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Comparative analysis of whole-genome sequences of African swine fever virus (Asfarviridae: *Asfivirus*) isolates collected on the territory of the left bank of the Dnieper River in 2023

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Abstract

Introduction. The lack of data on the whole-genome sequences of African swine fever virus (ASFV) variants circulating on the territory of the left bank of the Dnieper River complicates the understanding of the molecular evolution of the virus and the character of the epidemic process development in Russia and Ukraine. Understanding the genetic divergence and phylogenetic relatedness of isolates can largely adjust the strategy of general and specific prevention of the disease.

The aim of the study – search and description of unique mutations (deletions/insertions/substitutions) in isolates collected from domestic pigs in Donetsk, Luhansk and Zaporozhye regions in 2023; determination of relatedness and level of homology with reference strains of ASFV genotype II; sub-genotyping and clustering of isolates based on whole-genome analysis.

Materials and methods. The samples used were a culture suspension of porcine bone marrow (PBM) cells containing ASFV isolates obtained from pathologic material from domestic pig carcasses. Genomic DNA was prepared by purification and concentration of virus followed by phenol-chloroform extraction of total nucleic acid. The high-throughput sequencing process was performed using MGI technology. Consensus sequences were assembled by mapping reads to the reference genome of strain Georgia 2007/1.

Results. All isolates are assigned to genotype II, have a monophyletic origin, are phylogenetically close to the clusters «Europe» (4/5) and «Bryansk 2021» (1/5), and are divergent from the original parental genetic variants that make up the enlarged clades. In addition, numerous substitutions in the loci of the multigene family *MGF 110*, *505*, and *360*, encoding virulence proteins, were detected in 4 isolates from Donetsk and Zaporozhye regions.

Conclusion. The phylogeny of the genotype II ASFV, which originated from the reference strain Georgia 2007/1, is shown to be sufficient for isolate differentiation. The presented data are of theoretical and practical importance for domestic and international ASFV surveillance.

Keywords: *African swine fever; left bank of the Dnieper River; Donetsk, Lugansk, Zaporozhye region; whole-genome analysis; single nucleotide polymorphism; molecular epidemiology*

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Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

Ethics approval. Authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with Consensus author guidelines for animal use (IAVES 23 July 2010). The research protocol was approved by the Ethics Committee of Federal Centre for Animal Health (ARRIAH) (Protocol N Dnieper/2024 dated 15 May 2024).

ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ
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Сравнительный анализ полногеномных последовательностей изолятов вируса африканской чумы свиней (Asfarviridae: *Asfivirus*), выделенных на территории левобережья Днепра в 2023 году

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Резюме

Введение. Отсутствие данных о полногеномных последовательностях возбудителя африканской чумы свиней (АЧС), циркулирующего на территории левобережья Днепра, ограничивает понимание динамики молекулярной эволюции вируса и характера развития текущего эпизоотического процесса в центральной России и Украине. Определение степени генетической дивергентности и филогенетического родства вируса АЧС во многом может скорректировать стратегию общей и специфической профилактики болезни.

Цель работы – поиск и описание уникальных точечных мутаций (делеций/инсерций/замен) у изолятов, выделенных от домашних свиней на территории Донецкого, Луганского и Запорожского регионов в 2023 г.; установление родства и уровня гомологии с референтными штаммами вируса АЧС генотипа II; субгенотипирование на основе маркерных областей генома.

Материалы и методы. В качестве образцов использовали культуральную суспензию костного мозга свиньи, содержащую вирус АЧС. Подготовку геномной ДНК выполняли методом очистки и концентрирования вируса с последующей экстракцией тотальной нуклеиновой кислоты фенол-хлороформным методом. Процесс высокопроизводительного секвенирования осуществляли с помощью технологии MGI. Сборку консенсусных последовательностей проводили методом картирования прочтений на референс-геном штамма Georgia 2007/1.

Результаты. Все изоляты отнесены к генотипу II, имеют монофилетическое происхождение, филогенетически относятся к кластерам «Европа» (4/5) и «Брянск 2021» (1/5), а также являются дивергентными от исходных родительских генетических вариантов, составляющих укрупненные клады. Кроме того, обнаружены многочисленные замены в локусах мультигенного семейства *MGF 110*, *505* и *360*, кодирующих факторы вирулентности.

Заключение. На примере изучения филогении показана достоверная для дифференциации вариабельность вируса АЧС генотипа II, происходящего от референс-штамма Georgia 2007/1. Представленные данные обладают теоретической и практической значимостью при усовершенствовании национального и международного надзора за АЧС.

Ключевые слова: *африканская чума свиней; левобережье Днепра; Донецкая, Луганская, Запорожская области; полногеномный анализ; однонуклеотидный полиморфизм; молекулярная эпизоотология*

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Финансирование. Исследование выполнено в рамках государственного задания от 17.11.2023 № 081-00003-23-05 «Выявление возбудителей трансграничных заболеваний животных, изучение их биологических свойств, особенностей заноса и распространения болезней, вызываемых данными возбудителями, возможных факторов передачи».

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Этическое утверждение. Авторы подтверждают соблюдение институциональных и национальных стандартов по использованию лабораторных животных в соответствии с Consensus author guidelines for animal use (IAVES 23 July 2010). Протокол исследования одобрен Комиссией по биоэтике ФГБУ «ВНИИЗЖ» (протокол от 15.05.2024 № Dnieper/2024).

Introduction

In the 21st century, African swine fever (ASF) has become a worldwide problem of swine production in a relatively short period of time. Following the introduction of ASF genotype II virus from Africa to Georgia (2007), the disease was registered in 46 countries in Europe and Asia (2007–2024), the Caribbean (Haiti and the Dominican Republic, 2021), becoming panzootic and causing serious economic damage to the swine and hunting sectors of disadvantaged countries and re-

gions [1][1]. The rapid transboundary spread of ASF has contributed to the development of an outbreak investigation methodology alternative to the epizootologic survey.

The development of molecular biology (sequencing) and bioinformatics methods (phylogenetic and evolutionary analyses) has significantly influenced the formation of a new applied field – molecular epizootology, which studies the patterns of emergence and circulation of genetic variants and groups of pathogens of infectious animal diseases [2]. In this respect, spatio-temporal phylogenomics is an effective tool for domestic and international ASF surveillance.

On the Eurasian continent, a slow rate (1.14×10^{-5} substitutions/site/year) of ASF virus mutations was observed, limiting clustering [3]. Thus, genetic homogeneity of isolates isolated in 2007–2011 in the North Caucasian, Southern and Northwestern Federal Districts of the Russian Federation was observed in the study of marker fragments previously proposed to characterize genotype I [4]. In this regard, whole-genome analysis with high resolution is most preferable for detecting unique single changes and establishing phylogenetic relatedness [5]. A. Mazloum et al. (2021) demonstrated divergence between variants circulating in Central Russia and the Caucasus, Eastern Europe, and the Far East [6].

As a result of the disease spread, three groups showed significant genetic segregation from each other: ASF virus circulating at the beginning of the epizootic (Georgia, Armenia, Azerbaijan, Russia until 2019, Poland and Lithuania until 2015); isolates from the Baltic countries, eastern Europe and Germany (Romania, Poland and Lithuania from 2016, Latvia, Estonia, Kiev region of Ukraine and Kaliningrad region of Russia); ASF virus isolated in Belgium, Hungary, Czech Republic and Moldova and eastern countries (China, Vietnam, Republic of Korea, India, East Timor, as well as the Far Eastern Federal District of Russia) [7, 8]. The close phylogenetic relationship of the strains of pathogen, enzootic for European and Asian countries, is explained by its presumed introduction into China (2018) from Western Europe [9].

Since July 30, 2012, the territory of Ukraine is officially affected by ASF. During the epizootic outbreak (according to the World Animal Health Organization for 21.06.2024) 620 cases were registered, including 487 outbreaks among domestic pigs and 133 – among wild boar populations (**Fig. 1**)[2]. There is no reliable information on the circulation of genetic variants of the ASF pathogen in Ukraine and in the regions on the left bank of the Dnieper River (Donetsk, Luhansk and Zaporozhye regions). The only strain with an established nucleotide sequence of the genome, isolated on 11.04.2016 (Kyiv/131 2016) in the Kiev region of Ukraine, was described by G. Kovalenko et al. (2019) and assigned to a cluster characteristic of samples identified in Poland [10].

Difficulties in understanding the molecular evolution of ASF virus genotype II in Eurasia (2007 – present) are primarily due to the lack of sequencing data, which also limits the applied value of phylogenetic and spatio-temporal analyses in veterinary medicine. In this regard, molecular genetic studies devoted to the search for new and confirmation of the circulation of already characterized genetic variants in all administrative units of Euro-Asian countries are a relevant area of scientific research.

The objectives of the study were to perform whole-genome sequencing and analyze oligo- and single nucleotide polymorphism (SNP) of ASF virus isolated from biological material from domestic pigs kept in Donetsk, Lugansk and Zaporozhye regions in 2023; to identify unique point mutations that allow differentiating genetic variants; to establish phylogenetic relatedness of the studied isolates and their belonging to subgenotypes.

Materials and methods

ASF virus isolates. Samples of pathological material from fallen domestic pigs (muscle tissue, spleen, bone marrow) in Donetsk, Lugansk and Zaporozhye regions were sent to the ASF reference laboratory (FGBI ARRIAH, Vladimir, Russia) to confirm the laboratory diagnosis of ASF and to conduct research work (**Table 1**). After a positive result of real-time polymerase chain reaction, a 10% homogenized suspension was prepared from the samples and used for virus isolation. Identification and accumulation of isolates were performed in a primary culture of porcine bone marrow (PBM) cells according to a previously published protocol at a titer of at least 6.0 log10 HAD_{50}/cm^3 [11].

Whole-genome resequencing. Samples of ASF virus genomic DNA (gDNA) were prepared and its quality assessed in accordance with the guidelines for purification, concentration and isolation of ASF virus and capripoxvirus genome for whole-genome sequencing[3].

Purification and concentration were performed by method #1 (medium-speed centrifugation at 4 °C and 7000 rpm for 16 h).

Library preparation was performed using the MGIEasy Universal DNA Library Prep Set (MGI Tech, China). High-throughput sequencing (HTS) was performed on a DNBSEQ-G400 platform (MGI Tech) [12].

Assembling and analyzing sequences. Consensus sequence assembly was performed by mapping reads to the reference genome of Georgia 2007/1 strain (NC_044959.2) with con-

[1]Federal Service for Veterinary and Phytosanitary Surveillance. The epizootic situation of ASF in the territory of the Russian Federation, in Europe, Asia and America. WHO data from 2007 to 2023; 2023. Available at: https://fsvps.gov.ru/wp-content/uploads/2023/06/05_АЧС_2007_2023_мир.png (in Russian)

[2]Federal Service for Veterinary and Phytosanitary Surveillance. The epizootic situation of ASF in Ukraine; 2024. Available at: https://fsvps.gov.ru/wp-content/uploads/2023/06/АЧС-в-Украине-17.pdf (in Russian)

[3]Mazlum A., Chernyshev R.S., Krotova A.O., et al. Methodological recommendations for purification, concentration and isolation of the genome of the African swine fever virus and capripoxviruses for genome-wide sequencing. Vladimir; 2024. (in Russian)

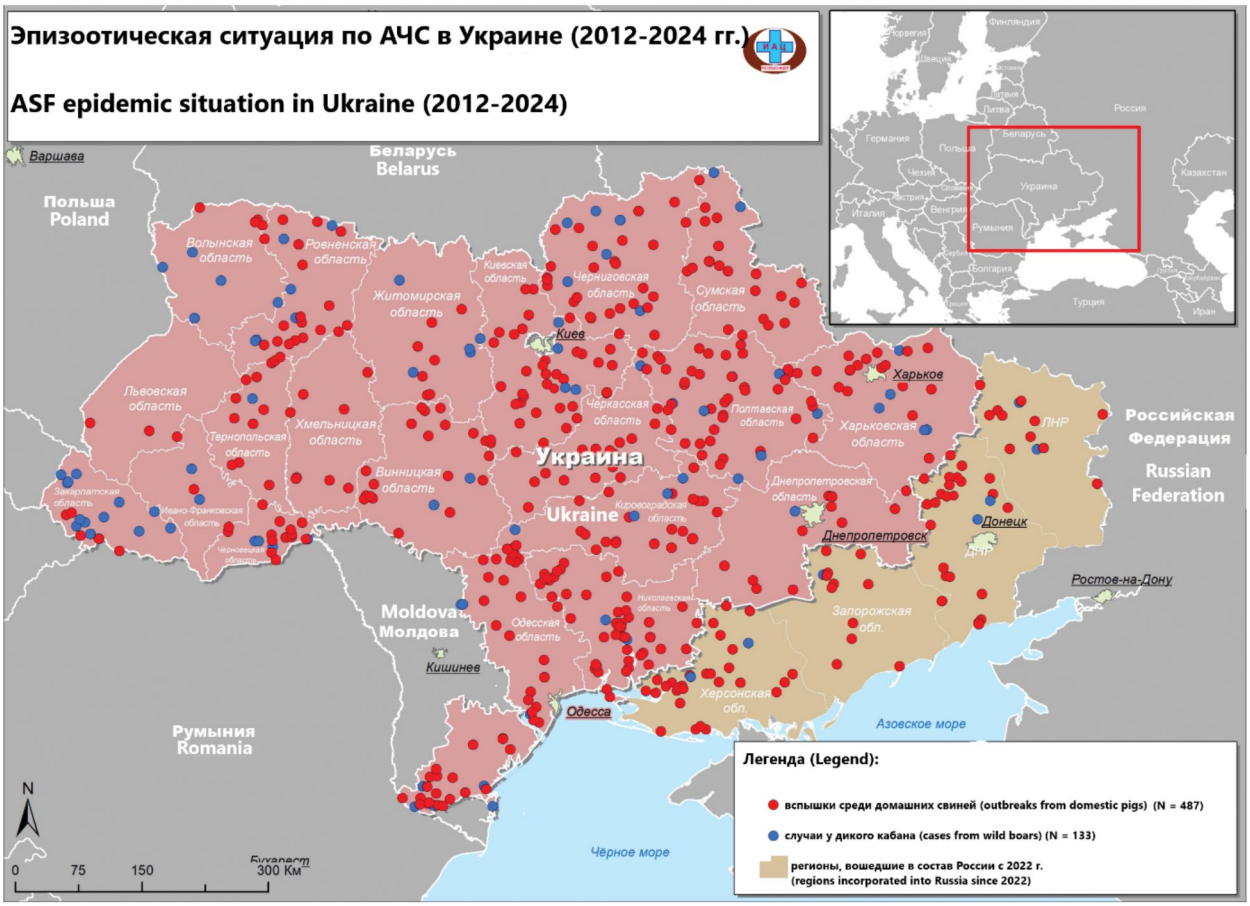

Fig. 1. Spread of ASF in Ukraine (2012–2024).
Рис. 1. Распространение АЧС на территории Украины (2012–2024 гг.).

Table 1. Brief characteristics of samples used in the study

Таблица 1. Краткие характеристики образцов, использованных в исследовании

Isolate name Наименование изолята	Date of outbreak registration Дата регистрации вспышки	Place of sample collection Место отбора образцов	Viral load in PBM cells by 3rd passage (log10 HAD_{50}/ml ± SD) Титр вируса в КМС к 3-му пассажу, (lg $ГАдЕ_{50}$/см^3 ± SD)
ASFV/DNR/DP2023/2466-1	22.09.2023	Donetsk region, Volnovakha Донецкий регион, г. Волноваха	8,62 ± 0,21
ASFV/DNR/DP2023/2466-3	21.09.2023	Donetsk region, Volnovakha district, Ivanovka village Донецкий регион, Волновахский район, с. Ивановка	7,95 ± 0,14
ASFV/DNR/DP2023/3343-2	16.10.2023	Donetsk region, Telmanovsky district, Andreevka settlement Донецкий регион, Тельмановский район, п. Андреевка	7,58 ± 0,14
ASFV/Zaporozskaya/DP2023/2896-5	01.11.2023	Zaporozhye region, Berdyansk district, Chernigovo-Tokmachansk settlement Запорожская область, Бердянский район, с. Чернигово-Токмачанск	8,81 ± 0,21
ASFV/LNR/DP2023/42-1	26.12.2023	Luhansk region, Starobelsky district, Podgorovka village Луганский регион, Старобельский район, с. Подгоровка	7,0 ± 0,14

tigs determined in Geneious Prime program (2024.0.5). The Genome Annotation Transfer Utility (GATU) on Java platform v. 8 was used for open reading frames (ORFs) prediction and genome annotation [13]. Multiple alignment of the obtained sequences with those retrieved from the GenBank database (**Appendix**) and nucleotide change analysis (NCA) were performed using the CLUSTAL W algorithm in the Geneious Prime program; translation and detection of amino acid changes were performed in the SnapGene v. 5.2.1 program. The level of homology was assessed on the Nucleotide BLAST NCBI online platform. The phylogenetic dendrogram was constructed in Mega X program using the recommended model T92 + G + I (BIC = 537644,659; AICc = 535583,3783) by Maximum Likelihood method with 100 iterations of initial bootstrap [14].

Subgenotyping of isolates was performed according to the methodological recommendations for molecular epizootological clustering of ASF virus by subgenotyping (modification of subgenotype classification by C. Gallardo et al., 2023)[4] [15].

Ethical approval. The authors confirm compliance with institutional and national standards for the use of laboratory animals in accordance with Consensus author guidelines for animal use (IAVES July 23, 2010). The study protocol was approved by the Bioethics Commission of FGBI ARRIAH (protocol dated 15.05.2024 No. Dnieper/2024).

Results

Quality assessment of gDNA and resequencing. Spectrophotometric indices (absorbance ratios at A260/A230 and A260/A280 wavelengths) and bioinformatic metadata were determined and presented in graphs (**Fig. 2**).

As shown in Figure 2, all samples had satisfactory purification values of gDNA from low molecular weight compounds, as A230/A260 values were ≥ 2.0. However, A260/A280 values were 1.77–1.80, which is less than the recommended value (≥ 1.8) and indicates insufficient purity due to protein based impurities. In turn, the proportion of specific reads ranged from 0.69 to 1.14% and the average depth of coverage was 407–1664 reads/nucleotide, exceeding the recommended value (> 30). The region with low coverage in 3 isolates from Donetsk region was located in positions 187,750 to 189,000 bp, while in ASFV/Zaporozskaya/DP2023/2896-5 – in positions 16,640 to 18,430 bp. When mapping reads to the reference genome of strain Georgia 2007/1 (190,584 bp), a single long contig was formed in the absence of scaffolds for each isolate, subsequently generating consensus sequences ranging in length from 190,573 to 190,596 bp.

Similar to strain Georgia 2007/1, 195 ORFs were identified in all genomes examined.

Based on the results of genome assembly and annotation, the sequences were deposited in the VGARus database under the identification numbers niiz000001-5.

SNF analysis. All studied isolates were found to belong to genotype II based on the C-terminal fragment of the *B646L* gene.

Multiple whole-genome alignment showed the presence of single and oligonucleotide polymorphisms. Unique (newly identified) and characterized synonymous and nonsynonymous substitutions were noted and indices presented in **Table 2**.

Thus, 63 substitutions (44 transitions and 19 transversions) were detected in 5 ASF virus isolates, 14 of which were synonymous and 38 were nonsynonymous, leading to a change in the amino acid composition of the encoded protein; 3 insertions: 1 in the ORF, 2 in intergenic regions; 1 single-nucleotide deletion in the intergenic region.

Ten nucleotide substitutions were unique for isolates from Donetsk region, 6 of them are contained in loci of *MGF 110*, *360*, *505* multigene families. A non-synonymous A → G transit in the *EP402R* gene encoding the hemadsorbing glycoprotein CD2v of ASF virus responsible for seroimmunotyping was registered exclusively in ASFV/DNR/DP2023/3343-2.

In ASF virus from the Zaporozhye region, numerous previously uncharacterized substitutions in the *MGF 360* multigene family (6 transversions and 16 transitions), which significantly changed the amino acid sequence of the identically named proteins, were observed. Two identical nonsynonymous G → A transitions repeated in the *R298L* gene resulted in the replacement of alanine (A) by valine (V).

ASF virus isolated in Lugansk region (ASFV/LNR/DP2023/42-1) had 9 unique substitutions and 6 previously characterized only for two samples isolated from domestic pigs at large enterprises of Bryanskaya region in 2021 (ASFV/Bryanskaya 2021/DP-18; ASFV/Bryanskaya 2021/DP-8823).

All 5 studied isolates belonged to genetic variants (II) with single substitutions at markers *I267L*, *NP419L*, *MGF 505-9R* and *MGF 110-1L*. Co-mutations were observed in all 4 genes in the alignment, except for the Odintsovo/WB/Russia/2014 strain (**Fig. 3**).

Analysis of the SNPs of the *E199L* and *DP60R* genes showed no correlation between the molecular evolution of ASF virus and spatio-temporal cluster distribution due to the registration of genetic variants different from the Georgia 2007/1 strain in geographically distant areas. Thus, C → T substitution in position 167062 of locus *E199L* and insertion A in position 190116 of *DP60R* gene were found in a number of isolates from Poland, Lithuania, China, Germany, Czech Republic, as well as from Kaliningrad region, Far Eastern Federal District and central regions of Russia.

The *I73R/I329L* intergenic region contained three 10-nucleotide tandem repeat insertions (TRS) and belonged to IGR-II, prevalent in Eurasia.

Homology level. Homology of the studied whole-genome sequences with the most studied (reference) ASF virus strains belonging to genotype II and isolated in different enzootic countries of Eurasia (Russia: Kaliningrad (West), Ulyanovsk (Center) and Amur (East) regions; Moldova,

[4]Chernyshev R.S., Mazlum A., Zinyakov N.G., et al. Methodological recommendations on molecular epizootological clustering of African swine fever virus isolates by subgenotyping. Vladimir; 2024. (in Russian)

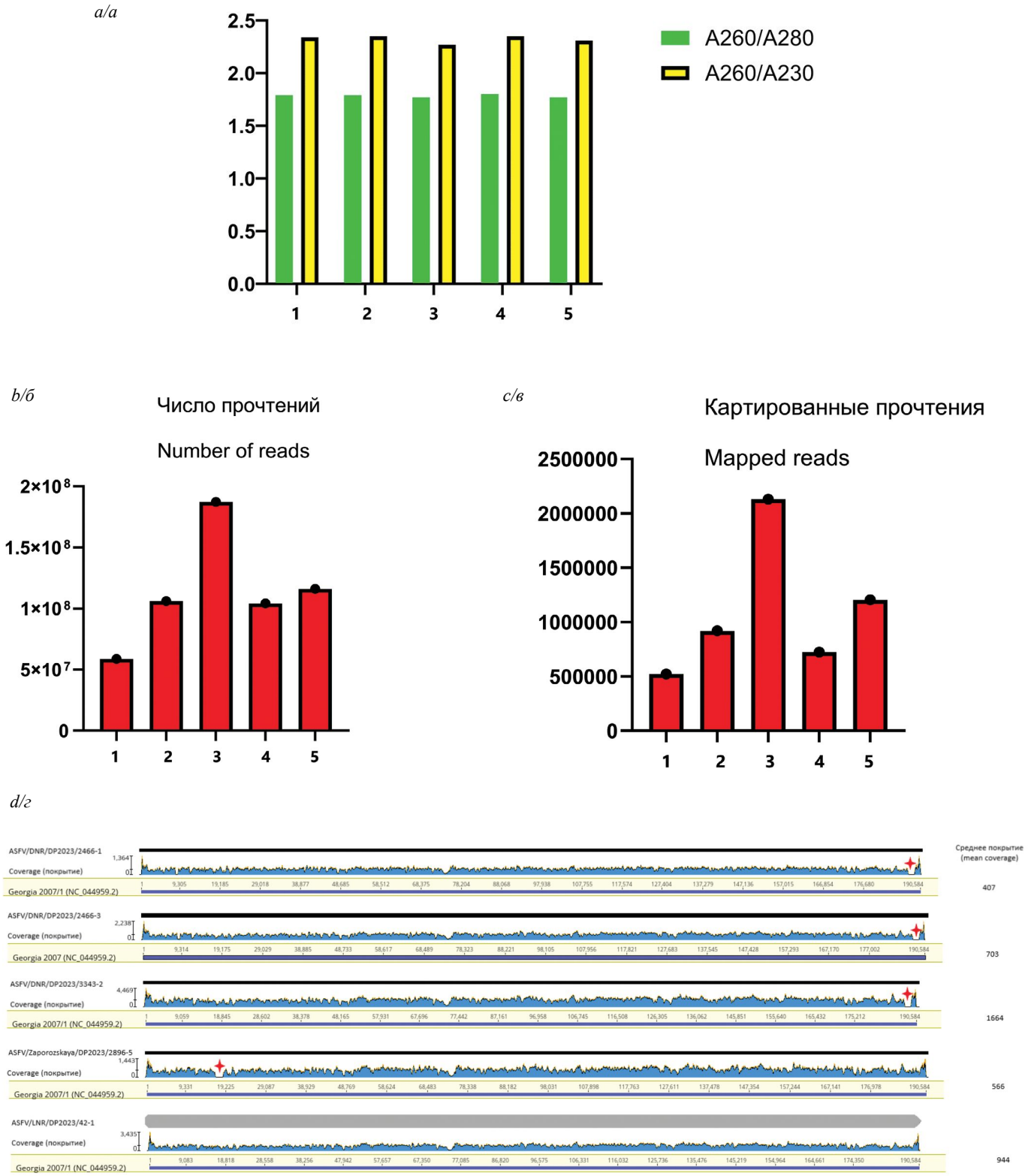

Fig. 2. Spectrophotometric indices (*a*) of gDNA samples and bioinformatic metadata: number of reads (*b*), specificity of reads (*c*), coverage (*d*) obtained after resequencing.

Note: fragments with l ow coverage are indicated ✦. 1 – ASFV/DNR/DP2023/2466-1; 2 – ASFV/DNR/DP2023/2466-3; 3 – ASFV/DNR/DP2023/3343-2; 4 – ASFV/Zaporozskaya/DP2023/2896-5; 5 – ASFV/LNR/DP2023/42-1.

Рис. 2. Спектрофотометрические показатели (*а*) образцов гДНК и биоинформатические метаданные: число прочтений (*б*), специфичность прочтений (*в*), покрытие (*г*), полученные после ресеквенирования.

Примечание: области с низким покрытием обозначены ✦. – ASFV/DNR/DP2023/2466-1; 2 – ASFV/DNR/DP2023/2466-3; 3 – ASFV/DNR/DP2023/3343-2; 4 – ASFV/Zaporozskaya/DP2023/2896-5; 5 – ASFV/LNR/DP2023/42-1.

Table 2. Comparative analysis of point mutations and amino acid changes

Таблица 2. Сравнительный анализ точечных мутаций и аминокислотных изменений

Nucleotide position (Georgia 2007/1) Нуклеотидная позиция	Type of SNP Характер ОНП	Variability of amino acid Аминокислотная изменчивость	Gene/Intergenic region Ген/Межгенная область	Occurrence Встречаемость
1572	T → C	–	Intergenic region Межгенная область	ASFV/DNR/DP2023/2466-1 ASFV/DNR/DP2023/2466-3 ASFV/DNR/DP2023/3343-2
1587	T → C	–	Intergenic region Межгенная область	ASFV/DNR/DP2023/2466-1 ASFV/DNR/DP2023/2466-3 ASFV/DNR/DP2023/3343-2
1611	A → T	–	Intergenic region Межгенная область	ASFV/DNR/DP2023/2466-1 ASFV/DNR/DP2023/2466-3
2817	A → T	L → stop	MGF 360-1Lb	ASFV/Zaporozskaya/DP2023/2896-5
2929	Делеция / Deletion A	–	Intergenic region *1L/2L* Межгенная область /	ASFV/LNR/DP2023/42-1 ASFV/Bryanskaya 2021/DP-18 ASFV/Bryanskaya 2021/DP-8823
7059	C → T	W → stop	*MGF 110-1L*	All of the isolates tested except Все исследуемые изоляты, за исключением Ulyanovsk/19/WB/5699 Kabardino-Balkaria/19/WB/ 964 Pol15/Podlaskie/Poland//2015 LT14/1490/Lithuania/2014
8280	G → A	H → Y	*MGF 110-3L*	ASFV/DNR/DP2023/2466-1 ASFV/DNR/DP2023/2466-3
8329	T → C	G = G	*MGF 110-3L*	ASFV/DNR/DP2023/2466-1 ASFV/DNR/DP2023/2466-3
9253	C → A	V → L	*MGF 110-4L*	ASFV/DNR/DP2023/2466-1 ASFV/DNR/DP2023/2466-3 ASFV/DNR/DP2023/3343-2
16283	A → G	–	PolyC-region	ASFV/LNR/DP2023/42-1
16683	C → T	A = A		
16692	A → G	V → I		
16694	C → T	I → V		
16703	T → C	I → V		
16718	T → C	V → I		
16719	G → A	A = A	*MGF 360-4L*	ASFV/Zaporozskaya/DP2023/2896-5
16727	G → T	Q → K		
16738	A → T	L → Y		
16739	G → A	L → Y		
16745	A → G	L = L		
16746	A → G	H = H		
17509	A → T	–	Intergenic region Межгенная область	ASFV/LNR/DP2023/42-1
18576	A → G	A = A		
18582	C → A	S → F		
18583	G → A	S → F		
18592	T → A	D → V	*MGF 360-6L*	ASFV/Zaporozskaya/DP2023/2896-5
18594	A → G	N = N		
18598	G → A	P → L		
18600	A → C	D → G		
18601	T → C	D → G		
24690	G → A	T → M	*MGF 360-8L*	ASFV/DNR/DP2023/2466-1 ASFV/DNR/DP2023/2466-3 ASFV/DNR/DP2023/3343-2
24967	A → G	S → P	*MGF 360-8L*	ASFV/Zaporozskaya/DP2023/2896-5
30904	C → T	L = L	*MGF 360-12L*	ASFV/Zaporozskaya/DP2023/2896-5
44576	A → G	K → E	*MGF 505-9R*	All of the isolates tested except Все исследуемые изоляты, за исключением Ulyanovsk/19/WB/5699 Kabardino-Balkaria/19/WB/ 964 Pol15/Podlaskie/Poland//2015 LT14/1490/Lithuania/2014
46135	C → A	F → L	*MGF 505-10R*	ASFV/DNR/DP2023/2466-1 ASFV/DNR/DP2023/2466-3 ASFV/DNR/DP2023/3343-2

Continuation of table 2 see on page 488.

Nucleotide position (Georgia 2007/1) Нуклеотидная позиция	Type of SNP Характер ОНП	Variability of amino acid Аминокислотная изменчивость	Gene/Intergenic region Ген/Межгенная область	Occurrence Встречаемость
46557	G → A	R → Q	*MGF 505-10R*	ASFV/LNR/DP2023/42-1 ASFV/Bryanskaya 2021/DP-18 ASFV/Bryanskaya 2021/DP-8823
48231	Insertion Инсерция CTAGCTATAG	–	Intergenic region Межгенная область	ASFV/Zaporozskaya/DP2023/2896-5
49085	T → A	N → Y	*A240L*	ASFV/LNR/DP2023/42-1
50655	G → A	A → T	*MGF 360-15R*	ASFV/LNR/DP2023/42-1 ASFV/Bryanskaya 2021/DP-18 ASFV/Bryanskaya 2021/DP-8823
50667	G → A	E → K	*MGF 360-15R*	ASFV/LNR/DP2023/42-1 ASFV/Bryanskaya 2021/DP-18 ASFV/Bryanskaya 2021/DP-8823
54369	C→ T	S → N	*A859L*	ASFV/LNR/DP2023/42-1
54758	G → A	–	Intergenic region Межгенная область	ASFV/Zaporozskaya/DP2023/2896-5
57427	G → A	H = H	*F334L*	ASFV/Zaporozskaya/DP2023/2896-5
74708	A → G	T → A	*EP402R*	ASFV/DNR/DP2023/3343-2
86659	G → A	A = A	*C257L*	ASFV/Zaporozskaya/DP2023/2896-5
106942	C → T	V → I	*B117L*	ASFV/LNR/DP2023/42-1
121744	G → A	I = I	*CP2475L*	ASFV/LNR/DP2023/42-1
131463	G → C	Q → E	*NP1450L*	ASFV/LNR/DP2023/42-1 ASFV/Bryanskaya 2021/DP-18 ASFV/Bryanskaya 2021/DP-8823
134514	T → C	R → S	*NP419L*	All of the isolates tested except Все исследуемые изоляты, за исключением Ulyanovsk/19/WB/5699 Kabardino-Balkaria/19/WB/ 964 Pol15/Podlaskie/Poland//2015 LT14/1490/Lithuania/2014
157272	G → A	A → V	*R298L*	ASFV/Zaporozskaya/DP2023/2896-5
157297	G → A	A → V	*R298L*	ASFV/Zaporozskaya/DP2023/2896-5
158805	C → G	E → Q	*Q706L*	ASFV/LNR/DP2023/42-1 ASFV/Bryanskaya 2021/DP-8823 ASFV/Zaporozskaya/DP2023/2896-5
167062	C → T	G → R	*I199L*	ASFV/Kaliningrad 18/WB-9767 ASFV/Kaliningrad 18/WB-12524 ASFV/Kaliningrad 18/WB-9766 Pol15/Podlaskie/Poland//2015 LT14/1490/Lithuania/2014
167188	C → G	A → P		ASFV/DNR/DP2023/2466-1, ASFV/Zabaykali 2020/WB-5314 ASFV/Zabaykaly_2020/DP-4905
167196	G → A	T → I		ASFV/LNR/DP2023/42-1
168627	T → A	F → I	*E248R*	ASFV/LNR/DP2023/42-1
170862	T → A	I → F	*I267L*	All of the isolates tested except Все исследуемые изоляты, за исключением Ulyanovsk/19/WB/5699 Kabardino-Balkaria/19/WB/ 964 Pol15/Podlaskie/Poland//2015 LT14/1490/Lithuania/2014 Odintsovo/WB/Russia/2014
173273	C → T	T = T	*I73R*	ASFV/LNR/DP2023/42-1
173408	Инсерция Insertion GGAATATATA	–	Intergenic region *I73R/I329L* Межгенная область	27 of the 45 isolates investigated, including the 5 described in this study 27 из 45 исследуемых изолятов, включая 5 описанных в настоящем исследовании
184404	G → A	A → T	*MGF 360-18R*	ASFV/DNR/DP2023/2466-1 ASFV/DNR/DP2023/2466-3 ASFV/DNR/DP2023/3343-2
187684	A → C	–		
187700	A → T	–		
187701	G → A	–	Intergenic region Межгенная область	ASFV/DNR/DP2023/2466-1 ASFV/DNR/DP2023/2466-3 ASFV/DNR/DP2023/3343-2
187702	G → T	–		
187703	G → A	–		
187718	T → C	–		
190116	Инсерция Insertion A	I → N	*DP60R*	All of the isolates tested except Все исследуемые изоляты, за исключением Ulyanovsk/19/WB/5699 Kabardino-Balkaria/19/WB/ 964 Pol15/Podlaskie/Poland//2015 LT14/1490/Lithuania/2014 Odintsovo/WB/Russia/2014 ASFV CzechRepublic 2017/1 ASFV Germany 2020/1

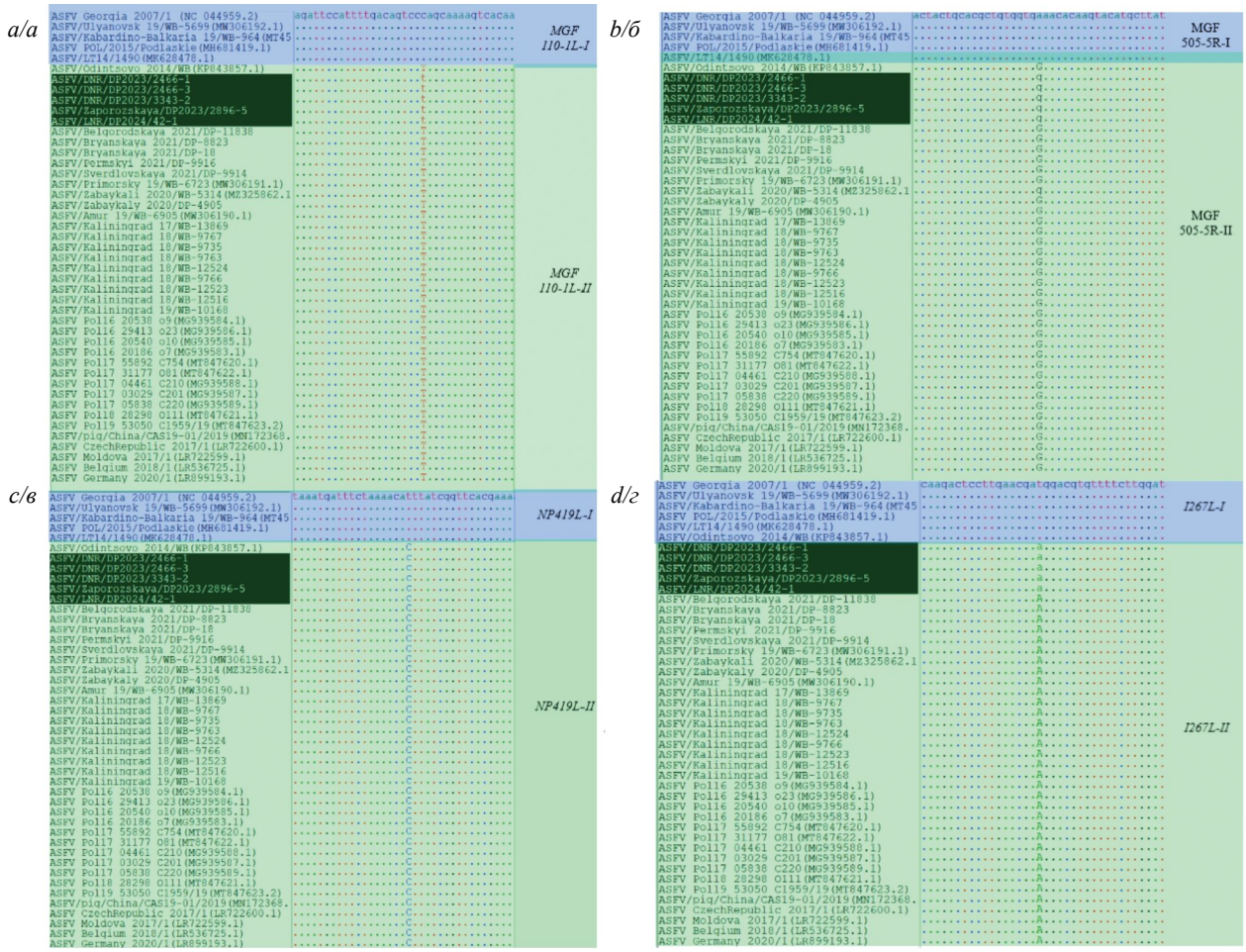

Fig. 3. Multiple alignment of *MGF 110-1L* (*a*), *MGF 505-9R* (*b*), *NP419L* (*c*), and *I267L* (*d*) genes showing non-synonymous substitutions.
Рис. 3. Выравнивание последовательностей генов *MGF 110-1L* (*а*), *MGF 505-9R* (*б*), *NP419L* (*в*) и *I267L* (*г*) с указанием несинонимичных замен.

Belgium, Poland: 2 heterogeneous genetic variants (before 2016 and after), China) was established when calculating the percentage of identity (**Fig. 4**).

According to Fig. 4, ASF virus circulating in Donetsk, Luhansk and Zaporozhye regions has high genetic affinity (99.95–99.99% identity) with genotype II strains isolated on the Eurasian continent during the current ASF epizootics.

Phylogenetic analysis. Phylogenetic relatedness of 45 strains and isolates of ASF virus retrieved from the GenBank database with the sequences under study was determined. The rest of the analysis results are presented in **Fig. 5**.

The rooted dendrogram (Fig. 5) identifies three large groups of isolates whose branches have one common internal node. The studied genetic variants can be divided into 6 clades (clusters) due to significant phylogenetic and geographic isolation. Thus, the isolates of the initial (root) clade are marked in blue and named «Georgia 2007» because they are characterized by an earlier origin and high relatedness to the parent strain Georgia 2007/1. The binary node on the tree divides ASF virus circulating in European countries into two clades named «Eastern Europe» (Kaliningrad Region, Poland, Germany, marked in red) and «Europe» (Moldova, Czech Republic, Belgium, marked in orange). Three sister clades of isolates are separated from the second major node: «Asia» (China and Far East Russia marked in yellow), «Center of Russia 2021» (Sverdlovsk and Belgorod regions, Perm Krai, marked with dark green shade) and «Bryansk 2021» (Bryansk region, marked with light green shade).

ASFV/LNR/DP2023/42-1 shows phylogenetic relatedness and belongs to the same group as the ASF virus from Bryansk region (20% – 1/5) that caused ASF outbreaks at pig farms in 2021.

The sequences of ASF virus isolated in Donetsk and Zaporozhye regions (80% – 4/5) are grouped into the clade «Europe». At the same time, all three isolates from Donetsk region form their own internal subcluster. The length of the ASFV/Zaporozskaya/DP2023/2896-5 branch is much longer than the other sequences, indicating a high number of additive substitutions.

All genotype II isolates isolated in Eurasian countries belong to the monophyletic group, i.e. they have a common ancestor (probably strain Georgia 2007/1).

Subgenotyping. Based on the distribution of genetic

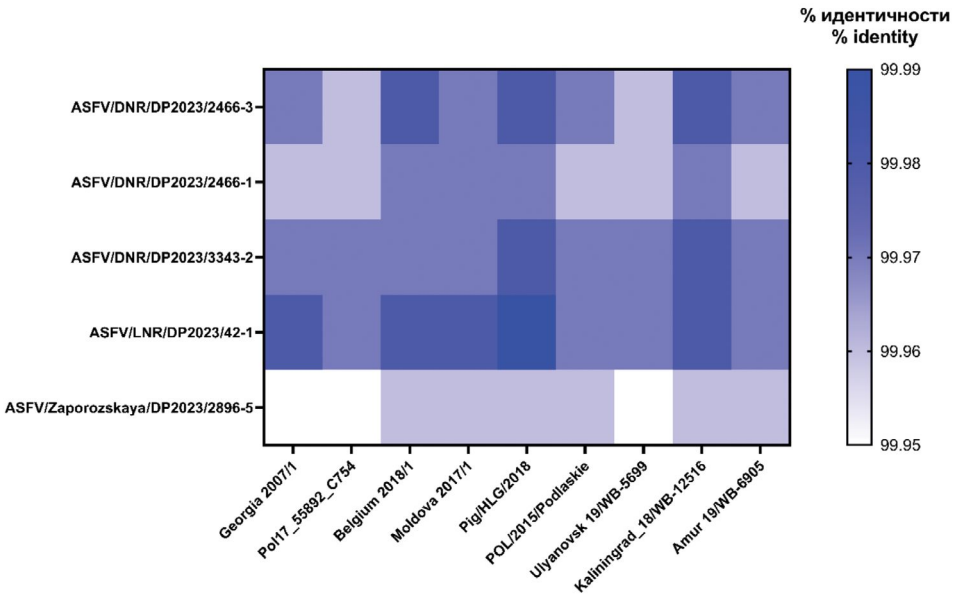

Fig. 4. Homology of isolates collected on the territory of the Dnieper left bank with strains characterized in geographically distant areas of Eurasia.

Рис. 4. Гомология изолятов, выделенных на территории левобережья Днепра, со штаммами, охарактеризованными в географически отдаленных друг от друга областях Евразии.

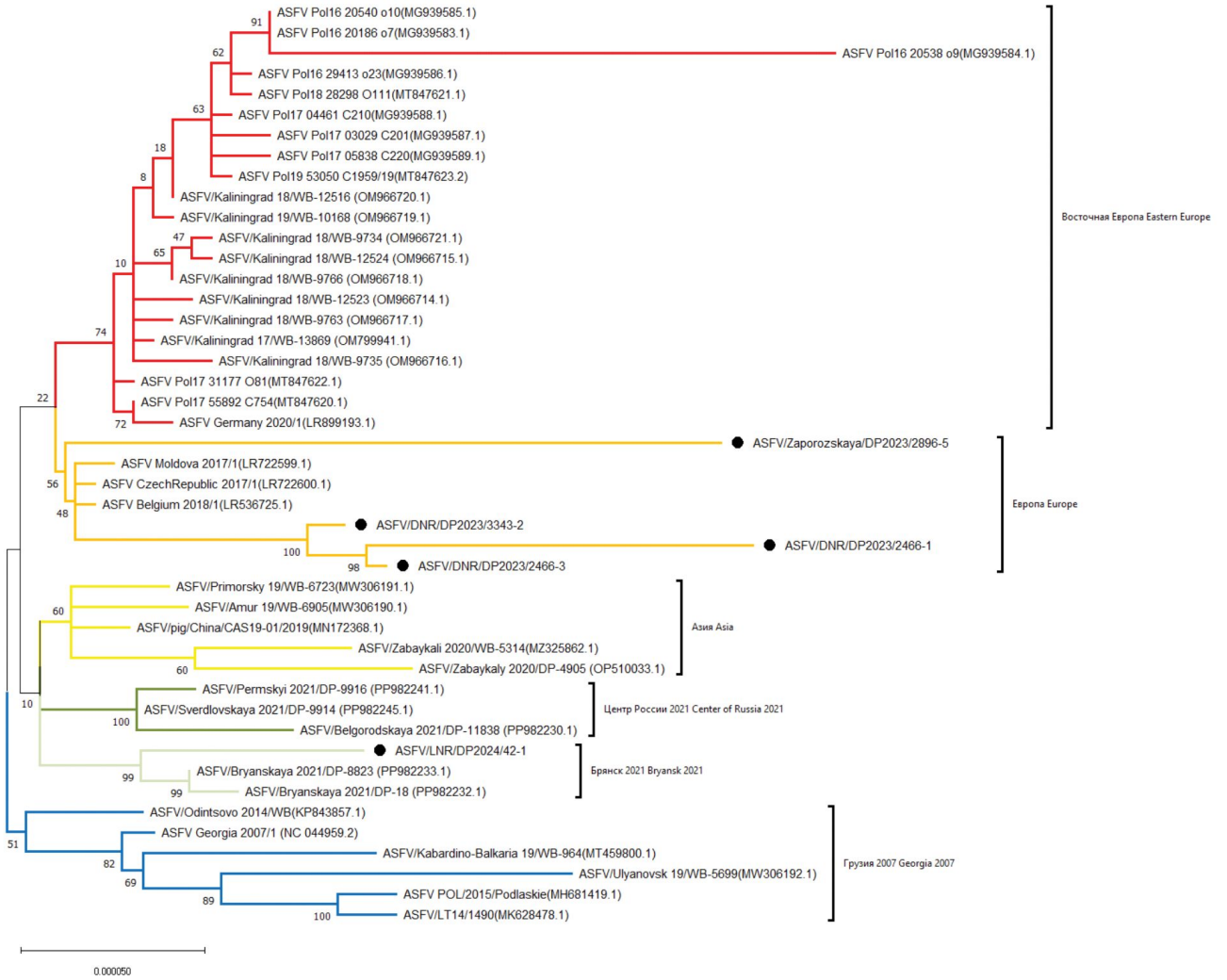

Fig. 5. Phylogenetic tree of ASFV isolates collected in Eurasia from 2007 to 2023.

Note: isolates obtained in this study are labeled ●.

Рис. 5. Филограмма изолятов вируса АЧС, выделенных в Евразии с 2007 по 2023 г.

Примечание: изученные в настоящем исследовании изоляты обозначены ●.

Table 3. Subgenotyping data of ASFV isolates studied in this research

Таблица 3. Данные субгенотипирования изолятов вируса АЧС, исследованных в работе

Features Характеристики	Isolate Изолят				
	ASFV/DNR/ DP2023/2466-1	ASFV/DNR/ DP2023/2466-3	ASFV/DNR/ DP2023/3343-2	ASFV/Zaporozskaya/ DP2023/2896-5	ASFV/LNR/ DP2023/42-1
CVR	I	I	I	I	I
IGR I73R/I329L	II	II	II	II	II
MGF 505-9R/10R	I	I	I	I	I
K145R	I	I	I	I	I
O174L	I	I	I	I	I
MGF 505-5R	I	I	I	I	I
MGF 360-10L	I	I	I	I	I
I267L	II	II	II	II	II
I215L	I	I	I	I	I
Genotype Генотип	II	II	II	II	II
Subgenotype Субгенотип	3b	3b	3b	3b	3b

variants on 9 marker fragments of the ASF virus genome, a subgenotype (genogroup; **Table 3**) was identified.

All 5 isolates studied belonged to subgenotype 3b, which is ubiquitous in all regions of the country, except for the Kaliningrad Region and the Far Eastern Federal District. In a comparative analysis of 45 isolates, 13 (28.9%: studied, Belgorodskaya 2021/DP-11838, Bryanskaya 2021/DP-8823, Bryanskaya 2021/DP-18, Permskyi 2021/DP-9916, Sverdlovskaya 2021/DP-9914, Belgium 2018/1, Moldova 2017/1, CzechRepublic 2017/1) belonged to genogroup 3b characteristic of Europe and Russia in the period from 2017 to 2023.

Discussion

To date, there have been no data on the whole-genome analysis of ASF virus isolated on the territory of the left bank of the Dnieper River in the open press. This study presents for the first time the results of point mutation detection and phylogeny in 5 isolates isolated in Donetsk, Luhansk and Zaporozhye regions in 2023.

The example of sequences retrieved from GenBank shows the formation of the following clades: «Georgia 2007» (a reference characteristic of the beginning of the ASF epizootic in the period from 2007 to 2015 and sporadically registered until 2019), «Center of Russia 2021», «Bryansk 2021», «Asia», «Europe» and «Eastern Europe» [5–9]. Four isolates from Donetsk and Zaporozhye regions were found to belong to the «Europe» cluster, which suggests independent and continuous molecular evolution of this ASF virus genotype II genetic variant on the territory of both Western (Belgium) and Eastern European (Czech Republic, Moldova, Ukraine) countries at least from 2017 to 2023 (Fig. 5).

The allele patterns at marker nucleotide sites characteristic of subgenotype 3b, the most common subgenotype in European countries and Central Russian subjects since 2016, also confirms enzooticity. It is worth noting that isolates of the «Europe», «Center of Russia 2021», and «Bryansk 2021» clusters belong to one subgenotype (3b), while the «Asia» clades belong to other subgenotypes (3d and 3i), despite the results of the whole-genome analysis showing close relatedness of the «Central» and «Asian» variants [15]. The weak correlation of the methods can be justified by different research priorities, since the phylogeny of whole-genome sequences mainly determines the indicators of the origin and degree of divergence of the virus, whereas fragmentary (local) analysis determines the circulation of the pathogen in a certain physiographic/administrative zone.

The Kyiv/131 2016 strain was not included in the comparative analysis because of the large number of sequencing errors that were found to contribute to unreliable conclusions [10]. However, the gene variants of the marker regions of the Kyiv/131 2016 genome are identical to isolates belonging to the Eastern Europe cluster [16].

Thus, ASF outbreaks in domestic pigs kept on the right-bank and left-bank territories of the Dnieper River were caused by ASF virus belonging to different subgenotypes (7 and 3b, respectively). In turn, the spatio-temporal analysis is significantly limited due to insufficient molecular epizootologic data on ASF spread in Ukraine in the period from May 2016 to August 2023.

The high level of homology (99.95–99.99%) of the characterized isolates with other genotype II strains isolated in Eurasian countries (Georgia, China, Poland, Moldova, Belgium and Russia) once again confirm the genetic conservatism of ASF virus infecting domestic pigs

and wild boars (one species – *Sus scrofa*) and divergent from the parental variant «Georgia 2007» [6]. It should be noted that such conclusions apply exclusively to ASF virus of genotype II and do not correlate with reports on the spread of the pathogen of genotype I or recombinant variant in the People's Republic of China [17, 18].

The majority of substitutions (6/10) in isolates isolated in Donetsk region are located in the *MGF 110*, *360*, *505* multigene families, which may encode virulence factors of ASF virus [19]. SNPs at the *EP402R* locus in the ASFV/DNR/DP2023/3343-2 isolate may lead to changes in seroimmunospecificity, which should be confirmed in animal immunoassay and in the delayed hemadsorption reaction (DHAR) using reference sera [20].

The ASFV/Zaporozskaya/DP2023/2896-5 isolate has a distinct polymorphism of *MGF 360* genes, manifested by the presence of 22 substitutions, 15 of which are nonsynonymous and 7 are synonymous. The identified SNP provides a basis for experimental work to evaluate immunobiological properties in naturally susceptible animals, since mutagenesis in *MGF* genes can lead to a decrease in the virulence properties of the virus [21].

Phylogenetic analysis of whole-genome sequences showed the relatedness of isolates isolated in Lugansk and Bryansk subjects (2021, Fig. 5). Thus, 6 out of 9 substitutions detected in ASFV/LNR/DP2023/42-1 were identical to those previously unique to ASF virus from Bryansk region, but 3 out of 9 substitutions were detected for the first time, which may indicate the origin of the genetic variant from the original, closely related clade «Bryansk 2021».

It should be noted that all 5 isolates studied on the phylogenetic dendrogram had a branch length significantly exceeding this parameter in the original clade-forming strains, indicating a larger number of additional SNPs (mutation rate in substitutions/site/year) and hypothetically a later origin of genetic variants, provided that sequencing was correct (Fig. 5). In this respect, the phylogeny results are supported by the known epizootologic data on ASF outbreaks presented in the study (Table 1).

The study of *E199L* and *DP60R* gene polymorphisms in 45 genotype II sequences, including those presented for the first time, revealed that there were no supporting prospects for using these loci as marker genome fragments to track the spread of ASF in Eurasia, in contrast to previous data [7]. The identical SNPs in isolates from geographically distant regions may be related to the presence in ASF virus of point genome repair provided through the functioning of Pol X DNA polymerase, since *E199L* encodes a crucial protein associated with the activation of autophagy of infected cells [22, 23]. In any case, the data from the analysis of *E199L* and *DP60R* genes, which refute the enzootic nature of ASF, contradict the results of the study of other fragments, including marker fragments, confirming the territorially restricted development of the epizootic process.

Furthermore, no point mutation was found that was exclusive to all isolates isolated on the territory of the left bank of the Dnieper River in 2023, indicating independent pathways of genetic variation of ASF virus infecting susceptible animals in these subjects. However, a number of unique SNPs were identified for each characterized isolate, allowing further identification of related genetic variants with those described in this study. The development of primers flanking fragments with identified specific mutations will help to accelerate the differentiation process in future research.

Conclusion

For the first time, a whole-genome analysis of ASF virus circulating on the territory of the left bank of the Dnieper River was carried out using 5 isolates isolated in Donetsk, Lugansk and Zaporozhye regions from domestic pigs in 2023. All of them were assigned to genotype II, have a monophyletic origin, and are phylogenetically close to the clades Europe (4/5) and Bryansk 2021 (1/5).

In general, on the territory of these subjects, the emergent nature of ASF is not noted, but intracluster distribution is characteristic. The studied isolates belong to subgenotype 3b and have a high level of homology (99.95–99.99%) with the majority of reference strains belonging to genotype II and registered in Eurasia. However, previously undescribed point mutations unique to each isolate were identified, which will allow identification of related variants.

Furthermore, numerous substitutions in the loci of the *MGF 110*, *505* and *360* multigenic families, encoding virulence factors, were detected in 4 isolates from Donetsk and Zaporozhye regions, which may also influence changes in the immunobiological properties of the ASF pathogen.

The phylogeny of ASF virus genotype II, which originated from the reference strain Georgia 2007/1, is shown to be sufficient for differentiation. The presented data are of theoretical and practical importance for the improvement and development of preventive measures, as well as can significantly expand the possibilities of domestic and international ASF surveillance.

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