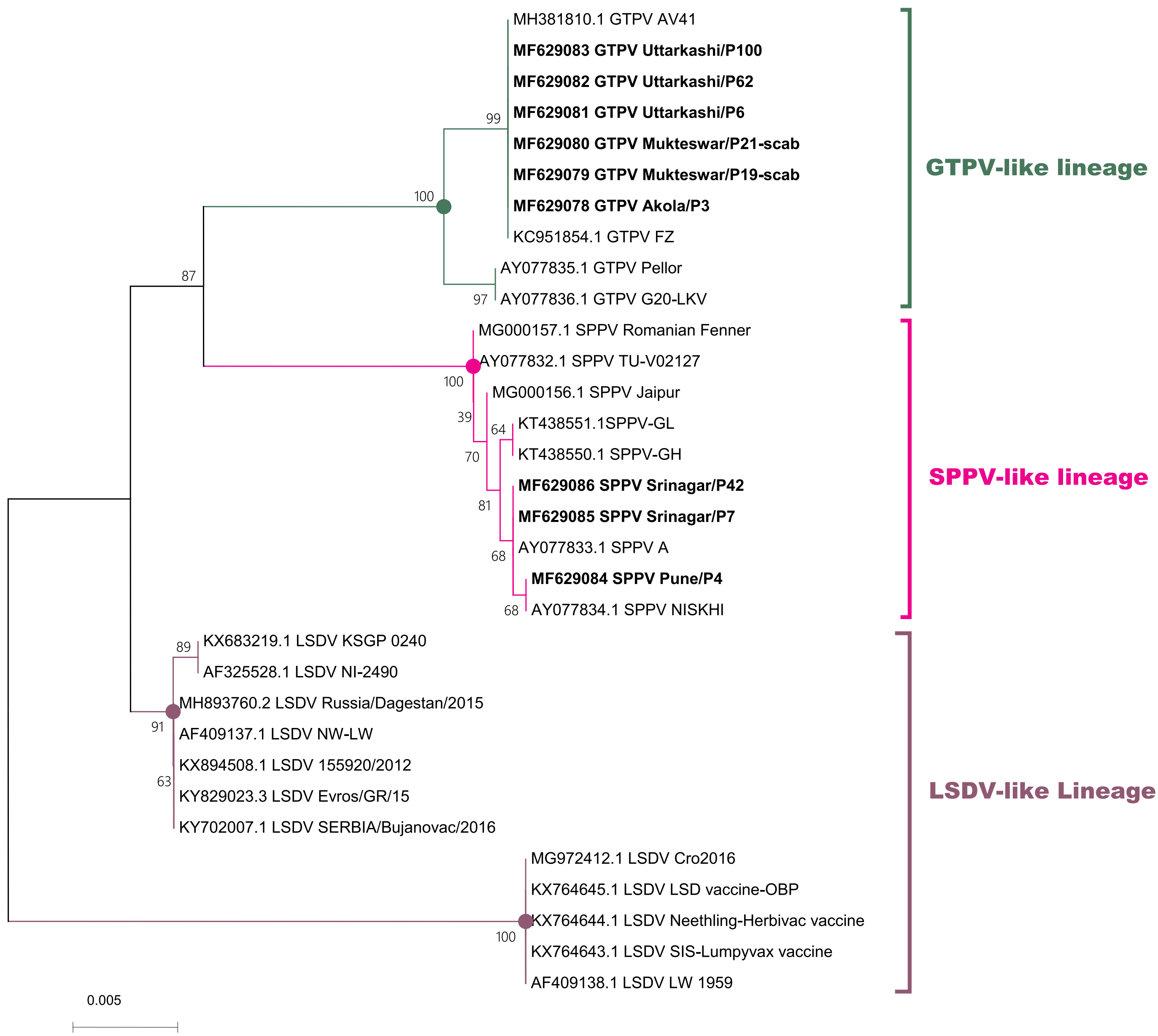

Fig. 1. Evolutionary relationship of ORF137 of Indian GTPV and SPPV with reference sequences. Isolates used in the current study are presented in bold letters.

changes in 14 positions between SPPV and GTPV which leads to aa changes at 8 positions. ORF138 (MF629132- MF629138) showed nts changes at 62 positions between SPPV and GTPV which leads to aa changes in 23 positions. ORF140 (MF629108-MF629113) showed nts changes at 44 positions between SPPV and GTPV which leads to aa changes at 20 positions. ORF141 (MF629114-MF629122) showed nts changes at 41 positions between SPPV and GTPV which leads to aa changes at 25 positions.

In virulence genes, ORF003 (MF629069-MF629077) showed nts changes in 27 positions between SPPV and GTPV which leads to aa changes in 13 positions. All GTPV including Indian isolates used in the present study had an ORF length of 513 nts whereas all the SPPV sequences including Indian isolates had a 507 nts due to the deletion of 6 nts of positions 46 to 51 (AGTTTG) which leads to deletion of 2 aa in SPPV (16 and 17; S and L, respectively). ORF012 (MF629051-MF629059) showed nts changes in 20 positions between SPPV and GTPV which leads to aa changes in 8 positions. ORF142 (MF629087-MF629095) showed nts changes in 53 positions between SPPV and GTPV which leads to aa changes in 23 positions.

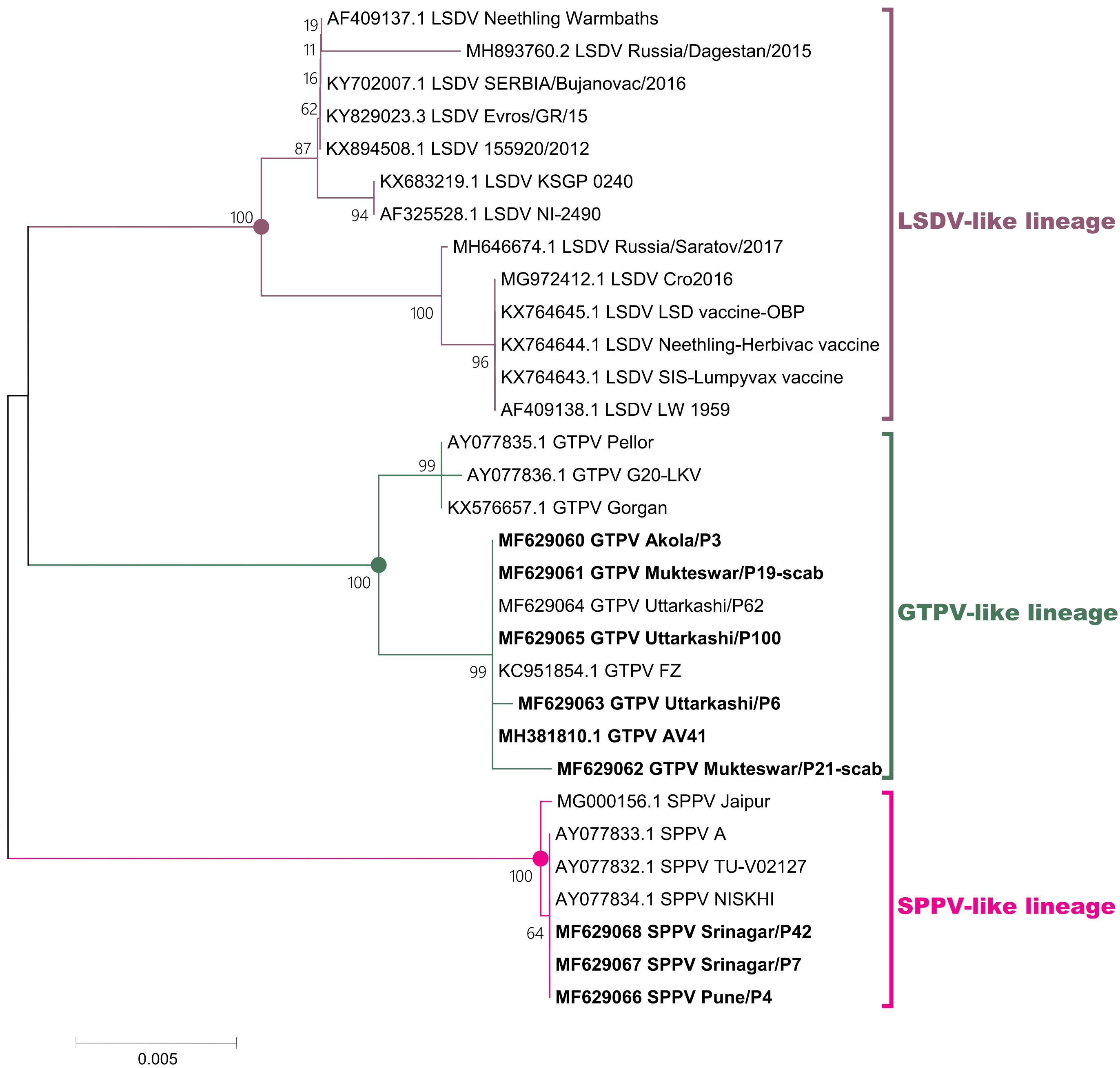

Fig. 2. Evolutionary relationship of ORF144 of Indian GTPV and SPPV with reference sequences. Isolates used in the current study are presented in bold letters.

ORF013 (MF629139-MF629147) showed nts changes in 14 positions between SPPV and GTPV which leads to aa changes in 6 positions. The GTPV sequences available in the GenBank had an ORF length of 291 nts whereas SPPV had 303 nts. Although the Indian SPPV analyzed in the present study had same size of ORF like other reference sequences, all Indian GTPV showed variation in ORF length; four isolates viz., GTPV Akola, GTPV Uttarkashi P6, GTPV Uttarkashi P62, and GTPV Uttarkashi P100 had a length of 285 nts whereas GTPV Mukteswar P19 and GTPV Mukteswar P21 had a length of 306 nts.

Overall, there were several nts and aa signatures were present between GTPV and SPPV isolates. However, none of these markers was able to differentiate between virulence and vaccine viruses of GTPV or SPPV.

3.2. Phylogenetic analyses

In the phylogenetic analyses of host-range and virulence genes with

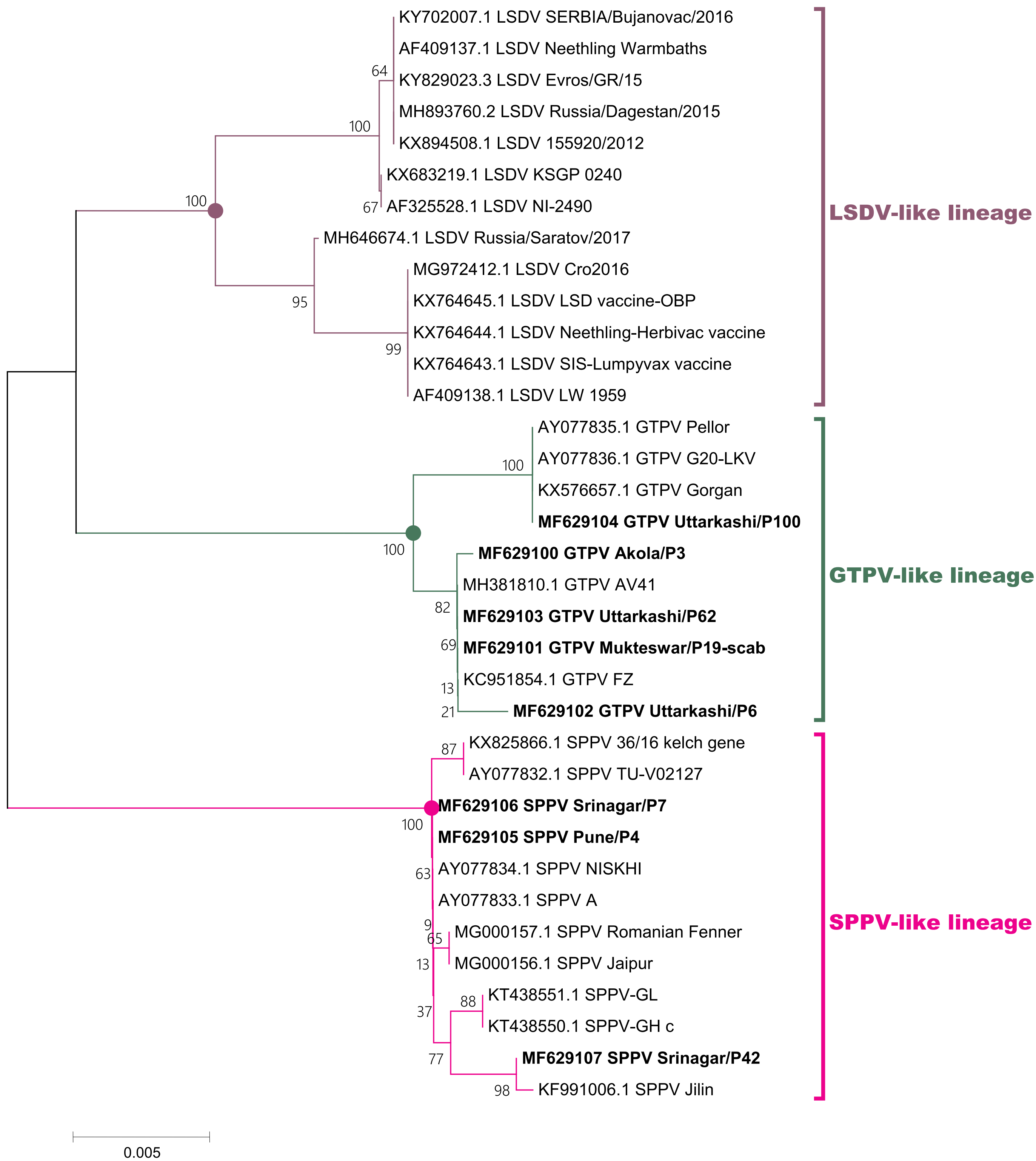

Fig. 3. Evolutionary relationship of ORF016 of Indian GTPV and SPPV with reference sequences. Isolates used in the current study are presented in bold letters.

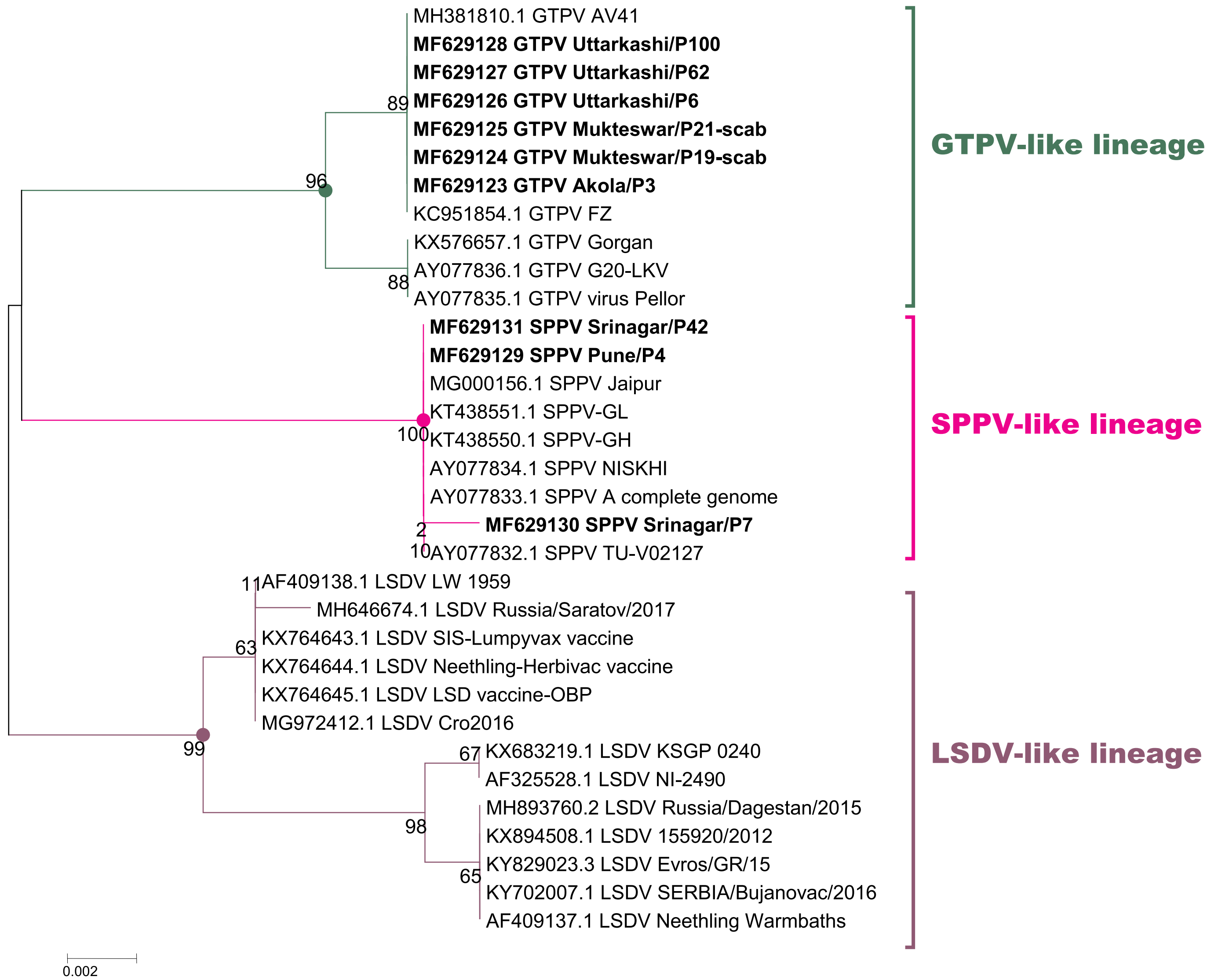

Fig. 4. Evolutionary relationship of ORF010 of Indian GTPV and SPPV with reference sequences. Isolates used in the current study are presented in bold letters.

reference sequences from SPPV, GTPV, and LSDV, they formed three clades viz., GTPV-like, SPPV-like, and LSDV-like lineages. All GTPV sequences formed two distinct subclades; Indian and Chinese (GTPV-FZ) GTPV sequences were clustered under the same subclade whereas all other sequences formed a separate subclade. However, all SPPV including Indian virus isolates grouped under a single clade (see Figs. 1-13).

4. Discussion

One of the possible reasons for vaccine failure is due to the usage of the genetically uncharacterized virus. For several decades, many countries used KSGP- O180, KSGP- O240 (KS-1) strain for vaccination in cattle, sheep, and goats. Once it was believed that these strains are sheeppox virus, however, later it was confirmed that these strains are LSDV. The LSDV-KS1 is failed to provide complete protection in several occasions possibly due to lack of attenuation for cattle usage (Gari et al., 2015; Gelaye et al., 2015; Tuppurainen et al., 2014). Further, in contrast to the earlier conception of capripoxviruses are host-specific, recent studies reveal that some strains may cause cross-species infections (Bhanuprakash et al., 2010; Davies, 1976; Kitching et al., 1986; Kitching and Taylor, 1985; Ramakrishnan et al., 2017; Santhamani et al., 2015; Yan et al., 2012). Therefore, genetic characterization of GTPV/SPPV of both vaccine and field strains is important. This will aid in understanding molecular epidemiology as well as differences between vaccines and viruses circulating in the field.

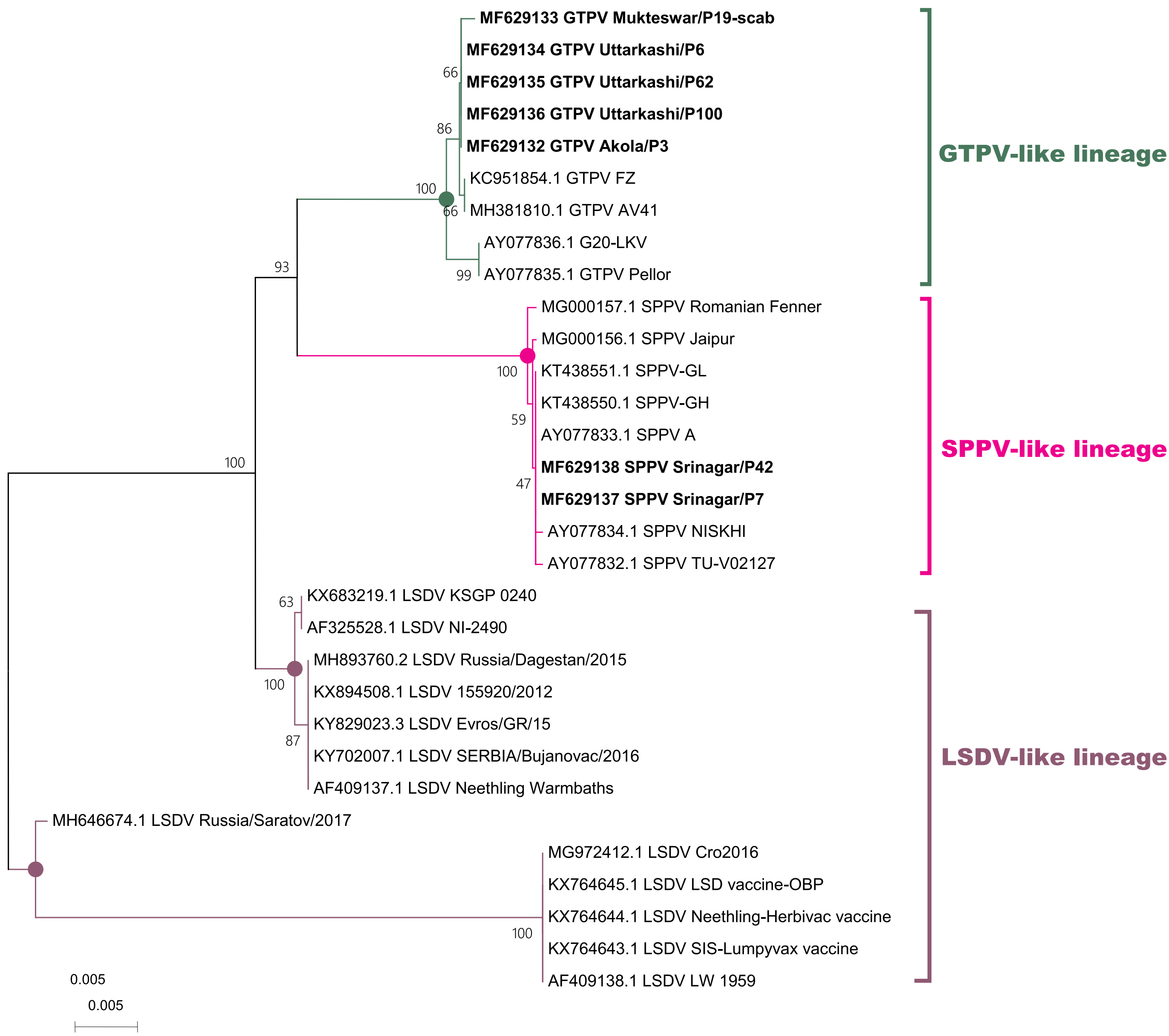

Fig. 5. Evolutionary relationship of ORF138 of Indian GTPV and SPPV with reference sequences. Isolates used in the current study are presented in bold letters.

In the genus *Capripoxvirus*, certain terminally located genes are possibly involved in virulence, immunomodulatory, and host-range functions. Genes potentially involved in host-range are divided into two major categories viz., ankyrin repeat proteins (ORFs 010, 138, 140, 145, and 141) and Kelch-like proteins (ORFs 016,137, and144). Genes affecting virulence are in broader categories which include IL- 10 (ORF003), IL-18 (ORF012), EGF-like protein (ORF013), and serpin (ORF142) (Tulman et al., 2002; Zeng et al., 2014). Therefore, in the present study, the above genes (except ORF145) were targeted for differentiation of GTPV and SPPV using vaccine and field strains.

The viral ankyrin (vANK) repeat proteins of poxviruses have been studied in detail and they involved in modulating intracellular signaling networks during viral infection (Guo et al., 2011). These proteins also inhibit virus-induced apoptosis (Tulman et al., 2002). Kelch-like proteins involved in virus virulence by affecting multiple cellular processes (Balinsky et al., 2007).

All the host-range genes analyzed in the current study showed several unique nts and aa signatures between SPPV and GTPV suggesting their possible role in host-range determination. Among the ankyrin repeat genes, ORF016 has shown a remarkable difference between SPPV and GTPV in terms of ORF length as well as nts and aa signatures. Narrowing of the host-range has been found with the loss of ankyrin genes (Kara et al., 2003). One of the recent studies with whole-genome sequencing of GTPV showed that low-level nucleotide identity of ORF016

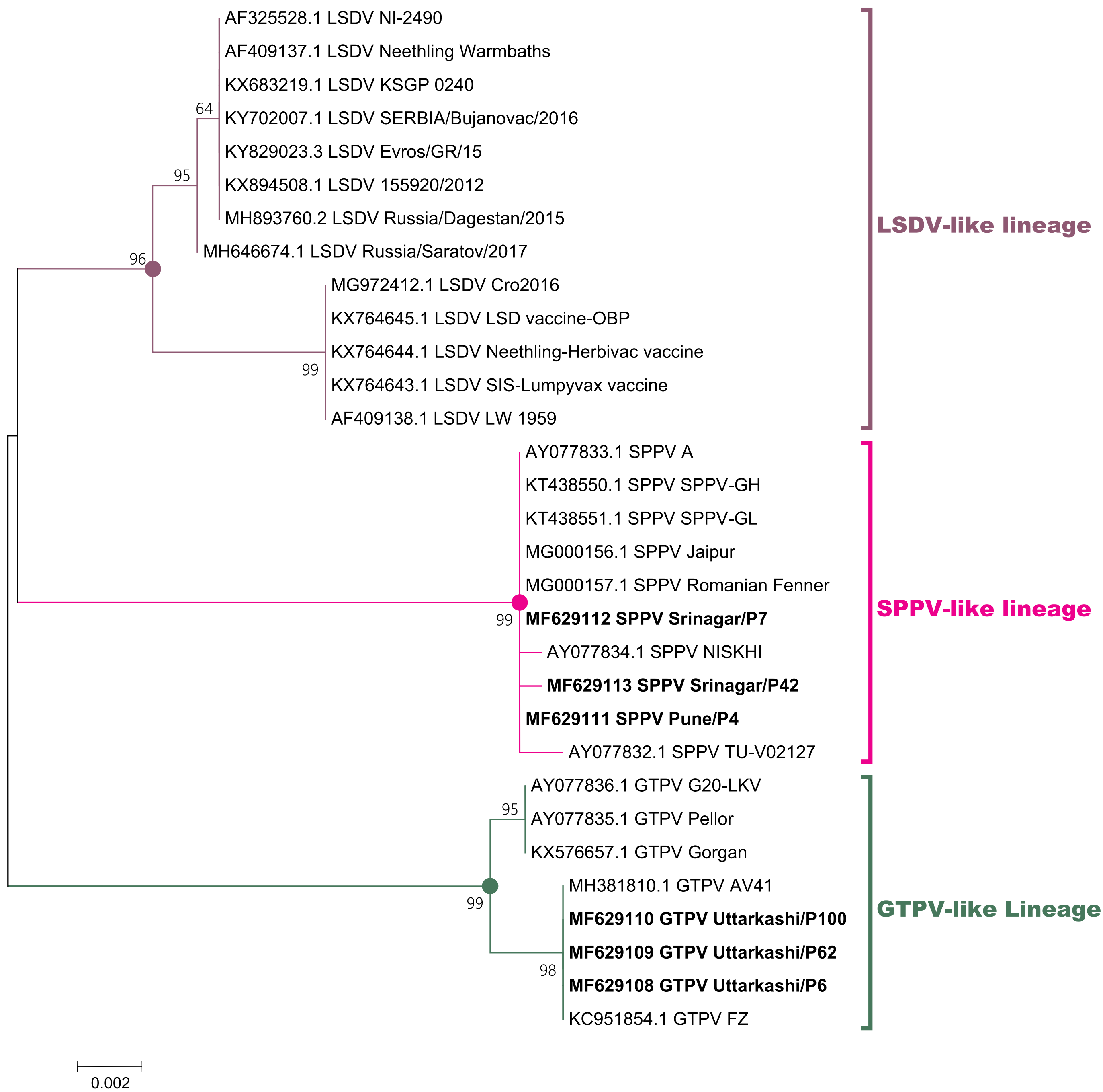

Fig. 6. Evolutionary relationship of ORF140 of Indian GTPV and SPPV with reference sequences. Isolates used in the current study are presented in bold letters.

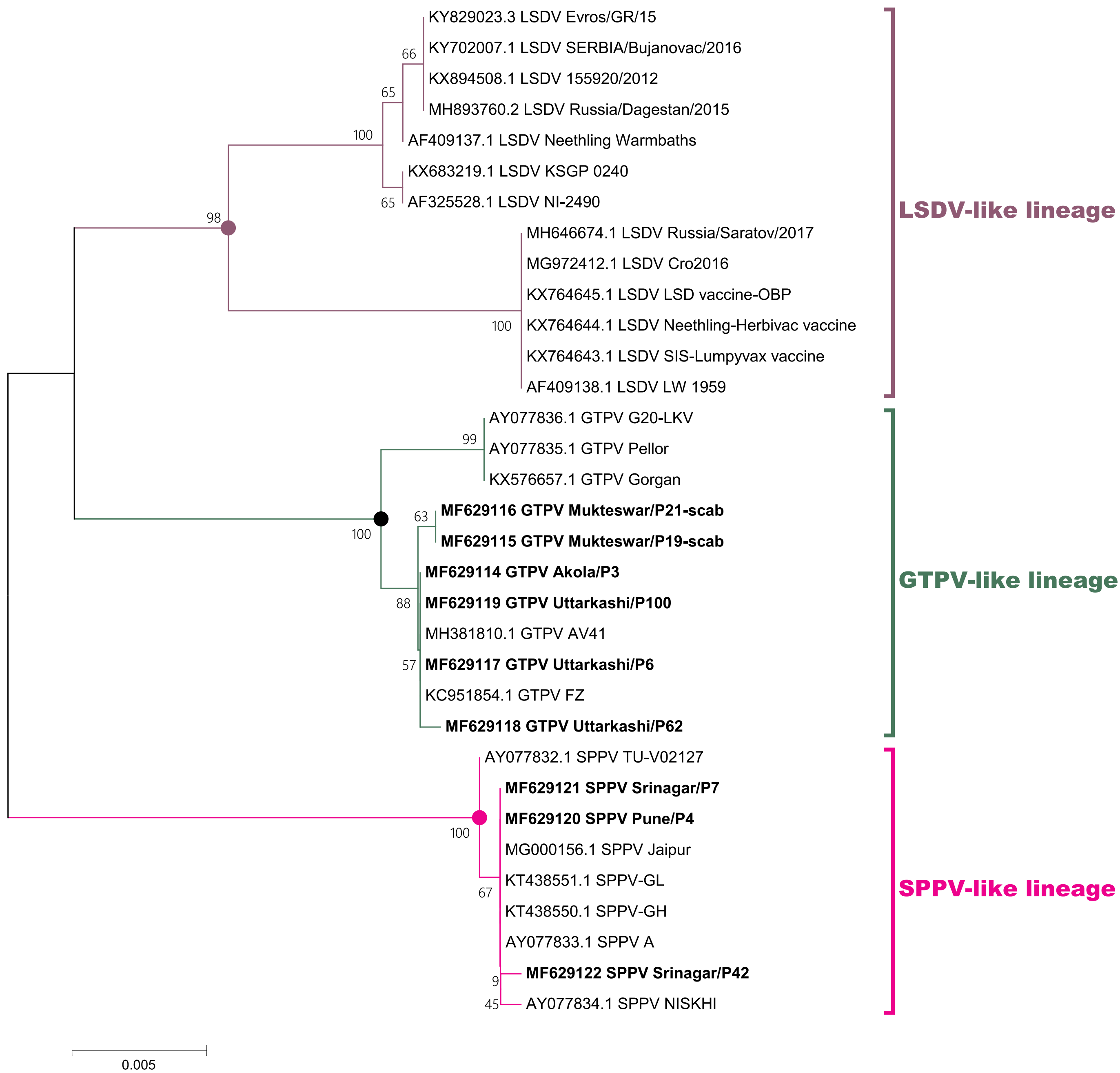

Fig. 7. Evolutionary relationship of ORF141 of Indian GTPV and SPPV with reference sequences. Isolates used in the current study are presented in bold letters.

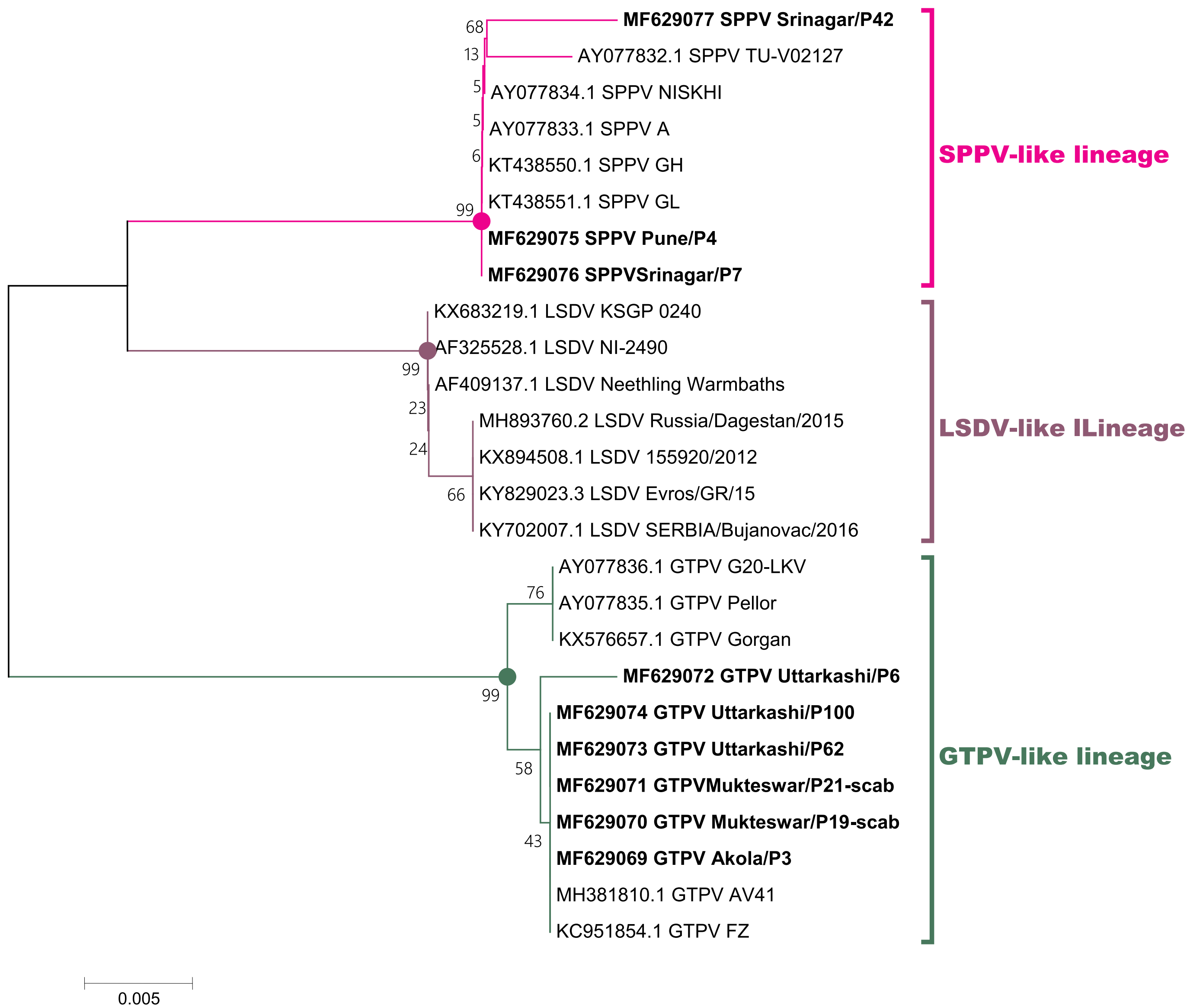

Fig. 8. Evolutionary relationship of ORF003 of Indian GTPV and SPPV with reference sequences. Isolates used in the current study are presented in bold letters.

(80.0–89.9%) with SPPVs suggesting that this gene could be targeted to differentiate SPPV and GTPV (Zeng et al., 2014). In the earlier studies, *RPO30* gene-based PCR has been used for the detection and differentiation of SPPV and GTPV based on 21 nts difference (Lamien et al., 2011; Santhamani et al., 2013). We observed that in ORF016, all SPPV had an insertion of 24 nts in contiguous and this region may be targeted for differentiation of GTPV and SPPV with higher resolution. In several studies, three genes viz., *RPO30*, *GPCR*, and *P32* were targeted for the identification of virus-lineages (Dutta et al., 2018; Lamien et al., 2011; Le Goff et al., 2009; Ramakrishnan et al., 2017; Santhamani et al., 2013, 2014, 2015; Tuppurainen et al., 2014). In addition to the above three genes, our study confirms that ankyrin repeat proteins genes (ORFs 010, 138, 140, and 141) and Kelch-like proteins genes (ORFs 016,137, and144) could also be useful in differentiating GTPV and SPPV.

Genes affecting virulence are in broader categories which include IL-10 (ORF003), IL-18 (ORF012), EGF-like protein (ORF013), and serpin (ORF142). Potent immunosuppressive effects exerted by IL-10 enable the virus to possess anti-inflammatory activities like suppression of cytokine release by macrophages and T-cells indicating its contribution to viral virulence (Fleming et al., 2015). Epidermal keratinocytes are major

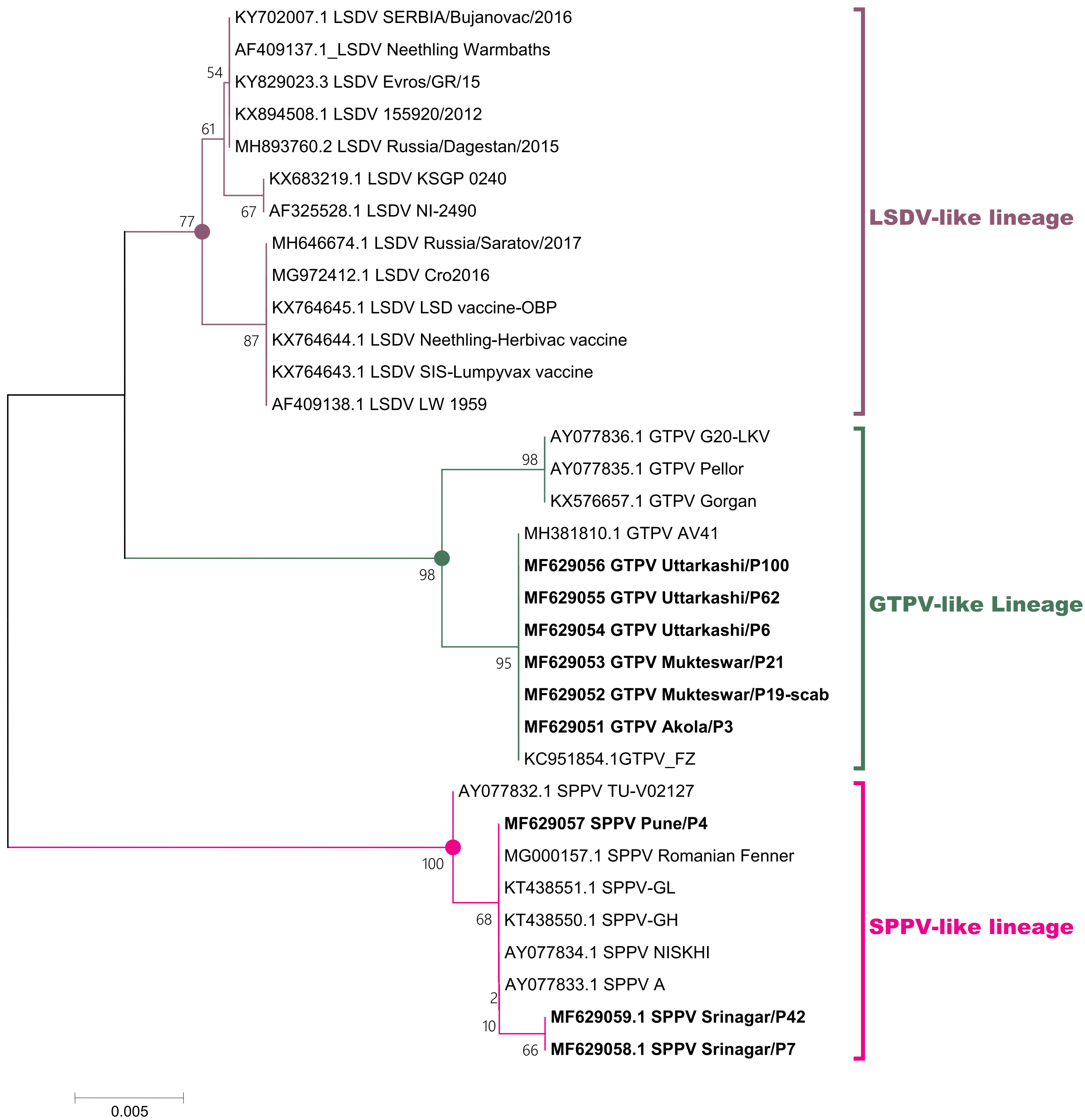

Fig. 9. Evolutionary relationship of ORF012 of Indian GTPV and SPPV with reference sequences. Isolates used in the current study are presented in bold letters.

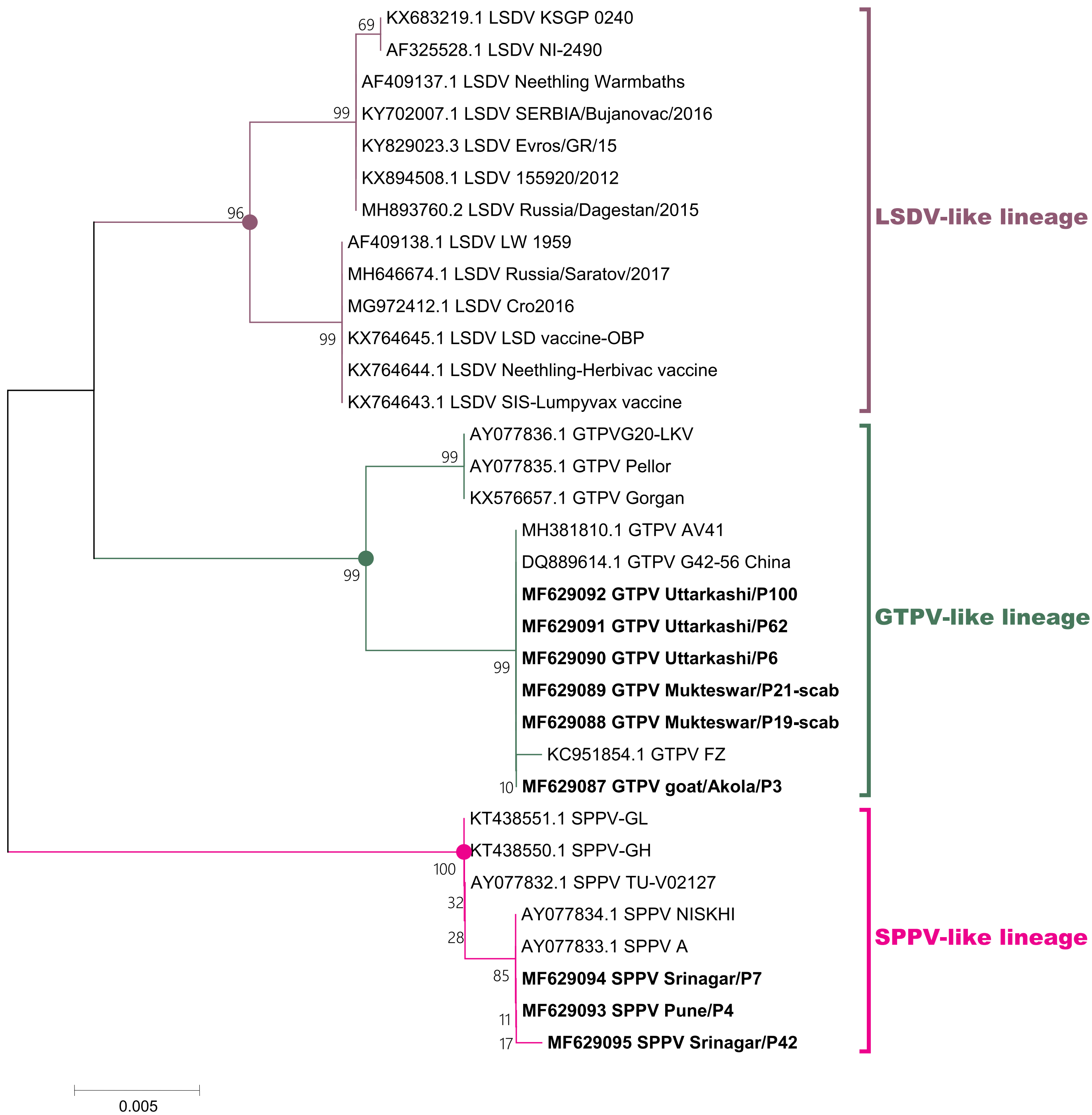

Fig. 10. Evolutionary relationship of ORF142 of Indian GTPV and SPPV with reference sequences. Isolates used in the current study are presented in bold letters.

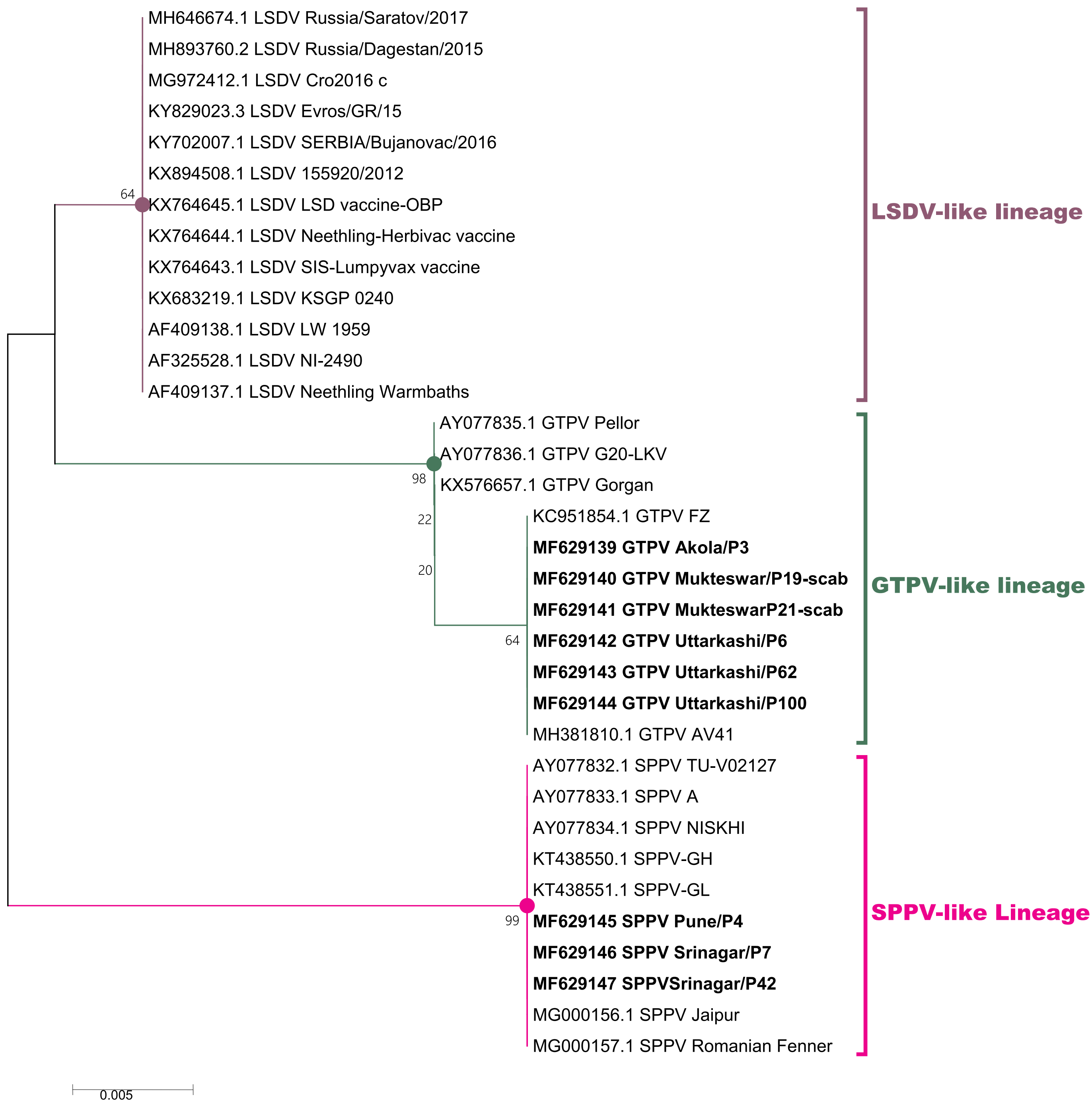

Fig. 11. Evolutionary relationship of ORF013 of Indian GTPV and SPPV with reference sequences. Isolates used in the current study are presented in bold letters.

producers of interleukin 18 (IL-18) stimulating interferon-gamma (IFN-ϒ) production and induce natural killer (NK) cell cytotoxicity. A gene knockout construct of LSDV by knocking out homologs of IL-10 and IFN-ϒ like genes have been found to be safe and provide protection emphasize the role of these genes in virulence (Kara et al., 2018). Poxvirus ORF012 encodes this IL18 and infects the host mainly through the cornified epithelium (Born et al., 2000). ORF013 encodes homologs of the cellular epidermal growth factor (EGF)/transforming growth factor (TGFα). It is stated that poxvirus EGF-like proteins influence in vivo virulence of the virus, and stimulate cell proliferation at sites of viral replication (Afonso et al., 2000). Serpins (Serine protease inhibitor) encoded by ORF142 have a role in the regulation of immune and inflammatory responses as well as cell death by acting as pseudosubstrates for their target proteinases, binding irreversibly and inhibiting their activity. Goatpox virus constructs deleted with the TK gene and ORFs 8–18 shows reduced virulence in goats (Zhu et al., 2018).

In the present study, analysis of virulence genes i.e. ORFs 003, 012, 013, and 142 from the vaccine and field SPPV and GTPV strains revealed signature markers between SPPV and GTPV. However, virulence/attenuation signatures could not be observed between virulent/field and vaccine viruses at both nts and aa level. In the 2010 nodular form of sheeppox epizootic in Morocco, when the samples were characterized by thymidine kinase (TK) and G protein-coupled receptor (GPCR) genes, the virus had a close association with vaccine virus (Zro et al., 2014). However, when the samples were analyzed with E3L (ORF034)/E5R (35)/E4L (036) and ORFs025/026 there was a difference between vaccine and field virus. It was observed that 3 single nucleotide polymorphisms in E3L/E5R (35)/E4L between vaccine and field viruses. In case of ORF026, there were 4 insertions and 3 deletions were observed in vaccine viruses (Haegeman et al., 2016). In our earlier study, *RPO30* (30 kDa RNA polymerase subunit) gene analyses, SPPV Roumanian Fanar, SPPV Ranipet, and SPPV Morocco vaccine strains had lysine (K) instead of asparagine (N) at position 190 (N190K) (Santhamani et al., 2014).

The expected nucleotide length of GTPV for ORF013 was 291 but the Indian GTPV isolates analyzed in the current study showed differences in the length; two isolates (Mukteswar P19, Mukteswar P21) had a length of 306 nts and remaining four isolates having 285 nts whereas all the Indian SPPV are had the length of 303 nts which is similar to the other SPPV sequences. It is unlikely that the changes observed in GTPV sequences especially in Mukteswar strain will have a role in virulence because other virulent strain (GTPV Uttarkashi) is not having similar signature molecules. In LSDV, it was reported that vaccine strain had a deletion of 28 bp in EEV glycoprotein gene (LW126 gene) (Menasherow et al., 2014). In a recent study, it was reported that there was an 84 bp deletion between DNA ligase gene and the VARV B22R homolog gene of two SPPV vaccine strains - the Romanian and Yugoslavian RM/65 (Chibssa et al., 2018).

5. Conclusion

Among the poxvirus researchers, there is a opinion that the terminal variable regions of the poxviruses involves in the determination of host-specificity and virulence/pathogenicity. The genes of terminal regions targeted in our current study showed that there are several virus lineage-specific nts and aa markers (i.e. differential marker for SPPV and GTPV) but no unique (nt or aa) signature could be observed between vaccine and challenge/field virus isolates. Therefore, it is reasonable to hypothesize that some other genes in the terminal/central regions also may play a role in determining virulence/pathogenicity and attenuation. The complete genome characterization of vaccine and field strains shed more light on genes involved in the pathogenicity/attenuation of capripoxviruses.

Declarations of Competing Interest

None.

Acknowledgments

The research work was supported by grants received from CAAST-ACLH (NAHEP/CAAST/2018-19) of ICAR-World Bank funded National Agricultural Higher Education Project (NAHEP) and ICAR-IVRI.

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