

The central region of the B646L gene supports African Swine Fever Virus Genotype II identification

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ABSTRACT

The B646L gene encoding the major capsid protein p72 is the standard genetic marker for African swine fever virus (ASFV) genotyping, with classification conventionally based on sequencing the C-terminal region. In contrast, the central region is routinely amplified during diagnostic PCR but has not been systematically evaluated for genotype assignment. Here, we assessed the genotyping utility of the central B646L region using laboratory-generated ASFV-positive sequences together with representative complete ASFV genomes. Phylogenetic analyses of either the complete B646L coding sequence (CDS), central region, and C-terminal region consistently assigned the laboratory-generated sequences to genotype II. Comparative sequence analyses further identified seven parsimony-informative sites within the central region that distinguished genotype II from its closest neighboring genotype, despite the fragment containing fewer informative sites than the complete CDS and the conventional C-terminal region. These findings demonstrate that the routinely amplified central B646L fragment retains sufficient sequence variation for ASFV genotype II identification and represents a practical source of molecular information that can complement routine molecular surveillance.

KEYWORDS: African swine fever virus; B646L; genotype identification; p72; phylogenetic analysis

I. Introduction

African swine fever (ASF) is a highly contagious hemorrhagic disease of domestic and wild pigs caused by African swine fever virus (ASFV), the sole member of the family *Asfarviridae* (Alonso et al., 2018). There are 24 known genotypes of this virus (Bastos et al., 2003). Among them, the rapid and widespread distribution of ASFV genotype II across Europe and Asia, caused substantial economic losses to the swine industry (Dixon et al., 2020; Mighell & Ward, 2021). Molecular characterization has therefore become an important component of ASF surveillance and outbreak investigations by supporting genotype identification and molecular epidemiological analyses.

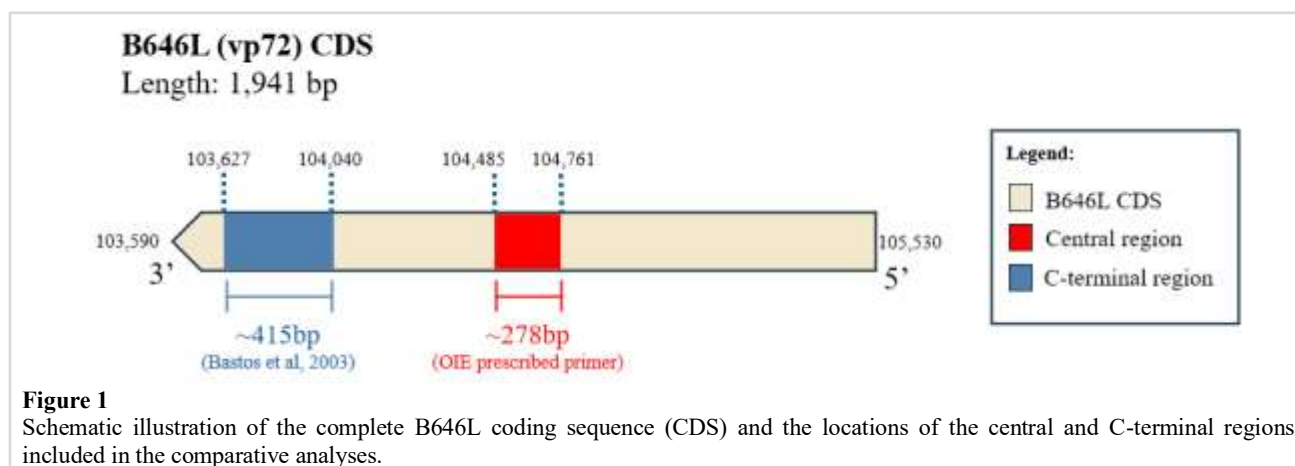
The B646L gene, which encodes the major capsid protein p72, is the most widely used genetic marker for ASFV genotype classification. The current p72 genotyping system is based on a partial C-terminal fragment of B646L described by Bastos et al. (2003), which is the frequently used marker for genotype assignment. Another region of the same gene, located at the central region of B646L, described herein as central region, is amplified by

the conventional diagnostic PCR assay described by Wilkinson (2000) and subsequently prescribed by the World Organization for Animal Health (WOAH) for routine diagnostic use (Wilkinson, 2000; Aguero et al., 2003). Consequently, different regions of B646L have become established for different applications in ASFV molecular investigations.

Although whole-genome sequencing and SNP analyses provide high-resolution genetic information for ASFV characterization (Chernyshev et al., 2024), partial sequencing of the B646L gene remains widely used in routine diagnostic laboratories, especially in settings with limited access to whole-genome sequencing. Consequently, the genotyping potential of the routinely sequenced central region remains to be established.

In brief, we evaluated the central region of the B646L gene using laboratory-generated sequences together with representative complete ASFV genome sequences retrieved from GenBank. Complete B646L coding sequences were extracted from annotated genomes, and the corresponding central and C-terminal regions were compared for sequence variation and genotype discrimination. This study provides new insight into the potential application of the central region of B646L for ASFV genotyping.

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II. Materials and Methods

Sequence datasets

Two sequence datasets were analyzed in this study. The first comprised laboratory-generated partial B646L gene sequences obtained from African swine fever virus (ASFV)-positive pork samples processed at the Animal Disease Diagnostics Research Facility (ADDRF), Pampanga State Agricultural University. These sequences were generated using the conventional PCR assay of Wilkinson et al. (2000). Briefly, PCR amplification was performed using the primer pair ASFV_Primer1 (5'-ATGGAT ACCGAG GGAATAGC-3') and ASFV_Primer2 (5'-CTTAC CGATGAAAATGATAC-3'), generating a 278-bp fragment from the central region of the B646L gene. PCR products were sequenced bidirectionally by Macrogen Inc. (Seoul, South Korea).

The second dataset comprised representative complete ASFV genome sequences retrieved from the National Center for Biotechnology Information (NCBI) GenBank database. Complete B646L coding sequences (CDS) were extracted from annotated genome records for comparative analyses.

Sequence assembly and similarity analysis

Forward and reverse chromatograms of the laboratory-generated sequences were manually inspected and assembled into consensus sequences using Geneious Prime 2025.1.3. Consensus sequences were examined using ORFfinder (NCBI) to verify that the amplified fragment corresponded to the B646L gene encoding the major capsid protein p72. Sequence similarity was assessed using BLASTn against the GenBank nucleotide database (Altschul et al., 1990).

Preparation of B646L sequence datasets

Representative complete ASFV genome sequences were retrieved from the National Center for Biotechnology Information (NCBI) GenBank

database. Sequence selection included representative genotypes described by Quembo et al. (2018), Qu et al. (2022), representative isolates recognized by the International Committee on Taxonomy of Viruses (ICTV), and complete genome sequences of ASFV genotype II from the Philippines. Complete B646L coding sequences were extracted from annotated genome records. For genomes lacking B646L annotations, the coding sequence was identified by pairwise sequence alignment with the ASFV Georgia 2007/1 reference genome (GenBank accession FR682468). The central and C-terminal regions were subsequently extracted from each B646L coding sequence for comparative analyses.

Sequence variation and phylogenetic analyses

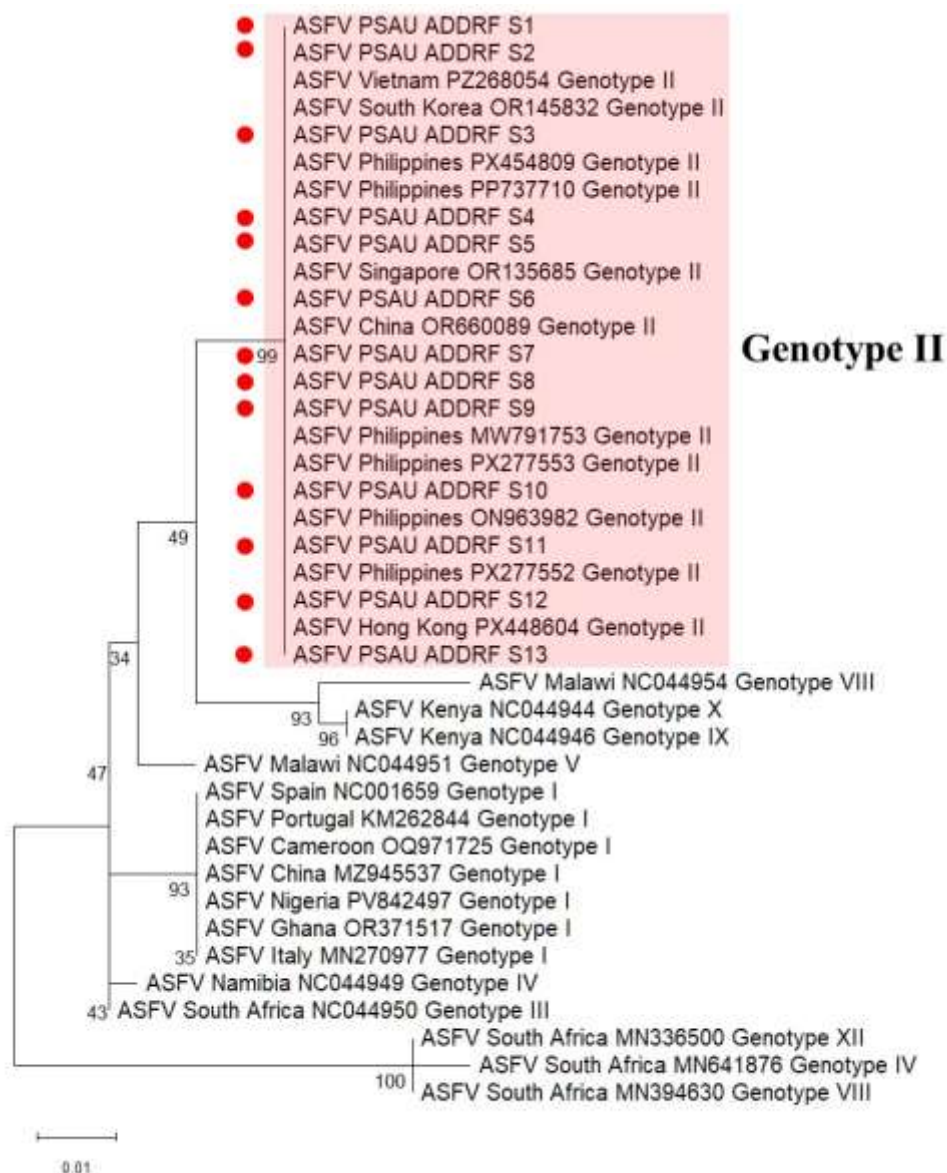
Independent multiple sequence alignments were generated for the laboratory-generated sequences, complete B646L coding sequences (CDS), and the central and C-terminal regions using Clustal Omega (Sievers et al., 2011) implemented in Geneious Prime.

The numbers of conserved, variable, and parsimony-informative sites were determined using MEGA version 12 (Tamura et al., 2021). Maximum-likelihood phylogenetic trees were reconstructed using the Tamura 3-parameter model with gamma-distributed rate variation (T92+G) for the complete B646L CDS and C-terminal region, and the Jukes–Cantor model with gamma-distributed rate variation (JC+G) for the central region. Branch support was assessed using 1,000 bootstrap replicates.

Laboratory-generated sequences were assigned to ASFV genotypes based on their phylogenetic clustering with representative reference sequences. Phylogenetic trees derived from the complete B646L CDS, central region, and C-terminal region were compared to evaluate the consistency of genotype assignment.

Table 1. Selected BLASTn matches of partial central region of B646L gene sequences generated in this study.

No.	BLAST Hit Accession No.	Country	Percent Identity (%)	Query Coverage (%)	E-value
1	PX277556	Philippines	100	94	0
2	PX277553	Philippines	100	94	0
3	PX454812	Philippines	100	94	0
4	PX454813	Philippines	100	94	0
5	PX119974	Thailand	100	94	0
6	PX119975	Thailand	100	94	0
7	PV592818	Vietnam	100	94	0
8	PV592817	Vietnam	100	94	0
9	PV592816	Vietnam	100	94	0
10	PV400257	China	100	94	0
11	PV400256	China	100	94	0
12	PV400255	China	100	94	0
13	PV400254	China	100	94	0

**Figure 2**

Maximum-likelihood phylogeny of ASFV inferred from partial central region of B646L (p72) gene sequences. All ASFV-positive samples from Central Luzon clustered within Genotype II. Node labels indicate bootstrap support values (1,000 replicates). Genotype II (red) is highlighted, and red circles denote sequences generated in this study

Table 2. Parsimony-informative sites within the partial central region of B646L (p72) between ASFV Genotype I and Genotype II isolates.

Representative Isolates	Parsimony-informative sites						
PSAU_Accession_GII	T	A	A	G	C	A	A
PSAU_Accession_GII	T	A	A	G	C	A	A
PSAU_Accession_GII	T	A	A	G	C	A	A
Philippines_MW791758_GII	T	A	A	G	C	A	A
Philippines_ON963982_GII	T	A	A	G	C	A	A
Philippines_PP737710_GII	T	A	A	G	C	A	A
Cameroon_OQ971725_GI	C	G	G	A	T	G	G
Ghana_OR371517_GI	C	G	G	A	T	G	G
Italy_MN270977_GI	C	G	G	A	T	G	G
Nigeria_PV842497_GI	C	G	G	A	T	G	G
Portugal_KM262844_GI	C	G	G	A	T	G	G
Spain_NC001659_GI	C	G	G	A	T	G	G
Position in the ASFV genome	104,490	104,517	104,534	104,637	104,697	104,706	104,727

III. Results

B646L regions evaluated

Figure 1 shows a schematic illustration for the three B646L regions evaluated in this study. Comparative analyses were performed using the complete B646L coding sequence (1,941 bp), the 278-bp central region amplified by the conventional diagnostic PCR assay (Wilkinson et al., 2000), and the 415-bp C-terminal region used for p72 genotyping (Bastos et al., 2003). These regions formed the basis of the subsequent sequence variation and phylogenetic analyses.

Laboratory-generated ASFV sequences were assigned to genotype II

BLASTn analysis of the 13 laboratory-generated partial B646L sequences identified African swine fever virus (ASFV) genotype II as the closest match for all isolates. Each sequence showed 100% nucleotide identity, 94% query coverage, and an E-value of 0 against the highest-scoring GenBank entries. Because the top 100 BLASTn hits exhibited identical alignment statistics, only representative matches from Asian countries are presented in Table 1 to provide geographical context relevant to the Philippines.

The central B646L region retains genotype II assignment

Maximum-likelihood analysis of the laboratory-generated partial B646L sequences placed all 13 laboratory-generated ASFV sequences within the genotype II clade together with representative reference sequences (Figure 2). The laboratory-generated sequences clustered with genotype II isolates from the Philippines, Vietnam, China, Singapore, Hong Kong, and South Korea, whereas representative sequences from genotypes I, IV, V, VIII, IX, X, and XII formed distinct clades. The genotype II cluster has a bootstrap value of 99%.

Because the analyses were based on partial central region of B646L sequences, the phylogenetic trees

Table 3. Parsimony-informative sites within the publicly available partial C-terminal region of B646L (p72) between ASFV Genotype I and Genotype II isolates.

Representative Isolates	Parsimony-informative sites			
Philippines_MW791758_GII	C	T	A	G
Philippines_ON963982_GII	C	T	A	G
Philippines_PP737710_GII	C	T	A	G
Cameroon_OQ971725_GI	T	C	C	A
Ghana_OR371517_GI	T	C	C	A
Italy_MN270977_GI	T	C	C	A
Nigeria_PV842497_GI	T	C	C	A
Portugal_KM262844_GI	T	C	C	A
Spain_NC001659_GI	T	C	C	A
Position in the ASFV genome	103,821	103,875	103,962	104,031

were interpreted with respect to genotype assignment and clustering rather than broader evolutionary relationships among ASFV genotypes.

Representative complete B646L coding sequences (CDS), together with their corresponding central and C-terminal regions, consistently placed the Philippine genotype II reference sequences within the genotype II clade (Figure 3), indicating that genotype II assignment was retained regardless of the B646L region analyzed.

Comparative nucleotide variation across the B646L gene

Comparison of representative ASFV genotype I and genotype II sequences identified seven parsimony-informative sites within the 278-bp central region of the B646L gene (Table 2). These sites were located at genome positions 104,490, 104,517, 104,534, 104,637, 104,697, 104,706, and 104,727 relative to the ASFV Georgia 2007/1 reference genome (FR682468). The laboratory-generated sequences shared identical nucleotide patterns with representative genotype II reference sequences at all seven positions, whereas genotype I reference sequences exhibited alternative nucleotides.

Alignment of the C-terminal region similarly identified four parsimony-informative sites distinguishing representative genotype I and genotype II sequences (Table 3). These sites were located at genome positions 103,821, 103,875, 103,962, and 104,031 relative to the ASFV Georgia 2007/1 reference genome. Representative genotype II sequences shared identical nucleotide patterns across all four positions, whereas genotype I reference sequences contained alternative nucleotides.

Comparison of sequence variation across the complete B646L coding sequence (CDS), central region, and C-terminal region revealed differences in the numbers of conserved, variable, and parsimony-informative sites (Table 4).

The complete B646L CDS contained 1,679 conserved sites, 262 variable sites, and 211 parsimony-informative sites. Among the two partial regions, the C-terminal region contained 76 variable and 61 parsimony-informative sites, whereas the central region contained 32 variable and 28 parsimony-informative sites.

Table 4. Comparison of sequence variations among the complete B646L coding sequence, central region, and C-terminal region of representative ASFV genotypes I, II, III, IV, V, VIII, IX, X, and XII

Region	Alignment Length (bp)	Conserved	Variable	Parsimony-informative
Complete CDS	1941	1679	262	211
C-terminal	414	338	76	61
Central	277	245	32	28

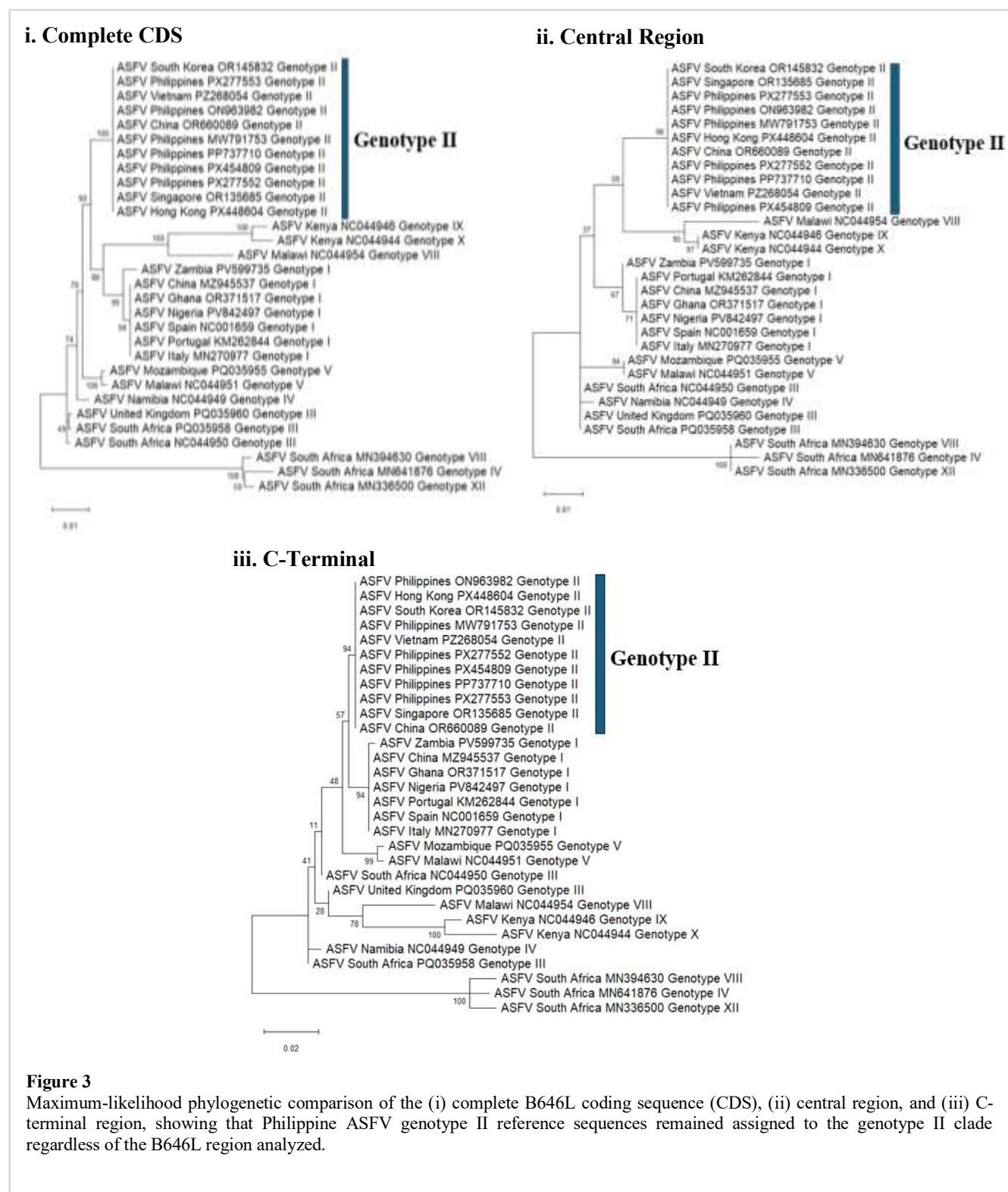


Figure 3

Maximum-likelihood phylogenetic comparison of the (i) complete B646L coding sequence (CDS), (ii) central region, and (iii) C-terminal region, showing that Philippine ASFV genotype II reference sequences remained assigned to the genotype II clade regardless of the B646L region analyzed.

IV. Discussion

The present study evaluated whether the central region of the B646L gene, routinely amplified during conventional African swine fever virus (ASFV) diagnosis, contains sufficient sequence variation for genotype assignment. Although ASFV genotyping has traditionally relied on sequencing the C-terminal region of B646L (Bastos et al., 2003), the routinely amplified central fragment consistently classified the laboratory-generated sequences as genotype II and produced the same genotype assignment as both the complete B646L coding sequence (CDS) and the conventional C-terminal genotyping region. These findings indicate that sequence information generated during routine diagnostic PCR can provide molecular information beyond confirmation of virus presence.

The B646L gene encodes the major capsid protein p72 and remains the principal genetic marker for ASFV molecular characterization because sequence variation within the gene forms the basis of the current classification of ASFV into 24 recognized genotypes (Bastos et al., 2003; Galindo & Alonso, 2017; Qu et al., 2022). Consequently, sequencing of the C-terminal region has become the standard approach for genotype assignment in molecular epidemiological investigations. In contrast, routine laboratory diagnosis relies on amplification of the central region using the conventional PCR assay of Wilkinson et al. (2000), subsequently optimized by Aguero et al. (2003) and recommended by WOAAH (2024). Despite its widespread use in diagnostic laboratories, the potential of this routinely generated amplicon for genotype assignment has remained largely unexplored.

Comparison of phylogenetic trees reconstructed from the complete B646L CDS, central region, and conventional C-terminal region demonstrated that Philippine genotype II reference sequences remained assigned to the genotype II clade regardless of the B646L region analysed. Because the phylogenetic analyses were based on partial gene regions, the trees were interpreted with respect to genotype assignment rather than broader evolutionary relationships among ASFV genotypes.

Notably, genotype I was consistently recovered as the nearest neighboring clade to genotype II in all B646L phylogenies. Sequence variation analyses therefore focused on these two genotypes, representing the most stringent comparison for evaluating the discriminatory capacity of the central region. Seven parsimony-informative sites were identified within the central region, compared with four in the conventional C-terminal fragment, demonstrating that the diagnostic amplicon retained

sufficient nucleotide variation to distinguish genotype II from its closest neighboring genotype.

Recent advances in whole-genome sequencing have substantially improved ASFV molecular epidemiology (Warr et al., 2021; Koltsov., 2022, and Chernyshev et al., 2024). These approaches provide considerably greater genetic resolution than single-gene analyses and remain indispensable for investigating virus evolution, transmission pathways, and outbreak dynamics. However, conventional PCR followed by Sanger sequencing continues to be widely implemented in routine veterinary diagnostic laboratories because of its accessibility, lower cost, and established diagnostic performance (Aguero et al., 2003; WOAAH, 2024). Rather than replacing whole-genome sequencing or the conventional C-terminal genotyping strategy, the present findings demonstrate that sequencing of the central region of B646L can provide complementary genotype information without additional laboratory procedures.

Overall, the central region of B646L, routinely amplified during ASFV diagnosis, retained sufficient sequence variation for consistent genotype II assignment in the dataset analyzed. Sequencing of this diagnostic amplicon may therefore provide a practical source of complementary genotype information, particularly where conventional PCR and Sanger sequencing remain the primary molecular tools.

Ethics statement

Ethical approval was not required because no live animals were involved. Samples used in this study were sold in public markets.

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Conflict of interest

The author declared that this work was conducted in the absence of any commercial or financial relationships.

Supplementary materials

A total of 13 partial B646L sequences were deposited in NCBI GenBank under accession numbers PZ668691–PZ668703.

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