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VARV B22R homologue as phylogenetic marker gene for *Capripoxvirus* classification and divergence time dating

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Abstract

Sheeppox disease is associated with significant losses in sheep production world over. The sheep pox virus, the goatpox virus, and the lumpy skin disease virus cannot be distinguished by conventional serological tests. Identification of these pathogens needs molecular methods. In this study, seven genes viz. EEV maturation protein—F12L, Virion protein—D3R, RNA polymerase subunit—A5R, Virion core protein—A10L, EEV glycoprotein—A33R, VARV B22R homologue, and Kelch like protein—A55R that cover the start, middle, and end of the genome were selected. These genes were amplified from Roumanian-Fanar vaccine strain and Jaipur virulent strain, cloned, and sequenced. On analysis with the available database sequences, VARV B22R homologue was identified as a marker for phylogenetic reconstruction for classifying the sheeppox viruses of the ungulates. Further, divergence time dating with VARV B22R gene accurately predicted the sheeppox disease outbreak involving Jaipur virulent strain.

Keywords Sheeppox virus · Goatpox virus · Lumpy skin disease virus · Phylogeny · *Capripoxvirus* · Divergence time dating

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Introduction

Sheep and goats are the small ruminants that contribute significantly to the agrarian economy in India. Adaptability to diverse climatic conditions makes sheep and goat farming possible in the arid, semi-arid, and mountain areas where crop and dairy farming are not economical [1]. India has the world's second and third highest population of goats (140.5 million) and sheep (72 million), respectively [2]. Among the diseases that affect small ruminants, sheeppox and goat pox are associated with the significant production losses in terms of reduced milk yield, damage to hide and mortality [3].

Sheeppox virus (SPPV) and goatpox virus (GTPV) belong to the genus *Capripoxvirus* (CaPV) of *Poxviridae* family. CaPV comprises sheeppox virus (SPPV), goatpox virus (GTPV) and lumpy skin disease virus (LSDV) [4]. CaPV infections are mainly characterized by fever, excess salivation, conjunctivitis, and rhinitis with ocular and nasal discharges. These symptoms are followed by eruption of pock lesions on the skin. The analysis of genome sequences of CaPV has already shown that, SPPV, GTPV, and LSDV are distinct from each other genetically, therefore should be defined as three different species [5]. CaPV seems to be host specific with SPPV, GTPV, and LSDV causing severe clinical disease principally in sheep, goats, and cattle, respectively. However, most of the sheeppox and goatpox virus isolates cause disease in either of two, but many of them seems to be equally infectious and pathogenic to both sheep and goats [6]. The classification of CaPV was based on the name of species and the country from which virus was isolated for the first time [7, 8]. Conventional serological tests cannot distinguish SPPV, GTPV and LSDV,therefore, identification of these pathogens needs molecular methods such as restriction profile analysis, RFLP, or nucleotide sequence analysis [9]. Earlier, genetic relatedness of SPPV and GTPV strains isolated from different outbreaks have been studied by phylogenetic analysis. These studies have utilized nucleotide sequence features of different genes from SPPV and GTPV isolates. The sequence of G-protein-coupled chemokine receptor (GPCR) gene was studied for differentiation and grouping of different CaPV isolates [10]. In other studies, P32 gene sequence was used for phylogenetic analysis of SPPV and GTPV isolated from disease outbreaks [6, 11]. Apart from single gene studies, multiple gene sequence analysis using P32 gene, RPO30, and GPCR were tested to identify genetic closeness of SPPV and GTPV isolates [12, 13]. Identification of candidate marker gene for classifying the CaPV will facilitate understanding the genetic relationships between isolates of sheeppox and goatpox disease outbreaks.

In this study, we have cloned and sequenced seven genes of SPPV vaccine (Roumanian-Fanar) and virulent (Jaipur) strains, analyzed the nucleotide diversity with the available data base sequences, constructed phylogeny, and identified the VARV B22R homologue to be the best gene for classifying the CaPV. Divergence time dating was also done with the available isolates using these gene sequences.

Materials and methods

Seven genes viz. EEV maturation protein gene (codes for a protein required for transport of intracellular viral particles to the cell membrane), the virion protein gene D3R (codes for capsid protein), the A5R gene (translates a protein for RNA polymerase subunit), A10L gene (codes for a protein essential for correct assembly of viral DNA into the nucleoprotein complex to form immature viral particles), gene A33R (codes for protein that works in infectivity of extracellular enveloped virus), VARV B22R homologue gene (codes for that functions in modulation or evasion of host immune response), gene A55R (codes for kelch like protein required for protein–protein interactions) covering the start, middle, and end of the genome and showing variations in the most frequently studied strains—TU, SPPV A, and Niskhi were selected. These gene fragments were amplified in SPPV Roumanian-Fanar vaccine and Jaipur virulent strains.

Viruses

The lamb testis adapted SPPV Jaipur and Roumanian-Fanar isolate were propagated in Vero cell culture in and Eagle's minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Invitrogen, Grand Island, NY, USA) was used.

DNA extraction

Viral genomic DNA was isolated using earlier described method [14]. Cells were washed with isotonic solution, dissolved in hypotonic solution and ultra-centrifuged after addition of TritonX-100 and β-mercaptoethanol. The pellet was re-suspended in a solution of TE buffer along with β-mercaptoethanol, proteinase K, and SDS. This was further incubated with 54% glucose at 55 °C for 2 h and 5M sodium chloride was added to the digested lysate. An additional step of extraction was done twice with chloroform and isoamyl alcohol. The aqueous phase was collected and precipitated with ethanol.

Cloning and sequencing

The selected regions of seven genes were PCR amplified using specific primers (Table 1) and high fidelity *Taq* polymerase. The PCR fragments were cloned, purified

Table 1 Primer sequences for amplification

ORF of SPPV	Name of gene	Forward primer	Reverse primer
27	EEV maturation protein gene	AAATTGATGGACCTAATGGAGTT	TTTGAAAGAGATTGGTCAGACA
81	Virion protein gene	CCAAAAATTGATTTGAATGACG	GATTAATAACAGCTTCCCAATCG
96	RNA polymerase subunit protein gene	GACGAAGATGCGAGTGATGA	TTCACCTGTTACGGATAACAATTC
101	Virion core protein gene	TAAATCCCATTGCCGAAAAA	AATGCTTGATGCAGCAAATG
122	EEV glycoprotein gene	TTGATGTTCCAAGGAGTGGA	TACCATCTTCTCCCCACGTC
134	VARV B22 homologue gene	AAAACAACTGACGACGATTTTG	GGCATCATACAAACCCTTGC
144	Kelch like protein gene	CGAATTACGTAAATACTGCGATG	TCCAAACGTTGATTCTCTGTG

and sequenced. The sequences were submitted to GenBank—EEV maturation protein gene [KY500994(RF), KY500993(JP)], Virion protein gene [KY500995(RF), KY500996(JP)], RNA polymerase subunit protein gene [KY501002(RF), KY500997(JP)], Virion core protein gene [KJ533136(RF), KJ533135(JP)], EEV glycoprotein gene [KY500998(RF), KY500999(JP)], VARV B22 homologue gene [MH593889(RF), KY501001(JP)], Kelch like protein gene [KJ638694(RF), KJ638693(JP)].

Sequence analysis

Sequences obtained in this study were analyzed with other CaPV sequences available in the NCBI database. Sequences were aligned in Megalign for identifying indels. Phylogenetic analysis was done using both MEGA 6.0 and Bayesian evolutionary analysis sampling trees (BEAST) software package v2.3.1 [15]. In MEGA 6.0, phylogeny was done by maximum likelihood method with best model fit (Table 2) obtained for each set of sequences using 1000 bootstrap replicates. In BEAST, phylogeny was constructed using Bayesian Markov chain Monte Carlo (MCMC) analysis for each gene. The XML input files for BEAST analysis were obtained in Bayesian evolutionary analysis utility software v2.3.1 by employing appropriate models of nucleotide substitution, a constant population size coalescent prior and strict clock model. The best model was selected by calculating the Bayes factor (BF), which is the ratio of marginal likelihood of one model to the marginal likelihood of the competing model. Evidence against the competing model is considered positive when $2\ln BF > 2$ [16]. Tree Annotator was used to summarize the posterior tree distributions after exclusion of the first 25% of the trees as burn-in. Annotated phylogenetic tree was visualized in FigTree software v1.4.0. Statistical uncertainty was reflected in estimates of the posterior distribution.

Table 2 Best substitution models for phylogenetic analysis using Mega 6.0

ORF of SPPV	Genes	Best model chosen
27	EEV maturation protein gene	T92 + I
81	Virion protein gene	T92 + G
96	RNA polymerase subunit protein gene	T92
101	Virion core protein gene	T92
122	EEV glycoprotein gene	T92
134	VARV B22 homologue gene	HKY
144	Kelch like protein gene	T92

T92 Tamura 3 parameter; *HKY* Hasegawa–Kishino–Yano

Molecular time dating

Molecular time dating was done using the available virulent VARV B22R gene sequences by Bayesian method implemented in BEAST v2.3.1 by employing GTR + Γ model of nucleotide substitution, a constant population size coalescent prior and strict clock model. The root was set to 800 years ago based on Babkin and Shchelkunov [17].

Results

PCR amplicons of 649, 660, 341, 808, 432, 729 and 552 bp of EEV maturation gene, Virion protein, RNA polymerase subunit, Virion core protein, EEV glycoprotein, VARV B22R homologue and Kelch like protein, respectively, were amplified both in RF and Jaipur strains, cloned and sequenced.

Sequence analysis

The percent identity across sequences of genes coding for EEV maturation (F12L), Virion protein (D3R), RNA polymerase subunit (A5R), Virion core protein (A10L), EEV glycoprotein (A33R), VARV B22R homologue, and Kelch

Table 3 Amino Acid substitutions across SPPV, GTPV and LSDV in all seven genes

EEV maturation														
Position	8	17–18	29	65	69	71	81	83	153	191	198			
SPPV	N	ET	E	Y	I M(RF)	M	S	I	D	E	K			
GTPV	–	KK	G	C	M	I	S	T	D	E	R			
LSDV	–	ET	G	Y	M	M	T	T	–	K	K			
Virion protein														
Position	1	15	30	76	79	85	144	232						
SPPV	K	R	D	D	K	V	A	L						
GTPV	K	K	N	N	T	I	S	L						
LSDV	N	K	D	N	T	I	A	V						
RNA polymerase subunit														
Position	8	28	29	34										
SPPV	Q	T	–	N										
GTPV	P	–	N	T										
LSDV	P	T	N	T										
Virion core protein														
Position	22	29	51	53	59	61	74	102	107	188				
SPPV	I	I	H	Y	F	A	I	R	N	L				
GTPV	V	M	Q	H	F	T	A	K	K	V				
LSDV	I	I	Q	Y	L	T	L	R	K	V				
EEV glycoprotein														
Position	3	5	36	48	55	63	67	101	107					
SPPV	A	T	M	I	–	N	Q	H	S					
GTPV	E	A	I	L	N	K	P	Y	L					
LSDV	A	A	M	L	–	K	P	Y	S					
VARV B22R														
Position	28	31	32	59	70	77	104	124	125	132	161	186		
SPPV	L	I	D	D	S	T	E	S	K	I	N	A		
GTPV	L	S	G	E	S	T	K	T	E	M	H	S		
LSDV	S	S	G	E	S/N	A	K	T	E	M	H	A		
Kelch like protein														
Position	2	4	19	31	33	69	87	92	120	129	155	157	158	170
SPPV	H	K	V	G	L	V	L	K	N	V	D	S	K	T
GTPV	H	K	A	G	I	I	F	K	N	L	N	L	R	T
LSDV	N	K/R	V	G/R	L	I	L	R/K	D	V	D	L	K	A

like protein (A55R) ranged from 47.9 to 96.3; 97.9–100; 96.5–100; 97.0–100; 96.8–100; 96.7–100; and 59.7–100, respectively.

Comparison of EEV maturation gene (SPPV ORF27) with TU as consensus showed that except for TU all SPPV strains possessed three nucleotide insertions at positions 21–23 (Fig. S1). This insertion led to an introduction of two asparagine residues at positions 7 and 8. Also, a change of G and C with A at positions 49 and 53 in the nucleotide sequence resulted in substitution of glutamic acid and threonine to lysine in all GTPV strains at positions 17 and 18 respectively (Fig. S1). Apart from this, GTPVs showed unique presence of Y65C, M71I, and K198R replacements (Table 3). Though there is a methionine present at position 69 of RF as in other *Capripoxvirus*, SPPVs showed isoleucine substituted at this position. At position 605 there is a

guanine residue present in place of adenine in TU (Fig. S1), resulting in a change of K202R. All the vaccine strains of LSDV showed a variation from field strains with K198R as in GTPVs (Fig. S2). Two prominent substitutions at positions G29E and T83I in the amino acid sequence differentiate SPPVs from other *Capripoxvirus*. All LSDVs marked the presence of threonine in place of serine at position 81. The vaccine strains of LSDVs seemed to diverge from field isolates by the presence of arginine instead of lysine at position 198. Also, deletion of one amino acid at position 153 was observed in field isolates of LSDVs (Fig. S2).

On comparing the virion protein (SPPV ORF81), all nucleotide substitutions are depicted in Fig. S3. SPPVs showed characteristic differences at positions K15R, N76D, T79K, and I85V (Table 3). GTPVs can be differentiated at D30N and A144S from the rest. The consensus sequence SPPV TU matches with GTPVs at A144S. The GTPV strain FZ showed two specific amino acid changes at D78N and N106D due to changes at position 232 and 318 at nucleotide level. LSDVs differed from other *Capripoxviruses* at position 202, with field strains carrying serine residue and vaccine strains showed presence of asparagine (Figs. S3 and S4).

For RNA polymerase subunit gene (SPPV ORF96), glutamine substitution to proline/threonine at position 8 was observed due to the replacement of A to C in all the isolates except the SPPVs in comparison to TU (Figs. S5 and S6). Three nucleotide deletions were observed in GTPV at position 82–84 and SPPV at position 85–87 leading to changes in amino acid at positions 28 and 29 (Table 3). Though there are specific substitutions at nucleotide level for all the three *Capripoxviruses* at position 60, 145, 153, 210, 234, 240, and 309 (Fig. S5), no changes were observed at the amino acid level. Vaccine strains of LSDV showed L38I substitution.

For virion core protein gene (SPPV ORF101), the vaccine strains (SIS Lumpywax, Ni-LW1959, Ni-Herbivac, Ni-OBP) showed difference at nucleotide level from field isolates (NW-LW, KSGP 0240, Ni-2490) at positions 72 and 130 (Fig. S7). The GTPV strains can be easily characterized from the rest by the replacements such as I22V, I29M, Y53H, and R102K (Table 3). The viruses also showed an important substitution at position 74, with SPPV carrying isoleucine, GTPV an alanine and LSDV a leucine (Fig. S8). The SPPV strains showed differences at Q51H, T61A, K107N and V188L from other *Capripoxviruses*. All LSDVs carried a phenylalanine to leucine substitution at position 59.

The EEV glycoprotein (A33R) gene (SPPV ORF122) sequences of FZ, G-20 LKV, Gorgan and Pellor showed an insertion of three nucleotides at position 164 and the TU showed a characteristic deletion of 3 nucleotides at position 172 (Fig. S9). With the changes in protein sequence at positions L48I, K63N, P67Q, and Y101H, our isolates along with most of the SPPV isolates seemed to be divergent from GTPV and LSDV (Table 3). GTPVs can be differentiated from other *Capripoxviruses* by the substitutions M36I and S107L. The vaccine strains of LSDV showed a specific change at position T37A in comparison to field isolates (Fig. S10).

For the VARV B22R (SPPV ORF134), the substitutions present in the stretch of nucleotides in the region from 87 to 96 (Fig. 1 and S11) led to differences in the amino acid sequence among various isolates (Table 3). The TU strain showed substitutions D30N and A159S in comparison to other SPPV strains (Fig. 2). SPPVs can be clearly differentiated from other

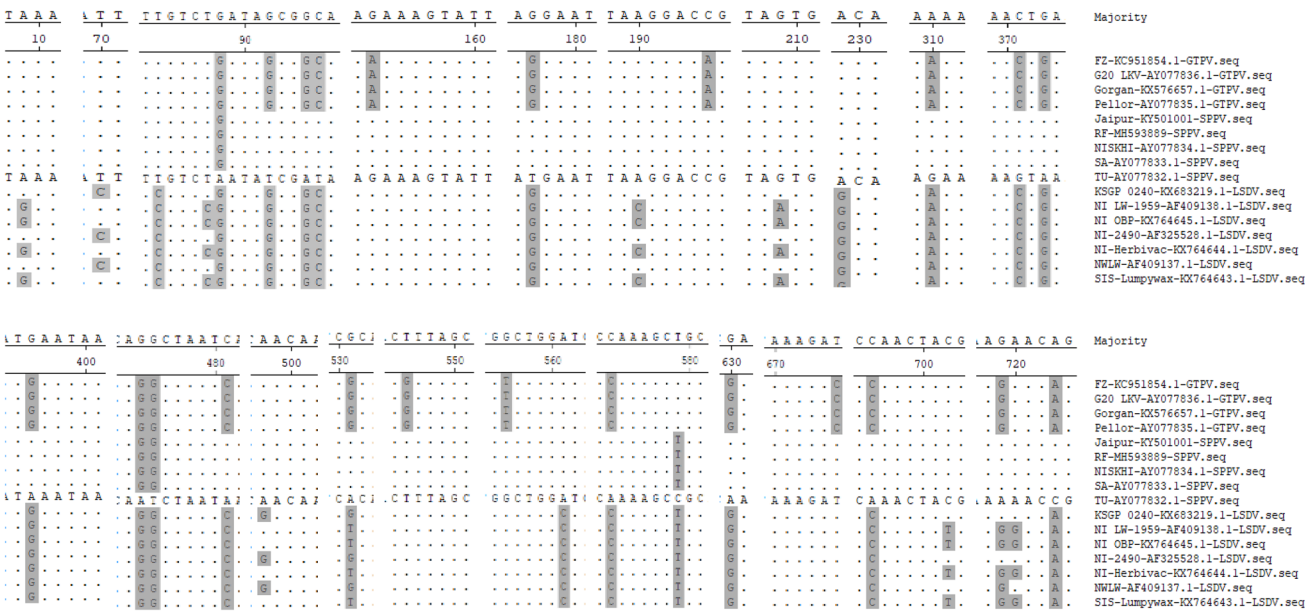

Fig. 1 Alignment (in sections) depicting nucleotide changes in partial gene sequence of VARV B22R homolog protein (ORF134)

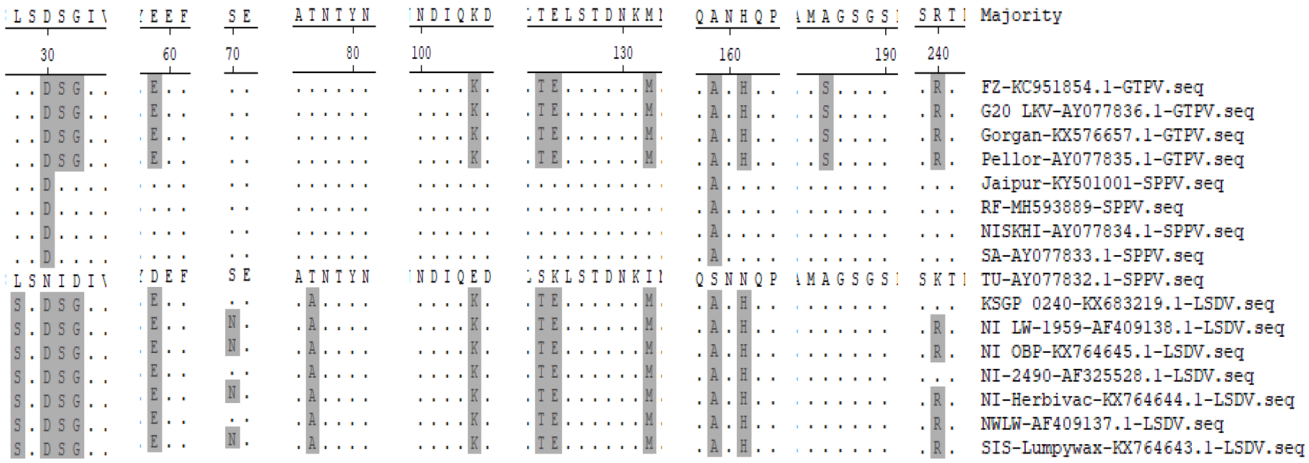

Fig. 2 Alignment (in sections) depicting amino acid changes in partial sequence of VARV B22R homolog protein (ORF134)

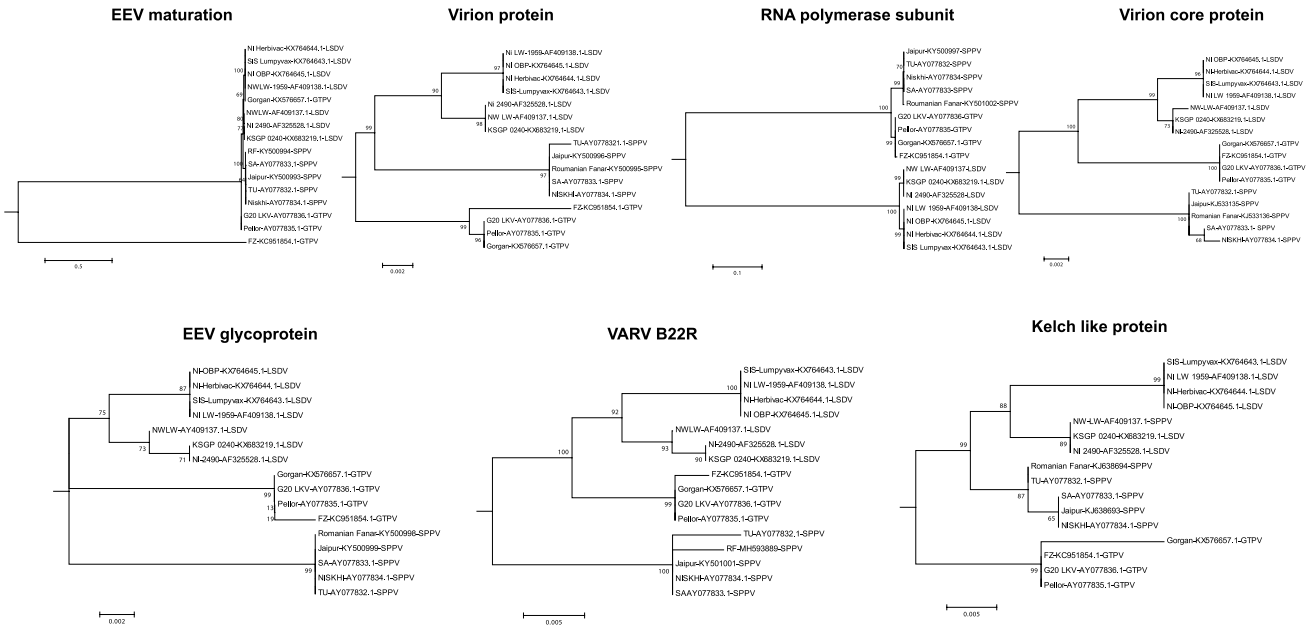

Fig. 3 Phylogenetic analysis of *Capripoxviruses* using Mega 6.0. Phylogeny was constructed for all the seven genes using appropriate model as mentioned Table 2. The bootstrap values at each node or sub-node indicate the reliability classifying the *Capripoxviruses* into distinct classes

Capripoxviruses at multiple positions with the substitutions of S31I, G32D, E59D, K104E, T124S, E125K, M132I, and H161N. LSDV seemed to differ from the rest with substitutions as L28S and T77A. GTPVs showed unique presence of serine in place of alanine at position 186 (Fig. S12). Though, quite identical, the vaccine strains of LSDVs marked the presence of asparagine in place of serine at position 70.

The Kelch like protein (SPPV ORF144) showed several single base substitutions at nucleotide level (Fig. S13). The vaccine strains of LSDV showed a characteristic presence of arginine at position 4 and 31 and alanine at position 148 (Fig. S14). GTPV isolates showed unique substitutions at V19A, L33I, and L87F. The substitution isoleucine to valine at position 69 was found specific to SPPVs. LSDVs can be differentiated from other *Capripoxviruses* at position H2N and T170A (Table 3).

Phylogenetic analysis

On phylogenetic analysis of nucleotide sequences, distinct clusters as per host origin were formed for all the genes. The strains in the present study (RF and JP) clustered as a

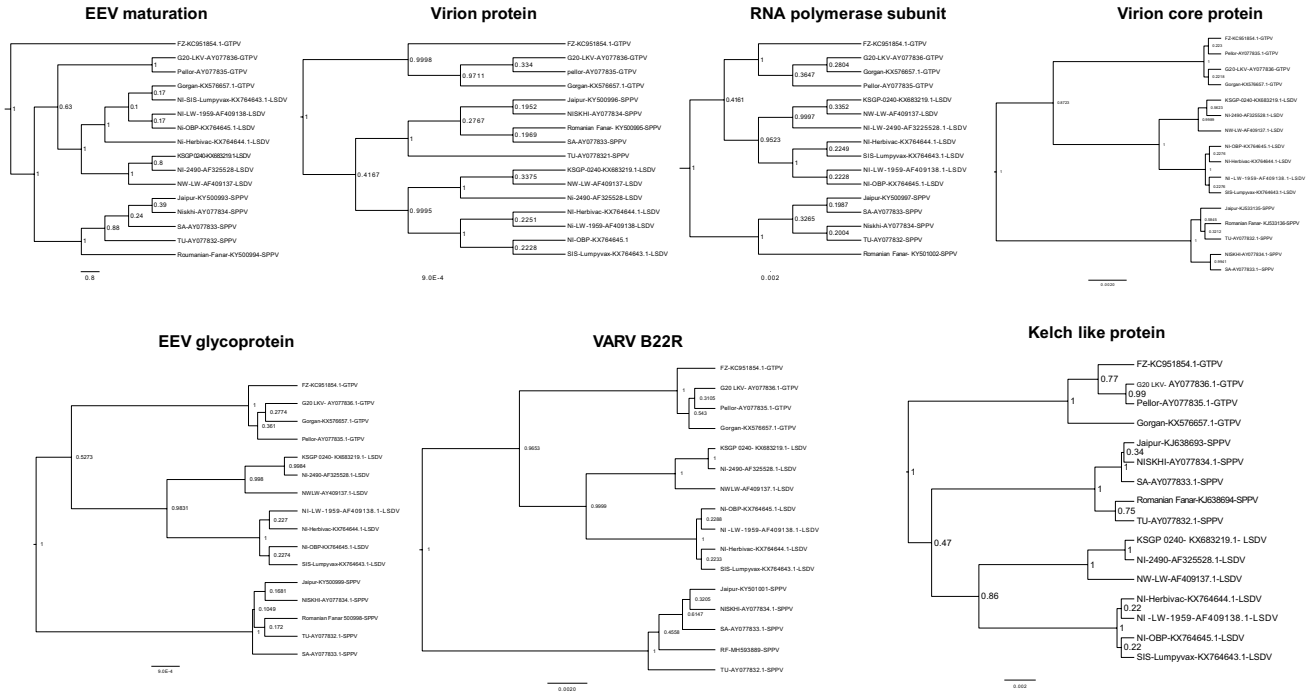

Fig. 4 Phylogenetic analysis of *Capripoxviruses* using BEAST v 2.3.1. Phylogeny was constructed for all the seven genes and the posterior probability values indicate the reliability of classifying the *Capripoxviruses* into distinct classes

single clade with the SPPV family in phylogeny of all the genes. The bootstrap values, which reflect the reliability of the tree, were found to be < 70 for most of the genes except for virion protein, VARV B22R, and EEV glycoprotein (Fig. 3). These three genes clearly clustered the strains into distinct groups with high bootstrap values. Among these three, the reliability was highest for the VARV B22R phylogeny followed by virion protein and EEV glycoprotein (Fig. 3). On phylogenetic analysis of all the genes in BEAST, it was observed that VARV B22R gene phylogeny had better posterior probability values than all the other genes (Fig. 4). The phylogenetic analysis of GPCR sequence for the *Capripoxviruses* in the study was done with best model—Tamura 3 parameter + G using the maximum likelihood method (Fig. S15).

Molecular time dating

On analysis, the Jaipur SPPV strain dated 35 years back in origin from the present data, which approximately coincided with the time of the earlier reported outbreak [18] (Fig. 5).

Discussion

Though both sheeppox and goatpox diseases are endemic in India, reports on molecular characterization of *Capripoxvirus* for differentiation of species are scanty. Also, the conventional serological tests cannot distinguish SPPV, GTPV, and LSDV. Here, we selected seven genes on the basis of their location on the genome. These genes cover the start, middle and end of the genome, and show variations in the most frequently studied strains, i.e., TU, SPPV A, and Niskhi. The gene fragments were cloned, sequenced and analyzed to identify the appropriate gene for classifying the CaPV.

Here the sequences were analyzed and phylogeny was constructed using both traditional Mega 6.0 and Bayesian - BEAST 2.0, approaches. The sequences clustered as per host origin for all the genes in both the approaches. The strains in the present study (RF and JP) clustered as a single clade with the SPPV family in phylogeny of all the genes. Bootstrapping is the most common method of assessing the reliability of phylogenetic analyses in the traditional approach [19, 20]. The low bootstrapping indicates the possibility of sub clustering of the taxa of a subnode along with the other taxa of a node. The bootstrap values, were found to be less than 70 for most of the genes except for virion protein, VARV B22R, and EEV glycoprotein. However, among these three, the reliability was highest for the VARV B22R phylogeny.

Bayesian approach of constructing a phylogeny uses a likelihood function to create a quantity called the posterior probability of trees using a model of evolution, based on some prior probabilities. The posterior probability gives the probability of a tree to be correct. The tree with the highest

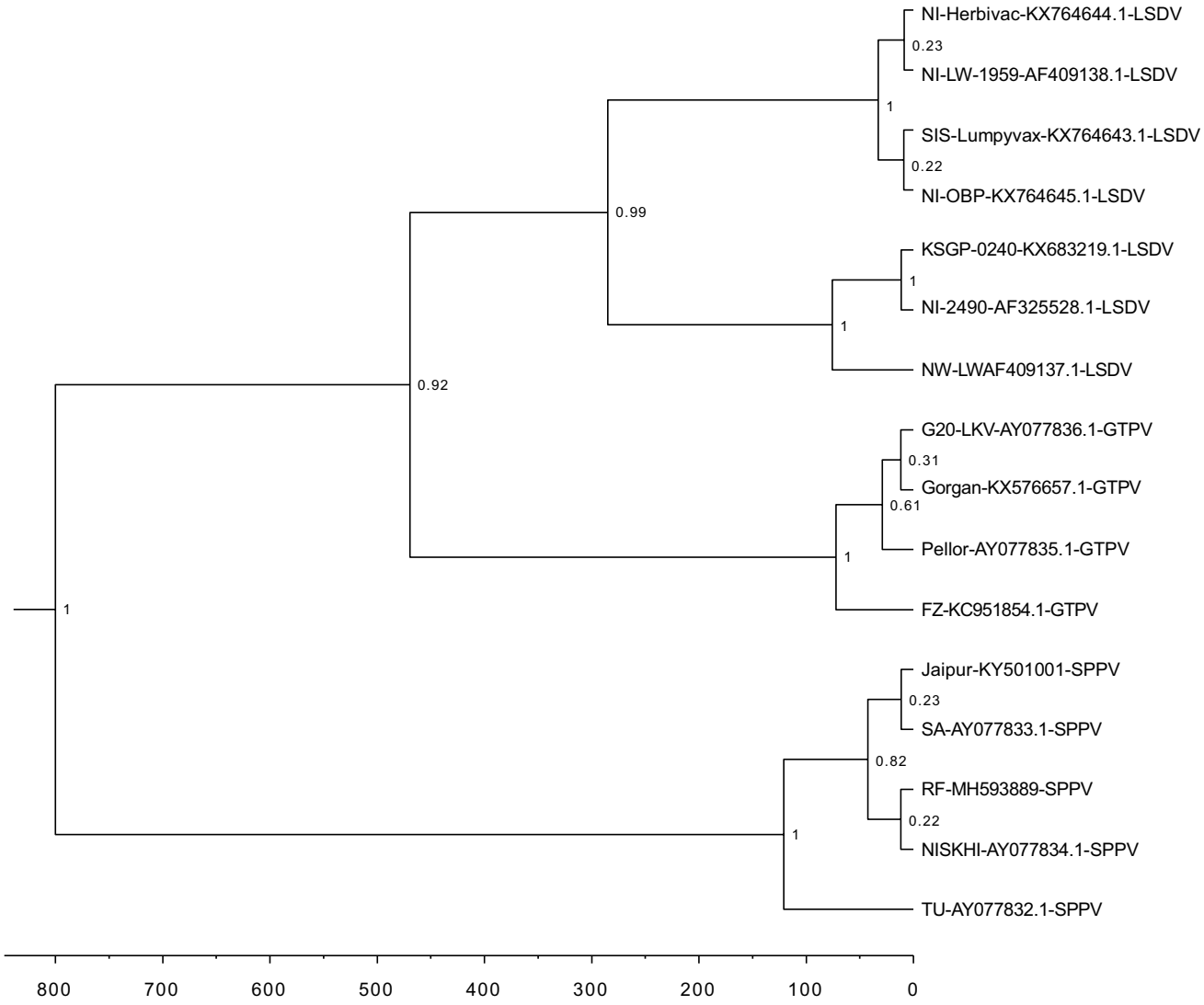

Fig. 5 Divergence time dating of virulent *Capripoxviruses* based on VARV B22R gene sequence

posterior probability is the one chosen to represent the best phylogeny [21]. In our study on comparing the posterior probability across the phylogenies constructed from all seven genes, it was observed that the VARV B22R phylogeny had the best posterior probabilities. On comparing with GPCR phylogeny, though GPCR classified the *Capripoxviruses* into distinct classes the reliability i.e., the bootstrap values of some of the nodes and sub-nodes were quite less in comparison to VARV B22R phylogeny. Also, the bootstrap values in VARV B22R phylogeny were closer to the bootstrap values of the whole genome phylogeny (Fig. S16) in comparison with all the other genes.

Time scale phylogeny has been used to estimate the probable divergence dating of important virus subtypes [22]. Earlier, Babkin and Babkina [8] analyzed 35 highly conserved pox virus genes to determine the rates of nucleotide substitution and divergence times for different subtypes of vertebrate poxviruses. Derived from these estimations, it was found that the *Capripox* and *Suipox* genera diverged 111 ± 29 thousand years ago. Also, Babkin and Shelkunov [17] estimated the origin of VARV strains to be around 800 years ago. Based on these estimates, in the present study SPPV, GTPV, and LSDV isolates were analyzed using VARV B22R sequence data. The origin of Jaipur isolate was predicted to be around 35 years back. This time scale coincides and agrees with the historical evidence of sheeppox outbreak at Jaipur [18]. Similarly, in previous studies, the evolutionary distances were calculated for different VARV pox viruses to determine the divergence time [23]. These predicted time scales correlated with the historical small pox occurrence in human beings.

In conclusion, though all the genes classified the CaPV sequences into distinct clusters as per host origin, VARV B22R is found to be the best phylogenetic marker gene for CaPV classification as VARV B22R classified the *Capripoxviruses* in distinct clusters with best bootstrap and posterior probability values. On divergence time dating using VARV B22R the predicted origin of the Jaipur isolates correlated with the occurrence of the outbreak.

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Author Contributions BM, GRK and RKS designed the study. BM, PM, CLP, IZ, RG, NS, DB and JS performed the laboratory work. IZ, ARS, MB, BS and GRK performed phylogenetic analysis and wrote the manuscript.

Compliance with ethical standards

Conflict of interest All authors in this paper declare they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Research involving human participants No human subjects were involved in this study.

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