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Genetic Characterization of African Swine Fever Virus in Various Outbreaks in Central and Southern Vietnam During 2019–2021

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Abstract

This study aimed to identify potential genetic diversity among African swine fever virus (ASFV) strains circulating in central and southern Vietnam. Thirty ASFV strains were collected from domestic pigs and convalescent pigs with ASFV-infected clinical signs from 19 different provinces of central and southern Vietnam during 2019–2021. A portion of the B646L (p72) gene and the entire E183L (p54), CP204L (p30), and B602L (CVR) genes were amplified, purified, and sequenced. Web-based BLAST and MEGA X software were used for sequence analysis. Analysis of the partial B646L (p72) gene, the full-length E183L (p54) and CP204L (p30) genes, and the central hypervariable region (CVR) of the B602L gene sequence showed that all 30 ASFV isolates belonged to genotype II and were 100% identical to the previously identified strains in Vietnam and China. Analysis of the p72, p54, and p30 regions did not indicate any change in the nucleotide and amino acid sequences among these strains in 3 years of research. No novel variant was found in the CVR within the B602L gene. Analysis of the CVR showed that these ASFV strains belong to subgroup XXXII. The results of this study revealed that these ASFVs shared high similarity with ASFV isolates detected previously in northern Vietnam and China. Taken together, the results of this study and a previous study in Vietnam showed high stability and no genetic diversity in the ASFV genome.

Introduction

African swine fever (ASF) is an infectious disease that is an extremely contagious viral hemorrhagic disease affecting domestic pigs and wild boars, with mortality rates reaching up to 100% [1]. ASFV, which is a notifiable disease as defined by the World Organization for Animal Health (OIE), has entailed severe repercussions for the entire global pig industry, as pigs are important sources of protein. Currently, a first new commercial ASF vaccine was introduced in Vietnam, but the use of this vaccine is still limited. Therefore, the biosecurity measure is essentially prevention, and rapid laboratory diagnosis and elimination of infected pig herds are required [2]. Since the first recognition of ASF in Kenya in the 1920s [3], the disease has been described in various countries around the world but remains endemic in Sardinia and eastern and southern Africa [4, 5]. Recently, countries in Europe and at least 11 countries in Asia have reported numerous ASF outbreaks [6]. In particular, in 2018, an outbreak of ASF was reported in China [7, 8] and then shortly after in Vietnam [9], which had a huge socioeconomic impact on the pork industry [10].

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The causative agent is a DNA virus that is presently the sole member of the Asfivirus genus, belonging to the family Asfarviridae [11]. ASFV is a large icosahedral virus with a double-stranded DNA genome composed of 170–193 kbp with 150–167 open reading frames depending on the strain [12]. The replication of ASFV has similarities with Poxvirus and Iridovirus, as all of them are DNA viruses that replicate in the cytoplasm [13]. The ASFV particle possesses a complex multilayered structure approximately 200 nm in diameter [14]. Twenty-four ASFV genotypes have been identified and designated I to XXIV [15, 16].

The key to understanding ASFV diversity, including its viral biology and evolution, is to dissect ASFV genetic and genomic variations [17]. Several specific genetic targets have been used to assess ASFV genetic diversity; however, three major and dominant gene fragments, the B646L gene-encoded capsid protein 72 (p72), the E183L gene-encoded envelope protein 54 (p54), and the CP204L gene-encoded phosphoprotein protein 30 (p30), are commonly used [18, 19]. Unfortunately, B646L (p72) genotyping analysis may not provide adequate typing resolution or the ability to discriminate between viruses of different biological phenotypes [20]. Intensifying genotypic resolution and genetic characterization has been achieved by the additional assessment of E183L (p54), CP204L (p30), and B602L (CVR) [19, 21–23]. The B602L gene (CVR) of ASFV is a hypervariable genetic marker that has been demonstrated to be useful for the high-resolution discrimination of ASFV. To achieve finer discrimination between the viruses, the variation and distribution of these amino acid repeats have been investigated using the primers CVR FL1/CVR FL2 [22].

In Vietnam, previous studies based on sequencing of the C-terminal region of the B64 6L (p72) gene have been reported [9, 24, 25] with samples collected mainly in North Vietnam at the beginning of the epidemic. Since the end of 2019, the disease has appeared in 63/63 provinces and cities and is a very widespread and complex epidemic in southern Vietnam. Therefore, a broader study of the genotypic characteristics of African swine fever virus in various outbreaks in central and southern Vietnam during 2019–2021 is essential to answer the question of the origin and diversity of the virus, in addition to providing the molecular epidemiology of the virus and a strategy for disease control.

Materials and Methods

Study Design and Samples

A total of 148 samples including bloods of sick pigs with typical clinical manifestations and tissues of pigs recovering from ASF were collected from 30 pig farms located in 19 provinces where outbreaks were reported from 2019 to 2021 (Fig. 1). The sick pigs showed signs of high fever, collapse, cyanosis and skin hemorrhage, and a high mortality. Meanwhile, 10 recovered pigs that continue to be kept until slaughter in four farms were collected tissues (lung, liver, spleen, kidney, and lymph nodes) at the slaughterhouse. A total of 30/118 ASFV strains were randomly selected from farms (one per farm), and 1–3 strains from each province were used for sequencing and genotypic analysis (Table S1). In addition, all previously published Vietnamese AFSV strains during this period were also included for the comprehensive analysis.

Extraction of Genomic DNA

Total DNA was extracted from each blood sample using the commercial Promega™ Wizard™ SV Genomic DNA Purification System (Promega, USA) according to the manufacturer's instructions.

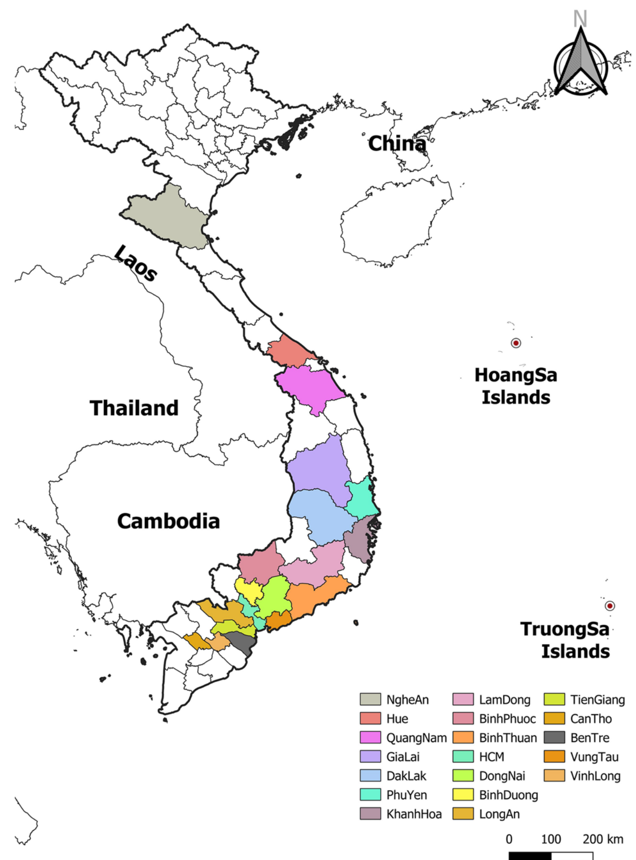

Fig. 1 Map showing location of ASF outbreaks in Vietnam where field AFSV strains were obtained and characterized

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Primers

Routine PCR was used to confirm ASF positivity, as recommended by the Office International des Epizooties (OIE, Paris, France) [26]. Then, primer pairs from previous studies were used to amplify the variable 3′-end of the B646L gene (p72), the entire E183L gene (p54), the CP204L gene (p30), and the B602L gene (CVR) for sequencing, as shown in Table S2.

PCR Amplification of the Viral DNA

The genes encoding the p72, p54, p30, and CVR proteins were amplified in a 25-µl reaction volume containing 12-µl MyTaqTM HS Mix (Bioline, USA), 5-µl DNA, 1 µl of each 10-µM primer, and 6-µl DNA-free water. A positive control (DNA) from Vietnamese AFSVs that were isolated and sequenced in another study (unpublished) was used.

The thermocycling conditions for PCR detection of ASFV included a 10-min initial denaturation step of 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 50 °C, and 30 s at 72 °C, with a 7-min elongation step at 72 °C [26]. Afterward, the PCR products were mixed with GelRed nucleic acid stain (Merck, Germany) and electrophoresed in a 1% agarose gel (Invitrogen, Thermo Fisher Scientific, USA) using a 1-Kb Plus Ladder (Invitrogen, Thermo Fisher Scientific, USA) to indicate the sizes of the amplification products.

Genetic Sequencing and Analysis

PCR products were purified using the TopPURE® PCR/Gel DNA Purification Kit (ABT Ltd.co, Vietnam) based on the manufacturer's recommendations. After purification, the products were sent to a sequencing laboratory (Macrogen, Korea). The raw (ABI) sequences were viewed and edited by Chromas version 2.6.6 to remove any ambiguous sequences and poor reads. A BLAST alignment was performed using our sequence in NCBI, and we then selected the homologous gene sequences for phylogenetic analysis. We tried to select reference sequences from different regions, years of publication, and publication authors. The nucleotide sequences of the B646L, E183L, CP204L, and B602L genes of the field ASFV strains were aligned to build a phylogenetic tree to compare with previously published Vietnamese ASFV strains and with other published global sequences available in GenBank (NCBI) (Table S3), including the vaccine strain ASF-G-Δl177L that was commercialized in Vietnam and some vaccine candidate strains.

Nucleotide/amino acid alignment was conducted using the CLUSTALW software program [27], while constructing and analyzing phylogenetic trees based on the p-distance model of the neighbor-joining method in MEGA X software [28]. Using this method, the branch lengths and the topology of a parsimonious tree can quickly be obtained. The principle of the neighbor-joining method is to find pairs of operational taxonomic units (OTUs) which minimize the total branch length at each stage of clustering of OTUs starting with a star-like tree [29]. In addition, to determine the degree of statistical support for each node in the resulting trees, the data were replicated 1000 times using the bootstrap method. The nucleotide sequences of the B646L (p72), CP204L (p30), E183L (p54), and B602L (CVR) genes from this study were deposited in GenBank under the accession numbers MW828850-MW828879, MW828820-MW828849, MW828760-MW828789, and MW828790-MW828819, respectively (Table S2).

Results

Molecular Characteristics

Based on sequence alignments of the B646L, E183L, CP204L, and B602L genes, the ASFV strains in this study shared 100% similarity at the nucleotide (nu) and amino acid (aa) levels, and the similarity with previous Vietnamese strains was 100%. The field ASFV strains had a high similarity (100%) at the nu and aa levels with strains belonging to genotype II, such as Chinese strains (Pig/HLJ/2018 and Wuhan/2019) and European and Eurasian strains (Belgium/Etalle/wb/2018, ASFV/LT14/1490, Pol18/28298/o111, and Georgia_2007/1). However, the field ASFV strains had differences at the nu and aa levels with genotype I strains in genes of B646L (0.84–1.05% and 0–0.63%, respectively), E183L (2.18–8.65% and 1.64–12.44%, respectively), CP204L (2.32–2.50% and 1.61–2.68%, respectively), and B602L (3.45–10.62% and 3.97–12.7%, respectively).

In addition, the number of aa tandem repeats in the B602L gene region is a highly variable genetic marker, which was used to differentiate closely related p72 genotypes. The B602L gene of 30 ASFV strains in this study was successfully amplified and sequenced, and the results showed nu sequences of 400 bp, which corresponded in size and aa sequence to other genotype II strains with 10 aa tetramers, except for ZAM/14/Chipata (LC174766) with 14 aa tetramers. Interestingly, the field ASFV strains from this study belonged to the CVR subgroup XXXII based on the length and sequence of the aa-tetramer repeats (Table 1). However, the quantity of aa tetramers of the CVR sequence differed from low to high among the field strains and the reference strains belonging to various genotypes, as presented in Table 1. ASFV strains with 10 aa-tetramer repeats have been described from previous outbreaks in Vietnam, China, Belgium, and Georgia, indicating the close relationship between these viruses. The genetic variation within genotype

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Table 1 The aa-tetramer sequence within the central variable region (CVR) of gene B602L within ASFV strains

Strains virus	Accession no	Isolates	P72 Genotype	Amino acid (aa) sequence of CVR fragment		CVR subgroup
				aa-tetramer sequence	Quantity	
VN/NA/2019-ASF[a]	–	Viet Nam	II	BNDBNDBNAA[b]	10	XXXII
Hanoi/2019	MT166692	Viet Nam	II	BNDBNDBNAA	10	XXXII
NgheAn/2019	MT180393	Viet Nam	II	BNDBNDBNAA	10	XXXII
Wuhan/2019	MN393476	China	II	BNDBNDBNAA	10	XXXII
Pig/HLJ/2018	MK333180	China	II	BNDBNDBNAA	10	XXXII
Pol18/28298/o111	MT847621	Poland	II	BNDBNDBNAA	10	XXXII
Georgia_2007/1	NC044959	Georgia	II	BNDBNDBNAA	10	XXXII
Belgium/Etalle/wb/2018	MK543947	Belgium	II	BNDBNDBNAA	10	XXXII
ASFV/LT14/1490	MK628478	Lithuania	II	BNDBNDBNAA	10	XXXII
ZAM/14/Chipata	LC174766	Zambia	II	BNABNDBTDBNAAG	14	NK
OURT_88/3	AM712240	Portugal	I	ABNAAAAAAAAAAAAACCBNAB-NAAAAACBNABNABNABTDBNAFA	46	NK
Benin97/1	AM712239	Benin	I	ABNAAAACBNAAAAACBNAAAAAC BNAAAACBNAFA	36	XIX
MUZUKI/1979	AY261362	South Africa	I	BVWAFNBNAAAF	12	NK
Warthog	AY261366	Namibia	IV	BNABUBMABUBMAAA	15	NK
Ken05/Tk1	NC044945	Kenya	X	AABNAABBA	9	XXVa
Ken06Bus	KM111295	Kenya	IX	AAABNABBNABBAABNABNABA	22	XXIV
ZAM/13/Kalomo	LC174767	Zambia	XIV	BNWNBVF	7	NK
R8	MH025916	Uganda	IX	AAAABNABBNABBAABBNABNABA	24	NK
RSA_2/2008	MN336500	South Africa	XXII	BVWVVNABNAABAGFFABAG	20	NK
Liv13/33	MN913970	France	I	BNADBNAFTBTDBNAF	16	NK
RSA/W1/1999	MN641876	South Africa	IV	BNABUBNABUBBMAAA	17	NK

[a]The ASFV strain was representative in this study as all field strains had identical nucleotide sequences

[b]Analysis of CVR amino acid sequences reveals tetrameric repeats that have been assigned single letter codes corresponding to each type. Tetramic amino acid repeats that have been previously characterized in ASFV isolates include repeat code A = CAST/CVST/CTST/CASI, B = CADT/CTDT, C = GAST/GANT, D = CASM, F = CANT, G = CTNT, J = GTDT, K = CTSP, L = YTNT, M = NEDT, N = NVDT/NVGT, O = NANI/NADI/NASI, H = RAST, S = SAST, T = NVNT, U = NIDT/NTDT, V = NAST/NADT/NANT/NAVT, W = SADT/SVDT, and X = NTDI [19, 22, 44]

NK Not known

II is barely detectable, even though the viruses in this study were sampled over 3 years.

Phylogenetic Trees

All ASFV strains in this study and the reference strains were used to build a phylogenetic tree based on the nu sequences of B646L (p72), E183L (p54), and CP204L (p30) (Fig. 2). All ASFV strains of this study were demonstrated to belong to genotype II, together with the previous strains reported from Vietnam (Hanoi/2019-MT166692 and NgheAn/2019-MT180393) and the first strains reported in China (Pig/HLJ/2018-MK333180 and Wuhan/2019-MN393476) and in particular had a molecular relationship with strains considered to be the source of recent ASF outbreaks globally (Belgium/Etalle/wb/2018-MK543947, ASFV/LT14/1490-MK628478, Pol18/28298/o111-MT847621, and Georgia_2007/1-NC044959). With absolute similarity in the gene segments of B646L (p72), E183L (p54), CP204L (p30), and B602L (CVR), the ASFV strains of this study were monophyletic, which showed they may have the same origin.

Genetic Similarity of the Field ASFV with Vaccine Strains

The ASFV strains of this study shared a high similarity at the nu and aa levels with vaccine candidate strains belonging to genotype II, such as Pig/HLJ/18 and Georgia_2007/1, but a lower similarity with those such as Benin 97/1 and OURT_88/3, which belong to genotype I (Table 2). In B602L (CVR), the Pig/HLJ/18 and Georgia_2007/1 strains were similar to the Vietnamese field ASFV strains with 10 aa tetramers, while the OURT_88/3 and Benin 97/1 strains had 46 and 36 aa tetramers, respectively.

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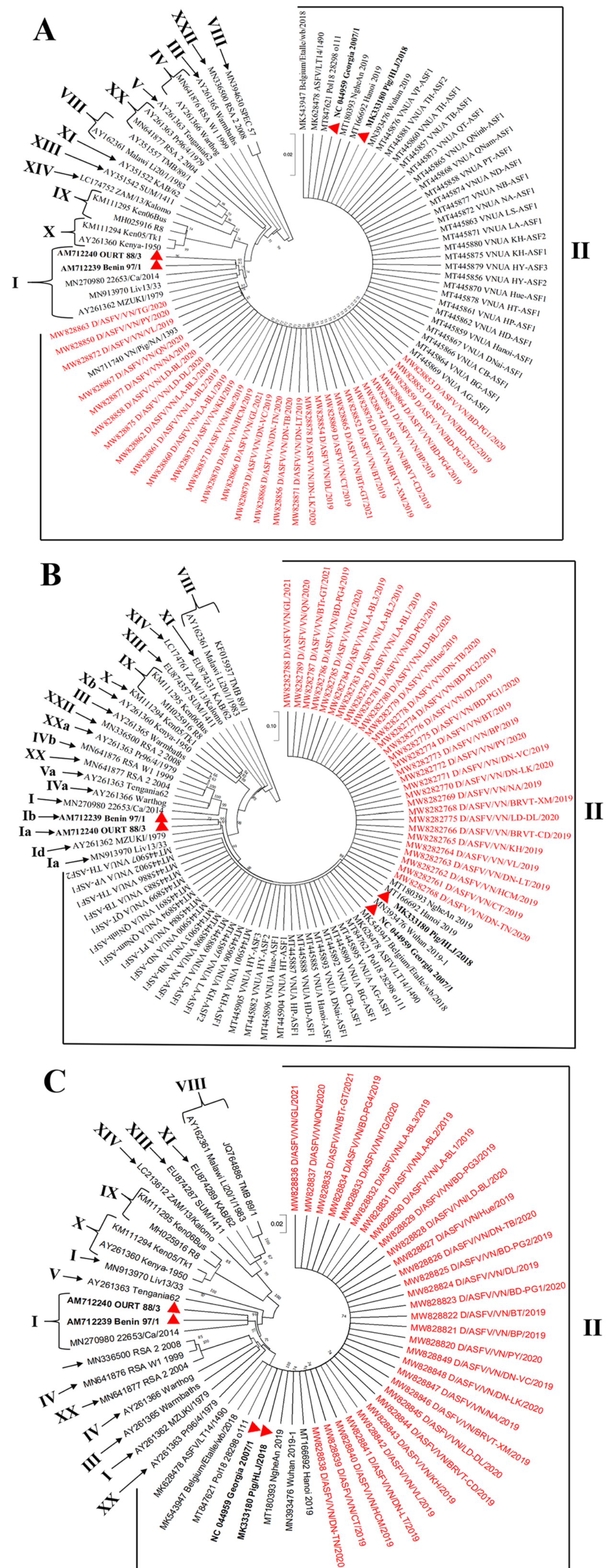

Fig. 2 The phylogenetic trees based on nucleotide sequences of genes encoding p72 **A**, p54 **B**, and p30 **C** of 30 field ASFV strains. The Neighbor-Joining method was used for construction of phylogenetic trees in MEGA X software. Numbers along branches indicate bootstrap values > 50% (1000 replicates). The red letters indicated ASFV strain in this study. The red triangles are the ASFV vaccine strains deposited in GenBank. Scale bars indicate nucleotide substitutions per site (Color figure online)

Discussion

African swine fever remains the major constraint on pig production in Vietnam, with outbreaks occurring throughout the year. There is a need for continuous characterization of ASFV strains during disease outbreaks to further understand ASF outbreak patterns and to map viral genotypes to the geographical regions within which they circulate. Continuous genotyping is important for giving insight into the virus origin, the epidemiological scenario, and the early detection of variants and/or novel strains. Furthermore, knowledge of the molecular epidemiology of ASFV strains is necessary to develop effective prevention and control strategies at both local and regional levels. Thus far, through this study, it can be seen that AFSV strains in Vietnam have the same origin and maintain stable genetic characteristics. In addition, this study provides a comparison of genetic homology among Vietnamese strains and global vaccine candidate strains and the ASF-G-Δl177L vaccine isolate that lacks the I177L gene compared to the Georgia 2007/1 strain [30]. The absolute genetic similarity on four independent gene sequences with the vaccine strains shows great promise for the protective efficacy of these vaccines against the field ASFV strains.

In this study, molecular diagnosis methods were used to confirm and characterize ASFV strains. The virus strains of this study were not only from pigs with characteristic clinical signs and from deaths reported from outbreaks in several farms and provinces but also from previous research in Vietnam and thus the study strains were highly representative of strains in Vietnam from 2019 to 2021. Phylogenetic analysis based on the nu sequences of the C terminus of the three dominant and major gene regions (B646L, E183L, CP204L) showed that all 30 field Vietnamese ASFV strains belonged to genotype II, with absolute homology. Although CP204L (p30) is considered as the gene fragment for differentiating among closely related viruses [31], the findings of this study showed that several ASFV strains obtained from many different epidemic areas in Vietnam showed a remarkable genetic stability and could not be distinguished from closely related strains (such as those from China and Georgia) [9]. In a previous study, 122 ASFV strains mainly collected from the North of Vietnam and they all belonged to p72 genotype II, p54 genotype II, CD2v serogroup 8, and CVR gene variant type I [32]. Besides, all field strains of our study came

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Table 2 Nucleotide (nu) and amino acid (aa) similarity (%) among Vietnamese ASFV strains compared with candidate vaccine strains

Vaccine strains		B646L/p72		E183L/p54		CP204L/p30		B602L/CVR	
Genotypes	Isolates	nu	aa	nu	aa	nu	aa	nu	aa
II	Pig/HLJ/18	100	100	100	100	100	100	100	100
	Georgia_2007/1	100	100	100	100	100	100	100	100
I	Benin 97/1	99.16	100	97.46	97.28	97.68	97.86	93.89	91.26
	OURT_88/3	99.16	100	97.46	97.28	97.5%	97.32	96.02	95.23

from the central and southern Vietnam and belonged to p72 genotype II and p54 genotype II. Our findings incorporated with the previous study provides a big picture about the Genetic Characterization of African swine fever virus in Vietnam.

The CVR is capable of providing further resolution than any of the genes encoding p72, p54, or p30 [33]. Fragment size analysis has identified B602L as the most variable genome region [19]. The variable region of B602L contains aa tetramers that vary in number and type. Sequence analysis of the B602L gene from the field ASFV strains identified five different aa-tetramer sequences encoded in this genome region. One of these tetramer sequences was CTST, which is one of the less common tetramer sequences [19]. With 10 aa-tetramer repeats in CVR (BNDBNDBNAA), the ASFV strains in this study were classified into subgroup XXXII, along with the ASFV isolates in circulation in Vietnam (2019), the Chinese ASFV strains from outbreaks in 2018–2019, and other strains in Europe. At the same time, a study in India that performed B602L variable region analysis also showed that the tetrameric aa repeats in CVR are similar to other post-2007-p72-genotype II viruses reported from other Asian and European countries [34]. Although analysis of the CVR has identified it as the most variable genome region, Estonia isolates, belonging to the same genotype II classification based on the p72 genes and showing no difference in size and aa tetramers in the CVR genes, had the aa change of Y instead of C compared with the reference strain (Georgia_2007/1) [35]. However, field strains in this study revealed high conservation and no variation over 3 years.

Therefore, the virus causing disease in pig production in the southern areas is highly derived from the viruses first reported in North Vietnam; however, we do not exclude the theory that the origin of the virus spreading from China followed an illegal route as a result of live pig transportation and pork products due to the sell-off of pigs when the disease was widespread in epidemic areas [10].

There are a few concerns, but the genetic analysis of the four independent regions of ASFV sequences in this study and previous reports in Vietnam showed homogeneity and maintenance of the Vietnamese ASFV population [9, 25]. In addition, one study reported a finding that conflicts with this study, and other previous studies have reported that the gene sequence encoding p72 from an ASFV strain (VN/Pig/NA/1393; MN711740) showed point mutations in its sequence [36].

In general, the mutation rate of DNA viruses is much lower than that of RNA viruses and ranges between 10^{-8} and 10^{-6} substitutions per nucleotide per cell infection [37]. However, unlike other nucleocytoplasmic large DNA viruses, ASFV has DNA proofreading repair enzymes (X-type polymerase and a DNA ligase) with low fidelity [38]. Therefore, the genetic diversity of field ASFV strains is postulated to arise from the activity of these enzymes. A previous study showed that African ASFV strains had a tremendous variety of ASFV variants in p72 and CVR gene sequences for 55 years. The mean nucleotide substitution rate of these African strains was around 3.31×10^{-4}/site/year [39]. ASFV can use multiple gene-related mechanisms to adapt, replicate, and persist under the changes of different host and ecological environments (domestic pigs, warthogs, and ticks) [40]. This indicated that ASFV may accumulate genetic mutations in order to spread and transmit within different host populations [38]. Many variations in ASFV genome have also been reported in Europe, where there is complex transmission between wild boars and domestic pigs [41, 42]. The genetic variations between ASFV strains mainly occurred in intergenic and terminal regions of each viral genome, which may affect the transcription of the related genes [43]. Currently, some different variants of ASFV were identified on sequences between I73R and I329L genes in domestic pig northern Vietnam [25, 32]. This indicates the continued introduction of Chinese ASFV strains through the illegal trade. However, these variants of ASFV could also be traced back to attenuated vaccine trials, which was reported in China [38].

Our results in this study can be an important basis to show the stability and genotype maintenance on four independent genes of the field ASFV strains over a short period of time. Interestingly, some of the field strains collected from the same outbreaks in the subacute to chronic phase in surviving pigs also showed no difference at the nu and aa levels (unreported results). Three years after the first time ASFV entered the Vietnamese pig herds, the epidemiological characteristics changed considerably, and the circulation of the virus was scattered everywhere. There were several outbreaks with mild clinical signs and lower mortality; therefore, it is very important to continue the surveillance of ASFV at the

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genetic level at wider and deeper scales in various groups of pig populations with different clinical manifestations. Until then, the results of this study provide valuable genotypic characteristics of the current ASFVs circulating in Vietnam during 2019–2021, which can be useful for ASF control programs in Vietnam and neighboring countries.

Conclusion

This study confirmed that the ASFVs circulating in central and southern Vietnam from 2019 to 2021 belong to genotype II, have complete genetic homology, and are derived from the same origin. The sequencing and analysis of the B646L(p72), E183L(p54), CP204L(p30), and B602L(CVR) genes over the years 2019, 2020, and 2021 showed the stability of this virus.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00284-022-03033-x.

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Author Contributions Co-first authors: NMN and TTNN. Conceived of or designed study: DTD, NMN, and TT. Nguyen. Performed research: NMN, DMTN, TTNN, HNN, TTPN, and DTD. Contributed new methods or models: DTD, NMN, and TTNN, HNN. Wrote the paper: TTNN, NMN, and DTD. Revise manuscript and response to comments of reviewers: DCL, NMN, and DTD.

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Data Availability The nucleotide sequences of the B646L (p72), CP204L (p30), E183L (p54), and B602L (CVR) genes from this study were deposited in GenBank under the accession numbers MW828850-MW828879, MW828820-MW828849, MW828760-MW828789 and MW828790-MW828819, respectively.

Code Availability Not applicable.

Declarations

Competing interest The authors declare that they have no competing interest.

Ethical Approval and Consent to Participate Permission to collect the sample and conduct the lab analysis and study were conducted in compliance with the institutional rules for the care and use of laboratory animals and using protocol approved by Ministry of Agriculture and Rural Development (MARD) Vietnam (TCVN 8402:2010) and under the acceptance and approval by Department of Animal Health, Vietnam (DAH) and the Ministry of Education and Training, Vietnam (MOET): 2867-QĐ BGDĐT, dated 01/10/2020.

Consent for Publication Not applicable.

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