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Regulated Emergence of the African swine fever virus: *B646L* (p72) Gene Based Bayesian Coalescent Analysis

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Abstract: African swine fever virus (ASFV) belongs to the genus of virus of the *Asfaviridae* family. ASFV infection causes hemorrhage and high death rate hence increased loss to the swine community. It is a complex infectious disease of swine, which constitutes devastating impacts on animal health and the economy of the pig farmers. It has been confirmed that virus infections has been spreading in swine population for many years. In this study, the evolutionary epidemiology analysis of ASF virus from the geographical regions Africa, Europe, and Asia, respectively were retrieved from GenBank for the analysis. The nucleotide gene sequences of the viral protein p72 encoded by *B646L* gene published during 1960-2020 was taken in to study. The Bayesian skyline model with uncorrelated randomized clock model was employed to reconstruct the evolutionary history of the virus, to identify virus population demographics. Results of the analysis suggested that ASFV exhibited a high evolutionary rate, as the divergence caused reduction in the population in the recent years. The *B646L* gene of ASFV had an evolutionary rate of 4.13 X 10^{-6} substitution/site/year and the tMRCA as 3.15 x 10^{5} with 95 percent HPD range in years (2.4 x 10^{4} to 1.23 x 10^{6}) was obtained. In conclusion, the evolutionary study of ASFV with p72 protein from the ASFV of the *B646L* genes indicated that they evolved at a faster rate and plays a major role in the evolutionary process. Further, this study may help in designing or developing vaccines to control the spread of the disease.

Keywords: ASFV, *B646L*, p72, evolutionary analysis, beast, India, tMRCA

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1. INTRODUCTION

African swine fever virus (ASFV), a causative agent of African swine fever (ASF), is a contagious and devastating hemorrhagic disease that affects the swine herd. The production and economy of swine and swine-based products are affected due to high mortality rates, serious socio-economic impact, high capacity for transboundary dissemination, and lack of an effective vaccine or treatment.[1-2] African swine fever (ASF) is a highly contagious hemorrhagic disease of pigs, American wild pigs, warthogs and European wild boar. All age groups are equally susceptible. ASF is characterized by high fever, loss of appetite, the skin and internal organs are hemorrhages and death within 2-10 days on average. The rate of mortality may be as high as 100%. Without any signs of the disease, the swine serves as a natural source for the virus. The spread is usually through the *Ornithodoros moubata* (a tick species) of various hosts. When taking a blood meal, the tick will ingest the virus and then pass it on to the susceptible animals while feeding on them. In the infected pigs the virus is usually found in body fluids and tissues. By close contact with contaminated pigs or by ingestion of garbage containing unprocessed tainted pig meat or pig meat products, pigs normally become infected. Based on clinical signs, ASF may be suspected and confirmation must be made through prescribed laboratory tests, especially to distinguish this disease from Classical Swine Fever (CSF) with respect to OIE, and OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.[3] Historically, ASF was first reported in Kenya in 1921 and considered to be endemic in many African countries. The disease is widespread in sub-Saharan Africa; OIE reported on May 21, 2020, a total of 11 outbreaks in Assam and Arunachal Pradesh states of India, wherein 3701 pigs were died due to ASF.[1] it is currently the only member of the family *Asfarviridae*.[4] ASFV have similar characteristics to other large nuclear DNA viruses such as Poxviridae that is highly endemic in most countries [4]. ASF is also endemic in Sardinia, where it was introduced in the 1970s.[5] In 2007, ASF had emerged in the Caucasus region of Georgia and then extended into the Russian Federation where the disease became endemic.[4-5] By 2014, ASF had spread to several Asian countries, including China, Mongolia, Vietnam and Cambodia, Hong Kong, the Republic of Korea, and Eastern European countries.[5] The origin and evolution of *Asfarviridae* are of special interest as common agricultural pathogens. The diversity of these viruses with 24 genotypes imposes difficulties in the collective evaluation of phylogenetic relationships.[5-6] ASFV replicates in the cytoplasm of infected cells and ASF viral genome is a linear double-stranded DNA molecule with covalently closed ends that are composed of identical terminal inverted repeats. The genome also comprises of a unique sequence, interrupted in some cases by short regions of tandem repeats.[5] usually variations are seen among different ASFV isolates i.e. 170-190kb with 160-234 Open Reading frames (ORFs).[6] Genome size variation is associated with three main regions *viz.* central region (approx. 125-130Kb), highly variable left region (40Kb) and right region (10-15Kb).[6-7] Among at least five multigene families (MGFs) situated at both ends of the genome, several length differences are associated with gene copy loss or gain. In addition, the number of tandem repeat sequences found at loci both within coding regions and in intergenic regions is associated with smaller length variations.[8] The virion protein's outermost shell is an icosahedral capsid, which is mainly assembled by protein p72, that is encoded by the viral gene *B646L*. p72 is the virion's most dominant structural element and comprises roughly ~31 % - 33 % of the virion's total mass, making it one of the main antigens found in infected pigs.[9] The protein structure of ASFV p72 molecule is double jelly roll (JR) fold.[10] The highest insertion is the F1-G1 region, and this region is much longer in ASFV p72 than in Fausto virus major capsid protein (FAUV MCP).[10] Ideally, 24 distinguishable genotypes have been identified by nucleotide sequence analysis of the variable region of the *B646L* gene encoding the main capsid protein p72.[11] No cure or vaccination for ASF has been developed so far. In order to ensure that the countries are disease free, strict import policies should be imposed. The rapid diagnosis, slaughter and disposal of all animals on infected premises, thorough cleaning and disinfection, disinfection, movement controls and surveillance have all involved successful eradication programs.[3] The phylogenetic analysis is used in applied and basic virological research, including epidemiology, diagnostics, forensic studies, phylogeography, evolutionary studies, and virus taxonomy. The analysis provide an evolutionary perspective on the variation in any trait that can be measured for a group of viruses.[5] The aim of the present study is mainly focused on molecular evolutionary analysis using Bayesian Evolutionary Analysis by Sampling Trees (BEAST) of the African swine fever virus that allows identification of the variation level of the p72 (*B646L*) gene sequence. The ability of standard modelling tools to achieve the nucleotide substitution rate and tMRCA for the data downloaded from the GenBank of NCBI in which the isolation dates were assessed and analysed.

2. MATERIALS AND METHODS

2.1 *Sequence Dataset and multiple alignments*

The protein sequence p72 encoded by *B646L* gene in ASFV were retrieved from the National Centre for Biological Information (NCBI) of GenBank public database.[12] Multiple Sequence alignment was performed using MEGA-X.[13-14] It is a sequence alignment tool used for analysis and editing of the sequences.

2.2 *Recombination Detection*

Recombination is a key evolutionary process that shapes the architecture of genomes and the genetic structure of populations. The affirmation of recombination prior to calculating selection pressures was carried out using RDP4 software. Evidence of putative local recombination events more specific to individual viruses was determined using the RDP4 software. Firstly, possible recombination breakpoints were examined using the RDP4 tool.[15] along with the seven recombination detection methods, Rescan/Bootscan, GENECOV, MaxChi, RDP, 3Seq, Siscan, and the Chimaera method to detect the recombination signals.[15-16]

2.3 *Model selection*

The Bayesian coalescent based method has been proposed in this study to delimit the species with multi locus genetic sequence data. This method accounts for species phylogenies and coalescent events in both extant and extinct species accommodating posterior probabilities of different species of delimiting models, lineage sorting and uncertainties in the gene trees. Under these models,the best-fit substitution model for the data was determined by Bayesian Information Criterion (BIC) using the tool jModelTest 2.1.10. [17-18] The best-fit models for the complete gene were selected and used for phylogenetic analysis.[17-98]jModel Test allows

optimization of base trees for every individual model, according to the model selection with BIC the model-averaged phylogenies are obtained.

2.4 *Phylogenetic and Evolutionary Analysis*

Nucleotide substitution rate estimates were determined using BEAST v.1.8.4, and also used for evolutionary analysis of the genes.[15-18] The BEAST software consists of a number of executable programs: beauti, beast, tree annotator, etc. The beauti program was used to set the parameters such as isolation dates in years for tMRCA calculation, DNA site model selection, clocks model, prior tab to insert values obtained from jModelTest, choosing the demographic tree model and finally the number of cycles for MCMC is chosen, which is utilized to obtain a .xml extension file, that is processed in the beast program.[17-20] The beast program utilizes the .xml file, which is processed to result in obtaining two output files (.log and .trees). Nucleotide substitution rates were accomplished using the Markov Chain Monte Carlo (MCMC) method. The basic model for substitution rate among the branches supported by BEAST with molecular model, it is specifying the substitution rate or date of the node.[18-21] The .log file was visualized with the tracer_v1.7.1[18] which contains the mean, median and with 95% Highest Posterior Density (HPD) intervals. The values of the substitution rate are obtained from mean rate/UCLD mean and divergence time tMRCA obtained tree-height/tmrca, each dataset was stimulated with the following options: generations are 100000000 cycles; Burn in: 10%; and ESSs is >200.[18-20] The Bayesian skyline reconstruction plot was also obtained from the analysis of tracer tool. Further, the .trees file was processed with the tree annotator to obtain the .tree file, which was visualized and edited with the Figtree 1.4.4.[15-18] The interpretation of the tree was done, with the nodes as the TMRCA ages.

3 STATISTICAL ANALYSIS

The Bioinformatics analysis were carried out for the 67 sequences of p72 using various bioinformatics tools such as MEGA-X**,** RDP4v.4.100, jModelTest 2.1.10, Beast v1.8.4 and results were obtained.

4 RESULTS

4.1 *Data set and Alignment*

A total of 67 *B646L* of ASFV nucleotide sequences with 1941 bp in length from Africa (13), Asia (11), Europe (40) and Eurasia (3) between 1960 and 2020 were downloaded from NCBI. The sequences with accession numbers, year of isolation and country of origin included in this study are listed in (Table.1 & Fig.1). The sequences were aligned using a clustalw algorithm and edited with MEGA-X tools.

4.2 *Homologous recombination detection*

A full exploratory scan with the seven recombination detection techniques in RDP4v.4.100 was employed on the multiple aligned fasta file to detect the recombinant regions. The RDP4 tool resulted with no recombination regions in *B646L* of ASFV.

4.3 *Model selection*

The best-fit substitution model under Bayesian coalescent models for the sequence data was determined by Bayesian Information Criterion (BIC) with jModelTest 2.1.10 tool that computed the likelihood scores, BIC scores for every single model. HKG+G were chosen to be the best-fitting model according to Bayesian Information Criterion (BIC) for *B646L* of ASF virus. The parameters kappa (10.3816) and alpha (0.2151) estimates were also obtained from this software.

4.4 *Phylogenetic and Evolutionary Analysis*

The evolutionary rate and time of the most recent common ancestor were estimated from the ASFV, *B646L* gene by applying MCMC technique in BEAST. Data with 67 sequences in nexus format was loaded, year of isolation from the sequences were taken as tip dates (1960-2020), HKY+G was chosen in priors, relaxed uncorrelated random clock model in clock rate was chosen, Bayesian skyline model was chosen under tree tab and MCMC chain lengths were changed accordingly and saved in .xml format using the BEAUti tool. The beast tool was used to generate the logarithmic (.log) and (.tree) files for the *B646L* ASF virus gene were obtained from the XML file. To estimate the evolutionary rates and tMRCA, the log file was used and to analyze the trees file, the Tree-Annotator tool was used. Tree file was visualized and modified using Figtree software to create the phylogenetic tree. The color of the branches suits the height of the tree with 95 % of the highest posterior density (HPD) figures, the individual datasets for the demographic model were chosen and with the use of uncorrelated relaxed clock model, these p72 sequences were used to estimate the tMRCA and substitution rate per site per year. Using the Markov chain Monte Carlo (MCMC) method available in BEAST v1.8.4, nucleotide substitution rate approximations were obtained. The tMRCA with 95% Highest Posterior Density (HPD) intervals of the substitution rate and divergence time parameters were calculated from the tree height/tMRCA, Mean clock rate/UCLD mean sample respectively. Each dataset was stimulated with the following options: generations is 100000000 MCMC cycle; Burn in: 10%; and ESSs is >150. The evolutionary rates of *B646L* gene from ASFV is 4.13×10^{-6} with 95 percent HPD range (3.41×10^{-7} to 1.02×10^{-5}). The tMRCA was reported as 4.39×10^{5} years with 95 percent HPD range (2.4×10^{4} to 1.23×10^{6}) in ages for the *B646L* gene of African swine fever virus was estimated (Table.2). The skyline plot is a graphical representation of historical effective population sizes as a function of time. In this analysis, the Bayesian Skyline Plot (BSP) was created for African Swine Fever Virus that shows changes in the median estimate of relative genetic diversity of the virus with time the plot also shows 95% highest probability density intervals and population size of the ASFV from 1960-2020 (Fig.2). The tree file was visualized and edited with Figtree software (Fig.3).

Table.1 List of the accession numbers, year of isolation/ collection and regions of ASFV p72 as obtained from NCBI

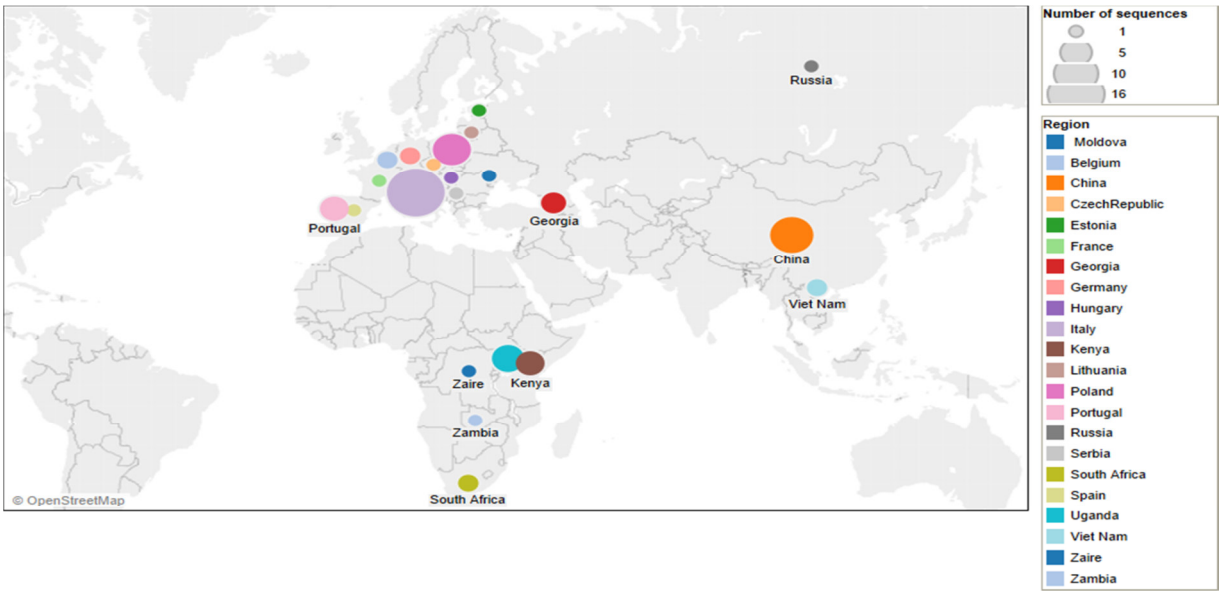
Accession number	Year	Region
NC044946.1	2006	Kenya
NC044945.1	2005	Kenya
MN641877.1	2004	South Africa
MN641876.1	1999	South Africa
MN630494.1	1977	Zaire
MN318203.2	1983	Zambia
MH025920.1	2015	Uganda
MH025919.1	2015	Uganda
MH025918.1	2014	Uganda
MH025917.1	2015	Uganda
MH025916.1	2015	Uganda
KM111295.1	2006	Kenya
KM111294.1	2005	Kenya
MT496893.1	2018	China
MT180393.1	2019	Viet Nam
MT166692.1	2019	Viet Nam
MN393477.1	2019	China
MN393476.1	2019	China
MK333181.1	2018	China
MK333180.1	2018	China
MN172368.1	2019	China
MK940252.1	2019	China
MK645909.1	2018	China
MK128995.1	2018	China
NC044955.1	2008	Italy
NC044947.1	2010	Italy
NC044943.1	1968	Portugal
NC044942.1	1971	Spain
NC044941.1	1960	Portugal
MT847623.1	2019	Poland
MT847622.1	2019	Poland
MT847621.1	2019	Poland
MT847620.1	2019	Poland
MT459800.1	2019	Russia
MT358368.1	2020	Serbia
MN913970.1	2017	France
MN715134.1	2018	Hungary
MN270980.1	2014	Italy
MN270979.1	2012	Italy
MN270978.1	2005	Italy
MN270977.1	2004	Italy
MN270976.1	1997	Italy
MN270975.1	1995	Italy
MN270974.1	1990	Italy
MN270973.1	1985	Italy
MN270972.1	1985	Italy
MN270971.1	1981	Italy
MN270970.1	1979	Italy
MN270969.1	1978	Italy
MK628478.1	2014	Lithuania
MK543947.1	2018	Belgium
MG939588.1	2017	Poland
MG939587.1	2017	Poland
MG939583.1	2017	Poland
LS478113.1	2014	Estonia
LR899193.1	2020	Germany
LR899131.1	2020	Germany
LR722600.1	2017	Czech Republic
LR722599.1	2017	Moldova
LR536725.1	2018	Belgium

KX354450.1	2008	Italy
KM262845.1	1968	Portugal
KM262844.1	1960	Portugal
KM102979.1	2010	Italy
NC044959.2	2007	Georgia
MH910495.1	2008	Georgia
FR682468.2	2007	Georgia

Table.2 Substitution rate and tMRCA of ASFV.

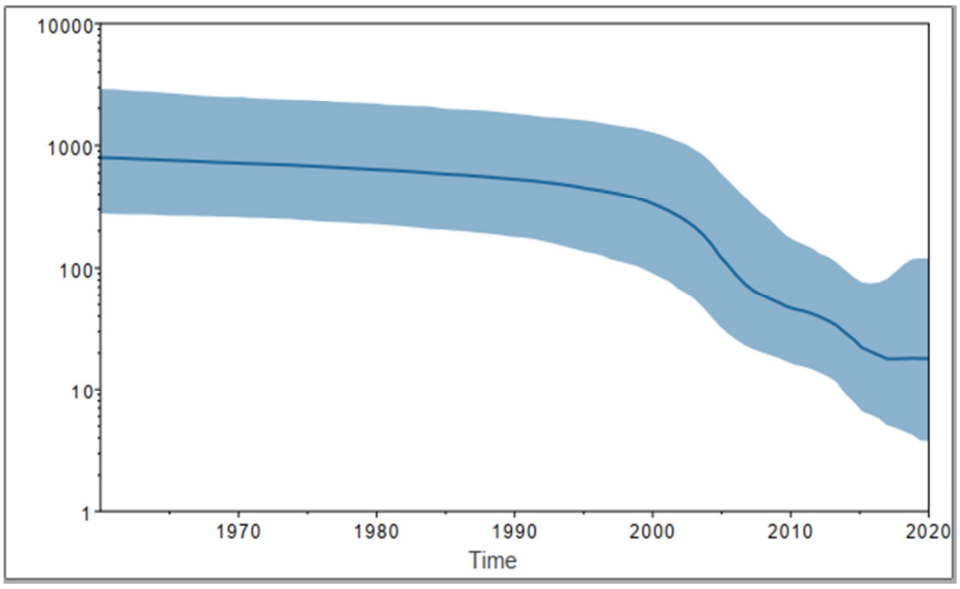
Gene	Host	Substitution rate/site/paper			tMRCA		
		Mean	HPD		Mean	HPD	
B646L (p72)	Sus scrofa (pig)	4.13×10^{-6}	3.41×10^{-7} (L)	1.02×10^{-5} (U)	4.39×10^{5}	2.40×10^{4} (L)	1.02×10^{6} (U)

HPD- 95% Highest posterior density, L- lower HPD, U- upper HPDtMRCA-time of the most recent common ancestor in ages.



Circles indicate locations of ASFV isolates, where their p72 gene segments were sequenced. The circles' size is proportional to the number of isolates.

Fig. 1: Geographical distribution of ASFV sequences isolated in Africa, Europe, Asia and Eurasia between 1960 and 2020 (N = 67).



The posterior median estimate is indicated by the blue line; this corresponds to the 95% HPD. The blue line represents the estimated time at which the population growth transitioned from higher to low rate.

Fig.2: Bayesian Skyline plot for temporal variation in the effective population size of ASF p72-B646L genes between 1960 and 2020.

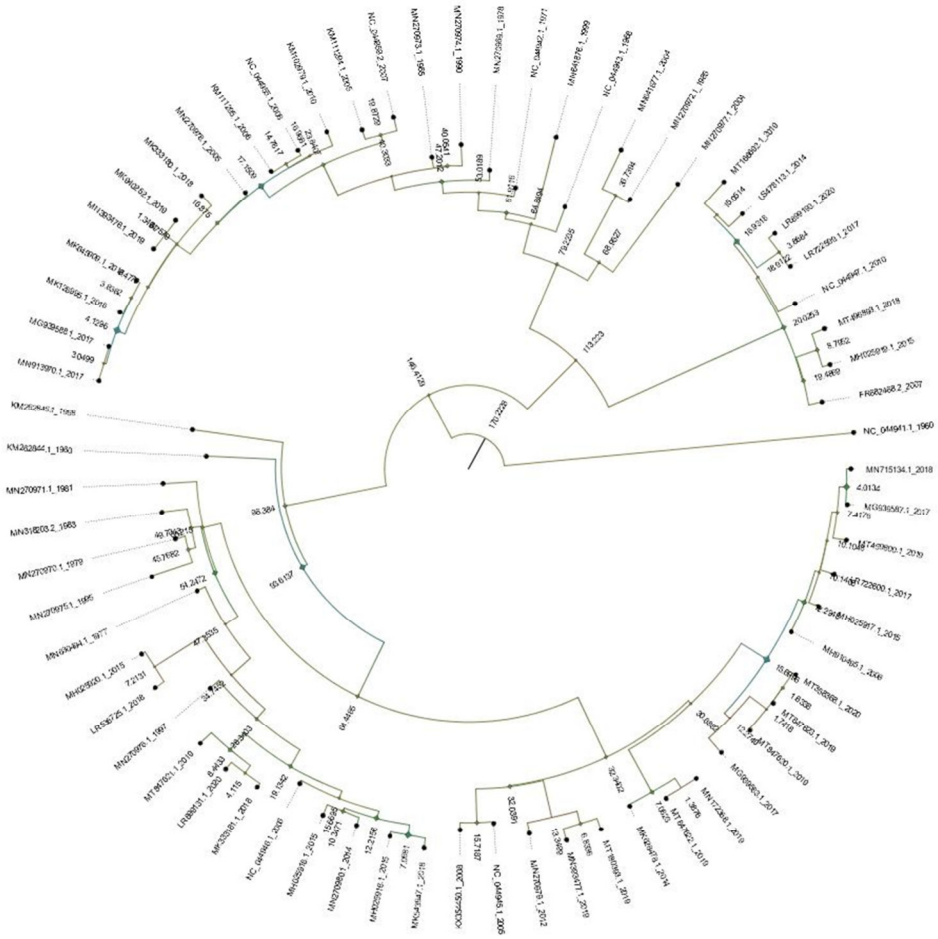


Fig.3: The MCC tree of ASFV p72-*B646L* gene obtained from beast software, showing tMRCA (in ages) at each node.

5 DISCUSSION

ASFV phylogenetic analysis is important for the classification of new viruses and for disclosing a past in evolution. The methods used in this study have helped to differentiate between ASFV isolates that are closely related. Population dynamics of DNA viruses is different from their RNA acquaintances. The virus mainly circulates within the domestic pig-to-pig cycle and in the population of wild boars.[5] The mutation rate among the DNA viruses is significantly lower than for error-prone RNA viruses.[22-23] Recombination is a key evolutionary process that shapes the architecture of genomes and the genetic structure of populations. The recombinant viruses with the increased fitness may spread like a wildfire within the susceptible population and have a great potential for a transboundary transmission among the native populations. Estimating the tMRCA among the population has always been a topic of great interest in population genetics, and there are presently a number of methods that leveraged on different genetic features and are built on a variety of statistical frameworks to perform this estimation, [24] in this regard this study mainly concentrated on using the beast tool to obtain the substitution rate and tMRCA. Furthermore, the *B646L* gene is widely agreed to induce virulence in the host, it is very informative to study the evolutionary analysis of the *B646L* gene ASFV. The analysis was based on the available nucleotide sequences of ASFV that were obtained from various geographical regions of the swine community. In the current study they concentrated on obtaining evolutionary rates and tMRCA of *B646L* gene from ASFV with the Bayesian approach. A total of 62 sequences of ASFV *B646L* genes were downloaded from the GenBank database, the sequences were retrieved based on the year of isolation. The sequences were multiple aligned and edited with MEGA-X software. In this study, there were no significant recombinant regions detected from the isolates of the gene *B646L* of ASFV. Since, ASFV is the only virus of *Asfarviridae* family belonging to the *Asfivirus* genus and till date there was no study based on evolutionary analysis pertaining to *B646L* gene from ASFV, this study mainly concentrated on this gene to analyze, estimate the substitution rate and tMRCA. Though there was a study on ASFV that estimated evolutionary rate of p72 of CVR gene was 3.31×10^{-4} substitutions/site/year only.[25] Comparing with virus from other family of the swine community- classical swine fever virus (CSFV) reported with an evolutionary rate of 3.2×10^{-4} substitutions/site/year.[26] Another study of CSFV has evolved at a rate of CSFV strains of 7.09×10^{-4} substitutions/site/year.[20] However, in current study estimated an evolutionary rate of 4.1368×10^{-6} substitutions/site/year between 1960 and 2020 taken as duration of the year of isolation. Compared with the previous study report , *B646L* gene from ASFV had evolved faster than CVR gene and a moderate evolutionary rate compared with the CSFV. The Bayesian skyline plot showed smooth decrease from 1960-2003 with a small growth rate and it was observed that there is a sudden decrease from 2003 to 2020 approximately also, the ASFV population has decreased compared to the starting of the plot (Fig.2). The substitution rate is mostly determined by various factors such as time of viral generation, method of transmission, mutation frequencies, and the selection pressure [27-28] hence,

eventually it is essential to record the influential contribution of changes in the evolutionary rate observed in ASFV. Also, the analysis revealed that the ASF virus prevailed in Africa and spread to Asia and Europe respectively. Further, the *B646L* gene evolved very fast from 2003 to 2020 revealing that the gene affected the immune system of the ASF virus. The Maximum clade credibility (MCC) tree obtained from the beast tool is a rooted tree that shows transmission cycle resulted in more significant diversification events of ASFV *i.e*., with the larger sub-tree. Node values represent the tMRCA in years, Tip labels are labeled with accession numbers and the variation in the color represents the evolutionary rate. The branches represent among the branch evolutionary rate with mean tMRCA of 4.39 x 10^5 in ages (Fig.3). The projected genetic diversity obviously illustrates the slow exponential growth inferred by ASFV over time. Genes are propagated by error-prone copying, and the resulting variation provides the basis for phylogenetic reconstruction of evolutionary relationships. dsDNA viruses can offer a deeper explanation of evolutionary and selection mechanisms and more precise predictions of various DNA viruses and distinct viral genes for divergence periods and origins. It is also possible that the DNA virus increases virus-host adaptation, existence and ability of fitness in faster spread of the virus to the different hosts and environments. This study mainly concentrates on the *B646L* gene that codes for p72 protein of ASFV to evaluate the evolutionary analysis of the virus. ASFV is regarded as the most epidemiological disease of the swine community with no accepted vaccine till date. Hence, to prevent and control of this virus, continuous surveillance along with various policies are necessary. The findings of this study, which were obtained using the time-calibrated Bayesian method, are consistent with historical, geographical, and epidemiolocal evidence of ASFV emergence. This research provides a framework for classifying the newly discovered ASF virus in future and also be used in the phylogenetic analysis of other viruses. Outcomes of this study were limited data with respect to the *B646L* gene as available, that may be essential for the control of the disease spread, predict and develop a novel vaccine, also expand the knowledge for awareness of epidemics of ASFV and their evolution.

6 CONCLUSION

In conclusion, the evolutionary study of ASFV with p72 protein from the ASFV of the *B646L* genes indicated that they play a vital role in the evolutionary process. The evolutionary rates and tMRCA observed in this study indicate heterogeneity among viral subtypes, as well as the nucleotide substitution rate assessments studied here, show a more composite image that the evolutionary force drives the evolution of the *B646L* gene from African swine fever virus. Moreover, it is remarkable that a single substitution rate does not work similarly, as the evolutionary rates in the former are consistently lower and can play a greater role in the environmental transfer. With the changing and evolving environmental factors, explains how it will help to interpret that the gene is mutated and adapted to the environment. Therefore, the study research was useful to consider the evolution of *B646L* virulence in the pigs. This study also helps other researchers to predict and develop vaccines or drugs which might alter the pathogenic characteristics of the ASFV and control the spread of viruses and disease. If it is possible we can eradicate the disease.

7 AUTHORS CONTRIBUTION STATEMENT

Dr. Kuralayanapalya Puttahonnappa Suresh and Dr. Sharanagouda S Patil Conceptualized and designed the work process; Mr. Prabhakarareddy Anapalli Venkataravana and Ms. Uma Bharathi Indrabalan collected, analyzed and interpreted the data. Mr. Prabhakarareddy Anapalli Venkataravana drafted the manuscript; Dr. Chandan Shivamallu revised and approved the manuscript, all the other authors contributed in editing and revising the manuscript.

8 CONFLICT OF INTEREST

Conflict of interest declared none.

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